Transformation of yeast

(gene exchange/hybrid plasmid/integration)

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ABSTRACT **A stable *leu2*$^-$ yeast strain has been transformed to *LEU2*$^+$ by using a chimeric ColE1 plasmid carrying the yeast *leu2* gene. We have used recently developed hybridization and restriction endonuclease mapping techniques to demonstrate directly the presence of the transforming DNA in the yeast genome and also to determine the arrangement of the sequences that were introduced. These studies show that ColE1 DNA together with the yeast sequences can integrate into the yeast chromosomes. This integration may be additive or substitutive. The bacterial plasmid sequences, once integrated, behave as a simple Mendelian element. In addition, we have determined the genetic linkage relationships for each newly introduced *LEU2*$^+$ allele with the original *leu2*$^-$ allele. These studies show that the transforming sequences integrate not only in the *leu2* region but also in several other chromosomal locations.**

Transformation is the process by which naked DNA is introduced into a cell, resulting in a heritable change. Transformation provides the link between the *in vitro* analysis of DNA and its *in vivo* function. Many recent advances in the analysis of eukaryotic DNA sequences have resulted from the facility with which these DNA segments can be attached to bacterial plasmids and subsequently introduced into bacterial cells by transformation. Such cloned sequences can be easily obtained in large amounts and can be altered *in vivo* by bacterial genetic techniques and *in vitro* by specific enzymatic modifications. To determine the effects of these experimentally induced changes on the function and expression of eukaryotic genes, the rearranged sequences must be taken out of the bacteria in which they are cloned and studied and introduced back into the eukaryotic organism from which they were originally obtained. Transformation back into eukaryotic organisms is the missing step in this sequence. There have been many attempts to transform fungi (refs. 1 and 2; reviewed in ref. 3). However, all failed to prove at the molecular level that exogenous DNA had become incorporated as a heritable component of the genome. In the final analysis, only by identification of the exogenous sequences in the recipient DNA can trivial explanations such as reversion be ruled out. Recent advances in recombinant DNA technology have provided the tools for a direct proof of transformation in yeast.

In the studies described here, a hybrid bacterial plasmid containing yeast DNA was used as a source of highly enriched genes as well as a molecular probe for the sequences introduced into the recipient yeast cells. The plasmid pY*eleu*10 is a hybrid composed of the *Escherichia coli* plasmid ColE1 and a segment of yeast DNA from chromosome III. This plasmid complements *leuB* mutants of *E. coli* and contains the yeast *LEU2*$^+$ gene which encodes the leucine biosynthetic enzyme β-isopropylmalate dehydrogenase (4, 5). Using the pY*eleu*10 plasmid, we can transform a stable *leu2*$^-$ mutant of yeast to *LEU2*$^+$. We have also determined that during the transformation event the ColE1 sequences together with the yeast DNA on the plasmid can integrate into the yeast chromosomes in several different places. Thus, through transformation, bacterial genes become a heritable component of the yeast genome and obey the rules of Mendelian inheritance characteristic of eukaryotic organisms.

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MATERIALS AND METHODS

Strains. All yeast strains used as recipients for transformation were derived from wild-type strain S288C. AH22 (*a leu2-3 leu2-112 his4-519 can1*) has a *leu2*$^-$ double mutation constructed by recombining the *leu2* alleles from strains 5463-8A (*a leu2-3 his4-519 can1*) and ICR112 (α *leu2-112*). MC333 (α *met8-1 trp1-1 leu2-2*) was obtained from M. Culbertson.

DNA Preparation. pY*eleu*10, a hybrid plasmid isolated by B. Ratzkin and J. Carbon, contains the *LEU2*$^+$ gene of yeast cloned on the *E. coli* plasmid vector ColE1 (4, 5). Plasmid DNA was isolated by a modification (5) of procedures described by Clewell (6) and Guerry *et al.* (7). Yeast DNA was isolated by the method of Cryer *et al.* (8).

