Sequence Requirements for Mitochondrial Import of Yeast Cytochrome c*

(Received for publication, November 17, 1995, and in revised form, January 9, 1996)

Xiaoye Wang†, Mark E. Dumont, and Fred Sherman§

From the Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Cytochrome c is synthesized in the cytoplasm, transported to the mitochondrial intermembrane space, and subsequently covalently attached to heme in a reaction catalyzed by the enzyme cytochrome c heme lyase. We have investigated the amino acid sequences in cytochrome c which are required for mitochondrial import, using a systematic series of site-directed alterations of the CYC7-H3 gene which encodes iso-2-cytochrome c in the yeast Saccharomyces cerevisiae. Import of the altered apocytochromes c was assayed in yeast strains that overexpressed cytochrome c heme lyase. Under these conditions, there was efficient mitochondrial accumulation of forms of apocytochrome c which are incapable of having heme covalently attached. In fact, all apocytochromes c containing deletions located to the carboxyl-terminal side of His19 efficiently accumulated in the mitochondria of strains overexpressing heme lyase, even though all but one of these deletion-containing proteins were incapable of heme attachment. A minimum length of polypeptide chain at the extreme amino terminus of cytochrome c, rather than any specific sequence element in this region, appears to be required for efficient mitochondrial import. Certain amino acid substitutions in the region extending from Gly15 to Leu18, at residue Phe19 and at residue His27, lead to reduced mitochondrial import of apocytochrome c, resulting from stalling of the altered apocytochrome c in partially imported states.

Most mitochondrial proteins, including cytochrome c, are encoded in the nucleus, synthesized in the cytoplasm, and then transported across one or more mitochondrial membranes. For several of these imported mitochondrial proteins, the sequences that direct them to mitochondria, and to the correct subcompartment of mitochondria, have been identified. Such sequences are generally located near the amino terminus of the imported protein, often as part of a presequence that is cleaved during or after import.

Cytochrome c is translocated into the mitochondrial intermembrane space from its site of synthesis in the cytoplasm along a pathway that differs in several respects from pathways followed by other, more extensively studied, mitochondrial proteins that are targeted to the same mitochondrial subcompartment. Cytochrome c is initially synthesized as apocytochrome c, lacking heme. During or after import into mitochondria, heme is covalently attached via stereospecific thioether linkages to two cysteine residues in the protein. This reaction is catalyzed by the enzyme cytochrome c heme lyase (CCHL), which is located in the mitochondrial intermembrane space, predominantly associated with the inner membrane. Heme attachment is accompanied by a transition from a partially extended conformation to the native compact structure. Cytochrome c does not contain any cleaved targeting sequence. No protease-sensitive receptor on the external surface of mitochondria mediates the initial binding and import of apocytochrome c. Cytochrome c import does not depend on the presence of an electrochemical potential across the inner mitochondrial membrane or on the presence of ATP (for review see Hartl et al., 1989; Stuart and Neupert, 1990; Kiebler et al., 1993; Hannavy et al., 1993; Dumont, 1995).

The signals in the cytochrome c sequence which are responsible for the mitochondrial import have not been well characterized previously. Since cytochrome c does not contain a cleavable signal presequence at the amino terminus of the protein, the import signal must be a part of the mature sequence. Furthermore, sequences important for import are unlikely to reside at the extreme amino terminus of cytochrome c, since deletions and replacements of up to 11 residues at the amino terminus of iso-1-cytochrome c of yeast (Sherman and Stewart, 1973; Baim et al., 1985; Hampsey et al., 1988) do not prevent normal targeting and maturation.

Sequences responsible for subcellular targeting of many proteins have been identified through mutational alteration of the relevant structural genes followed by analysis of the subcellular distribution of the altered protein. Application of this approach to cytochrome c has been problematic because of the difficulty of separating effects on heme attachment from effects on import. Failure to attach heme to cytochrome c leads to accumulation of the precursor, apocytochrome c, in the cytoplasm. Heme attachment can be blocked by competition with a heme analog that cannot be incorporated into holocytochrome c, by mutational alteration of cytochrome c, or by a lack of CCHL, the enzyme catalyzing the attachment (Hennig and Neupert, 1981; Dumont et al., 1988, 1991; Nargang et al., 1988). Such coupling between heme attachment and import is consistent with a mechanism in which apocytochrome c is reversibly transported across the mitochondrial outer membrane and subsequently trapped in the intermembrane space by the protein folding reaction accompanying CCHL-mediated heme attachment. However, direct participation of CCHL in import has not been completely ruled out (see Dumont, 1995; Mayer et al., 1995).

The abbreviations used are: CCHL, cytochrome c heme lyase; PCR, polymerase chain reaction.

* This work was supported by National Institutes of Health Grant R01 GM12702. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Center for Hemostasis and Thrombosis Research, Dept. of Medicine, Tufts-NEMC, Box 832, 750 Washington St., Boston, MA 02111.

‡ To whom correspondence should be addressed. Tel.: 716-275-2766; Fax: 716-271-2683; E-mail: fshrm@ghvax.biophysics.rochester.edu

§ To whom correspondence should be addressed. Tel.: 716-275-2766; Fax: 716-271-2683; E-mail: fshrm@ghvax.biophysics.rochester.edu
Two previous mutational studies of sequences responsible for targeting cytochrome c to mitochondria were conducted in systems where mitochondrial localization could be assayed independent of heme attachment. The first of these was carried out in vitro, assaying association of Drosophila melanogaster apocytochrome c with isolated mouse liver mitochondria in the absence of heme attachment. This study revealed that cytochrome c contains two targeting sequences that are at least partially redundant, one in the first 60 residues of the protein and the other in a more carboxyl-terminal region.

We have investigated the sequences involved in targeting cytochrome c to mitochondria using an in vivo system that is closely related to the authentic pathway of import in the yeast Saccharomyces cerevisiae. To separate the effects on heme attachment from effects on import, we have capitalized on a closely related to the authentic pathway of import in the yeast S. cerevisiae. The sequences of the mutant alleles that produce either normal or reduced levels of iso-2-cytochrome c and iso-2-cytochrome c are listed in Table I. The names of the plasmids are structural genes encoding iso-1-cytochrome c and iso-2-cytochrome c, respectively, which are the two isoforms of cytochrome c in yeast S. cerevisiae. The cyC1-Δ and cyc7-Δ null mutants completely lack, respectively, iso-1-cytochrome c and iso-2-cytochrome c. CYC7-H3 is an allele of CYC7 which produces abnormally high levels of iso-2-cytochrome c (McKnight et al., 1981). Mutant alleles that produce either normal or decreased levels of iso-2-cytochrome c are designated cyc7-H3, followed by the allele numbers, e.g. cyc7-H3-67, cyc7-H3-77. CYC3 encodes the yeast CCHL (Dumont et al., 1987).

