review

A second gene for peroxisomal HMG-CoA reductase? A genomic reassessment

Rainer Breitling and Skaidrite K. Krisans[1]

Department of Biology, San Diego State University, San Diego, CA

Abstract HMG-CoA reductase (HMGCR) catalyzes the conversion of HMG-CoA to mevalonate, the rate-limiting step of eukaryotic isoprenoid biosynthesis, and is the main target of cholesterol-lowering drugs. The classical form of the enzyme is a transmembrane-protein anchored to the endoplasmic reticulum. However, during the last years several lines of evidence pointed to the existence of a second isoform of HMGCR localized in peroxisomes, where mevalonate is converted further to farnesyl diphosphate. This finding is relevant for our understanding of the complex regulation and compartmentalization of the cholesterogenic pathway. Here we review experimental evidence suggesting that the peroxisomal activity might be due to a second HMGCR gene in mammals. We then present a comprehensive analysis of completely sequenced eukaryotic genomes, as well as the human and mouse genome drafts. Our results provide evidence for a large number of independent duplications of HMGCR in all eukaryotic kingdoms, but not for a second gene in mammals. We conclude that the peroxisomal HMGCR activity in mammals is due to alternative targeting of the ER enzyme to peroxisomes by an as yet uncharacterized mechanism.—Breitling, R., and S. K. Krisans. **A second gene for peroxisomal HMG-CoA reductase? A genomic reassessment. *J. Lipid Res.* 2002.** 43: **2031–2036.**

Supplementary key words genome duplication • HMG-CoA reductase isozymes • alternative subcellular localization • cholesterol metabolism

Subcellular distribution of classical HMG-CoA reductase

Hydroxymethyl-glutaryl-CoA reductase (HMGCR) catalyzes the conversion of hydroxymethyl-glutaryl-CoA to mevalonate (EC 1.1.1.34) or vice versa (EC 1.1.1.82; in mevalonate-feeding bacteria). In eukaryotes, HMGCR catalyzes the rate-limiting reaction of isoprenoid biosynthesis and is the main target of the favorite cholesterol-lowering drugs, the statins (1, 2). HMGCRs form a large family of proteins subdivided into two classes, one found in archaea+eukaryota (class I) and the other in bacteria (class II), with some cases of lateral gene transfer between the domains (3). Eukaryotic HMGCR is an eight-transmembrane span protein with a cytosolic catalytic domain anchored to the endoplasmic reticulum (ER) (4–7). A divergent soluble glycosome-specific form of HMGCR is present in the kinetoplastid *Trypanosoma cruzi* (8). Two homologous isoforms of the enzyme are found in several organisms, and in the case of yeast are reported to have complementing functions in specialized subregions of the ER (9).

Over the course of the last decade, our laboratory has presented evidence indicating that mammals also have a second organelle-specific HMGCR that is restricted to the matrix of peroxisomes. HMGCR activity was first detected in rat liver peroxisomes by immunoelectron microscopy and enzyme activity assays on purified peroxisomes (10). The activity in peroxisomes was less than 5% of the total HMGCR in the cell under control conditions, but reached up to 30% after treatment with cholestyramine (11). The presence of significant amounts of peroxisomal HMGCR has been confirmed by other groups (12, 13) and in other tissues and organisms [hamster ovarian epithelium (CHO cells) (14–16); rat brain (17)].

Evidence for a second, peroxisome-specific HMGCR. During the detailed examination of the peroxisomal HMGCR activity, a number of observations indicated that the peroxisomal HMGCR may be coded by a second independent gene.

