The EMBO Journal vol.6 no.1 pp.235–241, 1987

# Identification and sequence of the gene encoding cytochrome *c* heme lyase in the yeast *Saccharomyces cerevisiae*

**Mark E.Dumont[1], Joachim F.Ernst[2,3], D.Michael Hampsey[2] and Fred Sherman[1,2]**

Departments of [1]Biochemistry and [2]Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA, and [3]Biogen S.A., Division of Molecular Biology, CH-1227 Geneva, Switzerland

Communicated by G.Schatz

**Mitochondrial cytochrome *c* contains a heme group covalently attached through thioether linkages to two cysteinyl residues of the protein. We demonstrate here that the nuclear gene, *CYC3*, in the yeast *Saccharomyces cerevisiae*, encodes cytochrome *c* heme lyase (CCHL), the enzyme catalyzing the attachment of heme to apocytochrome *c*. Mitochondrial extracts from *cyc3*$^-$ mutants are deficient in CCHL activity compared with extracts from normal strains, whereas strains carrying multiple copies of the *CYC3* gene exhibit high levels of the activity. The *CYC3* gene was cloned by functional complementation of a *cyc3*$^-$ mutant using a previously isolated plasmid containing the gene *PYK1*, which is tightly linked to *CYC3*. An open reading frame encoding a protein of 269 amino acids was identified from the DNA sequence of a fragment encompassing the *CYC3* gene, and the corresponding transcript shown to be ~0.9 kb in length. CCHL appears to be a single polypeptide chain which acts specifically on the two forms of cytochrome *c*, but not on cytochrome $c_1$.**

*Key words:* cytochrome *c* heme lyase/cytochrome *c* synthetase/*CYC3*/mitochondrial import

## Introduction

Cytochrome *c* contains two stereospecific thioether bonds linking the heme (protoheme IX) to the protein. Formation of these bonds, which does not occur spontaneously under physiological conditions, is tightly coupled to the folding of cytochrome *c* (Fisher *et al.*, 1973), as well as to the transport of cytochrome *c* from its site of synthesis in the cytoplasm into mitochondria (Hennig and Neupert, 1981). An enzymatic activity capable of adding heme to apocytochrome *c* has been observed in a number of cell-free systems derived from mitochondrial fractions (Korb and Neupert, 1978; Matsuura *et al.*, 1981; Basile *et al.*, 1980). However, low abundance and the lability of this activity, denoted cytochrome *c* heme lyase (CCHL) (Harmey and Neupert, 1985) or cytochrome *c* synthetase (Basile *et al.*, 1980), have hindered its purification and further characterization (Taniuchi *et al.*, 1983; Visco *et al.*, 1985).

*CYC2* and *CYC3* are nuclear genes of *Saccharomyces cerevisiae* that affect the levels of the two isozymes of cytochrome *c*, but not other mitochondrial cytochromes. These genes are not linked to *CYC1* or *CYC7*, the structural genes encoding, respectively, iso-1-cytochrome *c* and iso-2-cytochrome *c* (Rothstein and Sherman, 1980). Thus, *CYC2* and *CYC3* have been presumed to encode cellular components required for some aspect of cytochrome *c* biosynthesis. Because *cyc3*$^-$ strains contain the normal wild-type levels of cytochrome *c* mRNA, this gene cannot be acting at the transcriptional level (Laz *et al.*, 1984). The detection of apo-iso-2-cytochrome *c* in *cyc3*$^-$ mutants (Matner and Sherman, 1982) demonstrated that this mutation does not block synthesis of apocytochrome *c*. While apo-iso-1-cytochrome *c* was lacking in the *cyc3*$^-$ mutants, the apo form of this isozyme appears to be unstable and, unlike apo-iso-2-cytochrome *c*, cannot be detected in normal or other mutant strains grown under a variety of conditions (Matner and Sherman, 1982). These findings, together with the nearly total absence of holocytochrome *c* found in some *cyc3*$^-$ mutants, suggested that *CYC3* might encode an obligatory step in the post-translational processing of *cyc3*$^-$, such as amino-terminal processing, methylation of lysine residues or addition of the heme. Only the amino-terminal methionine residue is removed from iso-1-cytochrome *c*, and mutations in the *CYC1* gene which prevent cleavage of this methionine do not significantly affect iso-1-cytochrome *c* levels (Sherman and Stewart, 1973; Stewart and Sherman, 1974). Furthermore, *cyc3*$^-$ mutants contain normal levels of cytochrome *c* methylase activity (Liao and Sherman, 1979). Thus, heme attachment seemed a possible function for *CYC3*.

To find out whether *CYC3* encodes CCHL, we have examined the ability of cell extracts from certain yeast strains to add heme to apocytochrome *c*. In this paper, we report that mitochondrial fractions from *cyc3*$^-$ mutants are deficient in CCHL activity whereas such fractions from a strain carrying multiple copies of the *CYC3* gene exhibit increased CCHL activity. These findings demonstrate that *CYC3* encodes CCHL. Furthermore, we have isolated and sequenced genomic DNA carrying *CYC3*, allowing identification of an open reading frame corresponding to this protein.

## Results

### *Demonstration that CYC3 encodes cytochrome c heme lyase*

The phenotype of *cyc3*$^-$ strains had previously led to the suggestion that *CYC3* encodes CCHL, the enzyme catalyzing the addition of heme to apocytochrome *c* (Matner and Sherman, 1982). To test this possibility, CCHL activity was measured in mitochondrial extracts from various related yeast strains having mutations at the *CYC3* locus. In contrast to the method originally described by Basile *et al.* (1980) and Veloso *et al.* (1981), the activities were determined by directly measuring incorporation of labeled heme into apocytochrome *c*, without determining the protease susceptibility of the labeled protein. This assay proved to be extremely sensitive and specific, as shown in Table I (Experiment II). If the mitochondrial fraction was heated to 90°C for 30 min before the incubation with heme and apocytochrome *c*, or if exogeneous apocytochrome *c* was omitted from the incubation, only very low levels of $^{55}$Fe co-purified with the cold carrier holocytochrome *c*. However, as previously observed (Veloso *et al.*, 1981), the efficiency of heme incorporation was low. Even in the presence of excess apocytochrome *c*, ~2% of the added $^{55}$Fe-heme was incorporated into holocytochrome