Construction of Plasmids—A series of mutations in the translated region of the CYC7-H3 gene was generated by the method of Kunkel et al. (1987), using the plasmid pB595 (Dumont et al., 1991) essentially as described previously (Das et al., 1989). The sequences of the mutagenic oligonucleotides are listed in Table I. The names of the plasmids and the corresponding mutagenic oligonucleotides are listed in Table I. Plasmid pAB1385, containing the 12CA5 epitope (Kodolizig and Young, 1991) YPYDVPDYA at the extreme amino terminus of iso-2-cytochrome c, was also derived from plasmid pAB595, using the same procedures described above with the oligonucleotide OL91.228. A 2.3-kilobase EcoRI-Sall fragment from plasmid pAB1385 containing the CYC7-H3 gene with the epitope was ligated into the EcoRI-Sall site of plasmid...
Import of Yeast Cytochrome c

TABLE II

| Allele       | Description | Hayploid series | Diploid series | Plasmid | Oligonucleotide no. | Parental plasmid |
|--------------|-------------|----------------|----------------|---------|---------------------|-----------------|
| CYC7-H3      | Normal      | —              | B-9609         | pAB595  |                     |                 |
| cyc-7Δ       | Deficiency  | —              | B-9610         | —       | —                   | —               |
| cyc-H3-67    | C2S C26S    | B-8118         | B-9687         | pAB599  | OL88.255            | pAB595          |
| cyc-H3-77    | Δ(A1–K14)   | B-9142         | B-9893         | pAB1317 | OL93.243            | pAB595          |
| cyc-H3-78    | Δ(G15–Q24)  | B-9143         | B-9589         | pAB1318 | OL93.240            | pAB595          |
| cyc-H3-79    | Δ(Q25–K36)  | B-9144         | B-9590         | pAB1319 | OL93.242            | pAB595          |
| cyc-H3-80    | Δ(V37–R47)  | B-9145         | B-9591         | pAB1320 | OL93.239            | pAB595          |
| cyc-H3-81    | Δ(H48–Y57)  | B-9146         | B-9592         | pAB960  | OL92.234            | pAB595          |
| cyc-H3-82    | Δ(T58–W68)  | B-9147         | B-9593         | pAB1321 | OL93.244            | pAB595          |
| cyc-H3-83    | Δ(D69–P80)  | B-9148         | B-9594         | pAB1322 | OL93.245            | pAB595          |
| cyc-H3-84    | Δ(K81–F91)  | B-9149         | B-9595         | pAB1323 | OL93.236            | pAB595          |
| cyc-H3-85    | Δ(A92–L103) | B-9150         | B-9596         | pAB1324 | OL93.246            | pAB595          |
| cyc-H3-86    | Δ(I104–K112)| B-9151         | B-9597         | pAB1325 | OL93.247            | pAB595          |
| cyc-H3-88    | Δ(T28–K36)  | B-9153         | B-9598         | pAB1327 | OL93.241            | pAB595          |
| cyc-H3-89    | Δ(G15–R22)  | B-9154         | B-9599         | pAB1328 | OL94.150            | pAB595          |
| cyc-H3-90    | Δ(G15–L18)  | B-9155         | B-9600         | pAB1329 | OL94.165            | pAB595          |
| cyc-H3-91    | Δ(F19–R22)  | B-9156         | B-9601         | pAB1330 | OL94.164            | pAB595          |
| cyc-H3-92    | F19A        | B-9157         | B-9602         | pAB1331 | OL94.091            | pAB595          |
| cyc-H3-93    | K20A        | B-9158         | B-9603         | pAB1332 | OL94.146            | pAB595          |
| cyc-H3-94    | R22A        | B-9159         | —              | pAB1333 | OL94.143            | pAB595          |
| cyc-H3-96    | C23A        | B-9160         | B-9603         | pAB1334 | OL94.092            | pAB595          |
| cyc-H3-96    | C26A        | B-9161         | B-9604         | pAB1335 | OL94.094            | pAB595          |
| cyc-H3-97    | H27A        | B-9162         | B-9605         | pAB1336 | OL94.095            | pAB595          |
| cyc-H3-101   | C25S        | B-9424         | B-9606         | pAB1450 | OL95.024            | pAB595          |
| cyc-H3-102   | C26S        | B-9425         | B-9607         | pAB1451 | OL95.025            | pAB595          |
| cyc-H3-99    | C23A C26A   | B-9173         | B-9608         | pAB1344 | OL94.098            | pAB595          |
| cyc-H3-100   | Ep Δ+ iso-2c | B-9208         | —              | pAB1386 | c                    | pAB1385         |
| cyc-H3-87    | Ep Δ+ (16–18) | B-9152     | B-9611         | pAB1326 | s                    | pAB1386         |

a From Dumont et al. (1991).
b Amino-terminal fusion of iso-2-cytochrome c or Δ(Ala1–Lys14) with the 12CA5 epitope YPYDVPDYA.
c See "Materials and Methods" for details.

YCP50 (Rose et al., 1987) to construct a plasmid pAB1386. For construction of the cyc-H3-87 allele (see Fig. 1) with the 12CA5 epitope replacing the first 14 amino acids of the protein, oligonucleotides OL91.072 and OL91.038 were used with plasmid pAB1386 in the polymerase chain reaction (PCR) to amplify the epitope-containing CYC7-H3 gene as described by Scharf (1990). The sample was cycled 30 times at 94°C for 1 min, annealing at 55°C for 2 min, and 72°C for 4 min. The PCR product was treated with restriction endonuclease Sall and self-ligated. Reverse PCR using this same protocol was then performed on the circular product with oligonucleotides OL91.300 and OL91.298 as primers, thereby creating the deletion. The PCR fragment was treated with restriction endonuclease BamH I and self-ligated. The circularized product was subjected to an additional round of PCR using oligonucleotides OL91.335 and OL91.257 as primers. Plasmid YCP50 was digested with restriction endonuclease Nru I, and a single T was added to each end of the DNA molecule with deoxy-TTP through the action of terminal deoxynucleotidyl transferase. Finally, the PCR fragment containing the CYC7-H3 allele with the epitope and the deletion was ligated to the modified YCP50 vector to construct a plasmid pAB1326.

