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Isolation of the nuclear yeast genes for citrate synthase and fifteen other mitochondrial proteins by a new screening method

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To isolate nuclear genes specifying imported mitochondrial proteins, a yeast genomic clone bank was screened by an RNA hybridization-competition assay. This assay exploited the fact that mRNAs for imported mitochondrial proteins are enriched in polysomes which are bound to the mitochondrial surface in cycloheximide-inhibited yeast cells. Clones selectively hybridizing to these enriched mRNAs were further screened by hybrid-selected translation and immunoprecipitation with monospecific antisera against individual mitochondrial proteins. Thirty-six clones were isolated which contained complete or partial copies of 16 different genes for imported mitochondrial proteins. Several of these clones caused expression of the corresponding precursor polypeptide in *Escherichia coli* or over-expression of the corresponding mature protein in yeast. The gene for the matrix enzyme citrate synthase was sequenced; the derived amino acid sequence of the precursor polypeptide revealed an amino-terminal extension containing basic but no acidic residues.

Key words: citrate synthase/cloning/mitochondria

Introduction

Virtually all of the genetic information specifying a mitochondrion resides in the cell's nucleus (Schatz and Mason, 1974). To understand how this information is used for mitochondrial biogenesis, it is necessary to study the structure and the expression of the individual genes. A general isolation procedure for yeast genes coding for mitochondrial proteins was recently described by Van Loon *et al.* (1982). Their procedure exploits the fact that clones carrying genes for mitochondrial proteins give a strong hybridization signal if challenged with total mRNA from yeast cells in which mitochondrial formation had been maximally induced by growth in the absence of glucose. This procedure has proved very successful but it is, of course, limited to genes whose transcription is strongly repressed by glucose.

This study describes a general screening procedure which is based on a somewhat different principle: mitochondria isolated from cycloheximide-poisoned yeast cells contain surface-bound cytoplasmic polysomes that are enriched in mRNAs for some, but not all, mitochondrial proteins (Kellems and Butow, 1972; Ades and Butow, 1980; Suissa and Schatz, 1982). Total mRNA isolated from these polysomes should thus preferentially hybridize to DNA sequences coding for many imported mitochondrial proteins. This hybridization should not be strongly competed against by excess mRNA from 'free' cytoplasmic polysomes since, in cycloheximide-poisoned yeast cells, those polysomes preferentially contain mRNA for non-mitochondrial proteins.

By this procedure we have identified 36 clones carrying complete or partial genes for 16 different mitochondrial proteins. These proteins include components of the outer membrane, the intermembrane space, the inner membrane and the matrix. At least some of the cloned genes appear to be complete since they cause synthesis of the appropriate polypeptide product in yeast and in *Escherichia coli*. One of the complete genes isolated in this screen codes for citrate synthase, an enzyme of the mitochondrial matrix space. The complete nucleotide sequence of this gene shows that the citrate synthase precursor polypeptide has a strongly basic amino-terminal region. This amino-terminal region is removed during import of the precursor into mitochondria (Böhni *et al.*, 1983) and may participate in targeting the precursor to the mitochondria.

Results

Isolation of yeast genes for imported mitochondrial proteins

We started from a clone bank containing fragments of yeast genomic DNA in the 'shuttle vector' pFL-1 (Chevallier *et al.*, 1980). This vector can be grown in either *E. coli* or yeast. To identify clones containing yeast DNA coding for imported mitochondrial proteins, 3 x 10^5 clones were hybridized to ^{32}P-labeled mRNA which had been isolated from polysomes bound to the mitochondrial surface. This mRNA preparation is enriched in mRNAs for mitochondrial proteins. To increase the specificity of the screen, hybridization was performed in the presence of a 10- to 20-fold weight excess of unlabeled mRNA from 'free' cytoplasmic polysome. This competitor mRNA preferentially codes for non-mitochondrial proteins. About 1% of the clones hybridized strongly to the labeled mRNA even in the presence of cold competitor mRNA. The strength of the hybridization signals and the signal-to-noise ratios were comparable with those depicted in Figure 3 of van Loon *et al.* (1982). After rescreening, 1200 clones were retained for further study. Of these 1200 clones, 300 were screened by 'hybrid-selected translation' as follows: (i) the clones were allowed to select mRNA from a total yeast mRNA preparation; (ii) the mRNA bound to each clone was eluted and used to program a nuclease-treated reticulocyte lysate; (iii) the ^{35}S-labeled translation products were subjected to immunoprecipitation with a mixture of monospecific antisera against 30 different mitochondrial proteins or with antisera against crude mitochondrial subfractions (matrix or outer membrane); (iv) one fourth (75) of the clones gave positive results and thus appeared to carry yeast nuclear genes coding for mitochondrial proteins; (v) the 75 clones were rescreened with individual monospecific antisera against mitochondrial polypeptides. As a result of this final screen, 36 of the cloned DNA sequences could be assigned to 16 different mitochondrial polypeptides (Figure 1 and Table I). Since only one fourth of the 1200 clones selected by competitive hybridization were analyzed, the remaining 900 clones are

