HFA1 Encoding an Organelle-specific Acetyl-CoA Carboxylase Controls Mitochondrial Fatty Acid Synthesis in Saccharomyces cerevisiae*

The Saccharomyces cerevisiae gene, HFA1, encodes a >250-kDa protein, which is required for mitochondrial function. Hfa1p exhibits 72% overall sequence similarity (54% identity) to ACC1-encoded yeast cytoplasmic acetyl-CoA carboxylase. Nevertheless, HFA1 and ACC1 functions are not overlapping because mutants of the two genes have different phenotypes and do not complement each other. Whereas ACC1 is involved in cytoplasmic fatty acid synthesis, the phenotype of hfa1Δ disruptants resembles that of mitochondrial fatty-acid synthase mutants. They fail to grow on lactate or glycerol, and the mitochondrial cofactor, lipoic acid, is reduced to <10% of its normal cellular concentration. Other than Acc1p, the N-terminal sequence of Hfa1p comprises a canonical mitochondrial targeting signal together with a matrix protease cleavage site. Accordingly, the HFA1-encoded protein was specifically assigned by Western blotting of appropriate cell fractions to the mitochondrial compartment. Removal of the mitochondrial targeting sequence abolished the competence of HFA1 DNA to complement hfa1 null mutants. Conversely and in contrast to the intact HFA1 sequence, the signal sequence-free HFA1 gene complemented the mutational loss of cytoplasmic acetyl-CoA carboxylase. Expression of HFA1 under the control of the ACC1 promoter restored cellular ACC activity in ACC1-defective yeast mutants to wild type levels. From this finding, it is concluded that HFA1 encodes a specific mitochondrial acetyl-CoA carboxylase providing malonyl-CoA for intraganellar fatty acid and, in particular, lipoic acid synthesis.

Yeast contains two distinct fatty-acid synthases (FAS)† acting specifically in the cytoplasm and in the mitochondrial matrix, respectively (1, 2). Cytoplasmic FAS is a multifunctional enzyme (type I-FAS) and synthesizes the bulk (>95%) of cellular fatty acids. In contrast, mitochondrial FAS is structurally similar to the non-aggregated FAS enzymes found in most bacteria where each component activity is represented by a distinct protein (type II-FAS) (2–4). Both FAS systems are encoded by nuclear genes. Although mutational loss of cytoplasmic FAS gives rise to a fatty acid-requiring phenotype (5), mutants defective in one of the mitochondrial FAS-encoding genes are fatty acid-prototrophic but fail to grow on non-fermentative media (2–4). In accordance with these findings, Brody et al. (2) and Wada et al. (6) working with either yeast or plant systems suggest that a major function of mitochondrial FAS refers to lipoic acid synthesis by providing the octanoyl-acyl-carrier protein precursor of this cofactor. Because the product spectra of both FAS systems are very similar in vitro (7), it remains to be shown whether, apart from octanoic acid, mitochondrialy produced long-chain fatty acids serve a specific function as well. Neither octanoic nor lipoic acid is capable of healing the mitochondrial FAS defect when added to the growth medium (2, 3). This suggests that either the uptake or the activation of these acids is impossible for mitochondria or intact yeast cells.

In all of the known FAS systems, chain extension depends on malonyl-CoA as a substrate. Thus, carboxylation of acetyl-CoA to malonyl-CoA is a key step of fatty acid synthesis. The respective enzyme, acetyl-CoA carboxylase (ACC), comprises three functional components, i.e. biotin carboxylase, biotin-carboxyl-carrier protein, and transcarboxylase (8). Depending on the organism, these components represent either three distinct proteins or they are contained as functional domains within difunctional or trifunctional ACC proteins (9). In yeast, cytoplasmic ACC is a single trifunctional polypeptide of 2233 amino acids and with an approximate molecular mass of 250 kDa (10–12). The ACC-encoding gene has been isolated and was designated as ACC1 and FAS3, respectively (11, 12). Unlike fatty-acid synthase mutants, which grow upon fatty acid supplementation, ACC1 disruption is lethal and cannot be compensated by external long-chain fatty acids (12, 13). This characteristic is commonly attributed to the malonyl-CoA requirement of cellular very long-chain fatty acid biosynthesis. Fatty acid elongation appears to be an essential cellular function not supplementable by exogenous very-long-chain fatty acid. Other than ACC1 null mutants, however, ACC1 missense mutants are potentially viable and grow upon fatty acid supplementation. The low level of ACC activity eventually retained in some of these mutants obviously fulfills the limited malonyl-CoA requirement of very long-chain fatty acid synthesis (14). These mutants therefore provide valuable tools for biochemical studies.

