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3-Hydroxy-3-methylglutaryl coenzyme A synthase-1 of *Blattella germanica* has structural and functional features of an active retrogene

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Abstract

Blattella germanica has two cytosolic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase genes, HMG-CoA synthase-1 and -2. HMG-CoA synthase-1 gene shows several features of processed genes (retroposons): it contains no introns but has a short direct-repeat sequence (ATTATTATT) at both ends. An atypical feature is the presence at both ends of the gene of short inverse repeats flanked by direct repeats. There is neither a TATA box nor a CAAT box in the 5′ region. Comparative analysis with other species suggests that the HMG-CoA synthase-1 gene derives from HMG-CoA synthase-2. Cultured embryonic *B. germanica* UM-BGE-1 cells express HMG-CoA synthase-1 but not HMG-CoA synthase-2, suggesting that the intron-less gene is functional. In addition, it can complement MEV-1 cell line, which is auxotrophic for mevalonate. We show that compactin and mevalonate do not significantly affect the mRNA levels of HMG-CoA synthase-1 in UM-BGE-1 cells. Compactin induces a 6.7-fold increase in HMG-CoA reductase activity, which is restored to normal levels by mevalonate. HMG-CoA synthase activity is not modified by either of these effectors, suggesting that the mevalonate pathway in this insect cell line is regulated by post-transcriptional mechanisms affecting HMG-CoA reductase but not HMG-CoA synthase.

Keywords: HMG-CoA synthase; HMG-CoA reductase; *Blattella germanica*; Intron-less gene; Compactin; Mevalonate; Retrogene

1. Introduction

Enzymes

3-Hydroxy-3-methylglutaryl Coenzyme A synthase (E.C. 4.1.3.5)

3-Hydroxy-3-methylglutaryl Coenzyme A reductase (E.C. 1.1.1.34)

Since insects lack squalene synthase and lanosterol synthase (Beenakkers et al., 1985), they cannot produce cholesterol de novo. However, their mevalonate pathway leads to specific isoprenoids such as juvenile hormones (JHs), which have a critical role in the maintenance of the larval form and in vitellogenesis. During the gonadotrophic cycle, *Blattella germanica* 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase activities change more than 10-fold in corpora allata and fat body, and peak activities correspond to maximum rates of JH and vitellogenin, respectively (Casals et al., 1996; Couillaud and Feyereisen, 1991; Feyereisen and Farnsworth, 1987).

The regulation of HMG-CoA reductase and cytosolic HMG-CoA synthase expression is best understood in mammals, in the context of cholesterol synthesis. The two genes are subject to multivalent feedback suppression mediated by sterols and non-sterol substances derived from mevalonate either by transcriptional or post-transcriptional mechanisms (Goldstein and Brown, 1990; Wang et al., 1994). Nevertheless, the non-sterol mevalonate inhibitor is still unknown (Petras et al., 1999; Peffley and Gayen, 1997).

In insects, regulation of HMG-CoA reductase activity by mevalonate has been shown in an established *Droso-*

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phila cell line (Kc cells), which neither synthesized nor required cholesterol for growth (Silberkang et al., 1983). In Kc cells, HMG-CoA reductase activity was not affected by cholesterol. However, mevalonate and compactin, a competitive HMG-CoA reductase inhibitor, respectively suppress or increase HMG-CoA reductase activity (Brown et al., 1983), indicating that a decrease in a product of the mevalonate pathway probably serves as a signal to increase HMG-CoA reductase activity.

Our group has reported the cloning and characterization of HMG-CoA reductase cDNA (Martínez-González et al., 1993a) and two cytosolic HMG-CoA synthases cDNAs from the German cockroach *B. germanica*, HMG-CoA synthase-1 (Martínez-González et al., 1993b), and HMG-CoA synthase-2 (Buesa et al., 1994). Other genes of the mevalonate pathway reported in insects are HMG-CoA reductase of *Drosophila melanogaster* (Gertler et al., 1988), farnesyl diphosphate synthase and HMG-CoA reductase of the moth *Agrotis ipsilon* (Castillo-Gracia and Couillaud, 1999; Duportets et al., 2000). The physiological significance of the occurrence of two cytosolic HMG-CoA synthases in *B. germanica* is unknown.

We now show that *B. germanica* HMG-CoA synthase-1 is an intron-less gene that encodes an active enzyme able to support the mevalonate pathway.

2. Materials and methods

2.1. Materials

The oligonucleotides used in PCR–RACE experiments and in the construction of a reading-frame cassette were synthesized by Amersham (Amersham Ibérica, Madrid, Spain). Radioactive compounds were obtained from Amersham. The pCMV-2 eukaryotic expression vector was a gift from Dr M. Stinsky (Department of Microbiology, School of Medicine, University of Iowa). The CHO-derived Mev-1 cells were a gift from Dr M. Sinensky (Eleanor Roosevelt Institute, Denver, CO). UM-BGE-1 is a *B. germanica* cell line kindly provided by Dr T. J. Kurtti (Department of Entomology, University of Minnesota). Clone λBgST-11 contains the longest HMG-CoA synthase-1 CDNA from *B. germanica* (Martínez-González et al., 1993b). Acetoacetyl-CoA and acetyl-CoA were from Sigma (St Louis, MO). [1-^{14}C]acetyl-CoA was from ICN (Irvine, CA).

2.2. Construction and screening of B. germanica genomic libraries

Construction of *B. germanica λDashII* genomic library: 9–25 kb fragments of *B. germanica* genomic DNA partially digested with Sau3A were ligated to λ*Dash*II/*Bam*HI arms (Stratagene, La Jolla, CA) and packed with Gigapack II Gold extracts (Stratagene) yielding a titer of 10^6 plaque-forming units (pfu)/ml.

Construction of *B. germanica* Charon 35 library: 9–20 kb fragments of *B. germanica* genomic DNA partially digested with *Eco*RI were ligated to previously purified arms of Charon 35. The recombinant phages were packed with Gigapack II Gold extracts (Stratagene) yielding a titer of 10^5 plaque forming units (pfu)/ml.

