The EMBO Journal vol.14 no.14 pp.3472–3479, 1995

# Peroxisomal and mitochondrial carnitine acetyltransferases of *Saccharomyces cerevisiae* are encoded by a single gene

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**Carnitine acetyltransferase (CAT) is present in mitochondria and peroxisomes of oleate-grown *Saccharomyces cerevisiae*. Both proteins are encoded by the same gene, *YCAT*, which encodes a protein with a mitochondrial targeting signal (MTS) at the N-terminus, and a peroxisomal targeting signal type 1 (PTS-1) at the C-terminus. Deletion of both motifs revealed the presence of an additional internal targeting sequence. Import of CAT via this internal signal was shown to be dependent on PAS10, a protein which is required for the import of PTS-1 containing proteins. An interaction of PAS10 with this internal targeting signal was demonstrated using the yeast two-hybrid technique. Expression of the *YCAT* gene behind a heterologous promoter resulted in loss of peroxisomal targeting, indicating that differential targeting is controlled at transcriptional or translational level. Determination of the 5′-ends of *YCAT* mRNAs revealed that *YCAT* transcripts initiating after the first AUG were present in oleate-grown cells. These transcripts were virtually absent in acetate- or glycerol-grown cells. We propose that in response to oleate, shorter transcripts are produced from which the peroxisomal form of CAT is translated, resulting in a CAT protein without a MTS, which can be targeted to peroxisomes.**

*Key words*: β-oxidation/mitochondrial import/peroxisomal import/targeting signal/transcriptional control

## Introduction

The degradation of fatty acids in vertebrate cells takes place in both mitochondria and peroxisomes. In general, the two organelles are involved in the β-oxidation of distinct fatty acids. Long-chain fatty acids are oxidized primarily in mitochondria whereas very-long-chain fatty acids are handled by peroxisomes. Since peroxisomes are not capable of oxidizing fatty acids to completion, very-long-chain fatty acids are chain-shortened and the resulting acyl-CoA esters are then transported to mitochondria for final oxidation to $CO_2$ and $H_2O$. It has been suggested that transport of acyl-CoA esters from peroxisomes to mitochondria is probably mediated by peroxisomal carnitine acetyltransferase and carnitine octanoyltransferase, which convert acyl-CoA esters into acylcarnitine esters, followed by transport across the mitochondrial membrane via the carnitine/acylcarnitine carrier (reviewed by Bieber, 1988).

In yeast, degradation of fatty acids takes place exclusively in peroxisomes, where they are oxidized completely to acetyl-CoA (Kawamoto *et al.*, 1978a; Kunau *et al.*, 1988). To allow further metabolism, acetyl units have to be transported from peroxisomes to cytoplasm and/or mitochondria. In the accompanying paper, two pathways for the transport of acetyl units have been proposed (van Roermund *et al.*, 1995), both preventing the depletion of free coenzyme A within the peroxisomes and facilitating the transport of acetyl units from peroxisomes to mitochondria. First, acetyl-CoA may enter the peroxisomal glyoxylate cycle, resulting in the formation of succinate, which could subsequently be transported to mitochondria via putative dicarboxylic acid carriers. Second, acetyl-CoA may be converted into a carnitine ester through the action of carnitine acetyltransferase (CAT), and subsequently transported out of the peroxisomes. This latter mechanism requires the presence of carnitine acetyltransferase in both peroxisomes and mitochondria. For *Candida tropicalis* and *Saccharomyces cerevisiae* it has been shown that both peroxisomes and mitochondria indeed contain CAT activity (Kawamoto *et al.*, 1978b; Atomi *et al.*, 1993). Two carnitine acetyltransferases have been cloned from *S.cerevisiae*. The major carnitine acetyltransferase (encoded by the *YCAT* gene) is reported to be located in the mitochondria, and contributes for >95% of the total CAT activity in yeast (Kispal *et al.*, 1993). The second CAT protein (encoded by the *YAT1* protein) is located at the outer surface of mitochondria and is probably responsible for the remaining 5% of the cellular CAT activity (Schmalix and Bandlow, 1993). Surprisingly, disruption of either gene did not lead to a phenotype on any carbon source tested. This suggests that either the carnitine-dependent transport system of acetyl-CoA can be bypassed via another pathway, that the isozymes might be able to take over each other's function, or that additional CAT genes are present in *S.cerevisiae*.

Here we report the presence of CAT in peroxisomes of *S.cerevisiae* and the identification of the gene encoding this protein. We demonstrate that the *YCAT* gene encodes both the peroxisomal and the bulk of the mitochondrial CAT protein, and we show how differential targeting to these two organelles is achieved.

## Results

### *CAT activity is induced by growth on fatty acids*

To test a possible role for CAT in fatty acid metabolism, we investigated the induction of CAT in cells grown on oleate, which is a well-known inducer of peroxisomes in

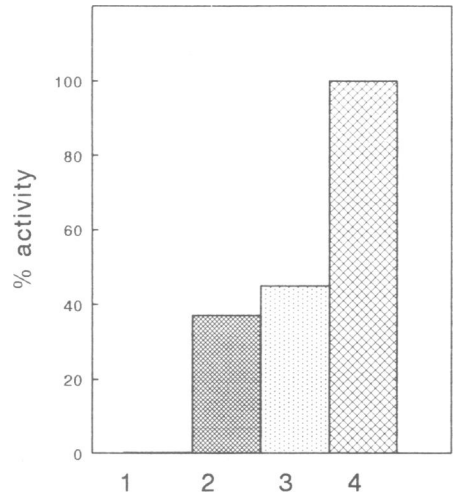



**Fig. 1.** Carnitine acetyltransferase (CAT) activity in cells grown on various carbon sources. The bars represent CAT activity of cells grown on glucose (1), acetate (2), glycerol (3) or oleate (4). CAT activity was measured in cell-free lysates after disrupting the cells with glass beads in the presence of 0.05% Triton X-100. The specific activity of cells grown on oleate was set to 100%.

