Molecular Biological Approaches to Study the Function and Regulation of Citrate Synthase Genes in *Saccharomyces cerevisiae*

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Introduction

Almost all of the aerobic organisms contain citric acid cycle (or, tricarboxylic acid cycle). This cycle is involved both in energy metabolism and biosynthetic reactions; generation of NADH which derives the synthesis of chemical energy, ATP, and provision of intermediates needed for the biosynthesis. Because of its importance in the cellular metabolism, the regulation of the TCA cycle and its component enzymes has been extensively studied by many biologists (7, 28). Citrate synthase is resposible for the initial step of the cycle and has been recognized as the rate limiting step (14, 121, 26). Understanding of the mechanism of the expression of citrate synthase should be a key step for the elucidation of the regulation of the TCA cycle in the cell metabolism.

Citrate Synthase is Highly Regulated

As a key enzyme in cell metabolism, citrate synthase is highly regulated in response to various metabolic signals in different type of organisms (28). Specific regulation of citrate synthase differs significantly from one organism to another. For example, this enzyme is usually inhibited by ATP in eukaryotic cells (15, 16) and by NADH in Gram negative bacteria (27, 28). But the activity of this enzyme is rather universally affected by the carbon sources (2, 7). In yeast, the activity of citrate synthase seems to be modulated by ATP level (10, 17). It is further regulated by the oxygen availavility (16, 23, 25) and by the carbon sources (5, 28, 25). The exact mechanism of these regulations is not clearly underatood. Recently, Hoosein and Lewin (11) indicated that the derepression of citrate synthase may occur at the transcriptional level in *Saccharomyces cerevisiae* by showing that the amount of translatable RNA increases as the enzyme is induced by the exhaustion of glucose in media.

Molecular Cloning of Structural Genes Encoding Citrate Synthase in *Sccharomyces cerevisiae*

The most plausible way to clone yeast genes is mutant complementation by using plasmid vectors. This approach has been impossible for the isolation of the gene encoding citrate synthase in *S. cerevisiae* because of the lack of availavility of cit syn mutant (1). A cit syn mutant of *S. cerevisiae* has been reported by Burand *et al.* (3), but it seems not to encode the structural gene (20). An alternative to bypass this situation is immunological screening by using antibody specific to yeast citrate synthase. In an effort to isolate nuclear genes for mitochondrial proteins, Suissa *et al.* (24) succeeded in isolating several genes including one encoding citrate synthase. Its sequence data revealed significant homology with those encoding *E. coli* and pig heart citrate synthases. We also have tried to isolate the gene coding for citrate synthase by immunologically screening λgt11 genomic bank (27). Three clones were isolated and appeared to contain an identical region by southern hybridization analysis and restriction mapping (12). This gene was referred to *CIT2*, and shown to encode a yeast citrate synthase by the following criteria (12). First, the gene was

isolated by immunological screening of yeast genomic library in λgt11 with antibody which reacted with a single protein of the correct size for citrate synthase (50 to 55 KDa) in extracts of yeast cells (1) and of *E. coli* cells infected with λgt11 phage bearing *CIT2*. Second, the gene cmplemented an *E. coli* mutation that abolished citrate synthase activity. From this experiment, transcriptional direction of *CIT2* gene could also be deduced. Third, simultaneous disruption in the yeast genome of both *CIT2* and another gene encoding citrate synthase, *CIT1* (24), produced the citrate synthase-deficient phenotypes of glutamate auxotrophy and poor growth on rich medium containing lactate, a nonfermentable carbon source. Disruption of both genes was necessary to produce these phenotypes (Fig.1). Our results agree with a recent report that disruption of *CIT1* dose not eliminate citrate synthase activity or produce citrate synthase-deficient growth phenotypes (20).

We therefore conclude that citrate synthase in *S. cerevisiae* is encoded by the two loci *CIT1* and *CIT2* and that the product of either gene can supply a level of citrate synthase sufficient for biosynthesis of glutamate and for optimal growth on rich lactate medium.