Although Acc1p provides the malonyl-CoA used in cytoplasmic fatty acid synthesis, the origin of this substrate in mitochondrial fatty acid synthesis is unclear. Mitochondrial membranes are unlikely to be permeable for malonyl-CoA. In higher plant mitochondria, malonic acid was suggested to be imported into the organelle with subsequent activation and transacycla-
tion to acyl carrier protein (15). On the other hand, fatty acid synthesis in plant chloroplasts relies on a specific organellar ACC, which is distinctly different from its cytoplasmic counterpart (16–18). Similarly, identification of the unassigned yeast reading frame, HFA1, encoding a putative protein with striking similarity in its length and amino acid sequence to cytoplasmic ACC suggested the existence of a second and functionally differentiated acetyl-CoA carboxylase providing malonyl-CoA for organellar fatty acid biosynthesis. For many years, however, the biochemical function of HFA1 remained elusive because hfa1Δ disruptants were, other than acc1 mutants, fatty acid-prototrophic and exhibited no obvious ACC deficiency. Accordingly, the fatty acid-requiring phenotype of hfa1Δ mutants was not compensated by functional HFA1 DNA (12, 13). A first clue to the physiological function of HFA1 came from an observation in our laboratory that HFA1 mutants failed to grow on non-fermentable carbon sources. As will be demonstrated in the present study, the HFA1 gene product is located in the mitochondria and the phenotype of HFA1 mutants conforms to that of yeast mitochondrial FAS mutants. For complementation of hfa1Δ null mutants, the putative mitochondrial targeting sequence of HFA1 proved indispensable. On the other hand, signal-free HFA1 DNA restored cytoplasmic ACC activity in acc1 Δ-mutant mitochondria. 

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Media—The Saccharomyces cerevisiae strain X2180-1A (R. Mortimer, Yeast Genetic Stock Center, Berkeley, CA) served as a haploid wild type reference. The ACC1-missense mutant acc1-31845 was from our own collection (14) The ACC1::HIS3 heterozygous diploid, Y25391, was obtained from Euroscarf (Frankfurt, Germany). The diploid JS95.3 (MATa/α his3Δ his3 Δ ura3/URA3 leu2/LEU2) was from H.-J. Schütter (Greifswald, Germany) and served for chromosomal disruption of HFA1. Plasmid pSE303 containing the N-terminal portion of HFA1 together with its 5' flanking chromosomal DNA was obtained from S. E. Kerssey (20). Plasmid pYE6 (21) was used for PCR amplification of S. cerevisiae HIS3-DNA. Plasmid p48.8.3 containing intact ACC1-DNA was isolated from a YEpl4-based yeast gene bank by acc1-mutant complementation (Dr. Lilian Schweizer). For HFA1 disruption, the 2684-bp XbaI fragment of pSE303 was subcloned into pUC19 giving plasmid pRSHFA1. In pRSHFA2, the 386-bp EcoRI/SstI fragment was replaced by S. cerevisiae HIS3 DNA, which was prepared by PCR amplification from YEpl4 (cf. Fig. 1). From the resulting construct, the inserted HIS3 gene together with its flanking HFA1 sequences was isolated as NcoI/SpeI fragment. Using the one-step gene disruption procedure of Rothstein (22), the NcoI/SpeI fragment served for disruption of chromosomal HFA1 DNA in the diploid JS95.3 (Fig. 1). Thus, the heterozygous diploid SC1383 (MATa/α, hfa1Δ::HIS3/HFA1 his3Δ/Δ his3 Δ ura3/URA3 leu2/LEU2) was ob-

**Fig. 1.** HFA1 disruption strategy. In plasmid pRSHFA1, part of the HFA1 start region originally contained in pSE303 was subcloned. Replacement of the SstI/EcoRI fragment in pRSHFA1 by the S. cerevisiae HIS3 gene resulted in pRSHFA2. The inserted SstI/EcoRI HIS3 DNA fragment was obtained by PCR amplification from YEpl4 using appropriate primers. From pRSHFA2, the indicated NcoI/SpeI fragment was isolated and used for integrative transformation, i.e. disruption of chromosomal HFA1 DNA in the diploid JS 95.3 according to the procedure of Rothstein (22). S, SstI; E, EcoRI; X, XbaI; N, NcoI; Sp, SpeI.

