

[23] Manipulating Yeast Genome Using Plasmid Vectors

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The yeast *Saccharomyces cerevisiae* has proved to be a popular and successful experimental system for biological research. It is in many ways a typical eukaryote, sharing the most fundamental aspects of cell biology with the cells of multicellular organisms, yet it stands alone among eukaryotic organisms in the ease with which it can be experimentally manipulated. The current high status of yeast as an experimental system is in large part due to the work of the many geneticists who recognized early on the virtues of this organism. Features such as a life cycle with essentially isomorphous haploid and diploid phases, the formation of tetrads in meiosis, a short generation time, and the ability to grow in defined media formed the fundamentals of yeast methodology, and are still important today.

For those interested in expression of heterologous genes, yeast holds particular advantages, evident in the articles in this volume devoted to the topic. The sequence elements required for expression of a number of genes have been intensely studied and are well characterized. From these studies have come a variety of useful promoters, both regulated and constitutive, that make it relatively easy, to a first approximation, to achieve expression of a foreign gene in yeast. In addition, study of the yeast secretory pathway has led to a knowledge of both the *cis*-acting sequences necessary for secretion and the components that make up the secretion apparatus.

Two particular advances in yeast technology have directly led to the current high level of experimental tractability: first, the advent of DNA transformation^{1,2} and the ensuing proliferation of vector systems; second, the demonstration that recombination of transforming DNA with the chromosome usually occurs via homology¹ and that the recombination can be "directed."³ We will describe the range of yeast vectors available and discuss methods for manipulating both the yeast genome and yeast vectors using homologous recombination. Some of these methods have been reviewed in a previous volume of this series.⁴

¹ A. Hinnen, J. B. Hicks, and G. R. Fink, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1929 (1978).

² J. D. Beggs, *Nature (London)* **275**, 104 (1978).

³ T. L. Orr-Weaver, J. W. Szostak, and R. J. Rothstein, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6354 (1981).

⁴ R. J. Rothstein, this series, Vol. 101, p. 202.

Yeast Vector Systems

Yeast vectors are of four general types. These differ in the manner in which they are maintained in yeast cells. YIp (yeast integrating plasmid) vectors lack a yeast replication origin, so must be propagated as integrated elements in a yeast chromosome, usually in a single copy per genome. YRp (yeast replicating plasmid) vectors have a chromosomally derived autonomously replicating sequence (ARS) and are propagated as medium-copy-number, autonomously replicating, unstably segregated plasmids. YCp (yeast centromere plasmid) vectors have both a replication origin and a centromere sequence and are propagated as low-copy-number, autonomously replicating, stably segregated plasmids. YEp (yeast episomal plasmid) vectors have a fragment of the yeast 2- μ m plasmid and, are propagated as high-copy-number, autonomously replicating, irregularly segregated plasmids.

All of these plasmids are “shuttle vectors” that allow propagation in *Escherichia coli* for convenient manipulation and large-scale preparation of their DNA. We will concentrate on YIp and YCp plasmids, as YEp plasmids are reviewed elsewhere in this volume,⁵ and YRp plasmids are now rarely used because of their extreme instability.

Yeast Vector Components

There are two components shared by all yeast vectors: a backbone derived from a bacterial plasmid, allowing selection of *E. coli* transformants and subsequent propagation of the DNA; and a yeast selectable marker, allowing selection of yeast transformants. YCp, YRp, and YEp plasmids have additional components required for autonomous replication in yeast.

The backbone of most yeast vectors is derived from the *E. coli* plasmid pBR322.⁶ This plasmid has a bacterial replication origin that allows maintenance at high copy number, and two selectable antibiotic resistance genes. The *bla* gene encodes resistance to the β -lactam ampicillin, and the *tet* gene encodes resistance to tetracycline. In addition, pBR322 has a number of unique restriction sites for cloning DNA of interest. Because almost all yeast vectors share this common backbone of pBR322 DNA, they are all homologous to each other over some portion of their length. This homology is both a blessing, in that it can be exploited in the creation of new plasmids *in vivo*, as discussed later, and a curse, as homologous

⁵ A. B. Rose and J. R. Broach, this volume [22].

⁶ F. Bolivar, R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Cross, and S. Falkow, *Gene* 2, 95 (1977).

TABLE I
YEAST SELECTABLE MARKERS AND THEIR
COGNATE MUTATIONS

Selectable marker gene	Stable yeast mutation	Stable bacterial mutation
<i>HIS3</i>	<i>his3-Δ200</i>	<i>hisB463</i>
<i>LEU2</i>	<i>leu2-3,112</i>	<i>leuB6</i>
<i>LYS2</i>	<i>lys2-201</i>	—
<i>TRP1</i>	<i>trp1-Δ901</i>	<i>trpC9830</i>
<i>URA3</i>	<i>ura3-52</i>	<i>pyrF::Tn5</i>

recombination can occur among virtually all members of this family of plasmids, even when unwanted.

The yeast selectable marker allows selection of yeast cells that have received the plasmid by transformation. There are a number of markers available, the most common being yeast genes that complement a specific auxotrophy. For example, the *LEU2* gene encodes β -isopropylmalate dehydrogenase⁷ and complements the leucine auxotrophy of a *leu2*⁻ mutant. Of equal importance to the selectable marker is the cognate chromosomal mutation that causes the auxotrophy. Ideally, this mutation must be completely recessive, and nonreverting; the commonly used *leu2-3,112* mutation is a double frameshift mutation that reverts extremely rarely ($< 10^{-10}$) and is completely complemented by the wild-type *LEU2* gene.¹ Additionally, four of the most widely used selectable markers (*HIS3*, *LEU2*, *TRP1*, and *URA3*) can be selected in *E. coli*. Table I lists commonly used pairs of yeast selectable markers and chromosomal mutations and the bacterial mutations that are complemented by the yeast genes.