Construction of Strains—Plasmid pAB790 is a multicopy plasmid containing TRP1 and the CYC3 gene fused to the actin promoter (Dumont et al., 1991). Yeast strain B-6748 (MATa cyc-1 Δ cyc-7 ura3-52 his3-11 leu2–3,112 trpl–289) was transformed with the plasmid pAB790 to construct a strain B-9077, using the transformation procedures described by Ito et al. (1983). Plasmids containing altered CYC7-H3 alleles were integrated at the chromosomal CYC7 locus of strain B-9077 through transformation (see Table II). Plasmids pAB1326 and pAB1386 were transferred into strain B-6748, resulting in strains B-9152 and B-9208, respectively.

Subcellular Fractionation—For preparation of subcellular fractions, all strains except B-9152 were cultured in S.D. medium containing 0.1% casamino acids plus histidine and leucine (Sherman et al., 1987) to an A600 of 3.0–4.0. Strain B-9152 was grown in uracil omission medium (Sherman et al., 1987) to an A600 of 3.0–4.0. Subcellular fractions were prepared as described by Daum et al. (1982) and Dumont et al. (1991, 1993), with the following modifications: (i) Zymolyase 101 (ICN Biomedicals, Inc.) was used to make spheroplasts. (ii) Cells were lysed in 0.5 M mannitol, 20 mM HEPES/KOH, 1 mM EDTA, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4. (iii) cytochrome c purified anti-iso-1-cytochrome c antibodies were used at 1:1,000 for 2 h. The membranes were then washed with phosphate-buffered saline and equilibrated in 10 mM sodium citrate, 10 mM EDTA, pH 5.0, for 5 min. After an additional 10-min incubation with 10 mM sodium citrate, 10 mM EDTA, 1% dextran sulfate, pH 5.0, the membranes were again washed, three times for 5 min each, with 10 mM sodium citrate, 10 mM EDTA, pH 5.0. Finally the membranes were incubated in 10 mM sodium citrate, 10 mM EDTA, 1% Bovine serum albumin, 1 mM mM tetramethylammonium bicarbonate and 0.003% hydrogen peroxide until colors were developed (McKimm-Breschkin, 1990). Quantitative comparisons of cytochrome c levels in different subcellular fractions were performed by comparing the intensities of a series of dilutions of the different fractions on immunoblots (Dumont et al., 1991).

Determination of Cytochrome c Levels—The strains, described in Fig. 2 and Tables II and III, were grown on 1% sucrose medium (Sherman et al., 1974) at 30 °C for 3 days, and the absorption spectra were recorded as described previously (Hickey et al., 1991). A low temperature...
Control strains

B-7908 MATα CYC3 cyc1–783::lacZ cyc7–67 ura3–52 his3–11 leu2–3,112 trp1–289 met8–1 ilv3–1

B-6945 MATα CYC3 CYC1 cyc7–67 ura3–52 leu2–3,112 trp1–289 cyh2

B-9609 B-7908 × B-6945

B-6748 MATα CYC3 cyc1–783::lacZ cyc7–67::CYH2 ura3–52 his3–11 leu2–3,112 trp1–289 cyh2

B-9610 B-6748 × B-6945

B-6442 MATα CYC3 cyc1–363 cyc7–67::CYH2 ura3–52 his3–11 leu2–3,112 trp1–289

B-7682 MATα CYC3 cyc1–31 cyc7–67 his3–1 ilv2–1 trp1–289

B-8442 B-6444 × B-7682

RESULTS

Experimental Design: Isozymes of cytochrome c System—Normal strains of S. cerevisiae contain two isozymes of cytochrome c, iso-1-cytochrome c and iso-2-cytochrome c, encoded, respectively, by the CYC1 and CYC7 chromosomal genes (Sherman et al., 1966; Downie et al., 1977). These two isozymes constitute, respectively, 95% and 5% of the total cellular complement of cytochrome c (Sherman et al., 1965). Although both are approximately equimolar in their function in electron transport, the properties of each of the apo-iso-cytochromes c differ. In particular, studies of mutants defective in heme attachment revealed that apo-iso-1-cytochrome c is rapidly degraded if it is not converted to holocytochrome c, but that apo-iso-2-cytochrome c is stable under the same conditions (Dumont et al., 1990). As a result, cytochrome c is a deletion of the structural gene that encodes CCHL, are deficient in apo-iso-1-cytochrome c but contain apo-iso-2-cytochrome c; that at a level that is approximately the same as the level of holocytochrome c in a related CYC3 strain containing CCHL. Thus, to allow analysis of the subcellular distribution of apo-cytocrome c, all of our import studies were conducted with altered iso-2-cytochrome c.

Because iso-2-cytochrome c normally constitutes only about 5% of the total complement of cytochrome c, making it difficult to detect, we have used the CYC7-H3 allele, which constitutively overproduces iso-2-cytochrome c to a level that is approximately the same as the normal level of iso-1-cytochrome c (McKnight et al., 1981). This increased expression is due to a deletion that removes the normal promoter and fuses the translated region of iso-2-cytochrome c to an upstream promoter (McKnight et al., 1981; Melnick and Sherman, 1993).

Haploid Series 1: Analysis of the Subcellular Distributions of Altered Apocytochromes c—Oligonucleotide-directed mutagenesis of the CYC7-H3 allele on plasmid pAB595 was used to generate a series of deletions and amino acid replacements of iso-2-cytochrome c (Table II). Strain B-9077 (cyc1–31 cyc7–xx CYC3 pCYC7-H3) was transformed with these p(cyc7-H3-xx) plasmids containing the series of cyc7-H3-xx alleles that encode the altered forms of iso-2-cytochrome c. The resulting series of haploid strains, containing an integrated copy of each of the p(cyc7-H3-xx) plasmids, are referred to as haploid series 1 and are abbreviated as follows (Table III):

cyc1–31 cyc7–H3-xx CYC3 pCYC3

Each strain of haploid series 1 has the following relevant genetic properties: a single copy of the cyc7-H3-xx allele integrated into the chromosome; a cyc1–31 deletion causing a total lack of iso-1-cytochrome c; a normal copy of the CYC3 gene encoding CCHL; and a multicopy plasmid pCYC7-H3 leading to overproduction of CCHL driven by the actin promoter. As described previously (Dumont et al., 1991) and discussed above, overproduction of CCHL leads to efficient accumulation of apo-cytochrome c inside mitochondria when heme attachment does not occur. Such accumulation appears to be mediated by a direct interaction between apocytochrome c and CCHL in a nonproductive enzyme-substrate complex located in the mitochondrial intermembrane space (Dumont et al., 1991; Mayer et al., 1995; Dumont, 1995). The combination of genetic elements in haploid series 1 allows examination of the effects of cyc7-
H3-xx alleles on mitochondrial accumulation of apocytochrome c under conditions where heme is not attached to the protein. Mitochondrial accumulation of mutant cytochromes c was assayed by immunoblotting of subcellular fractions derived from the relevant yeast strains. The antibodies used recognize both holocytochrome c and apocytochrome c.