In total, 400,000 pfu of each library were screened in LE392 host cells using λBgST-11 as a probe. The screening produced one positive clone in each library, called respectively λS1-13 and λS1-14. The positive phages were purified by two additional rounds of plaque screening and then amplified.

2.3. DNA sequencing

Genomic DNA clones were subcloned into pBluescript vectors (Stratagene) and sequenced by the dideoxy-nucleotide chain termination method with modified T7 DNA polymerase (Stratagene).

2.4. Sequence alignments and comparisons

For comparative purposes, in addition to the sequence studied in the present paper (*B. germanica* HMG-CoA synthase-1, GenBank accession number P54961), we used the previously published complete amino acid sequences of HMG-CoA synthase of the following species (GenBank accession number in parenthesis): *B. germanica* HMG-CoA synthase-2 (P54870), *Arabidopsis thaliana* (Mouse-ear cress) (P54873), *Saccharomyces cerevisiae* (Baker's yeast) (P54839), *Schizosaccharomyces pombe* (Fission yeast) (P54874), *Gallus gallus* (Chicken) (P23228), *Cricetulus griseus* (Chinese hamster), cytoplasmic (P13704), *Mesocricetus auratus* (Syrian hamster), cytoplasmic (L00334), *Mus musculus* (Mouse), mitochondrial (P54869), *Rattus norvegicus* (Rat), cytoplasmic (P17425), *R. norvegicus*, mitochondrial (P22791), *Sus scrofa* (Pig), mitochondrial (O02734), *Homo sapiens*, mitochondrial (P54868), *H. sapiens*, cytoplasmic (Q01581). We have also included in our analyses the CG4311 gene product of the fruit fly, *D. melanogaster*, which has been proposed as a putative HMG-CoA synthase (Q9V7N8).

Software from Genetics Computer Group (GCG, version 9.1), University of Wisconsin (Devereux et al., 1984) was used for sequence analysis. Sequence alignments were carried out with PILEUP and were not further hand-refined. Percentage similarity and percentage identity between sequences were estimated using the application BESTFIT. Parsimony analyses were carried out with the Phylogeny Inference Package (PHYLIP, version 3.57c) (Felsenstein, 1995), using amino acid sequences (PROTPARS program) (Swofford and Olsen, 1990). Bootstrap analyses were carried out with the

application SEQBOOT in the PHYLIP package, and the procedure was repeated 100 times.

2.5. Expression of B. germanica HMG-CoA synthase-1 in Mev-1 cells

HMG-CoA synthase-1 was transfected in Mev-1 cells, which are defective CHO mutants cells for cytosolic HMG-CoA synthase (Schnitzer-Polokof et al., 1982). Mev-1 cells were first cultured in Hams F12 medium supplemented with 5% fetal calf serum (FCS) and 430 mM of mevalonate. The cDNA clone of HMG-CoA synthase-1 (Martínez-González et al., 1993b) was digested with appropriate restriction enzymes and the fragment isolated, which contained the 5′ region, the coding region and a fragment of the 3′ region, was subcloned into a pCMV-2 expression vector previously linearized with the same enzymes. The new plasmid, pCMV-HMGS1ex, was used to perform stable transfection of Mev-1 cells by the standard calcium phosphate method, and a stable revertant cell line (Mev-HMGS1) was established as described previously for the second HMG-CoA synthase (Mev-HMGS2) from *B. germanica* (Buesa et al., 1994).

2.6. UM-BGE-1 cell culture

UM-BGE-1 cells were grown in suspension at room temperature. The medium was L15 (GIBCO, Grand Island, NY) modified as recommended by Munderloh and Kurtti (1989); the pH was adjusted to 6.5 with NaOH and the medium was complemented with 5% of fetal bovine serum (GIBCO), 10% tryptose phosphate broth (Difco, Detroit, MI), 10 μg/ml cholesterol (Sigma) and 50 μg/ml gentamicin (GIBCO).

2.7. Compactin and mevalonate treatment of UM-BGE-1 cells

Cells were plated at a density of 5×10^5 cells per ml. The experiments began 24 h after transferring the cells. Compactin (Sigma) was added at different concentrations in some plates for 24 h. In other plates, after the treatment with compactin cells were washed twice in medium and treated with 10 mM mevalonate for 24 h. Cells were collected by centrifugation at 1200**g** for 10 min.

2.8. Northern-blot

Samples (10 μg) of total RNA from UM-BGE-1 cells were subjected to agarose-gel electrophoresis and Northern transfer. The RNA was fixed to Nytran filters by u.v. at 254 nm for 2 min at 12 cm. After 6 h pre-hybridization, hybridization was carried out with the cDNA of the gene of interest as a probe. Filters were washed in 0.1×SSC/0.5% SDS at 68°C and placed in contact with X-ray film.

2.9. PCR–RACE conditions for determination of transcription start point

The transcription start point was determined by RACE (rapid amplification of cDNA ends), as described by Mizobuchi and Frohman (1993). The reverse transcription was performed on 2 μg of *B. germanica* poly $(A)^+$ RNA primed with 5′-CGACTGACGACGGTGAGA-CACAG-3′, a reverse primer located at position 267–245 on HMG-CoA synthase-1 cDNA. A poly (A) string was added with nucleotide terminal transferase (Amersham) and the cDNA was primed with the forward 35-mer primer 5′-CCTCCCTGTCCGTGCAGA-3′ located at position 233–211 on the cDNA. The amplification product was subcloned in Bluescript by digestion with *Xho*I enzymes, and seven independent clones were analyzed.

2.10. Assay of HMG-CoA synthase and reductase activities

UM-BGE-1 cells were collected by centrifugation, washed twice in PBS and re-suspended in a buffer composed of 100 mM sucrose, 40 mM K_2HPO_4 pH 7.2, 30 mM EDTA, 50 mM KCl, 10 mM PMSF, 0.25% (v/v) Triton X-100 and 10 mM DTT. Two aliquots were assayed in parallel and the protein content before the assay was determined. HMG-CoA reductase activity was determined in UM-BGE-1 cultured cells by the radiometric method described by Goldstein et al. (1983). The assay was performed with 100 μg of protein for 40 min; in these conditions the assay was linear and the substrate consumed was less than 5%. One unit of HMG-CoA reductase is defined as the amount of the enzyme that converts 1 nmol HMG-CoA into mevalonate in 1 min at 37°C.

