Insect Molecular Biology (2000) **9**(4), 385–392

Molecular cloning and structural analysis of 3-hydroxy-3-methylglutaryl coenzyme A reductase of the moth *Agrotis ipsilon*

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Abstract

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which plays a key role in isoprenoid biosynthesis, catalyses the synthesis of mevalonate from HMG-CoA. Insects do not synthesize cholesterol *de novo*, rather mevalonate derivatives lead to non-sterol isoprenoids which are essential for development and reproduction. In this paper, we describe an HMG-CoA reductase of the moth *Agrotis ipsilon* and we report its expression in fat body, ovary, muscle, brain and corpora allata tissues of adult specimens. The analysis of the cDNA reveals that it encodes a polypeptide of 833 amino acids (M_r = 89785). Alignments of this HMG-CoA reductase from *A. ipsilon* with the homologous sequences of other eukaryotes shows a high degree of conservation in all species studied. Parsimony analysis based on these alignments produced dendrograms congruent with the current systematic schemes. This suggests that, during eukaryote evolution, HMG-CoA reductase diversified in parallel with taxonomic splitting.

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Keywords: insect, isoprenoid, juvenile hormone, molecular phylogeny, *Agrotis ipsilon*, 3-hydroxy-3-methylglutaryl coenzyme A reductase, HMG-CoA reductase.

Introduction

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (EC 1.1.1.34) is a key enzyme in isoprenoid biosynthesis, catalysing the transformation of HMG-CoA to mevalonate, the common precursor for several classes of essential metabolites, including dolichols, ubiquinones, protein prenyl moieties, and sterols in many organisms (Brown & Goldstein, 1980; Goldstein & Brown, 1990). Due to its central role, HMG-CoA reductase has been extensively studied in mammals, plants and prokaryotes, but surprisingly to a lower rate in invertebrates including insects. In insects, however, the studies of the isoprenoids pathway is important since it plays an important role in physiology and it represents a unique model system for several reasons.

Insects cannot synthesize cholesterol *de novo* as they lack the two enzymes required beyond farnesyl pyrophosphate formation, namely squalene synthase and lanosterol synthase (Clayton, 1964). In mammals, HMG-CoA reductase has been shown to be precisely downregulated by cholesterol, at the level of both enzyme activity and gene expression (Rosser *et al.*, 1989; Wilkin *et al.*, 1990). Such a mechanism is unlikely to exist in insects as they obtain cholesterol from dietary sterols, and hence HMG-CoA reductase only participates in the synthesis of linear sesquiterpenes. Furthermore, there does not seem to be any regulation by sterols from exogenous origin (Brown *et al.*, 1983).

In insects, mevalonate is the precursor for juvenile hormones (JH), specific non-sterol sesquiterpene derivatives playing important roles in development and reproduction (Schooley & Baker, 1985). JH biosynthesis occurs in the corpora allata (CA), a small pair of retrocerebral endocrine glands. The brain regulates CA activity via stimulatory and inhibitory neuropeptides that act, at least in part, on

enzymes of the isoprenoid biosynthetic pathway (Stay & Woodhead, 1993). YXFGL-amide allatostatins, a family of brain–gut peptides which inhibit JH biosynthesis, also appear to inhibit isoprenoid synthesis in the fat body, a tissue that does not produce JH (Martin *et al*., 1996). This suggests that regulation of isoprenoid synthesis by neuropeptides may be an interesting singularity of insects.

To study these mechanisms of regulation, HMG-CoA reductase has been characterized by way of specific radiochemical tests (Kramer & Law, 1980; Law & Monger, 1982; Schooley & Baker, 1985; Bhaskaran *et al*., 1987; Feyereisen & Farnsworth, 1987). Notwithstanding, the small size of the CA makes biochemical assays difficult, and both the regulating factors and the molecular mechanisms sustaining them remain essentially unknown. Molecular characterizations of HMG-CoA reductase have been successfully accomplished in the fruit fly *Drosophila melanogaster* (Gertler *et al*., 1988) and in the German cockroach *Blattella germanica* (Martinez-Gonzalez *et al*., 1993), although the small size of the CA did not permit detection of the messenger RNA (mRNA) corresponding to these enzymes in the CA using Northern blot techniques. In addition to structural information, the comparison of insect HMG-CoA reductase sequences revealed some interesting features, like the relative isolation of that from *B. germanica* with respect to other sequences (Martinez-Gonzalez *et al*., 1993). In this context, cloning an HMG-CoA reductase from a lepidopteran species would be interesting also because CA from Lepidoptera produce a variety of additional JH homologues resulting from the synthesis of homomevalonate instead of mevalonate catalysed by HMG-CoA reductase (Schooley & Baker, 1985).