Initially, the studies described below were performed with the cyc7-H3-xx alleles to which a 12CA5 epitope tag from influenza hemagglutinin had been added (Kolodziej and Young, 1991). However, in some cases, the presence of this tag appeared to alter the intracellular distribution of cytochrome c (see below). Comparison of the immunologic reactivity of anti-epitope-tag antibodies with that of anti-cytochrome c antibodies demonstrated that the affinity-purified polyclonal anti-cytochrome c antibodies efficiently recognized all of the mutant cytochromes c that we tested. Thus, anti-cytochrome c antibodies rather than anti-epitope-tag antibodies were used in the assays of intracellular cytochrome c distributions presented below.

Haploid Series 2: Analysis of Heme Attachment and Function of Altered Iso-2-cytochromes c—The function and degree of heme attachment of the cyc7-H3-xx altered iso-2-cytochromes c were determined with the following haploid series 2 (Table III) which did not overproduce CCHL:

\[ \text{cyc1-}\Delta \text{cyc7-H3-xx CYC3}^+ \]

The level of hol-iso-2-cytochrome c in each member of this series was estimated by low temperature (−196 °C) spectroscopic examination of intact yeast cells. The ability of each altered iso-2-cytochrome c to function in electron transport was estimated from the ability of each strain to grow on media containing nonfermentable carbon sources, such as glycerol or ethanol. The levels and functions were assigned values in comparison with the control strain cyc1-Δ CYC7-H3 CYC3+.

Diploid Series: Assay of Competition between Normal Cytochrome c and Altered Iso-2-cytochromes c in the Mitochondrial Import Pathway—Heterozygous diploid strains were used to determine if the altered apo-iso-2-cytochromes c retained sufficient integrity to compete with normal cytochrome c or if the altered forms even had abnormally high affinities for any components of the mitochondrial import pathway which could lead to displacement of normal cytochrome c. The extent of the displacement was estimated from the levels of holocytochrome c in the following series of diploid strains (Table III):

\[ \text{cyc1-}\Delta \text{cyc7-H3-xx CYC3}^+ \times \text{CYC1}^+ \text{cyc7-}\Delta \text{CYC3}^+ \]

Thus, each of the diploid strains contains the normal level of CCHL, a single copy of the cyc7-H3-xx encoding one of the altered iso-2-cytochromes c, and a single copy of CYC1+ encoding normal iso-1-cytochrome c.

Using low temperature spectroscopic of intact cells, the level of holocytochrome c in each of the strains of this series was compared with the level in two control strains. One control strain contains two normal cytochrome c alleles. The other contains one normal cytochrome c allele accompanied by a deletion of the other allele. The level of holocytochrome c in the double hemizygous CYC7-H3/cyc7-Δ CYC1+/cyc1-Δ control strain was defined as 100%. The level of holocytochrome c in the heterozygous cyc7-Δ/cyc7-Δ CYC1+/cyc1-Δ control strain corresponded to 50% of the previous one. We used diploid series in which the normal cytochrome c allele was iso-1-cytochrome c (cyc1-Δ cyc7-H3-xx CYC3+ × CYC1+ cyc7-Δ CYC3+) rather than diploid series in which the normal cytochrome c allele was iso-2-cytochrome c (cyc1-Δ cyc7-H3-xx CYC3+ × cyc1-Δ CYC7-H3 CYC3+). Thus, each of the diploid strains contains the normal level of holocytochrome c encoding iso-1-cytochrome c, and no other cytochrome c genes. The data in Fig. 2 also demonstrate similar effects when alanine, rather than serine, was substituted for the two cysteine residues. Substitution of alanine or serine for either one of the two cysteine residues resulted in a phenotype that was indistinguishable from substitutions at both positions (Fig. 2C).
Import of Yeast Cytochrome c

Table 1: Summary of the mutations in the iso-2-cytochrome c and their effects.

| Mutation | Holo-Cyt. c | Internal-Cyt. c | Asso. Cyt. c | Holo. in 2N |
|----------|-------------|----------------|-------------|-------------|
| Wild Type | 100% | 0% | 0% | 50% |
| Cys23     | 10% | 10% | 0% | 0% |
| Cys26     | 0% | 0% | 0% | 0% |

These mutations having Cys23 or Cys26 replacements were assigned to Class I.

Six of the 10 deletions spanning the CYC7 gene gave rise to phenotypes typical of Class I mutations. The deletions in this class included Δ(Val17–Arg27) and the five deletions encompassing the region from Thr26 to Lys112, comprising most of the carboxyl-terminal half of the molecule. Each of these mutant apo-iso-2-cytochromes c lacked heme attachment. Furthermore, each was imported into mitochondria and interfered with the formation of holo-iso-1-cytochrome c in diploid strains with approximately the same efficiency as the mutations involving substitutions for the cysteine residues involved in heme attachment. Thus it appeared that these deletions did not reside in sequences required for mitochondrial import or binding to CCHL.

The Δ(Leu104–Lys112) deletion has been included in this class of mutants although the percentage of this form of apocyto-
The Δ(Gly\textsuperscript{15}–Arg\textsuperscript{22}) deletion was similar to the Δ(Gly\textsuperscript{15}–Gln\textsuperscript{26}) deletion. Thus, loss of Gln\textsuperscript{24} and Cys\textsuperscript{23}, which is one of the sites of covalent linkage of heme, does not explain the Class III phenotype. The lack of a role for Cys\textsuperscript{3} was also supported by analysis of the Cys\textsuperscript{23} → Ser\textsuperscript{23} and Cys\textsuperscript{23} → Ala\textsuperscript{27} mutations, which did not prevent mitochondrial accumulation of apocytochrome \( c \).

The Δ(Gly\textsuperscript{15}–Leu\textsuperscript{18}) and Δ(Phe\textsuperscript{19}–Arg\textsuperscript{22}) deletions each exhibited only partial defects in the accumulation of mitochondrial apocytochrome \( c \) compared with the entire Δ(Gly\textsuperscript{15}–Arg\textsuperscript{22}) deletion. This indicated that sequences from each of these two smaller regions might be important for this process. The phenotypes of the Δ(Phe\textsuperscript{19}–Arg\textsuperscript{22}) deletion and the single substitution Phe\textsuperscript{19} → Ala\textsuperscript{19} were indistinguishable, indicating that Phe\textsuperscript{19} might be the critical residue in the Phe\textsuperscript{19}–Arg\textsuperscript{22} region. Thus, Phe\textsuperscript{19} and one or more residues in the Gly\textsuperscript{15}–Leu\textsuperscript{18} region appeared to be required for efficient mitochondrial accumulation of apocytochrome \( c \).