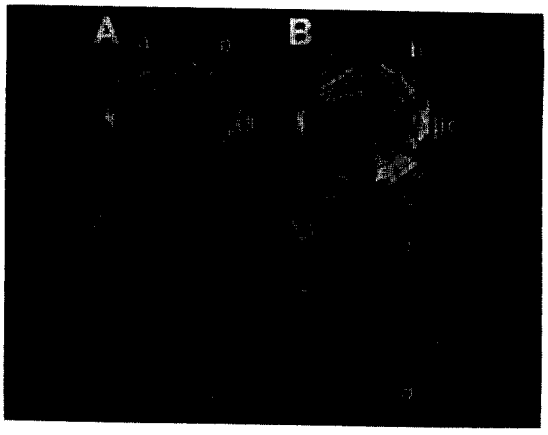

Fig. 1. Growth phenotypes of strains in which *CIT1* or *CIT2* or both were disrupted. Wild-type and disrupted strains were struck on glucose YMM(A), glucose plus glutamate YMM(B), lactate YEP(C), and acetate YEP(D). YMM plates were supplemented with histidine, leucine, adenine, and uridine. Strains were (a) 1-7A, (b) 1-7A-U, (c) 1-7A-L, (d) 1-7A-UL, and (e) 1-7A[*rhon*].

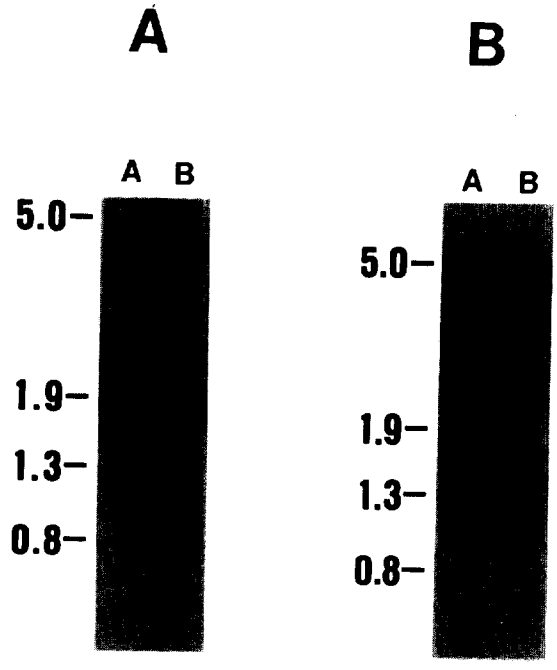

Fig. 2. Identification of *CIT1* and *CIT2* transcripts by Northern hybridization. RNA (8μg) from strains in which the *CIT1* or *CIT2* gene had been disrupted with the *LEU2* or *URA3* gene, respectively, was electrophoresed, blotted to nitrocellulose, and hybridized with a ^{32}P-labeled RNA probe. (A) RNA isolated from strains 1-7A(lane A) and 1-7A-L(lane B) was probed with ^{32}P-labeled antisense RNA transcribed from the 0.7kbp *Eco*RI-*Hin*dIII fragment of pFCS2 carrying a portion of *CIT1*. (B) RNA isolated from strains 1-7A(lane A) and 1-7A-U(lane B) was probed with ^{32}P-labeled antisense RNA transcribed from the 3.5-kbp *Eco*RI fragment of CS10 carrying *CIT2*. All strains were grown in lactate YMM supplemented with histidine, leucine, adenine, and uridine. The numbers indicate the positions of various DNA fragments (in kilobase pairs) generated by restricting lambda DNA with *Hin*dIII and *Eco*RI and probing with ^{32}P-labeled lambda DNA.

Modulation of Citrate Synthase Level by Carbon Source and Glutamate Occurs at the Transcriptional Level.