Transformation of *Saccharomyces cerevisiae*. Spheroplasts were prepared as described by Hutchinson and Hartwell (9). A fresh logarithmic phase culture (80 ml; 2×10^7 cells per ml) was concentrated to $^1/_{10}$ volume by centrifugation and treated with 1% Glusulase (Endo Laboratories) in 1 M sorbitol for 1 hr at 30°. Spheroplasts were washed three times with 1 M sorbitol and resuspended in 0.5 ml of 1 M sorbitol/10 mM Tris·HCl/10 mM $CaCl_2$, pH 7.5. Plasmid DNA was added to a final concentration of 10–20 μg/ml and incubated for 5 min at room temperature. Then, 5 ml of 40% polyethylene glycol 4000 (Baker Chemical Co., Phillipsburg, NJ)/10 mM Tris·HCl/10 mM $CaCl_2$, pH 7.5, was added as recently described by van Solingen and van der Plaat (10). After 10 min the spheroplasts were sedimented by centrifugation and resuspended in 5 ml of the sorbitol/Tris/$CaCl_2$ mixture; 0.2-ml aliquots were added to 10 ml of regeneration agar and poured on minimal agar plates [regeneration agar is Difco yeast nitrogen base without amino acids, supplemented with 1 M sorbitol, 2% glucose, 2% YEPD, and 3% agar (10)].

Hybridization Analysis of Restriction Digests. Total yeast DNA was digested with restriction endonuclease *Hin*dIII (New England Biolaboratories, Beverly, MA). Restriction digests were separated on 0.8% agarose gels and transferred to nitrocellulose filters (Millipore HAWP) according to the method of Southern (11). Details of the blotting procedures and hybridization conditions have been described (5). ^{32}P-Labeled plasmid DNA was prepared by nick translation with *E. coli* DNA polymerase I (Worthington Biochemical Co.) (12).

Yeast Colony Hybridization. Colony hybridization was performed as described by Grunstein and Hogness (13) with

the following modifications developed by J. Walsh in our laboratory. Colonies were grown on Millipore HAWP filters and lysed *in situ* by placing the filter sequentially on blotting paper saturated with the following solutions: 50 mM EDTA/2.5% 2-mercaptoethanol, pH 9.0 for 15 min; 1 mg/1 ml of Zymolyase 60000 (Kirin Brewery) for 2–3 hr at 37°; 0.1 M NaOH/1.5 M NaCl for 1 min; 0.2 M Tris·HCl, pH 7.5/0.30 M NaCl/0.03 M Na citrate for two changes, 2 min each. All treatments were at room temperature unless specified. Filters were air-dried briefly and baked under vacuum at 80° for 1 hr.

Genetic Techniques and Media. Genetic techniques and media, with the exception of regeneration agar (above), have been described (14).

Biohazard Consideration. This work was carried out under P2 laboratory conditions as approved by the local biohazards committee and the National Science Foundation. All yeast and bacterial strains harboring recombinant DNA were destroyed by autoclaving or exposure to Clorox solution before disposal as specified by the *NIH Guidelines for Recombinant DNA Research*, July 1976.

RESULTS

Transformation to *LEU2*$^+$ Depends on the pYe*leu*10 DNA. A sensitive detection system for transformation requires that the recipient strain not give rise to colonies in the absence of DNA. For this reason, strains carrying deletions are ideal recipients. However, none exists for the *leu2* region of yeast. Instead, we constructed a stable *leu2*$^-$ strain, functionally equivalent to a deletion, by combining within the *leu2* gene two different mutations, each of which has a low background rate of reversion to Leu$^+$. This stable double-mutant strain, AH22, fails to revert either spontaneously or by mutagenesis with ICR170 or UV irradiation ($<10^{-10}$).

Spheroplasts of strain AH22 were mixed with pYe*leu*10 DNA, Ca^{2+}, and polyethylene glycol, and the treated spheroplasts were regenerated in 3% agar (10). Ten percent of the spheroplasts plated under these conditions gave rise to colonies. When spheroplasts were plated on medium lacking leucine, colonies appeared after 3–5 days at 30° at a frequency of $1/10^7$ regenerated spheroplasts. No colonies were obtained in controls where spheroplasts were plated without DNA. Putative transformants were purified and subjected to further genetic and biochemical analysis.