Even though the Δ(Gly\textsuperscript{15}–Gln\textsuperscript{24}) apocytochrome \( c \) did not accumulate inside mitochondria to any appreciable extent, expression of this altered apocytochrome \( c \) in the heterozygous diploid strain displaced the formation of the normal holocytochrome \( c \) to the same extent as the Class I apocytochromes \( c \), which were accumulated efficiently inside mitochondria (see Fig. 2).

Class IV Mutations Affecting His\textsuperscript{27} Prevent Translocation across the Mitochondrial Outer Membrane—The Δ(Gln\textsuperscript{25}–Lys\textsuperscript{36}) deletion resulted in normal association of apocytochrome \( c \) with mitochondria. However, in contrast to Class I mutants, which were accumulated efficiently inside mitochondria, most of apocytochrome \( c \) containing the Δ(Gln\textsuperscript{25}–Lys\textsuperscript{36}) deletion remained protease-accessible on the mitochondrial surface. Expression of this mutant in diploid cells led to some inhibition of hol-o-iso-1-cytochrome \( c \) formation, although not to the same extent seen for the Class I mutants.

Since even normal apocytochrome \( c \) did not associate with mitochondria that lacked CCHL (Dumont et al., 1991), it seemed likely that binding the Δ(Gln\textsuperscript{25}–Lys\textsuperscript{36}) apocytochrome \( c \) on the mitochondrial surface would depend on CCHL. This was tested by measuring mitochondrial association of this mutant in a strain expressing a single copy of CYC3 (encoding CCHL), instead of the overexpressing strains used in the other subcellular localization study. The lower level of CCHL reduced the amount of mitochondrially associated cytochrome \( c \) from 40 to 30% of the total cellular level. Thus, interaction with CCHL was required to maintain even surface-exposed precursor in association with mitochondria.

His\textsuperscript{27} appears to be the key residue in this region responsible for the accumulation of apocytochrome \( c \) in a protease-inaccessible compartment of mitochondria. Apocytochrome \( c \) containing a Δ(Thr\textsuperscript{28}–Lys\textsuperscript{36}) deletion accumulated in a protease-protected state with the same efficiency seen for Class I mutants. Furthermore, the single substitution His\textsuperscript{27} → Ala\textsuperscript{27} exhibited a phenotype indistinguishable from deletion of the entire region from Gly\textsuperscript{25} to Lys\textsuperscript{36}.

Class V Deletion of His\textsuperscript{48}–Ty\textsuperscript{57} Allows Holocytochrome \( c \) Formation—The Δ(His\textsuperscript{48}–Ty\textsuperscript{57}) mutant produced holocytchrome \( c \) at 40% of the normal level. The deleted region corresponds to a loop in the three-dimensional structure. Thus, the structure appeared to be able to accommodate a rearrangement in which two residues that were in close proximity (Arg\textsuperscript{47} and Thr\textsuperscript{56}) were directly joined instead of being connected by a loop. Two additional deletions in the corresponding region of iso-1-cytochrome \( c \) were previously shown to allow formation of holocytochrome \( c \). These two iso-1-cytochrome \( c \) deletions lacked the following residues (iso-2-cytochrome \( c \) numbering system):

![Fig. 5. Low temperature (--196°C) spectrophotometric recordings of a series of diploid strains.](image-url)
residues 49–62 (cyc1–453) (Sherman et al., 1975; Hampsey et al., 1988) and residues 52–59 (cyc1–817) (Fetrow et al., 1989). In addition, this region is absent in the S-type bacterial cytochromes c (Matsuura et al., 1982), including cytochrome \( c_{SS} \), which lacks residues corresponding to positions 46–68 of iso-2-cytochrome c.

Not all of the apocytochrome c containing the \( \Delta (\text{His}^{48}, \text{Tyr}^{57}) \) deletion was converted into holocytochrome c. Approximately 30% of the immunologically detectable protein was found in the cytoplasm. Since strains expressing this mutant contained a normal overall cellular level of immunologically detectable cytochrome c, 70% of which was mitochondrial, there appeared to be additional apocytochrome c inside mitochondria. The existence of this population of mitochondrial apocytochrome c, which was presumably bound to CCHL, was consistent with the observed partial displacement of normal holo-iso-1-cytochrome c by the \( \Delta (\text{His}^{48}, \text{Tyr}^{57}) \) mutant in diploid strains.

**DISCUSSION**

The sequences in cytochrome c which are responsible for directing the protein from its site of synthesis in the cytoplasm to its functional site on the outer surface of the mitochondrial inner membrane have not been well characterized previously. A major difficulty in identifying such sequences has been the requirement for heme to be covalently attached to apocytochrome c to observe efficient import. This makes it difficult to distinguish sequence determinants that are important for heme attachment and concomitant folding of the protein from sequence determinants that are actually involved in subcellular targeting.

To circumvent these difficulties, we have made use of a previous observation that altered forms of apocytochrome c which are incapable of having heme covalently attached can be imported efficiently into mitochondria that have an abnormally high level of CCHL (Dumont et al., 1991). Determination of the subcellular distribution of apocytochrome c in such CCHL-overproducing strains has the following advantages for the identification of sequences involved in targeting. (i) The assay is performed in vivo with a system that is known to be capable of efficiently importing and maturing normal cytochrome c. (ii) Comparisons can be made among different forms of cytochrome c which are incapable of undergoing covalent heme attachment. (iii) The assay does not involve the creation of fusion proteins that could alter targeting. (iv) Apo-iso-2-cytochrome c is stable in the cytoplasm and mitochondria, allowing immunologic assay of the subcellular distribution of the protein.

**Protein Folding and Heme Attachment**

Deletions covering 75% of the sequence of iso-2-cytochrome c (Thr\(^{28}\)-Lys\(^{112}\)) accumulated in mitochondria to the same level as cytochrome c containing substitutions only for the two cysteine residues, Cys\(^{23}\) and Cys\(^{26}\), which are the sites of covalent heme attachment. However, with the exception of \( \Delta (\text{His}^{48}, \text{Tyr}^{57}) \), where the deletion apparently happened to coincide with a dispensable loop in the three-dimensional structure of the protein, none of the deletion-containing apocytochromes c that were efficiently imported could be converted into holocytochrome c. This suggests that mutations that interfere with the native structure of the mature protein also render cytochrome c incapable of having heme covalently attached. Since apocytochrome c appears to be an unfolded protein (Babul and Stellwagen, 1972; Fisher et al., 1973), either CCHL recognizes native-like structures in the precursor, or protein folding is coupled to heme binding so that the formation of the thioether linkages cannot proceed unless the protein-heme product can adopt a stable native conformation.

