Cloning and Disruption of a Gene Required for Growth on Acetate but not on Ethanol: the Acetyl-Coenzyme A Synthetase Gene of *Saccharomyces cerevisiae*

CLAUDIO DE VIRGILIO, NIELS BÜRCKERT, GEROLD BARTH*, JEAN-MARC NEUHAUS, THOMAS BOLLER AND ANDRES WIEMKEN†

Department of Botany, University of Basel, Hebelstr. 1, CH-4056 Basel, Switzerland
*Department of Microbiology, Biozentrum, University of Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland

A DNA fragment of *Saccharomyces cerevisiae* with high homology to the acetyl-coenzyme A (acetyl-CoA) synthetase genes of *Aspergillus nidulans* and *Neurospora crassa* has been cloned, sequenced and mapped to chromosome I. It contains an open reading frame of 2139 nucleotides, encoding a predicted gene product of 79·2 kDa. In contrast to its ascomycete homologs, there are no introns in the coding sequence. The first ATG codon of the open reading frame is in an unusual context for a translational start site, while the next ATG, 24 codons downstream, is in a more conventional context. Possible implications of two alternative translational start sites for the cellular localization of the enzyme are discussed. A stable mutant of this gene, obtained by the gene disruption technique, had the same low basal activity of acetyl-CoA synthetase as wild-type cells when grown on glucose but completely lacked the strong increase in activity upon entering the stationary phase, providing direct proof that the gene encodes an inducible acetyl-CoA synthetase (*ACS1*) of yeast. As expected, the mutant was unable to grow on acetate as sole carbon source. Nevertheless, it showed normal induction of isocitrate lyase on acetate media, indicating that activity of acetyl-CoA synthetase is dispensable for induction of the glyoxylate cycle in *S. cerevisiae*. Surprisingly, disruption of the *ACS1* gene did not affect growth on media containing ethanol as the sole carbon source, demonstrating that there are alternative pathways leading to acetyl-CoA under these conditions.

KEY WORDS — Acetyl-coenzyme A synthetase; carbohydrate metabolism; *Saccharomyces cerevisiae*; chromosome I.

INTRODUCTION

Like many other organisms, *Saccharomyces cerevisiae* can use acetate as sole carbon source. Growth of microorganisms on acetate requires the presence of the anaplerotic glyoxylate-cycle enzymes and of essential gluconeogenic enzymes (Kornberg, 1966). The first step of acetate utilization, after the uptake of acetate, involves the activation of free acetate to acetyl-coenzyme A (acetyl-CoA). This step is performed by the acetyl-CoA synthetase (EC 6.2.1.1.):

Acetate + coenzyme A + ATP → acetyl-CoA + AMP + PP$_i$
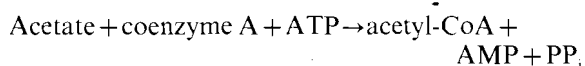
Recently, the genes of the acetyl-CoA synthetases in *Neurspora crassa* and *Aspergillus nidulans* have been isolated and sequenced (Thomas *et al.*, 1988;

†To whom correspondence should be addressed.

Sandeman and Hynes, 1989). Transcriptional analysis showed that the transfer of those fungi from sucrose as carbon source to acetate resulted in a high induction of mRNA synthesis of the acetyl-CoA synthetase.

Acetyl-CoA synthetase is also thought to be involved in ethanol utilization, converting the acetate formed by oxidation of ethanol (through the action of alcohol dehydrogenase and acetaldehyde dehydrogenase) to acetyl-CoA. Acetyl-CoA can then enter either the glyoxylate cycle or alternatively, be used as substrate in the tricarboxylic acid cycle (for review see Wills, 1990).

In the present report we describe a genomic DNA fragment of *S. cerevisiae* showing high homology to the acetyl-CoA synthetase genes of *N. crassa* and *A. nidulans*, and we present evidence, using a gene disruption mutant, that this fragment encodes the inducible acetyl-CoA synthetase in yeast. As expected, the gene disruption mutant does not grow

on acetate as the sole carbon source, but surprisingly, it grows normally on ethanol as the sole carbon source.

MATERIALS AND METHODS

Bacterial strains and media

Escherichia coli JM 101 (*supE, thi,* Δ(*lac-proAB*), [F', *traD36, proAB, lacI^{q}ZΔM15*]) was used as host for plasmid constructions. Bacterial media and general methods were used as described by Sambrook *et al.* (1989).

Yeast strain and media

The *S. cerevisiae* strain 144-3A (α *ura3, leu2, his4, bar1*) was kindly provided by H. Riezman. *S. cerevisiae* media and general methods were applied according to Sherman *et al.* (1986). Solid SD-medium contained 6·7 g/l yeast nitrogen base (Difco), 2% agar, the appropriate base or amino acids (uracil, 20 mg/l; histidine, 10 mg/l; leucine, 60 mg/l) and either 2% glucose, 2% ethanol or 60 mM-acetate (pH 5·8) as carbon source. In fluid SD-media, 2% glucose, 0·4% sodium acetate or 1% ethanol were used as carbon sources.

