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Genetics

Construction and genetic characterization of temperature-sensitive mutant alleles of the yeast actin gene

(*in vitro* mutagenesis/gene replacement/*Saccharomyces cerevisiae*/fine-structure mapping)

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ABSTRACT **Two temperature-sensitive mutations have been constructed in the single actin gene (*ACT1*) of the yeast *Saccharomyces cerevisiae* by *in vitro* mutagenesis of the cloned gene followed by integrative transformation of mutagenized DNA into yeast cells. A strategy of allele replacement was used that allowed recessive mutations to be phenotypically expressed in the initial transformants, thus simplifying the screening of large numbers of independently transformed cells. After confirming that several *ts* mutations were located within the actin structural gene by genetic methods, these mutant alleles were cloned, and the altered amino acid residues were defined by DNA sequence analysis. The two unique mutations resulted in substitution of proline-32 with leucine and alanine-58 with threonine. In the course of isolating these mutations, the observation was made that a high proportion of yeast cells transformed with exogenous DNA by the spheroplast method are temperature sensitive for growth because of genetic changes unrelated to the transforming DNA.**

Actin is a major component of the eukaryotic cytoskeleton, forming the basic structural unit of a complex and dynamic network of microfilaments (1). In most eukaryotic cells, actin is typically present at levels of 5%–10% of soluble protein (2) and is encoded by multiple genes (3). In the yeast *Saccharomyces cerevisiae*, however, actin constitutes only 1% of the soluble cell protein and is encoded by a single gene, which has been cloned and sequenced (4–6). The amino acid sequence of yeast actin is highly homologous to vertebrate non-muscle actin, and the purified protein can undergo polymerization *in vitro* to form 7-nm filaments (7–9).

Little is known about the function of actin in yeast cells, although it is reasonable to presume that its function is similar to the function of actin in non-muscle cells of higher eukaryotes. Recently, it has been shown that function is essential in yeast by constructing a null mutation using the technique of integrative gene disruption and showing it to be a recessive lethal mutation (10).

The knowledge that a null mutation results in lethality is, unfortunately, insufficient to allow any real study of the mutant phenotype. Furthermore, it is by itself of little use in devising either a screening or a selection procedure for conditional-lethal alleles; all one knows is that one can look for conditional lethality, a phenotype that can result from mutations in any of several thousand genes. Therefore, it was necessary to use a different approach to obtain conditional-lethal mutations in the single yeast actin gene.

This paper reports the construction and genetic characterization of conditional-lethal mutations in the yeast actin gene. These mutations were obtained by mutagenizing *in vitro* the cloned gene and then inserting the mutagenized DNA into the yeast chromosome in such a way as to allow expression of the mutant phenotype even when it is recessive. The method of insertion is a modification of the strategy of integrative gene disruption (10). The route used to find actin mutants in yeast is applicable, quite generally, to any essential gene in yeast or bacteria.

MATERIALS AND METHODS

Strains. *Escherichia coli* strain BD1528 (*met⁻ hsdR⁻ hsdM⁺ supE supF ung⁻1 nadB7*) was a gift from Bruce Duncan. Yeast strains DBY947 (*α ade-201 ura3-52 SUC2⁺*), DBY1091 (*a/α +/his4 +/can1 +/ade2-101 ura3-52/ura3-52*), and JT150 (*a his4 ura3-52 tub2-104*) are essentially isogenic with strain S288C.

Yeast Methods. Growth medium, tetrad analysis, and testing for markers were essentially as described in the Cold Spring Harbor yeast manual (11). Benomyl resistance was scored on YPD plates containing 40 μg of benomyl per ml at 30°C (12). DNA transformation of yeast was by the spheroplast method (13) with the modified protocol given in ref. 11 but with 3% agar in the regeneration mixture. Fifty micrograms of sonicated salmon sperm DNA was routinely added to each transformation.