In a previous analysis of single amino acid replacements at 16 different sites throughout iso-1-cytochrome c which caused deficiency of function, only replacements at Cys\(^{23}\), Cys\(^{26}\), and His\(^{57}\) (iso-2-cytochrome c numbering system) completely prevented the formation of holocytochrome c (Hampsey et al., 1988). Thus, except for these three amino acid residues that comprise and adjoin the actual site of covalent heme attachment, it seems likely that no short motifs are required for the covalent attachment of heme. On the other hand, the failure of the numerous deletion-containing cytochromes c to be converted into holocytochrome c indicates that successful catalysis of heme attachment by CCHL requires the existence of an overall structure. Since most of the deletion-containing apocytochromes c accumulate efficiently inside mitochondria, structural distortions that prevent heme attachment do not necessarily prevent binding to CCHL, and furthermore, the actual site of heme attachment in cytochrome c may not be the same as the sequence determinants involved in the binding to heme attaching enzyme.

The failure of most of the deletion-containing alleles of iso-2-cytochrome c to undergo covalent heme attachment is in sharp contrast to the report of Veloso et al. (1984) that partially purified CCHL is capable of attaching heme to a peptide corresponding to residues 1–25 of horse apocytochrome c. Either the efficiency of the reaction in vitro is very low, or the sequence requirement for CCHL action is much more stringent in vivo.

**Sequences Involved in Import**

Altered cytochromes c that do not accumulate in mitochondria in the presence of excess CCHL could be defective in any of the following aspects: (i) they could interact improperly with a hypothetical cytoplasmic machinery, failing to reach the mitochondrial surface; (ii) they could fail to undergo the correct initial interaction with a hypothetical mitochondrial receptor or import apparatus; (iii) they could be incapable of being translocated across the mitochondrial outer membrane; or (iv) they could fail to bind to CCHL. By combining subcellular fractionation with competition studies in diploid strains, we have distinguished the following four different types of sequence alterations with different effects on import.

1. **The Amino Terminus—Deletion of the first 14 amino acids of the protein reduces the efficiency of association of precursor with mitochondria or CCHL but still allows inefficient conver-
Import of Yeast Cytochrome c

A minimal requirement for the length of the amino-terminal region of a cleavable mitochondrial matrix targeting signal fused to dihydrofolate reductase has been observed by Ungermann et al. (1994). In that case, the minimal length appeared to be required for the ATP-dependent interaction of the precursor with mitochondrial Hsp70. In the case of cytochrome c, the length could be required for stable interaction with the mitochondrial outer membrane, with CCHL, or with some yet-to-be-identified factors. However, it is surprising that the length requirement for cytochrome c resides at the extreme amino terminus of the protein, since cytochrome c contains no cleavable targeting signal, and the other targeting signals that we have uncovered are located at least 15 residues from this end. This suggests either that there is nonspecific anchoring of the end of the chain in order to expose some more specific signals farther from the end or that the extreme amino terminus is involved in stabilizing a translocation-compliant conformation. The amino-terminal region of apocytochrome c could also enhance membrane-active properties that would facilitate penetration across the mitochondrial outer membrane (Iordi et al., 1998b).

2. Region Encompassing Gly15–Arg22—The Δ(Gly15–Arg22) apocytochrome c did not associate with mitochondria, although expression of this mutant cytochrome c in a heterozygous diploid strain led to inhibition of formation of coexpressed normal holocytochrome c. Two possible explanations for this observation are: (i) that mutations in the Gly15–Gln24 region lead to formation of a stalled complex between apocytochrome c and a cytoplasmic factor involved in import, thereby titrating the amount of factor available for import of normal cytochrome c; or (ii) that mutations in this region allow a loose association with mitochondrial components that can dissociate during subcellular fractionation but is still tight enough to allow competition in vivo for maturation of normal cytochrome c.

There is one previous report of a cytoplasmic factor involved in cytochrome c import in an in vitro system (Hakvoort et al., 1990). Furthermore, a cytoplasmic factor called mitochondrial import stimulation factor from rat liver has been found to promote mitochondrial import of proteins other than cytochrome c. The ATPase activity of mitochondrial import stimulation factor was stimulated by the presence of unfolded mitochondrial precursors, including apocytochrome c but not holocytochrome c. This stimulation of ATPase was reversed by the presence of mitochondrial outer membranes, apparently because of binding of the mitochondrial import stimulation factor-precursor complex to sites on the membranes (Hachiya et al., 1994). Import of cytochrome c into mitochondria does not appear to depend on cytoplasmic heat shock-related proteins to the same extent as import of other mitochondrial proteins, since, in contrast to other mitochondrial precursors, apocytochrome c can be imported efficiently into isolated mitochondria from wheat germ translation systems, which are at least partially deficient in such heat shock-related proteins (Dumont et al., 1988; Murakami et al., 1988).

There appear to be at least two sequence determinants in the region Gly15–Arg22, which affect mitochondrial accumulation, including Phe19 and at least one residue in the region Gly15–Leu18. The equivalent of Phe19 is found in the sequence of every known eukaryotic cytochrome c (see Hampsey et al., 1988), in agreement with the possible importance of this residue. The residues found at the equivalent of positions Ala16, Thr17, and Leu18 vary considerably among eukaryotic species and have been mutated with little effect on cytochrome c function. Gly15 is invariant among eukaryotic cytochromes c, suggesting that it could play an important role. However, replacements of Gly15 by alanine, serine, cysteine, or aspartic acid residues allowed the formation of substantial levels of holocytochromes c which were at least partially functional (Hampsey et al., 1988; Auld and Pielak, 1991). Thus, deletion of the region Gly15–Leu18 might lead to the loss of functionally redundant sequence elements or disrupt a local conformation that was not affected by replacement of any single amino acid residue.

