

Lethal Disruption of the Yeast Actin Gene by Integrative DNA Transformation



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gate the mechanism of the antileishmanial effect, the rapid immobilization and destruction of promastigotes by chlorpromazine suggest that this phenothiazine may act at the level of the protozoal membrane, possibly by inhibiting enzymes in one or more metabolic pathways (14, 15).

Whether phenothiazines can be used to treat visceral leishmaniasis or related protozoal infections remains to be determined. Chlorpromazine is highly protein bound; maximal concentrations of the parent compound in human serum are usually attained only after 2 weeks of oral administration. Furthermore, the concentration of chlorpromazine that kills parasites after a single dose in vitro is greater than the concentration achieved in the plasma of psychiatric patients (0.1 to 0.5 µg/ml). However, phenothiazines are lipophilic and concentrate in tissues, and in psychiatric patients their concentrations in serum are poorly correlated with clinical responses. In dogs, the tissue-to-plasma ratio of chlorpromazine is 68:1 in brain, 26:1 in spleen, and 13:1 liver (16). This characteristic of the phenothiazines is important. For example, we have found that chlorpromazine given by gavage (20 mg/kg per day for 14 days) to *L. donovani*-infected Syrian hamsters significantly reduces the number of amastigotes in the liver and spleen (17). Although the optimal dosage and duration of therapy required to obtain adequate protozoacidal levels in the human reticuloendothelial system are unknown, our data indicate that adequate concentrations may be achievable. Normally, the incidence of adverse effects from phenothiazines in humans is related to dosage and the duration of therapy. Additional studies are warranted to determine whether a phenothiazine such as chlorpromazine can be used safely and effectively in the treatment of visceral leishmaniasis or related protozoal diseases.

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Lethal Disruption of the Yeast Actin Gene by Integrative DNA Transformation

Abstract. A mutant allele of the chromosomal locus corresponding to the cloned actin gene of the yeast *Saccharomyces cerevisiae* has been constructed by DNA transformation with a hybrid plasmid which integrates into, and thereby disrupts, the protein-encoding sequences of the gene. In a diploid strain of yeast, disruption of the actin gene on one chromosome results in a mutation that segregates as a recessive lethal tightly linked to a selectable genetic marker on the integrated plasmid. The actin gene, therefore, must encode an essential function for yeast cell growth.

The genome of *Saccharomyces cerevisiae* contains a single actin gene (1, 2). This gene, which was cloned via its homology to actin genes of other eukaryotes, could encode a protein with exten-

sive amino acid sequence homology with higher eukaryotic actins (2, 3). In addition, a protein that resembles actins from other sources in molecular weight, polymerization ability, and peptide map has