Hybridization Tests. In the DNA hybridization experiments, two probes were used: hybrid plasmid pYe*leu*10 and its parent bacterial plasmid ColE1. There are no ColE1 sequences in the original recipient strain, AH22, or in any untransformed yeast strain that we have examined (see AH22 in Fig. 1 *right*). Therefore, the presence of ColE1 sequences in yeast DNA is unambiguous evidence of transformation. We tested each of 42 transformants for the presence of ColE1 sequences by using a modification of the Grunstein–Hogness colony hybridization technique (13). In this case, yeast colonies rather than bacterial colonies were lysed on nitrocellulose filters and subjected to hybridization with ^{32}P-labeled ColE1 DNA. Of 42 putative transformants tested, 35 were shown to contain ColE1 sequences by this method.

The arrangement of ColE1 and pYe*leu*10 sequences in the transformed strains was demonstrated by hybridization of the native plasmids to specific restriction fragments from the total DNA restriction digest of these strains. There are no *Hin*dIII restriction sites in either the yeast or the bacterial DNA carried by pYe*leu*10; therefore, a *Hin*dIII digest of total yeast DNA contains a single restriction fragment of $\sim 10^7$ daltons capable of hybridizing to this plasmid (ref. 5; see also AH22 in Fig. 1 *left*). Furthermore, as shown in Fig. 1 *right*, no sequences in the recipient strain hybridized with ColE1 DNA by itself. Thus, any fragment that hybridizes to ColE1 must contain sequences introduced by transformation. The sequences present in the restriction digests of yeast DNA that were complementary to the transforming DNA were visualized by hybridization with ^{32}P-labeled pYe*leu*10 or ColE1 plasmid DNA prepared by nick translation.

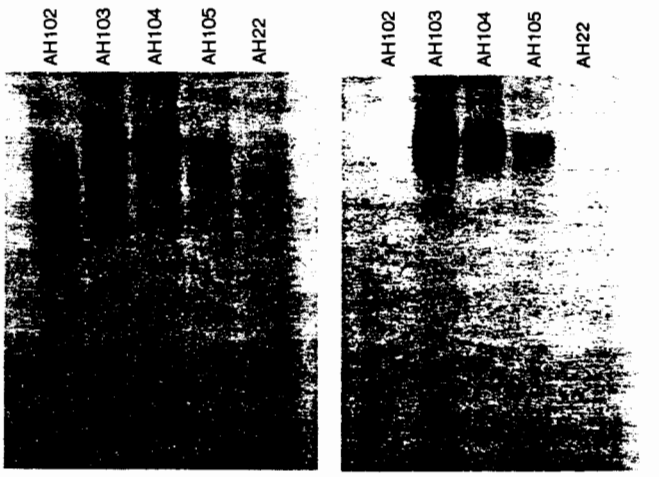

FIG. 1. Radioautographs of ^{32}P-labeled DNA hybridized to a "Southern blot" containing *Hin*dIII restriction digests of DNA from the recipient strain AH22 and four transformants, AH102–AH105. (*Left*) Hybrid plasmid pYe*leu*10 as the labeled probe. (*Right*) Plasmid ColE1 DNA as probe.

Approximately 5 μg of total DNA extracted from four representative transformants and the recipient strain AH22 were digested with *Hin*dIII and the restriction fragments were displayed on a 0.8% agarose gel. The restriction digest in the gel was then transferred to a nitrocellulose filter by the Southern blotting procedure (11). Radioautographs of two such hybridization experiments are shown in Fig. 1. The differences in the hybridization patterns of these strains indicate that at least three distinct types of transformation events had occurred. Each of these events is described further below.