Soon after the first detection of HMGCR in peroxisomes, Keller et al. (11) showed that cholestyramine treatment produced a 6- to 7-fold increase in the specific activity of peroxisomal HMGCR, whereas the microsomal HMGCR specific activity increased only by about 2-fold. They determined that between 20% and 30% of the total HMGCR activity is located in the peroxisomes of cholestyramine-treated animals compared with less than 5% of the HMGCR activity detected in peroxisomes under control conditions. They also demonstrated that this increase in activity is paralleled by an increase in immunolabeling of peroxisomes by HMGCR antibodies. While the different extent of upregulation could indicate that two

Manuscript received 15 July 2002, in revised form 17 July 2002, and in re-revised form 8 August 2002.

Published, JLR Papers in Press, August 16, 2002.
DOI 10.1194/jlr.R200010-JLR200

Abbreviations: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; HMGCR, HMG-CoA reductase.

[1] To whom correspondence should be addressed.
e-mail: skrisans@sunstroke.sdsu.edu

genes are involved, the immunoelectron microscopy data (with different antisera) showed that the peroxisomal protein is at least very similar to the ER HMGCR.

Rusnak and Krisans (18) extended the observations by Keller et al. (11) by showing that the activity (and immunoreactivity) of HMGCR in ER and peroxisomes has different diurnal rhythms. All of these initial observations (and also those of Appelkvist and Kalen, 12) came from normal and cholestyramine-treated rat liver cells. None of them is mandatory evidence for a second HMGCR gene and none of them was interpreted as such in the original publications.

Engfelt et al. (15) showed that HMGCR purified from rat liver peroxisomes is smaller than the ER enzyme (about 90 kDa vs. 97 kDa), but stated that the separation of the two forms by SDS polyacrylamide gel electrophoresis "requires specific conditions" (a high amount of protease inhibitors and high concentrations of urea, SDS, and MeSH). It is possible that the smaller size is the result of a highly specific, uninhibited proteolysis. This is difficult to reconcile with the demonstrated lack of a precursor-product relationship between the two forms (see below), and hence Engfelt et al. (15) for the first time explicitly postulate the existence of a second gene for the peroxisomal enzyme. However, their results could also be explained if processing and targeting proceed co-translationally, although this would require a very unusual mechanism.

Engfelt et al. (14, 15) used an alternative system, mutated CHO cells with a severe HMGCR deficiency (UT2 cells). These cells completely lack ER HMGCR, but still retain a small amount of HMGCR activity, which was suggested to represent the production of a second isozyme that is not detectable in wild-type cells (19). When maintained in medium without mevalonate, HMGCR enzymatic activity in these cells is increased and peroxisomal HMGCR becomes detectable by cell fractionation and immunoblotting (the cells are then called UT2* to distinguish them from the original cells in mevalonate complemented medium). This protein is slightly smaller than the usual ER enzyme (90 kDa vs. 97 kDa, comparable to the rat liver peroxisomal enzyme) but is nonetheless highly similar to ER HMGCR. It shows a cross-reaction with a monoclonal antibody against ER HMGCR as well as with polyclonal antisera against the whole protein or specific regions (cytosolic domain, Arg440-Ala888; C-terminus, Ile874-Ala888; peptide G, Arg224-Leu242; peptide H, Thr284-Glu302). The last two antibodies have been shown to detect epitopes on different sides of the membrane (7). In addition, peroxisomal HMGCR has highly similar saturation curves for HMG-CoA and NADPH and shows almost the same pH optimum. The increase of HMGCR activity in UT2* cells is accompanied by a strong upregulation of HMGCR mRNA levels as detected by Northern blot. However, all of the various transcripts cloned by Engfelt et al. (14) were splice variants of a mutated HMGCR allele and none of them could code for the 90 kDa protein found in peroxisomes. Northern blots probed with exon 11, which is absent in the mutated message, did not show intact transcripts in the UT2* cells. No experimental explanation for the presumed transcriptional silencing of the wild-type allele that is also present in the UT2* cells was provided, but Southern blotting showed that both alleles are present in their normal genomic context. In addition, the presence of the smaller protein in "normal" CHO cells was demonstrated, and a pulse-chase experiment showed that there is no precursor-product relationship between the two forms of HMGCR. Unfortunately, the UT2* system is highly artificial, which makes a detailed interpretation of the results very hard. Some of the unresolved issues are especially relevant for the question of whether a second HMGCR gene is indeed responsible for the observed changes in UT2 cells after mevalonate withdrawal: why and how is the second allele of the classical HMGCR silenced? How does mevalonate withdrawal cause a stable [inheritable? see (16)] change in HMGCR expression? Do the cloned transcripts really represent the complete diversity of existing transcripts, given that so many different splice variants are observed in UT2* cells, although the mutations are localized and well defined? The data clearly suggest the existence of a second gene and demonstrate again that peroxisomal HMGCR is very similar to the ER enzyme.