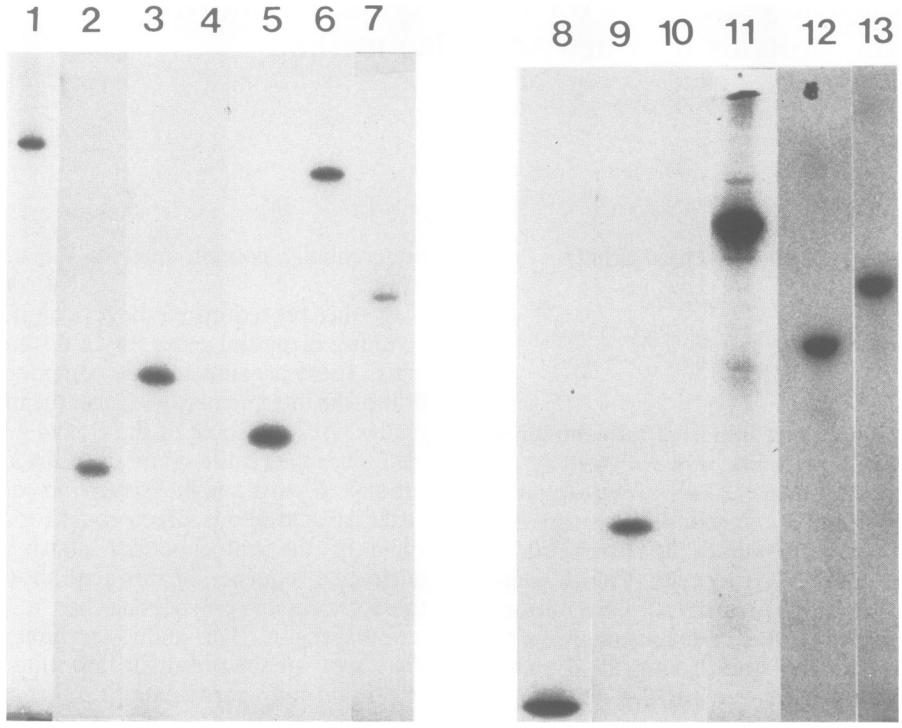

Fig 1. Identification of cloned nuclear genes for mitochondrial proteins: hybrid-selected translation coupled with immunoprecipitation. Each clone selected by the competitive RNA-hybridization assay was tested by hybrid-selected translation with a mixture of different antibodies (Materials and methods); clones giving a positive signal were then retested with antisera against individual mitochondrial polypeptides or mitochondrial subfractions. The figure illustrates the result of this second immunological screening. Two clones failing to respond to individual antisera are included (**lanes 4** and **10**) to illustrate the absence of background. Antisera against the following mitochondrial proteins were used: **(1)** ATPase *α-subunit;* **(2)** ATPase subunit V; **(3)** 33-kd outer membrane protein; **(5)** 29-kd outer membrane protein ('porin'); **(6)** 57-kd outer membrane protein; **(7)** cytochrome *c* peroxidase; **(8)** cytochrome *c* oxidase subunit VII; **(9)** 25-kd matrix protein; **(11)** ATPase *β*-subunit; **(12)** cytochrome c_1; **(13)** cytochrome bc_1-complex subunit I ('core I' protein). The two sets of lanes (**1–7** and **8–13**) were derived from two separate slab gels. Each gel was separately calibrated with unlabeled mol. wt. markers (not shown).

a potential source of many additional genes for imported mitochondrial proteins.

Many of the cloned yeast genes are expressed in E. coli and yeast

Since the cloned DNA sequences were selected by their ability to hybridize to mRNA, they could include partial genes or genes rearranged by the cloning procedure (cf. e.g., Riezman *et al.*, 1983). To identify complete genes lacking major rearrangements, several of the 36 clones listed in Table I were introduced into *E. coli* or yeast by transformation and the transformants were screened by immune blotting, pulse-labeling coupled with immune precipitation, or enzyme measurements. Aberrant DNA sequences would not be expected to direct the synthesis of the corresponding authentic protein product. Indeed, only some of the cloned genes caused expression of the corresponding mitochondrial protein *in vivo*. For example, only one of the three 'cytochrome c_1 clones' isolated (clone 113) caused expression of the cytochrome c_1 precursor in *E. coli* (Figure 2A, C) and over-expression of mature cytochrome c_1 in yeast (Figure 2B). Over-expression in yeast reflects the fact that the DNA sequences cloned into the vector pFL-1 exist in 10–50 copies in transformed yeast cells (van Loon *et al.*, 1983). As noted previously with other cloned yeast genes (van Loon *et al.*, 1983; Riezman *et al.*, 1983), the increase in steady-state level of cytochrome c_1 is less than the increase in gene copy number. This could perhaps reflect increased turnover of the excess cytochrome c_1 molecules as they are probably not present in an assembled cytochrome bc_1 complex. Another

Table I. Identity of cloned genes coding for mitochondrial proteins

Location of polypeptide in mitochondria	Polypeptide	Number of clones obtained
matrix	citrate synthase	2
	25-kd polypeptide[a,d]	1
	63-kd polypeptide[a]	1
	90-kd polypeptide[a]	1
inner membrane	ATPase complex subunit I (α)	7
	subunit II (β)	5
	subunit III (γ)	1
	subunit V[d]	3
	cytochrome *c* oxidase subunit VII[b,d]	4
	cytochrome bc_1 complex subunit I	1
	cytochrome c_1[d]	3
intermembrane space	cytochrome *c* peroxidase	1
outer membrane	29-kd polypeptide ('porin')[d]	1
	57-kd polypeptide[c,d]	3
	33-kd polypeptide[c,d]	1
	63-kd polypeptide[c]	1

[a]Function unknown; identified by using antiserum directed against total matrix. The mol. wts. refer to the precursors synthesized *in vitro*.
[b]'Subunit VII' may consist of more than one polypeptide (G.Schatz, unpublished).
[c]Function unknown; identified by using antiserum directed against total outer membrane. The mol. wts. refer to the polypeptides synthesized *in vitro*. Since none of the outer membrane proteins studied so far is made as a larger precursor, these mol. wts. should also be those of the corresponding protein *in vivo*.
[d]Shown in Figure 1.