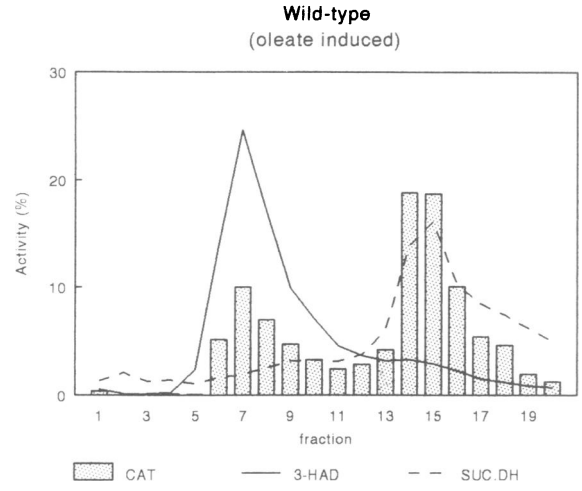



**Fig. 2.** Subcellular location of CAT in oleate-grown *S.cerevisiae*. A homogenate of oleate-grown cells was subjected to differential centrifugation to prepare an organellar pellet, followed by density gradient centrifugation of this preparation on Nycodenz. Activity (%) is relative amount of activity in each fraction as compared with the total amount of activity which is present in the organellar pellet. Fraction 1 corresponds to the bottom fraction, whereas fraction 20 reflects the top fraction. Succinate dehydrogenase (SUC.DH) was used as mitochondrial marker enzyme, and 3-hydroxyacyl-CoA dehydrogenase (3-HAD) as peroxisomal marker enzyme. Approximately 95% of total CAT activity as measured in the homogenate was present in the organellar fraction.

*S.cerevisiae*. To that end, CAT activity was measured in lysates of *S.cerevisiae* grown on different carbon sources. CAT activity was hardly detectable in glucose-grown cells, but was strongly increased in cells grown on acetate or glycerol (Figure 1). The highest CAT activity, however, was present in oleate-grown cells, suggesting a role for CAT in fatty acid metabolism.

### *CAT is present in peroxisomes*

To study whether carnitine acetyltransferase is present in peroxisomes of *S.cerevisiae*, a homogenate of oleate-grown cells was first subjected to differential centrifugation to obtain an organellar pellet. This material was further fractionated by density gradient centrifugation on Nycodenz. Figure 2 shows good resolution between mitochondria (marker enzyme succinate dehydrogenase) and peroxisomes (marker enzyme 3-hydroxy-acyl-CoA dehydrogenase activity of multifunctional enzyme). Measurement of CAT in each of the fractions revealed a bimodal profile with activity in mitochondrial as well as peroxisomal fractions. Although the peroxisomal contribution to organellar CAT activity varied somewhat from experiment to experiment, we found on average that at least 25% of the organellar CAT activity was present in peroxisomes of oleate-grown cells.

### *Peroxisomal and mitochondrial CAT are encoded by a single gene*

Since disruption of the *S.cerevisiae YCAT* gene resulted in a 95% decrease in total cellular CAT activity (Kispal *et al.*, 1993), we reasoned that this gene might encode both the peroxisomal and mitochondrial forms of CAT. Moreover, inspection of the amino acid sequence of the *YCAT* gene indicated the presence of an N-terminal amphiphilic α-helix, which can serve as a mitochondrial targeting signal (MTS). In addition, the C-terminus of this protein consists of the amino acids AKL. This tripeptide is known to be a functional variant of the peroxisomal targeting signal type 1 (PTS-1) (Gould *et al.*, 1989; Swinkels *et al.*, 1992). We obtained the *YCAT* gene by polymerase chain reaction (PCR) on genomic DNA, cloned it into a plasmid and replaced the major part of the reading frame by the *LEU2* gene. Transformation of cells with this linearized plasmid resulted in several $Leu^+$ transformants, from which we selected the *YCAT* gene disruptions using PCR analysis.

CAT enzyme activity was measured in cell lysates of oleate-grown Δ*ycat* cells and wild-type parental cells. The CAT activity in Δ*ycat* cell lysates was found to be 10- to 30-fold decreased as compared with wild-type cell lysates (Table I). The remaining activity probably reflects the contribution of the YAT1 protein (Schmalix and Bandlow, 1993). No significant CAT activity was present in either the peroxisomal or the mitochondrial fractions of a Nycodenz gradient prepared from Δ*ycat* cells as described for Figure 2 (data not shown). This demonstrates that mitochondrial and peroxisomal forms of CAT are encoded by a single gene.

The high level of CAT activity in peroxisomes of oleate-grown cells suggests that this enzyme may play an important role in fatty acid metabolism. This led us to test whether the *YCAT* disruption exerts an effect on the growth rate on oleate plates. However, no difference with parental cells was observed on this medium, indicating that *YCAT* is dispensable for growth on fatty acids (accompanying paper, van Roermund *et al.*, 1995).