Plasmids. Plasmid pRB147 (see Fig. 2) was constructed by substitution of the 375-base-pair (bp) *Eco*RI/*Bam*HI fragment of YIp5 (14) with the 1.8-kilobase (kb) *Bam*HI/*Kpn* I fragment of the cloned actin gene of Ng and Abelson (6) with regeneration of the *Eco*RI site. Plasmid pRB151 was derived from YIp5 by insertion of a 3.8-kb *Eco*RI fragment, which includes the entire yeast gene plus ≈1 kb of flanking sequence on both ends. Plasmid pRB152 was generated by excision of the 322-bp *Bgl* II fragment from pRB151. Restriction enzyme cleavage, transformation of *E. coli*, and preparation of plasmid DNA were essentially as described (15).

***In Vitro* Mutagenesis.** Several micrograms of the 1.2-kb *Xho* I/*Eco*RI fragment from pRB147 was purified by gel electrophoresis (16). To isolate the sense strand of DNA from this fragment, the *Eco*RI end was selectively blocked to the action of exonuclease III by incorporation of dATP(αS) onto this end using the Klenow fragment polymerase (17), and the antisense strand was degraded from the *Xho* I end by the 3′-to-5′ action of exonuclease III. Likewise, the antisense strand was isolated by selective blockage of the *Xho* I end by incorporation of dTTP(αS). D-loops were formed between the single-stranded fragment and covalently closed circular pRB147 DNA using the *E. coli* RecA protein according to the reaction conditions and assay described (18). After phenol extraction and ethanol precipitation, ≈500 ng of DNA (95% of which had a D-loop structure) was incubated in 1 M $NaHSO_3$ (pH 6.0) for 20 min at 37°C (≈1% deamination), and it was then processed according to the standard protocol (18).

Preparation of High-Molecular-Weight Yeast DNA. Cells were harvested from 5 ml of an overnight stationary culture,

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Abbreviations: bp, base pair(s); kb, kilobase(s).

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resuspended in 0.35 ml of a solution containing 1 M sorbitol/0.1 M sodium citrate, pH 7.0/60 mM EDTA/50 mM 2-mercaptoethanol/0.5 mg of zymolase per ml (60,000 units/mg). After 40 min at 37°C, the resulting spheroplasts were pelleted and resuspended in 0.3 ml of 4.5 M guanidine hydrochloride/0.1 M EDTA/0.15 M NaCl, pH 8.0; 0.015 ml of 1% sarkosyl was added and the lysate was heated to 65°C for 10 min. The nucleic acid was precipitated by addition of 0.3 ml of cold ethanol and was redissolved in 0.32 ml of 100 mM Tris·HCl/10 mM EDTA, pH 8.0. Proteinase K was added to a final concentration of 0.2 mg/ml. After 1 hr at 65°C, the nucleic acid was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. The yield from 10^9 cells is ≈5 μg of DNA.

RESULTS

The Integrative Replacement/Disruption Strategy. Knowing that the actin gene, when disrupted, results in a recessive lethal mutation presented both opportunities and problems for the design of a scheme to isolate point mutations affecting actin. The major opportunity was the expectation that mutations with a conditional-lethal phenotype (e.g., temperature-sensitive growth) could be found. There were two major problems. (*i*) Conditional lethality is hardly a specific phenotype, and (*ii*) any scheme for screening phenotype involving introduction of mutagenized actin DNA into yeast would have to take into account the expected recessiveness of the mutations.

After mutagenizing the cloned gene *in vitro*, we sought actin mutants by replacing the normal gene with the mutagenized one and then screening for conditional-lethal mutations. We expected that by limiting mutagenesis to the actin gene we could circumvent the lack of a distinguishing phenotype beyond lethality: we expected that spontaneous temperature-sensitive mutations in other nuclear genes would be relatively rare in comparison to mutations induced in the actin gene.