Genetic Identification of "New" *leu2* Regions. To determine the linkage relationships of the *LEU2*$^+$ regions in the transformants two sets of crosses were performed. (*i*) The transformants were crossed by a *leu2*$^-$ strain (MC333) carrying the centromere-linked *trp1* allele. The diploids were heterozygous for *his4*, *leu2*, and *trp1*, permitting the detection of linkage between *his4* and *LEU2*$^+$ as well as between *LEU2*$^+$ and its centromere. (*ii*) The transformants were crossed by a *LEU2*$^+$ strain (S288C). This cross allowed us to determine whether the *leu2*$^-$ region of the recipient was still present in the transformed strains. The results of these crosses (Tables 1 and 2) again define three classes of transformants congruent with the classes defined by hybridization studies. Each of these three types is described below along with hybridization and genetic mapping data obtained from representative transformants.

Classification of Transformants. Of the more than 100 transformants obtained, 42 have been subjected to both genetic analysis and colony hybridization with ColE1. Representatives of each of the three types were also subjected to restriction enzyme analysis (Fig. 1) and more detailed genetic mapping.

Recipient strain. The recipient strain AH22 showed a single

Table 1. Linkage of *LEU2*$^+$ to *his4* and *trp1**

	leu2–his4			*leu2–trp1*		
	PD	NPD	TT	PD	NPD	TT
Expected	50	<1	50	45	45	10
Observed:						
AH-102 × MC-333	18	0	22	17	22	10
AH-103 × MC-333	17	1	15	17	16	6
AH-104 × MC-333	31	0	22	27	25	6
AH-105 × MC-333	2	6	31	10	7	27

These data represent the results of crosses between the transformants (*LEU2*$^+$ *his4*$^-$ *TRP1*$^+$ *MET8*$^+$) by strain MC333 (*leu2*$^-$ *HIS4*$^+$ *trp1*$^-$ *met8*$^-$). In nontransformed strains, *his4* and *leu2* give a ratio parental ditype (PD)/nonparental ditype (NPD)/tetratype (TT) of 1:0.01:0.7, indicating 20% linkage. Thus, in the first three crosses above, *LEU2*$^+$ is linked to *his4*. If PD:NPD:TT is approximately 1:1:4, then two markers are considered unlinked. In the last cross, *his4* and the *LEU2*$^+$ region are unlinked. If the ratio PD:NPD:TT is 1:1:<4, two markers are considered linked to their centromeres. *leu2* and *trp1* in untransformed strains show centromere linkage. In the first three crosses the new *LEU2*$^+$ region shows centromere linkage, whereas in the AH-105 × MC333 cross it does not.

* Numbers represent both complete asci and three-spored asci that could be scored unambiguously.

Table 2. Segregation of *LEU2*$^+$ in transformants × wild type (S288C) crosses

	Leu$^+$:Leu$^-$		
	4:0	3:1	2:2
Expected:			
New Leu$^+$ at *leu2*	100	0	0
New Leu$^+$ unlinked to *leu2*	17	66	17
Observed:			
AH-102 × S288C	41	0	0
AH-103 × S288C	38	2	0
AH-104 × S288C	42	2	0
AH-105 × S288C	4	19	6

All strains used in these crosses are phenotypically Leu$^+$. The appearance of Leu$^-$ segregants therefore means that the original *leu2* region of the recipient is still present in the transformed strain. The frequency of Leu$^-$ segregants (3:1 and 2:2 asci) is a measure of the linkage between the old *leu2*$^-$ region and the new *LEU2*$^+$ region introduced by transformation. In the last three crosses, the *leu2*$^-$ region was clearly present. In the second and third crosses, *LEU2*$^+$ and *leu2*$^-$ were closely linked, whereas in the last cross they segregated independently.

*Hin*dIII fragment capable of hybridization with pY*eleu*10 (Fig. 1 *left*). No hybridization with ColE1 could be found (Fig. 1 *right*). Crosses with strain AH22 showed the expected linkage relationships for *his4* and *leu2*.