### *Targeting of CAT can be manipulated*

To gain insight into the targeting aspects of the YCAT protein, we made constructs expressing the complete YCAT protein, or the YCAT protein from which the presumed peroxisomal and/or mitochondrial targeting signals were deleted. These genes were expressed under the

**Table I.** Activity of CAT in oleate-grown wild-type cells, Δ*ycat* cells and Δ*ycat* cells expressing CAT constructs behind the CTA1 promoter

| Strain | CAT activity (nmol/min/mg) |
|---|---|
| Wild-type | 300 ± 30 |
| Δycat | 18 ± 5 |
| Δycat + CTA1- CAT-WT | 1280 |
| Δycat + CTA1- ΔN-CAT | 3910 |
| Δycat + CTA1- CAT-ΔC | 1150 |
| Δycat + CTA1- ΔN-CAT-ΔC | 3350 |

**Table II.** Import of ΔN-CAT-ΔC and PTS-1 (3-HAD) and PTS-2 (3-keto-acyl-CoA thiolase) containing proteins in control cells, *pas7* cells and *pas10* cells

| Strain | Activity in organellar fraction (%) | | |
|---|---|---|---|
| | 3-HAD (PTS-1) | Thiolase (PTS-2) | ΔN-CAT-ΔC |
| Δ*ycat* | 90–98 | 70–90 | 40–60 |
| *pas7*/Δ*ycat* | 90–98 | 1–10 | 40–60 |
| *pas10*/Δ*ycat* | 5–15 | 50–70 | 5–15 |

Subcellular fractionations were performed on Δ*ycat*, *pas7*/Δ*ycat* and *pas10*/Δ*ycat* cells, expressing the ΔN-CAT-ΔC protein.

control of the peroxisomal catalase A (CTA1) promoter. The CAT activity of these proteins expressed in oleate-grown Δ*ycat* cells is presented in Table I. The results show that expression of CAT-WT behind the CTA1 promoter resulted in a total CAT activity which was 4-fold higher than in untransformed wild-type cells. Deletion of the presumed mitochondrial signal (ΔN-CAT) resulted in another 3-fold higher expression, whereas deletion of the presumed peroxisomal signal (CAT-ΔC) led only to a slight decrease in CAT activity.

In one construct (CAT-ΔC) a deletion of the C-terminal AKL motif was made, which forms the putative PTS-1. As a result, the C-terminus now ended with the amino acids Lys–Arg–Lys. This tripeptide is not known as a variant of the PTS-1 signal. As expected, expression of this gene under the control of the CTA1 promoter resulted in the expression of a YCAT protein which was targeted exclusively to the mitochondria (Figure 3A). Surprisingly, however, an exclusive mitochondrial localization of this protein was also observed when we expressed the complete *YCAT* reading frame, including the PTS-1, behind the CTA1 promoter (CAT-WT) (Figure 3B). We concluded that the MTS probably overrules the PTS of YCAT. In a following section we demonstrate that the endogenous promoter is essential for the dual localization of the wild-type YCAT protein. The YCAT protein could be directed exclusively to peroxisomes by making a 66 bp deletion at the 5′ end of the *YCAT* gene, resulting in a *YCAT* gene starting at the second in-frame ATG codon, just behind the MTS (ΔN-CAT) (Figure 3C).

The presence of two targeting signals could also be demonstrated by fusing the N-terminal 136 amino acids or the C-terminal 28 amino acids of YCAT to the bleomycin reporter protein (BLE). We previously demonstrated that cells in which the BLE protein is targeted to peroxisomes become more sensitive to the drug phleomycin as compared with cells in which the BLE protein resides in the cytoplasm (Elgersma *et al.*, 1993). Cells expressing a fusion protein of BLE fused to the C-terminal 28 amino acids of YCAT [BLE-CAT(642–670)] were very sensitive to phleomycin whereas resistance was restored when we deleted the PTS-1 in this construct [BLE-CAT(642–667)] (Figure 4). In addition, cells expressing a fusion protein of BLE with the N-terminal 136 amino acids of YCAT [(1–136)CAT-BLE] were very sensitive to phleomycin (Figure 4). All constructs provided comparable resistance when expressed in *Escherichia coli* (not shown), proving that the BLE domain in these fusion proteins remained functional. Taken together, these results suggest that the decreased resistance in yeast is due to import of the chimeric proteins into the respective organelles and demonstrate that the *YCAT* gene product contains all the required topogenic information to be directed to both mitochondria and peroxisomes.

### *YCAT has an additional PTS*

As a control, a *YCAT* construct was made from which both the MTS and PTS were deleted (ΔN-CAT-ΔC). The localization of this protein in Δ*ycat* cells was analysed by subcellular fractionation. Remarkably, 40–60% of CAT activity was still present in the organellar pellet fraction. This is slightly less than we found for ΔN-CAT (70–80%), indicating that deletion of the C-terminal AKL motif only has a minor effect. Nycodenz gradient analysis revealed that the ΔN-CAT-ΔC activity coincides with the peroxisomal peak fractions (Figure 3D). This suggests that in addition to the AKL tripeptide (PTS-1), YCAT contains an internal peroxisomal targeting signal. It remains to be established whether the observed increased activity in the supernatant (cytosolic) fraction is due to leakage of YCAT from peroxisomes, or to inefficient import.