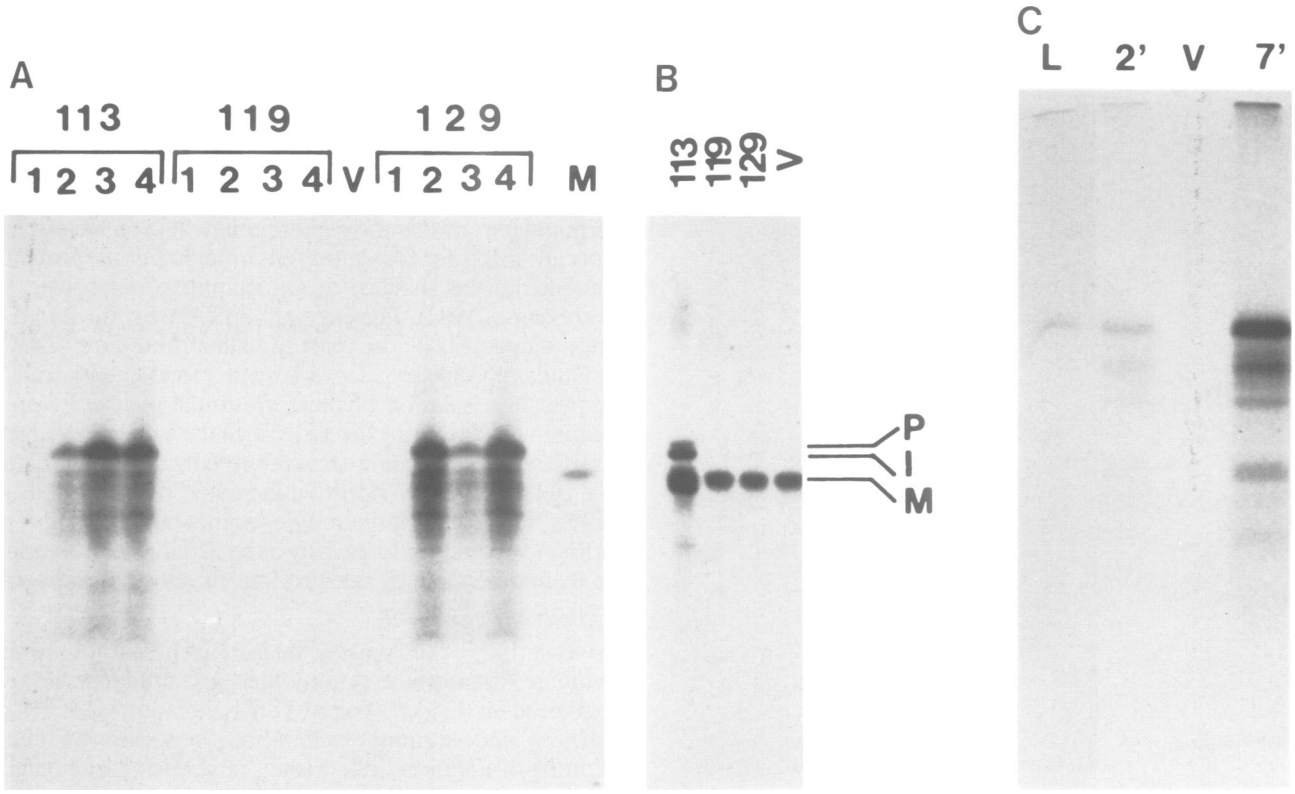

Fig. 2. The cloned yeast cytochrome c_1 gene: expression in *E. coli* and yeast. Three plasmids (113, 119 and 129) which selectively hybridize to yeast cytochrome c_1 mRNA were tested as follows. **(A)** The three plasmids were introduced into *E. coli*; the transformed cells were grown, treated overnight with chloramphenicol to amplify the plasmid, washed free of chloramphenicol, incubated for 0 **(lane 1)**, 10 **(lane 2)**, 45 **(lane 3)** and 90 min **(lane 4)** at 37°C and analyzed by immune blotting with an antiserum against yeast cytochrome c_1. V, *E. coli* transformed with the pFL-1 vector only, M, yeast mitochondria (used as a standard for mature cytochrome c_1). The structure of pFL-1 is given in Figure 5. **(B)** The three plasmids were introduced into yeast; the transformed yeast cells were grown on SD medium and analyzed by immune blotting with antiserum against yeast cytochrome c_1. V, yeast cells transformed with the pFL-1 vector only. P, I and M: precursor-, intermediate- and mature form of cytochrome c_1 (Ohashi *et al.*, 1982). These forms were identified by their immunological reactivity and their electrophoretic mobility relative to mol. wt. markers in neighboring gel lanes (not shown). **(C)** Same as **A** except that the *E. coli* cells transformed with plasmid 113 were labeled with [^{35}S]methionine for 2 and 7 min at 37°C and then subjected to immunoprecipitation with antiserum against yeast cytochrome c_1. L, pre-cytochrome c_1 immunoprecipitated from a reticulocyte lysate labeled with [^{35}S]methionine in the presence of yeast mRNA; V, *E. coli* transformed with the pFL-1 vector only and labeled for 21 min. Labeling of cells transformed with pFL-1 was longer to emphasize the absence of any labeled cytochrome c_1-crossreacting polypeptide. The faster migrating bands in the 7 min lane probably are proteolytic breakdown products of apocytochrome c_1 or reflect incorrect initiation or termination of transcription/translation in the heterologous *E. coli* system.

'cytochrome c_1 clone' (clone 129) caused expression in *E. coli* but no over-expression in yeast, perhaps because some upstream activating sequences necessary for expression in yeast are missing or altered (cf. Guarente and Mason, 1983). The clone carrying DNA sequences coding for the outer membrane 'porin' appears to carry the full gene since it caused over-expression of 'porin' in yeast (Figure 3A) and expression of 'porin' in *E. coli* (Figure 3B). Mitochondrial 'porin' is not made as a larger precursor (Schatz and Butow, 1983).

The two clones carrying sequences specifying mitochondrial citrate synthase were characterized in greater detail. When introduced into *E. coli*, both clones caused synthesis of a polypeptide of mol. wt. 52 000 that was recognized by antiserum against yeast citrate synthase and that co-electrophoresed with the citrate synthase precursor (Böhni *et al.*, 1983) made in a reticulocyte lysate (Figure 4A). A cross-reacting polypeptide of much smaller size was also made. The identity of this smaller product is unknown; it could be a specific proteolytic breakdown product of the citrate synthase precursor. When introduced into yeast, both clones caused 2-fold over-expression of mature citrate synthase antigen, most of which was found in mitochondria (Figure 4B and Table II). As shown in Figure 4B, other mitochondrial polypeptide antigens were not overproduced in the transformants.

Both clones also caused a significant increase of citrate synthase enzymic activity in the transformed yeast cells (Table III). The increase (~3.5-fold in the homogenate) was greater than the 2-fold increase determined by immune blotting (Figure 4B); in view of the many pitfalls of quantitative immune blotting (Haid and Suissa, 1983), this discrepancy was not explored further. Again, the bulk of the over-expressed citrate synthase activity was found in the mitochondrial fraction. Taken together, these results argue strongly that these two clones carry complete and authentic copies of the yeast nuclear gene for mitochondrial citrate synthase.

Nucleotide sequence of the yeast citrate synthase gene

Figure 5 shows the restriction map of citrate synthase clone 1. It carries a 4.5-kb piece of yeast DNA inserted into the single *Bam*HI site of the cloning vector pFL-1 (the *Bam*HI site is lost during cloning). The second citrate synthase clone (not shown) contains a 5.5-kb insert which is identical to the insert of clone 1 with two exceptions: (i) it contains a 1-kb extension at the end marked by the *Cla*I and *Pst*I sites in clone 1; (ii) it is present in the opposite orientation.

The nucleotide sequence of the cloned yeast citrate syn-

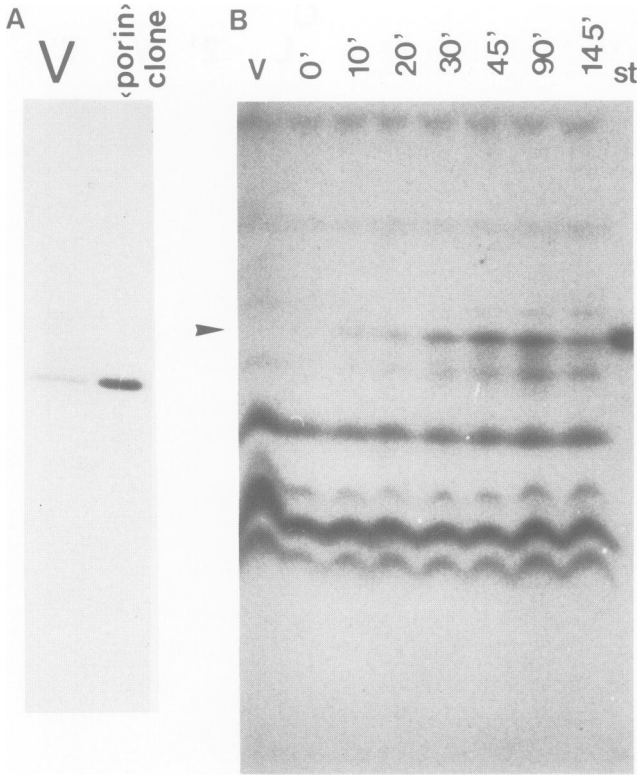

Fig. 3. The cloned outer membrane 'porin' gene: expression in yeast and *E. coli*. The plasmid which selectively hybridizes to mRNA for yeast outer membrane 'porin' was tested as follows. **(A)** Yeast cells transformed with the 'porin'-plasmid or with the control vector pFL-1 (V) were grown on SEG medium, labeled for 2 min with [^{35}S]methionine, extracted with SDS and subjected to immunoprecipitation with antiserum against yeast outer membrane 'porin'. The immunoprecipitate was analyzed on a 12% SDS-polyacrylamide gel and the gel was fluorographed. **(B)** E. coli cells transformed with the 'porin'-plasmid were grown, treated with chloramphenicol and washed as in Figure 2. The washed cells were incubated at 37°C for the indicated times and then tested by immunoblotting with antiserum against yeast mitochondrial outer membrane 'porin'. Cells transformed with the control vector pFL-1 (V) were incubated for 145 min and then treated as above. st, yeast mitochondrial outer membrane. The arrow denotes the position of the 29-kd 'porin' subunit. Even though the antiserum reacts with several endogenous *E. coli* proteins, time-dependent expression of yeast 'porin' is clearly evident.

thase gene was determined by subcloning fragments into phage M13 derivatives mp8 and mp9 and sequencing the subcloned fragments by the dideoxy method (Figure 6).