Sprinkle et al. (1990) found that substitutions of glutamine, glutamic acid, and asparagine for lysine residues at the equivalent of positions 14, 16, and 17 (iso-2-cytochrome c numbering system) in D. melanogaster cytochrome c led to loss of import. The lysine residues at positions 16 and 17 are not conserved in iso-2-cytochrome c. The third of these lysine residues, at position 14, is not indispensable based on the results of aminoterminal deletions described above. Nonetheless, our identification of a range of mutations over the aminoterminal 25% of the sequence of cytochrome c which lead to defects in mitochondrial accumulation targeting agrees with the identification by Sprinkle et al. (1990) of lesions in this region which are capable of preventing targeting. In contrast to these results, Nye and Scarpulla (1990b), assaying for targeting of an iso-1-cytochrome c chloramphenicol acetyltransferase fusion protein, found that they could delete as much as the first 67 amino acids of the cytochrome c sequence without affecting targeting to mitochondria. The results reported in the present manuscript do not support the proposal by Nye and Scarpulla (1990b) that there is functional redundancy between amino and carboxyl regions of the protein.

Because single localized deletions were examined in our study, we have not ruled out the possibility that, in addition to the sequences we uncovered, additional alterations at two or more nonadjacent sites might lead to impaired mitochondrial accumulation.

3. Role of His27—His27 serves as a ligand for the heme ion in native holocytochrome c and is conserved in all eukaryotic cytochromes c. Previously, alteration of this residue was shown...
to prevent mitochondrial accumulation of holocytochrome c, but the effects on import could not be distinguished from those on heme attachment (Dumont et al., 1988). In the present experiments, where import was examined in the absence of heme attachment, replacement or deletion of His27 led to association of the altered apocytochrome c with mitochondria, but most of the mitochondrially associated precursor remained protease-accessible on the mitochondrial outer surface. The bound precursor appears to represent a form of the protein which is stalled in the import process, either because His27 is required for interaction with translocation machinery of the mitochondrial outer membrane or because the histidine is involved in recognition of the precursor by CCHL. Since no binding to mitochondria is observed in the absence of CCHL (Dumont et al., 1991), simple failure to bind would not result in accumulation of the partially translocated form. Instead, the altered precursor must be entering either directly or indirectly into some aberrant interaction with CCHL.

The observation that apocytochrome c lacking His27 fails to associate with mitochondria unless they contain high levels of CCHL confirms the role of CCHL in maintaining the stalled form of the precursor. The simplest explanation of this result is that the altered apocytochrome c adopts a membrane-spanning topology, rendering it accessible to external proteases at the same time as it is bound to CCHL inside the mitochondrial outer membrane. However, CCHL has been reported to be associated predominantly with the mitochondrial inner membrane, not the outer membrane (Dumont et al., 1991). There are a number of ways of reconciling these findings. (i) CCHL may not interact directly with the stalled precursor but, rather, act in an indirect way to maintain the association of the altered apocytochrome c with mitochondria. (ii) CCHL may transiently become associated with the outer membrane in vivo, or it may be localized to sites where the two membranes are in close contact. (iii) The apparent localization of CCHL to the inner membrane in isolated mitochondria may result from redistribution during fractionation.

4. The Carboxyl Terminus—We detect only a slight diminution in mitochondrial accumulation resulting from deletion of the nine amino acid residues at the carboxyl-terminal region of iso-2-cytochrome c. However, like most of the deletions studied, removal of the carboxyl terminus effectively blocks conversion into holocytochrome c. These results contradict earlier claims that critical targeting sequences reside at the carboxyl terminus of cytochrome c (Matsuura et al., 1981; Stuart et al., 1987). Such claims were based either on competition with a carboxyl-terminal peptide of mitochondrial import in an in vitro system, which could be subject to nonspecific interference at high peptide concentrations, or on studies with a mutant cytochrome c containing 27 unrelated amino acid residues fused to the carboxyl terminus, which could lead to spurious mistargeting. Furthermore, neither of these previous studies clearly distinguished effects on heme attachment from effects on import. On the other hand, our finding that the carboxyl terminus is not critical for mitochondrial accumulation of cytochrome c is in agreement with the two previous systematic studies of cytochrome c sequences involved in targeting (Nye and Scarppulla, 1990b; Sprinkle et al., 1990).

We have not resolved the question of whether the same regions of cytochrome c which we have determined to be necessary for mitochondrial accumulation are also sufficient for accumulation. Expression of amino-terminal regions of apocytochrome c as short fragments in vivo does not result in accumulation of sufficient quantities of protein for detection using an epitope tag, presumably because of rapid degradation (data not presented). On the other hand, fusion of apocytochrome c fragments to a larger carrier protein can lead to mistargeting (see Nye and Scarppulla, 1990a).

Pathway of Cytochrome c Import into Mitochondria

Based on the results presented above and on previous studies, we present in Fig. 6 a tentative model for cytochrome c targeting, emphasizing several of the following unresolved aspects of the process.

1. After synthesis in the cytoplasm, apocytochrome c may associate with a cytoplasmic factor. The existence of such a factor is suggested by our observation that expression of altered forms of apocytochrome c which do not bind or enter mitochondria inhibits the formation of the coexpressed normal cytochrome c.

2. Apocytochrome c, either alone or in conjunction with a cytoplasmic factor, associates with mitochondria and adopts a membrane-spanning topology. Upon becoming exposed to the intermembrane space, sequences near the amino terminus of apocytochrome c bind either directly to CCHL or to a CCHL-associated factor. Such a factor would normally be present in excess relative to CCHL, or its level would increase upon overexpression of CCHL. Both the amino and carboxyl termini exhibit membrane-active properties, although only amino-terminal fragments are able to translocate across the lipid bilayer (Zhou et al., 1988; Ordi et al., 1989a, 1989b). However, it is not currently known whether the pathway of import of cytochrome c involves a direct interaction with the lipid phase.

3. His27 of apo-iso-2-cytochrome c interacts with CCHL or some other CCHL-associated component of the translocation machinery in order to allow the carboxyl-terminal portion of the protein to be translocated into the mitochondrial intermembrane space.

4. If CCHL has remained on the inner membrane, it associates with the precursor following diffusion across the intermembrane space.

5. Altered apocytochromes c that are unable to have heme covalently attached remain trapped in the intermembrane space in a dead end complex with CCHL. Normal precursors...
are covalently linked to heme in a reaction catalyzed by CCHL. Folding to the native globular structure occurs concomitant with this reaction.

6. Finally, matured normal precursors are released into the intermembrane space. In their folded form, they cannot translocate back across the outer membrane (see Fig. 6).

Acknowledgment—We thank Linda Comfort for the synthesis of oligonucleotides.