Two yeast selectable markers have the additional advantage of both positive and negative selection. For both *URA3* and *LYS2* the positive selection is for complementation of an auxotrophy, and the negative selection is for ability to grow on medium containing a compound that inhibits the growth of cells expressing the wild-type function. For the *URA3* gene the negative selection is growth in medium containing 5-fluoroorotic acid,⁸ for the *LYS2* gene the negative selection is growth in medium containing α -amino adipate.⁹ Thus, cells that have lost the selectable marker can be selected from a population; this is very useful, as many experiments that

⁷ B. Ratzkin and J. Carbon, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 487 (1977).

⁸ J. D. Boeke, F. Lacroute, and G. R. Fink. *Mol. Gen. Genet.* **197**, 345 (1984).

⁹ B. B. Chatoo, F. Sherman, T. A. Fjellstedt, D. Mehnert, and M. Ogur, *Genetics* **93**, 51 (1979).

we discuss below require that one be able to find the rare cells that have undergone a recombination or loss event to remove the plasmid DNA sequences. Unfortunately, the *LYS2* gene is quite large, and contains sites within the coding sequence for many of the most common enzymes used in plasmid construction.¹⁰ There are ways to get around this limitation, however, by using *in vivo* homologous recombination to construct plasmids with this marker (see below).

A Starter Set of Yeast Vectors

For most experiments we use the *URA3* gene as the selectable marker, resorting to other genes only when the experiment requires more than one marker. In addition to the advantage of negative selection noted above, the *URA3* gene is small and lacks sites for the most commonly used restriction enzymes. A trio of plasmids that contain the *URA3* gene are particularly useful (Fig. 1). These are the integrating vector YIp5 and two vectors derived from it: YEp420, derived by addition of a fragment of the 2- μ m plasmid that contains the replication origin, and YCp50, derived by addition of *ARS1* and *CEN4*. With these three plasmids all of the experiments described here can be performed.

Yeast Transformation

Before describing methods for manipulating the genome of yeast, a protocol for transforming yeast in a reproducible manner must be presented. The following is a distillation of various modifications to the lithium acetate method of Ito *et al.*¹¹ and works well with most laboratory strains. Transformation by this protocol consistently yields 1,000–10,000 transformants/ μ g of DNA. This protocol can be scaled up or down to provide competent cells for one or many transformation experiments. Approximately 2×10^8 cells are needed for each transformation, so about 10 ml of 2×10^7 cells/ml culture is sufficient per transformation. For example, for five transformations, grow 50 ml of culture to a density of 2×10^7 cells/ml. The transforming DNA can be from *E. coli* minipreps as well as from CsCl-purified preparations. DNA that is digested with a restriction enzyme(s) prior to transformation need not be purified again; we routinely heat-kill the enzyme(s) and use the reaction mixture to transform yeast. In many of the examples that follow, the desired transforming DNA is a restriction fragment of a plasmid. The fragment can be purified

¹⁰ D. A. Barnes and J. Thorner, *Mol. Cell. Biol.* **6**, 2828 (1986).

¹¹ H. Ito, Y. Fukuda, K. Murada, and A. Kimura, *J. Bacteriol.* **153**, 163 (1983).

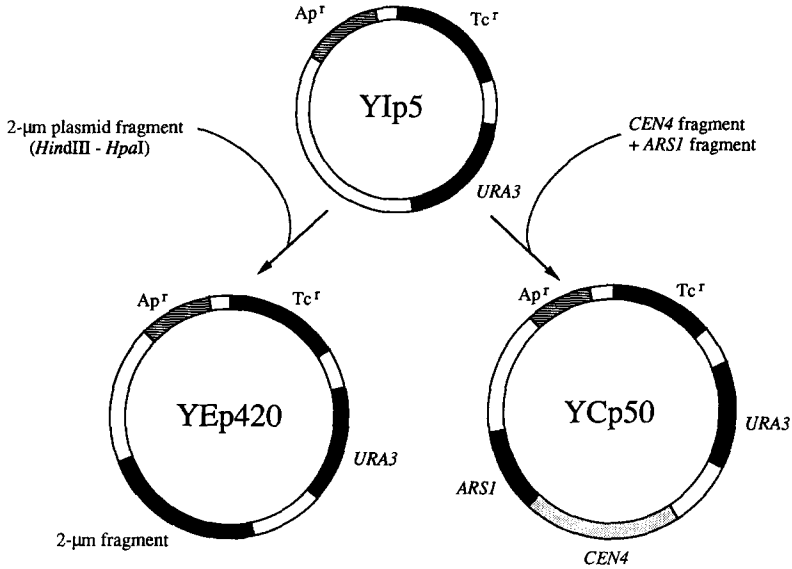


FIG. 1. Three commonly used yeast vectors that employ the *URA3* gene as a selectable marker. YIp5 was constructed by inserting the *URA3* gene into the *AvaI* site of pBR322. YEp420 was derived from YIp5 by insertion of a *HindIII*-*HpaI* fragment of the yeast 2- μ m circle into the YIp5 *PvuII* site. YCp50 was derived from YIp5 by insertion of both *ARS1* and *CEN4* into the YIp5 *PvuII* site.

from away from the plasmid backbone if desired, but this is often not necessary, such as when the backbone fragment cannot give transformants under the selection condition used. All steps are carried out at room temperature, except where noted.