To study the nature of the internal peroxisomal targeting signal of YCAT we expressed (ΔN-CAT-ΔC) in the peroxisome assembly (*pas*) mutants *pas10* and *pas7*, in which the endogenous *YCAT* gene had been deleted (*pas10*/Δ*ycat* and *pas7*/Δ*ycat*). The PAS10 and PAS7 gene products are essential for the import of peroxisomal proteins containing a PTS-1 or PTS-2 respectively (Van der Leij *et al.*, 1992, 1993; Marzioch *et al.*, 1994). Subcellular fractionation showed that ΔN-CAT-ΔC was present in the pellet (organellar) fraction of the *pas7*/Δ*ycat* cells (Table II). In addition, Nycodenz gradients showed that ΔN-CAT-ΔC co-localized with the peroxisomal marker in *pas7*/Δ*ycat* cells (data not shown), indicating that ΔN-CAT-ΔC import in peroxisomes was independent of the PAS7 protein. Subcellular fractionation of *pas10*/Δ*ycat* cells revealed that (ΔN-CAT-ΔC) remained in the supernatant (cytosolic) fraction in these cells (Table II), implying that import in peroxisomes via the internal PTS of YCAT requires the PAS10 protein.

Recently we have demonstrated a direct interaction between the PAS10 protein and PTS-1 signals (M.M.Franse and H.F.Tabak, personal communication) using the yeast two-hybrid system developed by Fields and Song (1989). This technique employs the DNA binding and transactivation domains of the GAL4 transcription factor. When these domains are fused to two proteins that interact with each other, the activation of transcription is restored and can be monitored by measuring β-galactosidase expression, which serves as reporter gene (Figure 5). To test whether PAS10 can interact with the internal

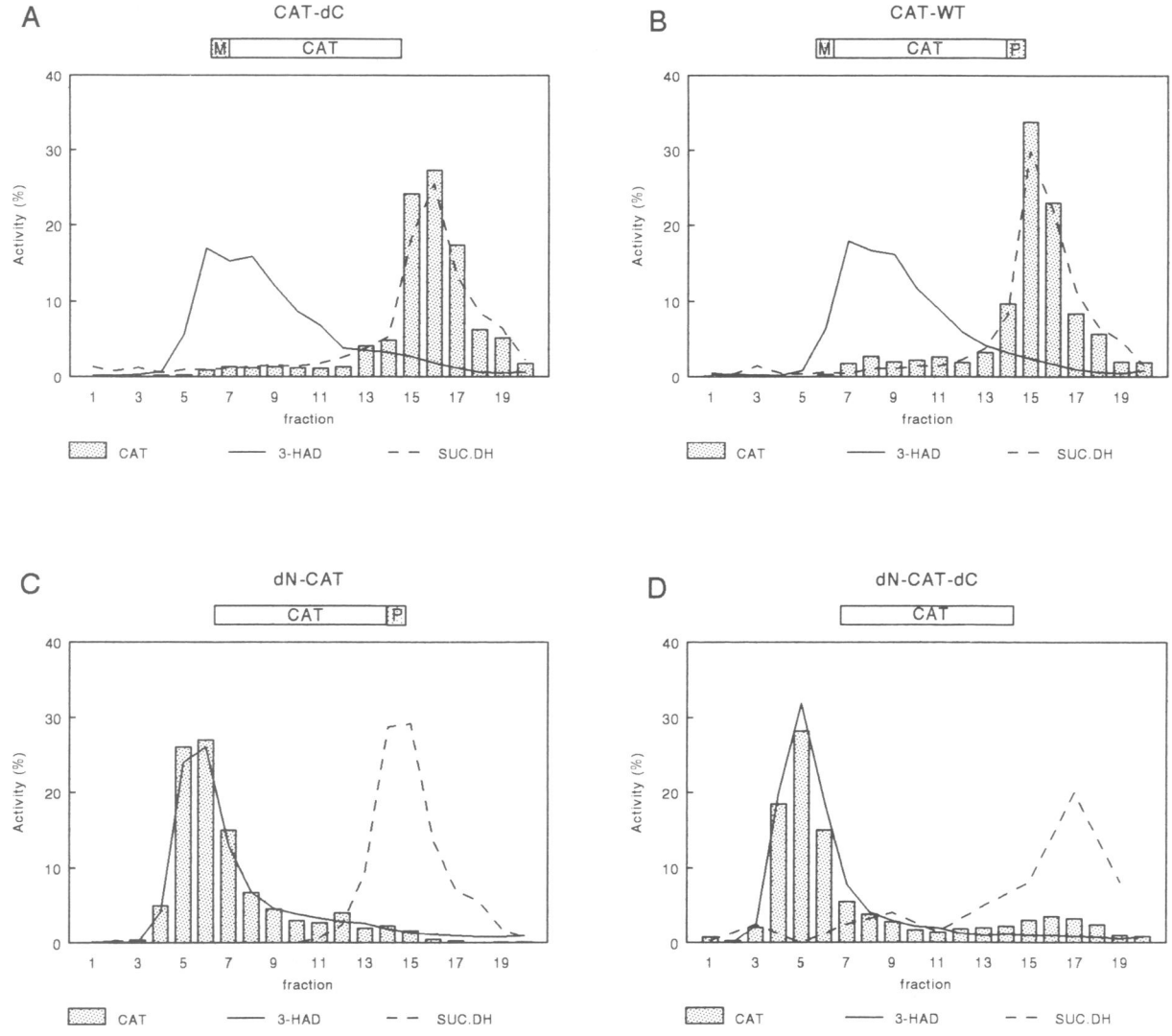



**Fig. 3.** Subcellular location of various modified CAT proteins in oleate-grown Δ*ycat* cells. The results shown are obtained as described for Figure 2. The constructs expressed under the control of the CTA1 promoter are: (**A**) CAT-ΔC; (**B**) CAT-WT; (**C**) ΔN-CAT; (**D**) ΔN-CAT-ΔC. The amount of CAT activity in the organellar fraction was 90% for CAT-ΔC and CAT-WT, 70% for ΔN-CAT and 60% for ΔN-CAT-ΔC.

