UCSF
UC San Francisco Electronic Theses and Dissertations

Title
Transformation of yeast with an autonomous plasmid carrying the recessive lethal suppressor SUP61

Permalink
https://escholarship.org/uc/item/60t195mm

Author
Bunin, Greta Renee

Publication Date
1982

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

Transformation of Yeast with an Autonomous Plasmid Carrying the Recessive Lethal Suppressor SUP61

by

Greta Renee Bunin

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

TRANSFORMATION OF YEAST WITH AN AUTONOMOUS PLASMID CARRYING THE RECESSIVE LETHAL SUPPRESSOR SUP61

A THESIS

Presented to the Faculty of the Graduate School

of the University of California, San Francisco

in Partial Fulfillment for the Degree of

Master of Science

by

Greta Renee Bunin

January, 1982

ABSTRACT

TRANSFORMATION OF YEAST WITH AN AUTONOMOUS PLASMID CARRYING THE RECESSIVE LETHAL SUPPRESSOR SUP61

Greta Renee Bunin, M.S.

University of California, San Francisco 1982

Mutations within tRNA genes which reverse the effects of chain termination alleles are known as nonsense suppressors. One such nonsense suppressor in Saccharomyces cerevisiae (SUP61) can only be maintained in diploid (heterozygous) strains; the gene has a lethal phenotype in haploids. Although suppression itself does not cause the lethality of SUP61, work with other nonsense suppressors suggested that oversuppression can be deleterious. To increase the efficiency of suppression by SUP61, we wanted to introduce the suppressor gene into a haploid on an autonomous plasmid. Since SUP61 was not available as a cloned fragment, the suppressor allele was recovered from yeast by the method of Broach et al. (1981). After integration of a sup^+-containing plasmid at the SUP61 locus, appropriate digestion of DNA from the transformant yielded the SUP61-containing plasmid, pBH6. An origin-containing, 2μ fragment was inserted into the plasmid. Two control plasmids - pTE33-2μ, containing the wild-type sup gene, and YIp5-2μ, containing the selectable marker $URA3^+$ but no tRNA gene - were constructed.

The transformation efficiency with pBH6-2μ did not differ appreciably from that with YIp5-2μ, suggesting that an unaltered SUP61 gene on an autonomous plasmid resulted in viable transformants. However, the expression of SUP61 in these transformants, as measured by phenotypic suppression, was reduced below that of one integrated chromosomal copy of the suppressor. The stability of pBH6-2μ was reduced relative to that of YIp5-2μ, suggesting a deleterious effect of suppression. Interestingly, pTE33-2μ was also less stable than YIp5-2μ, suggesting a deleterious effect of the wild-type tRNA gene on an autonomous plasmid. No reproducible effect on growth rate of either sup-containing plasmid was observed.

The reduced expression and stability of pBH6-2μ present an apparent paradox. That is, at a reduced level of expression, SUP61 exhibits instability suggestive of a deleterious effect of the gene. No instability or other suggestion of a deleterious effect is observed in SUP61-containing diploids which suppress more efficiently than the pBH6-2μ transformants. Speculation on the relationship between SUP61 expression and stability, and on the possibly deleterious effects of extra copies of the wild-type tRNA gene, is advanced.

ACKNOWLEDGMENTS

To Christine Guthrie, I am extremely grateful for her encouragement throughout this project and especially for her patient, thorough and timely help in the preparation of this manuscript. Tina Etcheverry introduced me to the intricacies of yeast genetics and techniques, helped me innumerable times in the lab, and provided a wonderful example of thoughtful, calm research. Without Tina and Christine, the project would never have been completed.

I'd also like to thank the other members of the Guthrie lab for their help and support. I extend a special thanks to Robert Bishop for his help in numerous parts of this work and for his generosity with his time and his plasmids.

Sue Adams and Janice Babula helped make this thesis possible by working out the administrative details of my somewhat unusual status. I appreciate the encouragement of Reg Kelly to pursue the project and the help of Elizabeth Lundburg in the completion of the manuscript.

Finally, I'd like to thank my friends and family for their encouragement, sympathy, and assurances that one day soon I'd think it was worth it.

TABLE OF CONTENTS

Page

Abstract..... i

Acknowledgments.....iii

Table of Contents.....iv

List of Figures.....v

List of Tables.....vi

List of Abbreviations.....vii

INTRODUCTION.....2

MATERIALS AND METHODS.....8

RESULTS

I. Plasmid Construction.....21

II. Transformations.....30

III. Stability Experiments.....33

IV. Growth Rates.....40

DISCUSSION.....48

REFERENCES.....59

LIST OF FIGURES

Figure		Page
1.	Structure of the Plasmid Vectors.	22
2.	Restriction Analysis of the Constructed Plasmid pTE33-2μ.	25
3.	Schematic Representation of the Two Possible Integration Events.	26
4.	Restriction Analysis of the Constructed Plasmid pBH6-2μ.	29
5.	Stability Curves of pBH6-2μ, pTE33-2μ, and YIp5-2μ Transformants in Non-selective Medium.	35-37
6.	Growth Curves of pBH6-2μ, pTE33-2μ, and YIp5-2μ Transformants in Selective and Non-selective Media.	42-44

LIST OF TABLES

Table		Page
1.	Genotypes and Phenotypes of Yeast Strains	10
2.	Generation Times and Proportion of Ura^+ Colonies During Exponential Growth	45

LIST OF ABBREVIATIONS

amp^r	ampicillin resistance
DNase	deoxyribonuclease
kb	kilobase pairs
OD	optical density
RNase A	pancreatic ribonuclease A
RNase T1	ribonuclease T1
SDS	sodium dodecyl sulfate
SSPE	0.18M NaCl, 10mM NaH_2PO_4, 1mM EDTA, pH 7.4

INTRODUCTION

A nonsense suppressor is derived by a mutation within a tRNA gene that reverses the effect of a chain termination codon within another gene. Without the suppressor, the cell cannot make the product coded for by the gene containing a termination codon; the suppressor tRNA inserts an amino acid and results in a full-length product. A tRNA can mutate to a suppressor only if the normal function(s) performed by the tRNA is (are) dispensable. This condition is presumably met when the genome contains other copies of that tRNA gene (or a gene for another tRNA species that can decode the same base triplet by wobble reading).

To overcome the limitation that only non-essential tRNA genes can give rise to suppressors, Soll and Berg (1969) and Miller and Roth (1971) isolated suppressors in merodiploids of *E. coli* and *S. typhimurium*, respectively. They isolated suppressors that were viable only in cells that also contained the wild-type allele. Such mutations are called recessive lethal suppressors. In *E. coli* the mutation was found to be a single base change in the anticodon of the $tRNA^{Trp}$ gene, allowing the tRNA to read UAG rather than the UGG triplets read by the wild-type allele (Soll, 1974). Since the *E. coli* genome contains only a single $tRNA^{Trp}$ gene (Hirsh, 1971), the mutation left the cell without the ability to read UGG, a lethal loss.

Recessive lethal suppressors have been isolated in yeast as well; i.e. these suppressors cannot be derived from or maintained in haploid strains. Two of these suppressors, *SUP61* and *SUP-RL1*, arose from the gene which codes for $tRNA^{Ser}_4$ (Piper, 1978; Etcheverry, 1980), *SUP61* from a secondary mutation in the *SUP-RL1* allele. The two suppressors are

readily distinguishable; SUP-RL1, an amber suppressor, reads UAG condons while SUP61, an ochre suppressor, reads UAA triplets.

The work of Etcheverry et al. (Etcheverry, 1980; Etcheverry et al. 1981) elucidated the cause of SUP61 lethality by distinguishing between two models. The first model attributes the recessive lethality to efficient suppression of natural termination codons. According to the second model, the wild-type gene from which SUP61 and SUP-RL1 are derived is indispensable to the cell; loss of that essential gene by mutation to a suppressor is thus a lethal event in haploid strains.

Etcheverry et al. demonstrated that excessive suppression cannot account for the lethality of SUP61 (nor presumably that of SUP-RL1, the amber-specific allele of the same gene), since second mutations within the $tRNA^{Ser}_4$ gene that eliminated all detectable suppressor activity did not eliminate the recessive lethal phenotype. Etcheverry et al. then postulated that the SUP61 mutation leads to the loss of an essential function and that this function is the ability to decode one of the six (UCN, AGU/C) serine codons, UCG.

If the lost function is UCG decoding, two predictions can be made: only one $tRNA^{Ser}_4$ gene exists, and the UCA decoding $tRNA^{Ser}_3$ cannot read UCG because wobble reading is restricted. $tRNA^{Ser}_3$ gene number has been inferred from the correlation of Southern hybridization data, DNA sequence analysis, and the relative amounts of $tRNA^{Ser}_3$ and $tRNA^{Ser}_4$ RNA. When a mixed population of $tRNA^{Ser}$ (species 3 and 4) is used as a probe for Southern hybridizations only four bands in the entire yeast genome hybridize to the tRNAs (Olson et al., 1981). This can be interpreted to mean that there are four genes in yeast which produce the mixed $tRNA^{Ser}$ population. When combined with the finding that the $tRNA^{Ser}_3$ species is

present in about three times the amount of $tRNA^{Ser}_4$ (Etcheverry, 1980), it seems likely that there are three genes for $tRNA^{Ser}_3$ and only one gene for $tRNA^{Ser}_4$. The cloning and sequencing of the four genes support the conclusion. The three $tRNA^{Ser}_3$ genes have the anticodon (5')TGA(3') (Broach et al., 1981; Olson et al, 1981) while the $tRNA^{Ser}_4$ gene has the anticodon (5')CGA(3') (UCG-decoding) (Olson et al., 1981).

Although there appears to be only one gene for a UCG reading tRNA, one would expect the products of the $tRNA^{Ser}_3$ genes to have UCG decoding ability because of wobble reading by the UCA reading tRNA. Etcheverry et al., (1979), however, found a modified U in the anticodon of $tRNA^{Ser}_3$ and postulated that the modified base prevents U:G wobble reading and would therefore prevent decoding of UCG. Work on other yeast tRNAs supports such a model (see Guthrie and Abelson, 1982). A modified U in the wobble position of a tRNA has been shown in one case (Yoshida et al., 1970; Sekiya et al., 1969) and postulated in another (Kuntzel et al., 1975) to recognize the codon with an A in the wobble position but not the same codon with a G in that position. The data on gene number and base modification in the anticodon are thus consistent with the hypothesis that the essential function of $tRNA^{Ser}_4$ is UCG reading.

As a final test of the model, a SUP61-containing diploid was transformed with the cloned wild-type $tRNA^{Ser}_4$ (sup$^+$) gene. All four spores were viable upon dissection of the sporulated transformed diploid. These data, together with Southern hybridization analysis, demonstrated that the cloned DNA had integrated in tandem to the SUP61 gene. This result supports two aspects of the second model. First, presence of this efficient suppressor per se is not lethal in haploid strains; i.e. transformation with cloned wild-type DNA "cured" the

lethal phenotype in the presence of suppression. The second part of the model proposes that the essential function lost upon mutation of the gene to a suppressor is the ability to decode UCG. In the transformation experiment, the cloned DNA that cured the lethality of SUP61 contained the $tRNA^{Ser}_4$ gene which can decode UCG. Again, the data suggest but not prove that the essential function of the $tRNA^{Ser}_4$ gene is UCG reading. The possibility that the $tRNA^{Ser}_4$ gene serves a function other than, or in addition to, UCG decoding has not been excluded.

While these data demonstrate that the presence of one SUP61 gene is not a lethal event, work with other suppressors nevertheless suggested that this high efficiency suppressor might be deleterious under certain circumstances. Yeast strains with two tyrosine-inserting suppressors grow poorly or not at all (Gilmore, 1967), though strains with one such suppressor grow normally. Loss of an essential function cannot explain this result, since S. cerevisiae has eight $tRNA^{Tyr}$ genes. The most plausible explanation is that two or more copies of the suppressor gene lead to a harmful level of suppression of natural termination codons (Gilmore and Mortimer, 1966; Mortimer and Gilmore, 1968). Other evidence that too much suppression can be deleterious or lethal comes from the study of yeast strains with ochre suppressors and the non-Mendelian factor psi [Ψ^+] (Cox, 1971; Liebman et al., 1975). [Ψ^+] acts to increase the efficiency of ochre suppression; for example, SUQ5, a weak serine-inserting ochre suppressor, is detectable phenotypically only in [Ψ^+] strains. Moreover, a single tyrosine-inserting ochre suppressor is lethal in [Ψ^+] strains.

Since increased copy number would likely increase the efficiency of suppression, we introduced multiple copies of SUP61 into a cell to study

the effect of increased suppression. The SUP61 gene copies were introduced by transformation with an autonomously replicating plasmid. Transformation with the SUP61-containing plasmid that generated no viable transformants with suppressor activity would be suggestive evidence of the lethality of oversuppression by SUP61. Viable transformants that contained a functional suppressor, on the other hand, would suggest that efficient suppression is not necessarily lethal. In this case, the suppressor might have sub-lethal effects that could be observed by studying growth rate or plasmid stability.

