

# Cloning of a cDNA Encoding Cytosolic Acetoacetyl-Coenzyme A Thiolase from Radish by Functional Expression in *Saccharomyces cerevisiae*<sup>1,2</sup>

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A cDNA coding for radish (*Raphanus sativus* L.) acetoacetyl-coenzyme A thiolase (AACT) was cloned by complementation of the *erg10* mutation affecting AACT in yeast (*Saccharomyces cerevisiae*). The longest reading frame encodes a protein of 406 amino acids with a predicted relative molecular weight of 42,032, with significant similarities to eukaryotic and prokaryotic thiolases. There is no evidence for the presence of a leader peptide characteristic, e.g. of glyoxysomal thiolase. Yeast transformants expressing the radish AACT gene placed under the control of the *GAL1* promoter exhibited a 10-fold higher enzyme activity than a wild-type yeast strain after induction by galactose. This enzyme activity is exclusively localized in the soluble fraction but not in membranes. These data indicate that we have cloned a gene encoding cytoplasmic (biosynthetic) AACT. Genomic DNA gel blot analysis suggests the presence of a single AACT gene, which is expressed in all parts of the seedling. Expression in cotyledons appears to be light-stimulated. We present preliminary evidence that a smaller transcript represents an antisense species being read from the same gene.

For yeast and mammalian tissue, it has been well documented (see Bach et al., 1990, 1991, and literature cited therein) that the conversion of three units of acetyl-CoA to HMG-CoA is catalyzed by two enzymes: AACT (acetyl-CoA acetyl transferase, EC 2.1.3.9), catalyzing a Claisen-type condensation, and HMGS ([S]-3-hydroxy-3-methylglutaryl-CoA:acetoacetyl-CoA lyase, CoA acylating, EC 4.1.3.5), catalyzing an aldol condensation. HMG-CoA serves two functions: first, as the substrate for mevalonate biosynthesis, catalyzed by HMGR (3-hydroxy-3-methylglutaryl-CoA reductase, mevalonate:NADP<sup>+</sup> oxi-

doreductase, CoA acylating, EC 1.1.1.34), and second, as a putative intermediate in the degradation of Leu via HMG-CoA lyase ([S]-3-hydroxy-3-methylglutaryl-CoA lyase, EC 4.1.3.4).

Two different types of thiolase are found in both eukaryotes and in prokaryotes: AACT (EC 2.3.1.9) and 3-ketoacyl-CoA thiolase (= OACT, EC 2.3.1.16). OACT (also called thiolase I) has a broad chain-length specificity for its substrates and is involved in degradation pathways, such as fatty acid  $\beta$ -oxidation. AACT (also called thiolase II) is specific for the thiolysis of acetoacetyl-CoA and is involved in biosynthetic pathways such as poly- $\beta$ -hydroxybutyrate synthesis in certain bacteria (see Murphy, 1994; Lee et al., 1995, and refs. cited therein) or isoprenoid biogenesis in eukaryotes. In eukaryotes, there are two forms of OACT, one located in mitochondria and the other located in peroxisomes (for literature citations, see Igual et al., 1992; Kanayama et al., 1994). In yeast, the formation of acetoacetyl-CoA appears to be an important step in the regulation of growth (Kornblatt and Rudney, 1971) and of ergosterol biosynthesis (Trocha and Sprinson, 1976; Servouse and Karst, 1986).

Until recently, little was known about the enzymology and genetics of the reactions leading from acetyl-CoA to mevalonic acid via HMG-CoA in plants (Gray, 1987; Kleinig, 1989; Bach et al., 1990, 1991; Alam et al., 1991; Van der Heijden et al., 1994). Although genes encoding various plant HMGR isozymes have been cloned from several species (for recent literature citations, see Enjuto et al., 1994; Stermer et al., 1994; Chappell, 1995; Weissenborn et al., 1995) according to the databases, full-length cDNA clones of plant genes encoding biosynthetic AACT have not been characterized. While this paper was under revision, an Arabidopsis cDNA sequence with significant similarities to vertebrate HMGS was made available (accession no. X83882, Montamat et al., 1995).

For the cloning of plant AACT, the complementation technique was chosen, using suitable *erg* (*ergosterol*-

<sup>1</sup> Part of this study at the University of Karlsruhe was supported by the Deutsche Forschungsgemeinschaft (Ba 871/3). Ongoing studies at the Institut de Biologie Moléculaire des Plantes (Strasbourg) have been made possible by the support of the Centre National de la Recherche Scientifique. K.-U.V. gratefully acknowledges the award of his stipend by the State of Baden-Württemberg.

<sup>2</sup> Essential results of this study were presented at the 11th International Plant Lipid Meeting (Paris, June 26–July 1, 1994).

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Abbreviations: AACT, acetoacetyl-CoA thiolase; DIG, digoxigenin; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; OACT, 3-oxoacyl-CoA thiolase; PQQ, pyrroloquinoline quinone; Ura, uracil.

deficient) and thermolabile yeast mutants (Karst and Lacroite, 1974). These *erg* mutants, which are impaired in AACT, consist of two linked complementation units, *erg10A* and *erg10B* (Servouse et al., 1984). The conditional *erg10* mutant has been successfully used to clone an AACT gene from the industrial yeast *Saccharomyces uvarum* (Dequin et al., 1988b). More recently, the AACT gene was isolated from *Saccharomyces cerevisiae* (Hiser et al., 1994) using another yeast *erg10* mutant. This paper describes the isolation of a radish (*Raphanus sativus* L.) cDNA encoding the cytosolic AACT by complementation of the temperature sensitivity of the *erg10A* mutation in yeast.

## MATERIALS AND METHODS

### Strains and Media

*Escherichia coli* strains DH10B (BRL) and XL1-Blue MRF' (Stratagene) were used as plasmid or phage hosts. Yeast strains F2sp5 [*MATa*, *erg10A*, *ura3-52*, *his3-D200*, *leu2-3,112*, *gal2<sup>-</sup>*] and W303-1B [*MAT $\alpha$* , *ura3-52*, *his3-D200*, *leu2-3,112*, *trp*, *ade*] were kindly provided by F. Karst (Université de Poitiers, France). F2sp5 is derived from strain FL100, which is not able to grow on Gal. Therefore, it was crossed with W303-1B (Thomas and Rothstein, 1989) to produce ergosterol-auxotrophic yeast strains, in which plant cDNAs placed under the control of the *GAL1* promoter can be expressed, depending on the ability to use Gal as a carbon source (Schneider and Guarente, 1991). Sporulation was induced according to the protocol of Sprague (1991). Meiosis and sporulation of diploids and random spore analysis were carried out as described by Kassir and Simchen (1991) and by Dawes and Hardie (1974), respectively. The resulting haploids were replica-plated and incubated (at either 28 or 37°C) on a medium containing Gal to select them for the trait of thermolability that is linked to AACT deficiency and the phenotype of ergosterol auxotrophy. Thus, strain KV5 [*MATa*, *erg10A*, *ura3-52*, *his3-D200*, *leu2-3,112*, *Gal2<sup>+</sup>*] was chosen for the following complementation experiments. Media for the growth of yeast were as described by Sherman (1991): YPGlu contained 2% Glc; YPGal contained 2% Gal; and YNB consisted of 0.67% yeast nitrogen base with ammonium sulfate, 2% Glc, and appropriate supplements for selection. Ergosterol was dissolved in detergent (Tergitol NP-40 [Sigma]:ethanol (1:1, v/v) and was added to liquid and solid media at a concentration of 4 and 80  $\mu\text{g}/\text{mL}$ , respectively, for the propagation of ergosterol-auxotrophic yeast strains F2sp5 and KV5.