Aboushadi et al. (16) provide additional circumstantial evidence (all experiments in UT2* cells) for a second peroxisome-specific gene for HMGCR.

They show that the peroxisomal protein is not phosphorylated and its activity not altered by phosphorylation inhibitors. Degradation of the peroxisomal enzyme is less accelerated by mevalonate, its degradation is not blocked by *N*-acetyl-leu-leu-norleucinal, and it is much less sensitive to inhibition by statins; but peroxisomal activity is decreased by 25-OH-cholesterol and upregulated by LPDS medium (15), as is the ER enzyme.

Again, these data do not necessarily indicate a second gene, but can also be explained by differences of conformation and accessibility in the peroxisomal compartment and small differences in the protein sequences due to point mutations and/or the larger difference indicated by the size discrepancy; e.g., difference in susceptibility toward mevalonate might be explained by the localization of the peroxisomal enzyme in the organellar matrix (instead of being attached to the membrane). Accessibility to the ubiquitin pathway and to specific kinases will also be different for a protein inside the peroxisomal matrix than for one localized in the ER.

In the meantime, we and others have accumulated consistent evidence for an almost exclusively peroxisomal activity of several enzymes immediately downstream of HMGCR in the isoprenoid biosynthesis pathway, including the identification of functional peroxisomal targeting signals in several of the enzymes (**Fig. 1**) (20). In addition, minor levels of HMG-CoA synthase activity and immunoreactivity have also been detected in peroxisomes (21). However, all attempts to clone the peroxisomal HMGCR from UT2* cells by yeast complementation, expression library screens, or affinity purification/microsequencing have been unsuccessful (Krisans et al., unpublished observations), and there still is no conclusive evidence for or against a second isoform of HMGCR in humans. Therefore, we decided to re-investigate this issue by a comprehensive analysis of the recently published complete