HMG-CoA synthase activity was determined by the radiometric method described by Clinkenbeard et al. (1975), as modified by Gil et al. (1986a). The enzymatic activity was measured as incorporation of [1-^{14}C]acetyl-CoA into HMG-CoA at 30°C in 30 min. The reaction was initiated by adding 100 μg of protein to a reaction mixture (final volume 200 μl) composed of 100 mM Tris–HCl, 1 mM EDTA, and 20 μM of acetoacetyl-CoA and 200 μM acetyl-CoA (specific activity between 4000 and 8000 cpm/nmol). Radioactivity was counted in a cocktail containing 67% toluene, 33% Triton X-100, and 0.56% PPO (v/v/w). One unit of HMG-CoA synthase is defined as the amount of enzyme that catalyzes the formation of 1 μmol HMG-CoA per minute.

3. Results and discussion

3.1. Isolation and characterization of a DNA clone for B. germanica HMG-CoA synthase-1

The screening of the two genomic libraries from *B. germanica*, using the λBgST-11 cDNA clone of HMG-CoA synthase-1 (Martínez-González et al., 1993b) as a probe, isolated two overlapping genomic clones, λS1-13 and λS1-14. A Southern blot of these fragments showed that while λS1-13 (8 kb) was truncated in the 3′ region, λS1-14 (13 kb) contained the whole coding region and additional 5′ and 3′ flanking regions (Fig. 1). The restriction map of the coding region of the gene for HMG-CoA synthase-1 coincided with that described for the cDNA (Martínez-González et al., 1993b). Sequencing of the *Eco*RI–*Eco*RI fragment showed that the coding region of the genomic DNA was colinear to the mRNA, i.e. that the gene for HMG-CoA synthase-1 from *B. germanica* does not contain introns. *B. germanica* is the only animal system in which two putative cytosolic HMG-CoA synthases have been reported. We had shown previously that HMG-CoA synthases-1 and -2 are present in *B. germanica* as single-copy genes (Martínez-González et al., 1993b; Buesa et al., 1994). Our preliminary data on the genomic structure of *B. germanica* HMG-CoA synthase-2 pointed to a gene spanning 20 to 30 kb, with similar organization (unpublished data) to that of known vertebrate genes (Gil et al., 1986b). In this context, the finding that HMG-CoA synthase-1 is devoid of introns is surprising. There are no other HMG-CoA synthases in *B. germanica*, as deduced by the relaxed screening of different cDNA and genomic libraries and experiments of Southern blot performed by our group (data not shown).

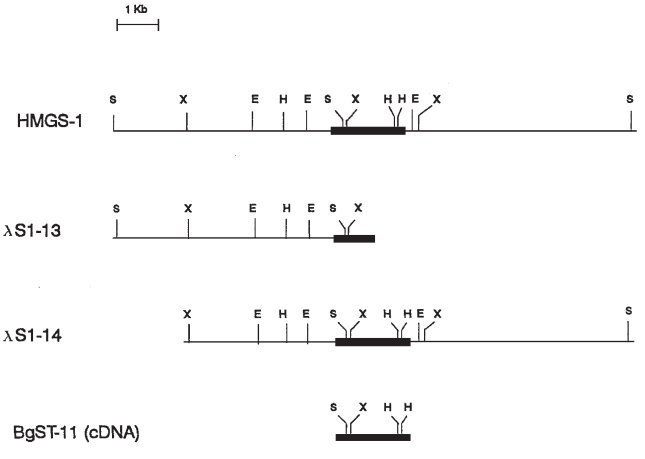

Fig. 1. Restriction endonuclease map of the HMG-CoA synthase-1 (HMGS-1) gene from *Blattella germanica*. The restriction map of the HMG-CoA synthase-1 gene deduced from λS1-13 and λS1-14 genomic clones was compared with that obtained from the BgST-11 cDNA clone. S=SacI; X=XhoI; E=EcoRI; H=HindIII. The bold line represents the coding region of the gene.

Fig. 2A shows the sequence of 832 bp of the 5′ flanking region of the HMG-CoA synthase-1 gene, and Fig. 2B shows the untranslated 3′ region and 187 bp of the 3′ flanking region. The transcription start sites were determined by PCR–RACE analysis. Three different sites were obtained. The most abundant transcription start site was considered as +1. The sequence ATCATTC at −17 bp (heavily underlined in Fig. 2A) is identical to the consensus for the transcription start site of many genes in *D. melanogaster* (Hultmark et al., 1986). Analysis of the 5′ flanking region showed several sequences homologous to those recognized by several known transcription factors. Among them, the consensus sequence of the transcription factors AP-1 (Lee et al., 1987), AP-2 (Williams and Tijan, 1991), E1A-F (Higashino et al., 1993) and a long sequence of A and T (marked in brackets in Fig. 2A) that binds to different homeotic proteins were also found (Biggin and Tijan, 1989). In addition, the sequence GTCAGC (boxed by number $_6$ in Fig. 2A) at +24 bp is identical to the major late promoter of adenovirus type 2 (Carthew et al., 1985) and also to a footprint of the hamster HMG-CoA reductase promoter (Reynolds et al., 1984). Neither the sterol response element (SRE), nor a TATA box, nor a CAAT box was found.

In addition, we found direct and inverse repeats at both ends of the gene. There is a perfect direct repeat of 9 bp (ATTATTATT) located at −595, at −198 and +1671. There is also an imperfect direct repeat of 18 bp located at −605 and +1662. An inverse repeated sequence of 16 bp located at −186 bp and +1656, which is flanked by a direct repeat of 4 bp (TTAT). All these sequences are indicated in Fig. 2A and B. The absence of introns, the occurrence of short direct repeats in the 5′ and 3′ regions of the gene and the remains of a poly-A tail are hallmarks of retroposons (Löwer et al., 1996; Weiner, 1986). The fact that HMG-CoA synthase-1 fits two of those hallmarks and that it is actively transcribed support the hypothesis that HMG-CoA synthase-1 is a retrogene.