However, this strategy left us with the problem of the expected recessiveness. With the method of Scherer and Davis (ref. 19; Fig. 1*A*), mutagenized actin DNA is introduced by integration of a circular vector into the chromosomal copy of the gene. Such integration results in a duplication of the gene, one copy being wild type and the other mutant. Therefore, recessive mutations would not be detected until the plasmid vector is excised in such a way as to leave the mutation on the chromosome. One must select integration (i.e., Ura^+ transformants if one uses the vector shown in Fig. 1*A*) and then excision (i.e., Ura^- segregants) before screening for temperature-sensitive lethality. Such a two-step procedure is feasible, but excessively laborious.

To avoid the step of screening for excision of the plasmid from independent transformants prior to screening, a new strategy, which we call "integrative replacement/disruption," was applied. As shown in Fig. 1*B*, the vector used for the construction of actin gene mutations carries, instead of the intact actin gene, a fragment of the gene that has one end within the gene and the other end outside the gene. Integration results in a duplication, but only one of the repeats has the entire actin gene. If the single crossover that results in integration of the vector occurs between a point mutation in the plasmid and the deleted end of the gene, then the mutation will be recombined into the intact copy on the chromosome, as shown in Fig. 1*B*. The wild-type information originally present on the chromosome is in the incomplete (disrupted) copy of the gene. Thus, the mutation on the plasmid, when integrated in this way, can be expressed even if it is recessive. To obtain conditional-lethal actin mutants, therefore, we only have to select URA^+ transformants and screen for temperature sensitivity. After isolation, the mutation can

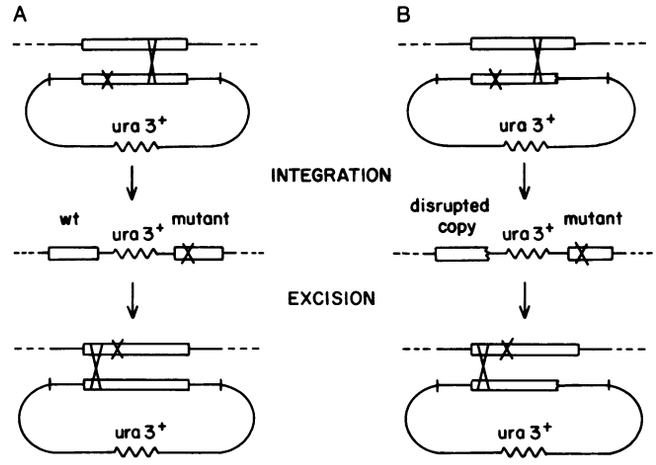


FIG. 1. Two strategies for recombining mutations constructed *in vitro* into the cellular gene.

be recovered in an actin gene of normal structure by excision of the plasmid, as with the method of Scherer and Davis (19). When the excisive crossover occurs in the region shown in Fig. 1*B*, the result is a single chromosomal copy of the actin gene bearing the mutation.

It is important to recognize that the position of the integrative crossover is significant. When the crossover occurs on one side of the mutation, as shown in Fig. 1*B*, the mutation ends up in the intact copy of the gene; however, when the crossover occurs on the other side of the mutation, then the integration results in the placement of the mutation in the disrupted copy of the gene. Consequently, only that fraction of transformed cells in which the mutation has been recombined into the intact copy will express the mutant phenotype. Similarly, the excisive crossover must fall in the correct interval if the mutation is to be left on the chromosome rather than on the plasmid. In practice, several isolates that have excised the plasmid must be screened to find those that have retained the mutation.

Isolation of Temperature-Sensitive Actin Mutants. Fig. 2 shows plasmid pRB147, which was designed to be used in integrative replacement/disruption mutagenesis of the actin gene. The plasmid was constructed from the standard *URA3* integrating vector YIp5 (14) and contains a 1.8-kb *Bam*HI/*Kpn* I fragment of the yeast actin gene (6). As shown diagrammatically in Fig. 2, this DNA fragment extends from a *Bam*HI site located several hundred base pairs upstream of the probable transcriptional control site (i.e., outside the gene) and extends through the amino-terminal four-fifths of the structural gene, ending at codon 301 (the *Kpn* I site). The carboxyl-terminal 73 amino acids are therefore missing from this fragment. When cleaved at the unique *Hin*dIII site located 128 bp in from the deleted end of the actin gene sequences, plasmid pRB147 transforms either haploid strain DBY947 or diploid strain DBY1091 at efficiencies of 1000 to 10,000 URA^+ transformants per μg of DNA. Except for their growth in the absence of uracil, such transformants are phenotypically indistinguishable from the $ura3^-$ parent, suggesting that the disrupted copy of the actin gene does not by itself have a significant phenotypic effect.