To carry out the experiment, we first had to construct an autonomously replicating plasmid which contained the SUP61 gene. Construction of such a plasmid was not trivial since the SUP61 gene had not been cloned. Broach et al. (1981), however, had provided a strategy for cloning mutant alleles. If a plasmid containing the wild-type allele is integrated at the mutant locus, a plasmid containing the mutant allele can be isolated by appropriate digestion of the genomic DNA from the transformed strain. We also wanted to select transformants by a means not related to SUP61 activity in order to permit comparison with transformation by a sup^+-containing plasmid. Therefore, the vector had to include a selectable marker and control plasmids - one containing the sup^+ gene and one without any tRNA gene at all - had to be constructed. Finally, a fragment conferring autonomous replication had to be inserted into all three plasmids.

We found viable transformants with SUP61 activity, corroborating Etcheverry's conclusion that the lethality of SUP61 is not due to the presence of the suppressor *per se*. The frequency at which the SUP61 transformants arose and the rates at which they grew were similar to the

same parameters of control transformants. Interestingly, the efficiency of suppression in the SUP61 transformants was apparently reduced in comparison with cells containing a single integrated copy of SUP61. In spite of apparently reduced expression, the transformants demonstrated high mitotic instability, suggesting deleterious effects of multiple copies of SUP61. We also present data on sup^+-containing transformants suggesting that an increase in the copy number of a wild-type tRNA can be harmful, and speculate on why this might be so.

MATERIALS AND METHODS

MEDIA

The yeast culture media used in this study have been described previously (Sherman, Fink and Lawrence, 1974). The media for bacteria have been described by Miller (1972). The recipes for the media and the general purposes are listed below.

Rich medium for yeast (YEP): 1% Difco Bacto yeast extract, 2% Difco Bacto peptone, 2% glucose, 2% Difco Bacto agar (which is omitted in liquid media). This is a complete medium and is used for the routine propagation of cultures, with the exception of the scoring of auxotrophic markers.

Minimal medium for yeast: 0.7% Difco yeast nitrogen base without amino acids, 2% glucose, 2% Bacto agar (omitted in liquid media). When required the following concentrations of supplements were added: adenine sulfate, 20mg/l; L-histidine-HCl, 20mg/l; L-leucine, 30mg/l; L-lysine-HCl, 30mg/l; L-methionine, 20 mg/l; L-phenylalanine, 50mg/l; L-tryptophan, 20mg/l; L-tyrosine, 30 mg/l; L-threonine, 200mg/l; uracil, 20mg/l; and L-canavanine sulfate, 10mg/l. Supplemented minimal media lacking individual constituents ("drop-out" plates) were used for the scoring of auxotrophic markers and the maintenance of several strains. The yeast transformants were grown in the absence of uracil in order to maintain the transformed (Ura^+) phenotype.

L broth for bacteria: 1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl, 0.1% glucose, 1.3% Bacto agar (omitted for liquid media). This is a complete medium and is used to culture the bacterial strains in most cases. When this media is used to test drug resistance, it was

supplemented with ampicillin (Wyeth) 120mg/l. Ampicillin containing plates were stored at room temperature and used within three weeks. Bacterial strains which contain plasmids can spontaneously lose the plasmid DNA. Therefore the transformed bacterial strains were usually grown in the presence of ampicillin to force the maintenance of the plasmid.

Supplemented M9 medium for bacteria: M9 salts as described by Miller (1972) were supplemented with 0.2% glucose, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 1mg/l B_1, 100mg/l tryptophan, 50mg/l uracil and 120mg/l ampicillin. Supplemented M9 medium was used to grow bacterial cells for the amplification and isolation of large amounts of plasmid DNA. M9 media which lacked uracil was used to select for the presence of the yeast URA3 gene following transformation of bacteria with various plasmids.

YEAST STRAINS

The heterothallic strains of Saccharomyces cerevisiae used in this work are listed in Table I. The symbols and nomenclature are those proposed at the IVth and Vth International Yeast Genetics Conference (von Borstel, 1969). SUP is the dominant suppressor mutation and sup^+ refers to the wild-type (suppressor-less) condition. The ochre suppressible auxotrophic markers ade2-1, leu2-1, his4-1176, and can1-100 were employed in scoring for the presence of the suppressor gene in the cells.

BACTERIAL STRAINS AND PLASMIDS

The bacterial strain which was used as a recipient for transformation events is a derivative of E. coli K12. The phenotype of

TABLE I. GENOTYPES AND PHENOTYPES OF YEAST STRAINS

Name	Genotype	Phenotype
204	his4-1176 leu2-1 a sup^+ can1-100 ade2-1 trp1-1 ura3-52 aro7-1	His^- Leu^- Can^r Aro^- Ade^- Trp^- Ura^-
209	his4-1176 leu2-1 a SUP61 / his4-1176 leu2-1 α sup^+ can1-100 / can1-100 + / ade2-1 trp1-1 / + ura3-52 / ura3-52 lys1-1 / lys1-1	His^+ Leu^+ Lys^+ Can^s Ade^+ Ura^- Trp^+

OCHRE SUPPRESSIBLE ALLELES

leu2-1
lys1-1
can1-100
ade2-1
his4-1176 (serine compatible only)

ura3-52 is a partial deletion of the URA3 gene.

this strain (MB1000) is as follows: rK^- mK^+ lac^- trp^- $pyrF^-$ (D. Botstein, pers. comm.). The $pyrF^-$ locus is complemented by the $URA3^+$ gene from yeast (Struhl et al., 1979). This strain was provided by R. Davis.

The plasmids that were used are all derivatives of the E. coli vector pBR322 (Bovier et al., 1977). These plasmids are listed below.

Plasmid	Mol.Wt.	Phenotype	Additional Features
YIp5	5.4 kb	Amp_r^r, Ura_+^+	
pTE33	8.2 kb	Amp_r^r, Ura_+^+	contains the sup^+ allele of SUP61
pBH6	7.7 kb	Amp_r^r, Ura_+^+	contains the SUP61 allele
pRB1240	8.8 kb	Amp^r, His^+	contains a 3.7 kb 2μ fragment and the HIS3 gene
YIp5-2μ	9.1 kb	Amp_r^r, Ura_+^+	contains a 3.7 kb 2μ fragment
pTE33-2μ	11.9 kb	Amp^r, Ura^+	contains the sup^+ allele and a 3.7 kb 2μ fragment
pBH6-2μ	11.4 kb	Amp^r, Ura^+	contains the SUP61 allele and a 3.7 kb 2μ fragment

The construction of YIp5 has been described by Struhl et al., (1979). R. Bishop supplied pRB1240 and constructed YIp5-2μ. The construction of pTE33 was reported by Etcheverry (1980).

DNA PURIFICATION

Miniscreens of Transformants

In order to quickly screen transformants by gel electrophoretic analysis of the DNA, the plasmid DNA was purified by modification of a procedure (Meagher et al., 1977) that facilitates the simultaneous preparation of multiple samples. Overnight cultures were grown in 3ml of L-broth with 120µg/ml ampicillin. 1.0ml aliquots of cells were removed to store as glycerol stocks. The subsequent steps were all carried out in 1.5ml polypropylene tubes; centrifugations were performed at 4°C in the Eppendorf microfuge. The remaining cells were pelleted for 5 minutes and resuspended in 0.1ml 25% sucrose, 50mM Tris-HCl, pH8, at 4°C. Then 50µl of 200mM EDTA and 10µl of lysozyme at 10mg/ml (in 250mM Tris-HCl, pH 8) were added. Then 10µg/ml of heat-treated (10 minutes at 95°C) RNase A was added, followed by a 15 minute incubation on ice. With gentle mixing, 0.16ml of a 2X-Triton lytic mix (0.1% Triton, 62mM EDTA, 50mM Tris-HCl,pH8) was added. After incubation on ice for 5 to 10 minutes, the lysate was cleared by sedimentation for 30 minutes. The viscous pellet was removed with a Pasteur pipette. The supernatant was extracted with an equal volume of phenol two times. The aqueous phase was recovered and extracted two or three times with ether. The DNA was precipitated in 0.35M LiOAc, 50mM $MgAc_2$, by the addition of two volumes of ethanol and kept at -70°C for 1 hour. The brown gelatinous pellet was resuspended in 0.5ml of 10mM Tris-HCl, pH8, 1mM EDTA and reprecipitated with two volumes of ethanol. This should yield a white pellet, and should allow subsequent digestion of the DNA with restriction enzymes. There is sufficient material in one-tenth of each sample for analysis by gel electrophoresis.

Rapid Plasmid Toothpick Screen

This quick screen was used to determine the approximate size of plasmids contained in bacterial colonies. DNA from colonies identified this way was then isolated and studied further to determine the structure of the plasmid.

Colonies, two to three millimeters in diameter, were picked with flat toothpicks and suspended in 20µl of 10 mM Tris, pH8 at 4°C in 1.5ml polypropylene tubes. The suspended cells were frozen in either dry ice-ethanol or a -70°C freezer and then thawed rapidly at 37°C. The tubes were then placed in an ice-water bath at 0-4°C. To each tube was added 3µl of a solution made up of one part 0.25M EDTA, pH8; one part lysozyme, 5mg/ml in 0.25M Tris, pH8; and one part RNase A, 10mg/ml in 50mM NaOAc, pH5 which had been heated to 85°C for 10 minutes to inactivate any DNase.

After incubating for 30 minutes, 5µl of 5% SDS, 25mM EDTA pH8, 25% glycerol and 0.05% bromphenol blue was added. The samples were mixed and then heated at 65°C for 10 minutes. Each sample was vortexed vigorously for about 15 seconds to break the chromosomal DNA. The very viscous samples were loaded with a Hamilton syringe onto a 0.8% agarose Tris-acetate gel.

Plasmid DNA

For the purification of plasmid DNA, cells were grown in supplemented M9 with ampicillin to an OD_{420} of 0.4-0.5, at which time chloramphenicol (Sigma) was added to a final concentration of 150mg/l to amplify the plasmid DNA. The cultures were harvested approximately 20 hours after the chloramphenicol addition by pelleting for 10 minutes at 5,000 rpm in the Sorvall GSA or GS3 rotor. A method derived from one

described by Clewell and Helinski (1969) was used for the preparation of cleared lysates as follows. (The volumes refer to 1 liter cultures; 100ml cultures were processed in one-half volumes.) Cell pellets were resuspended in 15ml of cold Tris-sucrose (50mM Tris-HCl, pH8.0, 25% sucrose) and frozen at -20°C for at least 15 minutes. This step enhances the subsequent lysis of cells. Then 3ml of lysozyme (Sigma) at 5mg/ml in 25mM Tris-HCl, pH8, was added and the cells were incubated on ice for 5 minutes. 1.5ml of 250mM EDTA, pH8, was added and incubated for 15 minutes on ice. To lyse the cells, 15ml of cold 3X-Triton lytic mix (0.3% Triton X100, 186mM EDTA, 150mM Tris-HCl, pH8.0) was added and mixed gently. This was kept on ice for 15-30 minutes until the suspension became viscous but not clear.

The suspension was cleared by centrifugation for 30 minutes at 23,000 rpm in a Beckman type 30 rotor. The centrifuge temperature was controlled to 4°C. The supernatant was decanted into a 30ml glass centrifuge tube, being careful to avoid the extremely viscous pellet which contains bacterial chromosomal DNA, cells and cellular debris. The plasmid DNA in the supernatant is about 90% pure at this stage, but still contains proteins, chromosomal DNA fragments, RNA and other cellular components. To degrade the RNA, an equal volume of water and 5µl of DNase-free RNase (Worthington) at 10mg/ml in 50mM NaOAc, pH4.8 was added and incubated at room temperature for 10 minutes or longer.

The DNA was then extracted with an equal volume of phenol, buffered with 50mM Tris-HCl, pH7.5, 0.2M EDTA. The aqueous phase containing the DNA was then precipitated with two volumes of cold ethanol. The precipitated DNA (along with the Triton X100) was collected by centrifugation at 7,500 rpm for 30 minutes in the SS34 rotor. The white

precipitate was resuspended in 2-5ml of 50mM Tris-HCl, pH7.5, 0.5M NaCl. The DNA solution was then applied to an A50 agarose (BioRad) column (2 x 20cm), which had been equilibrated in the same buffer used for the resuspension of the DNA. The plasmid DNA (monitored by A_{260}) plus chromosomal DNA fragments eluted in the exclusion volume. Fragments of RNA, Triton X100, phenol and other debris were retarded by the agarose network. The DNA fractions were pooled, two volumes of ethanol were added and the DNA was collected by centrifugation. This DNA was then resuspended in 10mM Tris-HCl, pH7.4, 1mM EDTA at a concentration of 100µg/ml or greater.

Yeast DNA

Yeast DNA was isolated by the method of Cryer et al. (1975). One liter of yeast cells was harvested in late log phase of growth (OD_{600}=1.0) and washed with 30ml of 50mM EDTA (pH7.5). The cell pellet was suspended in 20ml of 50mM EDTA, pH9.0 plus 0.5ml of β-mercaptoethanol, and incubated for 15 minutes at room temperature. The treated cells were collected by centrifugation at 6,000 rpm and suspended in 1M sorbitol, 0.1M EDTA, pH7.5 at a ratio of 2ml/gram of cells. Zymolyase 60,000 (Kirin Brewery) in sorbitol-EDTA was added at a final concentration of 0.1-0.5mg enzyme per gram of cells. This was incubated at 37°C for 1-2 hours until spheroplasts were formed. Spheroplast formation could be visually checked by adding one drop of 10% SDS to a 0.2ml aliquot of culture and checking for clearing of the solution. An equal volume of 0.15mM NaCl, 0.1M EDTA, pH8.0 was added with 50-100µg/ml of Proteinase K (E.M. Laboratories). The spheroplasts

were then lysed by making the suspension 1% with SDS and incubating at 37°C for 3-4 hours.