To investigate the mechanism of citrate synthase regulation, we used the cloned genes as hybridization probes in the Northern blotting analyses. Anti-sense RNA's which correspond to 0.7kbp fragment of *CIT1* gene or 3.5kbp insert DNA of phage CS10 for *CIT2* gene were made on the basis of the DNA sequence of *CIT1* (28) and the transcriptional direction of *CIT2* gene. *CIT1* and *CIT2* transcripts were identified by comparing RNA from wild and disrupted strains (Fig. 2). It appeared that *CIT1* and *CIT2* transcripts are approximately 1.9 and 1.7kb, respectively. It is to be noted that disruption of each gene revealed a weak band which is probably due to cross hybridization with RNA of the other gene. Our results showed that regulation of citrate synthase cocurs at the transcriptional level in yeast (Fig.3). But *CIT1* and *CIT2* genes showed both similarities and dissimilarities in their regulation. First, both genes showed increase in the RNA level about 2-to 4-fold when galactose was used as carbon source instead of glucose, but only *CIT1* RNA was increased about 6-to 7-fold in cells grown with lactate. Secondly in both genes, RNA levels were reduced about 5-to 10-fold by addition of glutamate to glucose or galactose media and were not reduced by addition of glutamate to lactate meidun. This form of synergistic regulation by catabolite repression and exogenous glutamate has also been observed for the bacterial citrate synthase

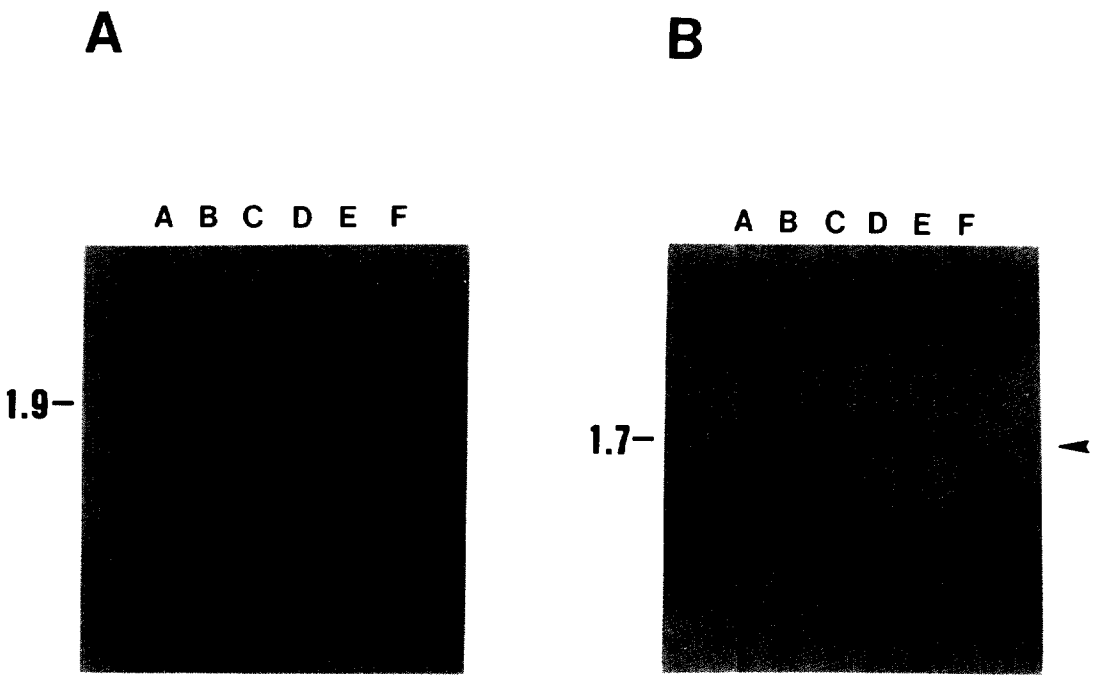

Fig. 3. Regulation of expression of *CIT1* and CIT2 detected by Northern hybridization. Strain *1-7A* was grwon in YMM medium containing glucose (lanes A), glucose plus glutamate (lanes B), galactose(lanes C), galactose plus glutamate(lanes D), lactate(lanes E), or lactate plus glutamate(lanes F). YMM was supplemented with histidine, leucine, adenine, and uridine. RNA isolated from these cultures (8μg) was electrophoresed, blotted to nitrocellulose, and hybridized with a ^{32}P-labeled RNA probe. (A) Blot probed with ^{32}P-labeled antisense RNA transcribed from the 0.7-kbp *Eco*RI-*Hin*dIII fragment of pFCS2 carrying a portion of *CIT1*. (B) Blot probed with ^{32}P-labeled antisense RNA transcribed from the 3.5-kbp *Eco*RI fragment of CS*10* carrying *CIT2*. The arrow indicates the *CIT2* transcript defined in Fig.6. Sizes are indicated in kilobase pairs.