**Fig. 2.** HFA1 constructs used for expression studies. Details of the plasmid construction protocols are described under "Experimental Procedures." HFA1 coding sequences are indicated in white. Non-coding HFA1, ADH1, or ACC1 upstream DNA sequences are shaded and marked appropriately. Numbering refers to the first ATG codon in the HFA1 reading frame (black asterisk), ATG initiation codons provided by the ADH1 or ACC1 promoter sequences are indicated as white asterisks. The C-terminal hexahistidine tag in pMA3, pMA1, and pUH33.3 is indicated in black.
tained. The haploid HFA1 null mutant SC 1517 (MATa Δhfa1::HIS3 his3) was a meiotic segregant of SC1343. The multicopy yeast expression vector, pVT100-U, of Vernet et al. (23) served as a recipient of the HFA1 coding sequence in pHFA1. The latter plasmid was restituted from three different PCR fragments representing nucleotides 216 to 1974 (fragment 1), 1975–3885 (fragment 2), and 3886–6423 (fragment 3) relative to the first ATG codon in the HFA1 reading frame. In pHFA1, an in-frame ATG start codon in combination with the ADH1 promoter was contributed by the vector (cf. Fig. 2). Fragments 1–3 were flanked by HindIII/SpeI (fragment 1), SpeI/XhoI (fragment 2), and XhoI/NheI (fragment 3) restriction sites, respectively, allowing their appropriate ligation and integration into the vector. A second HFA1 expression plasmid, pMA1, was prepared accordingly but using a C-terminally hexahistidine-tagged fragment 3 (Fig. 2). Another HFA1 expression plasmid, pMA3, contained the complete HFA1 reading frame in combination with its genuine promoter sequence and a C-terminal hexahistidine-encoding nucleotide sequence. According to Fig. 2, pMA3 was obtained by inserting the XbaI fragment of pMA1 into the unique XbaI site of pMA2. In pMA2, a 589-bp PCR fragment of the HFA1 upstream region had been cloned into the yeast expression vector Yep 352 (24).

According to Fig. 3, the HFA1 expression plasmid pUH 33.3 was constructed from pMA3 by replacing the S'-terminal 3439-1 bp HindIII/SphI fragment of the inserted HFA1 DNA by a shorter 2779-bp PCR-generated HindIII/SphI fragment from the same region. The resulting construct, pUH32, was used for inserting the 846-bp HindIII/HindIII PCR fragment of the ACC1 promoter DNA into the unique HindIII site of pUH32 (cf. Fig. 3). The inserted DNA comprises the first 11 bp of the ACC1 reading frame together with 835 bp of its 5'-flanking upstream region.

Yeast cells were routinely grown on complex YPD medium containing 1% yeast extract (Invitrogen), 2% peptone (Invitrogen), and 2% dextrose. Respiratory competence was examined on complex medium containing, alternatively, 3% glycerol (YPG), 3% lactate (YPL), or 3% ethanol (YPE) instead of dextrose. The fatty requirement of yeast mutants was analyzed by replica plating from fatty acid containing YPDFA plates (YPD supplemented with 0.5% Tween 40 and 0.015% each of 14:0 and 16:0 fatty acids) onto YPD agar. The uracil-free (SCD/-U) or histidine-free (SCD/-H) synthetic complete medium used for selecting and identifying pHFA1 transformants and hfa1Δ disruptants, respectively, was prepared as described previously (25).
RESULTS