© IRL Press Limited, Oxford, England

**Table I.** CCHL activity of mitochondrial fractions from various yeast strains

| Strain | Pertinent genotype | Cytochrome *c* heme lyase | | Approximate relative cytochrome *c* content (%) | $CYC3^+$ copy number |
|---|---|---|---|---|---|
| | | Specific activity (c.p.m./mg) | Relative activity (%) | | |
| | Experiment I | | | | |
| D311-3A | $CYC1^+$ $CYC2^+$ $CYC3^+$ | 30 | 100 | 100 | 1 |
| B-614 | $CYC1^+$ $CYC2^+$ *cyc3-10* | 4 | 13 | 5 | 0 |
| B-619 | $CYC1^+$ $CYC2^+$ *cyc3-15* | 1 | 3 | 5 | 0 |
| B-466 | *cyc1-17* $CYC2^+$ $CYC3^+$ | 12 | 40 | 5 | 1 |
| B-620 | $CYC1^+$ *cyc2-8* $CYC3^+$ | 12 | 40 | 15 | 1 |
| | Experiment II | | | | |
| B-7034 | $CYC1^+$ $CYC2^+$ $CYC3^+$ | 32 | 100 | 100 | 1 |
| B-6868-1 | $CYC1^+$ $CYC2^+$ $CYC3^+$ [pAA268] | 270 | 840 | 100 | Multiple |
| B-6868-1 (Heated) | $CYC1^+$ $CYC2^+$ $CYC3^+$ [pAA268] | 5 | 16 | 100 | Multiple |
| B-6868-1 (No apocytochrome *c*) | $CYC1^+$ $CYC2^+$ $CYC3^+$ [pAA268] | 0.1 | 0.3 | 100 | Multiple |

*c* in assays of extracts from normal strains. Incorporation rose to 10% in the assays of extracts from the strain overproducing *CYC3* (see below). This may reflect low levels of CCHL activity in the mitochondrial extracts or the fact that conditions for heme incorporation into cytochrome *c* have not yet been optimized in this *in vitro* system.

Experiment I of Table I demonstrates that mitochondrial extracts from two independently isolated $cyc3^-$ yeast strains exhibit an ~10-fold decrease in CCHL specific activity, relative to extracts from the parent strain. This substantial decrease cannot be a secondary effect of the lack of holocytochrome *c* from the mitochondria of these strains because two $CYC3^+$ strains that partially lack cytochrome *c* retain significantly higher levels of CCHL activity. However, a lesser reduction in CCHL activity observed in mitochondrial extracts of $cyc1^-$ and $cyc2^-$ strains may reflect a minor dependence of CCHL biosynthesis on cytochrome *c* levels. Strain B-466, carrying a *cyc1* mutation, is completely deficient in iso-1-cytochrome *c*, but contains iso-2-cytochrome *c* at ~5% of the total wild-type levels of holocytochrome *c*. This is about the same amount of holocytochrome *c* found in one of the $cyc3^-$ mutants, yet B-466 mitochondria retain 40% of the wild-type activity of CCHL, a much higher level than that seen in the $cyc3^-$ strains. Similarly, the $cyc2^-$ strain B-620, contains only 15% of the normal complement of holocytochrome *c*, yet it also exhibits ~40% of the CCHL activity seen in normal strains.

Mitochondrial extracts from $cyc3^-$ strains exhibited low but significant levels of CCHL activity compared with assays from which apocytochrome *c* was omitted. This low activity is consistent with the fact that the $cyc3^-$ strains used in these experiments are not completely deficient in cytochrome *c*. These strains, rather than strains completely lacking cytochrome *c*, were chosen because they are more physiologically related to the control strains, allowing for more meaningful comparisons.

Transformation of a replicating plasmid containing the *CYC3* gene under control of the actin promoter (see below) into the normal yeast strain, B-7034, resulted in a significant increase in CCHL activity (Table I, Experiment II). In order to be able to compare the growth conditions of the transformed and untransformed strains, mitochondria were harvested from yeast containing the plasmids under growth conditions where ~40% of the plasmids had been lost through growth under non-selective conditions (see Materials and methods). Thus, the CCHL activity in cells containing the full complement of plasmids would probably be even higher than that shown in Table I.

Significant levels of CCHL specific activity were found in cell fractions other than mitochondria. For instance, activities only slightly lower than the mitochondrial levels were found in the cell debris, as was also reported by Taniuchi *et al.* (1983). The levels of activity in the various fractions were roughly proportional to those of the mitochondria when comparing different strains (results not shown). The presence of activity in other than mitochondrial fractions cannot be interpreted as indicating the presence of the enzyme in other cellular compartments *in vivo* until other explanations, such as redistribution of CCHL and its substrates and co-factors during cell fractionation, can be ruled out. However, the presence of significant activities in non-mitochondrial fractions, and the alterations in these activities with the gene dosage of *CYC3* demonstrate that the observed differences between strains do not result from variations in the details of the cell fractionations from strain to strain. In addition, differences between $CYC3^+$ and $cyc3^-$ strains comparable with those shown in Table I have been observed using mechanical disruption of cells (Mason *et al.*, 1973) rather than the lysis of protoplasts (Duell *et al.*, 1964) (results not shown).

### *Identification of the CYC3 gene*

*CYC3* had previously been shown (Rothstein and Sherman, 1980) to map on chromosome I, ~2 cM from *PYK1*, the gene encoding pyruvate kinase. Thus, it seemed possible that *CYC3* might fortuitously be present on previously isolated plasmids containing *PYK1* (Kawasaki and Fraenkel, 1982; Burke *et al.*, 1983). The plasmid pPYK1, obtained from D.Fraenkel, contains a 10.4-kb fragment of yeast genomic DNA encompassing the gene *PYK1*. To test for the ability of this plasmid to complement a defect in *CYC3*, pPYK1 was transformed into the $cyc3^-$ yeast strain ER53/155. Transformants selected for leucine prototrophy were then tested for the ability to grow on lactate as the sole carbon source in liquid culture and on plates. Such transformants grew well under these conditions while the untransformed, $cyc3^-$, host cells showed only the residual growth typical of cells with low amounts of cytochrome *c*. Low temperature (−196°C) spectroscopic examination (Sherman and Slonimski, 1964) of cells grown on synthetic medium lacking leucine, to prevent plasmid loss,