These reasons led us to isolate and characterize a cDNA encoding a lepidopteran HMG-CoA reductase. In this paper, we present the cloning of a full-length cDNA of a putative HMG-CoA reductase isolated from a cDNA library of *Agrotis ipsilon* brain/suboesophageal ganglion complex, and the study of its expression in a number of adult tissues, namely the fat body, the ovary, the muscles, the brain and the CA. Finally, a comparative analysis with HMG-CoA reductase sequences of other species is also reported.

Results and discussion

Isolation of HMG-CoA reductase cDNA from Agrotis ipsilon *library*

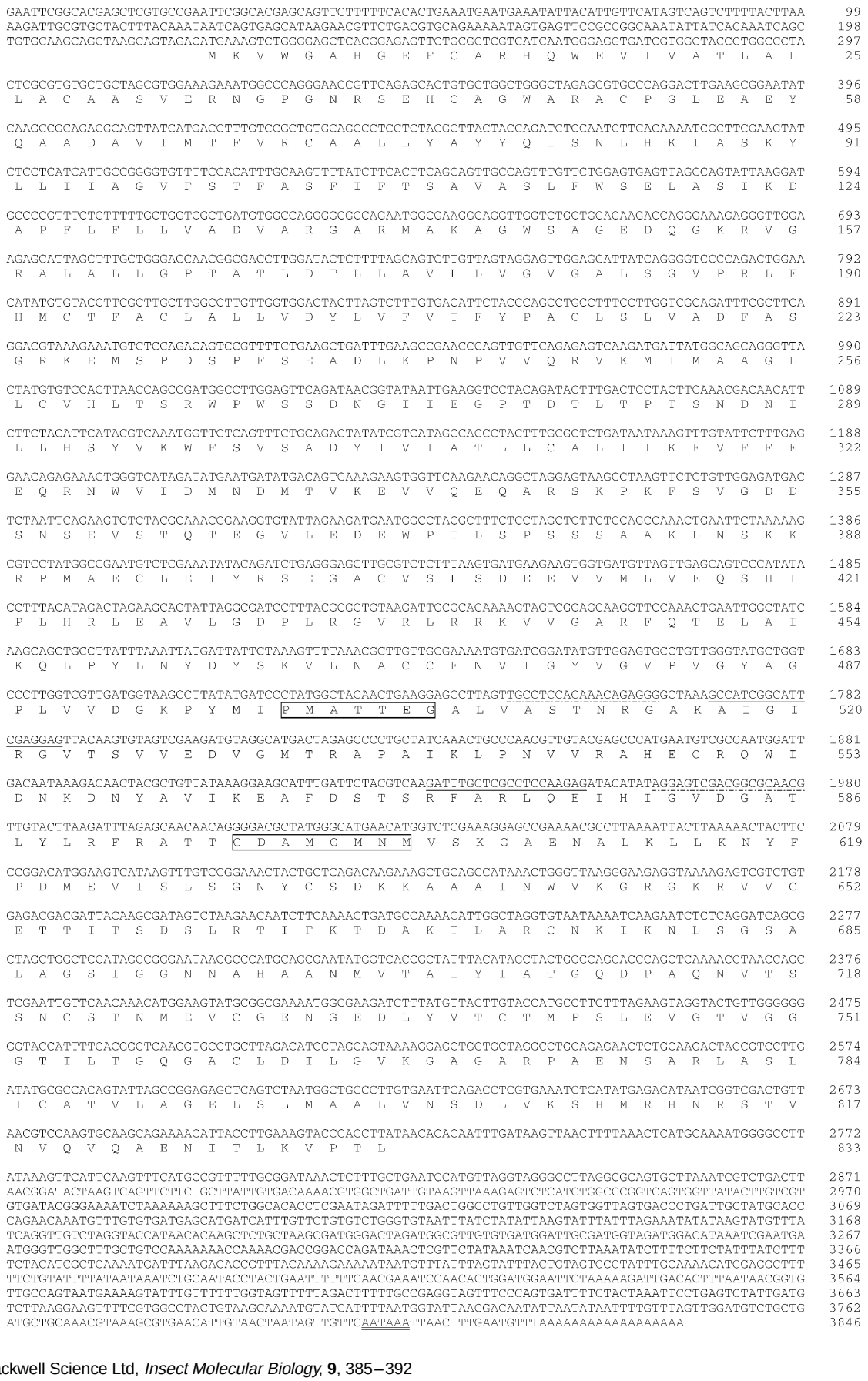
To clone HMG-CoA reductase cDNA from *A. ipsilon*, we postulated that the moth enzyme would contain highly conserved regions in the catalytic domain. We thus used degenerate primers previously designed to isolate HMG-CoA reductase cDNA from the cockroach *B. germanica* (Martinez-Gonzalez *et al*., 1993). Total RNA from brain–suboesophagal ganglion complexes of adult moth was used as template for reverse transcription polymerase chain reaction (RT-PCR). This strategy generated two PCR products with only one having the expected size for HMG-CoA reductase. Purification, reamplification, cloning and sequencing provided a nucleotide sequence from which the deduced amino acid sequence showed a high similarity with other HMG-CoA reductases. We postulated that this PCR product corresponded to part of the cDNA sequence of *A. ipsilon* HMG-CoA reductase.