Demonstrating HFA1 Transcription and Mapping the Transcriptional Start Site—Using total RNA from S. cerevisiae wild type cells as a template, HFA1 transcription was verified by RT-PCR amplification of the HFA1 transcript (Fig. 4). Using appropriate controls, it was shown that formation of the PCR product was strictly dependent on the addition of reverse transcriptase. From this result and from the fact that product formation proved to be insensitive to DNase pretreatment, it is evident that RNA, rather than traces of residual DNA, served as a template (Fig. 4A). Using two different HFA1-specific upstream primers (A and B) in combination with the same downstream primer (J), the size difference of the resulting products was according to expectation (Fig. 4A). The cDNA thus prepared from the 5’ end of HFA1-mRNA subsequently served as a template for a series of PCR experiments. These “primer walking” experiments were designed to map the 5’ end of HFA1-mRNA. When the same downstream primer (H) was used in combination with six differently located upstream primers (B–G), only the combinations containing upstream primers B–E generated PCR products of the expected successively increasing lengths (Figs. 4, B and C). In contrast, the two most distal primers, G and F, were ineffective in the PCR assay (Fig. 4B). Therefore, the transcriptional initiation site of HFA1 is suggested to be located at a position between 607 (primer E) and 633 nucleotides (primer F) upstream of the first ATG in the HFA1 coding sequence (Fig. 2 and 4C). This corresponds to a distance of 152–183 nucleotides upstream of the last stop codon in front of the HFA1 reading frame (Figs. 2 and 4C).

HFA1 Disruptants Are Respiratory Defective and Fail to Synthesize Lipoic Acid—One of the two HFA1 alleles in the diploid strain, JS953.3, was disrupted by inserting the S. cerevisiae HIS3 gene according to the scheme depicted in Fig. 1. After sporulation of the resulting heterozygous diploid, SC1343, HFA1 disruptants were isolated by tetrad analysis and subsequently characterized both biochemically and by their growth requirements. The mutants proved to be viable on glucose-containing medium and segregated according to their histidine-protoporphoric character in a regular Mendelian fashion (Fig. 5, A–C). Other than mutants of the sequentially related ACC1 gene, growth of HFA1 disruptants was independent of fatty acid supplementation (Fig. 5A). However, when cultivated on non-fermentable carbon sources such as lactate, glycerol, or ethanol, HFA1 disruptants exhibited the characteristics of respiratory-defective “petite” (Fig. 5, B, D, and E). Thus, HFA1 appears to be necessary for maintaining functional mitochondrial in yeast. When different non-fermentative carbon sources were compared, growth of HFA1 mutants on lactate was strictly negative, whereas growth on ethanol was retarded although not completely abolished (Fig. 5, D and E). In this respect, HFA1 mutants differ from yeast mutants defective in one of the respiratory chain or ATP synthase functions. Instead, they rather resemble the recently described mutants of mitochondrial fatty acid synthesis lacking the mitochondrial cofactor, lipoic acid (2). To confirm the similarity between

TABLE I

| Strain                  | Cellular lipoic acid content (mg/g cells) |
|-------------------------|------------------------------------------|
| SC1343-derived spore tetrad |                                           |
| Spore A                  | 0.44 ± 0.02                              |
| Spore B                  | 0.10 ± 0                                 |
| Spore C                  | 0.12 ± 0.01                              |
| Spore D                  | 0.55 ± 0.17                              |
| X2180-1A                 | 0.50 ± 0.09                              |

Fig. 5. Growth characteristics of HFA1 deletion mutants on different carbon sources. The array indicates six spore tetrads from the heterozygous diploid, SC 1343, after replica plating onto YPD (A), YPL (B), SCYD (C), YPG (D), and YEP (E) solid media.