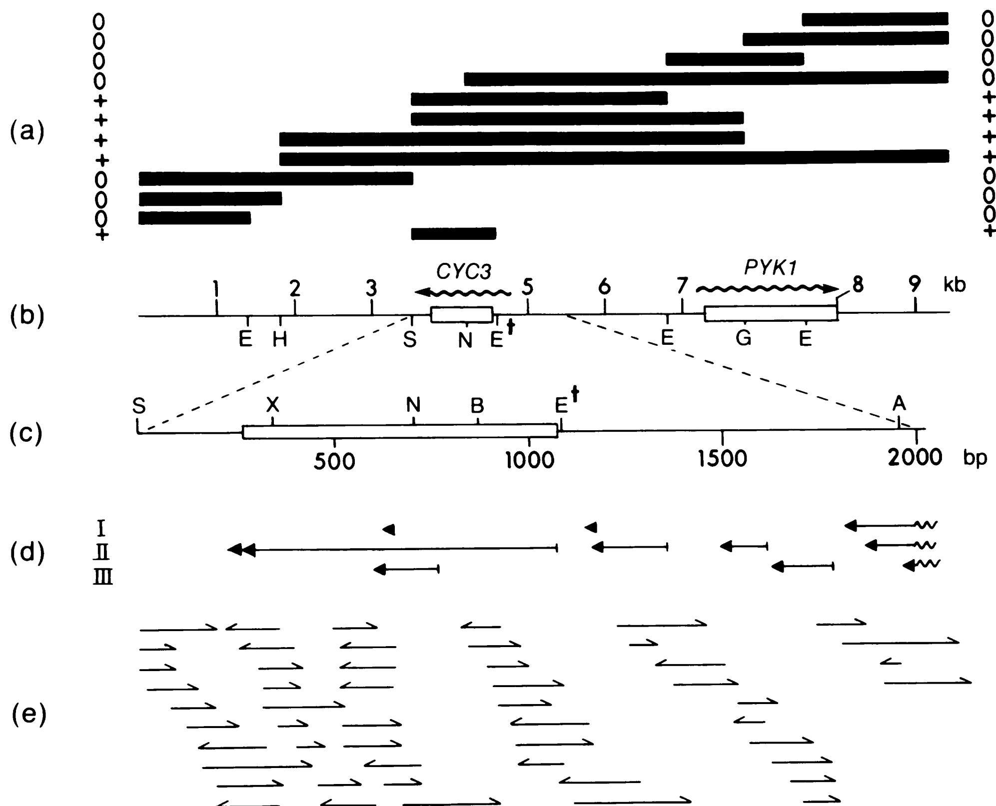


**Fig. 1.** Cloning and characterization of the *CYC3* gene. **(a)** The indicated restriction fragments were cloned into replicating plasmids. These plasmids were transformed into the yeast strain ER53/155 to test for the ability to complement the *cyc3* mutation. The plasmids used for cloning the 12 fragments shown in the figure were, from top to bottom: pAB107 (see text); YEp13 (Broach *et al.*, 1979b); pAB107; YEp13; pEX-6 (J.Ernst, unpublished results); YEp13; YEp13; YEp13; YRp7 (Botstein *et al.*, 1979); YEp13; pAB107; and pEX-7 (see text). A '+' signifies that the plasmid could complement a *cyc3* mutation, whereas a 'O' signifies that it could not. **(b)** Map of the region containing *CYC3* on chromosome I. The open boxes indicate the translated regions of *CYC3* and *PYK1*. Wavy arrows represent the corresponding mRNAs. Restriction sites are abbreviated as follows: E, *Eco*RI; $E^+$, *Eco*RI site introduced by *in vitro* mutagenesis; H, *Hin*dIII; S, *Sal*I; N, *Nco*I; G, *Bgl*II; X, *Xba*I; B, *Bal*I; and A, *Ava*I. **(c)** The region of the genome that was sequenced. The open box shows the position of the *CYC3* open reading frame. **(d)** The open reading frames of all three reading frames (I, II and III) of the transcribed strand. **(e)** The fragments that were sequenced.

```
-357  CTGCGACAAAAGTGTGACCGAATTACAAATTTAGAAGGCAAAAAAAAAAAAAAAGAAAAAAAAGTTGTACGTATGGTAGCGCCGCATATACTATGTAGTAGTAATATTGAAATATCTCT
-238  TGCGCGGCGTGGGATCTTCCGCCCCCCCCACCCAAATTTTCTATTTTTTCTTTTTCTTTTCGCTTACCTCTTTTTATCGACTTTTTCCCTTTTTTCTTTTCATCGGGAAATGATTATCA
-119  AAAAATTTCTCAGCTTTAAATGCGAATACTAAGAGAAATGAAATAGGCAGCTAATAAGATAGCATACACATACGAGTAGCACGAATAAAGCAGAGCAAATACAACAGAAACTACAAAAA

   1  ATG GGT TGG TTT TGG GCA GAT CAA AAA ACT ACG GGC AAA GAT ATT GGT GGG GCA GCA GTA TCA TCC ATG TCA GGG TGC CCA GTC ATG CAC
      Met Gly Trp Phe Trp Ala Asp Gln Lys Thr Thr Gly Lys Asp Ile Gly Gly Ala Ala Val Ser Ser Met Ser Gly Cys Pro Val Met His   30

  91  GAG TCG TCG TCG TCG TCG CCA CCA TCC TCT GAG TGC CCC GTT ATG CAG GGA GAT AAC GAT AGA ATA AAC CCG CTG AAC AAT ATG CCG GAG
      Glu Ser Ser Ser Ser Ser Pro Pro Ser Ser Glu Cys Pro Val Met Gln Gly Asp Asn Asp Arg Ile Asn Pro Leu Asn Asn Met Pro Glu   60

 181  TTG GCA GCA TCC AAA CAG CCT GGC CAA AAG ATG GAC TTG CCC GTT GAT CGG ACC ATC TCC AGC ATC CCC AAG AGT CCA GAC AGT AAC GAG
      Leu Ala Ala Ser Lys Gln Pro Gly Gln Lys Met Asp Leu Pro Val Asp Arg Thr Ile Ser Ser Ile Pro Lys Ser Pro Asp Ser Asn Glu   90

 271  TTC TGG GAG TAT CCT TCT CCA CAA CAG ATG TAC AAT GCT ATG GTT AGA AAG GGC AAG ATT GGC GGT AGC GGC GAA GTC GCC GAA GAT GCA
      Phe Trp Glu Tyr Pro Ser Pro Gln Gln Met Tyr Asn Ala Met Val Arg Lys Gly Lys Ile Gly Gly Ser Gly Glu Val Ala Glu Asp Ala  120

 361  GTG GAG TCC ATG GTG CAG GTC CAC AAC TTT CTA AAT GAA GGG TGC TGG CAG GAA GTG CTC GAA TGG GAA AAA CCG CAC ACA GAT GAA AGC
      Val Glu Ser Met Val Gln Val His Asn Phe Leu Asn Glu Gly Cys Trp Gln Glu Val Leu Glu Trp Glu Lys Pro His Thr Asp Glu Ser  150

 451  CAC GTG CAG CCT AAG TTG CTG AAA TTC ATG GGG AAA CCG GGC GTA TTG AGC CCT CGT GCT CGC TGG ATG CAC CTG TGC GGC CTA CTG TTT
      His Val Gln Pro Lys Leu Leu Lys Phe Met Gly Lys Pro Gly Val Leu Ser Pro Arg Ala Arg Trp Met His Leu Cys Gly Leu Leu Phe  180

 541  CCG TCC CAT TTT AGC CAA GAA CTA CCA TTC GAC AGG CAC GAC TGG ATT GTA CTC CGA GGC GAG CGC AAA GCG GAA CAA CAA CCT CCA ACC
      Pro Ser His Phe Ser Gln Glu Leu Pro Phe Asp Arg His Asp Trp Ile Val Leu Arg Gly Glu Arg Lys Ala Glu Gln Gln Pro Pro Thr  210

 631  TTC AAG GAA GTT AGA TAC GTC TTG GAT TTC TAC GGA GGG CCC GAC GAC GAA AAC GGA ATG CCT ACT TTC CAC GTG GAT GTC CGT CCT GCC
      Phe Lys Glu Val Arg Tyr Val Leu Asp Phe Tyr Gly Gly Pro Asp Asp Glu Asn Gly Met Pro Thr Phe His Val Asp Val Arg Pro Ala  240

 721  CTA GAT AGT CTA GAC AAT GCT AAG GAC CGG ATG ACC CGT TTC TTG GAC CGG ATG ATC TCG GGT CCG TCC TCT TCG TCC TCC GCC CCT TAA
      Leu Asp Ser Leu Asp Asn Ala Lys Asp Arg Met Thr Arg Phe Leu Asp Arg Met Ile Ser Gly Pro Ser Ser Ser Ser Ser Ala Pro End  269

 811  ATGATATACAGCCAGCGTAAGTACGTGTAAATAGCCAAGTAAGAAATAATGATGTCCTAGTGCAGCCACAATCAATTTCACTTTTTCATATATATTTTTCCACCTTATTATCACAAGGT
 930  GCACCTTTATCTGTGCCACGGCGGTAAAAAACTACCTTCAGGGGTACGATACATTCTGTGCTGGCGACCACGGGGCTGACAGAGACACCCGTAGAGGCTACATTACTGATTTGGGAAAT
```