The lysate was next heated to 60°C for 30 minutes. Lipids and proteins were removed by adding an equal volume of chloroform:isoamyl alcohol (24:1, v/v) and gently shaken for several minutes. The phases were separated by centrifugation at 10,000xg for 15 minutes, and the aqueous portion was removed to a 100ml beaker. Two volumes of 95% ethanol were layered over the aqueous phase and the DNA was collected by spooling with a 0.1ml glass pipette. Spooling was continued until the two phases were mixed. The DNA was resuspended in 0.1XSSC at approximately 2ml/gram of cells. The solution was adjusted to 1XSSC and heat-treated RNase A was added at a concentration of 50µg/ml. The DNA was incubated at 37°C for one hour. The deproteinization and ethanol precipitation steps were repeated. Then polysaccharides were removed by highspeed centrifugation in the Type 30 rotor at 29,000 rpm for 90 minutes. The optical density of the supernatant was determined. At this point the DNA was concentrated (if necessary) by ethanol-precipitation and stored at a concentration of 100-200µg/ml in 10mM Tris-HCl, pH7.5, 1mM EDTA.

RESTRICTION ENDONUCLEASE DIGESTIONS

The restriction enzymes used for these studies, along with their reaction conditions, are listed below. All restriction enzyme digestions were at 37°C, usually with approximately one unit of enzyme per µg DNA for at least 60 minutes. The enzyme was inactivated by heating at 65°C for 10 minutes prior to loading the DNA on a gel or ligation.

Enzyme	Source	Reaction Conditions
EcoR1	Pat Greene	0.1M NaCl, 50mM Tris-HCl, pH7.4, 10mM $MgCl_2$
HindIII	New England Biolabs/ Boehringer Mannheim	10mM Tris-HCl, pH7.6, 50mM NaCl, 10mM $MgCl_2$, 14mM DTT
BamH1	(same as above)	(same as above)

For digestions involving more than one enzyme, the HindIII buffer was used and both enzymes added at the start of the incubation period.

AGAROSE GEL ELECTROPHORESIS

Vertical gels (14 x 15 x 0.2cm) for analytical purposes containing 1% agarose (Seakem) were used for the separation of restriction enzyme treated fragments of DNA. Either of the following two buffers was used: Tris-acetate buffer, 40mM Tris, 20mM Na-acetate, 2mM EDTA, adjusted to pH8.1 with glacial acetic acid (Loening, 1967); Tris-borate buffer, 80mM Tris, 80mM boric acid, 1mM EDTA, pH8.3. The samples were loaded on the gel with an equal volume of 25% Ficoll, 0.05% bromphenol blue. Electrophoresis was conducted at room temperature at 30mAmps for the large gels and 100volts (15mAmps) for the analytical gels. The length of time of electrophoresis was varied depending on the size of the DNA.

DNA was visualized by staining the gel in 0.5µg/ml ethidium bromide in H_2O for about 15 minutes. Following a brief destaining in H_2O, gels were photographed over an ultraviolet light of wavelength 254nm, using a yellow filter (Kodak #9 Wratten gelatin filter) and Polaroid film (Type 55).

TRANSFORMATION OF BACTERIA

In preparation for bacterial transformation, restriction cut linear DNA molecules with cohesive ends were joined with T4 DNA ligase (New England Biolabs). This joins two or more DNA termini, converting the linear molecules to the closed circular form. The reaction conditions, which are a modification of those described by Modrich and Lehman (1970) were as follows: to a reaction mixture containing 10mM Tris-HCl, pH7.6, 10mM $MgCl_2$, 50mM NaCl, 10mM DTT, 1mM ATP and an equal mass of vector and insert DNAs, T4 DNA ligase was added at a ratio of 0.1units/µg DNA. DNA for the bimolecular reactions was normally at a concentration of 20µg/ml. The concentration of the linear DNA fragments can be varied to promote either polymerization or circularization. After incubation of the reaction mixture at 4°C for six to 24 hours, the extent of ligation of the reaction was checked by gel electrophoresis.

The procedure used for bacterial transformation is based on that described by Cohen et al. (1972), with modifications suggested by R. Bishop. The cells were made competent to take up DNA by treatment with $CaCl_2$ as follows. The cells were grown in L-broth to an optical density of 0.3-0.5 at 420nm. 30ml of cells were chilled quickly to 0°C followed by centrifugation at 4°C for 5 minutes at 7,000 rpm in the Sorvall SS34 rotor. (All subsequent steps were conducted at 0-4°C except as noted.) The pellet was resuspended in 15ml of cold 0.05M $CaCl_2$, in a volume approximately 0.1 times the original volume of cells. For transformation, 0.3ml of this cell suspension was combined with 50-75ng DNA and 0.2ml of transformation buffer (10mM Tris-HCl, pH8, 10mM $CaCl_2$, 10mM $MgCl_2$). Incubation for 25 minutes at 0°C was followed by a heat pulse at 42°C for 75 seconds to two minutes. The cells were allowed to

remain at 25°C for 10 minutes and then diluted with 1ml of L-broth. These cells were grown for 60 minutes at 37°C, diluted and plated on selective plates containing 120μg/ml ampicillin.

TRANSFORMATION OF YEAST

The procedure of Hinnen et al. (1978) was followed with modifications used by R. Bishop. Spheroplasts were prepared as described by Hutchinson and Hartwell (1967).

A logarithmic culture (100ml at OD_{650}=0.8) was harvested by centrifugation at 5000 rpm at 0°C for 5 minutes in a SS34 rotor. After the cells were resuspended in 5-10ml of water, water was added to fill the Oak Ridge tubes. The cells were centrifuged again, resuspended in a total of 10ml of 1M sorbitol with 1% Glusulase (Endo Laboratories), and incubated at 30°C for one hour or until spheroplasts had formed.

These spheroplasts were centrifuged at 3,000 rpm at 0°C for 5 minutes in a HB4 rotor. They were then washed three times with 1M sorbitol. The spheroplasts were centrifuged as above after each wash and gently resuspended. After the final wash, the spheroplasts were resuspended in 1-2 ml of 1M sorbitol, 10mM Tris-HCl, pH7.5, 10mM $CaCl_2$ (STC) and divided into 200μl aliquots.

Plasmid DNA was added to each aliquot to a final concentration of 10-20μg/ml and incubated for 5 minutes at room temperature. Then 2ml of 40% polyethylene glycol 4000 (Baker Chemical Co.) was added with gentle mixing. After 10 minutes at room temperature, the spheroplasts were collected by a 5 minute centrifugation at 3000 rpm at 0°C in a HB4 rotor and each aliquot resuspended in 0.8ml of STC. 0.2ml aliquots from each original aliquot of cells were added to 10ml of regeneration agar at

46°C and quickly poured onto uracil "drop-out" plates to select for cells with the plasmid containing the uracil gene. Regeneration agar is composed of Difco yeast nitrogen base without amino acids, supplemented with 1M sorbitol, 2% glucose, 2% YEP, and 3% agar (van Solingen and van Der Plaat, 1977).

BACTERIAL COLONY HYBRIDIZATIONS

Colony hybridization was performed as described by Grunstein and Hogness (1975). Cells were grown on nitrocellulose filters (Schleicher and Schuell) overlaid on L-broth plates overnight. The filters were then prepared for hybridization by sequentially treating with the following solutions: 500ml of 0.5N NaOH for 7 minutes; 150ml of 1.0M Tris-HCl, pH7.4 for 10 minutes; 150ml of 1.5M NaCl, 0.5M Tris-HCl, pH7.4 for 5 minutes; 150ml of 2XSSPE, 0.2M Tris-HCl, pH7.4 for 2 minutes. The filters were air dried and baked for 2 hours at 80°C *in vacuo*.

For the hybridization, a solution containing at least $1x10^5$ cpm of ^{32}P-labelled tRNA in 5XSSPE, 0.5% SDS, 50% formamide was applied to the DNA-containing filters. After an incubation of 12-14 hours at 40°C, the filters were washed 3 times in 2XSSPE, 0.5% SDS at 42°C, air dried, and exposed by autoradiography.

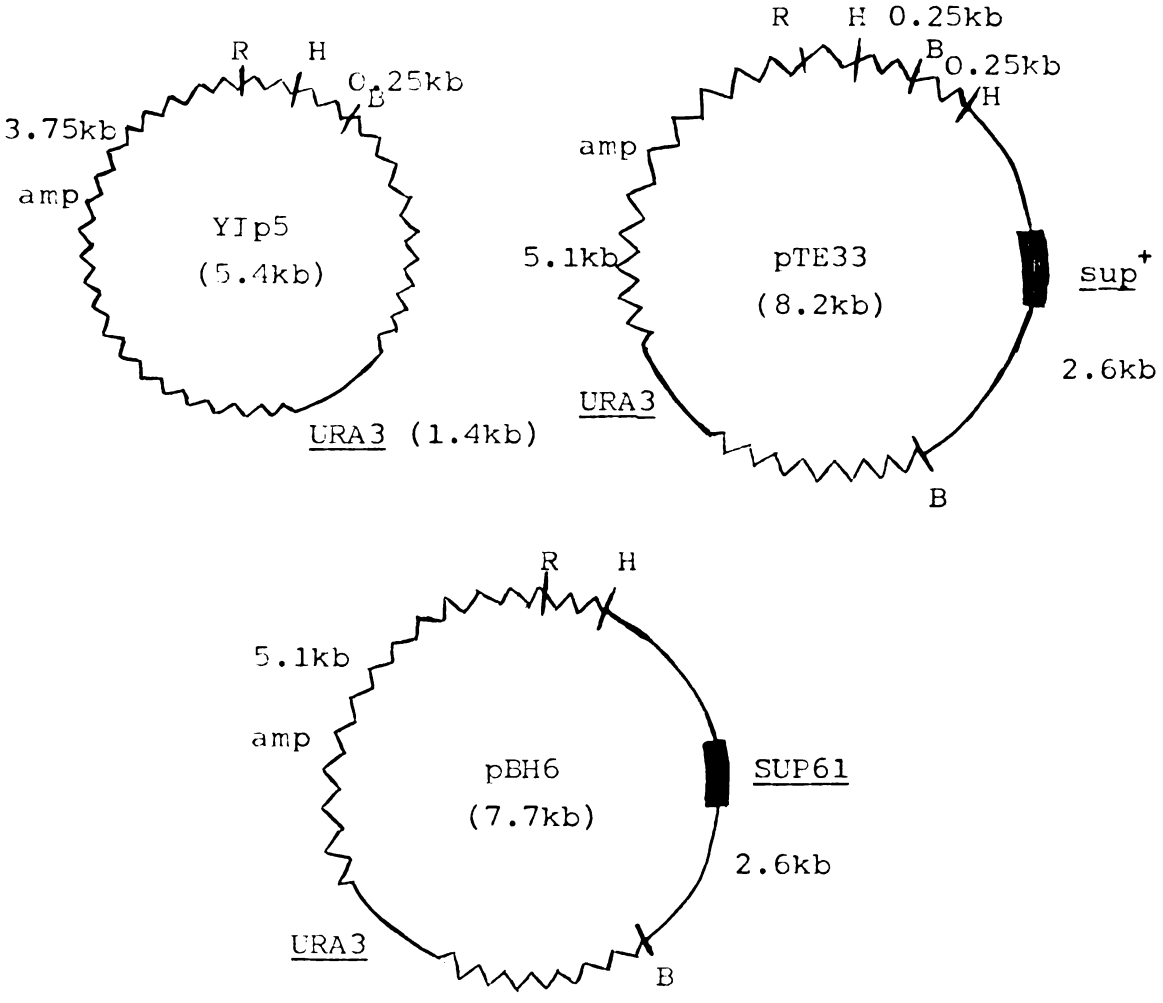
RESULTS

I. Plasmid construction

We chose to study possible effects of oversuppression on stability and growth rate by introducing the *SUP61* gene on a plasmid into an appropriate yeast strain. The experiment required two characteristics of the plasmid. First, since multiple copies of the gene would be likely to increase the efficiency of suppression, we needed a plasmid which could replicate autonomously and could therefore be maintained in the cell in high copy number. A 3.7 kb fragment of the circular yeast plasmid called 2μ DNA was used to confer autonomous replication. Cells transformed with plasmids containing this fragment contain autonomously replicating copies of the vector, presumably as a result of the presence of a yeast replicon. The second characteristic required of the plasmid was that it contain a selectable marker not requiring expression of the suppressor gene. If efficient suppression were lethal, only transformants whose plasmids contained mutated or otherwise inactivated *SUP61* genes would be viable. Such transformants would not be recovered by selection for the ability to suppress genes containing termination codons. The *URA3* allele was included on the plasmid and used to select transformants of a yeast strain with a *ura3*$^-$ deletion.

Two other plasmids were needed as controls. By replacing the *SUP61* with the *sup*$^+$ allele, we could compare the effects of the suppressor and the wild-type tRNA on a high copy number plasmid. A plasmid identical to the other two but containing no tRNA gene was used to control for effects due to the presence of the plasmid *per se*.