To generate a pool of mutant actin gene sequences that could be screened for conditionally lethal phenotypes by integrative replacement/disruption, *in vitro* mutagenesis was directed to the segment of pRB147 that extends from the *Xho* I site to the *Eco*RI site. This DNA segment includes most of the 309-bp intervening sequence (which interrupts codon 4) plus codons 5–301. By isolating each DNA strand from purified 1.2-kb *Xho* I/*Eco*RI fragment and using it as substrate, D-loops were formed with covalently closed pRB147 DNA

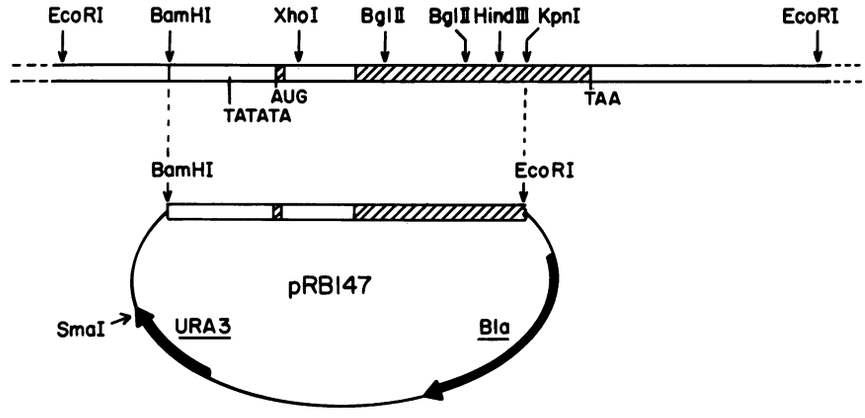


FIG. 2. Physical map of the yeast actin locus and plasmid pRB147.

in a reaction catalyzed by the recA protein of *E. coli*. Mutations were then directed to the nucleotide sequences exposed in the single-stranded loop by reaction with the mutagen sodium bisulfite.

To generate a pool of mutagenized plasmids with all mutational changes fixed in a homoduplex form, the bisulfite-treated DNA was transformed into *E. coli* strain BD1528, which is deficient in the repair enzyme uracil-*N*-glycosylase (*ung*$^-$). Plasmid DNA (pool 1) was prepared from a mixture of 4000 transformants obtained with pRB147 mutagenized to a level of 1–2 mutations per molecule on the sense strand within the *Xho* I/*Eco*RI interval. Similarly, pool 2 was derived from 8000 transformants with pRB147 mutagenized to a comparable level on the antisense strand.

In the first screen for conditionally lethal mutants, haploid strain DBY947 was transformed separately with 1 μg of *Hin*dIII-cleaved pool 1 or pool 2 DNA, and URA$^+$ transformants were selected at 26°C. Six hundred transformants from pool 1 and 600 from pool 2 were individually recovered from the regeneration agar by stabbing with a toothpick, and they were then scored for growth on minimal plates at 37°C, 26°C, and 17°C. Seven temperature-sensitive isolates were recovered from the pool 1 transformants (1.2%), and four were recovered from the pool 2 transformants (0.7%). No isolates that were cold-sensitive for growth were identified in this experiment.

To determine whether the temperature-sensitive mutation was genetically linked to the integrated plasmid, as predicted for actin gene mutations induced by the scheme outline in Fig. 1*B*, each *ts* isolate was mated to haploid strain JT150, and the linkage of the *ts* mutation to the *URA3*$^+$ marker and to the benomyl resistance marker [which marks the *TUB2* (12) locus approximately 1 cm from the actin gene] was determined by tetrad analysis. Of the 11 transformants analyzed, only 1 isolate from pool 1 showed the expected linkage. The remaining 10 unlinked *ts* mutations, which appeared at an exorbitantly high frequency among *Ura*$^+$ transformants, were induced or selected by the DNA transformation process.