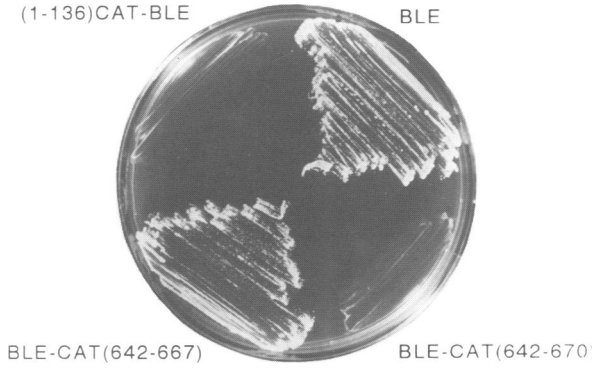
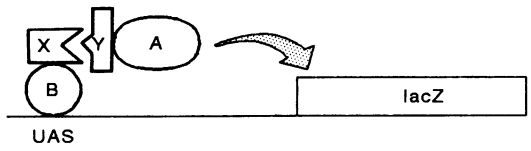



**Fig. 4.** Phleomycin resistance of cells expressing the wild-type bleomycin resistance protein (BLE-WT) or fusion proteins of BLE and CAT under the control of the CTA1 promoter. The abbreviations of the constructs are explained in detail in the text. The transformed cells are plated on rich glycerol plates containing 8 μg/ml phleomycin.

PTS in YCAT, we made the two-hybrid constructs as indicated in Figure 5. The results show that PAS10 does interact with YCAT in this two-hybrid system. However, the recognition was not only dependent upon the presence of the PTS-1 signal in YCAT, because deletion of the tripeptide AKL still resulted in blue colonies. These results strongly suggest that not only the PTS-1 but also the internal PTS in YCAT is recognized by the PAS10 protein. The exact position of this internal PTS in the protein remains to be determined.

### *Targeting of YCAT is controlled at the transcriptional level*

Since expression of the CAT-WT gene under the control of the CTA1 promoter resulted in a loss of targeting to peroxisomes, the authentic *YCAT* promoter is likely to be essential for the targeting of YCAT to two organelles. In addition, the exclusive mitochondrial localization of the CAT-WT protein suggests that the YCAT protein can only be directed to peroxisomes when the protein lacks the N-terminal MTS. We therefore analysed the positions of RNA initiation in response to different growth conditions. Total RNA was isolated from cells grown on acetate, glycerol or oleate. The 5′ start sites were determined by primer extension analysis, using reverse transcriptase and




| X | Y | Colour |
|---|---|---|
| - | PAS10 | White |
| CAT(+AKL) | - | White |
| CAT(+AKL) | PAS10 | Blue |
| CAT(-AKL) | PAS10 | Blue |

**Fig. 5.** Analysis of PAS10 and YCAT interaction using the yeast two-hybrid system. PAS10 was fused in frame to the GAL4-activation domain, whereas ΔN-CAT and ΔN-CAT-ΔC were fused in frame to the GAL4 DNA-binding domain. Plasmids with the GAL4-activation domain (A) fused to the PAS10 gene and plasmids with the GAL4 DNA-binding domain (B) fused to the YCAT gene were co-transformed to PCY2 cells, followed by a plating on selective plates. A two-hybrid plasmid without a *YCAT* or *PAS10* insert is denoted as (–). Colony colour 'blue' means blue-staining within 15 min, whereas colonies denoted 'white' remained white.

a primer complementary to a region downstream of the second AUG of *YCAT* mRNA. Multiple start sites were found upstream of the first AUG (Figure 6), nearly always at an adenine nucleotide. Most of the transcripts initiated at the TATA repeat which is present from base pairs –44 to –28. Besides all the start sites upstream of the first AUG, *YCAT* mRNA isolated from oleate-grown cells showed additional transcription initiation sites between the first and second AUG. Also these initiation sites were nearly always at adenine nucleotides, and they were virtually absent in *YCAT* mRNA isolated from cells grown on acetate or glycerol. Similar results were obtained using two other primers, and with independently isolated RNA preparations (data not shown). It is therefore likely that peroxisomal localization of YCAT results from a translational start at the second AUG remaining in the shorter mRNA. The absence of the MTS allows the translation product to target to peroxisomes, free of competition by the MTS.

The unique presence of the shortened transcripts in oleate-grown cells predicts an exclusively mitochondrial localization of YCAT in glycerol- or acetate-grown cells. We tested this by performing a subcellular fractionation on glycerol- and acetate-grown cells, followed by a Nycodenz gradient of the organellar pellet. Although mitochondria of cells grown on these carbon sources tend to give a broad peak, a sharp peroxisomal peak was obtained as concluded from the activity profile of the peroxisomal marker enzyme (Figure 7). Figure 7 further shows that CAT co-localized with the mitochondrial marker enzyme in glycerol-grown cells, as well as in acetate-grown cells (not shown). In spite of the sharp peroxisomal peak, bimodal distribution is no longer observed for CAT (compare Figure 2 with Figure 7). This strongly suggests that the targeting to peroxisomes is indeed a result of the induction of shorter *YCAT* transcripts in oleate-grown cells.