FIGURE 1 Structures of the Plasmid Vectors



DNA sequences are diagrammed as follows:
wavy line, E. coli plasmid pBR322
solid line, yeast chromosomal DNA
solid bar, approximate position of the sup gene
Restriction endonuclease sites are designated:
R, EcoR1
B, BamH1
H, HindIII

A yeast vector, YIp5, constructed by K. Struhl (1979) was used. Its structure is shown in Figure 1. YIp5 replicates in *E. coli* and confers ampicillin resistance. It also contains the structural gene for *URA3* which complements both *ura3*$^-$ yeast and *pyrF*$^-$ *E. coli*. The vector had to be altered for the purpose of this study so that it contained a tRNA gene and replicated autonomously in yeast.

The control plasmid containing no *sup* allele, YIp5-2μ, was constructed by R. Bishop by insertion of the 2μ fragment into YIp5.

The *sup*$^+$-containing autonomously replicating plasmid was constructed from pTE33, whose structure is also shown in Figure 1. pTE33 resulted from the insertion of a 2.6 kb *sup*$^+$-containing fragment into the YIp5 vector. In addition, pTE33 contains an extra copy of a small 0.25 kb, *Bam*-*Hin*dIII fragment of YIp5; the two small fragments are present as an inverted repeat near the *Eco*R1 site of pTE33.

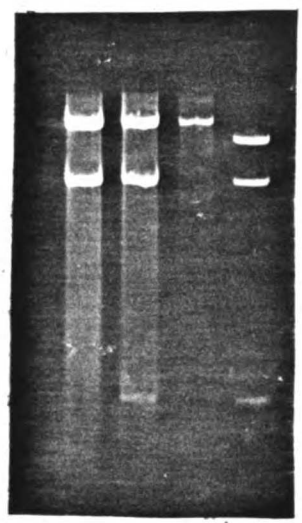
For this experiment, the 3.7 kb *Eco*R1 2μ fragment from pRB1240 had to be inserted into pTE33. (pRB1240, obtained from R. Bishop is a pBR322 derived plasmid containing a 1.1 kb *HIS3* insert and a 3.7 kb 2μ insert.) Both pTE33 and pRB1240 were cut with *Eco*R1. The resulting DNA fragments were ligated. This DNA was then used to transform an *E. coli* strain, MB1000-5, which is ampicillin sensitive and requires uracil for growth because of a *pyrF*$^-$ mutation. After transformation, Amp^r colonies were selected and screened for Ura$^+$ phenotype. Since any plasmid containing the pTE33 vector would be Amp^r and Ura$^+$, further screening was necessary. A rapid toothpick screen procedure (see Methods) was used to identify colonies containing plasmids that were about 11.9 kb in size, the expected size of the desired hybrid plasmid. The screening of 39 colonies revealed two plasmids of the size of the

pTE33 vector with a 2μ insert. Plasmid DNA was isolated from both colonies. Cleavage with EcoR1 generated fragments of 8.2 kb and 3.7 kb that migrated on gel electrophoresis with pTE33 and the 2μ fragment from pRB1240, respectively (Figure 2).

Since the SUP61 allele was not available as a cloned fragment, a different approach was necessary to construct the suppressor containing plasmid. By the method of Broach et al. (1981), the mutant allele of a cloned wild-type gene can be recovered directly. The method requires that the cloned DNA fragment, sup^+ in this case, be inserted into a vector so that the junctions constitute two different restriction sites, each of which appears only once in the transforming vector. Subsequent to integration of the plasmid into the genome at the site of the mutant allele (SUP61), the genomic DNA can be digested with either of two restriction enzymes to yield equivalent linear fragments containing plasmid sequences linked either to the wild-type gene or to the mutant allele. Each fragment can be recovered by ligation to circularize the fragment followed by transformation in E. coli. Because of the randomness of the location of the recombination event leading to integration, the fragment which will contain the mutant allele cannot be predicted. However, after their recovery, the two plasmids can be distinguished by transformation of a yeast strain with, in this case, the suppressible ochre markers his4-1176 and leu2-1.

As a source of the SUP61 gene, we used a SUP61/sup^+ haploid daughter of a diploid transformed with pTE33. Etcheverry transformed a SUP61/sup^+ diploid with pTE33 and showed by tetrad analysis that the plasmid had integrated onto the chromosome carrying the SUP61 allele at

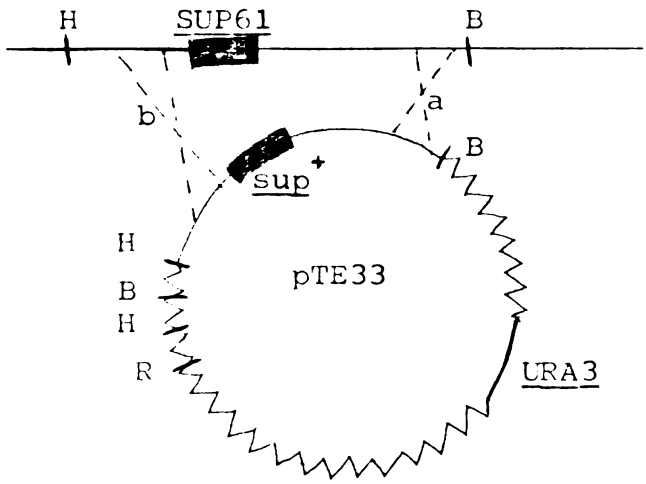
FIGURE 2 Restriction Analysis of the Constructed Plasmid pTE33-2μ.

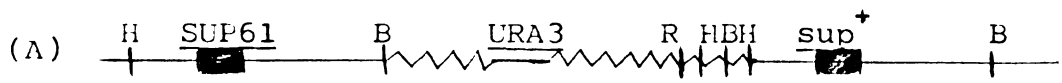


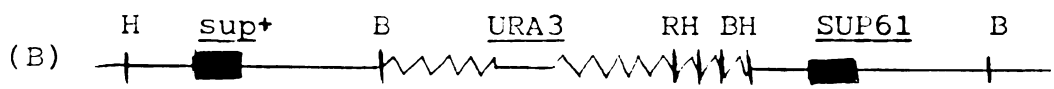
A B C D

Each lane contains plasmid DNA cleaved by EcoR1.
(A) pTE33-2μ; (B) spurious pTE33-2μ candidate;
(C) pTE33; (D) pRB1240.
The DNA fragments were electrophoretically separated in 1.25% agarose. The upper and lower fragments of pTE33-2μ (A) migrate the same distance as pTE33 (C) and the middle, 2μ-containing fragment of pRB1240 (D), respectively.

FIGURE 3 Schematic Representation of the Two Possible Integration Events.







The crossover event between the sup+ plasmid, pTE33, and the SUP61 chromosome can occur to the right (a) or to the left (b) of the sup gene. Crossover at (a) results in chromosomal structure (A); crossover at (b) results in structure (B).

DNA sequences are diagrammed as follows: wavy line, E. coli plasmid pBR322; solid line, yeast chromosomal DNA; solid bar, approximate position of the sup gene. Restriction endonuclease sites are designated: R, EcoR1; B, BamH1; H, HindIII.

the suppressor locus. Since pTE33 contains two BamHl and two HindIII sites, the recovery of SUP61 was not a certainty as described above. The two possible structures of the SUP61/sup^+ chromosome, shown in Figure 3, depend on where the crossover event leading to integration occurred in relation to the SUP61 allele. In neither case would complete cleavage with BamHl generate any fragments containing both plasmid and sup DNA. If integration had occurred as shown in possibility B, cleavage with HindIII would result in a fragment containing sup^+ as well as plasmid sequences. In this case, that fragment would correspond precisely to that DNA which had integrated, except that it would not contain the 0.2 kb inverted repeat of pTE33. Only if integration occurred as in possibility A is the recovery of SUP61 with plasmid DNA possible. Cleavage with HindIII would generate a fragment containing SUP61 and all of the pTE33 DNA except for the sup^+ fragment and the 0.5 kb inverted repeat. Upon ligation, the HindIII-generated fragment would yield a SUP61 version of pTE33 without the latter's small inverted repeat.

DNA from a SUP61/sup^+ haploid daughter of the pTE33 transformed strain was cleaved with HindIII. After ligation, the DNA was used to transform the bacterial strain MB1000-5, selecting for Amp^r colonies. All HindIII fragments containing the allele for ampicillin resistance should also contain a sup allele. To confirm this and to eliminate contaminants, eight colonies were screened by colony hybridization using ^{32}P-labelled $tRNA^{Ser}_{3+4}$ as a probe. Several colonies containing a $tRNA^{Ser}_{4}$ allele were identified.

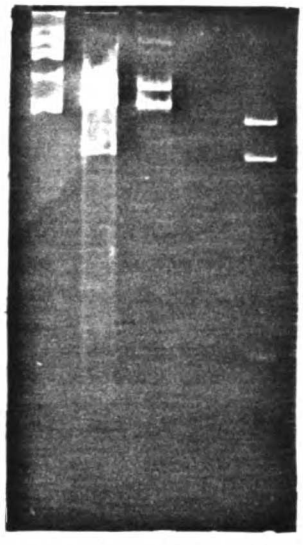
Plasmid DNA from one such colony was isolated and found to be 7.7

kb in size, the size expected for the sup-containing plasmid. The plasmid, called pBH6, was cleaved with EcoR1 and ligated with EcoR1 cleaved pRB1240, which contains the 2μ origin fragment. The ligated DNA was used to transform bacteria. Amp^r colonies were selected and about 25 screened by the quick toothpick method to identify plasmids of about 11.4 kb. A candidate for pBH6-2μ was identified and isolated. Restriction analysis demonstrated that it contained pBH6 and the 2μ fragment (Figure 4).

Whether the plasmid contained the wild-type or the SUP61 allele of $tRNA^{Ser}_4$ was still unknown, but this could be determined by using the plasmid to transform a yeast strain containing suppressible ochre markers. The generation of transformants with SUP61 activity would unambiguously identify the plasmid allele as the suppressor. If, on the other hand, all the transformants lacked suppressor activity, we would conclude either that the plasmid contained the wild-type allele or that a functional suppressor carried on an autonomous plasmid is lethal. The two possibilities can be distinguished by comparison of the pBH6-2μ transformation frequency with the frequencies of the sup^+ and control plasmids. If pBH6-2μ contained the wild-type allele, the transformation frequency should be similar to that of the other plasmids. The transformation frequency of pBH6-2μ should be lower than that of the other plasmids if it contains SUP61 but inactivation of the suppressor by mutation (a rare event) is required for a viable transformant.

Cells transformed with pHB6-2μ showed SUP61 activity proving that the plasmid contained the suppressor and not the wild-type allele. The next section discusses the transformation experiment and its results in detail.

FIGURE 4 Restriction Analysis of the Constructed Plasmid pBH6-2μ.



A B C D

Lanes B, C, and D contain plasmid DNA cleaved by EcoR1. (A) pBH6-2μ, not cleaved; (B) pBH6-2μ, partially cleaved; (C) pBH6, partially cleaved; (D) pRB1240, cleaved. The DNA fragments were electrophoretically separated in 1.25% agarose. The uppermost band in the EcoR1 cleaved pBH6-2μ lane (B) corresponds to a band in the uncleaved plasmid (A). The second and third bands of the cleaved pBH6-2μ migrate with the (lower) cleaved band of pBH6 (C) and the middle, 2μ-containing fragment of pRB1240 (D), respectively.

II. Transformations

To determine the effects of SUP61 when present on an autonomous plasmid, we first needed to compare the transformation frequency of the SUP61-containing plasmid to that of the two control plasmids. If the presence of the functional suppressor on an autonomously replicating plasmid was lethal, only cells in which the plasmid integrated into the genome and/or cells in which the suppressor on the plasmid could not function due to a mutation, for example, should be recovered as transformants. Since selection of transformants required expression of the URA3 gene but not of SUP61, we would thus expect to be able to recover transformants with inactivated suppressor genes. If integration, mutation, or another rare event were required for recoverable transformants, the observed transformation frequency of the SUP61 containing plasmid should be much less than that of the other two plasmids.

The haploid strain used as the recipient for the transformations, 204, has a ura3$^-$ deletion which is complemented by the URA3$^+$ gene contained on the plasmid. The use of the ura3$^-$ deletion eliminates the possibility that Ura$^+$ revertants would be selected along with actual transformants. The strain also contains four ochre suppressible alleles, his4-1176, leu2-1, ade2-1, and can1-100, and an amber suppressible allele, trp1-1. This combination of markers permits the serine-inserting ochre suppressor, SUP61, to be distinguished from possible newly arising suppressors. Newly arising amber suppressors would be recognized by their suppression of the trp1-1 allele. It is unlikely that a new ochre suppressor would result in a His$^+$ phenotype because his4-1176 requires serine insertion for function. Since there

are eight $tRNA^{Tyr}$ genes, a tyrosine inserting suppressor is the most likely ochre suppressor to arise. It would be distinguishable from SUP61 because his4-1176 requires serine insertion for function. Leucine- and serine-inserting ochre suppressors that might arise usually require [Ψ^+] for phenotypic detection and thus would not be detected in the strain used.

One transformation experiment yielded 62 pBH6-2μ, 347 pTE33-2μ, and 43 YIp5-2μ Ura^+ transformants with transformation efficiencies of 23, 2300, and 24 transformants/μg DNA, respectively. The smallest amount of DNA used, about 1/10 that of the other two plasmids, resulted in the highest transformation frequency (pTE33-2μ), a frequency similar to that achieved with other 2μ DNA containing plasmids, i.e. 10^3-10^4 per μg DNA (Thomas and James, 1980).