3.2. Comparative analysis with other species

The protein sequence of HMG-CoA synthase-1 of *B. germanica* is between 60 and 78% similar to that of other animal species. Maximal similarity was found with the HMG-CoA synthase-2 of the same species (78%), and then with the sequence from *D. melanogaster* (76%). Percentages of similarity with vertebrate, and plants or yeast sequences were lower (between 72 and 74%, and between 60 and 62%, respectively).

The PILEUP alignment of the HMG-CoA synthase-1 of *B. germanica* with the sequences mentioned in Section 2 (not shown) reveals a high degree of conservation, especially in the catalytic domain. A parsimony analysis carried out with the PILEUP alignment gave a dendrog-

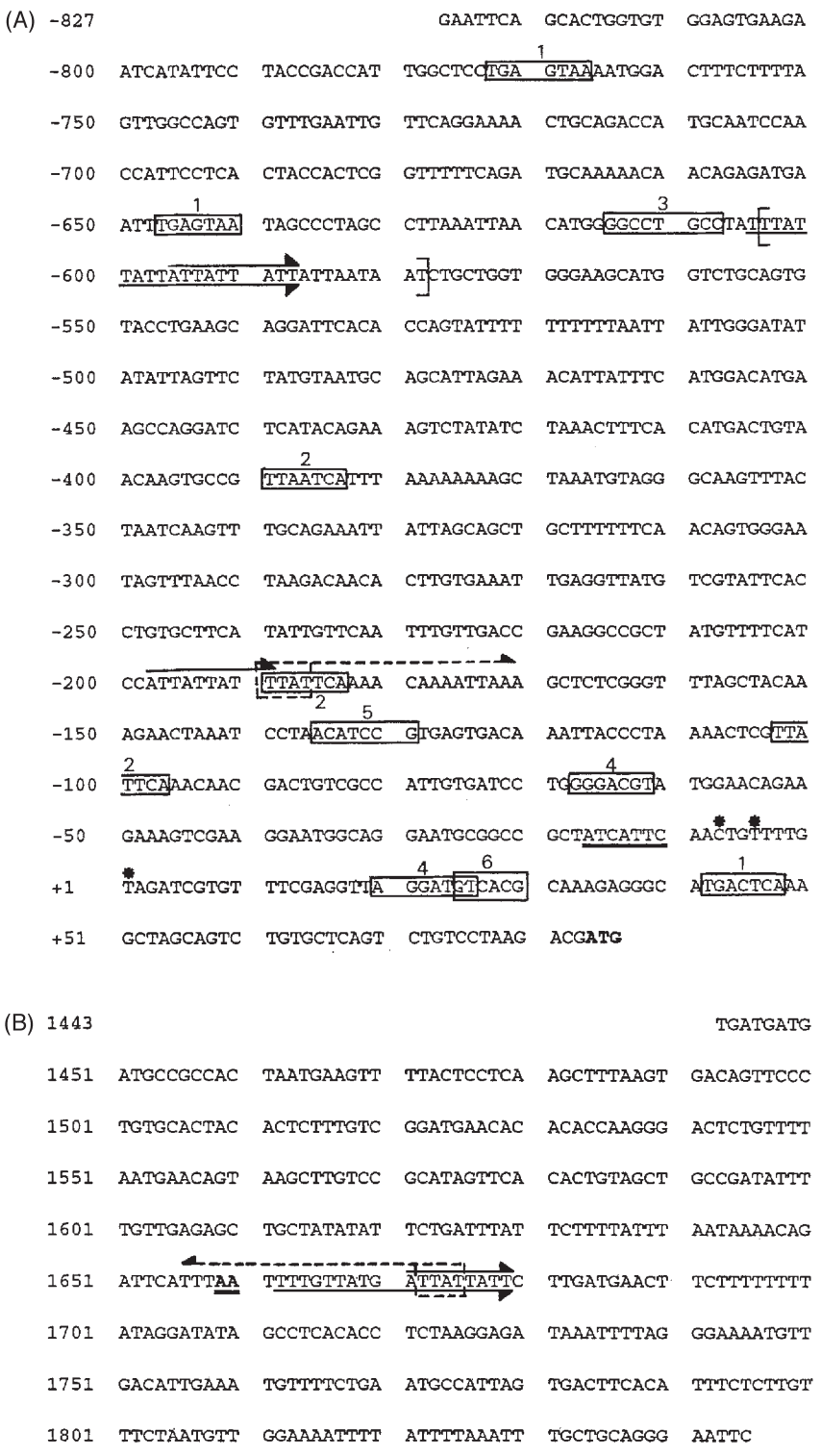

Fig. 2. Nucleotide sequence of the 5′ and 3′ flanking regions of the HMGS-1 gene. 827 nucleotides of the 5′ flanking region (A) and the 3′ untranslated region and 187 nucleotides of the 3′ flanking region (B) of the gene are shown. The transcription start points are depicted by asterisks. The sequence is numbered starting from the major transcription start site. The consensus sequence for the transcription start site in *D. melanogaster* and other insects is heavily underlined. Short (9 bp) direct repeats are superscripted with continuous arrows. Long (18 bp) imperfect (16/18 bp) direct repeats are underlined with continuous arrows. Long (16 bp) inverted repeats flanked by 4 bp direct repeat are superscripted with broken arrows. A seven (TTA) repeat is located at position −604 to −584 and it is a long part of a sequence (marked in the figure by brackets) that binds different homeotic proteins (Biggin and Tijan, 1989). The first ATG of the protein is marked in boldface capital letters. At position +1659, two adenines are underlined and marked in boldface capital letters, as a possible remains of the poly A tail of the putative incorporated transcript. The consensus sequences for the union of different transcription factors are boxed and marked as follows: 1, AP1 (activator protein 1); 2, inverted AP1; 3, inverted AP2 (activator protein 2); 4, E1A-F (adenovirus E1A enhancer binding protein); 5, inverted E1A-F; 6, inverted footprint of the hamster HMG-CoA reductase promoter.