REFERENCES

Auld, D. S., and Pielak, G. J. (1991) Biochemistry 30, 8684–8690
Babul, J., and Stellwagen, E. (1972) Biochemistry 11, 1195–1200
Bairn, S. B., Pietras, D. F., Eustice, D. C., and Sherman, F. (1985) Mol. Cell. Biol. 5, 1839–1846
Das, G., Hickey, D. R., McElenon, D., McElenon, G., and Sherman, F. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 496–499
Dauw, G., Boviri, P., and Schatz, G. (1992) J. Biol. Chem. 257, 13028–13033
Downie, J. A., Stewart, J. W., Brockman, N., Schweingruber, A. M., and Sherman, F. (1977) J. Mol. Biol. 113, 369–384
Dumont, M. E. (1996) Adv. Mol. Cell. Biol. 43, in press
Dumont, M. E., Ernst, J. F., Hampsey, D. M., and Sherman, F. (1987) EMBO J. 6, 235–241
Dumont, M. E., Ernst, J. F., and Sherman, F. (1988) J. Biol. Chem. 263, 11928–11937
Dumont, M. E., Mathews, A. J., Nall, B. T., Bairn, S. B., Eustice, D. C., and Sherman, F. (1990) J. Biol. Chem. 265, 2733–2739
Dumont, M. E., Cardillo, T. S., Hayes, M. K., and Sherman, F. (1991) Mol. Cell. Biol. 11, 5487–5496
Dumont, M. E., Schlichter, J. B., Cardillo, T. S., Hayes, M. K., Bethelndy, G., and Sherman, F. (1993) Mol. Cell. Biol. 13, 6442–6451
Fetrow, J. S., Cardillo, T. S., and Sherman, F. (1989) Proteins 6, 372–381
Fisher, W. R., Taniuchi, H., and Anfinsen, C. B. (1973) J. Biol. Chem. 248, 3188–3195
Hachiya, N., Konoya, T., Alam, R., Iwashashi, J., Sakaguchi, M., Omura, T., and Miihara, K. (1994) EMBO J. 13, 5146–5154
Hakvoort, T. B. M., Sprinkle, J. R., and Margoliash, E. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 4996–5000
Hampsey, D. M., Das, G., and Sherman, F. (1988) FEBS Lett. 231, 275–283
Hannavy, K., Rospert, S., and Schatz, G. (1993) Curr. Opin. Cell Biol. 5, 694–700
Hartl, F.-U., Pfanner, N., Nicholson, D. W., and Neupert, W. (1989) Biochim. Biophys. Acta, 988, 1–45
Hennig, B., and Neupert, W. (1981) Eur. J. Biochem. 121, 203–212
Hickey, D. R., Jayaraman, K., Goodhue, C. T., Shah, J., Fingar, S. A., Clements, J. M., Hosokawa, Y., Tsunawaisa, S., and Sherman, F. (1991) Gene (Amst.) 105, 73–81
Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
Jordi, W., de Krujff, B., and Marsh, D. (1989a) Biochemistry 28, 8998–9005
Jordi, W., Li-Xin, Z., Pilon, M., Demel, R. A., and de Krujff, B. (1989b) J. Biol. Chem. 264, 2292–2301
Kiebler, M., Becker, K., Pfanner, N., and Neupert, W. (1993) J. Membr. Biol. 135, 115–127
Kolodziej, P. A., and Young, R. A. (1991) Methods Enzymol. 194, 508–519
Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 372–378
Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Rev. Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Matsuura, S., Arpin, M., Hannum, C., Margoliash, E., Sabatini, D. D., and Morimoto, T. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4368–4372
Matsuura, Y., Takano, T., and Dickerson, R. E. (1982) J. Mol. Biol. 159, 389–409
Mayer, A., Neupert, W., and Lill, R. (1990) J. Biol. Chem. 270, 12390–12397
McKimm-Breschkin, J. L., and Sherman, F. (1991) J. Mol. Biol. 233, 372–378
Moore, G. R., and Pettigrew, G. W. (1986) Cytochromes c: Evolutionary, Structural and Physicochemical Aspects (Springer Series in Molecular Biology), pp. 116–127, Springer Verlag, Berlin
Murakami, H., Pain, D., and Bidist, G. (1988) J. Cell Biol. 107, 2051–2057
Nargang, F. E., Drygas, M. E., Kwong, P. L., Nicholson, D. W., and Neupert, W. (1988) J. Biol. Chem. 263, 9388–9394
Nye, S. H., and Scarpulla, R. C. (1990a) Mol. Cell. Biol. 10, 5753–5762
Nye, S. H., and Scarpulla, R. C. (1990b) Mol. Cell. Biol. 10, 5763–5771
Rose, M. D., Novick, P., Thomas, J. H., Botstein, D., and Fink, G. R. (1987) Gene (Amst.) 60, 237–243
Schafer, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
Scharf, S. J. (1990) PCR Protocols: A Guide to Methods and Applications (Innis, M. A., Gelfand, D. H., Siwsky, J. J., and White, T. J., eds) pp. 84–91, Academic Press Inc., San Diego
Sherman, F., and Stewart, J. W. (1973) The Biochemistry of Gene Expression in Higher Organisms (Pollack, J. K., and Lee, J. W., eds) pp. 56–86, Australian 363- New Zealand Book Co., Sydney, Australia
Sherman, F., Taber, H., and Campbell, W. (1965) J. Mol. Biol. 13, 21–39
Sherman, F., Stewart, J. W., Margoliash, E., Parker, J., and Compbell, W. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 1498–1504
Sherman, F., Stewart, J. W., Jackson, M., Gilmore, R. A., and Parker, J. H. (1974) Genetics 77, 255–284
Sherman, F., Jackson, M., Liebian, S. W., Schweingruber, A. M., and Stewart, J. W. (1975) Genetics 81, 517–528
Sherman, F., Fink, G. R., and Hicks, J. B. (1987) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Sprinkle, J. R., Hakvoort, T. B. M., Koshi, T. I., Miller, D. D., and Margoliash, E. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 5729–5733
Stuart, R. A., Neupert, W., and Tropschug, M. (1987) EMBO J. 6, 2131–2137
Stuart, R. A., and Neupert, W. (1990) Biochimie (Paris) 72, 115–121
Ungrmann, C., Neupert, W., and Cyr, D. M. (1994) Science 266, 1250–1253
Veloze, D., Jullierat, M., and Taniuchi, H. (1981) J. Biol. Chem. 256, 6067–6073
Zhou, L. X., Jordi, W., and de Krujff, B. (1988) Biochim. Biophys. Acta 942, 115–124
Sequence Requirements for Mitochondrial Import of Yeast Cytochrome c
Xiaoye Wang, Mark E. Dumont and Fred Sherman

J. Biol. Chem. 1996, 271:6594-6604.
doi: 10.1074/jbc.271.12.6594

Access the most updated version of this article at http://www.jbc.org/content/271/12/6594

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 48 references, 23 of which can be accessed free at http://www.jbc.org/content/271/12/6594.full.html#ref-list-1