Vectors, recombinant DNA methods and sequencing

The plasmids utilized in this study are summarized in Table 1. The original clone containing the complete sequence of the acetyl-CoA synthetase was isolated from a YCp50 genomic bank (Rose *et al.*, 1987). For sequencing and functional expression in yeast, a 3·7 kb *Eco*RI-*Kpn*I fragment was subcloned into the multi-cloning site of YCplac33 (Gietz and Sugino, 1988). The resulting plasmid was called pYCACS (Table 1). Recombinant DNA procedures followed standard methods (Sambrook *et al.*, 1989). Sequences were done on plasmids, prepared by CsCl gradients, using the dideoxy chain-termination method (Sanger *et al.*, 1977) with sequenase (United States Biochemical Corporation, Ohio) and [^{35}S]dATP (Amersham). Oligonucleotides were synthesized with an Applied Biosystem DNA Synthesizer. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim, Germany.

Computer methods

Sequence analyses were carried out with the GCG package (Devereux *et al.*, 1984).

Table 1. Plasmids used in this study.

Plasmid	Source
YCp50 genomic bank (C3)	Rose *et al.* (1987)
YEp13	Broach *et al.* (1979)
YCplac33	Gietz and Sugino (1988)
pYCACS	This study: *Eco*RI-*Kpn*I fragment (*ACS1*) cloned into the multi-cloning site of YCplac33

Gene disruption and Southern analysis

Gene disruption was accomplished by the one-step procedure of Rothstein (1983). A 2·8 kb *Bgl*II-*Bgl*II fragment containing *LEU2* (obtained from a YEp13 vector) was cloned at the *Bam*HI site within the coding sequence of *ACS1*. The disrupted sequence was then excised from the corresponding plasmid with *Xba*I and *Pst*I and transformed into strain 144-3A. The resulting gene disruption mutant was called MM67 (α *ura3, leu2, his4, bar1, acs1::LEU2*). Gene disruption was confirmed by Southern analysis (Southern, 1975; Sambrook *et al.*, 1989) of genomic DNA digested with either *Bam*HI or *Eco*RI. The probe for the Southern analysis was prepared by labeling the *Xba*I-*Pst*I fragment of the *ACS1* gene with [α-^{32}P]dATP (Amersham) using the Random Primed DNA Labeling Kit of Boehringer Mannheim, Germany. For the chromosomal localization of the *ACS1* gene, yeast chromosomal DNA (BioRAD) was separated by pulse-field electrophoresis and subsequently analysed by Southern blot, using the above-mentioned labeled *Xba*I-*Pst*I fragment as probe.

Preparation of cell-free extracts, enzyme assays and determination of protein

Preparation of cell-free extracts was performed as previously described (De Virgilio *et al.*, 1991). Acetyl-CoA synthetase (acetate:CoA ligase, EC 6.2.1.1) was assayed according to Jones and Lippmann (1955), except that potassium fluoride was omitted. The assay of isocitrate lyase (threo-D-isocitrate glyoxylate lyase, EC 4.1.3.1.) was done according to Barth and Weber (1987), except that 60 mM-Tris/HCl buffer was used instead of phosphate buffer. Protein content was determined by the method of Lowry *et al.* (1951).

RESULTS

Cloning, sequencing and chromosomal localization of ACS1

In a study of a mutant defective in trehalose metabolism, we obtained and partially sequenced a clone from the YCp50 genomic bank (C3) of Rose *et al.* (1987) that appeared to complement the defect (De Virgilio *et al.*, unpublished results). Although closer analysis showed the complementation results to be an artefact, we serendipitously found that the clone contained a DNA sequence highly similar to known sequences of the acetyl-CoA synthetase genes of *A. nidulans* and *N. crassa* deposited in the EMBL data bank. For further analysis of the gene, we constructed a subclone (pYCACS, Table 1) containing the flanking regions and the whole coding sequence of *ACS1* (Figure 1).
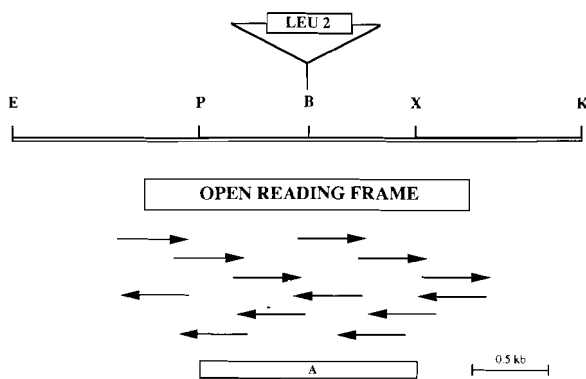

Figure 1. Restriction map, sequencing strategy and gene disruption of the *ACS1* gene of *S. cerevisiae*. On top is shown the position of the gene disruption of *ACS1* by insertion of a *Bg*lII-*Bg*lII fragment containing *LEU2*. The horizontal arrows indicate the direction and extent of each individual sequencing reaction. Oligonucleotides were used to promote DNA synthesis for the sequencing, thus allowing complete coverage of both strands. The fragment used as probe for Southern analysis is shown below (A). B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; X, *Xba*I.