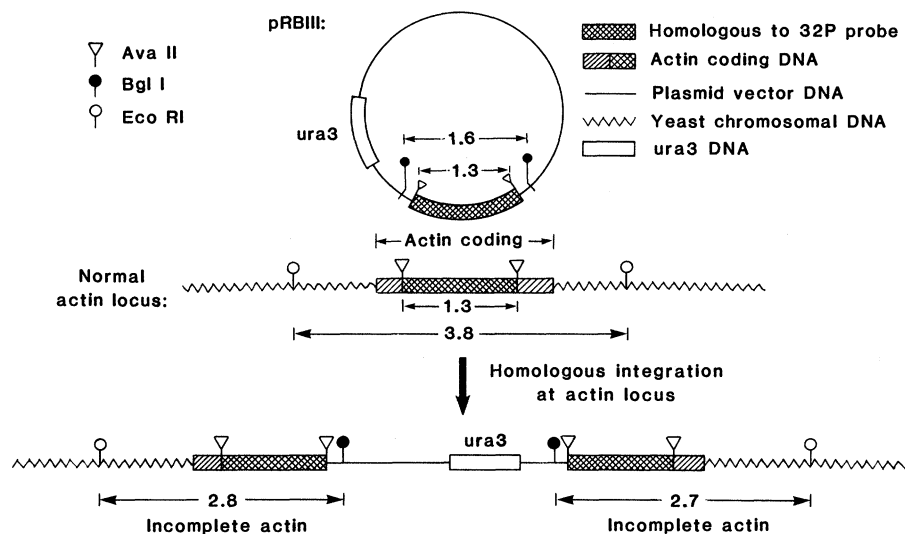


Fig. 1. Structure of the yeast actin locus before and after recombination with the hybrid plasmid pRB111. Positions of the Eco RI and Ava II restriction sites are taken from (2). Distances between relevant restriction sites are indicated in kilobase pairs. The structure predicted for the actin locus after recombination with pRB111 is confirmed by the data shown in Fig. 2. Plasmid construction involved ligation of the 1.3-kb Ava II fragment of the cloned actin gene (which contains the entire protein-encoding sequence except for deletion of the first $3\frac{1}{2}$ codons plus 50 base pairs of intervening sequence from the amino terminal end and the last nine codons from the carboxyl terminal end) into the yeast integrative cloning vector YIp-5 which had been linearized by Ava II cleavage in the presence of ethidium bromide (100 µg/ml). From among the amp^r tel^s bacterial transformants with this ligation mixture, pRB111 was identified as a hybrid plasmid with a single 1.3-kb fragment inserted into the Ava II site at position 1135 in the tetracycline resistance gene of pBR322.

Table 1. Results of tetrad analysis on the six yeast diploids transformed with pRB111. Procedures for growth of cells, sporulation, micromanipulation, and scoring of genetic markers were carried out by standard methods (15).

Trans-formant	Locus of integration	Viable spores per tetrad				Ratio of spores ura ⁺ :ura ⁻
		4	3	2	1	
1	Actin	0	1	11	3	0:28
2	Actin	0	0	16	1	0:33
5	Actin	0	0	9	0	0:18
6	Actin	0	0	10	1	0:21
3	Ura 3	13	3	0	0	30:31
4	Ura 3	5	3	0	0	15:14

been purified from extracts of yeast by deoxyribonuclease I affinity chromatography (4). Although the biological function of actin in a simple, nonmotile eukaryote-like yeast is not known, it may involve one or more of the activities attributed to cytoplasmic actins of higher organisms, such as maintenance of a cytoskeleton, organization of cytoplasmic membrane proteins, transport of materials within cells, or chromosomal movement (5, 6). Given the ease with which yeast can be manipulated both by classical genetics and recombinant DNA methods, analysis of the yeast actin gene through the use of mutations provides an unusual opportunity to study the molecular biology of this important eukaryotic protein.

If the function of actin is essential to the growth of yeast, it should be possible to induce both lethal and conditional lethal mutations in the actin gene. Conversely, the induction of recessive lethal mutations in the actin gene would suffice to demonstrate that the gene's function is an essential one.

A null mutation in the chromosomal locus corresponding to the cloned yeast actin gene was constructed (Fig. 1). A 1.3-kb Ava II restriction fragment which is internal to the protein coding region of the gene was inserted into the yeast plasmid vector YIp5. This transformation vector, a derivative of the *Escherichia coli* plasmid pBR322, carries the yeast *ura3* gene and can transform *ura3*⁻ yeast to prototrophy only by integration, via homologous recombination, into the yeast genome (7). Likewise, the hybrid plasmid (designated pRB111) carrying the Ava II actin fragment also can only transform *ura3*⁻ yeast to uracil-independence by integration. Since pRB111 carries sequences homologous to both the actin and *ura3* genes, integration can occur into either of these two chromosomal loci. Integration at the actin locus disrupts the protein-coding portion of the actin gene (Fig. 1). In effect, integration by a single homologous recombinational

event between the chromosomal actin gene and the internal fragment in pRB111 (which can be viewed as a double deletion mutant of the gene) results in a direct repeat of actin gene sequences in which only that portion of the gene carried by the plasmid is duplicated. Consequently, each of the repeated copies of the actin gene is now incomplete.

Disruption of an essential gene in this way should be a lethal event in a haploid strain. However, if the loss of gene function is, as expected, recessive, disruption of the gene in a diploid strain would result in a recessive lethal mutation, a genetic lesion easily detected by standard tetrad analysis. For this reason the diploid yeast strain DBY1091 (*a/α ade2/ + his4/ + can1-101 ura3-53/can^S ura3-52*) was transformed to uracil independence with pRB111 (8), and six independent transformants were studied further.