ram (Fig. 3) showing that *B. germanica* HMG-CoA synthase-1 clusters with *B. germanica* HMG-CoA synthase-2, and both have the sequence of *D. melanogaster* as a sister group. These relationships and the results of the structural analysis suggest that the intron-less HMG-CoA synthase-1 gene derives from HMG-CoA synthase-2. These results do not agree with the hypothesis of a horizontal gene transfer. We suggest that HMG-CoA synthase-1 derives from HMG-CoA synthase-2 by retrotransposition. Moreover, the general dendrogram topology is congruent with the conventional phylogenetic schemes, and the bootstrap values are high in all nodes. Using the plant (*A. thaliana*) as the sister group of all other species, fungi (the yeasts *S. cerevisiae* and *S. pombe*) appear as the sister group of animals, as in other protein-based phylogenies reported recently (see, for example, Doolittle et al., 1996). Animals are well grouped in insects and vertebrates. As expected, the cytoplasmic and mitochondrial forms of the enzyme cluster separatedly in the vertebrate node, which suggests that the separation of these forms occurred before the splitting of the species.

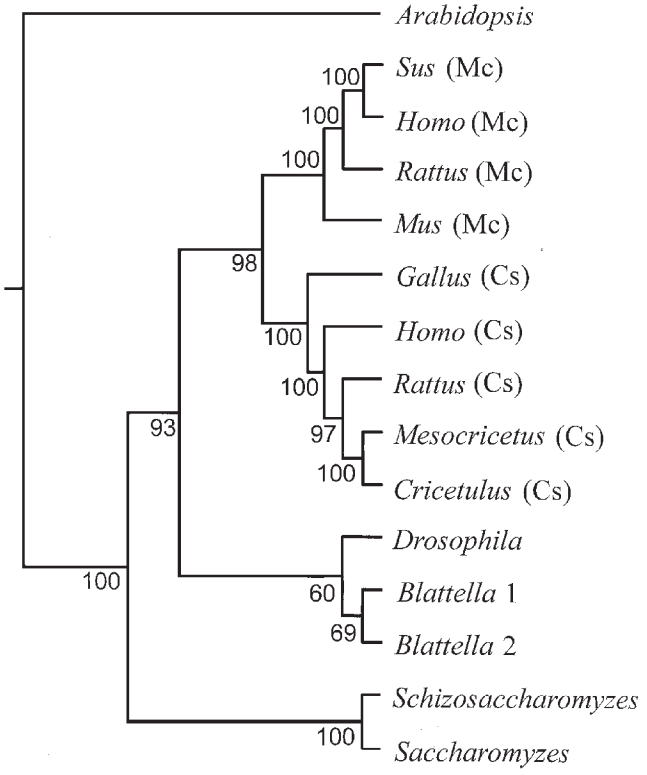

Fig. 3. Parsimony analysis of the amino acid sequences of HMG-CoA synthase available, using the plant *Arabidopsis thaliana* as outgroup. The analysis was carried out with the PROTPARS program from PHYLIP package, from an alignment generated by the PILEUP program from the GCG package which was not further refined. Values from bootstrap analysis (100 replications using SEEQBOOT from PHYLIP package) are also indicated. Complete specific names and the corresponding GenBank accession numbers have been mentioned in Section 2. Cs and Mc mean cytoplasmic and mitochondrial, respectively.

3.3. HMG-CoA synthase-1 encodes for a functional HMG-CoA synthase

The fact that *B. germanica* has two cytosolic HMG-CoA synthases and that HMG-CoA synthase-1 is an intron-less gene with characteristics of a retrogene led us to analyze whether the encoded protein was functional. The whole coding region and 140 bp of the 3′ UTR were subcloned into the pCMV-2 eukaryotic expression vector. The transfection of this cDNA in a CHO-derived cell line, Mev-1, which is a defective mutant for HMG-CoA synthase activity and auxotrophe for mevalonate, produced colonies able to grow in the absence of mevalonate. The new cell line was called Mev-HMGS-1. When Mev-1 cells were transfected only with the control plasmid and transferred to a medium without mevalonate, all the cells died in a few hours (Fig. 4). This result demonstrates that HMG-CoA synthase-1 is a functional protein. The same conclusion is obtained with HMG-CoA synthase-1 gene expression in UM-BGE-1 cells (see below) and with HMG-CoA synthase-1 overexpression in *Escherichia coli* (Cabañó et al., 1997). The differential expression pattern between synthase-1 and synthase-2 in the cockroach ovary (Buesa et al., 1994), fat body (Casals et al., 1996) and during the embryonic and larval development stages (Martínez-González et al., 1993b) suggests that HMG-CoA synthase-1 retrogene codes for a functional HMG-CoA syn-

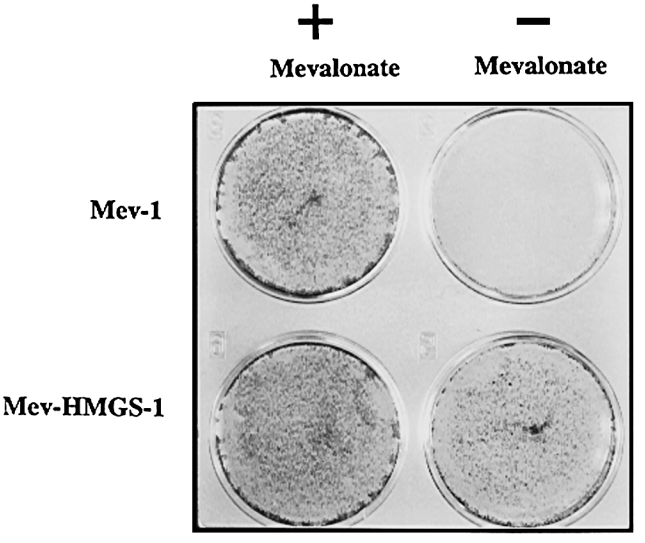

Fig. 4. Validation of HMG-CoA synthase-1 clone as a functional eukaryote HMG-CoA synthase. A 1380-bp fragment of HMG-CoA synthase-1 containing the whole coding region was subcloned into pCMV-2 eukaryotic expression vector. Mev-1, a cytosolic HMG-CoA synthase mutant CHO cell line, was transfected with this construction and with the plasmid control. HMG-CoA synthase-1 restored the mutant phenotype as a result of a stable transfection. This new line, Mev-HMGS-1, grew in the absence of mevalonate, whereas Mev-1 transfected with control plasmid died in a few hours. Cells were stained with 0.1% methylene blue in 50% methanol.

thase enzyme with specific physiological functions in *B. germanica*.