**Fig. 2.** The DNA sequence of the *CYC3* gene and the deduced amino acid sequence of CCHL. The numbers at the left refer to nucleotide positions, with the A of the ATG initiation codon designated as number 1. The numbers at the right refer to amino acid positions. An asterisk appears above every tenth nucleotide of the untranslated regions.

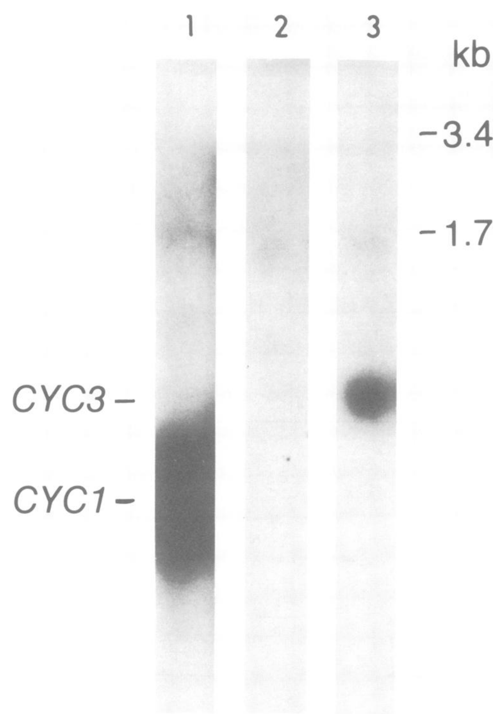


**Fig. 3.** Identification of the *CYC3* transcript using strand-specific probes; 1.7 and 3.4 kb denote the positions of 18S and 25S rRNAs, respectively. **Lane 1** was probed with single-stranded bacteriophage M13 containing an insert complementary to *CYC1* mRNA. **Lane 2** was probed with M13mp8 containing a 2.9-kb *Eco*RI–*Sal*I insert from the region of plasmid pPYK1 encompassing the *CYC3* gene. **Lane 3** was probed with M13mp19 containing a 2.9-kb *Eco*RI–*Sal*I insert consisting of the opposite strand of DNA from the insert used in the probe for **lane 2**, thus establishing that this M13mp19 insert is complementary to *CYC3* mRNA.

also demonstrated the presence of wild-type levels of cytochrome *c* in the transformants, compared with 5% or less in the untransformed host.

The *CYC3* gene was localized by subcloning pPYK1 DNA restriction fragments and testing for the ability of these fragments to complement the *cyc3* defect. The results of this work are shown in Figure 1. The smallest such fragment capable of complementing the *cyc3* defect in ER53/155 was a 2.9-kb *Eco*RI–*Sal*I fragment situated 5′ to the *PYK1* gene.

The DNA sequence of a 2-kb segment from the 2.9-kb pPYK1 insert was determined as shown in Figure 1. The DNA was fragmented by sonication; fragments ~0.5 kb in length were purified by agarose gel electrophoresis and inserted into the *Sma*I site of bacteriophage M13mp8. The sequenced region contained two major open reading frames, one on each strand. Northern analysis was used to determine which of the two strands is transcribed. Strand-specific probes were constructed as described in Materials and methods. Autoradiograms of these probes hybridized to blots of RNA gels clearly show the presence of RNA sequences complementary to only one of the probes (Figure 3). This is consistent with transcription of the strand of DNA containing the reading frame shown in Figure 2. The observed transcript is ~0.9 kb in length, which is just sufficient to encode the protein shown in Figure 1. This *CYC3* mRNA is present in low abundance, ~1% of the level of the *CYC1* mRNA (Figure 3). The open reading frame shown in Figure 2 is the only one on this strand that is large enough to encode an enzyme. The next largest could encode only 71 amino acids, and is also excluded for other reasons (see below).

To facilitate further manipulations involving the major open reading frame, an *Eco*RI site was constructed by site-directed mutagenesis between bases −9 and −12 using the numbering scheme shown in Figure 2. The 1.1-kb *Eco*RI–*Sal*I fragment of the modified plasmid was inserted into a yeast expression vector created by one of us (J.F.Ernst, unpublished results) such that transcription of the open reading frame shown in Figure 2 was driven by the yeast actin promoter. This plasmid, pAA268, which contained both 2$\mu$ and *ARS1* origins of replication, as well as $amp^R$ and *URA3* markers, was transformed into the $cyc3^-$ yeast strain ER53/155. Such transformants showed growth on lactate medium, demonstrating expression of *CYC3*. In addition, as described above, high levels of CCHL activity were observed in normal strains into which this plasmid, pAA268, had been transformed. Thus, *CYC3* must be contained within the 1.1-kb *Eco*RI–*Sal*I fragment, ruling out expression of the second largest reading frame on the transcribed strand (see Figure 1).