Nucleotide sequencing of *ACS1* revealed an open reading frame (ORF) of 2139 nucleotides (Figure 2). In addition to the ORF, 162 nucleotides at the 5' non-coding region and 202 nucleotides at the 3' non-coding region were sequenced. The first two methionine codons were situated at base 1 and 73 (Figure 2). In the 5' region of the ORF, five TATA boxes were found 137, 122, 67, 54 and 36 base pairs in front of the first predicted translational start. Of these, the first sequence TATAAA (at base -136) matched with the eukaryotic consensus sequence TAT(A/T)A(A/T). The other five TATA boxes

were less well conserved. On the basis of a recently published overview about regulatory DNA-binding proteins in yeast (Verdier, 1990), no obvious regulatory sequences were found in the 162 nucleotides of the 5' non-coding region. At bases 2286 and 2311 were the sequences AAGAAA and CATAAA, which are similar to the conserved polyadenylation sequence AATAAA of eukaryotes. According to Sharp and Cowe (1991; see also Bennetzen and Hall, 1982), we calculated a codon bias index of 0·29. Southern blot analysis of yeast chromosomal DNA, using the labeled *Xba*I-*Pst*I fragment as probe, revealed that *ACS1* is localized on chromosome I (data not shown).

Deduced amino acid sequence

The ORF (from the first initiation codon) codes for a protein of 713 amino acids and 79 168 daltons in molecular weight (Figure 2). The predicted pI value of the acetyl-CoA synthetase is 6·68. Hydropathy plots show that the acetyl-CoA synthetase gene contains no sequence of sufficient length and hydrophobicity to be considered a transmembrane domain (data not shown).

The deduced amino acid sequence was searched for homology with sequences in the EMBL data bank. This search revealed significant homology to the deduced sequences of the acetyl-CoA synthetases of *A. nidulans*, *N. crassa* (Connerton *et al.*, 1990) and *Methanothrix soehngenii* (Eggen *et al.*, 1991). The ascomycetes *A. nidulans* and *N. crassa* showed 78% and 76% similar (conservative replacements of amino acids) or 62% and 61% identical residues in 713 and 642 amino acids respectively, when compared with the sequence of *S. cerevisiae* (Figure 3). The archaebacterial sequence of *M. soehngenii* revealed 62% similarity (40% identity) in 709 residues with the *S. cerevisiae* sequence.

Gene disruption of ACS1

In order to determine the phenotype of a null mutant of the gene we have identified, a gene disruption was carried out (see Methods and Figure 1). Southern blot analysis confirmed that integration of the 2·8 kb *Bg*lII-*Bg*lII fragment containing *LEU2* occurred at the *ACS1* locus (Figure 4). In addition, Southern analysis indicated that the *ACS1* gene is a single copy gene in *S. cerevisiae*. In accordance with this observation, disruption of the *ACS1* gene resulted in the complete loss of the ability to grow on a medium with acetate as sole carbon source

-161   AAATAGGTTCTCCCTTCATTTTGTATATATAAACTGGGCGGCTATTCTAAGCATATTTCTCCCTT

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-98    AGGTTATCTGGTAGTACGTTATATCTTGTTCTTATATTTTCTATCTATAAGCAAAACCAAACATATCAAAACTACTAGAAAGACATTGCCCACTGTGCT
       *                                                          *
   1   ATGTCGCCCTCTGCCGTACAATCATCAAAACTAGAAGAACAGTCAAGTGAAATTGACAAGTTGAAAGCAAAAATGTCCCAGTCTGCCGCCACTGCGCAG
   1   M   S   P   S   A   V   Q   S   S   K   L   E   E   Q   S   S   E   I   D   K   L   K   A   K   M  -S   Q   S   A   A   T   A   Q

 100   CGAAAGAAGGAACATGAGTATGAACATTTGACTTCGGTCAAGATCGTGCCACAACGGCCCATCTCAGATAGACTGCAGCCCGCAATTGCTACCCACTAT
  34   R   K   K   E   H   E   Y   E   H   L   T   S   V   K   I   V   P   Q   R   P   I   S   D   R   L   Q   P   A   I   A   T   H   Y