### cDNA Library Construction

Conditions for the hydroponic culture of radish (*Raphanus sativus* L.) seedlings were as described previously (Bach and Lichtenthaler, 1983). Total RNA from 10 g of 4-d-old etiolated radish seedlings (cotyledons and stems without roots) was extracted according to Dean et al. (1985). Radish mRNA was enriched by oligo(dT)-cellulose affinity chromatography (Aviv and Leder, 1972), slightly modified as a batch procedure. Radish cDNA was synthesized from 5  $\mu\text{g}$  of mRNA using the cDNA SYNTHESIS kit from Stratagene

with some modifications. After blunt-end conversion, *XbaI* linkers were ligated to both ends of the cDNAs. Double digestion with restriction endonucleases *XhoI* and *XbaI* created an *XbaI* site upstream and an *XhoI* site downstream of the cDNAs, respectively. Digested or excess linkers and adaptors, as well as cDNA fragments smaller in size than 500 bp, were removed by chromatography on a 1-mL Sephacryl S-500HR spin column. A total yield of approximately 600 ng of cDNA was estimated from the color intensity on ethidium bromide-containing agarose plates. *XhoI/XbaI*-predigested arms of  $\lambda$ MAX1 from Clontech (1  $\mu\text{g}$ ) were ligated to 100 ng of radish cDNA and in vitro packaged with phage particles of the Stratagene GiGAPack Gold kit. The primary titer of the library was determined as  $1.5 \times 10^6$  independent clones with an average insert size of 0.9 kb. The library was amplified in *E. coli* XL1-Blue MRF', resulting in a titer of  $2.2 \times 10^9$  plaque-forming units  $\text{mL}^{-1}$ . The conversion from the  $\lambda$ MAX1 phage library to the pYEUra3 plasmid library was performed as described in the user's manual of the Exassist/Solr System from Stratagene. Plasmid DNA isolation followed standard procedures (Sambrook et al., 1989). Plasmid DNA was purified using fast-performance liquid chromatography on a Superose 6 prep-grade column purchased from Pharmacia (McClung and Gonzales, 1989). Alternatively, plasmid DNA was purified using Qiagen (Chatsworth, CA) columns according to the manufacturer's protocol.

### Complementation of the *S. cerevisiae erg10* Mutation

About 10  $\mu\text{g}$  from the radish cDNA library were used to transform AACT-deficient yeast strain KV5 by the lithium acetate method as previously described (Becker and Guarente, 1991), but without carrier DNA in the transformation assays. *Ura3<sup>+</sup>*-transformants were pooled, and aliquots were plated on YPGal-complete medium. The Petri dishes were incubated at 37°C to select transformants expressing Gal-induced radish AACT, and thus complementing the mutation, which causes thermolability. Plasmid DNA was recovered (Kaiser and Auer, 1993) from six yeast transformants that showed thermoresistance after induction by Gal and were transformed into *E. coli* DH10B by electroporation. Restriction analysis revealed all six clones carrying an identical 1.5-kb cDNA (referred to as cRS10) inserted into the *XhoI/XbaI* cloning site of pYEUra3. The corresponding plasmid, referred to as pYRS10, was then retransformed into the yeast strain KV5, thus generating strain KV10 to confirm the Gal-dependent phenotype of thermoresistance at 37°C in 100% of the transformants.

### DNA Sequencing and Sequence Analyses

pCRS10 was constructed by ligating the 1.5-kb *XhoI/XbaI* fragment cRS10 into pBluescript (SK<sup>+</sup>) for sequence analysis by the dideoxynucleotide method (Sanger et al., 1977) using Hyperpaper<sup>35S</sup> (Amersham). The complete sequence of cRS10 was determined with a set of two flanking pBluescript (SK<sup>+</sup>) primers (T3 and M13-20) by creating nested deletion mutants of cRS10 with exonuclease III. Double-

stranded plasmid DNA was sequenced using Sequenase 2.0 from United States Biochemical or *Taq* polymerase from Promega's fmol cycle sequencing kit, both according to the manufacturers' instructions. The nucleic acid sequence of cRS10 and the deduced amino acid sequence were used to search database libraries for homologies with the DNASIS/PROSIS software from Hitachi (Tokyo, Japan). Further sequence analyses were performed at the Institut de Biologie Moléculaire des Plantes (Strasbourg, France) with the BLAST network service (Altschul et al., 1990). Sequence alignments and additional analyses were performed using the Genetics Computer Group sequence analysis software package (version 7.0, April 1991) for the Vax workstation installed at the Institut de Biologie Moléculaire des Plantes.

### RNA Isolation and RNA Gel Blot Analyses

Total RNA from radish tissue was isolated according to the protocol of Groppe and Morse (1993), developed for RNase-rich marine mollusks, with some modifications. Approximately 10 g of plant material were deep-frozen in liquid nitrogen and pulverized with a mortar and pestle. Additional steps in the protocol were adjusted to this quantity of tissue. DNA prepared in parallel could be precipitated by means of isopropanol and was used for genomic DNA gel blot analyses.

For the isolation of RNA from yeast, cultures of 100 mL ( $A_{695} = 1.5$ ) were centrifuged in 50-mL Falcon (Becton Dickinson) tubes (4000g, 5 min) and washed twice with diethyl pyrocarbonate-treated (RNase-free) water. The pellet was resuspended in 10 mL of lysis buffer (Groppe and Morse, 1993) plus 10 mL of phenol, followed by the addition of 10 mL of glass beads (diameter 0.5 mm). Cells were broken by vortexing ( $5 \times 1$  min, maximum speed, interrupted by 1-min cooling periods on ice). After that, 2 mL of chloroform:isoamyl alcohol (49:1, v/v) were added, and the sample was processed further, as described by Groppe and Morse (1993).

cDNA probes were labeled by the incorporation of non-radioactive DIG-11-dUTP using random-primed labeling, according to the manufacturer's protocol (Boehringer Mannheim). Oligonucleotides were end-labeled with DIG-dUTP by means of terminal dideoxynucleotide transferase (Schmitz et al., 1991).

Sense and antisense mRNA species were detected with DIG-UTP-labeled RNA probes that exhibited single-strand target specificity. They were labeled with the hapten DIG by the aid of the DIG RNA labeling kit from Boehringer Mannheim, according to the manufacturer's protocol, and pRS10 (a derivative of pBluescript SK) as the template. T3 RNA polymerase thus generated an antisense-specific RNA probe, and T7 RNA polymerase generated a sense-specific probe.

Total RNA (usually 10  $\mu$ g per lane) was electrophoretically separated in 1.2% agarose gels (Sambrook et al., 1989). However, the concentration of formaldehyde was reduced to 0.41 M (see Chomczynski, 1992). Downward alkaline capillary transfer to positively charged nylon membranes (Appligene, Heidelberg, Germany) followed the protocol of Chomczynski (1992). Hybridizations and detection of

DIG-labeled probes were carried out as described by Engler-Blum et al. (1993). The usual hybridization and washing temperature was 68°C (60°C when oligonucleotide probes were used). Disodium 3-(4-methoxyspiro{1,2-dioxetane - 3,2' - (5' - chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan} -4-yl)phenyl phosphate (Tropix, Bedford, MA) was used as a chemiluminescent substrate for membrane-based detection of alkaline-phosphatase conjugates (Düring, 1991).