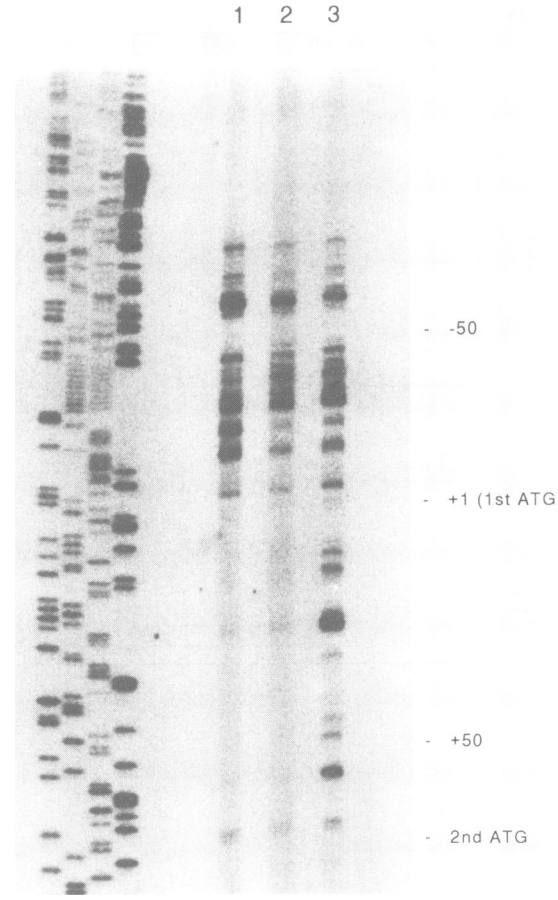



**Fig. 6.** Determination of the 5′ initiation sites of YCAT mRNA in response to various carbon sources. Primer extension analysis was performed on RNA isolated from cells grown on acetate (1), glycerol (2) or oleate (3). The primer used was CAT-RO3 which anneals to region +217 to +240 bp with respect to the first ATG. The sequence ladder is obtained with the same primer, and was performed on pCAT-WT(+45) DNA. The numbers indicate the position (in bp) relative to the first ATG.

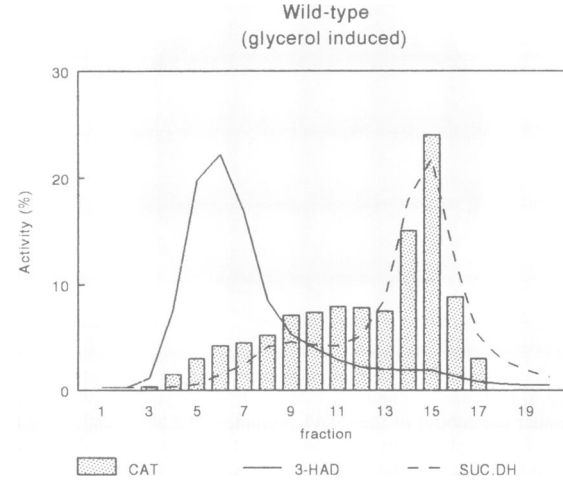



**Fig. 7.** Subcellular location of CAT in glycerol-grown *S.cerevisiae*. The results shown are obtained as described for Figure 2. Approximately 80% of total CAT activity as measured in the homogenate was present in the organellar fraction.

## Discussion

It has been demonstrated that the YCAT protein plays an important role in the transport of acetyl-CoA from peroxisomes (accompanying paper, van Roermund *et al.*, 1995). Here, we demonstrate the presence of CAT in both peroxisomes and mitochondria, and show that both forms are encoded by the same gene (*YCAT*). The encoded protein possesses, besides a mitochondrial targeting signal, a PTS-1-like tripeptide at its C-terminus (AKL). Remarkably, this PTS-1 is able to direct a reporter protein to peroxisomes, but is not essential for import of YCAT itself, indicating the presence of an internal peroxisomal targeting signal in YCAT. An internal PTS has also been found in acyl-CoA oxidase of *C.tropicalis* (Small *et al.*, 1988), and in catalase A of *S.cerevisiae* (Kragler *et al.*, 1993). Peroxisomal import of YCAT without its PTS-1 was not affected in *pas7* cells (defective in import of PTS-2-containing proteins), whereas this protein could not be imported in peroxisomes of *pas10* cells (defective in import of PTS-1-containing proteins). This indicates that PAS10, but not PAS7, is required for import of YCAT via this internal PTS. A similar result was found for catalase A (Van der Leij *et al.*, 1992, 1993). Moreover, making use of the yeast two-hybrid system (Fields and Song, 1989), we have demonstrated that PAS10 might interact directly with the internal PTS signal of YCAT. Alternatively, import of YCAT via this internal PTS is dependent upon the interaction with other PTS-1-containing proteins. Considering these findings, we like to designate this internal PTS as PTS-1i, since import is dependent on the presence of the PTS-1 receptor.