## Discussion

We report here the identification of *CYC3* as a gene encoding a protein involved in covalent attachment of heme to cytochrome *c*. This conclusion is based on the significant decrease in CCHL activity observed in mitochondrial extracts from yeast carrying the *cyc3* mutations, as well as the increase in CCHL activity observed in yeast transformed with the *CYC3* gene on a multicopy plasmid under control of the actin promoter. The simplest interpretation of these results is that CCHL is encoded entirely by the *CYC3* gene. If *CYC3* encoded a subunit of a larger complex that catalyzed heme attachment to cytochrome *c*, overproduction of the *CYC3* gene alone would not be expected to lead to an increase in CCHL activity. There remains the possibility, however, that *CYC3* encodes a regulatory component of a multi-protein complex, or that the activity of the *CYC3* protein is a rate-determining step in a multi-step pathway for mitochondrial import and heme attachment. Since extracts from $cyc2^-$ strains contained much higher levels of CCHL activity than those from $cyc3^-$ strains, *CYC2* does not appear to be directly involved in heme addition to cytochrome *c*.

Visco *et al.* (1985) have suggested that yeast may contain two different forms of CCHL, each with its own substrate specificity. Although both of these would be capable of adding heme to apocytochrome *c*, only one would carry out heme addition to apocytochrome $c_1$. Since some *cyc3* mutations exhibit a complete absence of detectable holocytochrome *c*, <0.1% of the normal amount, it is unlikely that there are two enzymes capable of converting apocytochrome *c* to holocytochrome *c in vivo*. Loss of a co-factor or modification of the enzyme during the solubilization used to resolve the two activities (Visco *et al.*, 1985) might explain the different specificities. In addition, the presence of normal levels of cytochrome $c_1$ in the $cyc3^-$ mutants, including those mutants exhibiting the most complete deficiencies in cytochrome *c*, demonstrates that the CCHL activity described here is not involved in heme attachment to cytochrome $c_1$.

The sequence of *CYC3* shown in Figure 2 contains several features that are characteristic of coding regions of yeast genes. An A-rich tract 24 bp in length is found starting at position −318. A T-rich region consisting of short T tracts extends from position −202 to −140 (Struhl, 1985). The 24-base region immediately 5′ to the ATG is A-rich and contains only a single G. The −3 position contains an A (Kozak, 1981). Possible TATA boxes are found at positions −270, −256 and −68 (Hahn *et al.*, 1985). Typical yeast transcriptional termination sequences are seen be-

**Table II.** UAS-like sequences of the *CYC3* gene compared with identified UAS sequences of the *CYC1* and *CTT1* genes

| Gene | Position of the 5′ nucleotide[a] | Sequence | Reference |
|---|---|---|---|
| *CYC3* | (−232) | C T C T T G C G C G G C G T G G G A | (This paper) |
| *CYC3* | (−170) | C T C T T T T T A T C G A C T T T T | (This paper) |
| *CYC1* | (−271) | C T C T T T G G C C G G G G T T T A | (Guarente *et al.*, 1984) |
| *CYC1* | (−228) | C T C T T T G G C G A G C G T T G G | (Guarente *et al.*, 1984) |
| *CTT1* | (−437) | C T C T C C T G C G T G C T T T C A | (Spevak *et al.*, 1986) |
| *CTT1* | (−388) | C T C T T T T T C A A G G G G A T C | (Spevak *et al.*, 1986) |

[a]The indicated sequence numbers refer to the numbering scheme in the referenced publication.

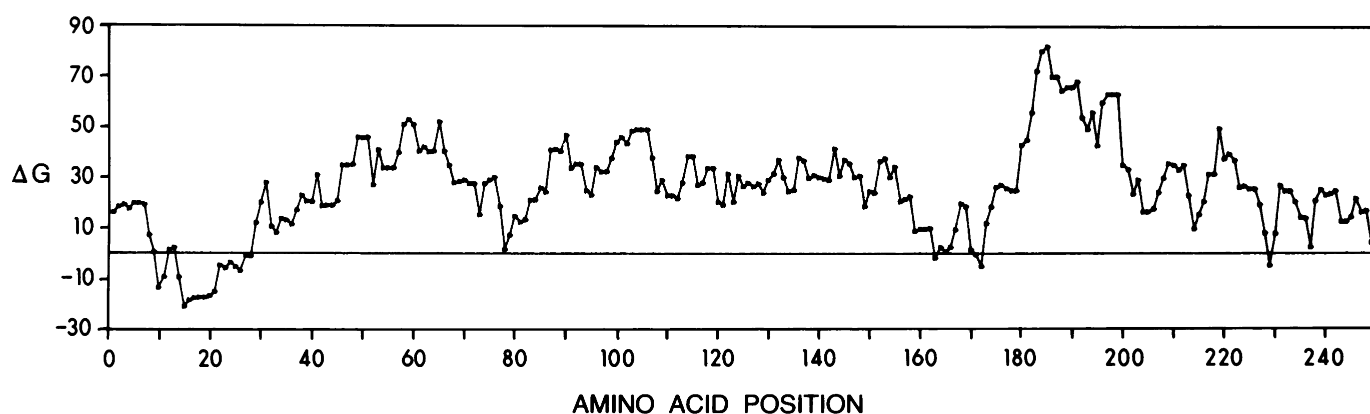


**Fig. 4.** Hydrophobicity profile of the *CYC3* protein. The program of Engelman *et al.* (1982) was used to calculate the free energy of insertion of 20 amino acid segments of the protein into membranes, assuming folding as an α-helix.

ginning at positions 842 (TAG), 868 (TAGT) and 886 (TTT) (Zaret and Sherman, 1982).

Two sequences, starting at −232 and −170, exhibit considerable homology with UAS (upstream activator sequence) segments which have been implicated in controlling transcription of *CYC1* (iso-1-cytochrome *c*) (Guarente *et al.*, 1984) and *CTT1* (catalase T) (Spevak *et al.*, 1986), in response to the levels of catabolites and heme (see Table II). We are currently investigating the possibilty that such upstream sequences regulate *CYC3* expression in parallel with the regulation of the gene for its substrate, cytochrome *c*.

The open reading frame we have identified for *CYC3* codes for a protein of 269 amino acids with a predicted mol. wt of 30 081 daltons. Codon usage of the *CYC3* protein is not biased (58 of 61 possible coding triplets are used), suggesting that the protein is not abundant (Bennetzen and Hall, 1982). This is in agreement with the low abundance of the *CYC3* mRNA seen in Figure 3. The amino terminus of the protein contains both acidic and basic residues, in contrast to the basic amino termini of many proteins that are imported into mitochondria (Douglas *et al.*, 1986). At least one imported protein, however, is known to have acidic residues at the amino terminus (van Loon *et al.*, 1984). An unusual feature of the predicted *CYC3* sequence is a run of serine residues starting at residue 32. A similar run of five serines is found at the COOH end of the protein, starting at residue 263.