RT-PCR Amplification of HFA1-mRNA—Total RNA from yeast strain X2180-1A grown in YPD medium was extracted with TRIzol (Invitrogen) as described by the manufacturer. 10-µg RNA fractions were incubated with 1 unit of RNase-free RQ1-DNase (Promega) for various time intervals ranging from 0 to 35 min. 0.5 µg of DNase-treated RNA was reverse-transcribed for 50 min at 42 °C with 1 unit of Superscript II reverse transcriptase (Invitrogen) and 0.5 µM HFA1-specific primer (Fig. 4). The reaction mixture contained in a volume of 20 µl, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 5 mM MgCl₂, 0.5 mM dNTP, and 1 mM dithiothreitol. 0.5 µl of the cDNA synthesis mixture were subsequently applied to PCR amplification using in a total volume of 50 µl the Expand High Fidelity PCR system (Roche Applied Sciences) and 200 ng of primers. 30 cycles of 94 °C annealing (2 min), 52 °C denaturing (1.5 min), 52 °C extension (2 min) were run in an automated thermal cycler (Biometra, Gottingen, Germany). The following primers were used: primer A, 5’-atgaggaactatattac-3’; primer B, 5’-aagggaggaactacacac-3’; primer C, 5’-ACGCGTCGACcattggttattattttcctttgt-3’; primer D, 5’-ACGCGTCGACTTTCGAagtaataatagct-3’; primer E, 5’-ACGCGTCGACTTTCGAAcatatgattcatcattcatc-3’; primer F, 5’-ACGCGTCGACTTTCGAagtaataatagct-3’; primer G, 5’-ACGCGTCGACTTTCGAagtaataatagct-3’; primer H, 5’-ACGCGTCGACTTTCGAagtaataatagct-3’. Capitola letters indicate non-HFA1 sequences. HFA1 sequences are indicated in small letters, and restriction sites are underlined. 5 µl of each reaction mixture were applied to 1% agarose gel electrophoresis. The synthetic oligonucleotides used in this study were supplied by MWG-Biotech (Ebersberg, Germany).

Acetyl-CoA Carboxylase Assay—Cells were grown in 500 ml of uracil-free SC-URA medium containing 0.5% Tween 40 and 0.15% of free SCD/-U liquid medium containing 0.5% Tween 40 and 0.15% of

Mitochondrial Acetyl-CoA Carboxylase

Lipoic acid content of wild type and HFA1-disrupted yeast cells

The heterozygous diploid, SC 1343, was sporulated, and the resulting asci were dissected by micromanipulation. Individual spores (A–D) were grown to early stationary phase in 50 ml of YPD media each. The harvested cells were assayed for lipoic acid content as described previously (2). As a control, X2180-1A wild type cells were subjected to the same assay. Values are the average of three different tetrads.
Mitochondrial Acetyl-CoA Carboxylase

HFA1-defective and mitochondrial FAS-defective mutants further, several meiotic segregants of the hfa1Δ/HFA1 heterozygous diploid, SC1343, were analyzed for their lipoic acid content. As indicated in Table I, cellular lipoic acid was indeed reduced drastically in hfa1Δ disruptants. This finding compares well to the lipoic acid deficiency observed in mitochondrial FAS-defective cprΔ or ppt1Δ mutants (2, 28). To definitively correlate these characteristics to the loss of HFA1 function, hfa1Δ disruptants were transformed with the multicopy HFA1 expression plasmids, pHFA1 and pMA3, respectively. For transformation, both the heterozygous diploid, SC1343, and the haploid hfa1Δ-disruptant, SC1517, were used as recipients. It turned out that the extrachromosomal HFA1 DNA of either plasmid complemented the lactate-negative phenotype of the HFA1 disruption both in haploid transformants (Fig. 8) and in the meiotic segregants of the transformed diploid (data not shown). From these findings, it is evident first that the mutant phenotype is indeed related to the loss of HFA1 function. Secondly, HFA1 was functionally expressed both with its genuine promoter and with the heterologous ADH1 promoter when the latter was fused in pHFA1 to an artificial ATG codon 220 nucleotides upstream of the first ATG triplet were included in this analysis, the characteristics of a typical yeast mitochondrial import signal became apparent within this sequence (cf. Fig. 6) (29). Besides many hydrophobic and hydroxy amino acids, this extended N-terminal part of Hfa1p comprises 27 basic and only 3 acidic amino acids. Furthermore, a putative signal peptide cleavage site is as predicted by the algorithm of Claros and Vincens (30) maps between the HFA1-specific leader and the subsequent ACC1-homologous Hfa1p sequence (cf. Fig. 6).