 199   TCTCCACACTTGGACGGGTTGCAGGACTATCAGCGCTTGCACAAGGAGTCTATTGAAGACCCTGCTAAGTTCTTCGGTTCTAAAGCTACCCAATTTTTA
  67   S   P   H   L   D   G   L   Q   D   Y   Q   R   L   H   K   E   S   I   E   D   P   A   K   F   F   G   S   K   A   T   Q   F   L

 298   AACTGGTCTAAGCCATTCGATAAGGTGTTCATCCCAGACCCTAAAACGGGCAGGCCCTCCTTCCAGAACAATGCATGGTTCCTCAACGGCCAATTAAAC
 100   N   W   S   K   P   F   D   K   V   F   I   P   D   P   K   T   G   R   P   S   F   Q   N   N   A   W   F   L   N   G   Q   L   N

 397   GCCTGTTACAACTGTGTTGACAGACATGCCTTGAAGACTCCTAACAAGAAAGCCATTATTTTCGAAGGTGACGAGCCTGGCCAAGGCTATTCCATTACC
 133   A   C   Y   N   C   V   D   R   H   A   L   K   T   P   N   K   K   A   I   I   F   E   G   D   E   P   G   Q   G   Y   S   I   T

 496   TACAAGGAACTACTTGAAGAAGTTTGTCAAGTGGCACAAGTGCTGACTTACTCTATGGGCGTTCGCAAGGGCGATACTGTTGCCGTGTACATGCCTATG
 166   Y   K   E   L   L   E   E   V   C   Q   V   A   Q   V   L   T   Y   S   M   G   V   R   K   G   D   T   V   A   V   Y   M   P   M

 595   GTCCCAGAAGCAATCATAACCTTGTTGGCCATTTCCCGTATCGGTGCCATTCACTCCGTAGTCTTTGCCGGGTTTTCTTCCAACTCCTTGAGAGATCGT
 199   V   P   E   A   I   I   T   L   L   A   I   S   R   I   G   A   I   H   S   V   V   F   A   G   F   S   S   N   S   L   R   D   R

 694   ATCAACGATGGGGACTCTAAAGTTGTCATCACTACAGATGAATCCAACAGAGGTGGTAAAGTCATTGAGACTAAAAGAATTGTTGATGACGCGCTAAGA
 232   I   N   D   G   D   S   K   V   V   I   T   T   D   E   S   N   R   G   G   K   V   I   E   T   K   R   I   V   D   D   A   L   R

 793   GAGACCCCAGGCGTGAGACACGTCTTGGTTTATAGAAAGACCAACAATCCATCTGTTGCTTTCCATGCCCCCAGAGATTTGGATTGGGCAACAGAAAAG
 265   E   T   P   G   V   R   H   V   L   V   Y   R   K   T   N   N   P   S   V   A   F   H   A   P   R   D   L   D   W   A   T   E   K

 892   AAGAAATACAAGACCTACTATCCATGCACACCCGTTGATTCTGAGGATCCATTATTCTTGTTGTATACGTCTGGTTCTACTGGTGCCCCCAAGGGTGTT
 298   K   K   Y   K   T   Y   Y   P   C   T   P   V   D   S   E   D   P   L   F   L   L   Y   T   S   G   S   T   G   A   P   K   G   V

 991   CAACATTCTACCGCAGGTTACTTGCTGGGAGCTTTGTTGACCATGCGCTACACTTTTGACACTCACCAAGAAGACGTTTTCTTCACAGCTGGAGACATT
 331   Q   H   S   T   A   G   Y   L   L   G   A   L   L   T   M   R   Y   T   F   D   T   H   Q   E   D   V   F   F   T   A   G   D   I

1090   GGCTGGATTACAGGCCACACTTATGTGGTTTATGGTCCCTTACTATATGGTTGTGCCACTTTGGTCTTTGAAGGGACTCCTGCGTACCCAAATTACTCC
 364   G   W   I   T   G   H   T   Y   V   V   Y   G   P   L   L   Y   G   C   A   T   L   V   F   E   G   T   P   A   Y   P   N   Y   S

1189   CGTTATTGGGATATTATTGATGAACACAAAGTCACCCAATTTTATGTTGCGCCAACTGCTTTGCGTTTGTTGAAAAGAGCTGGTGATTCCTACATCGAA
 397   R   Y   W   D   I   I   D   E   H   K   V   T   Q   F   Y   V   A   P   T   A   L   R   L   L   K   R   A   G   D   S   Y   I   E

1288   AATCATTCCTTAAAATCTTTGCGTTGCTTGGGTTCGGTCGGTGAGCCAATTGCTGCTGAAGTTTGGGAGTGGTACTCTGAAAAAAATAGGTAAAAATGAA
 430   N   H   S   L   K   S   L   R   C   L   G   S   V   G   E   P   I   A   A   E   V   W   E   W   Y   S   E   K   I   G   K   N   E