Several internal homologies have been found in the coding region of the *CYC3* protein. A 13-bp duplicated sequence, with one mismatch, occurs at nucleotides 744−756 and 765−777 coding for the repeated amino acid sequence Asp-Arg-Met. A second in-frame duplicated sequence of 12 bp with one mismatch occurs at nucleotides 302−313 and 734−745, coding for the amino acids Asn-Ala. A third, out-of-frame 9-bp duplication appears at nucleotides 709−717 and 782−790.

Figure 4 shows the free energy for insertion of 20 amino acid segments of the sequence into the membrane (Engelman *et al.*, 1982). From this representation, it is apparent that *CYC3* is predominantly hydrophilic. Only residues in the region 14−40 would be expected to show a significant tendency to insert into the membrane as an α-helix. van Loon *et al.* (1986) have proposed that hydrophobic stretches near the carboxy-terminal ends of presequences of proteins destined for mitochondria act as 'stop transfer' sequences, thereby targeting these proteins to the intermembrane space. However, a 20-residue membrane-inserted segment in this region would have to contain at least a glutamic acid and a histidine as well as a minimum of five serine residues. Although some membrane proteins with relatively hydrophilic sequences are capable of interacting with membranes (Dumont and Richards, 1984; Chen *et al.*, 1979), the lack of obvious membrane-spanning regions in *CYC3* is consistent with the view presented by Harmey and Neupert (1985) of CCHL as a soluble protein residing in the mitochondrial intermembrane space. Addition of heme to partially transported apocytochrome *c*, according to these authors, could trigger cytochrome *c* folding, providing energy to pull the molecule through the membrane. The isolation, sequencing and overexpression of the gene for *CYC3*, reported here, should facilitate purification and characterization of CCHL, allowing experimental verification of such hypotheses.

## Materials and methods

### *Yeast strains*

The strains B-614, B-619, B-466 and B-620, listed in Table I, contain independent *CYC* mutations derived from the normal laboratory strain, D311-3A (*MATa lys2 his1 trp2*) (Rothstein and Sherman, 1980). B-466 contains an ochre mutation, *cyc1-17*, and is devoid of iso-1-cytochrome *c* (Sherman *et al.*, 1974). B-7034 (*MATa ura3-52 leu2-3, 112 his3-Δ1 trp1-289 cyh$^{R}$2*) contains wild-type *CYC* loci (S.Baim, unpublished results). B-6868-1 is the strain resulting from the transformation of B-7034 with the plasmid pAA268, which contains *CYC3* under control

of the actin promoter. ER53/155 (*MATa cyc3-10 can1-100 lys2 his1 his3-Δ1 leu2-3,112 ura3-52 trp1-289*) is a meiotic segregant from a strain obtained by crossing B-614 with strains containing *ura3-52* and *leu2-3,112* markers.

The cytochrome *c* contents of the strains listed in Table I were estimated by low temperature (−196°C) spectroscopy of whole cells (Sherman and Slonimski, 1964). Intensities of the $c_\alpha$ bands were standardized against spectra of strains containing known amounts of cytochrome *c*.

*Plasmids*

The plasmid pPYK1 was obtained from D.Fraenkel (Kawasaki and Fraenkel, 1982). pAB107 was a derivative of YIp5 (Struhl *et al.*, 1979) containing an *Eco*RI−*Hin*dIII fragment of the yeast origin of replication *ARS1* (S.Baim, unpublished results).

The replicating plasmid pAA268 was derived from the plasmid pEX-2 (Ernst and Chan, 1985) by way of a second plasmid, pEX-7. To make pEX-7, the *Eco*RI site bordering *ARS1* in pEX-2 was destroyed by cleavage and filling in the cohesive ends. The actin promoter was then introduced into this plasmid on a *Bam*HI−*Eco*RI fragment as a replacement for the *CYC1* promoter. To create the *Eco*RI site in the actin gene, the 'A' at the −4 position preceding the actin ATG codon was changed to a 'C' by joining a filled-in *Eco*RI site to a *Bal*31 end (J.F.Ernst, unpublished results). In the final step in the construction of pAA268, the short *Eco*RI−*Hin*dIII fragment containing *CYC3* was cut from the *in vitro* mutagenized plasmid described in Results and ligated to the large *Eco*RI−*Hin*dIII fragment of pEX-7.

*Growth media*

Lactate medium (Sherman *et al.*, 1974), 'dropout' medium lacking leucine or uracil (Sherman *et al.*, 1981), and YPD (Sherman *et al.*, 1981) were used as described previously. YP1%D medium was identical to YPD except that glucose was present at 1%. Fermentor medium contained 2% yeast extract, 2% bactopeptone, 1% glucose, 0.01% streptomycin, 0.005% ampicillin, 0.5% ethanol and 0.2% polyethylene glycol (P2000, MCB Chemicals).

*Isolation of yeast cellular fractions*

The strains from which extracts were to be assayed for CCHL activity were first grown overnight in 1 l of YP1%D. Aliquots of this pre-culture were used to inoculate an 11-l fermentor (New Brunswick Scientific, Model SF-116). Fermentor growths were allowed to go through 5−6 generations before being harvested at an optical density of 8−9 at 600 nm, corresponding to a cell density of 0.5−1 × $10^8$ cells/ml.

The strain B-6868-1, containing *CYC3* on a replicating plasmid, was maintained on a synthetic medium lacking uracil in order to prevent loss of the plasmid. The host strain, B-7034, was also initially cultured in synthetic medium, but in this case containing uracil. However, the final growth of both these strains was carried out in the fermentor medium described above.

Mitochondrial fractions were isolated essentially as described by Duell *et al.* (1964). After two washes in distilled water, a portion of the cells was set aside for the determination of total cellular protein by the boiling Biuret method of Strickland (1951), standardized against bovine serum albumin (BSA). Each cell fractionation was begun with cells corresponding to 10−12 g of protein at a concentration of 50−60 mg/ml. Following spheroplasting, cell lysis was carried out in 3 ml of 'lysis buffer' [0.25 M sucrose, 0.05 M potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)] per gram of wet cells. All subsequent steps except the final resuspension of the mitochondrial pellet was carried out on ice and in the presence of 1 mM PMSF. Mitochondria and other cellular fractions, as described by Basile *et al.* (1980), were frozen in 'M buffer' (20% sucrose, 50 mM potassium phosphate, pH 7.0, 1 mM EDTA) at −70°C and within 24 h of harvesting the cultures.