To determine the site of plasmid inte-

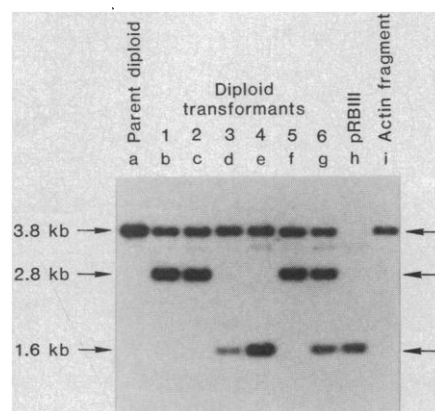


Fig. 2. Gel transfer analysis of six *ura3*⁻/*ura3*⁻ diploid yeast transformed to *ura3*⁺ with pRB111. Total DNA (2 μg) from each transformant was cleaved with restriction endonucleases Eco RI plus Bgl I. After electrophoresis on a 1.2 percent agarose gel, DNA fragments were transferred to nitrocellulose filter paper (10) and hybridized with a ³²P-labeled (10⁶ count/min) 1.3-kb Ava II fragment derived from the cloned yeast actin gene. (Lane h) 100 pg of plasmid pRB111 DNA cleaved with Eco RI and Bgl I; (lane i) 40 pg of the 3.8-kb Eco RI actin fragment cloned by Ng and Abelson (2).

gration in these transformants (that is, either the *ura3* or the actin locus), gel-transfer hybridization experiments with the method of Southern (9, 10) were carried out. The actin gene sequences on pRB111 are contained within a 1.6-kb segment flanked by Bgl I sites (Fig. 1). Integration into the actin locus has the effect of separating these two Bgl I sites. In a gel-transfer experiment using the Ava II actin fragment as probe, the 1.6-kb fragment should be absent, and in its place, two new fragments should appear. Integration into the *ura3* gene has a different consequence: the 1.6-kb Bgl I fragment should remain intact. In practice the experiment was carried out with total DNA isolated from the six diploid transformants and cleaved with Bgl I and Eco RI. The expected sizes of the fragments hybridizing with the radioactive probe, given the known arrangement of Bgl I and Eco RI cleavage sites at the actin locus are 3.8 kb for the intact locus, 2.8 and 2.7 kb for the disrupted gene after integration of pRB111, and 1.6 kb for the plasmid, either intact or integrated at *ura3*. Since the transformation recipient is diploid, all transformants should have the 3.8-kb band for the intact actin locus, while integrants at actin should acquire the 2.8- and 2.7-kb doublet as well; integrants at *ura3* should retain the 1.6-kb band.

The results of the gel-transfer analysis are shown in Fig. 2. Transformants 1, 2, and 5 show the loss of the band corresponding to the plasmid and the appearance of a new band estimated at about 2.8 kb relative to marker fragments on the gel. The 3.8-kb band representing the intact actin gene is present in these three transformants, and its intensity is about half that of the 2.8-kb band, supporting our conclusion that the latter is the expected, though unresolved, doublet. Thus transformants 1, 2, and 5 represent cases in which pRB111 was integrated into the actin gene. Transformants 3 and 4 show the pattern expected for integration at the *ura3* locus; namely the 1.6-kb and 3.8-kb bands are present. Transformant 6 shows an anomalous pattern: both the 1.6-kb band and the 2.8-kb doublet are present along with the 3.8-kb band. Since the 2.8-kb doublet can only arise by integration at the actin locus, we interpret this pattern as indicative of integration of two or more copies of the plasmid in tandem at the actin locus; the genetic data described below support this interpretation. Tandem integration of multiple copies of integrating vectors in yeast has been observed (11).

The crucial test of the idea that disruption of the actin gene is a lethal event is

the survival of haploid spores resulting from meiosis of diploids containing pRB111 integrated at actin. If such a null mutation is lethal, the spores that inherit the disrupted gene (and therefore the *ura3*⁺ allele carried on the plasmid) should fail to grow, while the spores that inherit the intact actin gene should grow and show a *ura*⁻ phenotype. The diploids carrying the plasmid integrated into the *ura3* gene serve as controls; they should all be viable and the *ura*⁺ phenotype should segregate 2:2.