1387   ATCCCCATTGTAGACACCTACTGGCAAACAGAATCTGGTTCGCATCTGGTCACCCCGCTGGCTGGTGGTGTTACACCAATGAAACCGGGTTCTGCCTCA
 463   I   P   I   V   D   T   Y   W   Q   T   E   S   G   S   H   L   V   T   P   L   A   G   G   V   T   P   M   K   P   G   S   A   S

1486   TTCCCCTTCTTCGGTATTGATGCAGTTGTTCTTGACCCTAACACTGGTGAAGAACTTAACACCAGCCACGCAGAGGGTGTCCTTGCCGTCAAAGCTGCA
 496   F   P   F   F   G   I   D   A   V   V   L   D   P   N   T   G   E   E   L   N   T   S   H   A   E   G   V   L   A   V   K   A   A

1585   TGGCCATCATTTGCAAGAACTATTTGGAAAAATCATGATAGGTATCTAGACACTTATTTGAACCCTTACCCTGGCTACTATTTCACTGGTGATGGTGCT
 529   W   P   S   F   A   R   T   I   W   K   N   H   D   R   Y   L   D   T   Y   L   N   P   Y   P   G   Y   Y   F   T   G   D   G   A

1684   GCAAAGGATAAGGATGGTTATATCTGGATTTTGGGTCGTGTAGACGATGTGGTGAACGTCTCTGGTCACCGTCTGTCTACCGCTGAAATTGAGGCTGCT
 562   A   K   D   K   D   G   Y   I   W   I   L   G   R   V   D   D   V   V   N   V   S   G   H   R   L   S   T   A   E   I   E   A   A

1783   ATTATCGAAGATCCAATTGTGGCCGAGTGTGCTGTTGTCGGATTCAACGATGACTTGACTGGTCAAGCAGTTGCTGCATTTGTGGTGTTGAAAAACAAA
 595   I   I   E   D   P   I   V   A   E   C   A   V   V   G   F   N   D   D   L   T   G   Q   A   V   A   A   F   V   V   L   K   N   K

1882   TCTAGTTGGTCCACCGCAACAGATGATGAATTACAAGATATCAAGAAGCATTTGGTCTTTACTGTTAGAAAAGACATCGGGCCATTTGCCGCACCAAAA
 628   S   S   W   S   T   A   T   D   D   E   L   Q   D   I   K   K   H   L   V   F   T   V   R   K   D   I   G   P   F   A   A   P   K

1981   TTGATCATTTTAGTGGATGACTTGCCCAAGACAAGATCCGGCAAAATTATGAGACGTATTTTAAGAAAAATCCTAGCAGGAGAAAGTGACCAACTAGGC
 661   L   I   I   L   V   D   D   L   P   K   T   R   S   G   K   I   M   R   R   I   L   R   K   I   L   A   G   E   S   D   Q   L   G

2080   GACGTTTCTACATTGTCAAACCCTGGCATTGTTAGACATCTAATTGATTCGGTCAAGTTGTAATGATGATTTCTTTCCTTTTTATATTGACGACTTTTT
 694   D   V   S   T   L   S   N   P   G   I   V   R   H   L   I   D   S   V   K   L   *   *   *