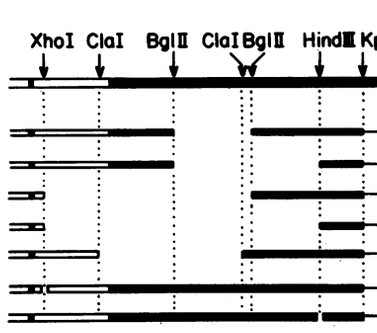
The temperature-sensitive mutant showing tight linkage to the integrated plasmid rapidly lost its Ts phenotype when scored at 37°C on YPD plates. In one experiment, 24 independent temperature-resistant revertants arising spontaneously on rich medium were checked for growth on minimal medium, and 23 were found to be *Ura*$^-$. Thus, loss of the temperature-sensitive phenotype correlates strongly with loss of the integrated plasmid. To obtain *ts*$^-$*ura*$^-$ segregants, the *ts* mutant was grown at 26°C in the presence of uracil, plated for single cells on uracil-containing plates, and screened for *ura*$^-$*ts*$^-$ colonies by replica plating. From ≈2000 cells screened, two such isolates were recovered, both of which no longer gave rise to temperature-resistant papillae on YPD plates. One of these isolates was mated to strain JT150, and linkage of the *ts* mutation to the *tub2* mutation was confirmed [parental ditype (PD)/nonparental ditype (NPD)/tetratype (T); 12:0:0]. The *ts* mutation in this strain was designated *act1-1*.

In the second screen for mutations in the actin gene, diploid strain DBD1091 was transformed separately with 1 μg of *Hin*dIII-cleaved pool 1 DNA and pool 2 DNA. Approximately 10,000 URA$^+$ colonies from each transformation were pooled and sporulated *en masse*. Random haploid spores were selected on canavanine plates without uracil, and colonies were screened by replica plating to three YPD plates and scored for growth at 37°C, 26°C, and 13°C. On retesting of several dozen candidates, three *ts* mutants were recovered from pool 1 transformants and two were recovered from pool 2 transformants. Again, no cells with a cold-sensitive phenotype were found. Tetrad analysis revealed one *ts* mutant from pool 2 (*act1-2*) and one from pool 1 (*act1-3*), which demonstrated tight linkage to the *URA3*$^+$ marker on the plasmid and to the benomyl resistance marker. Ts$^-$Ura$^-$ segregants of each mutant were isolated and linkage of the *ts* mutation to the *tub2* locus was confirmed for *act1-2* (PD/NPD/T; 31:0:1) and for *act1-3* (PD/NPD/T; 19:0:2).

Genetic Analysis of *act1-1*, *act1-2*, and *act1-3*. From a backcross of each *act1-ts* mutant to strain JT150, which is *ACT1*$^+$, a diploid heterozygous for the mutant was recovered and tested for growth at 37°C. In each case, growth at the high temperature was indistinguishable from that of an essentially isogenic *ACT1*$^+$/*ACT1*$^+$ diploid, indicating that all three mutations are recessive.

Haploid strains of opposite mating type were recovered from the above crosses, and diploids heteroallelic for each combination of actin mutations were constructed. When these three diploid strains (*act1-1/act1-2*; *act1-1/act1-3*; *act1-2/act1-3*) were tested at 37°C, growth was either extremely slow or absent, indicating that no significant complementation occurs among the three *act1* alleles and thus formally placing them in the same complementation group.