Yeast Lithium Acetate Transformation Protocol

Solutions required:

LiAc buffer: 100 mM Lithium acetate, 10 mM Tris, 1 mM EDTA, pH 8.0

Carrier DNA: commercial chick blood, salmon sperm, or calf thymus DNA made up as a 10 mg/ml stock solution and sonicated to reduce viscosity

Transforming DNA: usually 0.1–1.0 μ g of circular or linear plasmid DNA per transformation, in a volume of 20 μ l or less

PEG buffer: 40% Polyethylene glycol (PEG) 3350 (Sigma Chemical Co., St. Louis, MO, #P-3640) in 100 mM lithium acetate, 10 mM Tris, 1 mM EDTA, pH 8.0

1. Grow cells to approximately 2×10^7 /ml.

2. Harvest by centrifugation, and wash once with water.
3. Resuspend cells in LiAc buffer so that total volume is 0.01 of original culture volume (i.e., 0.5 ml for 50 ml of culture).
4. Incubate for 30 min at 30° with agitation (any type of agitation that keeps cells from settling to the bottom is sufficient).
5. Split cells into 100- μ l aliquots in sterile 1.5-ml Eppendorf tubes; one aliquot for each transformation. Add 5 μ l of carrier DNA to each tube. Add transforming DNA to each tube. Vortex briefly.
6. Incubate for 30 min at 30° with agitation.
7. Add 700 μ l of PEG buffer, vortex.
8. Incubate for 60 min at 30° with no agitation required.
9. Heat shock for 5 min at 42°.
10. Spin in Eppendorf centrifuge for approximately 5 sec, pour off supernatant.
11. Resuspend cell pellet in 200 μ l of sterile water and plate by spreading on selective medium.

Notes: Transformants should be purified by picking single colonies from the transformation plate and streaking for single colonies on selective plates.

Frozen competent yeast cells can be prepared after step 4 by adding sterile glycerol to 15% (v/v) and freezing at -70° . When needed, these cells are thawed on ice and used in the protocol as for fresh cells, starting at step 5; the transformation efficiency of such cells is usually reduced 2- to 5-fold from fresh cells.

On occasion, a particular strain does not transform well by the lithium acetate method, or an experiment requires a very high frequency of transformation; in these cases, the more efficient, but more tedious, spheroplasting method can be used.^{1,12}

Integrating Plasmids: Uses of Homologous Recombination

Integrating plasmids lack a yeast origin of replication; the only way they can be propagated in yeast is by integration into the host genome. Recombination of introduced DNA with the chromosome in yeast occurs extremely efficiently, for all practical purposes exclusively by homologous recombination;¹ (the "illegitimate recombinants" found by Hinnen *et al.* turned out to be homologous crossovers within a repeated element). A very useful technique allows directed integration by homology³: this is particularly important when the plasmid contains more than one yeast gene,

¹² F. Sherman, G. R. Fink, and C. W. Lawrence, "Methods in Yeast Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1979.

namely, any instance in which a yeast gene has been cloned into a vector with a yeast selectable marker. Cleavage in one of these regions of homology with the genome greatly stimulates the frequency of integration in the immediate vicinity of the cleavage site. Thus, the investigator, using YIp plasmids, can manipulate the integration of exogenous DNA with considerable exactitude. A list of commonly used YIp plasmid vectors is given in Table II.

The simplest form of experiment using YIp vectors is integration into the yeast genome using the homology in the cloned fragment, directed, if desired, to the chromosomal locus of the cloned fragment by cleaving within the cloned sequences. This results in a direct duplication of the cloned DNA in the chromosome with vector sequences between the repeats (Fig. 2). The duplication event has increased the copy number of the cloned fragment from one to exactly two in the genome. If the cloned fragment contains a functional gene, then the copy number of that gene has been increased, and the effect of the increased gene dosage can be

TABLE II
YEAST INTEGRATING PLASMID (YIp) VECTORS

Plasmid	<i>E. coli</i> markers	Yeast markers	Comments ^a	Ref. ^b
YIp1	<i>amp^r</i>	<i>HIS3</i>		1
YIp5	<i>amp^r, tet^r</i>	<i>URA3</i>		1
YIp25	<i>tet^r</i>	<i>HIS4</i>		1
YIp26	<i>amp^r</i>	<i>URA3, LEU2</i>	All orientations of markers available	1
YIp30	<i>amp^r</i>	<i>URA3</i>	Available in both orientations	1
YIp32	<i>amp^r</i>	<i>LEU2</i>	Available in both orientations	1
pRB61	<i>amp^r</i>	<i>HIS3</i>		2
pRB290	<i>amp^r, tet^r</i>	<i>URA3</i>	YIp5 without <i>HindIII</i> site	3
pRB315	<i>amp^r</i>	<i>TRP1</i>	No <i>ARS1</i>	4
pRB328	<i>amp^r</i>	<i>HIS3</i>	Polylinker (1) flanks <i>HIS3</i>	4
pRB331	<i>amp^r</i>	<i>URA3</i>	Polylinker (1) flanks <i>URA3</i>	2
pRB388	<i>amp^r, tet^r</i>	<i>URA3</i>	13-kb <i>URA3</i> fragment	2
pRB502	<i>amp^r, tet^r</i>	<i>URA3</i>	Polylinker (2) flanks <i>URA3</i>	2
pRB506	<i>amp^r</i>	<i>LYS2</i>	Polylinker (1) flanks <i>LYS2</i>	2
pRB758	<i>amp^r</i>	<i>LEU2</i>	Polylinker (3) flanks <i>LEU2</i>	2
pRB759	<i>amp^r</i>	<i>URA3</i>	Polylinker (3) flanks <i>URA3</i>	2

^aThe polylinkers are (1) pPL7 (J. Mullins, personal communication; sites are *EcoRI*-*ClaI*-*HindIII*-*XbaI*-*BglII*-*MboII*-*PstI*-*BamHI*), (2) pT7-2 (United States Biochemical Corporation), (3) pUC19 (Yanisch-Perron *et al.*, 1985).

^b1, D. Botstein, S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis, *Gene* 8, 17 (1979); 2, this laboratory (Dept. of Biology, Massachusetts Institute of Technology); 3, Schatz *et al.* (1986); 4, H. Ma, S. Kunes, P. J. Schatz, and D. Botstein, *Gene* 58, 201 (1987).

assayed. It is important to realize also that this strain now carries a selectable genetic marker, *URA3* in this case, at the chromosomal locus of the cloned DNA; this construction can be used to map the locus in relation to other genetic markers, even if the cloned fragment has no function. This duplication is quite stable in comparison to autonomously replicating plasmids; the integrated sequences excise at a frequency of approximately $1 \times 10^{-3} - 1 \times 10^{-4}$.