The complete nucleotide sequence together with the derived protein sequence is shown in Figure 7. The mol. wt. calculated for the citrate synthase precursor protein is 53 419.

Discussion

The cloning procedure

The procedure used in this study has led to the isolation of 1200 yeast genomic DNA clones enriched in genes for imported mitochondrial proteins. The method does not require appropriate yeast mutants deficient in individual mitochondrial functions and is limited mainly by the availability of antibodies against defined mitochondrial polypeptides. With the relatively small number of antibodies used here, 36 out of 300 clones tested (12%) proved to contain genes for imported mitochondrial proteins. The actual percentage is probably several-fold higher since mitochondria contain hundreds of different imported polypeptides.

The screening principle used here, while successful, is not completely understood since it is unclear how cytoplasmic polysomes are bound to the mitochondrial surface. Most likely, binding is mediated by the nascent chains (Kellems and Butow, 1972) of those mitochondrial protein precursors whose 'addressing sequences' are formed by the amino-terminal part of the polypeptide chain. As pointed out earlier, not all mRNAs for imported mitochondrial proteins are enriched in this subclass of mitochondrial polysomes (Suissa and Schatz, 1982). For example, mRNA for the 29-kd pore-forming protein of the yeast mitochondrial outer membrane is almost completely recovered with 'free' cytoplasmic polysomes. The isolation of the corresponding gene by our procedure was, thus, unexpected. Since the two mRNA preparations used for screening are undoubtedly cross-contaminated, the isolation of the 'porin' clone could be accidental. Alternately, mitochondria-bound polysomes might contain 'porin' mRNA which is preferentially damaged during isolation so as to be undetectable by our previous *in vitro* translation assay.

Expression in E. coli

At least three of the yeast genes isolated here (those for citrate synthase, cytochrome c_1 and outer membrane 'porin') can be expressed in *E. coli*. Expression is not only seen by pulse-labeling and immunoprecipitation, but even by immunoblotting of unlabeled cell extracts. Expression of cloned yeast genes in *E. coli* has been described before (Ratzkin and Carbon, 1977). The products of the genes for citrate synthase and cytochrome c_1 accumulate as the uncleaved precursors; *E. coli* is thus unable to process these precursors. The yeast citrate synthase precursor which accumulates in pulse-labeled transformed *E. coli* cells can be imported into isolated yeast mitochondria (R.Hay and M.Suissa, unpublished); it is thus closely similar to, and probably identical with, the precursor made in yeast cells. Expression of cloned yeast genes in *E. coli* may be a promising approach for isolating chemically or radiochemically pure mitochondrial precursor proteins and for testing the catalytic activity of mitochondrial enzyme precursors.

Structure of yeast citrate synthase

The amino acid sequence of yeast citrate synthase is highly homologous to that of pig heart citrate synthase (Bloxham *et al.*, 1981); homology to the *E. coli* enzyme (Ner *et al.*, 1983) is less marked, but still highly significant (Figure 8).

All the earlier-noted regions of homology between the porcine and the *E. coli* enzyme (Ner *et al.*, 1983) are also found with the enzyme from yeast; these regions include residues His 235, His 238, His 274, His 320, Arg 329 and Asp 375 which are essential for catalysis in the pig-heart enzyme (Bell *et al.*, 1983). The most striking region of homology is a perfectly conserved stretch of 20 amino acids (312–331); X-ray analysis of the crystalline pig heart enzyme indicates that this region binds the adenine ring of CoA by several hydrogen bonds from the peptidyl backbone of the polypeptide chain (Remington *et al.*, 1982).

The exact size of the transient 'presequence' remains uncertain. The strong amino-terminal homology between the mature enzymes from pig (Bloxham *et al.*, 1981) and chicken (Beckmans and Kanarek, 1983) and a corresponding region of the yeast enzyme (Figure 7) suggests that the presequence of the yeast precursor polypeptide terminates with the serine-alanine bond preceding the above-mentioned region of homology. However, this is unlikely for two reasons. First,