2179   TTTTTTCGTGTGTTTTTGTTCTCTTATAACCGAGCTGCTTACTTATTATTATTTCACCTTCTCTTTTTATTTATACTTATAATTATTTATTCTTTACAT

2278   ACTGTTACAAGAAACTCTTTTCTACATTAATTGCATAAAGTGTCAATCAGCACA
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Figure 2. Nucleotide sequence and deduced amino acid sequence of the acetyl-CoA synthetase of *S. cerevisiae*. The predicted amino acid sequence is shown in single letter-code, TATA boxes and potential polyadenylation sites are underlined. The two potential start codons are marked with asterisks.

```
                                                                                          100
M.soehngenii   vlklagked.klkttvfqdetrifnp.kelveksivmqwmkkkgfktekem.awcs.d.hyle.wdem.ktyvd
N.crassa                                                         mrqt.rstrsytsspsps.tm.waer.rel.s
A.nidulans     msdgpiappkpavvaeahevdtfhvpkafdkhpsg...kn.de.kk.ye...rs.dv.war.rel.t
S.cerevisiae   MSPSAVQSSKLEEQSSEIDKLKAKMSQSAATAQRKKEHEYEHLHSVKIVPQRPISDRLQPAIATHYSPHLDGLQDYQRLHKESIEDPAKFFGSKATQFLN
               1                                                                                    200

M.soehngenii   .h..yt..mdds-emp-----yfh..tg.ei.it..a.....kgakkd.v.y.wipeptd.pvqk...gd.yk..nkf.ng.k-.l.lk...r.si....
N.crassa       ..d.qt.hsgslan.-----d...ve.e...s.....krh--.d..rv...y.a..vr--rnv..g...r..skl.mssp-twvi.........i.l..
A.nidulans     fd.d.qttr.gslen.----dv...pe.r..sf......i-.n..v...y.a...ne.-rt..g...r..sr..w..k-qr..k......i.l..
S.cerevisiae   WSKPFDKVFIPDKTGRPSFQNNAWFLNGQLNACYNCVDRHAL-KTPNKKAIIFEGDEPGQG-YSITYKELLEVCQVAQVLTYSMGVRKGDTVAVYMPM
               101                                                                                  300

M.soehngenii   i.qlp.am..cakl.vs.i...s....kg.m..aahcg.rai..v.gfy.r..pvpl.pna.e.agga.s.ekii..krag-vd.smkeg..vw.hdlv.
N.crassa       i....vam..ct.......d.....vv.aq........gk.....l.g..k...e..qqc.d..n....kr.g-.iismtpg...w.he.ve
A.nidulans     i....af..c......v......d....vl.ag......gk......g....eg.kqc.d.st...kr.g-ae.pwteg..iw.he.ve
S.cerevisiae   VPEAITTLLAISRIGAIHSVVFAGFSSNSLRDRINDGDSKVVITTDESNRGGKVIETKRIVDDALRETPGVRHVLVYRKTNPSVAFHAPRDLDWATEKK
               201                                                                                  400

M.soehngenii   gqsee--.e..wv.p.hr.yi.....t..k...ie.a.g.nav.paq.lhwv..lkdd..wwcta....v...s.i..a..il.mts.my..aad..dfg
N.crassa       ..pa.--y..ama...........k....a...g....r.-m.gk.v..i.dg.ry.cg..v.....ph..lsa...l.vs.v.....--.tn.
A.nidulans     ..pa.--ia.dsvn...........k....m.t......am.gk.v..i.dd.ry.cg..v........a...l.vs.v...s....df.
S.cerevisiae   KYKTYPCTPV--DSEDPLFLLYTSGSTGAPKGVQHSTAGYLLGALLTMRYTFDTHQEDVFFTAGDIGWITGHTYVVYGPLLYGCATLVFEGTPAYPNYS
               301                                                                                  500

M.soehngenii   .w.kn.qd....vl.t....v.mfmkq.aewpdkyd.s...l..........np.a.m..r.h..rg.lq.m..w....t.tf.ns..p--i..l....ct.
N.crassa       p....e.......s........hhvr-nem.h..v.......s....k..ydvv..aaaq.c......t..nvi....-.....t........
A.nidulans     ...v.ek........hh.h-.kmeh..v...........k..f..v..e.ah.c......t..nvi...-....t.......l
S.cerevisiae   RYWDIIDEHKVTQFYVAPTALRLIKRAGDSYIENHSLKSLRCLGSVGEPIAAEFVWEWYSEKIGKNEIPIVDTYWQTESGSHLVTPLAGGVTPMKPGSASF
               401                                                                                  600

M.soehngenii   ip.y.isi..e-e.n.vplgsggniv.l.-py..ml.af.gdke.fmke.wqf.wdvpgrr.v.la..k.qr.....ff.q..i...ls.a...ian..
N.crassa       ....epalv..v...irgndv.....f.qp...m..v.ga.k..me...hv.-----k.......r.he.fy..r....r...........
A.nidulans     ....epali..vs...isgndv.....f.qp...m..v.ga.k..m....qv.------k.........gr.he..y..r...............
S.cerevisiae   PFFGIDAVVLDPNTGEELNTSHAEGVLAVKAAWPSFARTIWKNHDRYLDTYLNPY------PGYYFTGDGAAKDKDGYIWLGRVDDVVNVSGHRLSTAE
               501                                                                                  700

M.soehngenii   v.s.lvah.ki..a....kp.evk.eslv..i.rv-----gnepspela.daia.-...tl..v..tevhf.n...........vvkaral.--
N.crassa       ....l..hhsi..a....va.e......n...av.e-----gtqindalr.espslq..rs......a.yi.p.....l..............v...e
A.nidulans     ...l..h.m..a...ia.e......n..s..e------gnetndqvr.d.-ilq...s......avfv....................s..e
S.cerevisiae   IEAAIIEDPIVAECAVVGFNDDLTGQAVAAFVVLKNKSSWSTATDDELQDIKKHL-VFTVRKDIGPFAAPKLILVDDLPKTRSGKIMRRILRKILAGES
               601                                                                                  729

M.soehngenii   npv..i...m..ea.dgipki.*
N.crassa       ...it....d.sv.aki..v.hahq*
A.nidulans     .s..si...d.sv.eri.at.hasrgk*
S.cerevisiae   DQLGDVSTLSNPGIVRHLIDSVKL*
               701
```

Figure 3. Comparison of the amino acid sequences of the acetyl-CoA synthetases of *M. soehngenii* (Eggen *et al.*, 1991), *N. crassa* (Connerton *et al.*, 1990), *A. nidulans* (Connerton *et al.*, 1990) and *S. cerevisiae*. Dots indicate identical amino acid residues. Gaps (—) were introduced to optimize the alignment.