### Genomic DNA Gel Blot Analysis

For genomic DNA gel blot analyses, 10  $\mu$ g of each genomic DNA were digested with various restriction endonucleases and electrophoretically separated in 0.8% agarose gels.  $\lambda$ DNA was used as a size marker, digested with *Hind*III and *Hind*III/*Eco*RI. The gel was washed with 0.5 M HCl for 15 min to depurinate the DNA, which facilitates the transfer. Capillary transfer, hybridization, and signal detection were carried out as described for the RNA gel blot analyses.

### Determination of AACT Activity in Yeast Transformants

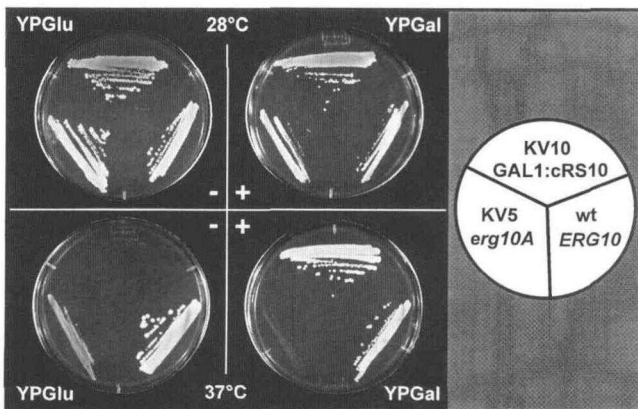
Yeast strain KV10, carrying the radish cDNA cRS10 under control of the *GAL1* promoter, the mutant strain KV5, and strain W303-1B as a reference were used to inoculate liquid cultures with 100 mL of minimal medium supplemented with ergosterol and with either Glc or Gal as a carbon source. The cultures were incubated at 26°C. The cells were harvested by means of centrifugation at 5000g for 5 min, at which point they had reached an  $A_{600}$  of 1.5. Disruption of yeast cells by glass beads, preparation of protein extracts, and membrane fractionation were performed as previously described (Vollack et al., 1994). AACT activity was determined by incorporation of [<sup>14</sup>C]acetyl-CoA into heat-stable and acid-stable [<sup>14</sup>C]HMG-CoA (Weber and Bach, 1994). This test is a modification of the method used for the assay of avian HMGS introduced by Clinkenbeard et al. (1975). In our method we use <sup>14</sup>C-labeled acetyl-CoA as the only substrate, which is then converted to acetoacetyl-CoA by action of AACT. Endogenous HMGS uses this intermediate for the condensation with an additional acetyl-CoA unit to yield S-HMG-CoA. After stopping the reaction by adding HCl, followed by heating of the samples to 110°C, the thioesters were cleaved. Unreacted substrate (in the form of acetate or acetoacetate converted into acetone) evaporated, but HMG acid remained. Hence, the incorporation of radioactivity into a heat-stable and an acid-stable product indicated the combined activity of AACT and HMGS. Protein was quantified by the Lowry method (Bensadoun and Weinstein, 1976) with some modifications (Bach et al., 1986).

## RESULTS

### Generation of a Suitable Yeast *erg10* Mutant and Phenotype of Transformants

The original ergosterol-auxotrophic yeast strain F2sp5 (Servouse et al., 1984) is a derivative of the strain FL100,

which bears a mutation in the *GAL2* gene encoding Gal transferase. Thus, for heterologous expression of plant cDNAs placed under the *GAL1* promoter, we had to generate an *erg10A* recipient strain with the wild-type allele *GAL2* being reconstituted. This was achieved by crossing the two haploid strains F2sp5 (Mat a) and W303-1B (Mat  $\alpha$ ; Thomas and Rothstein, 1989). The new strain thus generated, termed KV5, was capable of growing at the permissive temperature of 28°C on Glc or Gal as a carbon source, but did not grow on Gal at 37°C (inducing conditions; Fig. 1). Both ergosterol-auxotrophic strains, F2sp5 and its derivative KV5, exhibited rather low transformation efficiencies, with approximately  $1.3 \times 10^3$  Ura3<sup>+</sup>-transformants/ $\mu$ g DNA. Transformation assays using electroporation (Becker and Guarente, 1991) were unsuccessful (data not shown). The problem of low transformation rates and/or fragility of cells has also been seen with other yeast *erg* mutants deficient in mevalonate kinase (*erg12-1*, Riou et al., 1994) or deficient in  $\Delta^7$ -sterol-C5-desaturase (*erg3*, Gachotte et al., 1996). From a total of about  $10^5$  Ura3<sup>+</sup>-transformants, 127 clones were found to be thermoresistant, but only 6 of them showed the wild-type phenotype that was dependent on the selected carbon source. These clones, as well as the transformed strain KV10 (see Fig. 1), grew at 37°C in the presence of Gal, but not when Glc was the sole carbon source. A parental strain, W303-1B (Thomas and Rothstein, 1989), was used as an Erg10<sup>+</sup> control; this grew under all conditions applied (Fig. 1). Although the *erg10* strain KV5 exhibited some residual growth on Glc at 37°C, it did not develop on Gal at this temperature.



**Figure 1.** Culture of *erg10A* mutants and transformants expressing radish AACT and wild-type yeast cells. Cells were grown for 3 d at the permissive temperature of 28°C and at the nonpermissive temperature of 37°C on 2% Glc (YPGlu, noninducing conditions) and on 2% Gal (YPGal, inducing conditions), respectively. In each case, the medium contained 80  $\mu$ g/mL ergosterol. Top, Strain KV10 (*GAL1:cRS10* (*erg10A* [pYRS10])). Left, KV5 (*erg10A* [pYEUra3]), plasmid without cDNA insert as a control). Right, Wild-type (wt) strain W303-1B (*ERG10*). Note that strain KV10 cannot grow at 37°C in the absence of Gal, whereas some growth of yeast cells with strain KV5 was observed at 37°C on Glc but not on Gal.