3.4. HMG-CoA synthase-1, HMG-CoA synthase-2 and HMG-CoA reductase gene expression in UM-BGE-1 cells from B. germanica

Fig. 5 shows that *B. germanica* embryo cell line UM-BGE-1 cells express HMG-CoA reductase and only the HMG-CoA synthase-1 gene, while mRNA from HMG-CoA synthase-2 was undetectable in all treatments, indicating that in these cells only the retrogene HMG-CoA synthase-1 supports the synthesis of HMG-CoA in the mevalonate pathway. Fig. 5 also shows that neither compactin nor mevalonate modulates HMG-CoA synthase-1 mRNA levels. Moreover, compactin modestly increases (about 1.5–2 times) HMG-CoA reductase mRNA levels, although mevalonate is unable to reduce them to the initial level.

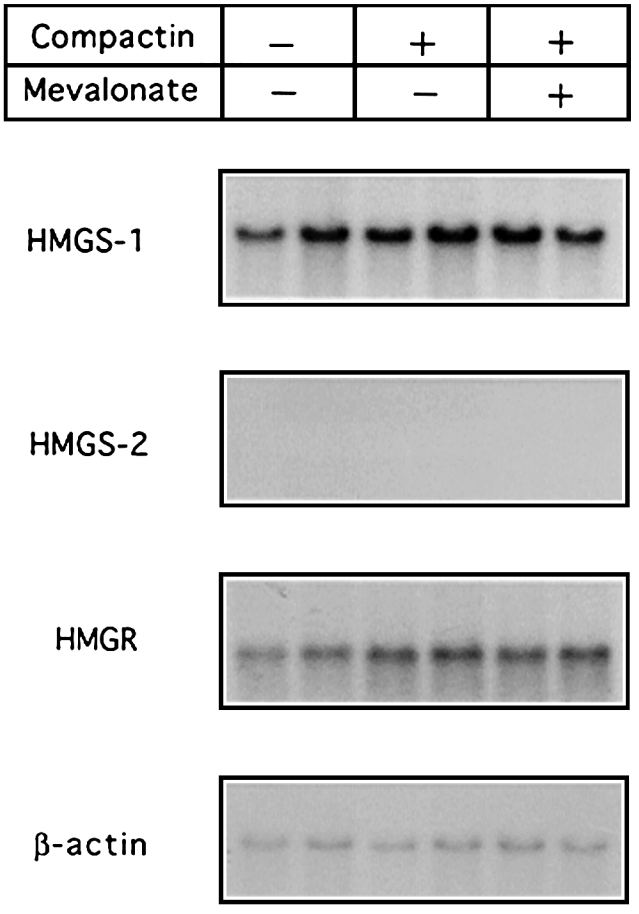

Fig. 5. mRNA levels of HMG-CoA synthase-1, HMG-CoA synthase-2 and HMG-CoA reductase in UM-BGE-1 cells after stimulation with compactin or compactin plus mevalonate. UM-BGE-1 cells (10^7 cells per plaque) were incubated with 2 μM compactin for 24 h in some cases or with 2 μM compactin for 24 h and thereafter with 10 mM mevalonate for 24 h. 10 μg of total RNA was processed by Northern blot. The probes were the full-length cDNA in each case. The intensity of the blots was compared with β-actin mRNA.

Fig. 6 shows that compactin, a competitive inhibitor of HMG-CoA reductase, increases HMG-CoA reductase activity but not HMG-CoA synthase activity in UM-BGE-1 cells. HMG-CoA reductase specific activity in cells grown in the presence of 0.02 μM compactin for 24 h was 6.7 times higher than in untreated cells (S.E.M.$_{(n=7)}$=0.75). The addition of 10 mM mevalonate for 24 h to UM-BGE-1 cells previously treated by compactin rapidly decreased HMG-CoA reductase activity, without affecting HMG-CoA synthase-1 activity.

Table 1 shows HMG-CoA synthase and reductase specific activity in cells grown at different concentrations of compactin. Dialysis of the samples eliminated compactin, an inhibitor of the HMG-CoA reductase activity. Dialysis also eliminated the cation Mg^{2+} from the samples so the HMG-CoA lyase activity was inhibited