The results of tetrad analysis are shown in Table 1. Transformants 3 and 4 (pRB111 integrated into the *ura3* gene) gave mainly four viable spores per ascus, with no tetrad having fewer than three. The *ura*⁺ phenotype segregated 2:2 and showed normal linkage to the *can1* locus, which resides on the same chromosome arm (linkage data not shown). Transformants 1, 2, 5, and 6 (all expected to have pRB111 integrated into the actin gene on the basis of the gel-transfer results) showed the pattern of viability expected for segregation of a recessive lethal mutation. Most importantly, none of the 100 spores were *ura*⁺, indicating very tight linkage of the lethal mutation to the integrated plasmid. The one exceptional tetrad with three viable spores was tested further and apparently represents a gene conversion event that resulted in removal of the lethal mutation as well as the *ura3*⁺ marker.

Our results indicate that disruption of the actin gene results in a recessive lethal mutation and justifies the conclusion that the actin gene encodes a function essential for germination of haploid spores. It seems very probable that the actin gene is also essential for vegetative growth of yeast cells, a conclusion supported by the construction (12) of three temperature-sensitive mutant alleles of the actin gene.

Since tetrad analysis is carried out by micromanipulation, the fate of individual spores that have inherited the disrupted actin gene could easily be followed by microscopy. Of 30 spores that failed to form microcolonies after 24 hours on a rich medium, 28 failed to bud at all and 2 possessed tiny buds. This result suggests a specific defect in bud emergence or in the initiation of a cycle of cell division.

The technique of gene disruption described above should provide a simple, definitive, and general method for inducing a null mutation in any yeast gene which has been cloned. In experiments very similar to those described above, it has been possible to demonstrate that the single yeast β -tubulin gene encodes an essential function (13). Gene disruption

results in an insertion mutation which is marked by the acquisition of a selectable character carried by the integrating plasmid. Null mutations of this type, therefore, can be stably maintained by selection in diploids and reliably scored in tetrad analysis. As a genetic tool, use of gene disruption mutants has many of the advantages characteristic of transposon insertion in prokaryotes (14), including complete loss of function, indissolubly linked selectable marker, and acquisition of additional DNA at the position of the mutation.

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Genetic Length of a Human Chromosomal Segment Measured by Recombination Between Two Fragile Sites

Abstract. *Two families were studied in which the same homolog of chromosome pair 10 expressed both the fragile sites on the long (q) arm at 10q23 and 10q25. Recombination between the fragile sites was observed in 3 of the 27 offspring in whom it could occur. The genetic length of chromosome between the fragile sites was 11 female centimorgans within a 95 percent probability interval of 4 to 28 centimorgans. This estimate of genetic length is comparable to those obtained with other methods.*

Fragile sites are morphological features of human chromosomes that can be recognized cytologically when lymphocytes are cultured under appropriate conditions (1). Since any fragile site is always expressed at the same locus in individuals from the same kindred, kindreds in which fragile sites are segregating can be used for gene mapping (2). Two fragile sites have been described on chromosome 10. One, in band 10q23, is expressed only under conditions of folic acid and thymidine deprivation (1), and the other, in band 10q25, is expressed only if bromodeoxyuridine or bromodeoxycytidine is present in the tissue culture medium for some hours before harvest (3). The discovery of two families in which both of these fragile sites were present on one chromosome (Fig. 1) provided an opportunity to measure the genetic length of the segment between the fragile sites by segregation analysis.

The two families are not known to be related to each other, although both are of British origin. The families were ascertained through mildly retarded girls, but other members of these families who have the fragile sites on chromosome 10 were normal. Examples of number 10 chromosomes expressing both the fragile sites are shown in Fig. 2. On G-banded preparations, one fragile site is at the distal end of 10q23 (1), probably at 10q23.32 or 10q23.33, and the other in the middle of 10q25 (3), probably at 10q25.2 (4).

The frequency of expression of the fragile sites is shown in Table 1. One member of family Ho, III-12, has been omitted from consideration because in the small number of cells available for examination, it was not possible to be sure that fra(10)(q25) was absent. Fragile sites were present on the same homolog of chromosome 10 in several individuals, including I-6 from family Ho and I-1