*Enzymatic assay for CCHL*

The assay for CCHL activity was a modification of that of Basile *et al.* (1980). Lyophilized apocytochrome *c* was dissolved in 0.15 M β-mercaptoethanol, 50 mM potassium phosphate, pH 8.0, to a concentration of 3.5 mg/ml and incubated at room temperature for 3 h. To begin the assay, 200 μl of freshly thawed mitochondrial fraction containing 20−50 mg of protein were mixed with 38 μl of $^{55}$Fe-hemin (5−7 × $10^4$ c.p.m., comprising ~1 nmol). To this, 75 μl of a solution of 6 mM NADPH (Sigma, type I, freshly dissolved), 24 mM isocitric acid (Sigma, freshly dissolved), 6.8 mg/ml isocitrate dehydrogenase (Sigma, type IV) and 24 mM $MgCl_2$ were added, followed by 38 μl of the apocytochrome *c* solution. This reaction mixture was incubated at 30°C for 40 min with occasional agitation.

Labeled cytochrome *c* was extracted from the assay mixture in the presence of cold carrier holo-iso-1-cytochrome *c* (Basile *et al.*, 1980; Sels *et al.*, 1965). Following the 30°C incubation, 15 μl of holocytochrome *c* (33 mg/ml in M buffer), 25 μl of 50% glycerol, 150 μl of ethyl acetate and 15 mg of solid $Na_2S_2O_4$ were added to each sample. Samples were subjected to vigorous agitation for 1 h at 32°C, followed by the addition of 0.75 ml of 0.75 M NaCl and 0.1 M potassium phosphate, pH 7.0, to each. The samples were centrifuged for 5 min at 4°C in an Eppendorf microfuge at 15 000 *g*. The supernatant solution was collected and the pellet resuspended in 0.4 ml of 0.5 M NaCl and centrifuged again under the same conditions. The two supernatants were pooled and centrifuged for 45 min at 15 000 *g* and 4°C. The supernatant fraction from this centrifugation was diluted in 25 ml of 1 mM $K_2Fe(CN)_6$, 10 mM potassium phosphate, pH 7.0, and subjected to ion-exchange chromatography (Basile *et al.*, 1980). Holocytochrome *c* was eluted in 1.5 ml of 0.5 M potassium phosphate, pH 8.0.

Cytochrome *c* was precipitated with trichloroacetic acid (TCA) in order to concentrate the protein and as an additional purification designed to remove non-covalently bound heme. Following addition of concentrated TCA to a final concentration of 10% (w/v), the samples were held on ice for 1 h, then centrifuged at 15 000 *g* for 15 min. The pellet was washed in 1 ml of 10% TCA, then twice in 1 ml of acetone, followed each time by centrifugation at 15 000 *g* for 5 min. The final pellet was resuspended in 40 μl of 50 mM sodium phosphate buffer, pH 8.0, containing 1% SDS, mixed by vortexing, then incubated at 90°C for 10 min. The radioactivity was measured by transferring the resuspended samples to scintillation vials containing Aquasol™ (New England Nuclear) and counting in a Beckman LS7000 set to the tritium counting mode. Specific activity of CCHL in a given yeast extract was calculated as c.p.m. per mg of protein in the extract. Protein concentrations of the yeast fractions were determined by the method of Peterson (1977), using BSA for calibration.

Iso-1-cytochrome *c* was purified from commercial bakers' yeast as described previously (Matner and Sherman, 1982). Apo-iso-1-cytochrome *c* was prepared from the holo-protein by the method of Fisher *et al.* (1973). Some difficulty was encountered in achieving complete removal of the heme from the yeast protein. For this reason, apocytochrome *c* to be used for the CCHL assays was cycled twice through the heme removal procedure. The sulfhydryl determination of Ellman (1959) demonstrated the presence of 2.5 free sulfhydryl groups per molecule of apocytochrome *c*, based on the protein concentration determined using the extinction coefficient for the apoprotein of 1.35 × $10^4$ at 276 nm (Basile *et al.*, 1980). Apocytochrome *c* contains three cysteine residues, but these cannot usually be quantitatively detected by this procedure (Basile *et al.*, 1980).

$^{55}$Fe-labeled heme was synthesized from protoporphyrin IX (Sigma) and $^{55}FeSO_4$ (New England Nuclear) using the method described by Falk (1964). The labeled product was extracted into ether at pH 4.0, washed with 5% (w/v) HCl, and concentrated to dryness as described by Colleran and Jones (1973). Labeled heme was stored dry at −20°C, but could be maintained frozen in 0.1 N NaOH for several weeks without affecting the efficiency of the CCHL assay. Radiochemical purity of the $^{55}$Fe-heme was checked by t.l.c. in lutidine:water (5:3.5) on silica gel (Kodak Chromogram). Autoradiograms of the chromatograms showed a single radioactive spot co-migrating with a standard of unlabeled hemin (Aldrich Chemical). No radioactivity could be detected at the origin of migration in exposures which were long enough to saturate the density of the spot corresponding to labeled hemin, demonstrating the absence of unreacted heme from the purified product.

*Recombinant DNA procedures*

Standard procedures were used for the construction and amplification of plasmids (Maniatis *et al.*, 1982). Transformation into yeast was accomplished as described (Sherman *et al.*, 1981). Sequencing was carried out using M13 shotgun techniques (Messing, 1983).

*In vitro* mutagenesis, to create an *Eco*RI site just upstream of the transcribed portion of the gene, made use of the 2.9-kb *Eco*RI−*Sal*I fragment cloned into pUC18 (Yanisch-Perron *et al.*, 1985). The gapped plasmid technique (Oostra *et al.*, 1983) was used to insert the mutation.

*RNA analyses*

RNA from the normal yeast strain was prepared as described by Broach *et al.* (1979a) except that washed cells were resuspended in buffer containing 10 mM iodoacetic acid and the cells were disrupted in the presence of phenol:chloroform: isoamyl alcohol (50:50:1). Northern analyses were performed as described by Zaret and Sherman (1982).

Strand-specific DNA probes were prepared by inserting the 2.9-kb *Eco*RI−*Sal*I DNA fragment encompassing *CYC3* (Figure 1) into the vectors M13mp8 and M13mp9 (Yanisch-Perron *et al.*, 1985). Single-stranded DNA was prepared and radiolabeled by primer extension using [α-$^{32}$P]dATP and a hybridization probe−primer (Biolabs) such that polymerization of the complementary strand was in a direction away from the *CYC3*-containing insert, leaving the insert single-stranded (Hu and Messing, 1982). Labeled probe was separated from unincorporated dATP by gel chromatography over a column of Sephadex G50. Final specific activities were 1−2 × $10^8$ c.p.m./μg.