To establish that this single complementation group represents the single actin gene and not an unidentified gene in the vicinity of *ACT1* or *TUB2*, a complementation test was carried out by DNA transformation on an *act1-1* strain with either an integrating plasmid carrying an intact actin gene (pRB151) or an identical plasmid except for a deletion of the 322 bp between the two *Bgl* II sites in the actin gene (pRB152) shown in Fig. 2. The integration of each plasmid was directed to the *URA3* locus by transforming with plasmid DNA cleaved at the unique *Sma* I site in the 1.1-kb *URA3* segment, thereby imposing a requirement for complementation in *trans* of the Ts phenotype.



	Ts⁺ transformants / Total transformants		
	act1-1	act1-2	act1-3
BglII	37/64	36/64	22/32
BglII + HindIII	20/32	28/32	26/32
XhoI + BglII	0/32	0/32	0/32
XhoI + HindIII	0/32	ND	ND
ClaI	0/64	0/32	0/64
XhoI	34/64	18/31	22/31
HindIII	27/32	31/32	29/32

FIG. 3. Deletion mapping of *act1* mutations by transformation with linear pRB147 DNA with defined ends.

All 13 Ura$^+$ transformants obtained with pRB152 (actin gene deletion) were temperature sensitive. However, of the 11 Ura$^+$ transformants with pRB151 (intact actin gene), 9 were Ts$^+$ and 2 were Ts$^-$. [The 2 Ts$^-$ transformants presumably were *Ura3*$^+$, as a result of a gene conversion event at the *URA3* locus instead of plasmid integration (20).] The site of plasmid integration was confirmed for 3 of the Ts$^+$ transformants by crossing to a *URA3*$^+$ strain and analyzing segregation of Ura3 and the Ts phenotype. As expected for plasmid integration at the *URA3* locus, all spores from each of these crosses were Ura$^+$. In addition, one-half of the benomyl-sensitive spores (which presumably also inherited the tightly linked *act1-1* allele) were temperature resistant, providing further evidence for an unlinked suppressor of the *act1-1* allele segregating 2:2. Because an integrated plasmid that has a defined deletion within the actin-coding sequences does not complement *act1-1*, whereas a similar plasmid with an intact actin gene does complement, the conclusion can be drawn that the Ts defect of *act1-1*, and therefore of *act1-2* and *act1-3*, is due to a mutational change in the structural gene for actin.

To localize the mutation in these three *ts* alleles to a defined segment of the actin gene, a deletion-mapping method suggested by Orr-Weaver *et al.* (20) was used. This method is based on two properties of the transformation of yeast with linear DNA molecules. (*i*) Circular plasmid DNA cleaved once within a region of yeast sequences is highly recombinogenic with homologous chromosomal sequences, and (*ii*) contiguous segments of DNA that have been deleted from the recombinogenic ends of a linear DNA are replaced by a copy mechanism during the integration of the plasmid, to yield two complete repeats. As shown in Fig. 3, several restriction enzymes that cleave plasmid pRB147 either once or twice can be used to delete specific segments of the actin gene sequences and to generate linear molecules with ends that are both still within the actin gene region. When the restriction enzyme cleavages excise sequences that are the wild-type counterpart of an actin gene mutant allele, the missing segment is copied from the chromosomal copy, and all transformants will remain mutant. However, when the restriction enzyme cleavage does not excise the wild-type counterpart to a particular mutant allele, recombination can potentially regenerate a wild-type gene during the integration (see ref. 20 for a more complete discussion). Thus, as with conventional deletion mapping, the absence of wild-type recombinants between a mutation and a specific deletion localizes the mutation within that deletion interval.

The results of mapping *act1-1*, *act1-2*, and *act1-3* using pRB147 that had been cleaved to remove defined segments of the actin gene are listed in Fig. 3. In all cases in which wild-type recombinants appeared, they represented >50% of the total transformants scored. For each of the mutant alleles, no *ts*$^+$ recombinants were formed in transformations involving deletion of the *Cla* I/*Bgl* II interval from 246 bp inside the intervening sequence to codon 93, a result that genetically positions all three mutations into this physical interval.