		348				393	
Sccaact	P...SKVNVY	<b>GGAV</b> ALGHPL	<b>GCSGAR</b> VVVT	LLSILQ <b>Q</b> EGG	K...IGVAAIC	*	
Sucaact	P...SKVNVY	<b>GGAV</b> ALGHPL	<b>GCSGAR</b> VVVT	LLSILQ <b>Q</b> EGG	K...IGVAAIC		
Ctpaact	L...EKLNVY	<b>GGAV</b> AMGHPL	<b>GCSGAR</b> IIVT	LLSVLT <b>Q</b> EGG	R...FGVAGVC		
<b>Rscaact</b>	P...EKVNVN	<b>GGAV</b> SLGHPL	<b>GMSGAR</b> ILIT	LLGILK <b>K</b> RRG	K...YGVGGVC		
Hsmaact	P...QKVNIN	<b>GGAV</b> SLGHPI	<b>GMSGAR</b> IVGH	LTHAL <b>KQ</b> .G	E...YGLASIC		
Hsmaoact	I...SKTNVN	<b>GGAI</b> ALGHPL	<b>GSGSR</b> ITAH	LVHEL <b>R</b> RRGG	K...YAVGSAC		
Rnmoact	P...SKTNVS	<b>GGAI</b> ALGHPL	<b>GSGSR</b> ITAH	LVHEL <b>R</b> RRGG	K...YAVGSAC		
Aecoact	T...SKVNVN	<b>GGAI</b> AIGHPI	<b>GASGCR</b> ILVT	LLHEM <b>K</b> RRDA	K...KGLASLC		
Cacaact	M...NKVNVN	<b>GGAI</b> ALGHPI	<b>GASGAR</b> ILVT	LVHAM <b>Q</b> KRDA	K...KGLATLC		
Zrcaact	P...SIVNVN	<b>GGAI</b> AIGHPI	<b>GASGAR</b> ILNT	LLFEM <b>K</b> RRGA	R...KGLATLC		
Hscaact	P...EKVNIE	<b>GGAI</b> ALGHPL	<b>GASGCR</b> ILVT	LLHTL <b>R</b> MGR	S...RGVAALC		
Ctpoact	E...EKLNVN	<b>GGAI</b> ALGHPL	<b>GETGAR</b> QYAT	IIP <b>L</b> KPG..	Q...IGLTSMC		
Ylpoact	E...SKVNPR	<b>GGAI</b> AIGHPL	<b>GATGAR</b> QFAT	LLSEL <b>K</b> ESGK	K...YGVVSMC		
Hspoact	P...EKVNPL	<b>GGAV</b> ALGHPL	<b>GCTGAR</b> QVIT	LLNEL <b>K</b> RRGK	RA...YGVVSMC		
Rnpoact	A...EKNVPL	<b>GGAI</b> ALGHPL	<b>GATGAR</b> CVAT	LLHEM <b>K</b> RRGK	DCRFV <b>I</b> SMC		
Cspoact	P...EKVNVN	<b>GGAI</b> AIGHPL	<b>GATGAR</b> CVAT	LLHEM <b>K</b> RRGK	DCRFV <b>I</b> SMC		
Eccoact	EQID <b>E</b> KINLN	<b>GGAI</b> ALGHPL	<b>GCSGAR</b> ISTT	LLNL <b>M</b> ERKDV	Q...FGLATMC		
Sccaact	N <b>GGG</b> GASSIV	IEKI.....	.....	.....	.....		
Sucaact	N <b>GGG</b> GASSVV	IEKL.....	.....	.....	.....		
Ctpaact	N <b>GGG</b> GASAVV	IEKID <b>A</b> DAKL	.....	.....	.....		
<b>Rscaact</b>	N <b>GGG</b> GASALV	LEVV.....	.....	.....	.....		
Hsmaact	N <b>GGG</b> GASAML	IQKL.....	.....	.....	.....		
Hsmaoact	I <b>GGG</b> GIAVI	IQSTA.....	.....	.....	.....		
Rnmoact	I <b>GGG</b> GISLI	IQNTA.....	.....	.....	.....		
Aecoact	I <b>GGM</b> GVALA	VERK.....	.....	.....	.....		
Cacaact	I <b>GGG</b> GTAIL	LEKC.....	.....	.....	.....		
Zrcaact	I <b>GGM</b> GVAMC	IESL.....	.....	.....	.....		
Hscaact	I <b>GGM</b> GIAMC	VQRE.....	.....	.....	.....		
Ctpoact	I <b>SGM</b> GSASI	LVRE.....	.....	.....	.....		
Ylpoact	I <b>GTM</b> GAAVL	VVAE.....	.....	.....	.....		
Hspoact	I <b>GTM</b> GAAAV	FEYPGN.....	.....	.....	.....		
Rnpoact	I <b>GTM</b> GAAAV	FEYPGN.....	.....	.....	.....		
Cspoact	I <b>GTM</b> GAAAV	FEYDCVDEL	CNA <b>K</b> VEGGV	NLL <b>S</b> KDAR	.....		
Eccoact	I <b>GLG</b> GIIATV	FERV.....	.....	.....	.....		

**Figure 2.** Sequence homologies between thiolases at the C-terminal end. Amino acid residues that are common to all sequences are in boldface. Only full-length sequences have been considered. Using the DNASIS/PROSIS software from Hitachi, the nucleic acid sequence of cRS10 and the deduced amino acid sequence were used to search database libraries for homologies. Additional sequence analyses were performed with the BLAST network service, and sequence alignments were carried out with the PileUp program of the Genetics Computer Group sequence analysis software package. The numbering of amino acid residues corresponds to the radish AACT. The catalytically important Cys residue in this domain is marked by an asterisk (\*). Putative subcellular localization: c, cytosolic; m, mitochondrial; p, peroxisomal. Database accession numbers are given in parentheses. Ae, *Alcaligenes eutrophus* (J04987); Ca, *Clostridium acetobutylicum* (U08465); Cs, *Cucumis sativus* (X67696); Ct, *Candida tropicalis* (AACT, D13471; OACT, D17321); Ec, *Escherichia coli* (fadA gene, J05498); Hs, *Homo sapiens* (AACT, mitochondrial, D90228 and M61117; OACT, peroxisomal, X12966); Rn, *Rattus norvegicus* (AACT, mitochondrial, D00512; OACT, mitochondrial, X05341, and peroxisomal, D90063 and J05269); Rs, *Raphanus sativus* (X78116); Sc, *Saccharomyces cerevisiae* (L20428); Su, *Saccharomyces uvarum* (X07976); Yl, *Yarrowia lipolytica* (X69988); Zr, *Zoogloea ramigera* (J02631).

**Sequence Analysis**

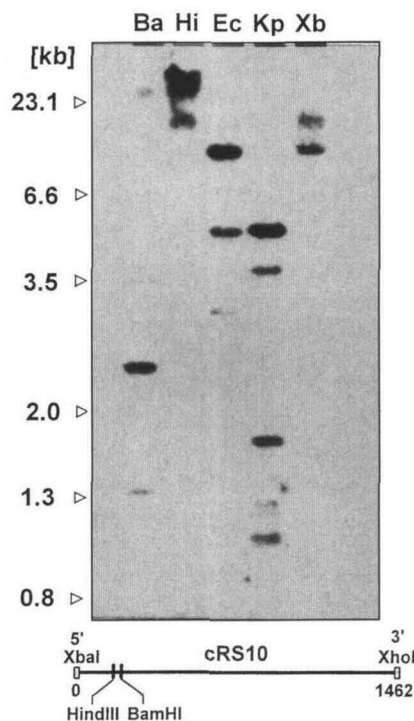
Sequence analysis of the 1462-kb insert of plasmid pYRS10 (EMBL accession no. X78116), which confers ergosterol autotrophy and thermoresistance to the yeast strain KV5 (*erg10*), revealed the existence of an open reading frame containing the entire coding unit for a protein of 406 amino acids ( $M_r$  42,032). There was no evidence of the presence of a leader peptide (Von Heijne et al., 1989; De Hoop and Ab, 1992), as in the peroxisomal OACT from cucumber (462 amino acids,  $M_r$  48,650). The precursor form of peroxisomal OACT was approximately 4 kD larger than the 45-kD subunit of the mature enzyme (Preisig-Müller and Kindl, 1993). At the amino acid level there exist significant sequence homologies (Fig. 2) (see Igual et al., 1992;

Kanayama et al., 1994; Mathieu et al., 1994; for additional refs. consult the accession numbers indicated in legend to Fig. 2) with domains of thiolases from mammals; from the yeasts *Saccharomyces uvarum*, *S. cerevisiae*, *Candida tropicalis*, and *Yarrowia lipolytica*; and from bacteria such as *Escherichia coli*, *Clostridium acetobutylicum*, and *Zoogloea ramigera* (Peoples et al., 1987). Homotetrameric (biosynthetic) thiolase from *Z. ramigera* has subunits of  $M_r$  40,598, in complete agreement with the value determined by SDS-PAGE (Davis et al., 1987).