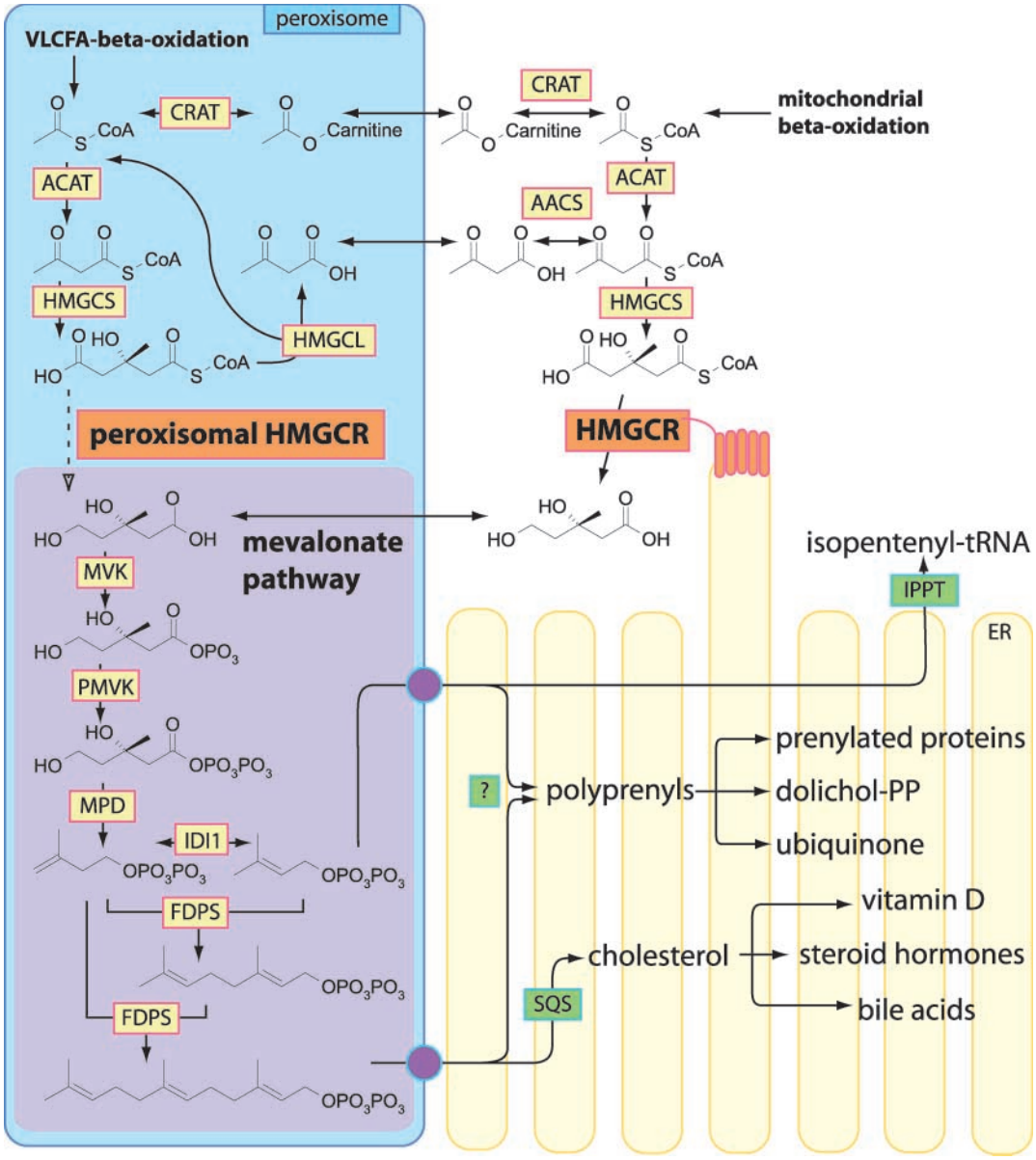

Fig. 1. Current view of the subcellular localization of isoprenoid biosynthesis in mammalian cells. Reactions between mevalonate and farnesyl diphosphate are assumed to be almost exclusively peroxisomal (purple shading). Several reactions downstream of farnesyl diphosphate are omitted for clarity. Enzyme names are abbreviated as follows: AACS, acetoacetyl-CoA synthase; ACAT, acetoacetyl-CoA lyase; CRAT, carnitine acetyltransferase; FDPS, farnesyl diphosphate synthase; HMGCL, HMG-CoA lyase; HMGCR, HMG-CoA reductase; HMGCS, HMG-CoA synthase; IDI1, isopentenyl diphosphate isomerase; IPPT, isopentenyl tRNA transferase; MPD, mevalonate diphosphate decarboxylase; MVK, mevalonate kinase; PMVK, phosphomevalonate kinase; SQS, squalene synthase; VLCFA, very long chain fatty acid.

genome sequences of several eukaryotes, as well as the mammalian genome drafts for human and mouse.