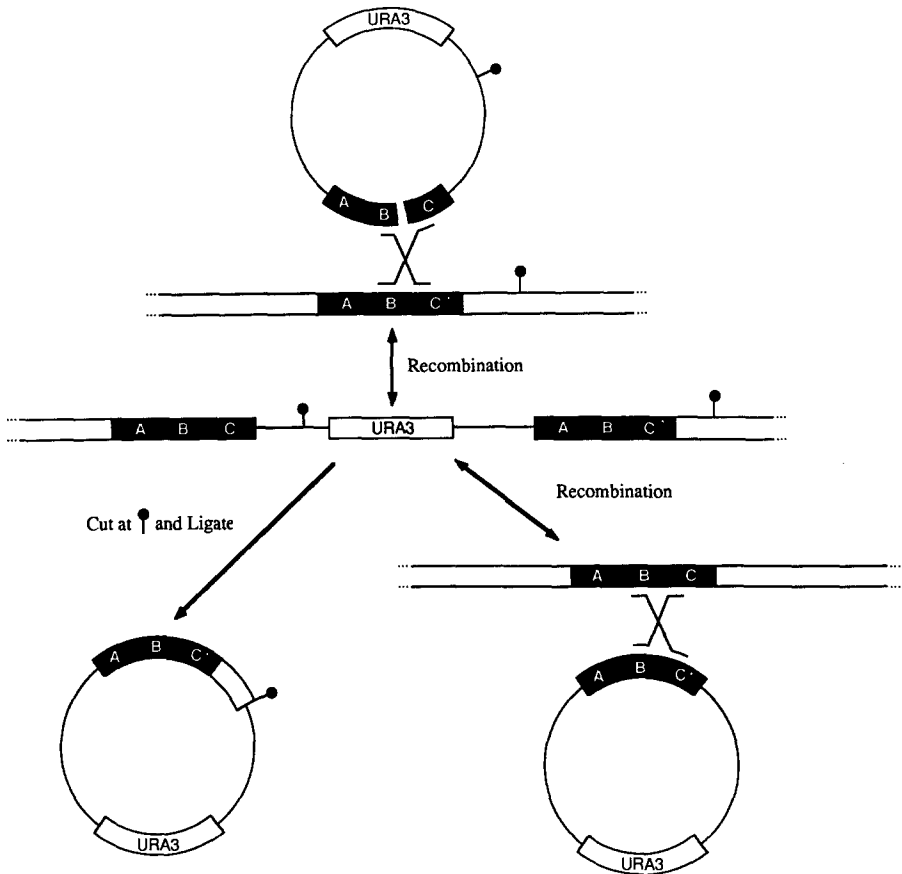


FIG. 2. Recombination of an integrating plasmid with the yeast genome. The plasmid and chromosome differ at position C (C versus C*). The recombination event is directed by cleaving the transforming DNA between B and C, leading to a direct duplication in the chromosome. If genomic DNA is isolated from this transformant, cleaved at an appropriate restriction site, and ligated, then a region of the chromosome, including the chromosomal C*, can be recovered in *E. coli*. Alternatively, recombination between the duplicated sequences will lead to recovery of cells that have looped out the plasmid sequences, occasionally leaving the plasmid C behind in the chromosome.

Of course, any such construction must be checked by physical means, such as restriction digestion and Southern hybridization, to determine that the desired event is actually what took place, and that only a single copy of the plasmid has integrated. In particular, one must be aware of the possibility that integration has taken place at the chromosomal locus of the selectable marker, as it also provides homology for recombination. Indeed, there are situations where this event is desired, and can be accomplished by cleaving the plasmid in the selectable marker sequences rather than the insert sequences. An example of this would be the introduction of a foreign gene into the yeast genome; as the foreign DNA provides no homology for recombination with the yeast chromosome, integration could be directed to the locus of the selectable marker.

Excision of the inserted DNA from the chromosome also takes place by homologous recombination. Therefore, recombination between the duplicated sequences in Fig. 2 will result in complete excision of the plasmid DNA sequences, including the cloned fragment, leaving the chromosome as it was before integration. This *in vivo* recombination event can take place at any point within the homology created by the duplicated segments. Thus, if the cloned fragment contains a mutation made *in vitro*, integration and subsequent excision will, at some frequency, lead to the replacement of the wild-type sequence with the mutant sequence, leaving the chromosome otherwise wild-type. This kind of perfect replacement of mutant for wild type (often called "transplacement") is the best way of testing the *in vivo* consequences of mutations made *in vitro*. As discussed above, the *URA3* and *LYS2* genes are particularly useful for this type of experiment as both positive and negative selections are possible. Thus, to isolate the rare cells that have undergone the excision event, one can grow the strain under nonselective conditions for a few generations to allow the recombination event to occur, then plate them under conditions that select against the function of the selectable marker gene. The colonies that appear can then be checked for the presence of the mutant phenotype.

Integration of YIp plasmids can also be exploited to clone mutations made *in vivo* so that their structure can be analyzed *in vitro*. In this case, a wild-type gene is integrated into a mutant allele, creating a duplication. If genomic DNA from this strain is then prepared, and cleaved with an appropriate restriction enzyme, the mutant DNA can be isolated by circularizing the cut DNA by ligation and selecting in *E. coli* for the vector marker (Fig. 2).^{13,14}

The examples thus far have involved integration of an intact copy of a

¹³ F. Winston, F. Chumley, and G. R. Fink, this series, Vol. 101, p. 211.

¹⁴ G. S. Roeder and G. R. Fink, *Cell* 21, 239 (1980).