There are two conserved Cys residues that have been recognized as being important for *Z. ramigera* thiolase activity (Thompson et al., 1989; Williams et al., 1992). One is located in the N-terminal section of the enzymes (presumably Cys<sup>91</sup> for radish AACT) and is involved in the formation of an acyl-enzyme intermediate already identified by Gehring and Harris (1970) using pig heart AACT. The other, located at the C-terminal extremity (corresponding to Cys<sup>390</sup> in radish AACT; see Fig. 2), is the active site base involved in deprotonation in the condensation reaction (see Thompson et al., 1989; Williams et al., 1992; Mathieu et al., 1994, for discussion and further literature citations). The hydrophathy profile of the deduced amino acid sequence of radish AACT, calculated according to Kyte and Doolittle (1982), exhibits the typical features of a soluble protein with internal hydrophobic core domains (not shown). The highly conserved Gly-rich domain in the C-terminal region (see Fig. 2) with the consensus sequence G<sub>355</sub>-G-A-I/V-S/V-L/I-G-H-P-I/L-G-X-S/T-G-X-R<sub>370</sub> (underlined and index numbers indicate the radish sequence) seems to be important for the stability of the thiolase protein (Fukao et al., 1991). Within this domain (radish AACT: L<sub>360</sub>-G-H-P-L-G-C<sub>366</sub>) we found a high similarity to a heptapeptide (five identical residues, two conservative exchanges) of human lecithin-cholesterol acyltransferase (EC 2.3.1.43), which has been discussed as forming an interfacial lipid binding site (McLean et al., 1986).

### Genomic DNA Gel Blot Hybridization Analysis

Genomic DNA gel blot hybridization (Fig. 3) with the radish cDNA (cRS10) suggests the presence of a single gene or a small family of genes. Only two bands were visible in the lanes containing *Bam*HI- and *Hind*III-digested DNA when hybridized under stringent conditions to the full-length cDNA probe. Because the cDNA sequence contains one internal restriction site each for *Bam*HI and for *Hind*III, and assuming that such restriction sites are not preserved in isogenes, this observation suggests a single-copy AACT gene in radish. Numerous bands in lanes in which DNA was digested with *Eco*RI, *Kpn*I, or *Xba*I and no internal restriction sites were present in the cDNA sequence most likely point to the occurrence of several introns within the radish AACT gene. When hybridizations were performed under less-stringent conditions, however, more signals could be detected in all lanes (not shown). This would be expected in view of the presence of strongly conserved regions in all AACT and OACT sequences isolated so far (see Kanayama et al., 1994; Mathieu et al., 1994). However, at this stage we would not exclude the presence of related



**Figure 3.** Gel blot analysis of genomic radish DNA. Genomic DNA (30  $\mu$ g each) was digested with *Bam*HI (Ba), *Hind*III (Hi), *Eco*RI (Ec), *Kpn*I (Kp), and *Xba*I (Xb). Electrophoresis was done in 0.8% agarose gels. As a probe the complete 1.46-kb *Xba*I/*Xho*I cDNA fragment of cRS10 was used, previously DIG-11-UTP-labeled by the random priming method.

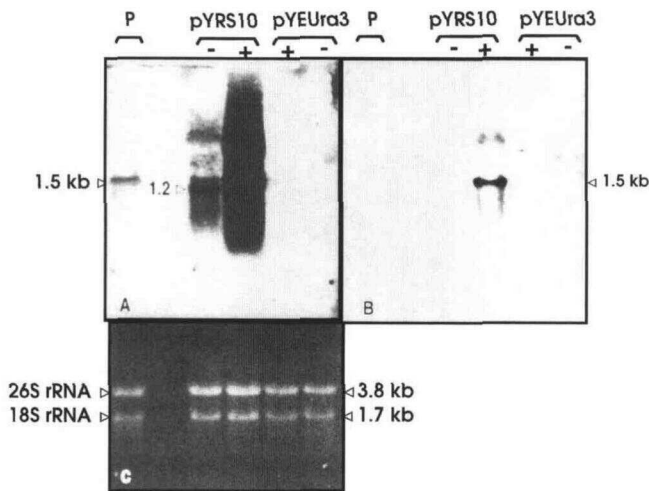
genes encoding isozymes with differential intracellular localization and biochemical function.

### Heterologous Expression in Yeast

Gel blot analysis of RNA isolated from yeast transformant strain KV10 and parental strain KV5, transformed with the plasmid without the cDNA insert (Fig. 4), reveals a strong overexpression of radish cDNA cRS10 at the transcriptional level and its dependence on Gal induction (Fig. 4, A and B). Exposure of the film for 2 h led to the masking of a distinct band and to a positive signal over the whole separation range of the agarose gel. After exposure for only 20 min, a prominent band of about 1.5 kb appeared, with total RNA isolated from KV10 exclusively under inducing conditions (Fig. 4B). After longer exposure an additional transcript of approximately 1.2 kb became visible with RNA isolated from KV10 under *GAL*1 promoter-repressing conditions, namely in the presence of Glc (Johnston and Davis, 1984). Since it could not be detected in the lane with RNA from parental strain KV5 transformed with the vector pYEUra3, a further transcript must have been read from cRS10, which, however, was not controlled by the *GAL*1 promoter.

### Measurement of Enzyme Activity in Yeast

For the assay of AACT in cell-free yeast extracts, we adopted the method of Weber and Bach (1994), which



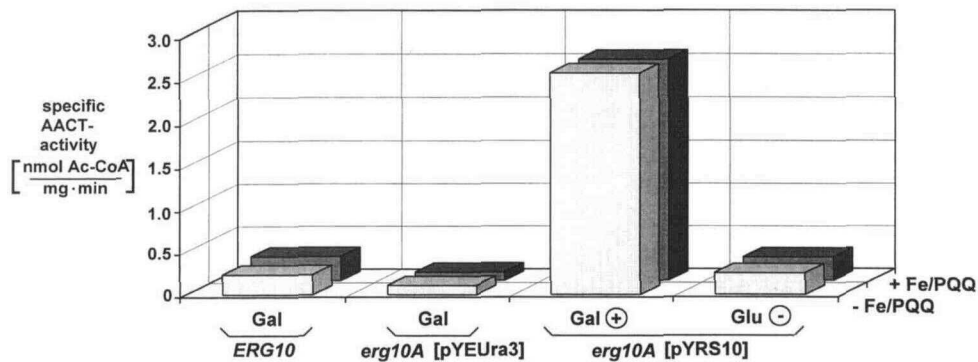
**Figure 4.** Transcription of radish cDNA cRS10 in yeast transformant KV10. Gel blot analysis of total RNA (10  $\mu$ g) from yeast cells grown under inducing (+, 2% Gal) and noninducing (–, 2% Glc) conditions. KV10, (*erg10A* [pYRS10]) containing the radish AACT coding unit; KV5p, (*erg10A* [pYEura3]) plasmid without insert as a control; P, RNA (10  $\mu$ g) from 4-d-old etiolated radish seedlings for comparison; A, Exposition time 3 h; B, exposition time 20 min; C, ethidium bromide staining of the 1.2% agarose gel after electrophoresis (rRNA bands are of similar intensity). As a probe the complete 1.46-kb *Xba*I/*Xho*I cDNA fragment of cRS10 was used, which was DIG-11-UTP-labeled by the random priming method.