(6, 8, 9).

DNA Sequence Analysis of *CIT1* and *CIT2* genes

Nucleotide sequence of *CIT2* gene was determined by the dideoxy chain-termination method (22). Whole insert DNA of phage CS4 was subcloned into M13 mp18, and M13 mp19, and serially deleted by T4 DNA polymerase to produce appropriately overalpping regions (4). The complate DNA sequence contained one major open reading frame of 459 codons for *CIT2* gene (13). This gives a calculated Mr for the protein of 51282.18 (13), which agrees fairly well with previous reports (1, 11). Strong homology (81%) was observed between *CIT1* and *CIT2* genes along the whole sequence except the N terminal region (Fig.4). Homology of 61 and 60% was observed between *CIT1* product and pig heart enzyme, and between *CIT2* product and pig heart enzyme, respectively. Homology to *E. coli* enzyme was much less significant. The most notable feature of Fig.4 is that *CIT1* gene product has longer stretches at its N terminal region. It probably functions as a signal sequence when it is imported into mitochondria (24).

Why 2 citrate synthase genes in yeast?

The TCA cycle occurs in the mitochondria of *S. cerevisiae*. When grown on media containing ethanol or acetate as a sole carbon source, S. cerevisiae also requires the glyoxylate cycle which occurs in the cytoplasm (5). Citrate synthase, therefore, is required both in mitochondria and in cytoplasm. This was actually observed by several groups (5, 18, 20).

Slight difference in the size of *CIT1* and *CIT2* transcripts was recognized in our Nothern blotting analyses (Fig.2). Our sequence analysis directly revealed the size difference of proteins encoded by *CIT1* and *CIT2* at their N terminal regions (Fig.4). Based on the molecular weight difference between the precusor and nature form of citrate synthase, Suissa *et al.* (24) indicated that the amino-terminal stretch of *CIT1* protein would be 10-20 amino acids. Thus, we conclude that the 19 extra amino acid stretch of *CIT1* protein represent the signal sequence which is required for the targeting of *CIT1* protein into mitochondria. Meanwhile, citrate synthase encoded by *CIT2* may function in the glyoxylate cycle, and represent the activity observed in the cytoplasm (5, 20, 25). Specifi-