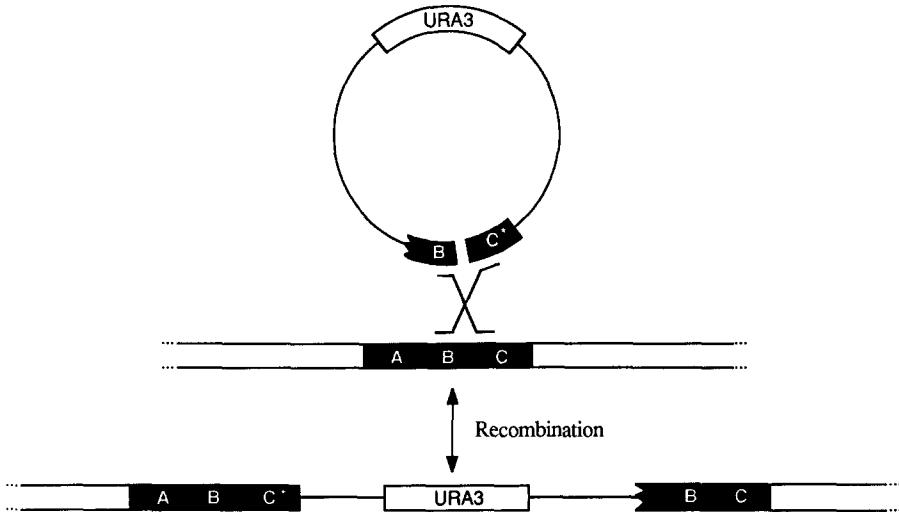


FIG. 3. Integration of a partial copy of a gene. The gene on the plasmid has been truncated such that it lacks one end, and it differs from the chromosome at C. Integration of this plasmid, directed by cleaving between B and C*, will lead to a direct duplication in the chromosome in which only one of the two copies of the gene is active. Because the integration was directed to occur between B and C*, the functional copy contains C*.

gene at its chromosomal locus; other useful approaches have been developed that make use of integration of an incomplete copy of a gene. By constructing the integrating plasmid so that the gene of interest lacks its 5' end, and integrating the plasmid at the locus, a partial gene duplication is created (Fig. 3). Only one of the copies is functional in this construct, thus the locus has been genetically marked without an accompanying change in gene dosage. Of course, this could also be done with a construct lacking the 3' end of the gene. If the plasmid contains a mutation, then, depending on the location of the mutation with respect to the restriction enzyme site used for directing recombination, the functional copy of the gene may contain the mutation. Thus, this is a useful method for isolating recessive mutations in one step; integration of the plasmid in the correct way will lead to expression of the mutant phenotype. This has been exploited in isolating conditional mutations in a number of genes: *ACT1*,¹⁵ *TOP2*,¹⁶ and *TUB2*.¹⁷

Homologous integration of a partial copy of a gene can also be used to create null mutations in a single step. If the integrating plasmid construct

¹⁵ D. Shortle, P. Novick, and D. Botstein, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4889 (1984).

¹⁶ C. Holm, T. Goto, J. C. Wang, and D. Botstein, *Cell* **41**, 553 (1985).

¹⁷ T. C. Huffaker, J. H. Thomas, and D. Botstein, *J. Cell Biol.* **106**, 1997 (1988).

contains an internal fragment of the gene, then, after recombination, the gene is split into two inactive fragments (Fig. 4A), assuming, of course, that neither truncated gene fragment has activity. Other methods for producing null mutations exist, the most common being single-step transplacement⁴ in which yeast is transformed with a linear DNA fragment that contains a gene or gene fragment into which a selectable marker has been inserted. The free ends of the fragment direct integration to the homologous chromosomal locus, resulting in direct replacement of the chromosomal sequences (Fig. 4B). For both of these methods of gene disruption it is important to know that the mutation is actually a null mutation and does not retain partial function or gain novel function, and that the construction does not interfere with surrounding genes.

One of the major advantages of yeast as a genetic organism is that there are stable haploid and diploid phases of the life cycle. Thus, null alleles of uncharacterized genes can be constructed as described above in a diploid strain; this diploid can then be induced to undergo meiosis to yield four haploid progeny. Two of these will be wild-type, and two will contain the mutation. If the two containing the mutation fail to grow, then the mutation is lethal. If they are viable, the phenotype of the null mutation can be studied. Again, the presence of the construction in the transformant must be confirmed by physical means.

Centromere Plasmids

Yeast centromere plasmids (YCp) stably replicate as autonomous plasmids. YCp vectors share the selectable markers of YIp vectors, but differ from YIp vectors in two important ways: they have an element that functions in yeast as replication origin, and they have a yeast centromere sequence that allows fairly regular mitotic and meiotic segregation. The replication origin sequence is termed ARS (autonomously replicating sequence); the most widely used YCp vectors contain *ARS1*, which is found immediately adjacent to the *TRP1* gene of yeast.¹⁸ The centromere sequence is termed CEN;¹⁹ *CEN4*, the centromere of chromosome IV, is commonly used. Interestingly, as few as 125 base pairs (bp) of authentic centromere DNA are required for proper mitotic and meiotic segregation of chromosomes, although the fragment commonly used in yeast vectors contains considerably more adjacent sequence. Table III is a listing of some available YCp vectors. We have included only those that are derived from

¹⁸ D. T. Stinchcomb, K. Struhl, and R. W. Davis, *Nature (London)* **282**, 39 (1979).

¹⁹ L. Clarke and J. Carbon, *Nature (London)* **287**, 504 (1980).