determines the combined activity of AACT and HMGS. When the yeast strain KV10 carrying the plasmid with the gene encoding radish AACT was grown on Glc minimal medium (supplemented with 4 mg/L ergosterol in liquid broth) at the permissive temperature of 26°C, there was practically no AACT activity measurable in total cellular protein extracts prepared using a detergent (Basson et al., 1986) (Fig. 5). As expected, wild-type yeast showed a similar AACT/HMGS activity when grown on either a Glc or a Gal medium. In strain KV10 expression of radish AACT

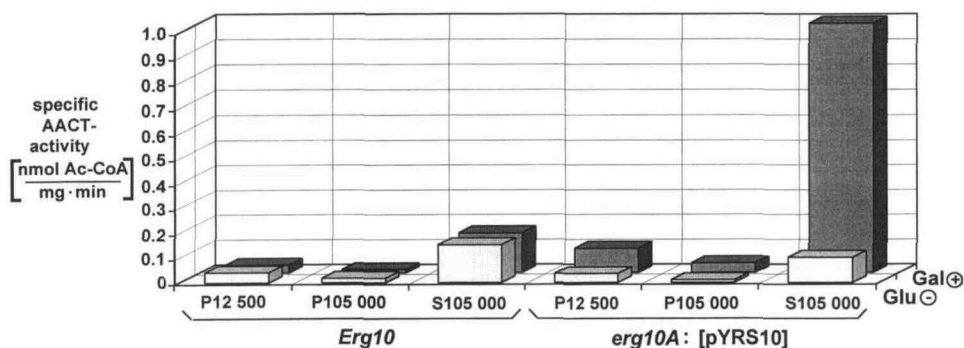
led to an 8- to 10-fold increase of apparent AACT/HMGS activity over that of the wild type (Fig. 5). In the presence of 1233A (also called F244 or L659,699), a potent inhibitor of HMGS (Greenspan et al., 1987; Tomoda et al., 1987), this activity was completely blocked (data not shown). The ergosterol-auxotrophic strain used for transformation apparently was slightly leaky, with about 20% of wild-type activity, as was shown by Dequin et al. (1988b) using strain F2sp5 (*erg10A*). Addition of PQQ and Fe(II), cofactors that stimulated the AACT/HMGS enzyme system solubilized from radish membranes (Weber and Bach, 1994), had no effect (Fig. 5). When yeast cell homogenates were subfractionated and assayed for AACT/HMGS, it was clearly revealed that specific enzyme activity was associated with the cytosolic fraction, but not with resuspended membrane pellets (Fig. 6). This is in clear contrast to the intracellular localization of two intact radish HMGR isozymes expressed in a mevalonate-auxotrophic yeast mutant originally generated by Basson et al. (1988), in which enzyme activity was exclusively associated with membranes (Vollack et al., 1994). The degree of overexpression in measurable enzyme activity was not as high as expected from the RNA blot analysis, but was still about 10 times higher than the values found in protein extracts from wild-type yeast. Similar values have been found in yeast cells by overexpression of the *ERG10* gene from *S. uvarum* cloned on a multi-copy 2- $\mu$ m plasmid (Dequin et al., 1988a).

#### Expression of AACT in Radish

RNA gel blot analysis of total RNA isolated from etiolated, intact radish seedlings (from d 2–8 after germination) indicated the presence of a 1.5-kb transcript, with little or no change in intensity (Fig. 7). In comparison, the same RNA samples were hybridized to two probes specifically recognizing radish *HMG1* (EMBL accession no. X68651, nucleotides 500–521) and *HMG2* (EMBL accession no. X68652, nucleotides 464–484). (The nomenclature we used for the characterization of HMGR-encoding genes in radish

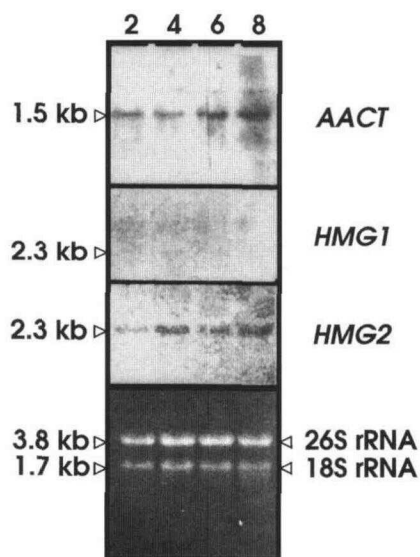


**Figure 5.** AACT activity in total protein extracts from yeast: strain KV10 (*erg10A* [pYRS10]); strain KV5p (*erg10A* [pYEura3]); and strain W303-1B (*ERG10*). Activity was measured by incorporation of [ $^{14}$ C]acetyl-CoA into heat-stable and acid-stable HMG acid via HMG-CoA (see Weber and Bach, 1994, for details). Medium: YNB [minimal medium; 0.17% yeast nitrogen base, 0.5% ( $\text{NH}_4$ ) $_2$ SO $_4$ , 50  $\mu$ g/mL His, adenine, Len, 4  $\mu$ g/mL ergosterol] + His + adenine + Leu, 4  $\mu$ g/mL ergosterol. Gal, 2% Gal; Glu, 2% Glc; temperature, 28°C. The tests were also performed in the presence of 25  $\mu$ M PQQ plus 50  $\mu$ M Fe(II), conditions that were described as stimulating the *in vitro* activity of the coupled enzyme system AACT/HMGS in extracts from radish membranes. Note that no difference is visible.



**Figure 6.** AACT activity in subcellular fractions isolated from transformed yeast. Homogenates were prepared from strain KV10 (*erg10A* [pYRS10]) and from wild-type strain W303-1B. Subcellular fractions were isolated by means of differential centrifugation at 12,500g (organelle fraction) and at 105,000g (microsomes). Soluble proteins were found in the following supernatant (S105 000). Medium: YNB [minimal medium; 0.17% yeast nitrogen base, 0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 μg/mL His, adenine, Leu, 4 μg/mL ergosterol] + His + adenine + Leu, 4 μg/mL ergosterol. Gal, 2% Gal; Glu, 2% Glc. Temperature during culture was 28°C (permissive conditions).

depended on the sequence of their cloning). *HMG2* is expressed to a considerable degree from d 2 to 8 of germination, whereas the signals for *HMG1* were much weaker. When we examined gene expression in different parts of 6-d-old etiolated seedlings, the 1.5-kb signal was found with RNA isolated from roots, stems, and, to some extent, from cotyledons of radish seedlings, suggesting a more or less constitutive expression of the corresponding AACT gene in all major parts (Fig. 8A). However, the signal intensity was higher with RNA isolated from cotyledons of 6-d-old light-grown seedlings than from etiolated seedlings

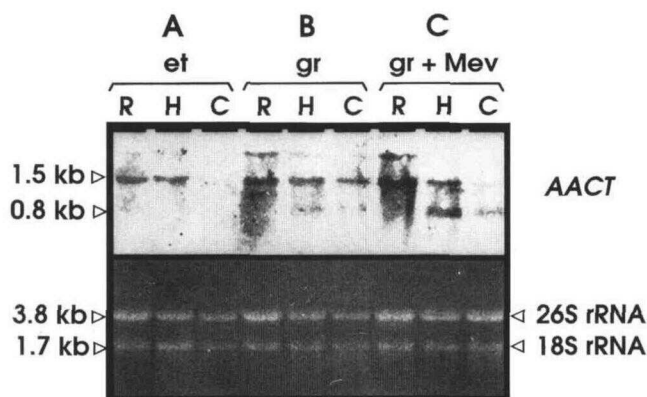


**Figure 7.** AACT gene expression in etiolated radish seedlings during germination. For RNA gel blot analysis, total RNA was isolated from seedlings from d 2 to 8 after germination in darkness. Ten micrograms of RNA each were hybridized to the 1.4-kb cDNA cRS10, which was labeled with DIG-11-dUTP by random priming. For comparison, the same RNA samples were hybridized to a 22-mer specifically recognizing radish *HMG1* and to a 21-mer recognizing *HMG2*. Oligonucleotides were end-labeled with DIG-11-dUTP. Bottom, Ethidium bromide-stained 1.2% agarose gel with rRNA bands.