## Acknowledgements

We thank Ms Sandra Consaul for technical assistance, particularly in the preparation of iso-1-cytochrome *c*. We are also grateful to Dr Michael Douglas of the University of Texas Health Science Center and Dr Hiroshi Taniuchi of the National Institutes of Health for critically reading the manuscript. This research was supported in part by the United States Public Health Service Grant R01 GM12702 and Training Grant T32 GM07098 from the National Institutes of Health.

## References

Basile,G., DiBello,C. and Taniuchi,H. (1980) *J. Biol. Chem.*, **255**, 7181–7191.
Bennetzen,J.L. and Hall,B.D. (1982) *J. Biol. Chem.*, **257**, 3026–3031.
Botstein,D., Falco,S.C., Stewart,S.E., Brennan,M., Scherer,S., Stinchcomb,D.T., Struhl,K. and Davis,R.W. (1979) *Gene*, **8**, 17–24.
Broach,J.R., Atkins,J.F., McGill,C. and Chow,L. (1979a) *Cell*, **16**, 827–839.
Broach,J.R., Strathern,J.N. and Hicks,J.B. (1979b) *Gene*, **8**, 121–133.
Burke,R.L., Tekamp-Olson,P. and Najarian,R. (1983) *J. Biol. Chem.*, **258**, 2193–2201.
Chen,R., Kramer,C., Schnidmayr,W. and Henning,U. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5014–5017.
Colleran,E.M. and Jones,O.T.G. (1973) *Biochem. J.*, **134**, 89–96.
Douglas,M.G., McCammon,M.T. and Vassarotti,A. (1986) *Microbiol. Rev.*, **50**, 166–178.
Duell,E.A., Inoue,S. and Utter,M.F. (1964) *J. Bacteriol.*, **88**, 1762–1773.
Dumont,M.E. and Richards,F.M. (1984) *J. Biol. Chem.*, **259** 4147–4156.
Ellman,G.L. (1959) *Arch. Biochem. Biophys.*, **82**, 70–77.
Engelman,D.M., Goldman,A. and Steitz,T. (1982) *Methods Enzymol.*, **88**, 81–88.
Ernst,J.F. and Chan,R.K. (1985) *J. Bacteriol.*, **163**, 8–14.
Falk,J.E. (1964) *Porphyrins and Metalloporphyrins.* Elsevier Publishing Co., NY, p. 135.
Fisher,W.R., Taniuchi,H. and Anfinson,C.B. (1973) *J. Biol. Chem.*, **248**, 3188–3195.
Guarente,L., Lalonde,B., Gifford,P. and Alani,E. (1984) *Cell*, **36**, 503–511.
Hahn,S., Hoar,E.T. and Guarente,L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8562–8566.
Harmey,M.A. and Neupert,W. (1985) In Martonosi,A. (ed.), *The Enzymes of Biological Membranes.* Plenum Press, NY, Vol. **4**, pp. 431–464.
Hennig,B. and Neupert,W. (1981) *Eur. J. Biochem.*, **121**, 203–212.
Hu,N. and Messing,J. (1982) *Gene*, **17**, 271–277.
Kawasaki,G. and Fraenkel,D.G. (1982) *Biochem. Biophys. Res. Commun.*, **108**, 1107–1112.
Korb,H. and Neupert,W. (1978) *Eur. J. Biochem.*, **91**, 609–620.
Kozak,M. (1981) *Nucleic Acids Res.*, **9**, 5233–5252.
Laz,T.M., Pietras,D.F. and Sherman,F. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4475–4479.
Liao,H.H. and Sherman,F. (1979) *J. Bacteriol.*, **138**, 853–860.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, NY.
Mason,T.C., Poyton,R.O., Wharton,D.C. and Schatz,G. (1973) *J. Biol. Chem.*, **248**, 1346–1354.
Matner,R.R. and Sherman,F. (1982) *J. Biol. Chem.*, **257**, 9811–9821.
Matsuura,S., Arpin,M., Margoliash,E., Sabatini,D. and Morimoto,T. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4368–4372.
Messing,J. (1983) *Methods Enzymol.*, **101**, 20–79.
Oostra,B.A., Harvey,R., Ely,B.K., Markham,A.F. and Smith,A.E. (1983) *Nature*, **304**, 456–459.
Peterson,G.L. (1977) *Anal. Biochem.*, **83**, 346–356.
Rothstein,R.J. and Sherman,F. (1980) *Genetics*, **94**, 871–889.
Sels,A.A., Fukuhara,H., Pere,G. and Slonimski,P.P. (1965) *Biochim. Biophys. Acta*, **95**, 486–502.
Sherman,F. and Slonimski,P.P. (1964) *Biochim. Biophys. Acta*, **90**, 1–15.
Sherman,F. and Stewart,J.W. (1973) In Pollack,J.K. and Lee,J.W. (eds), *The Biochemistry of Gene Expression in Higher Organisms.* Australia and New Zealand Book Co., Pty. Ltd., Sydney, Australia, pp. 56–86.
Sherman,F., Stewart,J.W., Jackson,M., Gilmore,R.N. and Parker,J.H. (1974) *Genetics*, **77**, 255–284.
Sherman,F., Fink,G. and Hicks,J. (1981) *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press,NY.
Spevak,W., Hartig,A., Meindl,P. and Ruis,H. (1986) *Mol. Gen. Genet.*, **203**, 73–78.
Stewart,J.W. and Sherman,F. (1974) In Prakash,L., Sherman,F., Miller,M.W., Lawrence,C.H. and Taber,H.W. (eds), *Molecular and Environmental Aspects of Mutagenesis.* Charles C.Thomas Publishers Inc., Springfield, IL, pp. 102–127.
Strickland,L.H. (1951) *J. Gen. Microbiol.*, **5**, 698–703.
Struhl,K. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8419–8423.
Struhl,K., Stinchcomb,D.T., Scherer,S. and Davis,R.W. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1035–1039.
Taniuchi,H., Basile,G., Taniuchi,M. and Veloso,D. (1983) *J. Biol. Chem.*, **258**, 10963–10966.
van Loon,A.P.G.M., De Groof,R.J., De Haan,M., Dekker,A. and Grivell,L. (1984) *EMBO J.*, **3**, 1039–1043.
van Loon,A.P.G.M., Brandli,A.W. and Schatz,G. (1986) *Cell*, **44**, 801–812.
Veloso,D., Basile,G. and Taniuchi,H. (1981) *J. Biol. Chem.*, **256**, 8646–8651.
Visco,C., Taniuchi,H. and Bartlett,B.S. (1985) *J. Biol. Chem.*, **260**, 6133–6138.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.
Zaret,K.S. and Sherman,F. (1982) *Cell*, **28**, 563–573.

*Received on 17 September 1986; revised on 28 October 1986*