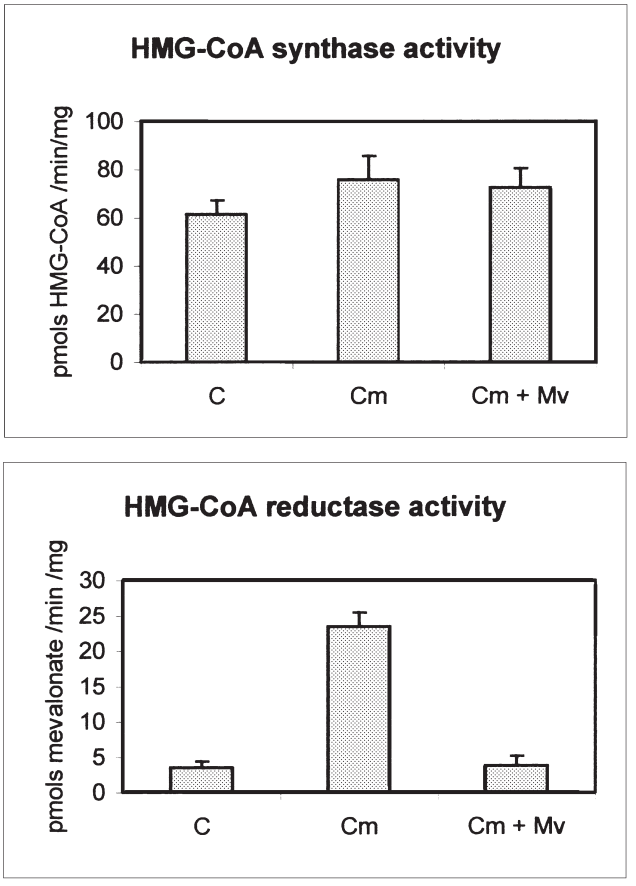

Fig. 6. HMG-CoA reductase and HMG-CoA synthase activity in UM-BGE-1 cells after stimulation with compactin or with compactin plus mevalonate. UM-BGE-1 cells were incubated with 0.02 μM compactin for 24 h or with 0.02 μM compactin for 24 h, and thereafter with 10 mM mevalonate for 24 h. Protein extracts were assayed for HMG-CoA synthase and HMG-CoA reductase activities. HMG-CoA synthase activity levels are indicated in μUnits (pmol HMG-CoA/mg/min). HMG-CoA reductase activity levels are indicated in μUnits (pmol mevalonate/mg/min). The figure bars represent the mean of seven independent experiments. The SEM is also indicated. C=control samples; Cm=compactin-treated samples; Cm+Mv=compactin-treated plus mevalonate-treated samples.

Table 1
HMG-CoA synthase and HMG-CoA reductase activity in UM-BGE-1 cells grown at different concentrations of compactin[a]

Compactin (μm)	HMG-CoA synthase (pmol HMG-CoA/min/mg)		HMG-CoA reductase (pmol mevalonate/min/mg)	
	−dialysis	+dialysis	−dialysis	+dialysis
–	75.7	89.3	3.2	3.5
0.002	61.3	92.6	10.3	5.7
0.02	83.3	91.8	25.1	18.4
0.2	74.8	120	15.7	22.2
2	88.6	81.9	1.8	17.5
20	70.0	83.9	0.2	18.6

[a] UM-BGE-1 cells were harvested 24 h after the addition of compactin. The protein crude extract was halved, and one of the portions was dialysed 1:1000 against KH_2PO_4 40 mM/KCl 50 mM/EDTA 30 mM for 24 h at 4°C with three changes of buffer. Enzymatic activity was assayed per duplicate in 100 μg of protein. The values shown in the table are the media of two independent experiments.

and did not interfere with the HMG-CoA synthase assay. No variations in HMG-CoA synthase activity were observed, while HMG-CoA reductase activity increased up to 0.2 μM of compactin in a dose-dependent manner, probably by a post-transcriptional mechanism which became saturated at high doses of compactin. The inhibition of HMG-CoA reductase activity observed at high doses of compactin in non-dialysed samples may be caused by the presence of compactin in the reaction assay. Table 1 indicates that at low compactin doses, no prior dialysis of the sample is required.

The data obtained in the UM-BGE-1 cells suggest that neither compactin nor mevalonate modulate the transcription rate or the enzymatic activity of HMG-CoA synthase-1. It remains to be determined whether this behaviour is peculiar to the retrogene, or whether it also affects HMG-CoA synthase-2. However, mevalonate and compactin modulate HMG-CoA reductase activity in the same way as that reported for vertebrate steroidogenic cells (Brown and Goldstein, 1980; Straka and Panini, 1995), yeast cells (Dimster-Denk et al., 1994) or *Drosophila* cells (Brown et al., 1983). Thus, increased mevalonate and/or its conversion to a non-sterol isopentenoid product in UM-BGE-1 cells signals a post-transcriptional decrease in *B. germanica* HMG-CoA reductase activity. This mechanism probably reflects a fundamental process, characteristic of all eukaryotic cells, and appears to be exclusive to HMG-CoA reductase without affecting HMG-CoA synthase-1.

The fact that UM-BGE-1 cells express only HMG-CoA synthase-1 makes this cell line an interesting system for future studies on the regulation of the expression of this gene. The question as to whether the presence of two HMG-CoA synthase activities in *B. germanica* is an isolated evolutionary event or a widespread molecular adaptive phenomenon will be enlightened when new data from other organisms become available.

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References

Beenakkers, A.T.M., Van der Horst, D.J., Van Marrewijk, W.J.A., 1985. Insect lipids and lipoproteins, and their role in physiological processes. Prog. Lipid Res. 24, 19–67.

Biggin, M.D., Tijan, R., 1989. Transcription factors and the control of *Drosophila* development. Trends Genet. 5, 377–383.

Brown, K., Havel, C.M., Watson, J.A., 1983. Isoprene synthesis in isolated embryonic *Drosophila* cells. II. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. J. Biol. Chem. 258, 8512–8518.

Brown, M.S., Goldstein, J.L., 1980. Multivalent feedback regulation of HMG-CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J. Lipid Res. 21, 505–517.

Buesa, C., Martínez-González, J., Casals, N., Haro, D., Piulachs, M.D., Bellés, X. et al., 1994. *Blattella germanica* has two HMG-CoA synthase genes. J. Biol. Chem. 269, 11707–11713.

Cabañó, J., Buesa, C., Hegardt, F.G., Marrero, P.F., 1997. Catalytic properties of recombinant 3-hydroxy-3-methylglutaryl coenzyme A synthase-1 from *Blattella germanica*. Insect Biochem. Mol. Biol. 72, 499–505.

Carthew, R.W., Chodosh, L.A., Sharp, P.A., 1985. An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell 43, 439–448.

Casals, N., Buesa, C., Piulachs, M.D., Cabañó, J., Marrero, P.F., Bellés, X. et al., 1996. Coordinated expression and activity of 3-hydroxy-3-methylglutaryl coenzyme A synthase and reductase in the fat body of *Blatella germanica* during vitellogenesis. Insect Biochem. Mol. Biol. 26, 837–843.