A GENOMIC SURVEY FOR HMGCR GENES IN MAMMALS AND BEYOND

Searching the mammalian genome databases (**Table 1**) with the protein sequence of human HMGCR revealed only a single HMGCR gene in all three genomes, i.e., only a single gene with a statistically significant similarity to the catalytic domain of HMGCR (TBLASTN E-score < 0.001). The human gene is localized at 5q13.3-q14.3 and the rodent homologs are found in the corresponding regions of conserved synteny at chromosome 13, 49cM from the centromere (mouse), and 2q14-q16 (rat). A small fragment of a processed pseudogene of HMGCR in the subtelomeric region of the long arm of human chromosome 19 codes for a peptide highly similar to about 50 amino acids of the catalytic domain of HMGCR. This peptide is, however, disrupted by a frameshift and a nonsense mutation, and no detectable similarity to other highly conserved regions of HMGCR is found in the neighboring regions of the genome. The EST databases contained 75 expressed sequence tags for the human gene and 36 hits for the mouse gene, all of them pertaining to the canonical gene

TABLE 1. Mammalian genome datasets

	Number of Sequences	Number of Nucleotides	Estimated Coverage[a]
Human genome contigs	2044	2,888,140,301	97.8% (http://www.ncbi.nlm.nih.gov/genome/seq/)
Human high-throughput genomic sequences	42,615	5,549,024,645	
Celera genome scaffold	483,997	3,279,602,164	
Mouse genome MGSCV3 supercontigs	42,620	2,590,790,854	96% (http://www-genome.wi.mit.edu/media/press/pr_mousegenome.html)
Mouse high-throughput genomic sequences	13,143	1,629,483,184	
Rat high-throughput genomic sequences	13,493	1,838,188,714	62% (http://www.hgsc.bcm.tmc.edu/projects/rat/coverage.html)
Takifugu rubripes genome contigs	12,403	332,496,529	90% (http://fugu.hgmp.mrc.ac.uk/News/FuguWebsite_17_05_02.html)
Tetraodon nigroviridis genome survey sequences	108,177	318,680,599	83% (http://www.genoscope.cns.fr/externe/tetraodon/Ressource.html)
Human EST sequences	4,455,050	2,220,752,380	>95%
Mouse EST sequences	2,585,930	1,106,162,284	>95%

[a] Estimated minimal coverage of the non-repetitive part of the genome as indicated on the web pages of the sequencing consortia. The coverage of the transcriptome by ESTs was extrapolated from Carmargo et al. (35) for genes that are at least moderately expressed. Genomic and EST databases were accessed at GenBank (www.ncbi.nlm.nih.gov) on May 30, 2002. All sequences, except the Celera genome, were examined using TBLASTN 2.2.3 with default parameters. The Celera human genome draft was examined using BLASTN 2.1.2, because TBLASTN searches are not available for the public part of their database.

without any indication of a second isoform. The only variation in the transcripts that could be detected by EST analysis is the differential use of polyadenylation sites (22). No alternative splicing of coding exons was detected. In contrast, both the fugu and the pufferfish genome drafts contain two closely related forms of HMGCR, which may be remnants of the whole-genome duplication supposed to have taken place early in teleost evolution (23). To test the sensitivity of the BLAST algorithm, we repeated the same searches using *Vibrio cholerae* HMGCR as template. This protein has only 34% amino acid identity with the human enzyme. Again, the searches detect one and only one HMGCR gene in the mammalian genomes and two copies each in the two fish species. Further searches, using a prototypical class II HMGCR from *Pseudomonas mevalonii* (22% identity) failed to identify any homologs in the vertebrate genomes. This indicates that the detection limit for a single-iteration TBLASTN search is somewhere between 22% and 34% identity.