The similar, though low, transformation efficiencies of pHB6-2μ and YIp5-2μ suggest that the presence of the SUP61 gene on an autonomously replicating plasmid is not lethal to the cell. In this situation, rare events inactivating the suppressor should be required for transformation and should thus result in a transformation frequency much lower than that seen with YIp5-2μ.

The phenotypes of the transformants were determined by replica plating to selective media. All the pTE33-2μ and YIp5-2μ transformants were His^-, Leu^-, Ade^-, Trp^-, Can^r, and Ura^+, i.e. they carried the plasmid and showed no suppressor activity. The pBH6-2μ transformants grew in the absence of histidine, leucine and uracil, but required tryptophan. This phenotype demonstrated that the pBH6-2μ transformants not only contained the plasmid (Ura^+), but also contained and expressed the suppressor allele (His^+, Leu^+). The suppression of the two ochre

markers, one (his4-1176) requiring serine insertion, but not the trp1-1 amber marker, identified the active suppressor as SUP61 and not a new suppressor. The pBH6-2μ transformants did not suppress the can1-100 or ade2-1 markers., i.e. they were Can^r and only small spots of growth appeared on plates lacking adenine. The suppression of some but not all of the markers by the SUP61 transformants can be interpreted in light of other work suggesting that various ochre suppressible markers require different degrees of suppression. Colby et al. (1981) characterized a mutant with a decreased quantity of suppressor-specific tRNA that suppressed several ochre alleles but not can1-100 or ade2-1. The SUP61 transformants thus appear to have reduced levels of suppressor activity.

The reduced suppressor activity of the transformants with autonomous plasmids was surprising since cells with one integrated copy of SUP61 show greater suppression. A SUP61/sup^+ diploid and a SUP61/sup^+ haploid both suppress ade2-1 and can1-100 as well as other suppressible markers (Etcheverry, 1980). Before hypothesizing about the transformants' reduced suppressor activity, we wanted evidence that the plasmids were indeed autonomous. We chose to study mitotic instability, a known property of autonomous plasmids (Beggs, 1978).

III. Stability Experiments

To investigate the mitotic instability of the SUP61 transformant, we wanted to determine whether the rate at which the plasmid was lost was higher than expected. By comparing the rate of loss of the SUP61 plasmid to the rates of the sup^+ and YIp5-2μ plasmids, we could control for effects due to extra copies of the tRNA gene per se and for the mitotic instability of the 2μ-containing vector, respectively.

We studied the loss of the plasmid under conditions that were non-selective for maintenance of the plasmid as well as non-selective for suppression, that is, in medium containing uracil. Any selective advantage of cells without the plasmid should be reflected by a reduction in the proportion of cells capable of forming colonies in medium lacking uracil.

The transformants were first patched from the regeneration agar of the original transformation plates onto selective (ura^-) plates. When possible, transformants from the same transformation experiment were used. (In experiment 2, the pTE33-2μ transformant came from a transformation done one week prior to the transformation that generated the pBH6-2μ and YIp5-2μ colonies.) After two to three days, one transformant for each of the three vectors was inoculated into 10ml of YEP liquid medium and grown for 24 hours with aeration at 30°C. The transformants were then reinoculated at a dilution of about 1:100 into fresh YEP. After 24 hours, the cultures were again reinoculated and allowed to grow for another 24 hours.

A reduction in the proportion of cells maintaining the plasmid could be monitored in one of two ways. One option was to plate cells directly onto both ura^+ and ura^- media. The statistical accuracy of

this method would be limited especially if differences among the transformants were small. The second option was to plate cells onto YEP, allow them to form colonies and then replica plate onto ura^+ and ura^- media. This method improves the statistical accuracy but increases the time interval, i.e. the number of divisions during which a cell could lose its plasmid. The second method is thus likely to overestimate the actual rate of plasmid loss of the culture. We chose to monitor the loss of the plasmid by the second method because of its greater statistical accuracy.

At the first inoculation and just before each subsequent inoculation, aliquots were removed from the cultures, diluted appropriately and plated onto YEP. All cells (i.e. those maintaining the plasmid as well as those which had lost the plasmid) should form colonies on YEP. After two to three days at 30°C, the colonies were replica plated onto selective medium (ura^-) and onto YEP. Only those colonies retaining the plasmid will grow on ura^- plates. The proportion of Ura^+ colonies was calculated as the number of colonies on a ura^- plate divided by the number of colonies on the corresponding YEP plate.

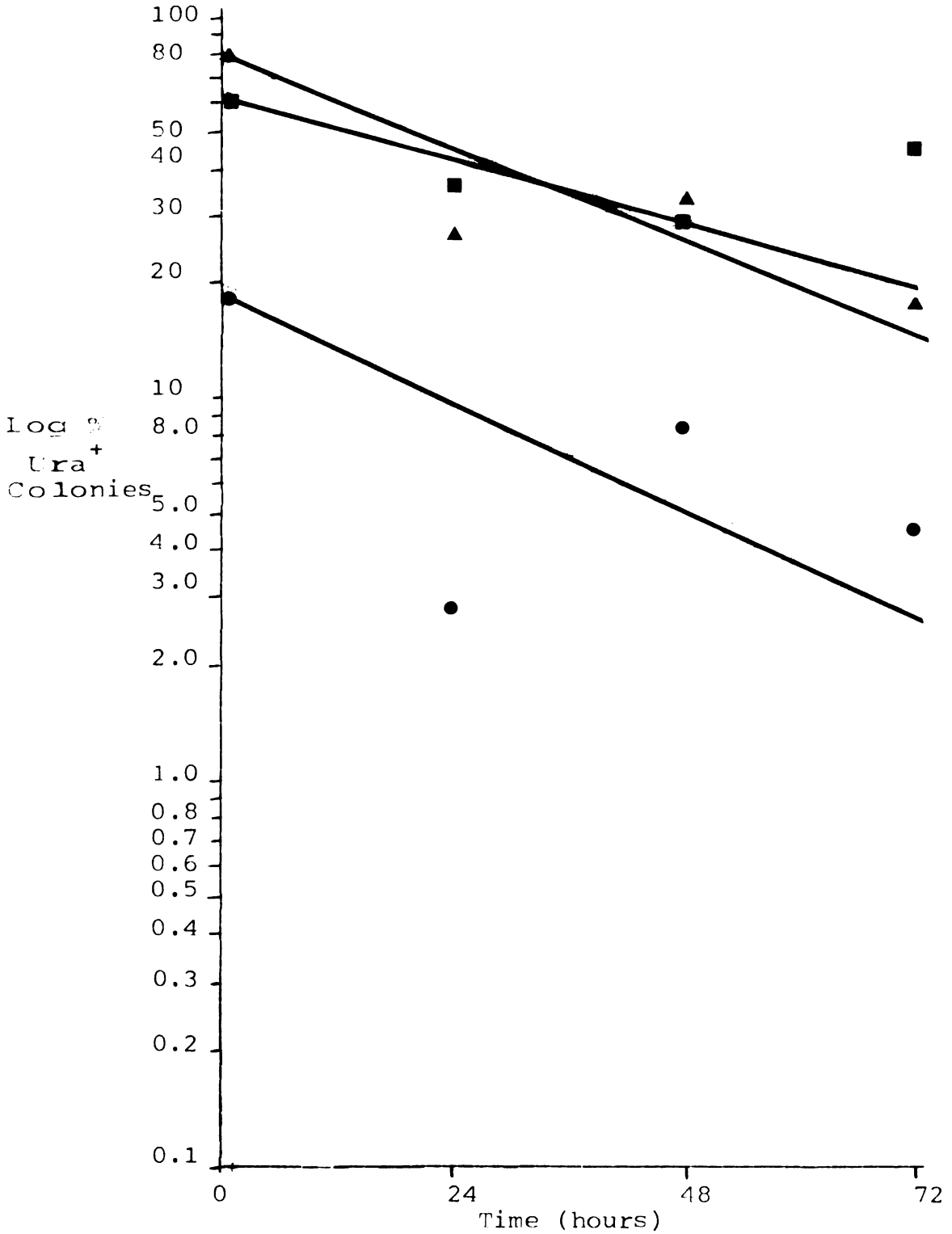
The graphs of the percentage of Ura^+ colonies versus time of two stability experiments are shown in Figure 5. Allowing for experimental error (only a single value was used for most points), a steady decline in the percentage of Ura^+ colonies occurred in most cases. The slopes calculated from Figure 5 suggest that the stabilities of pBH6-2μ and pTE33-2μ are reduced relative to that of YIp5-2μ. The logarithmic rate of decline of Ura^+ colonies in the SUP61-containing transformant was 1.7 and 6.7 times greater than that of YIp5-2μ in the first and second

FIGURE 5 Stability Curves of pBH6-2μ, pTE33-2μ, and YIp5-2μ Transformants in Non-selective Medium.

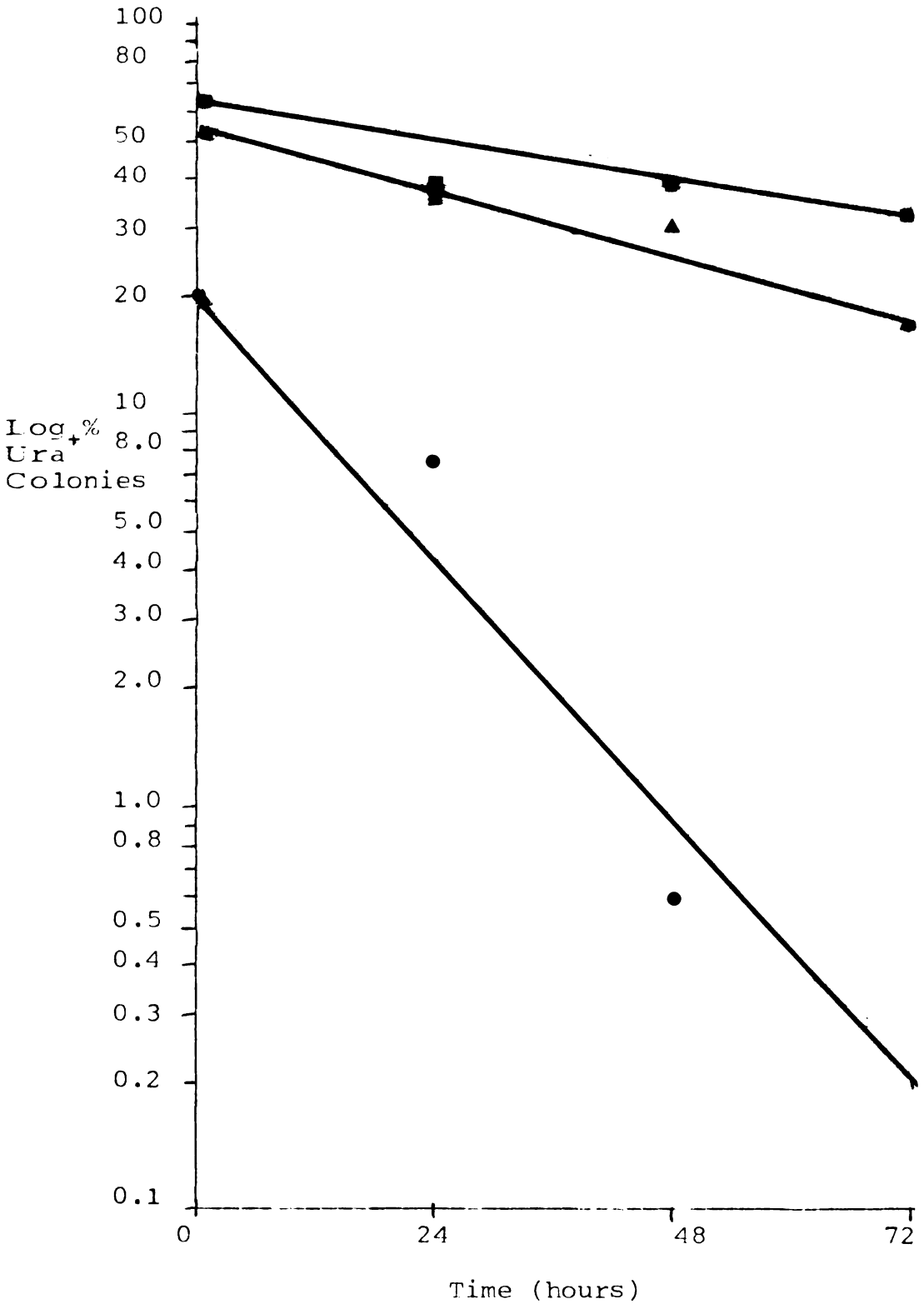
The loss of the plasmid during growth in non-selective medium was monitored by following the reduction in the proportion of Ura^+ colonies over time as described in Results, Section III. Since the number of doublings of the three transformant cultures did not differ significantly, the % Ura^+ colonies are plotted versus time.