Type I (duplication of leu2 *region of chromosome III).* The most frequent class of transformation event (30/42) appeared to involve the integration of the complete pY*eleu*10 plasmid into the region of chromosome III homologous with the yeast-derived portion of the plasmid and adjacent to the *leu2* gene. AH103 and AH104 are representatives of such a type I event. In type I transformants, ColE1 DNA was present and the *LEU2*$^+$ allele was closely linked to the original *leu2*$^-$ allele (Tables 1 and 2). Restriction analysis of the DNA from type 1 transformants AH103 and AH104 showed a single *Hin*dIII fragment that hybridized to both pY*eleu*10 and ColE1 DNA. The size of this fragment is consistent with that expected if the pY*eleu*10 plasmid had integrated into the original *Hin*dIII fragment. Fig. 2 illustrates the integration of pY*eleu*10 into the yeast genome near the *leu2* locus by a Campbell-like recombination event (15). Fig. 2 also contains a schematic representation of the hybridization pattern predicted by such a sequence arrangement.

Genetic tests confirmed our interpretation of the hybridization data. Type 1 transformants gave meiotic segregation patterns indicating that the newly introduced *LEU2*$^+$ region was adjacent to, but did not replace, the resident *leu2*$^-$ region. In crosses of the *LEU2*$^+$ transformants by standard *LEU2*$^+$ strains (Table 2) 1 in 20 tetrads showed a *leu2*$^-$ spore. Each Leu$^-$ spore was tested and shown to carry the *leu2-3leu2-112* double mutation present in the original recipient. We interpret the *leu2-3leu2-112* meiotic segregants of these strains to result from recombination events that separate the *LEU2*$^+$ region from the *leu2-3leu2-112* region. Thus, both a *LEU2*$^+$ and a *leu2*$^-$ region are present in chromosome III in type I transformants (Fig. 2).

The ColE1 sequences in transformant AH103 have been shown to be tightly linked to the *LEU2*$^+$ allele by colony hybridization analysis of the meiotic tetrads (unpublished data).

*Type II (*pY*eleu10 integrated into other chromosomes).* AH105 is representative of another class of transformants which contains the bacterial ColE1 sequences; 5 of 42 transformants were of this type. Fig. 1 *left* shows that AH105 has two *Hin*dIII fragments that hybridize with pY*eleu*10. We identified the lower band as the "old" *leu2*$^-$ region of the host genome and the higher band as the "new" region resulting from transformation. This conclusion is based on several facts. First, the lower band showed the same molecular weight as the single band in AH22. Second, the upper band hybridized with ColE1 as well as with pY*eleu*10. Finally, crosses of AH105 indicate that the *LEU2*$^+$ region in this strain is unlinked to the *leu2* region on chromosome III (Table 2). Moreover, this *LEU2*$^+$ region is unlinked to *his4* and the centromere of this chromosome (Table 1). These results can be explained by insertion of pY*eleu*10 into other yeast chromosomes as depicted in Fig. 2.

Type III (no bacterial sequences). Transformant AH102 showed the same gel hybridization pattern with ColE1 and pY*eleu*10 as did the original recipient AH22 (Fig. 1). This transformant, along with six others that failed to show colony hybridization with ColE1, acts genetically as if only a single *leu2* region were present. Transformants of this type showed the standard linkage relationships for *leu2* on chromosome III and, so far, have yielded no Leu$^-$ segregants in crosses with wild type (Table 2).

Several explanations are compatible with these facts. First, AH102 could result from a transformation event in which a double crossover occurred as shown in Fig. 2. Second, this type could arise by excision of the *leu2*$^-$ portion of a type I tandem duplication. Of course, there is no way to distinguish this type from a revertant to *LEU2*$^+$. However, we have never observed revertants in our controls without DNA.

Stability of the Leu$^+$ Transformants. Transformants containing the duplicated *leu2* region (AH103, AH104) were extremely unstable during mitotic growth; 1–2% of the vegetative haploid cells segregated Leu$^-$ clones. These are stable Leu$^-$ strains that have lost the ColE1 sequences but retained both *leu2*$^-$ alleles present in the original recipient. The mechanism responsible for the production of Leu$^-$ segregants is unknown. However, it is possible that the *LEU2*$^+$ region is excised by a reversal of the mechanism by which it became inserted (Fig. 2). No Leu$^-$ segregants have been observed in type II and III transformants.