In addition, we searched the protein section of the non-

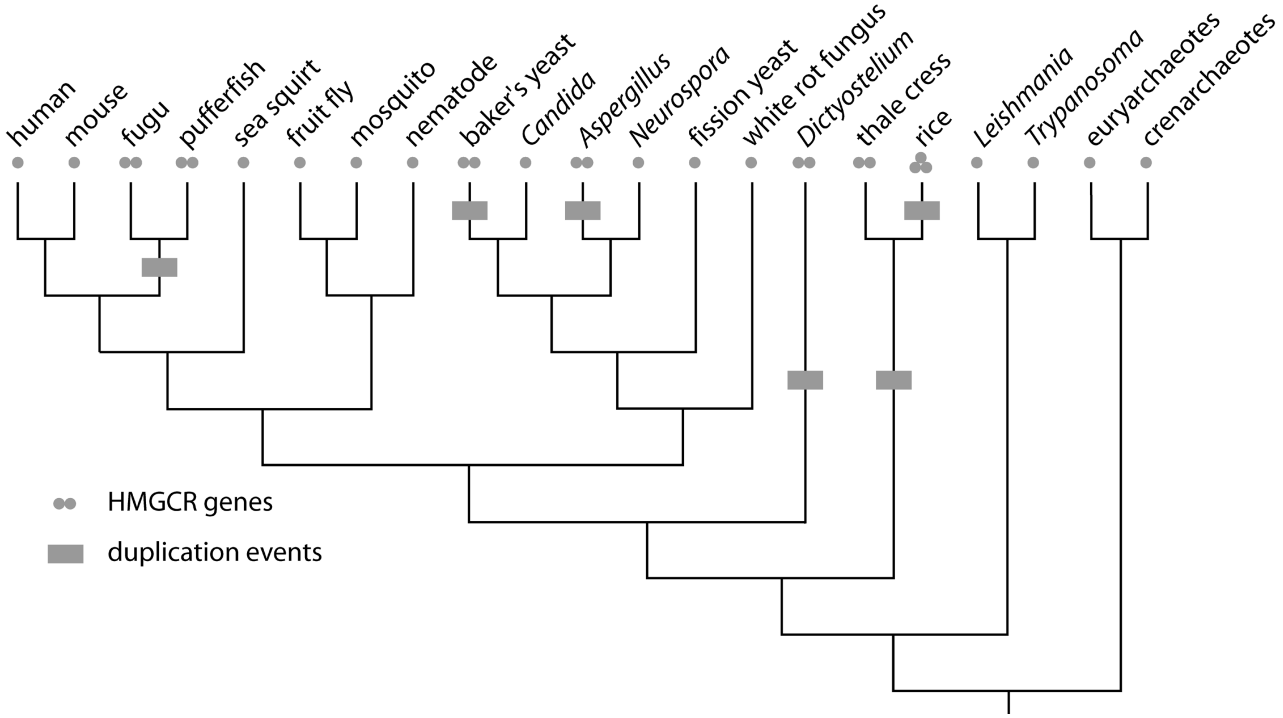

Fig. 2. Distribution of duplicated HMGCR genes in completely sequenced eukaryotic genomes mapped on a consensus phylogenetic tree. Gray bars indicate predicted gene duplication events that most parsimoniously explain the observed distribution of duplicates. The duplication events shown are most likely an underestimate, because duplicated genes are usually lost quickly without getting fixed in the genome.

redundant GenBank database for distant homologs of the human HMGCR catalytic domain using PSI-BLAST (24). This database contains among other sequences the translations of about 50,000 predicted open reading frames from the mouse and human genome. The search detected possible HMGCR proteins in 114 organisms (21 animals, 12 fungi, 27 green plants, five protozoa, 26 archaea, and 23 eubacteria) with high significance (E-values $< 10^{-15}$), including proteins of both classes of HMGCR, although some of the proteins have less than 25% identity with the human enzyme or are only known from partial sequences. This demonstrates the sensitivity of the search algorithm. Several plant species (e.g., *Lycopersicon esculentum* and *Hevea brasiliensis*) contain at least four HMGCR isoforms, ranging in similarity between 60% and 99% amino acid identity. Two isoforms of 60% identity are also found in the unicellular eukaryote *Dictyostelium discoideum*. However, no second HMGCR homolog was found in mammals, the best hits (with E-values > 1!) being two hypothetical proteins XP_093947 (human) and XP_146397 (mouse), both of which show no similarity to one another and are significantly related to other predicted proteins of unknown function. Mapping the distribution of duplicated HMGCR genes onto a consensus tree of eukaryotes predicts a large number of independent duplication events in animals, plants, and yeasts (Fig. 1).