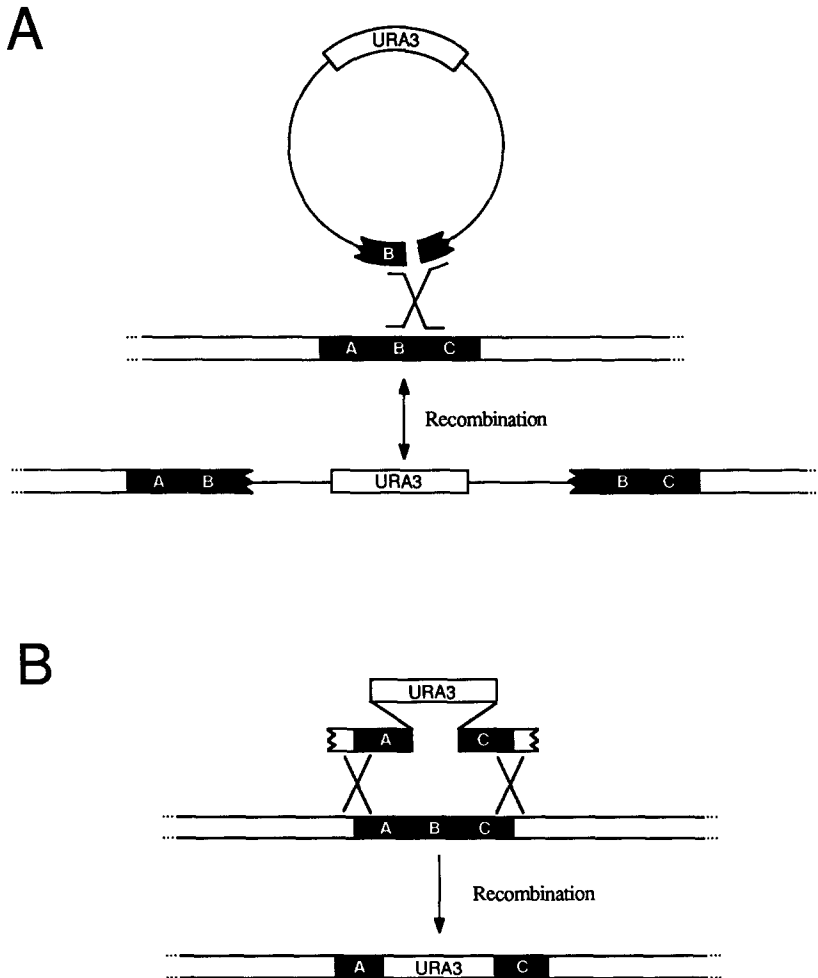


FIG. 4. Methods of gene disruption. In A, the plasmid-borne copy of the gene is truncated such that it lacks both ends. Integration of this construct into the chromosome leads to two nonfunctional copies of the gene, one lacking its 5' end, the other its 3' end. In B, a portion of the gene has been replaced with the *URA3* coding sequence. Integration of a linear fragment of DNA containing the insertion, homologous to the chromosome at both ends, leads to replacement of the chromosomal locus with the disrupted version. No plasmid sequences are left in the chromosome.

YCp50, the most frequently used centromere vector, as these are the ones of which we have had experience.

The presence of a yeast centromere on a plasmid does not ensure that the plasmid will remain single copy in the yeast cell. This is because plasmids bearing a centromere sequence are not as stable as natural linear

TABLE III
YEAST CENTROMERE PLASMID (YCp) VECTORS

Plasmid	<i>E. coli</i> markers	Yeast markers	Comments	Ref. ^a
YCp50	<i>amp^r, tet^r</i>	<i>URA3</i>	<i>CEN4</i> and <i>ARS1</i>	1
YCp401	<i>amp^r</i>	<i>LYS2, URA3</i>	Derived from YCp50	2
YCp402	<i>amp^r</i>	<i>LEU2</i>	Derived from YCp50	2
YCp403	<i>amp^r</i>	<i>HIS3</i>	Derived from YCp50	2
YCp404	<i>amp^r</i>	<i>HIS3, URA3</i>	Derived from YCp50	2
YCp405	<i>amp^r</i>	<i>LYS2</i>	Derived from YCp50	2
YCp406	<i>amp^r</i>	<i>URA3</i>	Derived from YCp50	2
YCp407	<i>amp^r</i>	<i>HIS3</i>	Derived from YCp50	2
YCp408	<i>amp^r</i>	<i>HIS3, URA3</i>	Derived from YCp50	2
YCp409	<i>amp^r</i>	<i>LEU2</i>	Derived from YCp50	2
YCp410	<i>amp^r</i>	<i>TRP1</i>	Derived from YCp50	2
YCp411	<i>amp^r</i>	<i>TRP1, URA3</i>	Derived from YCp50	2
YCp412	<i>amp^r</i>	<i>URA3</i>	YCp50 with pUC19 polylinker	3

^a1, C. Mann (see Ma *et al.*, 1987, for map); 2, H. Ma, S. Kunes, P. J. Schatz, and D. Botstein, *Gene* 58, 201 (1987); 3, this laboratory (Dept. of Biology, Massachusetts Institute of Technology).

chromosomes. For example, chromosome V is lost approximately once in 10^5 divisions,²⁰ while YCp vectors typically are lost approximately once in 10^2 divisions.²¹ This lowered fidelity of mitotic transmission results in some cells receiving two copies of the plasmid at mitosis, rather than one (actually, only about 20% of the loss events result in a daughter getting two copies; most often, one daughter receives one copy, and the other receives none). If cells carrying more than one copy of the plasmid have a growth advantage, then they may dominate the culture. It has been shown however, that a large excess of centromere-bearing plasmids causes sickness and inviability, presumably because the extra centromeres interfere with the normal process of chromosome segregation,²² so it can be assumed that the copy number of a sequence on a YCp vector in yeast will be on the order of one to a few copies.

YCp vectors are true shuttle vectors in that they can replicate freely in both *S. cerevisiae* and *E. coli*. Indeed, the shuttle can be quite rapid: plasmid DNA from standard *E. coli* "minipreps" can be used for transformation of yeast, and DNA from several yeast DNA miniprep procedures can be used to transform bacteria. The most rapid of these yeast minipreps

²⁰ L. H. Hartwell, S. K. Dutcher, J. S. Wood, and B. Garvik, *Rec. Adv. Yeast Mol. Biol.* 1, 28 (1982).

²¹ D. T. Stinchcomb, C. Mann, and R. W. Davis, *J. Mol. Biol.* 158, 157 (1982).

²² B. Futcher and J. Carbon, *Mol. Cell. Biol.* 6, 2213 (1986).

involves breaking cells open by agitation with glass beads, and requires only a few minutes.²³ We present here a version of this protocol that we have successfully used.