Despite the presence of two peroxisomal targeting signals in YCAT, the targeting to peroxisomes is dependent on the carbon source in the growth medium. In acetate- or glycerol-grown cells, YCAT is present predominantly in mitochondria, whereas in oleate-grown cells YCAT is present in both organelles. Expression of the entire YCAT protein behind a heterologous promoter provided a clue to how targeting is controlled: despite the presence of MTS and PTSs in the protein product, targeting to peroxisomes was no longer observed. We reasoned that most likely, the MTS in the YCAT protein overrules the PTS. This can be by-passed if alternative initiation of translation from the second methionine (residue 24) takes place, because this would give rise to a YCAT protein which lacks the MTS. Two mechanisms are known to result in alternative initiation of translation. First, alternative initiation of translation could take place from a single *YCAT* mRNA species in a process called leaky (ribosome) scanning. The first initiation codon is weakly recognized by the scanning ribosomes, resulting in a fraction of the ribosomes proceeding past the first AUG and initiating at the second AUG. Second, translation from two AUG codons can be the result of translation of different *YCAT* mRNA species, which arise by alternative initiation of transcription. Our results show that in oleate-grown cells, shorter transcripts of *YCAT* occur, which start between the first and second AUG. Since these transcripts are not present in acetate- or glycerol-grown cells, it is reasonable to assume that these transcripts encode the YCAT protein which can be targeted to peroxisomes, due to the absence of the MTS in this protein.

To rule out that YCAT targeting might be the result of a leaky ribosome scanning mechanism, we tested whether the targeting of YCAT would change if we added to our CAT-WT construct the 45 bp upstream of the first AUG [pCAT-WT(+45); data not shown]. In this way we assumed to better mimic the *YCAT* mRNAs as they are present in wild-type cells. However, expression of this gene under the control of the CTA1 promoter resulted in the targeting of YCAT to mitochondria only, indicating that alternative initiation of translation due to leaky scanning is probably not the basis for the regulation of targeting of YCAT. In addition, it should be noted that the sequence neighbouring the second AUG is not a more favourable translation initiation sequence context than that neighbouring the first AUG, which also argues against a leaky ribosome scanning mechanism.

The carbon source-dependent targeting of YCAT is very similar to the yeast invertase situation (Carlson and Botstein, 1982). This protein, encoded by the *SUC2* gene, is expressed constitutively as an intracellular protein. When the yeast is grown under low-glucose conditions, a larger mRNA is synthesized, including the information for the ER targeting signal, which encodes the secreted form of invertase.

A peroxisomal and mitochondrial form of CAT encoded by a single gene may also exist in the yeast *C.tropicalis* (Ueda *et al.*, 1982, 1984a,b). In addition, also in higher eukaryotic cells, the peroxisomal and mitochondrial forms of CAT may be derived from a single gene, since these proteins have very similar amino acid compositions and purification properties (Miyazawa *et al.*, 1983a,b). Recently, the sequence of the human *CAT* gene was reported (Corti *et al.*, 1994). Interestingly, two splicing variants were found, one clone contained both a MTS and PTS-1, whereas in the other clone the MTS was disrupted by an intron. Comparing this with our results, it is very likely that the first clone encodes a mitochondrial CAT protein, whereas translation of the latter clone presumably starts from the second in frame AUG, giving rise to a peroxisomal form of CAT. This would indicate that the remarkable property of one CAT gene encoding two differentially targeted proteins, is conserved from yeast to man. Only the mechanism to obtain this differential targeting is different between these two organisms.

Several other proteins are encoded by a single nuclear gene and possess a dual subcellular localization (reviewed by Surguchov, 1987). However, to our knowledge, the only other protein which is known to be targeted to both peroxisomes and mitochondria is the alanine–glyoxylate aminotransferase (AGT) protein. In both rat (Oda *et al.*, 1990) and marmoset (Purdue *et al.*, 1992), AGT is present in mitochondria and peroxisomes, whereas only one gene is present. Moreover, RNA transcripts of AGT are present in these organisms which initiate within the region encoding the MTS, such that initiation of translation must take place from the downstream AUG (corresponding to residue 23). This is very similar to what we have found for YCAT in this study. However, the distribution of AGT between mitochondria and peroxisomes seems to be fixed within an organism, whereas we have demonstrated that the targeting of YCAT is flexible and dependent on the growth conditions.

## Materials and methods

### Yeast strains and culture conditions

Yeast strains used in this study were *S.cerevisiae* BJ1991 (*MATα, leu2, trp1, ura3–251, prb1–1122, pep4–3*) for gene disruptions and gradients, and PCY2, which contains the GAL1-lacZ reporter gene integrated at the URA3 locus and GAL4 and GAL80 deletions, for two-hybrid assays (a kind gift of P.Chevray). The *pas7* and *pas10* mutants used were mutants isolated at our laboratory (Van der Leij *et al.*, 1992; Elgersma *et al.*, 1993). Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO)(DIFCO), 2% glucose and amino acids (20 μg/ml) as needed. The rich media used for growth for RNA isolation or subcellular fractionation contained 0.5% potassium phosphate buffer, pH 6.0, 0.3% yeast extract, 0.5% peptone, and 2% glucose, or 2% glycerol or 2% K-acetate or 0.1% oleic acid/0.2% Tween-40 (YPO) as carbon source. Before shifting to one of these media, cells were grown on minimal 0.3% glucose medium for at least 24 h. For RNA isolations, the cultures were inoculated at such a density that they reached $OD_{600}$ = 0.7–1.0 after ~15 h.