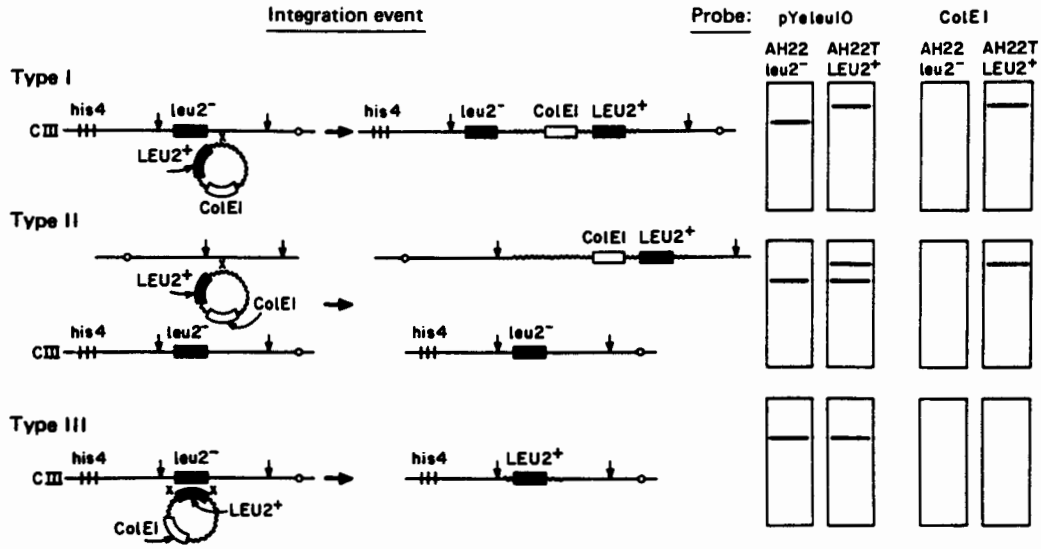

FIG. 2. Schematic interpretation of the integration events proposed for transformant types I, II, and III. Each type of integration event (*Left*) gives rise to a unique chromosome structure (*Center*) that can be visualized by hybridization of pY*eleu*10 and ColE1 DNA to *Hin*dIII restriction digests (*Right*). The arrows (↓) represent *Hin*dIII restriction sites. Type I: integration of plasmid pY*eleu*10 into chromosome III at a sequence complementary to a yeast sequence carried by the plasmid. Type II: integration of plasmid pY*eleu*10 into a chromosomal location genetically unlinked to the *leu2* region of chromosome III. Type III: integration of yeast DNA sequences of plasmid pY*eleu*10 into the *leu2* region by a double crossover event. These different integration events lead to predictable patterns when *Hin*dIII restriction digests of these strains are hybridized with pY*eleu*10 or ColE1 DNA. These hypothetical hybridization patterns are in agreement with the actual patterns shown in Fig. 1.

DISCUSSION

Direct proof that transformation has occurred in yeast comes from our hybridization studies that show that the bacterial ColE1 sequences from the pY*eleu*10 plasmid are present in the DNA of the transformed yeast strains. In a parallel fashion, genetic analysis confirms that the *LEU2*$^+$ gene from the DNA of the plasmid has integrated in several different places in the yeast genome. The hybridization results predict a genetic behavior of the newly inherited *LEU2*$^+$ sequences that is fully realized when the transformants are analyzed by standard genetic analysis. For example, the presence of a single large *Hin*dIII fragment in type I transformants capable of hybridization with pY*eleu*10 predicts insertion of pY*eleu*10 into the original *leu2*-containing *Hin*dIII fragment. In agreement with this prediction, genetic analysis shows that the newly obtained *LEU2*$^+$ region is closely linked to the old *leu2*$^-$ region of the recipient. Furthermore, the two *leu2* regions in the transformants can be separated by a rare meiotic recombination event. In the type II transformants the presence of two *Hin*dIII fragments capable of hybridization with pY*eleu*10 predicts two separable *leu2* regions. In agreement with this prediction, genetic analysis shows that the *LEU2*$^+$ region of these transformants is unlinked to the normal *leu2* region on chromosome III. In fact, the *leu2*$^-$ region of the recipient and the *LEU2*$^+$ region show independent assortment. The genetic results reinforce the hybridization data and lend an additional dimension to our studies: The pY*eleu*10 plasmid not only transforms yeast to Leu$^+$ but also can integrate into the yeast chromosomes at several locations.