- ● pBH6-2μ (SUP61)
- ▲ pTE33-2μ (sup^+)
- ■ YIp5-2μ (no *sup* gene)

A. Experiment 1



B. Experiment 2



experiments, respectively. The increased rate of plasmid loss in the *sup*$^+$-containing transformant was less dramatic but reproducible. The proportion of Ura$^+$ colonies declined 1.4 and 1.7 times as rapidly as that of YIp5-2μ in Experiment 1 and 2, respectively. Additional evidence for the reduced stabilities of the plasmids carrying a *sup* gene comes from the raw data. After 72 hours of growth in non-selective medium, the YIp5-2μ transformant culture had the greatest proportion of Ura$^+$ colonies and pBH6-2μ the smallest. This result was consistent between the two experiments. At all but two time points, the order of the proportion of Ura$^+$ colonies remained the same.

In the second experiment, the full phenotypes of the transformants were checked at 24 hour intervals. Cells were streaked on ura$^-$ plates and after two to three days, replica plated onto appropriate dropout and drug plates. Ura$^+$ colonies maintained their respective phenotypes, i.e. Ura$^+$ colonies from pTE33-2μ and YIp5-2μ cultures were His$^-$, Leu$^-$, Ade$^-$, Can^r, and Trp$^-$, while those from the pBH6-2μ culture were His$^+$, Leu$^+$, Ade$^-$, Can^r, and Trp$^-$. We can thus conclude that the pBH6-2μ transformants which maintained the plasmid still had a functional suppressor.

These experiments demonstrate the mitotic instability of all three plasmids, providing evidence that the transformants carry non-integrated, i.e. autonomous copies of the plasmids. *SUP61* transformants were more unstable than the other two transformants, suggesting a deleterious effect of the suppressor in high copy number. Despite the tendency of the *SUP61* transformants to lose the plasmid, transformants that retained the plasmid still had a functional suppressor. Interestingly, the pTE33-2μ transformant appears to be

somewhat less stable than the YIp5-2μ transformant, suggesting that there might also be a deleterious effect of extra copies of the wild-type tRNA gene.

IV. Growth rates

Transformants of the haploid strain 204 which contain the SUP61 gene on an autonomously replicating plasmid are viable and express the suppressor gene. However, the mitotic stability of these transformants is lower than that of the transformants containing either of the control plasmids, implying a deleterious effect of the suppressor at some level. We analyzed the growth rates of the transformants to investigate whether the long term selective disadvantage conferred by the SUP61 plasmid was reflected in decreased growth rates. It was necessary to compare the growth rates under selective conditions for the maintenance of the plasmid, i.e. in ura^- medium, because of the high mitotic instability of the transformants in non-selective medium.

Ten milliter cultures of selective medium were inoculated from the same patches of transformants used to start the stability experiments. After an 18-40 hour growth period, each transformant was inoculated into selective and non-selective media so that each culture had approximately the same OD. ODs of the six cultures were read at two to three hour intervals (i.e. about one generation time) until stationary phase was reached. At the beginning, end and one or two intermediate times, aliquots of each culture were diluted and plated on both complete and selective media to determine the percentage of Ura^+ colonies. As discussed in the previous section, this method of determining the percentage of Ura^+ colonies sacrifices some statistical accuracy for a shorter interval in which the transformant could lose its plasmid and be scored as Ura^-. As can be seen in Table 2, plasmid loss is apparently significant even on selective medium. Although one would expect all cells in aliquots from cultures grown in selective medium to be Ura^+,

the observed proportion of Ura$^+$ colonies is significantly lower. The transformants presumably lose the plasmid during the cell divisions necessary to form a colony.

The growth curves obtained in two experiments are shown in Figure 6. (Table 2 exhibits other data from these experiments.) Within a given experiment, the three transformants each reached the same final density and grew at approximately equal rates. The interpretation of generation times in non-selective medium is complicated by the loss of the plasmid during growth. In selective medium, the generation times were all within $\pm$ 10% of that of the YIp5-2μ control, with one exception; the SUP61 transformant during its fastest rate of growth had a generation time 30-50% longer than that of the other transformants in the second experiment. However, this result was not seen in the other experiment and thus does not appear to be reproducible.

The longer lag period of the SUP61 cultures in selective medium, however, is reproducible. In the first experiment, the SUP61 culture exhibited a lag of six hours before growing at a measurable rate and a lag of 10 hours before reaching the fastest growth rate. The sup$^+$ and YIp5-2μ cultures in the same experiment had lag times of less than two hours. In the second experiment, the SUP61 culture required about 20 hours to reach the fastest rate of growth, compared to 8-12 hours for the other two cultures.

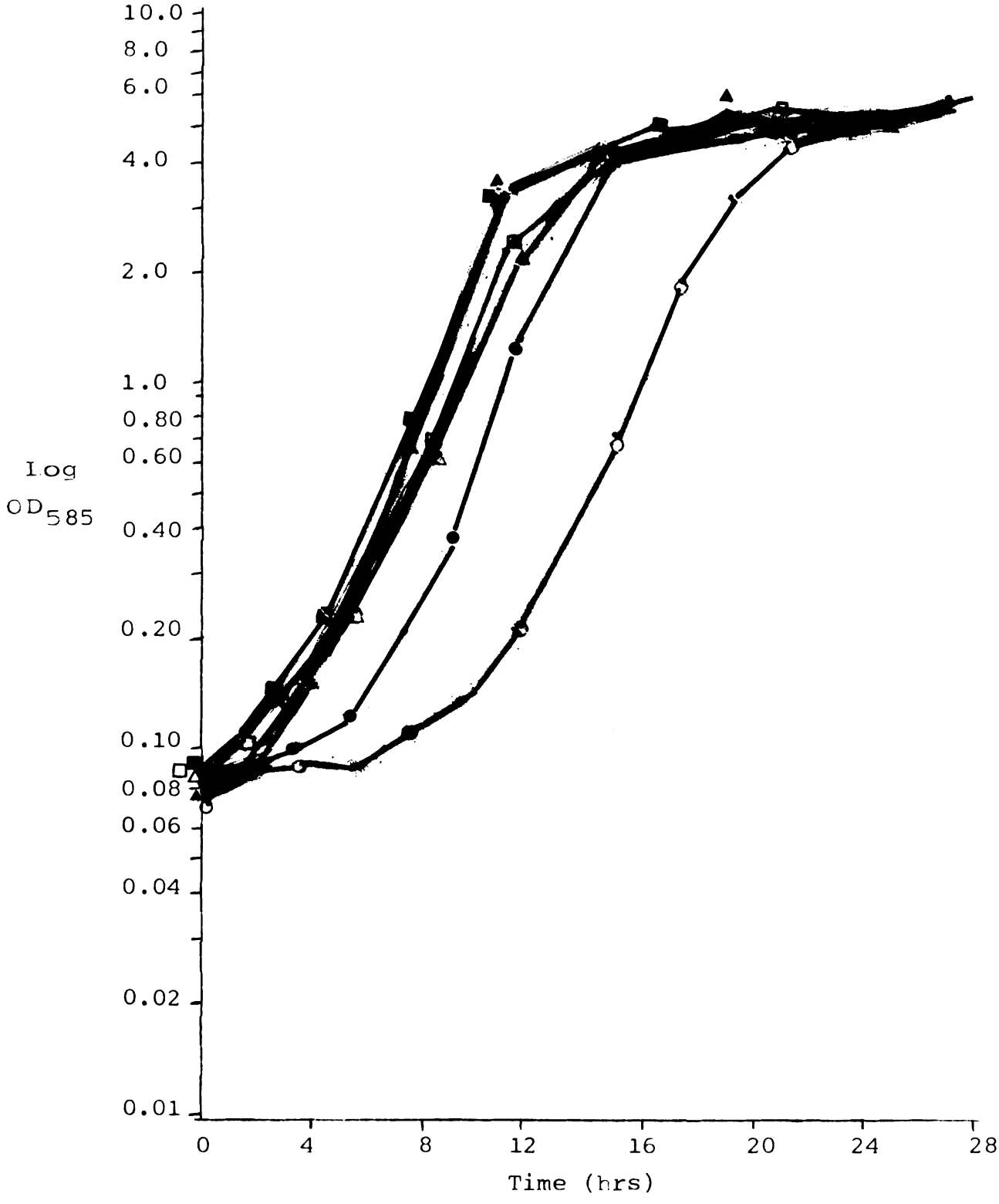
The percentage of Ura$^+$ colonies during exponential growth in selective medium demonstrates no striking or reproducible differences among the three transformants. In the first experiment, the percentage of Ura$^+$ colonies in all three transformants is about 60%. The YIp5-2μ

FIGURE 6 Growth Curves of pBH6-2μ, pTE33-2μ, and YIp5-2μ Transformants in Selective and Non-selective Media

The growth of the three transformants in ura^- and ura^+ media was monitored by optical density as described in Results, Section IV. The two media are supplemented minimal medium differing only in the presence or absence of uracil.

● pBH6-2μ, ura^+ medium
○ pBH6-2μ, ura^- medium
▲ pTE33-2μ, ura^+ medium
△ pTE33-2μ, ura^- medium
■ YIp5-2μ, ura^+ medium
□ YIp5-2μ, ura^- medium

A. Experiment 1



B. Experiment 2

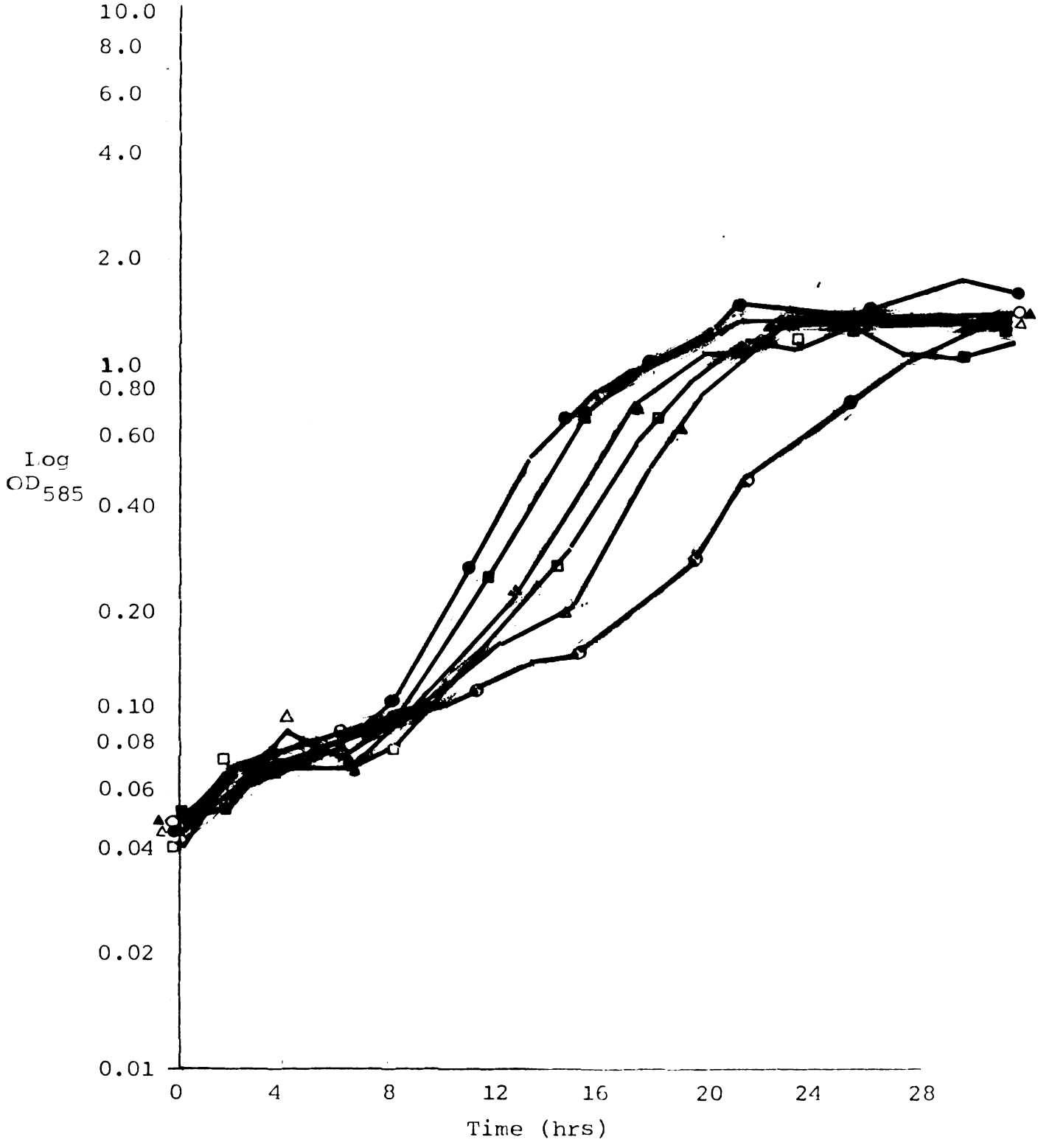


TABLE 2 Generation Times and Proportion of Ura$^+$ Colonies During Exponential Growth

Experiment	Plasmid	Ura$^-$ medium			Ura$^+$ medium		
		$t_{1/2}$ (hrs)	$t_{1/2}$ (normalized)	%Ura$^+$	$t_{1/2}$ (hrs)	$t_{1/2}$ (normalized)	% Ura$^+$
1	YIp5-2μ	2.1	[100%]	60	1.85	[100%]	41
	pTE33-2μ (*sup*$^+$)	2.0	96%	64	1.70	92%	46
	pBH6-2μ (*SUP61*)	2.15	102%	67	1.80	97%	24
2	YIp5-2μ	2.74	[100%]	56	2.28	[100%]	37
	pTE33-2μ	2.98	109%	67	2.69	118%	25
	pBH6-2μ	3.60	131%	35	2.28	100%	2.4

The growth of the three transformants in ura$^-$ and ura$^+$ media was monitored as described in Results, Section IV. The media are minimally supplemented and differ only in the presence or absence of uracil. The generation time during exponential growth (t1/2) was calculated from the growth curves of Figure 6 and normalized to the generation time of the YIp5-2μ transformant. The proportion of Ura$^+$ colonies during exponential growth (% Ura$^+$) was determined as the number of colonies on ura$^-$ medium divided by the number of colonies on YEP, as described in the text.

and pTE33-2μ cultures in experiment 2 showed similar percentages (56% and 67%, respectively) of Ura^+ colonies, while the percentage in the pBH6-2μ culture was somewhat lower (40%). Although the lower proportion of Ura^+ colonies in the pBH6-2μ culture was not a reproducible result, it is perhaps noteworthy that it was accompanied by a longer generation time. In fact, pBH6-2μ growth in the second experiment is apparently biphasic. From 8-17 hours, the culture grew with a generation time of 8.4 hours; 23% of the total colonies were Ura^+, i.e. retained the plasmid. From about 19-24 hours, the culture grew faster and more of the colonies were Ura^+; the generation time was 3.6 hours and the percentage of Ura^+ colonies was 35% at 19 hours and 45% at 24 hours.