(Table 2). Transformation of the *acs1* disruption mutant (MM67) with the plasmid pYCACS containing the complete sequence of the *ACS1* gene restored the ability to grow on acetate media.
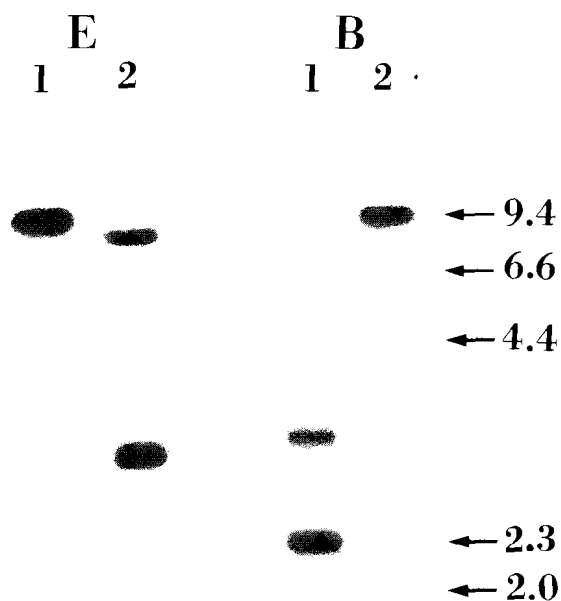

Figure 4.   Southern blot of yeast chromosomal DNA. Chromosomal DNA (10 µg) was digested with *Eco*RI (E) or *Bam*HI (B) and analysed by Southern blotting using the internal *Pst*1-*Xba*1 1·4 kb fragment of the *ACS1* gene as a probe. The positions of the molecular weight standards are indicated on the right (in kb). Yeast strains analysed are: 144-3A (wild-type), lane 1; MM67, lane 2.

Table 2.   Growth of *S. cerevisiae* wild-type and mutant strains on either acetate or ethanol.*

Strain	Acetate	Ethanol
144-3A (wild-type)	+	+
MM67 (*acs1::LEU2*)	–	+
MM67 + pYCACS	+	+

*Cells were grown on solid SD-media containing either 60 mM-acetate or 2% ethanol.

We measured the activity of acetyl-CoA synthetase in 144-3A and MM67 cells grown on YPD medium and harvested either in the logarithmic or in the stationary phase (Table 3). Logarithmically growing cells of 144-3A and MM67 had both hardly any detectable acetyl-CoA synthetase activity. In comparison, acetyl-CoA synthetase activity was about 19-fold induced in stationary wild-type cells. This would have been expected, since it has been reported that the acetyl-CoA synthetase of *A. nidulans* is under catabolite repression (Sandeman and Hynes, 1989). In contrast to the wild-type, MM67 completely lacked the stationary phase-induced activity increase of acetyl-CoA synthetase. However, transformation of MM67 with the plasmid pYCACS almost completely restored the stationary phase-induced increase of acetyl-CoA synthetase activity (Table 3).

Effect of the disruption of the ACS1 gene on the utilization of ethanol as sole carbon source and on the induction of isocitrate lyase

We examined growth of the *acs1* disruption mutant, MM67, on ethanol as the sole carbon source. Surprisingly, wild-type and mutant grew equally well on solid media containing 2% ethanol as the sole carbon source (Table 2).

A recent study has demonstrated that a certain level of acetyl-CoA is necessary for induction of the glyoxylate cycle enzymes in the yeast *Yarrowia lipolytica* (Kujau *et al.*, 1992). We examined induction of isocitrate lyase, a key enzyme of the glyoxylate cycle, in media containing acetate or ethanol as the sole carbon sources. Isocitrate lyase was induced in strain MM67 to the same extent as in wild-type cells after 8 h on both acetate and ethanol media (Table 4). Thus, the activity of acetyl-CoA synthetase is dispensable for the induction of the glyoxylate cycle in *S. cerevisiae*.

DISCUSSION

Sequencing of the *ACS1* gene of *S. cerevisiae* revealed an ORF of 2139 nucleotides. It is worth noting that the start codon of this ORF has a context of GTGCT<u>ATG</u>TCG, which is at variance with the consensus context A(A/Y)A(A/T)A<u>ATG</u>TCT for translational start in yeast (for reviews see: Cigan and Donahue, 1987; Linder and Prat, 1990). The next ATG downstream is in frame with the first and is surrounded by CAAAA<u>ATG</u>TCC, a sequence very similar to the consensus for translational start. Similarly, it has been reported that the nucleotide sequence of *N. crassa* contains two possible start codons (Connerton *et al.*, 1990). Thus,

Table 3.   Acetyl-CoA synthetase in *S. cerevisiae* wild-type and mutant strains.