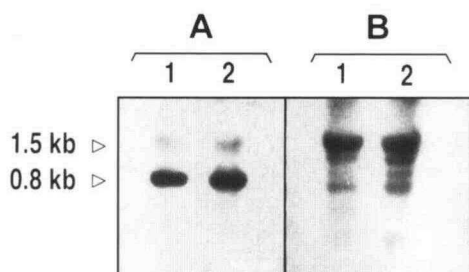
(Fig. 8B). In a further experiment, seedlings were grown for 6 d in the light in the presence of 10 μM mevinolin, a specific inhibitor of HMGR and, hence, of de novo phytoosterol biosynthesis (Bach and Lichtenthaler, 1983). In the RNA gel blot we noted the appearance of an additional transcript of about 800 bp with RNA isolated from hypocotyls and to some extent with RNA from cotyledons (Fig. 8C)

When we looked for a possible explanation for the appearance of another transcript from cRS10 we noted that on the noncoding strand we could identify putative TATA box-like elements that could give rise to the formation of antisense transcripts (the numbering corresponds to positions on the coding strand): (1) T<sub>1273</sub>ATAAAT<sub>1267</sub>; (2) T<sub>1299</sub>ATATAG<sub>1293</sub>; (3) T<sub>1345</sub>ATCAAAA<sub>1339</sub>; and (4) T<sub>1416</sub>ATAATA<sub>1410</sub>.

Therefore, we used strand-specific DIG-labeled RNA probes for the detection of putative sense and antisense



**Figure 8.** Tissue-dependent expression of radish AACT. RNA gel blot analysis of total RNA (10 μg each) isolated from roots (R), hypocotyls (H), and cotyledons (C) of 6-d-old radish seedlings cultivated in darkness (A, et), in light (B, gr), or in light in the presence of 10 μM mevinolin (C, gr + Mev). The light/dark cycles were 14 and 10 h, respectively. Light intensity was 500 μmol m<sup>-2</sup> s<sup>-1</sup>. Room temperature (25°C) and humidity (80%) were kept constant. Hybridization was as in Figure 7.



**Figure 9.** Sense- and antisense-specific detection of mRNA isolated from 6-d-old light-grown radish seedlings. Seedlings were grown on water (lanes 1, control) or on water in the presence of 10  $\mu$ M mevinolin (lanes 2). The light and culture conditions were as in Figure 8. Antisense (A) and sense mRNA species (B) were detected with DIG-UTP-labeled, single-strand-specific RNA probes using pcRS10 (derivative of pBluescript SK) as the cDNA template. T3 RNA polymerase generated the antisense-specific RNA probe, and T7 RNA polymerase generated the sense-specific probe.

transcripts in radish (Fig. 9), using 6-d-old light-grown radish seedlings cultivated on water (control) and on 10  $\mu$ M mevinolin. The antisense-specific probe (Fig. 9A) specifically hybridized to a band of approximately 0.8 kb. The signal arising from RNA isolated from mevinolin-treated light-grown seedlings (Fig. 9A, lane 2) was slightly more intense. The sense-specific probe (Fig. 9B) turned out to hybridize to the 1.5-kb transcript. The use of DIG-labeled RNA probes resulted in a sensitivity of RNA detection that was at least 10 times higher than that resulting from DIG-labeled cDNA probes. Some weak signals in experiment B might have resulted because total RNA was used and in some unspecific hybridization occurred.

## DISCUSSION

Several lines of evidence indicate that we have successfully cloned a cDNA containing the entire coding unit for a radish AACT, resulting in a protein of  $M_r$  42,032. There are significant sequence homologies to other eukaryotic and prokaryotic thiolase genes, but clearly more are related to AACT than to OACT encoding genes. A strong argument is the functional expression in yeast, and thereby complementation of the *erg10* mutation. Activity, determined as conversion of acetyl-CoA to HMG-CoA in the presence of AACT and endogenous HMGS, can be found only in the soluble fraction but not in membrane pellets isolated from yeast transformants. This and the complete lack of a typical transit peptide characteristic of glyoxysomal OACT recently cloned from cucumber (Preisig-Müller and Kindl, 1993), which the radish gene resembles significantly, but much less than cytosolic AACT from *S. uvarum* (Dequin et al., 1988b) or from *S. cerevisiae* (Hiser et al., 1994), led us to conclude that we have indeed cloned the first plant cDNA encoding cytosolic (biosynthetic) AACT.

Recently, a membrane-associated enzyme system was purified from radish, catalyzing the conversion of acetyl-CoA into a heat-stable and acid-stable product considered to contain HMG-CoA, which was thus referred to as AACT/HMGS. Its apparent *in vitro* activity is greatly stimulated in the presence of Fe(II) and a quinone cofactor

(Weber and Bach, 1994). However, when we used the same assay conditions, namely determination of [2- $^{14}$ C]acetyl-CoA incorporation in the presence of Fe(II) and PQQ as the most efficient quinone cofactor found so far, we could not observe any significant stimulation of enzyme activity with cell-free extracts of transformed yeast. From these observations we conclude that the product of the cloned gene is not identical to the membrane-associated AACT/HMGS system described before (Weber and Bach, 1994). Cloning of this radish AACT gene and of an Arabidopsis HMGS gene (Montamat et al., 1996) provides convincing evidence for the presence of separate AACT and HMGS enzymes in plant cells, although they seem to behave similarly during various purification steps (Alam et al., 1991; Van der Heijden et al., 1994).

Recently, Mathieu et al. (1994) published the crystal structure of peroxisomal OACT from *S. cerevisiae* refined at 2.8-Å resolution. Apparently, the homodimeric unliganded yeast OACT comprises three domains: two compact core domains that have the same fold and a loop domain. Each monomer contains an identical core domain: that is folded into a mixed, five-stranded  $\beta$ -sheet covered by helices on each side and assembled into a five-layered  $\alpha\beta\alpha\beta\alpha$  structure. Although peroxisomal OACT proteins isolated from seven organisms form a much closer family (152 identical residues out of 417) than the 40 identical residues found in all 21 thiolase sequences in databases (Mathieu et al., 1994), it is reasonable to assume that the three-dimensional structures, especially around the pocket of the active center shaped by highly conserved residues close to the C-terminal end, are being conserved in the same way. However, a final answer awaits the crystallization and x-ray analyses of plant AACT protein.