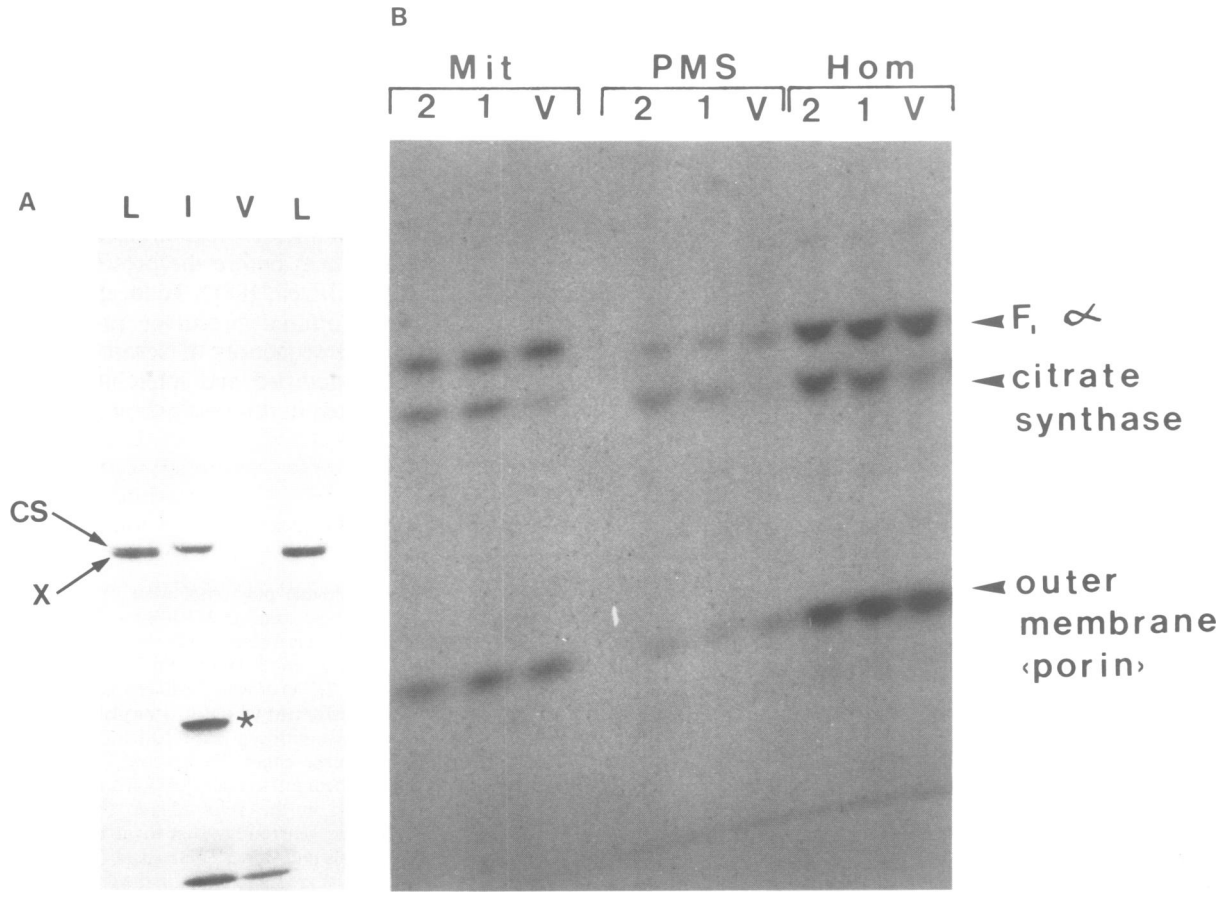

Fig. 4. The cloned yeast citrate synthase gene: expression in *E. coli* and yeast. Two plasmids (1 and 2) which selectively hybridize to yeast citrate synthase mRNA were tested as follows. **(A)** Plasmid 1 was introduced into *E. coli* and the transformed cells were grown, treated with chloramphenicol and washed as in Figure 2. They were then labeled with [^{35}S]methionine for 20 min. The labeled cells were subjected to immunoprecipitation with antiserum against yeast citrate synthase. V, cells transformed with the control vector pFL-1 and labeled for 20 min; L, pre-citrate synthase (CS) synthesized in a reticulocyte lysate programmed with yeast mRNA. The polypeptide (X) migrating slightly faster than pre-citrate synthase reflects the presence of a contaminating antibody in our citrate synthase antiserum. I, cells transformed with citrate synthase plasmid I; (the result obtained with the second citrate synthase plasmid was identical). The asterisk identifies a cross-reacting polypeptide made in *E. coli* carrying the yeast citrate synthase gene (see text). **(B)** Plasmids 1 and 2 as well as the control vector pFL-1 (V) were introduced into yeast, the cells were grown on SD medium, converted to spheroplasts, and fractionated into homogenate (Hom), mitochondria (Mit) and post-mitochondrial supernatant (PMS). Aliquots of the three fractions (derived from the same amount of yeast cells as determined by turbidity measurements at 600 nm) were tested by immunoblotting with antisera against the yeast polypeptides indicated on the right-hand margin.

Table II. Overproduction of citrate synthase in yeast cells transformed with plasmid-borne citrate synthase gene: immunoblotting

Yeast cells transformed with	Signal (c.p.m.) elicited by		Ratio of citrate synthase to F_1-ATPase α-subunit	Over-production (fold)
	citrate synthase	F_1-ATPase α-subunit[a]		
citrate synthase clone 1	616	899	0.685	1.9
citrate synthase clone 2	495	665	0.744	2.0
pFL-1 (vector only)	270	744	0.363	(1.0)

[a]This polypeptide was used as a reference for quantifying overproduction of citrate synthase. Control experiments showed that transformation of the strains with either of the two citrate synthase clones did not affect the intracellular levels of F_1-ATPase α-subunit (not shown). The immunoblot was quantified by excising the radiolabeled bands from the nitrocellulose replica and counting them in a gamma counter.

this assignment predicts that the mol. wt. difference between precursor and mature form of the yeast enzyme is >4 kd whereas the experimentally determined difference appears to be only ~2 kd (Figure 5A; see also Alam *et al.*, 1982; Böhni *et al.*, 1983). Second, a comparison by SDS-polyacrylamide gel electrophoresis indicates that the mature yeast enzyme is ~2 kd larger than the mature porcine enzyme (Alam *et al.*, 1982). Thus, we favor the view that the mature yeast enzyme differs from its mammalian counterparts by an amino-terminal stretch of 10–20 amino acids. (A similar situation also exists with yeast-iso-1-cytochrome *c*, see Dickerson and Timkovich, 1976.) The transient presequence would then be only about half as long as suggested by the homology shown in Figure 8. However, this uncertainty does not affect the conclusion that the presequence is strongly basic: the sequence upstream from the amino-terminal homology shown in Figure 8 contains only basic, but no acidic amino acids.

Two reports had claimed that yeast citrate synthase lacks sulfur-containing amino acids (Greenblatt and Sarkissian, 1974; Alam *et al.*, 1982), but our data do not support the claim. The sequence (starting with the amino-terminal region of homology shown in Figure 8) contains three methionine residues, two of which (Met 139 and Met 228) are invariant among the three enzymes. The mature yeast enzyme lacks cysteine, however; Cys at position −19 is most likely localiz-

Table III. Overproduction of citrate synthase in yeast cells transformed with plasmid-borne citrate synthase gene: activity measurements on subcellular fractions

Enzyme activity	Subcellular fraction	Specific activity in cells transformed with			Overproduction (fold)
		pFL-1 (vector only)	clone 1	clone 2	
citrate synthase	homogenate	0.44	1.57	1.51	3.4–3.6
	mitochondria	2.67	6.8	10.6	2.5–4.0
	post-mitochondrial supernatant	0.23	0.65	0.54	2.3–2.8
fumarase[a]	homogenate	0.14	0.11	0.09	<1
	mitochondria	0.24	0.22	0.19	<1
	post-mitochondrial supernatant	0.13	0.09	0.08	<

[a]Used as a control; since eukaryotic cells generally contain a mitochondrial as well as a cytosolic fumarase (Nakashima *et al.*, 1976), the enzymic activity is only moderately enriched in the mitochondrial fraction.
The yeast strain SF 747 was transformed with the plasmids listed in the Table, grown on SD, converted to spheroplast and fractionated into homogenate, mitochondria and post-mitochondrial supernatant. Citrate synthase and fumarase were assayed according to published procedures in the presence of 0.1% cholate to unmask latent mitochondrial activity.

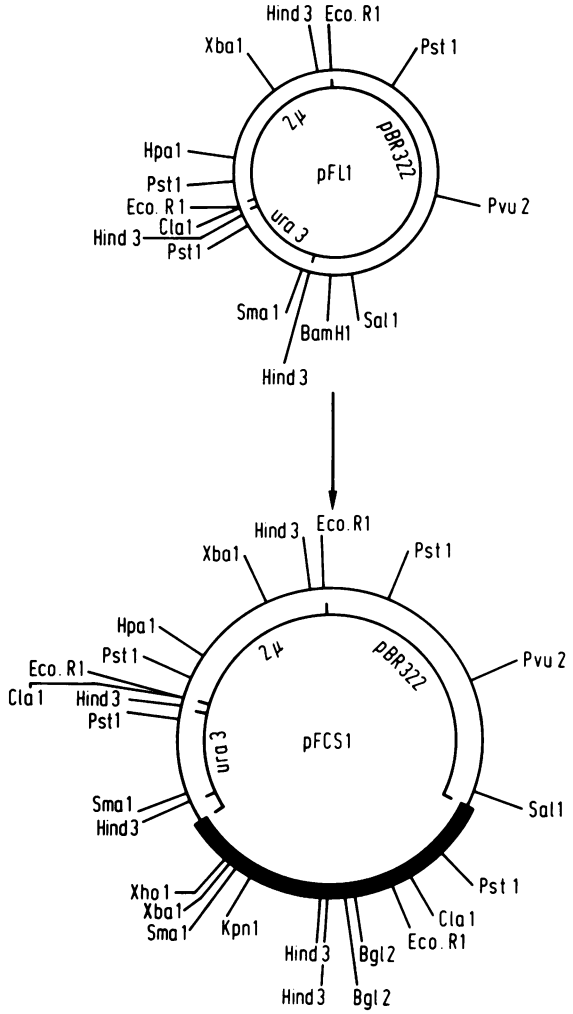

Fig. 5. Restriction map of the cloning vector pFL-1 and of citrate synthase plasmid 1 (pFCS 1). The yeast genomic DNA fragment inserted into the *Bam*HI-site of the cloning vector is indicated by the thick black bar. The thin lines identify sequences derived from the yeast 2 μm plasmid, pBR322 and the yeast *URA 3* gene (Chevallier *et al.*, 1980). The *Bam*HI site of the cloning vector is lost by the cloning procedure.

ed in the transient presequence.