Many questions remain concerning transformation in yeast. The mechanism of insertion of the pY*eleu*10 plasmid into yeast DNA is currently unknown. We have illustrated three types of integration events in Fig. 2. Type I and type II probably arise from Campbell-like recombination events which involve a circular transforming plasmid. This type of insertion would result in the integration of the ColE1 sequences together with the *LEU2*$^+$ sequences as was found for types I and II. If pY*eleu*10 can integrate by this mechanism, then yeast must contain enzymes analogous to the λ *int* system (16). The type III transformants have no sequences that hybridize to ColE1 and could result from the event shown in Fig. 2. Alternatively, the integration could result from an intrachromosomal crossover subsequent to a type I event. Because the *leu2* region is duplicated in type I transformants, an intrachromosomal crossover could "pop out" the *leu2*$^-$ region together with the ColE1 sequences.

The fact that most of the transformation events occur at or near *leu2* suggests that homology plays a role in the insertion event. The segment of yeast DNA in pY*eleu*10 is likely to carry several genes in addition to *LEU2*$^+$ and our evidence suggests that the insertion takes place within one of these genes adjacent to *leu2*$^-$ on chromosome III. Genetic analysis of the type II transformants shows that the pY*eleu*10 can also insert in at least three other locations. Thus, if the recombination event involves homology, pY*eleu*10 must carry a sequence that is repeated at several places on the yeast genome. Integration is not a unique property of the pY*eleu*10 plasmid, however. We have also obtained transformation using a plasmid carrying the yeast *his3* gene (pY*ehis1*) (4). These results will be presented elsewhere.

Transformation of yeast makes possible the cloning of eukaryotic genes in a eukaryotic host with a sophisticated genetic system. Baker's yeast has several advantages over enteric bacteria as a host for pharmacologically important genes such as insulin. It is not a pathogen under any known circumstances and, because it is a eukaryote, it probably will allow more efficient expression of such eukaryotic genes. Maximal expression would be especially desirable for commercial applications. Furthermore, transformation permits several novel approaches to the cloning of yeast genes. The association of bacterial sequences, such as ColE1, with yeast genes *in vitro* provides a specific probe for those genes. This bacterial sequence, juxtaposed with a gene of interest, would permit the identification

and isolation of that gene. This property could be exploited by transformation back and forth between yeast and *E. coli* as described below.

Many yeast genes of interest fail to function when cloned in *E. coli*. However, it is likely that these genes could be expressed upon transformation back into yeast. The existing banks of yeast–*E. coli* hybrid plasmids could be used to transform yeast auxotrophs. Presumably, the bacterial sequences could often integrate along with the yeast genes of interest (as in type I and type II transformants, Fig. 2) and the position of these sequences could be verified by tetrad analysis. The bacterial plasmid DNA along with the adjacent yeast gene could then be isolated by standard cloning techniques using the original bacterial vector as a hybridization probe. Alternatively, the yeast sequences could be obtained by transforming *E. coli* with a restriction digest of DNA from the yeast transformant. In such a digest, one fragment would contain the original bacterial vector along with the desired yeast sequences. Selection for the bacterial functions carried by the vector (drug or colicin resistance) after transformation into *E. coli* would permit the isolation of this fragment from all other yeast DNA. Thus, the bacterial sequences would provide the vehicle for cloning those yeast sequences that had been adjacent to it on the yeast chromosome.

The successful transformation of yeast by bacterial plasmid DNA raises the possibility that genetic exchange can take place between widely divergent species (17). The behavior of the bacterial plasmid DNA sequences once integrated into yeast chromosomes demonstrates that prokaryotic DNA can be maintained and transmitted by eukaryotes. Genetic exchange by interspecific transformation could lead to the acquisition of blocks of new genes that could contribute to the genetic diversity of the species.

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