Cloning and DNA Sequence Analysis. To determine the nucleotide-sequence change in each mutant allele, a fragment of DNA carrying the mutant actin gene was cloned by the strategy of Roeder and Fink (21). This strategy involves positioning of an *E. coli* plasmid plus a selectable marker into a chromosomal site near the actin gene by integrative transformation, followed by cleavage of DNA purified from transformed cells with a restriction enzyme that will generate one large fragment, which includes the *E. coli* plasmid, selectable marker, and the actin gene sequences. After cyclization of this fragment with DNA ligase and transformation into *E. coli*, a hybrid plasmid is recovered. To specifically clone the relevant segment of each mutant actin allele, integration of plasmid pRB123 (12) was targeted to the neighboring *TUB2* gene by cleaving the DNA at the unique *Bst*EII site located in the *TUB2* segment (12). An appropriate *act1* mutant strain was then transformed by selection for the *URA3*$^+$ marker, and high-molecular-weight DNA purified from individual transformants was cleaved with *Hin*dIII, ligated with T4 DNA ligase, and transformed into *E. coli* by selection for ampicillin resistance. From a ligation of 2 μg of *Hin*dIII-cleaved chromosomal DNA, a total of 240 transformants were recovered.

The predicted structure of the isolated hybrid plasmids, pNB12 (*act1-1*), pNB13 (*act1-2*), and pNB15 (*act1-3*), was confirmed by restriction analysis with several enzymes and by comparison with the known restriction map of the *TUB2–ACT1* region of yeast chromosome 6. Allele *act1-1* was also cloned into a λ phage vector, identified by plaque hybridization using the cloned gene as a probe, and then subcloned into pBR322.

The sequence in the *Cla* I/*Bgl* II interval was determined for each cloned mutant gene by the method of Maxam and Gilbert (22). For *act1-1* and *act1-3*, the same two mutational changes were found: proline-32 (CCA) to leucine (CTA) and a silent third position change asparagine-12 (AAC) to (AAT). Since both alleles were recovered from transformants with pool 1 DNA in which the sense strand had been mutagenized, they are consistent with bisulfite-induced C·G to T·A changes. For *act1-2*, the mutation alanine-58 (GCT) to threonine (ACT) was the only nucleotide sequence change found, a change that is consistent with a bisulfite-induced C·G to T·A mutation induced on the antisense strand in pool 2.

DISCUSSION

By application of *in vitro* mutagenesis to the cloned actin gene of yeast plus DNA transformation, two mutations have been constructed that alter single amino acids at the amino-terminal end of the actin protein and confer on cells a temperature-sensitive growth phenotype. Although both muta-

tions have been shown to be recessive, they were identified by their Ts phenotype within a population of transformed yeast cells. The expression of a recessive phenotype in the initial transformants resulted from the fact that, as it integrates into the genome, the transformation vector both inactivates the wild-type cellular gene and recombines the mutational change from a cloned gene fragment on the vector into the only intact gene copy. Such integrative replacement/disruption vectors with the now standard gene transplacement strategy (19) should be generally applicable to the targeted mutagenesis of any cloned yeast gene, particularly those essential genes for which no specific selection or screen is available.

Unfortunately, the large majority of mutations uncovered by screening transformants for temperature-sensitive growth were not in the gene undergoing mutagenesis. These untargeted mutations, which appeared at the extraordinary frequency of 1%, presumably represent a background of random mutagenesis either resulting from or selected by the DNA transformation protocol. Possible mutagenic agents are the sonicated carrier DNA used to enhance the transformation frequency or a transient state of uracil (and thymine) starvation. Unless this background of random mutations can be avoided, other genetic tests, such as tetrad analysis or complementation with known mutations, must be used to identify the subset of mutations that falls within the cloned gene.

Finally, temperature-sensitive mutations are powerful tools for analyzing the functional roles of a gene of interest. Not only can temperature-shift studies be applied to characterize the morphological and biochemical consequences of loss of gene function, but extragenic suppressors of the Ts phenotype can also be selected. Suppressor mutations that have a cold-sensitive phenotype can often be studied free of the mutation being suppressed in order to define and characterize new genes that encode functions related to the *ts* mutant gene (23).

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