Potential applications of the clone collection

Since import of proteins into mitochondria is a post-translational process, the 'addressing sequences' guiding the precursor into the mitochondria could be located in the presequence, the mature sequence, or in both. This is in marked contrast to the co-translational segregation of proteins across the endoplasmic reticulum in which transmembrane movement of the protein starts before the protein has been synthesized to completion (Kreil, 1981). To locate the 'addressing sequences' in mitochondrial precursor proteins, the presequences and mature sequences of several precursors will have to be deleted, modified and interchanged. The large number of genes isolated in this study should help in such efforts.

Materials and methods

Yeast cells and vectors

Mitochondria-bound cytoplasmic polysomes were isolated from the *Saccharomyces cerevisiae* wild-type strain D 273-10B (α; ATCC 25657). Transformation experiments were performed with the *S. cerevisiae* strain SF 747-19D (α *gal 2, his 4, leu 2, ura 3*; kindly provided by Charles Field and Randy Schekman, Berkeley, USA) or with the *E. coli* strain HB 101 (Kedes *et al.*, 1975). SF 747-19D transformed with pFL-1 or plasmids derived from it was grown on 0.67% yeast nitrogen base (Difco)-20 μg/ml L-histidine-20 μg/ml L-leucine containing either 2% glucose (SD medium) or 3% glycerol-2% ethanol (SEG medium) as major carbon and energy sources. The yeast genomic clone bank in the yeast/*E. coli* 'shuttle vector' pFL-1 (Chevallier *et al.*, 1980) was generously given to us by Francois Lacroute (Strasbourg, France). Details and original references for growth and transformation of yeast and *E. coli* are summarized in the paper by Riezman *et al.* (1983). Amplification of the plasmids in transformed *E. coli* cells was as described by Hershfeld *et al.* (1974).

Biochemical preparation and techniques

Published procedures were used for isolating RNA from mitochondria-bound and 'free' cytoplasmic polysomes (Suissa and Schatz, 1982), for extracting proteins from yeast cells (Riezman *et al.*, 1983), for immune blotting (Haid and Suissa, 1983), for pulse-labeling and fractionating yeast spheroplasts (Reid and Schatz, 1982), for preparing nuclease-treated reticulocyte lysate (Pelham and Jackson, 1976), for immunoprecipitation and analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis and fluoro-

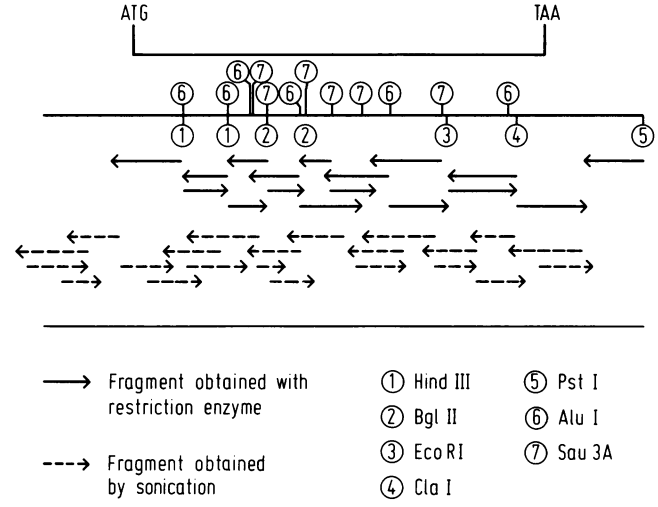

Fig. 6. Strategy for sequencing the yeast citrate synthase gene by the dideoxy-method. The coding region is indicated by the top line. Fragments of the gene were generated either by cleavage with restriction enzymes (solid arrows) or by sonication (broken arrows). The directions of the arrows specify the direction of sequencing. See Materials and methods for further details.

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                15                  30                  45                  60                  75                  90
AAA AGG CGT CAC GTT TTT TTC CGC CGC AGC CGC CCG GAA ATG AAA AGT ATG ACC CCC GCT AGA CCA AAA AAT ACT TTT GTG TTA TTG GAG

               105                 120                 135                 150                 165                 180
GAT CGC AAT CCC TTT GGA GCT TTT CCG ATA CTA CTC GAC TTA TCC GAC CTC TTG TTG TTT GAA AAT GTC AAT TGA TAT CCA TCC CAT TAT

               195                 210                 225                 240                 255                 270
AAA TGC TCA AAA CTT GCA GCA ACT ATT CTT TAC CCT TCC CCT GTT ATG GAT TGC TAG TCT TAA GGG GGA AAT TTG CTG TTT ACT AAA ATA

               285                 300                 315                 330                 345                 360
CAA ACC AGG TTT GTT TTG GCT TTT ATT TGC ATT TAA GTA ATT ACA ATT ACA ACC ATT AAA AAG AAA ATA AGG CAA AAC ATA TAG CAA TAT

               375                 390                 405                 420                 435                 450
AAT ACT ATT TCG AAG ATG TCA GCG ATA TTA TCA ACA ACT AGC AAA AGT TTC TTA TCA AGG GGC TCC ACA AGA CAA TGT CAA AAT ATG CAA
                   MET SER ALA ILE LEU SER THR THR SER LYS SER PHE LEU SER ARG GLY SER THR ARG GLN CYS GLN ASN MET GLN

               465                 480                 495                 510                 525                 540
AAG GCT CTT TTT GCA CTA TTG AAT GCT CGC CAC TAT AGT AGC GCC TCC GAA CAA ACG TTG AAG GAG AGA TTT GCT GAA ATT ATC CCA GCA
LYS ALA LEU PHE ALA LEU LEU ASN ALA ARG HIS TYR SER SER ALA SER GLU GLN THR LEU LYS GLU ARG PHE ALA GLU ILE ILE PRO ALA

               555                 570                 585                 600                 615                 630
AAG GCA CAA GAA ATT AAA AAA TTC AAG AAA GAA CAC GGT AAA ACC GTT ATT GGT GAA GTT CTT TTG GAG GAG CAA GCT TAT GGT GGT ATG
LYS ALA GLN GLU ILE LYS LYS PHE LYS LYS GLU HIS GLY LYS THR VAL ILE GLY GLU VAL LEU LEU GLU GLU GLN ALA TYR GLY GLY MET

               645                 660                 675                 690                 705                 720
AGA GGT ATT AAA GGC CTT GTT TGG GAA GGT TCC GTG TTA GAC CCC GAA GAA GGT ATT AGA TTT AGG GGT CGT ACT ATT CCA GAA ATT CAA
ARG GLY ILE LYS GLY LEU VAL TRP GLU GLY SER VAL LEU ASP PRO GLU GLU GLY ILE ARG PHE ARG GLY ARG THR ILE PRO GLU ILE GLN