To confirm the possible mitochondrial function of Hfa1p, we tested both the expression of HFA1 and the intracellular localization of its product biochemically. For this experiment, yeast cells were transformed with pMA3 encoding hexahistidine-tagged Hfa1p. After growth to mid-log phase, cells were broken and the homogenate was fractionated into cytoplasm and purified mitochondria. Subsequently, both cell fractions were analyzed by Western blotting for the presence of Hfa1p. As is evident from Fig. 7B, anti-pentahistidine antibodies elicited a strong signal with the mitochondrial fraction but not with the cytoplasm. The gel position of the signal conformed to a protein of a molecular mass of $\geq 250\,\text{kDa}$. A second signal corresponding to approximately half of this size probably represents a defined degradation product of Hfa1p. A comparable signal was not obtained with cells that had been transformed with the empty vector (Fig. 7B, lane 3). A minor band of $< 75\,\text{kDa}$ observed with the cytoplasm of both pMA3 transformants and control cells obviously reflects a nonspecific cross-reaction. These results together with the respiratory-defective phenotype of hfa1Δ mutants not only demonstrate that HFA1 is required for mitochondrial function but also show that its product is specifically restricted to the mitochondrial compartment.

Acetyl-CoA Carboxylase Activity of Hfa1p—The low level of HFA1 expression in yeast mitochondria together with the large excess of cytoplasmic acetyl-CoA carboxylase resembling Hfa1p both in its size and biochemical properties precludes Hfa1p
characterization by conventional methods. To overcome these difficulties, we intended to both increase HFA1 expression and target its product to the cytoplasmic compartment. By replacing the promoter and the putative mitochondrial targeting sequence of HFA1 by the upstream region of ACC1, both objectives were achieved. In pUH33.3, the HFA1 coding sequence is deprived of its 429 5'-terminal nucleotides and instead is fused to the first 11 nucleotides of the ACC1 reading frame (Figs. 2 and 6). Expression of pUH33.3 is thus controlled by the promoter of ACC1 encoding cytoplasmic acetyl-CoA carboxylase. Upon transformation of pUH33.3 to the hfa1Δ mutant, SC1517, it was found that in accordance with the loss of its mitochondrial import sequence and in contrast to the HFA1 DNA contained in pMA3 and pMA1, the pUH33.3-encoded HFA1 sequence lost the capacity to complement the hfa1Δ null mutant (Fig. 8). On the other hand, targeting of the truncated HFA1 gene product to the yeast cytoplasm compensated for the loss of ACC function in acc1Δ mutants (Table II). If the heterozygous acc1Δ/ACC1 diploid, Y25391, was transformed with pUH33.3 and subsequently sporulated, the acc1::kanMX allele providing G418 resistance to the respective spores segregated in the expected 2:2 fashion (Fig. 9). However, in contrast to segregants of the untransformed diploid, the acc1-defective spores of the pUH33.3-transformed diploid were not lethal (Fig. 9). Obviously, sufficient copies of the plasmid were provided to every segregant and, at the same time, the encoded HFA1 product was capable of substituting for the loss of ACC1 function. The genuine HFA1 DNA contained in plasmid pMA3, similar to the empty vector YEp352, proved ineffective in acc1Δ mutant complementation when subjected to the same experimental protocol (Fig. 9). These findings strongly suggest that in the cytoplasm of pUH33.3-transformed acc1Δ-mutants, a functionally competent acetyl-CoA carboxylase is expressed from the plasmid-encoded HFA1 DNA.

To corroborate the above conclusion by direct enzyme activity measurements, we assayed cellular ACC levels in extracts of untransformed and pUH33.3-transformed acc1-318/45 mutant cells. Acc1-318/45 is exceptional in being viable even though cellular ACC activity is below detectability (Table II). Again, pMA3 and the empty vector YEp352 were ineffective in this respect (Table II). Transformation of acc1-318/45 with p48.8.3 containing homologous acc1 DNA induced ACC levels, which were even higher than those in the untransformed wild type (Table II). Possibly, either the copy number of the plasmids or the specific activity of the encoded enzymes was different in the ACC1 and HFA1 expression constructs. Thus, both the genetic

![Fig. 7. SDS-PAGE (A) and Western blot analyzes (B) of cytosolic (lane 1) and mitochondrial (lane 2) fractions from pMA3-transformed yeast cells. Lane 3 contains the extract of untransformed hfa1Δ (SC1517) cells. 20 μg protein were applied to each lane of a 7.5% polyacrylamide gel. S, protein molecular weight standard. Western blotting was performed using anti-pentahistidine mouse antibodies (Qiagen) in combination with anti-mouse IgG horseradish peroxidase conjugate (Promega). Mitochondria were purified from mid-log phase yeast cells essentially as described by Glick and Pon (38).](image)