Castillo-Gracia, M., Couillaud, F., 1999. Molecular cloning and tissue

expression of an insect farnesyl diphosphate synthase. Eur. J. Biochem. 262, 365–370.
Clinkenbeard, K.D., Sugiyama, T., Lane, M.D., 1975. Cytosolic 3-hydroxy-3-methylglutaryl coenzyme A synthase from chicken liver. Methods Enzymol. 35, 160–167.
Couillaud, F., Feyereisen, R., 1991. Assay of HMG-CoA synthase in *Diploptera punctata* corpora allata. Insect Biochem. 21, 131–135.
Devereux, J., Haeberly, P., Smithies, O., 1984. A comprehensive set of sequences analysis programs for the VAX. Nucleic Acids Res. 12, 387–395.
Dimster-Denk, D., Thorsness, M.K., Rine, J., 1994. Feedback regulation of 3-hydroxy-methylglutaryl coenzyme A reductase in *Saccharomyces cerevisiae*. Mol. Biol. Cell. 5, 655–665.
Doolittle, R.F., Feng, D.F., Tsang, S., Cho, G., Little, E., 1996. Determining divergence times of the major kingdoms of living organisms with a protein clock. Science 271, 470–477.
Duportets, L., Belles, X., Rossignol, F., Couillaud, F., 2000. Molecular cloning and structural analysis of 3-hydroxy-3-methylglutaryl coenzyme A reductase of the moth *Agrotis ipsilon*. Insect Mol. Biol. 9, 385–392.
Felsenstein, J., 1995. PHYLIP (Phylogeny Inference Package) Version 3.57c. Distributed by the author. Department of Genetics, University of Washington, Seattle.
Feyereisen, R., Farnsworth, D.E., 1987. Characterization and regulation of HMG-CoA reductase during a cycle of juvenile hormone synthesis. Mol. Cell. Endocrinol. 53, 227–238.
Gertler, F.B., Chiu, C.-Y., Richter-Mann, L., Chin, D.J., 1988. Developmental and metabolic regulation of the *Drosophila melanogaster* 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mol. Cell. Biol. 8, 2713–2721.
Gil, G., Goldstein, J.L., Slaughter, C.A., Brown, M.S., 1986a. Cytoplasmic 3-hydroxy-3-methylglutaryl coenzyme A synthase from the hamster. I. J. Biol. Chem. 261, 3710–3716.
Gil, G., Goldstein, J.L., Slaughter, C.A., Brown, M.S., 1986b. Cytoplasmic 3-hydroxy-3-methylglutaryl coenzyme A synthase from the hamster. II. isolation of the gene and characterization of the 5′ flanking region. J. Biol. Chem. 261, 3717–3724.
Goldstein, J.L., Basu, S.K., Brown, M.S., 1983. Receptor mediated endocytosis of low-density lipoprotein in cultured cells. Meth. Enzymol. 98, 241–260.
Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. Nature 343, 425–430.
Higashino, F., Yoshida, K., Fujinaga, Y., Kamio, K., Fujinaga, K., 1993. Isolation of a cDNA encoding the adenovirus E1A enhancer binding protein: a new human member of the ets oncogene family. Nucleic Acids Res. 21, 547–553.
Hultmark, D., Klemenz, R., Gehring, W.J., 1986. Translational and transcriptional control elements in the untranslated leader of the heat-shock gene hsp22. Cell 44, 429–438.
Lee, W., Mitchell, P., Tjian, R., 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49 (6), 741–752.
Löwer, R., Löwer, J., Kurth, R., 1996. The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. Proc. Natl. Acad. Sci. U. S. A. 93 (11), 5177–5184.
Martínez-González, J., Buesa, C., Piulachs, M.D., Bellés, X., Hegardt, F.G., 1993a. Molecular cloning, developmental pattern and tissue expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase of the cockroach *Blattella germanica*. Eur. J. Biochem. 213, 233–241.
Martínez-González, J., Buesa, C., Piulachs, M.D., Bellés, X., Hegardt, F.G., 1993b. 3-Hydroxy-3-methylglutaryl-CoA synthase from *Blattella germanica*. Eur. J. Biochem. 217, 691–699.
Mizobuchi, M., Frohman, L.A., 1993. Rapid amplification of genomic DNA ends. Biotechniques 15, 214–216.
Munderloh, U.G., Kurtti, T.J., 1989. Formulation of medium for tick cell culture. Exp. Appl. Acarol. 7, 219–229.
Peffley, D.M., Gayen, A.K., 1997. Inhibition of squalene synthase but not squalene cyclase prevents mevalonate-mediated suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase synthesis at a post-transcriptional level. Arch. Biochem. Biophys. 337, 251–260.
Petras, S.F., Lindsey, S., Harwood, H.J. Jr., 1999. HMG-CoA reductase regulation: use of structurally diverse first half-reaction squalene synthetase inhibitors to characterize the site of mevalonate-derived nonsterol regulator production in cultured IM-9 cells. J. Lipid Res. 40, 24–38.
Reynolds, G.A., Basu, S.K., Osborne, T.F., Chin, D.J., Gil, G., Brown, M.S. et al., 1984. HMG CoA reductase: a negatively regulated gene with unusual promoter and 5′ untranslated regions. Cell 38, 275–285.
Schnitzer-Polokof, R.V., Logel, J., Torget, R., Sinensky, M., 1982. Isolation and characterization of a mammalian cell mutant defective in 3-hydroxy-3-methylglutaryl coenzyme A synthase. J. Biol. Chem. 257, 472–476.
Silberkang, M., Havel, C.M., Friend, D.S., McCarthy, B.J., Watson, J.A., 1983. Isoprene synthesis in isolated embryonic *Drosophila* cells. I. Sterol-deficient eukaryotic cells. J. Biol. Chem. 258, 8503–8511.
Straka, M.S., Panini, S.R., 1995. Post-transcriptional regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by mevalonate. Arch. Biochem. Biophys. 317, 235–246.
Swofford, D.L., Olsen, G.J., 1990. Phylogeny reconstruction. In: Hillis, D.M., Moritz, C. (Eds.), Molecular Systematics. Sinauer, Sunderland, Massachusetts, pp. 411–501.
Wang, X.S., Brown, M.S., Hua, X., Goldstein, J.L., 1994. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. Cell 77, 53–62.
Weiner, A.M., 1986. Nonviral retroposons: genes, pseudogenes, and transposable elements generated by the reverse flow of genetic information. Annu. Rev. Biochem. 55, 631–661.
Williams, T., Tijan, R., 1991. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes Dev. 5, 670–682.