               735                 750                 765                 780                 795                 810
AGG GAA CTA CCA AAG GCT GAG GGT AGT ACA GAA CCT TTG CCA GAA GCT TTA TTT TGG TTG CTT TTG ACT GGT GAA ATA CCT ACT GAC GCT
ARG GLU LEU PRO LYS ALA GLU GLY SER THR GLU PRO LEU PRO GLU ALA LEU PHE TRP LEU LEU LEU THR GLY GLU ILE PRO THR ASP ALA

               825                 840                 855                 870                 885                 900
CAA GTT AAA GCC CTT TCT GCT GAT TTA GCT GCC AGA TCA GAA ATT CCA GAG CAC GTT ATC CAA CTT TTA GAT AGC CTC CCA AAA GAT CTA
GLN VAL LYS ALA LEU SER ALA ASP LEU ALA ALA ARG SER GLU ILE PRO GLU HIS VAL ILE GLN LEU LEU ASP SER LEU PRO LYS ASP LEU

               915                 930                 945                 960                 975                 990
CAT CCA ATG GCG CAA TTT TCT ATT GCC GTG ACT GCT TTA GAA AGC GAG TCT AAG TTT GCC AAA GCA TAT GCT CAA GGT GTA TCC AAG AAA
HIS PRO MET ALA GLN PHE SER ILE ALA VAL THR ALA LEU GLU SER GLU SER LYS PHE ALA LYS ALA TYR ALA GLN GLY VAL SER LYS LYS

              1005                1020                1035                1050                1065                1080
GAA TAT TGG AGC TAT ACA TTT GAA GAT TCG TTA GAT CTG CTG GGT AAA TTA CCT GTT ATT GCT TCC AAA ATT TAT CGT AAT GTG TTC AAG
GLU TYR TRP SER TYR THR PHE GLU ASP SER LEU ASP LEU LEU GLY LYS LEU PRO VAL ILE ALA SER LYS ILE TYR ARG ASN VAL PHE LYS

              1095                1110                1125                1140                1155                1170
GAT GGT AAA ATT ACT TCA ACC GAT CCT AAT GCT GAC TAT GGT AAA AAT TTG GCC CAA CTT TTG GGC TAC GAA AAC AAG GAT TTT ATT GAC
ASP GLY LYS ILE THR SER THR ASP PRO ASN ALA ASP TYR GLY LYS ASN LEU ALA GLN LEU LEU GLY TYR GLU ASN LYS ASP PHE ILE ASP

              1185                1200                1215                1230                1245                1260
TTA ATG AGA CTA TAT TTA ACT ATT CAT TCT GAT CAT GAA GGT GGT AAC GTT TCT GCC CAT ACT ACA CAT TTA GTG GGT TCT GCC TTA TCT
LEU MET ARG LEU TYR LEU THR ILE HIS SER ASP HIS GLU GLY GLY ASN VAL SER ALA HIS THR THR HIS LEU VAL GLY SER ALA LEU SER

              1275                1290                1305                1320                1335                1350
TCG CCA TAC TTA TCT TTG GCC GCT GGT TTG AAT GGT TTA GCT GGC CCA TTA CAT GGT CGT GCC AAT CAA GAA GTT TTA GAA TGG CTA TTT
SER PRO TYR LEU SER LEU ALA ALA GLY LEU ASN GLY LEU ALA GLY PRO LEU HIS GLY ARG ALA ASN GLN GLU VAL LEU GLU TRP LEU PHE

              1365                1380                1395                1410                1425                1440
AAA TTG AGA GAA GAA GTG AAA GGT GAC TAT TCA AAA GAA ACA ATT GAA AAG TAC TTG TGG GAT ACT TTG AAC GCA GGG AGA GTT GTT CCT
LYS LEU ARG GLU GLU VAL LYS GLY ASP TYR SER LYS GLU THR ILE GLU LYS TYR LEU TRP ASP THR LEU ASN ALA GLY ARG VAL VAL PRO

              1455                1470                1485                1500                1515                1530
GGT TAT GGC CAT GCG GTT TTG AGA AAA ACT GAT CCT CGT TAT ACG GCT CAA CGT GAA TTC GCA TTG AAA CAT TTC CCA GAT TAC GAG TTA
GLY TYR GLY HIS ALA VAL LEU ARG LYS THR ASP PRO ARG TYR THR ALA GLN ARG GLU PHE ALA LEU LYS HIS PHE PRO ASP TYR GLU LEU

              1545                1560                1575                1590                1605                1620
TTT AAG TTG GTC TCC ACC ATT TAT GAA GTT GCC CCA GGG GTT TTA ACT AAG CAT GGT AAA ACT AAG AAC CCA TGG CCA AAT GTT GAT TCA
PHE LYS LEU VAL SER THR ILE TYR GLU VAL ALA PRO GLY VAL LEU THR LYS HIS GLY LYS THR LYS ASN PRO TRP PRO ASN VAL ASP SER

              1635                1650                1665                1680                1695                1710
CAT TCC GGT GTT TTA TTG CAA TAC TAT GGT CTA ACT GAG GCT TCG TTC TAC ACT GTA TTG TTT GGT GTT GCC AGA GCT ATT GGT GTG TTA
HIS SER GLY VAL LEU LEU GLN TYR TYR GLY LEU THR GLU ALA SER PHE TYR THR VAL LEU PHE GLY VAL ALA ARG ALA ILE GLY VAL LEU

              1725                1740                1755                1770                1785                1800
CCC CAA TTA ATC ATC GAT AGG GCT GTT GGT GCT CCA ATC GAA AGG CCA AAA TCA TTC TCC ACC GAA AAA TAC AAG GAG TTG GTA AAG AAA
PRO GLN LEU ILE ILE ASP ARG ALA VAL GLY ALA PRO ILE GLU ARG PRO LYS SER PHE SER THR GLU LYS TYR LYS GLU LEU VAL LYS LYS

              1815                1830                1845                1860                1875                1890
ATC GAA AGT AAG AAC TAA GGA AAA TTT GAT TTT TGA TTC AGG GTA TGC GGC TAT TCA AAC ACG TAT TTT CAT ATA CGA ATG AGT AGT TTA
ILE GLU SER LYS ASN xxx

              1905                1920                1935                1950                1965                1980
TAA TTT ATA TAA ATA TAT ATT ACA TAA GGA AAT GGC TAT GAC TTC ATG ATT TTT ACG CTT TGA GTC CCC TAT GCC TAC CGG TTC TGT TAT

              1995                2010                2025                2040                2055                2070
ATA CTT GGC AAA TGA TAT CAA TGA ATA GAA AAC AAA TGC TCT TAA ATT CCT GCA AAG CGC TTT CAA ATC GAA TTG TGG TGG CTG GCG CGC

              2085                2100                2115                2130                2145                2160
TGA TTT AGC AGT AAA ATC TAG CCT GTA GTC GAT ATC TAC GTC AAG GTG TAA CTA ACG TTT ATA TGT ATA TGA ACC TGC AGA AGA AGC AAA

              2175                2190                2205                2220                2235                2250
ATG ACA AGG TTA ATG CAT CTT TAT CTG TCC TAG AGT AGA AGA TAC ACT AAA AAG TTG ATA AAG CGA ATA TAA TAT GGT CAT GTG AAA AAA