![Fig. 8. Complementation of hfa1Δ disruptants by intact but not by N-terminally truncated HFA1 DNA. SC 1517 cells were transformed with the indicated plasmids. Transformants were selected on uracil-free SC/UD medium and subsequently grown for 4 days at 30 °C on either glucose (YPD) or lactate-containing (YPE) complex medium. Plasmids were as indicated under "Experimental Procedures."](image)

![Fig. 9. Complementation of acc1Δ-defect with ACC1 or HFA1 DNA. The ACC1/acc1Δ heterozygous diploid, Y25391, was transformed with vector DNA (YEp352), intact HFA1 DNA (pMA3), intact ACC1 DNA (p48.8.3) and ACC1 promoter-controlled HFA1 DNA (pUH33.3), respectively. After sporulation, tetrads were dissected by micromanipulation and individual spore segregants were screened for viability (YPD) and for Geneticin (G418) resistance. In each case, representative tetrads from a total of 5–10 investigated tetrads are shown. From YEp352 and pMA3 transformants, only 2 viable spores per tetrad were obtained.](image)

### Table II

| Strain          | Transformed plasmid | Insert in plasmid | Specific ACC activity (milliunits/mg) |
|-----------------|---------------------|------------------|-------------------------------------|
| Wild type       | None                | None             | 6.8                                 |
| acc1-318/45     | None                | None             | 0                                   |
| acc1-318/45     | YEp352              | None             | 0                                   |
| acc1-318/45     | p48.8.3             | ACC1             | 13.4                                |
| acc1-318/45     | pMA3                | HFA1             | 0                                   |
| acc1-318/45     | pUH33.3             | Truncated HFA1   | 7.2 with ACC1 promoter and start codon |

**YPD**

![YPD](image)

**YPD + G418**

- **YEps352**
- **pMA3**
- **p48.8.3**
- **pUH33.3**

**Mitochondrial Acetyl-CoA Carboxylase**
and enzymatic data agree in supporting the conclusion that HFA1 encodes a protein with ACC activity.

**DISCUSSION**

Based on the results reported in this study, it is suggested that yeast mitochondria contain an organelle-specific acetyl-CoA carboxylase encoded by the nuclear gene, HFA1. Both biochemical and genetic evidence support this conclusion. Accordingly, in a recent study of Sickmann et al. (39), Hfa1p has been identified by tandem MS spectrometry as one of the 750 different proteins present in highly purified yeast mitochondria. In analogy to cytoplasmic acetyl-CoA carboxylase mutants affecting fatty acid synthesis in the cytoplasm, mutation of the putative mitochondrial carboxylase, Hfa1p, abolishes mitochondrial fatty acid synthesis. As is known from previous studies in this laboratory, the failure of cytoplasmic fatty acid synthesis in either FAS or ACC1-defective mutants is characterized by a long-chain fatty acid-requiring phenotype. In contrast, mutants of mitochondrial fatty acid synthase are lipidic acid-negative and, consequently, respiratory-defective. Our finding that both defects are characteristically associated with hfa1Δ-negative mutants also strongly suggested the functioning of Hfa1p as a mitochondrial acetyl-CoA carboxylase. Direct proof for the ACC activity of Hfa1p was obtained after expression of recombinant Hfa1p in the cytoplasm of ACC1-defective yeast mutants. When targeted to the cytoplasmic compartment, Hfa1p was capable of complementing the acc1 mutational defect and, at the same time, the ACC activity of a partially purified Hfa1p preparation could be demonstrated in vivo. Nevertheless, even though ACC1 and HFA1 encode functionally identical enzymes, the two activities cannot replace each other in vivo. Mutation of neither one of the genes is complemented by the other. The impermeability of the mitochondrial membrane for malonyl-CoA may be responsible for this compartment-specific action of Acc1p and Hfa1p.