Gal-induced expression of radish AACT in the yeast *erg10* mutant reestablishes the wild-type phenotype, namely growth at 37°C and ergosterol autotrophy. Repression by Glc of the *GAL1* promoter results in the phenotype of the conditionally lethal *erg10* mutation. A surprising characteristic of strain KV10 (carrying pYRS10) when it was cultivated on Glc medium at 37°C with a high selection pressure was the complete absence of so-called "revertants," which are typical of the *erg10A* mutant (strain KV5; see also Dequin et al., 1988b). The revertants were observed more rarely in cells grown at the restrictive temperature of 37°C on a Gal medium than on a Glc medium. This might be explained by the fact that the cells used for these experiments had originally been taken from Glc plates and, as a consequence, had adapted to Glc as a carbon source. Even under restrictive "heat stress" at 37°C, the freshly plated Glc-adapted *erg10A* cells were able to convert Glc into energy, whereas on Gal an energy-consuming adaptation to the Gal metabolism was required. This lack of energy, together with the blocking of the isoprenoid biosynthesis, would lead to an immediate growth arrest on Gal, whereas on Glc some additional cell divisions would be possible, with a certain probability of back mutation.

Gel blot analysis of RNA isolated from pYRS10 transformants in which the *GAL1* promoter was induced showed a strong signal for a 1.5-kb transcript, which does not appear



in RNA isolated from control yeast cells (strain KV5 carrying the parental plasmid pYEUra3). However, under Glc-repressed conditions (Johnston and Davis, 1984) we noted the appearance of a smaller hybridizing transcript (about 1.2 kb). Since no signal was obtained with control cells, the only conclusion that could be drawn was that this transcript arose from a transcript of the radish cDNA cRS10, which had not been controlled by Glc repression of the *GAL1* promoter. Analysis of the nucleotide sequence of cRS10 in the downstream, nontranslated region showed the presence of TA-rich nucleotide stretches comprising several TATA-box elements on the complementary strand. In particular, the first TATA box (positions 1273–1267) corresponds exactly to the consensus sequence, which determines the transcription starting point in yeast (Hahn et al., 1985) and in other eukaryotes (Dyran and Tijan, 1985). If only one of the putative TATA elements on the antisense strand were recognized by the yeast's transcription initiation factors and, thus, the transcription of an antisense mRNA were initiated, this could lead to the hybridization with rather well-conserved transcripts of the mutated but still transcribed yeast *erg10A* allele. For yeast cells, the result would be something like a gene disruption.

In terms of measurable enzyme activity, we arrived at an overexpression of AACT about 8 to 10 times higher than the wild-type values. This observation corresponds well with that made by Dequin et al. (1988a), who overexpressed AACT from *S. uvarum* in a yeast *erg10* strain. These authors used an assay system that measures thiolase activity exclusively in the cleavage direction, which is thermodynamically favored. In our coupled AACT/HMGS assay, in which the HMGS has to arise from the yeast HMGS gene, such an apparent increase in enzyme activity can be interpreted in such a way, at least in *S. cerevisiae*, that AACT appears to catalyze the rate-limiting step for the two-step conversion of acetyl-CoA to HMG-CoA.

Overexpression of cytosolic AACT in yeast has no apparent negative effect on the growth behavior of its cells. This has already been shown with yeast overexpressing the AACT gene isolated from *S. uvarum* (Dequin et al., 1988a) or for yeast overexpressing other enzymes downstream on the sterol pathway, mevalonate kinase (Riou et al., 1994) and phosphomevalonate kinase (Tsay and Robinson, 1991). From the results obtained by Dequin et al. (1988a) it seems clear that overexpression of AACT does not significantly affect the sterol pattern nor its total content. Thus, other enzymatic steps downstream on the pathway must be responsible for substrate flow regulation from acetyl-CoA to ergosterol. On the other hand, a complete knockout of the yeast *ERG10* gene is lethal (Servouse et al., 1984; Hiser et al., 1994). Only thermolabile *erg10* mutants could be isolated; these grow at the permissive temperature of 26°C but not at 36°C (Servouse et al., 1984). It is noteworthy that the original *erg10* mutant exhibits about 20% of wild-type AACT activity (Dequin et al., 1988b), which indicates that the gene is not completely inactive. The product of the mutated gene could therefore exhibit a lower catalytic efficiency and/or a higher thermosensitivity. Such an amino acid exchange in the catalytic center has been demon-

strated for the *erg20* mutation of yeast farnesyl pyrophosphate synthase (Blanchard and Karst, 1993). For instance, the difference in the heat stability of the sweet protein mabinlin is a result of the replacement of a single amino acid residue (Nirasawa et al., 1994). The lethality of a complete lack of AACT might indicate a further role of this enzyme independent of its inclusion in the sterol pathway, e.g. in the biosynthesis of pre-squalene products that are essential for cell-cycle regulation.

Recently, an AU-rich RNA binding protein has been isolated from the flesh fly *Sarcophaga peregrina*, and this protein has subsequently been identified as a thiolase (Nanbu et al., 1993). The partial amino acid sequences of two peptides obtained from the 39-kD protein showed high similarities to rat and yeast OACT and in fact exhibited thiolase activity. Since rat mitochondrial OACT showed affinity to the AU-rich RNA, this RNA-binding activity might be an intrinsic character of thiolase (Nanbu et al., 1993). It is tempting to assume that in this way thiolase protein could somehow down-regulate its own synthesis once sufficient amounts have been formed. The presence of AU-rich regions in the untranslated, downstream region of our cDNA clone could possibly match such a requirement.

Especially when radish seedlings were grown in the presence of the HMGR inhibitor mevinolin, RNA blot analysis using DIG-labeled cDNA revealed the appearance of a hybridizing band of approximately 800 bp. Under such conditions, namely blockage of de novo phytosterol biosynthesis (Bach and Lichtenthaler, 1983), this might indicate some stress response of the plant that remains to be further characterized. This additional 800-bp band had already been barely visible with RNA isolated from control seedlings. It was initially interpreted as demonstrating the presence of a gene bearing considerable sequence homology to the AACT gene. However, this could be ruled out in view of the results from the genomic DNA gel blot analysis. It is also possible that we might have obtained an additional transcript that arises from alternative splicing. The above-mentioned TATA-box elements in the complementary strand might also give rise to another transcript. In a preliminary experiment, we were able to demonstrate the presence of a putative antisense transcript by strand-specific hybridization to DIG-labeled RNA. Synthesis of an AACT antisense mRNA would provide the means for a down-regulation of AACT activity, which could be rapidly reversed due to the small lifetime of RNA-RNA duplex hybrids (see Inouye, 1989). Such a mechanism, including the synthesis of antisense mRNA for in vivo expression, was described for  $\alpha$ -amylase from barley (Rogers, 1988; see also Mol et al., 1990). However, additional research, including characterization of corresponding genomic clones and high-resolution mapping of putative sense and antisense RNAs, is needed to clarify these questions.

#### ACKNOWLEDGMENTS

We thank Mrs. Sabine Zeiler for her expert technical assistance and Dr. Manfred Focke for support and stimulating discussions. We are especially grateful to Prof. Francis Karst for providing us with an original *erg10A* yeast strain and for introducing K.-U.V. to

the techniques of yeast genetics during a short research stay in his laboratory at the University of Poitiers. We are indebted to Dr. Michael Greenspan (Merck Research Laboratories, Rahway, NJ) for a generous gift of the inhibitors L659,699 (F244) and mevinolin. Thanks are due to the two anonymous referees for helpful suggestions. We thank Mr. John Ackerson for critically reading the English text.

Received March 12, 1996; accepted April 29, 1996.

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The EMBL accession number for the sequence described in this article is X78116.

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