              2265                2280                2295                2310                2325                2340
GCA ATC TCA TAA AAT TTT CTC TTT TTC CTT TTT TTC TTC TGT TCT TAC CTC AAG ATA AAT GTC ACT TTT ATA CTA ATT AAA AGC AGT GTT

              2355                2370                2385                2400                2415
TTA TAT TTC TAC CAG CAA CCT TTT GAG TTT AAT CAC ATA AAA ACT AGG AGC AGG GTA GCA TAA ACC TGC TGT CTA ATC CGT ATT CAT
```

Fig. 7. Nucleotide sequence of the yeast citrate synthase gene and derived amino acid sequence of the corresponding protein.

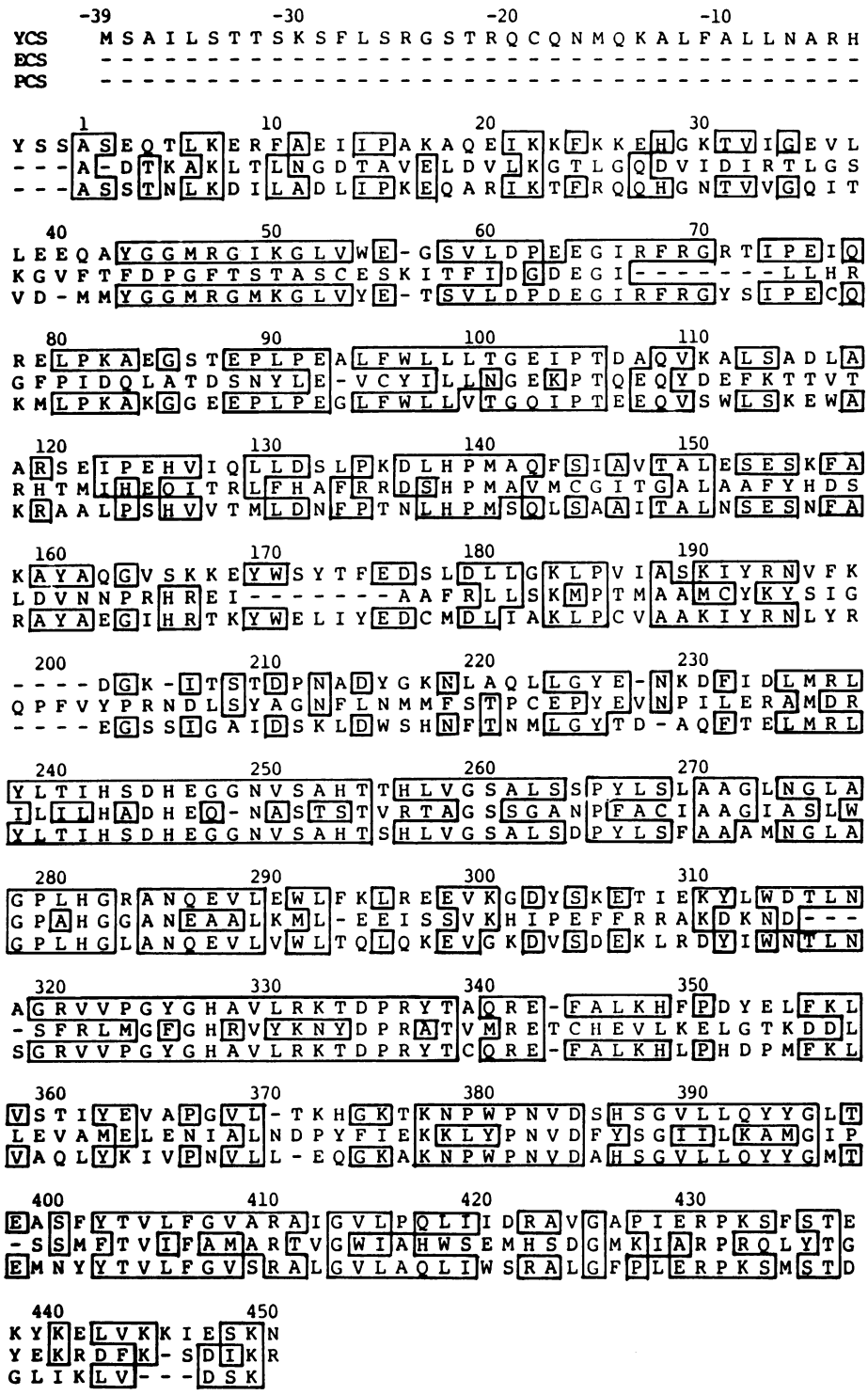

Fig. 8. Comparison between the amino acid sequences of citrate synthase from yeast (YCS), *E. coli* (ECS) and pig heart (PCS). The sequences were aligned to maximize homologies. Regions of homology are boxed.

graphy (Gasser *et al.*, 1982), for isolating yeast subcellular fractions (Daum *et al.*, 1982), for quantifying fluorograms (Suissa, 1983), for assaying citrate synthase (Srere, 1969) and fumarase (Racker, 1950) and for measuring protein (Lowry *et al.*, 1951). Most of the rabbit antisera used in this study were raised against individual polypeptide bands excised from SDS-polyacrylamide gels (Daum *et al.*, 1982). Monospecificity was checked by immunoblotting, using either whole mitochondria or yeast spheroplasts as antigens. Antisera against total outer membrane or matrix fraction were raised by injecting the undenatured fractions into rabbits.

Recombinant DNA techniques

Mitochondria-associated cytoplasmic polysomes were isolated from galactose-grown *S. cerevisiae* strain D 273-10B (Suissa and Schatz, 1982). Total polysomal RNA was prepared; polyadenylated RNA was isolated from it (Holland *et al.*, 1977), labeled with ^{32}P *in vitro* (Goldbach *et al.*, 1978) and used to screen a yeast genomic DNA bank in the 'shuttle' vector pFL-1 by a hybridization-competition assay (van Loon *et al.*, 1982). A 10- to 20-fold excess (by weight) of total RNA from 'free' yeast cytoplasmic polysomes of galactose-grown D 273-10B served as competitor RNA. Of the $\sim 3 \times 10^5$ clones screened, ~3000 (1%) hybridized strongly to the labeled RNA even in the presence of competitor RNA; these clones were screened further as described under Results. All other recombinant DNA manipulations involved standard procedures; details and original references are described in the papers by Riezman *et al.* (1983) and Hase *et al.* (1983).

DNA sequencing

Sequencing was done by the dideoxy method (Sanger *et al.*, 1977). Single-strand templates were prepared from either of the two citrate synthase clones as follows. (i) The smaller of the two *SalI/XhoI* fragments derived from clone 1 was circularized with DNA ligase and sonicated; random fragments (average size 200–500 bp) were isolated by electrophoresis in agarose gels and end-repaired with T4 polymerase. Alternatively, the *SalI/XhoI* fragment was further digested with either *AluI* or *Sau*3A. (ii) Specific fragments were isolated from clone I by digestion with *SalI/PstI*, *PstI/ClaI*, *ClaI/Eco*RI and *Eco*RI/*BglII*. (iii) The smaller of the two *SalI/BglII* fragments derived from clone 2 was further cut with *HindIII* or *HindIII/SmaI*. The resulting fragments were inserted into the appropriate cloning sites of the phage M13 derivatives mp8 and mp9 (Sanger *et al.*, 1980; Messing *et al.*, 1981; Messing and Vieira, 1982).

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