A possible explanation for the increased stability and decreased growth rate of the pBH6-2μ culture with time is the integration of the plasmid into the genome. In this model, the high mitotic instability of the SUP61 transformant can be correlated with a long lag period. That is, the culture fails to exhibit an increase in mass because a large portion of the cells lose the plasmid during early divisions. Rare integration events, however, result in mitotic stability of the transformed phenotype and thus confer a selective advantage on cells with an integrated plasmid. As cells with integrated plasmids become an ever larger proportion of the population, the culture shows more normal growth rates.

The differences between the two experiments are striking and remain unexplained, as the experiments were carried out identically (except that the pTE33-2μ transformant used in the second experiment originated from a transformation done one week earlier than the transformation producing the other two transformants). The transformant cultures of

the second experiment from which the monitored cultures were inoculated grew noticeably more slowly than those of the first experiment and required a growth period of 40 hours, instead of 20, to reach a density from which the cultures of the growth experiment could be inoculated. All the cultures of this experiment grew more slowly with longer lag times and lower final densities than those in the first experiment. For example, the cultures of the second experiment grew to final densities between 0.9 and 1.7 OD units compared to values of 5.0-6.5 of the earlier experiment. In addition, the SUP61 transformant was significantly less stable in the second experiment. During exponential growth in non-selective conditions, the proportions of Ura^+ colonies were 24% and 2.4% in the first and second experiments, respectively.

The unexplained differences between experiments complicate the interpretation of the findings but certain conclusions are possible. The growth rates of the three transformants under conditions that require maintenance of the plasmid do not vary dramatically. The largest difference from the YIp5-2μ control is a 30-50% increase in the generation time of the SUP61 transformant in one experiment. In contrast to the growth rate, the longer lag time of the SUP61 transformant is reproducible. The stability of the transformed phenotype of that culture increases when the lag period ends and increases further with concomitant increases in growth rate. Thus, the possibility that a large proportion of the SUP61 population undergoes rapid loss of the plasmid, which is reflected in a long lag period, is intriguing.

DISCUSSION

The transformation of *S. cerevisiae* with the *SUP61*-containing plasmid, pBH6-2μ, corroborates previous evidence of Etcheverry et al. (Etcheverry, 1980; Etcheverry et al., 1981) that suppression itself is not the cause of lethality in strains carrying *SUP61*. If this were the case, only Ura^+ transformants with no suppressor activity, i.e. those in which a mutation had occurred in either *SUP61* or a locus necessary for the expression of the suppressor, should be recovered. The requirement for such a mutation should significantly lower the transformation for pBH6-2μ relative to that for the control plasmid, YIp5-2μ. The observed efficiency of transformation frequency for pBH6-2μ, though low, was similar to that of YIp5-2μ, arguing that the pBH6-2μ transformants contain an unaltered *SUP61* gene. Similar findings have been reported for an *E. coli* tryptophan-inserting ochre suppressor, originally isolated as a recessive lethal (Yarus, 1979); in this work, the transformation frequency for a suppressor-containing plasmid present in transformants in high copy number (about 180 copies per cell) did not differ from that of the vector without the suppressor gene. The suppressor activity of the pBH6-2μ transformants, i.e. their ability to suppress two of the ochre suppressible markers in the recipient strain, adds further evidence that viable transformants contain an unaltered *SUP61* gene.

Although the presence of the suppressor gene on an autonomous plasmid was not lethal, it did decrease the relative stability of the plasmid, suggesting a deleterious effect of multiple copies of *SUP61*. Under non-selective conditions, the *SUP61* transformant lost its plasmid at a faster rate (up to seven-fold) than the transformant containing the

wild-type allele. The plasmids used for transformation were identical except for the sup allele and the presence of a 0.5 kb repeat in the sup^+ containing plasmid. Since the absence of this small repeat is unlikely to cause the instability of the SUP61-containing plasmid, the nature of the sup allele seems the most likely cause of the difference in stability between the two classes of transformants. Since the transformants containing the SUP61 gene were less stable than those containing the wild-type (sup^+) gene, oversuppression is presumably the deleterious effect of SUP61.

Thomas and James (1980) made similar observations on transformation and stability using SUP4.o, a tyrosine-inserting ochre suppressor. In addition to the suppressor gene, their plasmid, like ours, contained a 2μ fragment which conferred the ability to replicate autonomously. However, since their plasmid contained no selectable marker that did not require suppression, they did not have the option of selecting transformants with a plasmid carrying the wild-type gene. Therefore, in asking whether an alteration affecting the plasmid was required to generate viable transformants, they could not compare the transformation efficiencies of plasmids containing the suppressor and wild-type alleles. Nevertheless, the observed transformation frequency for the SUP4.O-containing plasmid, 10^3-10^4/μg DNA, was similar to frequencies of other 2μ-containing plasmids. This suggests that SUP4.o on an autonomous plasmid, like SUP61, yields viable transformants without alteration of the suppressor.

The comparison of our data on stability with those of Thomas and James is somewhat tenuous because we lack direct evidence on the subcellular location of our plasmids in the transformants. The SUP4.o

transformants fell into two classes - those with only autonomously replicating copies of the plasmid and those with both integrated and autonomously replicating copies. In our transformants, the absence of integrated copies of the plasmid in a substantial fraction of the population is inferred from the instability of the transformants. The stability of the suppressor phenotype in cells integratively transformed with SUP61 would be expected to be similar to that of cells containing endogenous SUP61 copies except that the transformants should lose the plasmid at a low "pop-out" frequency. Although no strain has been integratively transformed with a SUP61-containing plasmid, the sup^+-containing plasmid, pTE33, is indeed stable when integrated and lost only at a low frequency (less than 0.07% per generation) (Etcheverry, 1980). Since the rate of plasmid loss in our transformants was much higher for a similar number of generations (3-5% per generation), the plasmid is not likely to be integrated (unless the 2μ fragment greatly increases the "pop-out" frequency). Experiments are now being done in our laboratory to directly determine the location of the transforming DNA.

Similar to our results, Thomas and James report instability of the transformants, although they measured meiotic rather than mitotic stability. In both types of transformants, they found the autonomously replicating copies to be highly unstable meiotically, while the integrated copies were generally stable. Comparisons with our data would obviously be more meaningful if the mitotic stability of the SUP4.o transformants were known. However, Thomas and James do cite preliminary evidence that mitotic stability parallels meiotic stability,

i.e. that non-integrated copies of the SUP4.o gene are mitotically unstable and integrated copies are stable.

Despite the implied deleterious effect of SUP61, we were unable to show any significant, reproducible effect on growth rate under either selective or non-selective conditions. However, effects on growth rate are difficult to assess for several reasons. The high mitotic instability of the SUP61 transformants precludes measurement of their growth rate in non-selective medium. The possibility of integration into the genome and the selective advantage of such integrative transformants complicate the interpretation of growth experiments in selective medium. Thomas and James also found no evidence that SUP4.o transformants had a reduced growth rate, although those transformants contained both integrated and non-integrated copies of the plasmid. Similarly, experiments with an E. coli tryptophan-inserting suppressor on a high copy number plasmid showed no effect on growth rate (Yarus, 1979).

The most intriguing similarity between our data and those of Thomas and James is the markedly reduced expression of the suppressor genes carried on autonomous plasmids. Transformants with integrated and autonomous copies of SUP4.o as well as cells containing only integrated copies (meiotic segregants of crosses between transformants and wild-type), showed efficient suppression of the ade2-1 allele; transformants with only non-integrated copies showed weak suppression of ade2-1 but efficient suppression of other ochre markers. Similarly, we found that transformants carrying SUP61 on an autonomous plasmid suppressed the his4-1176 and leu2-1 markers efficiently, the ade2-1 locus weakly, and the can1-100 locus not at all. In a SUP61/sup$^+$

haploid and a SUP61/sup$^+$ diploid, all of these markers are efficiently suppressed.

The phenomenon of reduced expression is difficult to interpret. We cannot yet determine the degree to which expression per gene copy is reduced because we lack data on the actual copy number in our transformants. However, since the 2μ-fragment included in our plasmids results in transformants carrying multiple copies of other plasmids into which it has been inserted, our transformants are likely to contain multiple plasmid copies. Unpublished estimates place the average copy number per cell at 30-50 for 2μ-containing hybrid plasmids. In addition, the fact that we can follow the loss of the plasmid over many generations (as in Figure 5) suggests that the transformants initially carry multiple copies of the plasmid.

The reduced expression of SUP61 on autonomously replicating plasmids is paradoxical in light of the deleterious effect of such plasmids implied by their elevated instability. As judged by phenotypic suppression, the total expression of SUP61 genes from the autonomous plasmids, which we presume to exist in multiple copies, is less than the expression of a single integrated copy; yet deleterious effects due to suppression are implied by plasmid instability while haploids or diploids with an integrated copy of SUP61 are mitotically (and in the case of diploids, meiotically) stable. The resolution of this paradox is also of interest for interpreting our stability data on the sup$^+$ allele. That is, the small but reproducible decrease in stability observed in transformants carrying sup$^+$ on an autonomous plasmid suggests that an increase in copy number of the wild-type gene also confers a (small) selective disadvantage. If the expression of the sup$^+$

gene on an autonomous plasmid is also reduced, then we could infer that even the addition of less than the functional equivalent of one integrated *sup*$^+$ copy is deleterious. A deleterious effect of increased copy number might suggest a requirement for tRNA balance in growth and/or development.

One basis for considering the necessity for a balance among tRNAs is the maintenance of translational fidelity. In their analysis and interpretation of codon usage in yeast, Bennetzen and Hall (1981) suggest that divergence from the preferential codon usage observed could promote infidelity. Their analysis of seven *S. cerevisiae* genes showed that the genes coding abundant proteins are highly biased in favor of codons read by the most abundant isoacceptor tRNAs and do not contain any codons read by rare tRNAs. They consider the effect of extensive use of a codon corresponding to a rare isoaccepting tRNA in coding for a highly abundant yeast protein as follows. The intracellular pool of the charged rare tRNA must be small relative to the other tRNAs because of the small amounts of the tRNA. When the growth of the yeast demanded large amounts of the protein, translation of the RNA would draw heavily upon the pool of the charged rare tRNA, discharging a large fraction of these molecules. The lack of the charged tRNA could cause all ribosomes translating the corresponding codon at the moment to suffer a block in translation. Early termination and/or translational error would likely result. The cell would be effectively starved for the amino acid corresponding to the rare tRNA. Amino acid starvation has been shown to cause translation errors in both bacterial and animal cells (O'Farrell, 1978; Parker et al., 1978).

The UCG-reading $tRNA^{Ser}$ coded for by *sup*$^+$, $tRNA^{Ser}_4$, is rarely used. Bennetzen and Hall found only one UCG codon out of 116 serine codons in the genes analyzed. With an increase in the number of $tRNA^{Ser}_4$ gene copies, charging of the "preferred" $tRNA^{Ser}$ species might be reduced by depletion of the serine pool by competing $tRNA^{Ser}_{UCG}$. As in the previous example, the ribosomes translating a serine condon in the mRNA of an abundant protein might experience a block in translation. By this model, many copies of the *sup*$^+$ gene could result in early termination and/or translational error.

Other variations on this general theme can also be entertained. For example, Atkins et al. (1979) found direct evidence for deleterious effects of the tRNA imbalance on translational fidelity. They studied the translation of MS2 virus in an *E. coli* cell-free system before and after adding various tRNAs to the endogenous tRNA pools. Two of the 36 tRNAs tested, $tRNA^{Ser}_{AGU/C}$ and $tRNA^{Thr}_{ACU/C}$ promoted frameshifts. The results suggest the possibility that tRNA imbalance *in vivo*, such as that presumably caused by the addition of multiple $tRNA^{Ser}_4$ genes, could also reduce translational fidelity.