Yeast DNA Miniprep for Shuttling Plasmids

Materials

Lysis buffer: 2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0

Glass beads: Obtained from Sigma Chemical Co., (St. Louis, MO; Cat. No. G-9268, 425–600 μm glass beads); prepared by soaking in concentrated nitric acid for 1–2 hrs, washing extensively in distilled water, and baking until dry in a 200° oven

PCI: A 25:24:1 mixture of phenol:chloroform:isoamyl alcohol

Method

1. Grow 2-ml overnight cultures in a medium that selects for presence of the plasmid.
2. Fill a 1.5-ml microfuge tube with culture and spin for 5 sec to collect the cells. Pour off the supernatant.
3. Add 50 μl of lysis buffer and 50 μl of PCI. Then add 0.2 g of glass beads.
4. Vortex at top speed for 2 min.
5. Spin for 5 min in a microfuge.
6. Use $\sim 1 \mu\text{l}$ of the aqueous phase to transform competent *E. coli* cells.

Using *E. coli* cells that give approximately 10^6 transformants/ μg DNA, one can expect on the order of 10–30 transformants when transferring centromere plasmids from yeast to *E. coli*, and 100–300 transformants when transferring 2- μm -based plasmids. The number of transformants does not increase in proportion to the amount of aqueous phase added; no more than 3 μl should be added to the transformation.

A typical experiment with YCp vectors is to insert a DNA fragment into the plasmid and to determine the phenotype of yeast carrying this fragment at low copy number. This is very useful when subcloning a DNA fragment to determine the region required for complementation of a recessive mutant phenotype. An extension of this is to clone a gene by complementation of a mutant phenotype. In this case, a library of random yeast DNA inserts is made in the centromere vector, then the library is transformed into the mutant yeast. Cells that receive a plasmid carrying the wild-type gene will be able to grow at the restrictive condition. The use of a

²³ C. S. Hoffman and F. Winston, *Gene* 57, 267 (1987).

centromere vector for library construction is advantageous because the cloned wild-type gene is present in approximately the same copy number as when at its chromosomal locus. Indeed, some genes cannot be cloned from libraries made in YE_p vectors because they are lethal in high copy number: *KARI*,²⁴ *TUB2*,²⁵ and *ACT1* (unpublished observation). An excellent centromere–vector yeast DNA library in *E. coli*²⁶ is available from the American Type Culture Collection. This library consists of *Sau3A* partial fragments of ~ 15 kb inserted into the *Bam*HI site of YCp50. Once strains carrying complementing plasmids have been isolated, the plasmids can be examined by shuttling them from yeast to *E. coli*, and using physical means to determine their relationship to each other. To confirm that a given plasmid clone contains the authentic gene, and not a suppressor, the insert can be cloned into a YIp vector, integrated into the genome at the locus of the cloned fragment using the homology of the fragment to direct integration, and crossed to a strain bearing a mutation in the gene to be cloned. If the cloned sequence is the authentic gene, then tetrad analysis should show complete genetic linkage between the locus of the cloned fragment and the locus of the mutant gene.

Centromere plasmids can also be used to clone mutations from the chromosome onto a plasmid. The previously discussed method of doing this, using YIp vectors, requires that DNA be isolated from a transformant, cleaved, and recircularized by ligation. Using centromere vectors, this cloning can be done directly.²⁷ This is accomplished by introducing a linear centromere plasmid containing a gap that covers the region of the mutation into a mutant strain. *In vivo*, the homology on the ends of the gapped plasmid direct repair DNA synthesis such that the gap is repaired from the chromosomal sequence containing the mutation. The plasmid resulting from this repair can be transferred to *E. coli* as described above and analyzed.

Construction of New Vectors by *in Vivo* Homologous Recombination

One of the limitations of *in vitro* recombinant DNA techniques is that the researcher is often at the mercy of available restriction sites when making new constructions. This is especially apparent in yeast research because genetic analysis of any given gene now routinely involves moving the gene onto plasmids of different copy number and different selectable

²⁴ M. D. Rose and G. R. Fink, *Cell* **48**, 1047 (1987).

²⁵ J. H. Thomas, Ph.D., thesis, Massachusetts Institute of Technology (1984).

²⁶ M. D. Rose, P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink, *Gene* **60**, 237 (1987).

²⁷ T. Orr-Weaver and J. Szostak, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4417 (1983).

markers. A recently described alternative to the *in vitro* approach takes advantage of *in vivo* homologous recombination to create new plasmids.²⁸

This *in vivo* method can, in principle, be used to construct any plasmid with a yeast selectable marker and a yeast replication origin. Briefly, the method is based on the observation that two linear DNA molecules with overlapping homologies at or near the ends of the molecules can recombine efficiently after cotransformation into yeast to regenerate a circular molecule. In practice, one of the linear molecules is a plasmid linearized at a restriction site, the other is a DNA fragment that bears homology to the plasmid, but cannot itself circularize to form a replicating plasmid.

In the simplest case, a new selectable marker is introduced onto a plasmid either as a simple insertion or with concomitant loss of the original marker (Fig. 5). The homology for recombination is provided by the pBR322 backbone sequences common to most yeast plasmids. Because the linearized plasmid itself can give rise to transformants at a very low frequency by *in vivo* ligation, a small amount of this DNA is used in the transformation. Typically, a DNA mixture containing approximately 0.1 μg of a linearized plasmid DNA and 1.0 μg of a DNA restriction fragment is used to transform competent yeast cells, and the new marker is selected. We have observed that the frequency of transformation is quite high in these circumstances in comparison to transformation with closed-circular DNA controls. Plasmid DNA from the transformants is then isolated and shuttled to *E. coli* so that the structure of the plasmids can be determined.