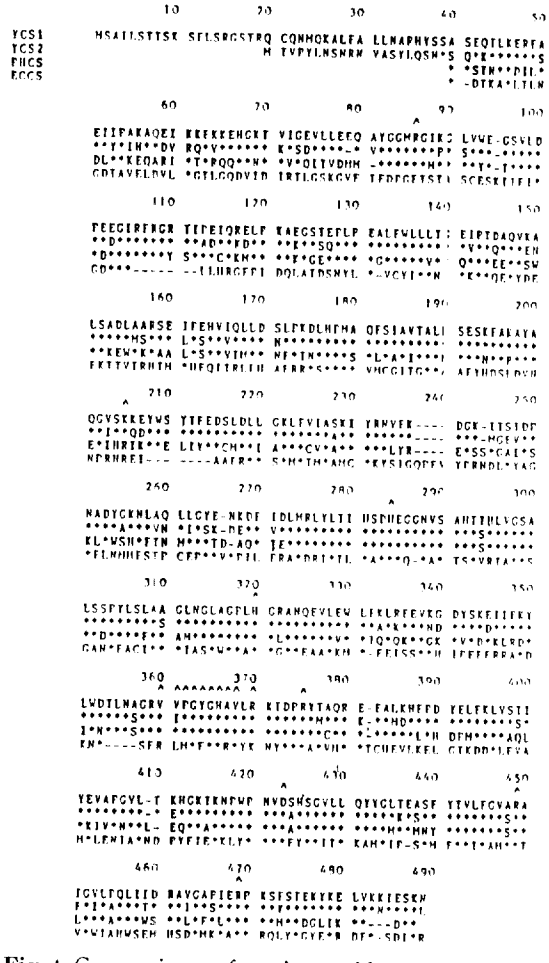

Fig. 4. Comparison of amino acid sequences of citrate synthases from yeast (*CIT1* = YCS1 : *CIT2* = YCS2), pig heart (PHCS) and *E. coli* (ECCS). The predicted amino sequence of the CIT2 product is shwon aligned with other citrate synthase sequences from yeast, pig (mature form) or *E. coli*. *Residues homologous with YCSI are indicated by asterisks* (*). *Gaps intr oduced to maximize homology are indicated by dashes* (—).

cally, almost of all citrate synthase activity of only the mitochondrial fraction was shown to be abolished in the strain where *CIT 1* gene was disrupted (20). We therefore conclude that *CIT 2* gene encode cytoplasmic citrate synthase which presumably functions in the glyoxylate cycle. Based on this conclusion, we propose a schematic model to explain the growth patterns of the disrupted strains on the media containing nonfermentable carbon source, lactate or acetate (Fig.1). Doubly disrupted strain, 1-7A-UL, showed some growth, if significantly reduced, on lactate -medium (Fig.1). By the action of lactate dehydrogenase, reducing power could be supplied in this strain, which possibly explains the partial growth. In singly disrupted strains, 1-7A-L or 1-7A-U, occurs either glyoxylate cycle or TCA cycle, respectively, which would supplement the cell's energetic need, and thus allows the strain's apparently optimal growth (Fig. 1 & 5). When 1-7A-UL was grown on medium containing acetate as a sole carbon source, it did not grow at all (Fig.1). There is no metabolic pathway or reactions by which reducing power or chemical energy could be produced. The growth pattern of singly disrupted strains shows that *CIT 1* gene product is essential for the growth on acetate media. According to our model, only the glyoxylate cycle occurs in 1-7A-L where *CIT 1* gene is disrupted. This cycle does not seem to meet the energetic need of the cell for its detectable growth (Fig.1). Actually, the primary function of the glyoxylate cycle has been known to be the provision of succinate for the further biosynthetic reactions. Meanwhile, the TCA cycle is supposed to occur in 1-7A-U because the citrate synthase coded by *CIT*1 could be imported into mitochondria. 1-7A-U therefore could be provided with sufficient chemical energy by the TCA cycle necessary for the growth on acetate medium (Fig.5). Here, possibility is that glyoxylate cycle is partially running due to the transient activity of *CIT 1* gene product before it is completely targeted into mitochondria. Thus, we conclude that it is not the nature of enzyme per se, but its localization in the cell that determines which gene product is essential for the growth on media containing acetate or pyruvate.

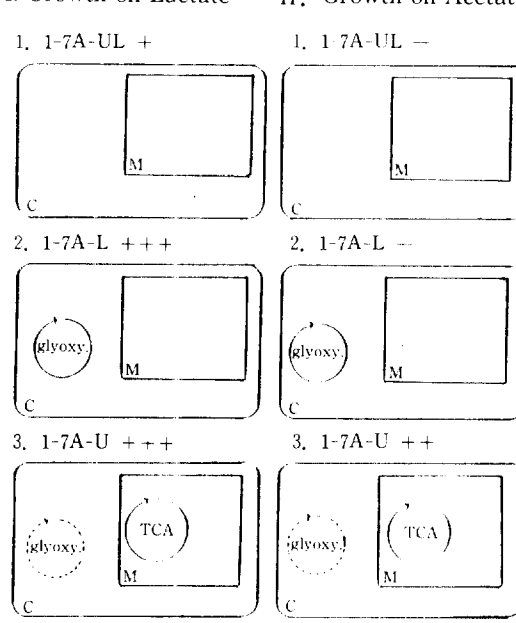

Fig.5. Schematic model showing the relationship between the growth of disrupted strains and the availability of metabolic cycles; − ; no growth, ++ ; partial growth, +++ : full growth. Circles with solid line indicate that the corresponding cycle is fully active, and that with dotted line indicate that the cycle possibly has partial activity.

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