In addition to maintaining the fidelity of translation, tRNA balance might also participate in the regulation of temporal or developmental changes in protein synthesis (see Guthrie and Abelson, 1982). Such a model suggests a reason for the conservation of minor codons and thus, minor isoacceptor tRNA species. By the proper placement of a rare triplet such as that decoded by *sup*$^+$, the rate of translation of a protein could be regulated. Since the concentration of a charged tRNA determines the step time of insertion of that amino acid, rare triplets (associated with rare tRNAs) would slow the rate of

translation. Very sensitive and specific regulation seems possible since the local context on the mRNA of the codon can change the speed of translation of that codon by an order of magnitude (Bossi and Roth, 1980). Thus, the proper placement of a rare triplet in one context would set the rate of translation of that mRNA specifically; another mRNA with the same triplet in a different context would be translated at a different rate. This model of regulation of protein synthesis by rare codon usage provides one means for modulating the quantities of proteins whose mRNAs contain rare codons. If the cell had mechanisms to control the tRNA balance, it could increase the level of a rare tRNA when it needed larger quantities of a protein whose mRNA contained the corresponding codon. Too little of the rare tRNA would result in an insufficient quantity of the protein. Too much of another tRNA might have the same effect by reducing the effective concentration of the rare species.

The possible roles of tRNA balance in such vital functions as preserving the fidelity of translation and regulating protein synthesis suggests the possibility that active mechanisms exist to control that balance. Further studies of cells transformed with pTE33-2μ, the *sup*$^+$-containing autonomous plasmid, could provide evidence on whether or not mechanisms to control the levels of tRNAs exist. For example, comparison of the number of transcripts per copy of the *sup*$^+$ gene and the quantity of precursor and mature tRNA in pTE33-2μ and YIp5-2μ (control) transformants might suggest the control of tRNA expression in response to gene copy number. If no such control existed, the transformant with multiple *sup*$^+$ genes should have that many times more transcript and mature product than the control transformants containing

only one sup$^+$ gene. Transcriptional control of tRNA balance, on the other hand, should reduce the quantity of transcript per sup$^+$ gene in the pTE33-2μ transformant relative to the YIp5-2μ transformant. If control were post-transcriptional, for example, related to splicing of the intervening sequence, the pTE33-2μ transformant should contain the same amount of transcript per gene copy as the YIp5-2μ transformant, but a reduced quantity of mature tRNA per copy. (That several of the minor isoacceptor tRNAs have intervening sequences has led to speculation that the level of these tRNAs might be modulated by a mechanism related to the requirement for splicing of the precursors (Guthrie and Abelson, 1981). Since little is known about the enzymology of splicing, specific mechanisms are difficult to predict at this time.)

It should be noted, however, that the interpretation of such data might be complicated by possible effects of differences in chromatin structure and availability for transcription, for example, between the chromosomal and plasmid genes. Some of these complications might be circumvented by doing the experiment with a recently constructed plasmid containing a yeast centromere (Clarke and Carbon, 1980). Plasmids carrying the centromere CEN3 in addition to a yeast replicator generally behave as minichromosomes in mitosis and meiosis. Thus, the insertion of the sup$^+$ gene into this plasmid would provide a stable, additional sup$^+$ copy to transformants. Since the CEN3 plasmids behave as chromosomes they are presumably located in the nucleus. Thus, any effect due to the possible cytoplasmic location of the 2μ-containing plasmid should be eliminated. If the CEN3 1.6kb fragment was inserted into the YIp5-2μ (control) and pTE33-2μ (sup$^+$) plasmids, we could compare the levels of sup$^+$ transcript, precursor, and mature product in

haploid cells with one and two copies, respectively of the *sup*$^+$ gene. The predictions of various models of control of tRNA balance would be the same as those described above.

The experiment on *sup*$^+$ expression using a *CEN3*-containing plasmid does not eliminate all the problems of using the autonomous plasmid pTE33-2μ; the *sup*$^+$ gene on the *CEN3* plasmid might still have a different chromatin structure and/or accessibility for transcription from the chromosomal copy of *sup*$^+$. To evaluate this possibility, one could compare the expression of *SUP61* in a haploid transformed with a *SUP61*-*CEN3* plasmid and a haploid containing an integrated copy of *SUP61*. Identical levels of suppression measured qualitatively by the degree of suppression of *ade2-1* and *can1-100* and quantitatively by amounts of suppressor tRNA would imply that location on a *CEN3* plasmid does not significantly affect that gene's expression.

SUP61 might also be useful in studying gene expression from an autonomous plasmid, since our data suggest that the expression of this gene is reduced. To determine at what level the reduction occurs, one could compare the ratios of *SUP61* transcripts, precursors, and mature tRNA in the haploid containing an integrated copy of the gene and the haploid transformed with the *SUP61* autonomous plasid. Similar ratios would indicate an effect at the level of transcription. For example, the structure or location of the plasmid might preclude efficient transcription, resulting in less tRNA. However, an effect due to the chromatin structure or location of the plasmid *per se* would be indistinguishable from active control of the suppressor gene at the level of transcription. A reduced ratio of the amount of precursor or mature tRNA to the amount of transcript in the transformant would imply

a post-transcriptional effect on expression. For example, the plasmids might be accessible to transcription but, the transcript, by virtue of the plasmids' location or a characteristic of the transcript itself, might be unavailable for processing. Alternatively, the cell might have active post-transcriptional mechanisms to reduce the level of the harmful suppressor tRNA. Ultimately, cellular localization studies of autonomous plasmids are needed to determine whether these plasmids are indeed accessible to transcription and processing enzymes.

Perhaps the most intriguing aspect of the work presented here is the paradox of the reduced expression of SUP61 concomitant with its instability, which implies a selective disadvantage. Small amounts of suppressor activity are associated with genetic instability, despite the fact that integrated copies of the fully functional suppressor gene are relatively stable. One partial resolution of the paradox might simply involve the differential ease with which genes on chromosomes and autonomous plasmids are lost. According to this model, even small degrees of suppression are disadvantageous. Cells with either integrated or autonomous copies of SUP61 would suffer deleterious effects but only in cells with autonomous copies can the genes be easily lost. This model explains the differential stability of cells with integrated and autonomous copies of SUP61 but not the reduced expression of the latter.

REFERENCES

Atkins, J.F., Gesteland, R.F., Reid, B.R., and Anderson, C.W. (1979). Normal tRNAs promote ribosomal frameshifting. Cell 18, 1119-1131.

Beggs, J.D. (1978). Transformation of yeast by a replicating hybrid plasmid. Nature 275, 104-109.

Bennetzen, J.L. and Hall, B.D. (1981). Codon selection in yeast. In press.

Bolivar, F., Rodriquez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., and Boyer, H.W. (1977). Construction and characterization of new cloning vehicles. Gene 2, 95-113.

Bossi, L. and Roth, J.R. (1980). The influence of codon context on genetic code translation. Nature 286, 123-127.

Broach, J.R., Friedman, L., and Sherman, F. (1981). Correspondence of yeast UAA suppressors to cloned $tRNA^{Ser}_{UCA}$ genes. J. Mol. Biol. 150, 375-387.

Clarke, L. and Carbon, J. (1980). Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature 287, 504-509.

Clewell, D.B. and Helinski, D.R. (1969). Supercoiled circular DNA-protein complex in E. coli: Purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. 62, 1159-1166.

Cohen, S.N., Chang, A.C.Y., and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of E. coli by R-factor DNA. Proc. Natl. Acad. Sci. 69, 2110-2114.

Colby, D., Leboy, P.S., and Guthrie, C. (1981). Yeast tRNA precursor mutated at a splice junction is correctly processed in vivo. Proc. Natl. Acad. Sci. 78, 415-419.

Cox, B.S. (1971). A recessive lethal super-suppressor mutation in yeast and other Ψ phenomena. Heredity 26, 211-233.

Cryer, D.R., Eccleshall, R. and Marmur, J. (1975). In Methods in Cell Biology, Ed. Prescott, D.M. (Academic, New York), Vol 12, 39-44.

Etcheverry, T., Colby, D., and Guthrie, C. (1979). A precursor to a minor species of yeast $tRNA^{Ser}$ contains an intervening sequence. Cell 18, 11-26.

Etcheverry, T. (1980). Characterization of the recessive lethal gene, SUP61, from Saccharomyces cerevisiae. Ph.D. dissertation, Cornell University.

Etcheverry, T., Salvato, M. and Guthrie, C. (1981). Recessive lethality of yeast strains carrying the SUP61 suppressor results from loss of a tRNA with a unique decoding function. J. Mol. Biol., submitted.

Gilmore, R.A. and Mortimer, R.K. (1966). Super-suppressor mutations in S. cerevisiae. J. Mol. Biol. 20, 307-311.

Gilmore, R.A. (1967). Super-suppressors in S. cerevisiae. Genetics 56, 641-658.

Grunstein, M. and Hogness, D.S. (1975). Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. 72, 3961-3965.

Guthrie, C. and Abelson, J. (1982). Organization and expression of tRNA genes in Saccharomyces cerevisiae. In The Molecular Biology of Yeast, Cold Spring Harbor Press, Cold Spring Harbor, New York, in press.

Hinnen, A., Hicks, J.B. and Fink, G.R. (1978). Transformation of yeast Proc. Natl. Acad. Sci. 75, 1929-1933.

Hirsh, D. (1971). Tryptophan tRNA as the UGA suppressor. J. Mol. Biol. 58, 439-458.

Hutchinson, H.T. and Hartwell, L.H. (1967). Macromolecular synthesis in yeast spheroplasts. J. Bacteriol. 94, 1697-1705.

Kuntzel, B., Weissenbach, J., Wolff, R.E., Tumaitis-Kennedy, T.D., Lane, B. and Dirheimer, G. (1975). Presence of the methylester of 5-carboxymethyl uridine in the wobble position of the anticodon of $tRNA^{Arg}_{III}$ from brewer's yeast. Biochimie 57, 61-70.

Liebman, W.W., Stewart, J.W. and Sherman, F. (1975). Serine substitutions caused by an ochre suppressor in yeast. J. Mol. Biol. 94, 595-610.

Loening, U.E. (1967). The fraction of high-molecular-weight RNA by polyacrylamide gel electrophoresis. Biochem. J. 102, 251-257.

Meagher, R.B., Tait, R.C., Betlach, M. and Boyer, H.W. (1977). Protein expression in E. coli minicells by recombinant plasmids. Cell 10, 521-536.

Miller, C.G. and Roth, J.R. (1971). Recessive-lethal nonsense suppressors in Salmonella typhimurium. J. Mol. Biol. 59, 63-75.

Miller, J. H. (1972). Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, New York.

Modrich, P. and Lehman, I.R. (1970). Enzymatic joining of polynucleotides. J. Biol. Chem. 245, 3626-3631.

Mortimer, R.K. and Gilmore, R.A. (1968). Suppressors and suppressible mutations in yeast. Adv. Biol. Med. Phys. 12, 319-331.

O'Farrell, P.H. (1978). The suppression of defective translation by ppGpp and its role in the stringent response. Cell 14, 545-557.

Olson, M.V., Page, G.S., Sentenac, A., Piper, P.W., Worthington, M., Weiss, R.B. and Hall, B.D. (1981). Only one of two closely related yeast suppressor tRNA genes contains an intervening sequence. Nature 291, 464-469.

Parker, J., Pollared, J.W., Friesen, J.D., and Sanners, C.P. (1978). Stuttering: High-level mistranslation in animal and bacterial cells. Proc. Natl. Acad. Sci. 75, 1091-1095.

Piper, P. (1978). A correlation between a recessive lethal amber suppressor mutation in S. cerevisiae and an anticodon change in a minor serine tRNA. J. Mol. Biol. 122, 217-235.

Sekiya, T., Takeishi, K. and Ukita, T. (1969). Specificity of yeast glutamic acid transfer RNA for codon recognition. BBA 182, 411-426.

Sherman, F., Fink, G.R., and Lawrence, C.W. (1974). Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Soll, L. and Berg, P. (1969). Recessive lethals: A new class of nonsense suppressors in E. coli. Proc. Natl. Acad. Sci. 63, 392-399.

Soll, L. (1974). Mutational alterations of tryptophan-specific tRNA which generate translation suppressors of UAA, UAG, and UGA nonsense codons. J. Mol. Biol 86, 233-243.

Struhl, K., Stinchcomb, D.T., Schere, S. and Davis, R.W. (1979). High-frequency transformation of yeast: Autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. 76, 1035-1039.

Thomas, D.Y. and James, A.P. (1980). Genetic analysis of Saccharomyces cerevisiae transformed by a plasmid containing a suppressor transfer ribonucleic acid gene. J. Bacteriol. 143, 1179-1186.

Van Solingen, P. and Van der Plaat, J.B. (1979). Fusion of yeast spheroplasts. J. Bacteriol. 130, 946-947.

Von Borstel, R.C. (1969). Yeast genetics supplement, Microbiol. Genetics Bulletin 38, 1-28.

Yarus, M. (1979). Isolation and properties of a plasmid which expresses the E. coli Su^+7 amber suppressor tRNA gene. Molec. Gen. Genet. 170, 291-298.

Yoshida, M., Takeishi, K., Ukita, T., (1970). Anticodon structure of GAA-specific glutamic acid tRNA from yeast. Biochem. Biophys. Res. Comm. 39, 852-857.

San Francisco
LIBRARY

UC San Francisco LIBRARY
UNIVERSITY OF CALIFORNIA · UNIVERSITY OF CALIFORNIA