The location of the cleavage site in the linearized plasmid and the extent of homology that the fragment has with the linearized plasmid determine the types of recombinants that will be recovered. If the cleavage site is outside of the selectable marker present on the linearized plasmid, then it is possible for that selectable marker to be maintained in the recombinational repair event. If the cut is made in the selectable marker gene of the linearized plasmid, then that selectable marker will be lost and replaced by the new marker. Similarly, if the ends of the restriction fragment carrying the new selectable marker do not cover the region of the selectable marker of the linearized plasmid, then the recombinant plasmid will contain both markers. If the ends of the restriction fragment do cover the site of the linearized plasmid's selectable marker, then the recombinant plasmid may or may not have the original marker, depending on the location of the recombination event (Fig. 5).

This method is also applicable to cases where the selectable marker itself is not exchanged. It may be necessary, for example, to exchange

²⁸ H. Ma, S. Kunes, P. J. Schatz, and D. Botstein, *Gene* 58, 201 (1987).

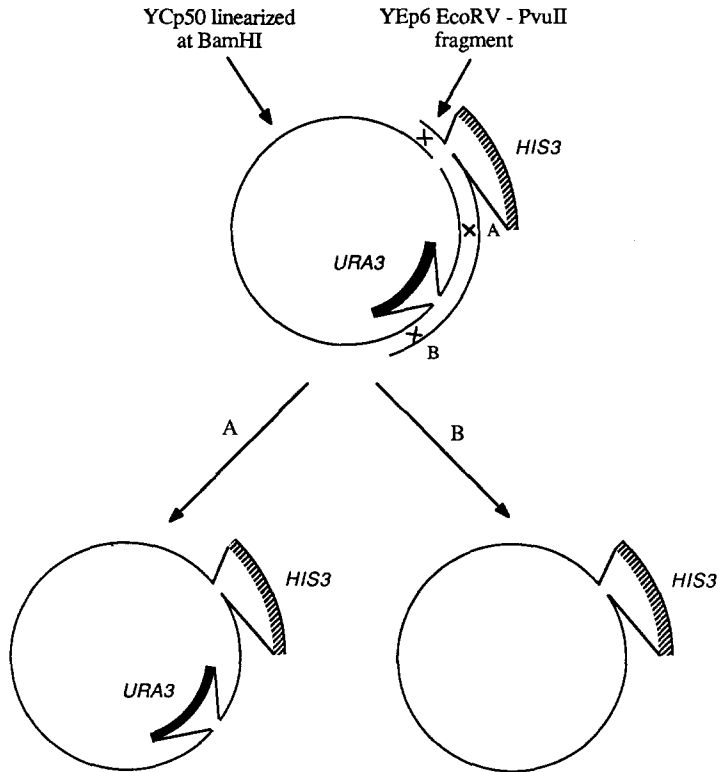


FIG. 5. Construction of new plasmids by *in vivo* recombination. In this example, YCp50 linearized at the *Bam*HI site is cotransformed into yeast with a fragment of YEp6. The *HIS3* marker of YEp6 is selected in the transformation. Because the YEp6 fragment cannot itself replicate, most transformants contain plasmids derived by recombination between YCp50 and the fragment. If one end of the recombination event takes place at A, then the recombinant plasmid will contain both the *URA3* and *HIS3* markers. If the recombination event takes place at B, then the plasmid will contain only *HIS3*.

alleles of the insert gene on the plasmid, from wild type to mutant or vice versa. In such a case, the plasmid is cleaved in the insert sequences to create a gap that covers the change in the new allele. This is then cotransformed with a fragment containing the new allele, and the marker of the original plasmid is selected. Because ligation of the plasmid to form a circle *in vivo* is infrequent compared to the frequency of recombination between the two cotransformed DNA fragments to form a functional plasmid, the majority of transformants will have the allele replacement. In this case, it is important that there be a deletion of the locus in the chromosome so that the plasmid cannot be repaired from the chromosomal copy.

In some cases, a restriction site needed to linearize the plasmid at the

desired position is not available, or is not unique in the plasmid. In such a case, the circular plasmid can be cotransformed, uncut, with the linear fragment, and the selectable marker of the linear fragment selected. This method will only work if there is a selectable marker on the linear fragment, as otherwise there is no selection for the recombination event.

Summary

The vectors and techniques described here enable one to manipulate the yeast genome to meet specific needs. Genes can be cloned, and the clone used to delete the wild-type gene from the chromosome, or replace it with mutant versions. Mutants derived by classical methods, such as mutagenesis of whole cells, or by reversion of a phenotype, can be cloned and analyzed *in vitro*. Yeast genes and foreign genes can either be inserted into autonomously replicating plasmid vectors that are reasonably stable or integrated into a yeast chromosome where they are maintained at one copy per genome. The combination of these techniques with the characterized promoter systems available in yeast make it possible to express almost any gene in yeast. Once this is achieved, the entire repertoire of yeast genetics is available to probe the function of the gene, or to engineer the expression in useful ways.

[24] Regulated *GAL4* Expression Cassette Providing Controllable and High-Level Output from High-Copy Galactose Promoters in Yeast

By LAWRENCE M. MYLIN, KATHRYN J. HOFMANN, LOREN D. SCHULTZ, and JAMES E. HOPPER

The *Saccharomyces* galactose-inducible promoters *GAL1*, *GAL7*, and *GAL10* have been extensively exploited for regulated and high-level production of foreign and nonforeign proteins in yeast. The inducibility of these promoters is based on a physical interplay between the *GAL4* protein, a transcriptional activator, and the *GAL80* protein, an antagonist of *GAL4* protein activity.^{1,2} The fact that the *GAL1*, *GAL7*, and *GAL10* promoters can be rapidly switched from a near-zero *off* state to a very high *on* state by addition of galactose to a nonglucose-containing growth medium makes these promoters especially attractive. In particular, when

¹ N. F. Lue, D. I. Chasman, A. R. Buchman, and R. D. Kornberg, *Mol. Cell. Biol.* **7**, 3446 (1987).

² M. Johnston, *Microbiol Rev.* **51**, 458 (1987).