NO SECOND GENE FOR HMGCR AFTER ALL?

The mammalian genome sequences analyzed so far do not contain a second HMGCR gene with more than about 35% identity to the canonical enzyme based on the detection limit of the TBLASTN search. They also lack any homolog of the class II HMGCRs described by Bochar et al. (25). Our analysis also indicates that the presence of duplicated HMGCR in a variety of organisms cannot be used as supportive evidence for a mammalian duplication, because the acquisition of a second HMGCR gene occurred independently in a number of evolutionary lineages (**Fig. 2**). Furthermore, the existence of these isoforms does not necessarily indicate an evolutionary advantage/function of an HMGCR duplication. The functional subunit of HMGCR is a homodimer, with residues from both monomers contributing to the active site (26–29), and this will lead to a prolonged retention of functional duplicates, e.g., after whole-genome duplications, to prevent dominant-negative mutations.

This leaves several options regarding the peroxisomal HMGCR of mammals: *1*) Our analysis might be incomplete. Peroxisomal HMGCR could be encoded by a second, very similar gene, but the genome sequences might have some unexpected deficiencies so that just by chance we don't find the second gene in all three mammalian genomes. In that case, it would also be necessary to postulate that the second gene has in some way escaped inclusion in the EST sequence databases. As the genome sequences are very comprehensive (Table 1) and are expected to be biased toward coding regions, it is very unlikely that a very large protein coding sequence would still be completely absent from all three mammalian genome assemblies (human, mouse, rat). And despite statistical limitations of the transcriptome coverage by ESTs, detection of ESTs for the peroxisomal HMGCR should be virtually guaranteed because the reported peroxisomal protein and activity levels are substantial in several tissues well represented in the EST databases (brain, liver) (10, 17). *2*) Our analysis might not be sensitive enough. Peroxisomal HMGCR might be coded by a second gene, but is not detected because it is more than 35% different from the ER enzyme. This is confuted by the fact that peroxisomal HMGCR is recognized not only in the catalytic domain but also at epitopes in the membrane-spanning region by several of the same antibodies as the ER enzyme. This is extremely unlikely if the two proteins are not very similar, and it is expected that BLAST searches should be far more "cross-reactive" than antibodies. *3*) Our analysis is correct, and peroxisomal HMGCR is encoded by the same gene as the ER enzyme. Evidence to the contrary has to be reinterpreted in the light of the genomic data.

If we accept the third alternative, it is necessary that a fraction of the ER HMGCR is alternatively targeted to peroxisomes by an as yet unknown mechanism. The observations in UT2* cells seem to argue against an involvement of alternative splicing, and targeting signals for peroxisomal membrane proteins are little understood in general. In any case, such a dual localization is not without precedence in the peroxisomal isoprenoid biosynthesis pathway. For example, mitochondrial acetoacetyl-CoA thiolase as well as HMG-CoA lyase contain both a mitochondrial targeting signal at the N-terminus and a functional peroxisomal targeting signal at the carboxy terminus (21, 30). The physiological relevance of the peroxisomal HMGCR activity is still unknown, and Fig. 1 indicates how the peroxisomal HMGCR step can be circumvented by the export of ketone bodies and import of mevalonate, which seems to be readily permeating the peroxisomal membrane (31). Another open question is the general relevance of the peroxisomal localization for the efficiency and regulation of isoprenoid biosynthesis, particularly with respect to peroxisome deficiency disorders. The recent availability of peroxisome-deficient mouse models offers great opportunities to address these issues (32–34).

This work was supported in part by National Institutes of Health Grants DK58238 and DK58040.

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