Apart from its 150 amino acid N-terminal extension and from the absence of an appropriately located translational start codon, Hfa1p exhibits over 96% of its length a very high sequence similarity to Acc1p. In particular, the three catalytic carboxylase domains, i.e. biotin carboxylase, biotin-carboxyl-carrier protein, and transcarboxylase, contain up to 90% of conserved positions. In fact, the similarity between Acc1p and Hfa1p is considerably higher than that between yeast pyruvate carboxylase on the one side and either one of the two acetyl-CoA carboxylase proteins, Acc1p or Hfa1p, on the other side. Regarding the N-terminal demarcation of the HFA1 reading frame, several lines of evidence suggest that the first ATG at position 450 of the stop codon-free sequence is actually not the translational initiation site of HFA1p. From the results of this study, we propose that not only transcription but also translation of HFA1 starts considerably further upstream of the first ATG. The HFA1 transcriptional initiation site was mapped >600 nucleotides upstream of this codon. The transcript includes at its 3' end a translatable sequence of 450 nucleotides preceding the first ATG triplet. A non-translated leader of this length is rather uncommon in yeast. More importantly, this pre-sequence encodes, other than the RNA distal to the first ATG, the signatures for both a mitochondrial targeting signal (29) and a subsequent peptide cleavage site (30). Both signatures should be required for the mitochondrial homologue of cytoplasmic acetyl-CoA carboxylase. These data strongly suggest that HFA1 translation starts upstream of the canonical ATG initiation codon and thereby allows for the biosynthesis of an N-terminal mitochondrial import sequence, which is clipped off upon the transfer of Hfa1p into the mitochondrial matrix. This model is strongly supported by our finding that for complementation of hfa1Δ mutants, the HFA1 DNA sequence upstream of position −128 (cf. Fig. 2) is required. This sequence should be irrelevant if the AUG at position +1 was the actual HFA1 initiation codon. The presence of another AUG initiation codon further upstream in the HFA1 reading frame was excluded since automated as well as manual re-sequencing of both the chromosomal and the respective cDNA confirmed the published HFA1 sequence (data not shown). Thus, apart from an eventual frameshifting event, it remains to be determined that HFA1-mRNA translation may represent one of the rare cases where a non-AUG initiation triplet is employed. The differential expression rates of ACC1 and HFA1 with the latter being extremely low may be due to this fact. Although eucaryotes and, in particular, yeast almost exclusively use AUG as an initiation codon, systematic studies with mutationally altered AUGs revealed that a series of alternative codons may initiate mammalian translation in vivo and in vitro (32, 33). Also in yeast, codons other than AUG were effective when combined with either appropriately mutated initiator tRNAs or initiation factors (32, 34). In particular with non-AUG initiation codons, specific nucleotides in the surrounding RNA context appear to be important determinants of initiation efficiency (35–37). In fact, some of the alternative initiation triplets identified in mammalian systems such as ACG, AUA, or AUA (33) are present within the HFA1 leader. It remains to be shown whether one of them acts, possibly together with an appropriate sequence context, as the in vivo initiation site of Hfa1p translation.

The occurrence of multiple acetyl-CoA carboxylases in the same cell is not restricted to yeast but has also been reported for plant and mammalian systems (16, 17, 31). In plants, two compartment-specific ACC isoforms were identified in the cytosol and in the chloroplast, respectively (16–18). In humans and in rats, at least two but possibly even more tissue-specific ACC isoforms are present (31). Similar to cytosolic ACC of yeast, the various animal enzymes and the cytosolic ACC of plants are homomultimeric high molecular weight multienzymes. In contrast, the molecular structure of plastid ACCs depends on the plant species. Dicots and most monocot plants contain a heteromer ACC in their plastids consisting of four dissociable subunits with one of them being encoded by the plastid DNA (16, 18). This structure of chloroplast ACC corresponding to the multisubunit composition of Escherichia coli ACC agrees with the putative prokaryotic origin of the organelle and compares well with the dissociated structures of chloroplast and mitochondrial fatty-acid synthases (2–4, 14). In contrast, the chloroplast ACC of Gramineae exhibits the multifunctional characteristics of cytoplasmic ACC enzymes (18). In these organisms, the prokaryotic ACC has obviously been lost and, instead, a variant of the cytoplasmic form seems to have acquired on organellar import sequence. According to the data reported here, a similar situation is proposed for the HFA1-encoded mitochondrial ACC of yeast.

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Mitochondrial Acetyl-CoA Carboxylase

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