	Specific activity (μmol min^{-1} per mg protein)*		
Strain	Log-phase	Stationary phase	Induction factor
144-3A (wild-type)	0·02	0·38	19·0
MM67 (*acs1::LEU2*)	0·02	0·02	1·0
MM67+pYCACS	0·03	0·48	16·0

*Cells were pre-grown on selective media and then transferred to YPD medium containing 2% glucose and either harvested in the log-phase (1×10^7 cells/ml) or in the stationary phase (1 day old).

Table 4.   Specific activity of isocitrate lyase in *S. cerevisiae* wild-type (144-3A) and *acs1* disruption mutant (MM67).*

	Specific activity (nmol min^{-1} per mg protein)		
Strain	2% glucose	1% ethanol	0·4% acetate
144-3A (wild-type)	21	126	134
MM67 (*acs1::LEU2*)	18	112	96

*Cells were incubated for 8 h in either glucose-, acetate- or ethanol-containing media.

depending on conditions, proteins with different N-termini might be formed from the gene. Interestingly, previous reports (Klein and Jahnke, 1968, 1971) have suggested that, according to the physiological state of the cell, the acetyl-CoA synthetase of *S. cerevisiae* was either localized in the microsomal or in the mitochondrial fraction. It is possible that different start sites yield enzymes with different subcellular locations (Slusher *et al.*, 1991). In this context, analysis of the COOH terminus of the acetyl-CoA synthetase protein revealed the sequence VKL, which resembles the eukaryotic targeting signal for microbodies: S/A/C-K/R/H-L (Gould *et al.*, 1989; Van der Klei *et al.*, 1991). Therefore, we suggest that one of the possible locations of the *ACS1* gene product is microbodies (either glyoxysomes or peroxisomes). Further analyses are needed to examine a potential mitochondrial or peroxisomal localization.

Earlier published data revealed that the transcribed sequence of *A. nidulans* contained six introns and that of *N. crassa* only one (Connerton *et al.*, 1990). In contrast, none of these introns were found in the sequence of *S. cerevisiae*. Thus, our data lend further support for the hypothesis that *S. cerevisiae* tends to loose introns through gene conversion by intron-less cDNA formed by reverse transcription (Fink, 1987).

Disruption of *ACS1* resulted in an almost complete loss of acetyl-CoA synthetase activity in stationary cells, demonstrating that the gene encodes a functional acetyl-CoA synthetase in yeast. Furthermore, the gene disruption mutant was unable to grow on acetate, supporting the notion that *ACS1* is an essential gene in *S. cerevisiae*. This is also in line with our finding that there is a single copy of *ACS1* in the yeast genome. The low basal level in exponentially growing cells is probably an artefact caused by interferences in the enzyme assay and of spurious activities of different enzymes, although it may also represent a different, constitutive acetyl-CoA synthetase unable to support growth on acetate. In wild-type cells, the activity rose up to 19-fold upon entrance into the stationary phase, suggesting that the *S. cerevisiae* acetyl-CoA synthetase is under catabolite repression, as has been proposed to be the case for the acetyl-CoA synthetase of *A. nidulans* (Sandeman and Hynes, 1989).

We are presently investigating whether acetate or other substances provide additional induction of the enzyme.

We were surprised to find that MM67 was not impaired in growth on ethanol, since it is generally assumed that no biochemical pathway exists in *S. cerevisiae* to circumvent a defect of the acetyl-CoA synthetase in order to enable gluconeogenesis (Hanes *et al.*, 1986). However, our results may be explained by one of the following hypotheses:

(i) There is a constitutive second enzyme, insufficient for growth on acetate but sufficient for growth on ethanol.

(ii) There may exist another way to activate acetate, as is known from *E. coli* (Nunn, 1986). Besides the acetyl-CoA synthetase in *E. coli*, two additional enzymes are present, which form acetyl-CoA in a two-step reaction: acetate kinase converts acetate to acetyl phosphate and phosphotransacetylase transfers the acetyl moiety to CoA. Both enzymes are constitutively expressed and are required for *E. coli* to grow optimally on glucose. However, it is not known whether the acetyl-CoA synthetase is needed for optimal growth of *E. coli* on glucose, since up to now no mutants are available with a defect in this enzyme. It may be that both pathways are also present in *S. cerevisiae* and that the acetyl-CoA synthetase is required for growth on acetate but neither for growth on ethanol nor for the induction of the glyoxylate cycle.

(iii) It might also be that the pyruvate decarboxylase, which converts pyruvate to acetaldehyde, is able to function in the inverse direction. This would mean that the pyruvate decarboxylase is able to convert CO_2 and acetaldehyde to pyruvate, which in its turn is the key substrate for gluconeogenesis.

Further studies of the regulation of the acetyl-CoA synthetase may shed light on the pathway of acetate utilization and also provide new insights into the mechanisms of the regulation of carbon metabolism in *S. cerevisiae*.

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