### Subcellular fractionation and Nycodenz gradients

Subcellular fractionations were performed as described by Van der Leij *et al.* (1992). Continuous 16–35% Nycodenz gradients were used (12 ml), with a cushion of 1 ml 42% Nycodenz dissolved in 5 mM MES, pH 6.0, 1 mM EDTA, 1 mM KCl and 8.5% sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor (MSE 8×35) at 19 000 r.p.m. (29 000 *g*) at 4°C.

### Cloning procedures

The yeast *YCAT* gene was amplified from genomic DNA using the 5′ CAT-A primer (5′-TTTGAATTCGAGAACTCTCTCAAAC-3′) and the 3′ CAT-B primer (5′- TTTCTGCAGCGTAAGCCCTTTTTTCTCCC-3′) oligonucleotides. The PCR reaction was performed using 55°C as annealing temperature. The resulting 2.1 kb *Eco*RI–*Pst*I fragment was subcloned into pUC19 (pEL72). The major part of the open reading frame was deleted, by replacing the *Acc*1–*Bgl*2 fragment (containing 1281 bp of the CAT open reading frame) by the LEU2 gene (pEL78). This plasmid was used to transform wild-type cells, *pas7* cells or *pas10* cells. $Leu^+$ transformants were selected for integration in the *YCAT* gene by PCR analysis.

For all the expression constructs described, the single-copy catalase A (CTA1) promoter expression plasmid was used (previously described by Elgersma *et al.*, 1993). The YCAT constructs were obtained by PCR, introducing a *Sac*I site at the 5′ end, and a *Pst*I or *Hind*III site at the 3′ end. In addition, a stop codon was introduced when a C-terminal deletion was made. CAT-WT (pEL98) was constructed by PCR with the 5′ CAT-C primer (5′-TTTGAGCTCATGAGGATCTGTCATTCGAGA-3′) and the 3′ CAT-B primer. CAT-ΔC (pEL96) was made by using the 5′ CAT-A primer and the 3′ CAT-D primer (5′-AAAAAGCTTATTTT-CGTTTATTCTCATTTTCCAAG-3′). ΔN-CAT (pEL97) was made by using the 5′ CAT-E primer (5′-TTTGAGCTCATGCATTCGGCC-ATTGTCAAT-3′) and the 3′ CAT-E primer. ΔN-CAT-ΔC (pEL99) was made by using the 5′ CAT-E primer and the 3′ CAT-D primer. CAT-WT(+45) was made by using 5′ primer (5′-TTTGAGCTCGT-ATATATATATATATATCCCTTAAAAAC-3′) and 3′ primer CAT-B. All the targeting signals were verified by sequencing. Two independent clones were used from every PCR.

The two-hybrid fusion proteins were made using the two-hybrid plasmids pPC86 and pPC97 (a kind gift of P.Chevray) by cloning the PAS10 gene in frame with the GAL4 activation domain (pMF6) (M.M.Franse, manuscript in preparation) and the ΔN-CAT and ΔN-CAT-ΔC in frame with the GAL4 DNA binding domain (resulting in pEL127 and pEL128 respectively).

### Primer extension analysis

RNA was isolated from acetate-, glycerol- and oleate-grown cells which were harvested at $OD_{600}$ = 1. For every assay, 15 μg (acetate- and glycerol-grown cells) or 10 μg (oleate-grown cells) of total RNA was used. Primers used were: CAT-RO1 (annealing to bp 75–94 with respect to the first ATG) (5′-TGGAGTAATTGACAATGGCC-3′), CAT-RO2 (bp 82–106) (5′-GGGCACGGGTAATGATGGTAAGTC-3′) and CAT-RO3 (bp 217–240) (5′-GGGCACGGGTAATGATGGTAAGTC-3′).

### CAT assays

CAT assays were performed at 30°C in 50 mM HEPES, pH 7.4, 5 mM carnitine, 0.3 mM acetyl-CoA (including $^{14}C$-labelled acetyl-CoA), 0.2% (w/v) Triton X-100 buffer. After 15 min, the reactions were terminated by addition of 5 vol ice-cold ethanol. The [$^{14}C$]acetylcarnitine produced was separated from the [$^{14}C$]acetyl-CoA on an AG IX-8 (CL) Biorad column, and quantified in a liquid scintillation counter.

### Phleomycin assays

The bleomycin resistance gene used for this study is the Sh-BLE gene. This gene and the phleomycin (yeast) and zeomycin (*E.coli*) antibiotics were obtained from CAYLA (Toulouse, France).

### β-oxidation measurements

Methods and strains used are described in van Roermund *et al.* (1995).

### Miscellaneous

Published procedures were used for 3-hydroxy-acyl-CoA dehydrogenase [an activity of multifunctional enzyme (MFE)] measurements (Wanders *et al.*, 1990), succinate dehydrogenase measurements (Munujos *et al.*, 1993) and β-galactosidase colony assay (Fields and Song, 1989). PCR, primer extension and other DNA techniques were carried out as described by Sambrook *et al.* (1989).

## Acknowledgements

We are grateful to P.Borst and I.Braakman for valuable and stimulating discussions, and to B.Distel for calling our attention to the putative targeting properties of YCAT. We thank L.IJlst for technical assistance, M.M.Franse for the Gal4–PAS10 plasmid, W.H.Kunau and co-workers for providing the Nycodenz gradient protocol and the thiolase antibody, P.Chevray for the two-hybrid plasmids and yeast-strain PCY2, and finally G.Tiraby and H.Durand for providing the Sh-BLE gene, antibodies, and helpful suggestions.

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*Received on October 20, 1994; revised on March 17, 1995*