Isolation of the full-length cDNA corresponding to the probe was performed by PCR screening of the cDNA library. Two primers (H1/H2) were designed on the basis of the probe sequence. Primers H1/H2 yielded a strong and specific amplification of a 183 bp fragment using a single rapid PCR method. The HI/H2 PCR was used to screen the cDNA library up to isolation of a single clone. One positive clone λ(AIHMGR-4) containing an insert of about 3820 bp was selected, and the corresponding phagemid (pAIHMGR-4) was excised and sequenced. The nucleotide sequence, starting at the most 5′ ATG triplet, reveals an open reading frame of 2499 bp and encodes for a polypeptide of 833 amino acid residues (M_r = 89 785 Da). It is flanked by untranslated regions 5′ and 3′ which are 222 and 1108 bp in length, respectively. The nucleotide sequence of this cDNA, and the deduced amino acid sequence, are shown in Fig. 1. A region containing the polyadenylation site was found at position 3793–3799 located 16 bp upstream of the poly(A) tail.

Expression of HMG-CoA reductase mRNA in different tissues

Expression of HMG-CoA reductase mRNA was assayed on different adult tissues. The RT-PCR procedure was chosen because Northern blot analysis is not sensitive enough to detect mRNA for an enzyme of the JH pathway in the CA (Castillo & Couillaud, 1999). Using two specific primers H3/H4 external to H1/H2 primers and surrounding the catalytic domain of HMG-CoA reductase, a PCR product of 236 bp was detected in all the tissues assayed, namely fat body, ovary, muscle, brain and CA from adult moths (Fig. 2). The 236 bp PCR product was used as template for a nested PCR using H1/H2 primers, and again a 236 bp product was detected in all the studied tissues (not shown), which affords additional arguments for the specificity of the amplification. Given that the analysis of PCR fragments was carried out after either thirty-five or forty cycles,

Figure 1. (Opposition) Complete nucleotide sequence and deduced amino acid sequence for *A. ipsilon* HMG-CoA reductase. Numbers on both sides refer to either the nucleotide sequence (1–3846) or to the amino acid sequence (1–833). Boxed amino acids correspond to the site used for the design of degenerate primers. Specific primers are underlined, H1/H2 used to screen the cDNA library (full line) and H3/H4 used for RT-PCR (dotted line).



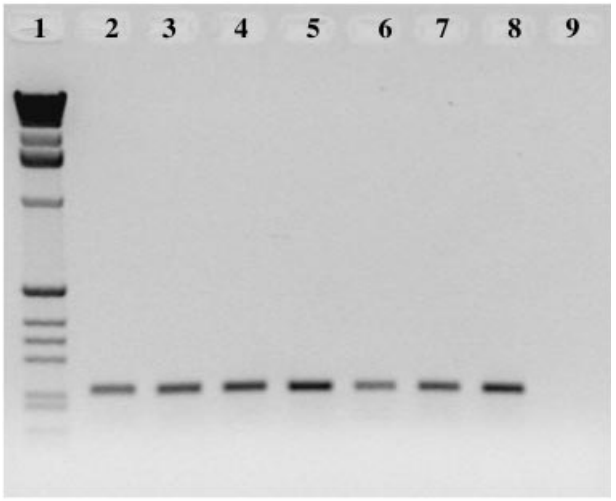


Figure 2. Expression of HMG-CoA reductase in different tissues of adult *A. ipsilon* determined by RT-PCR. Templates are mRNA from 5-day-old adult female fat body (1 mg, Lane 2), brain (1 brain, Lane 3), corpora allata (2 pairs, Lane 4), ovary (1 mg, Lane 5) or muscle (1 mg, Lane 6) or from 5-day-old adult male brain (1 brain, Lane 7) and corpora allata (2 pairs, Lane 8). No template was used in Lane 9 and 1 kb DNA ladder is shown in Lane 1.

a plateau phase was probably reached, thus leading to equivalent ethidium bromide fluorescence, and making quantification irrelevant. However, it is worth pointing out that starting material used for each sample corresponds to ≈ 1 mg of fresh tissues for fat body, ovary muscle and brain, but about 100 times less for male CA (two pairs, ≈ 10 μg) and 500 times less for female CA (two pairs, ≈ 2 μg). Furthermore, it is also worth noting that the PCR products cannot result from amplification of contaminant genomic DNA because if reverse transcription is omitted, then amplification does not occur (results of control experiments not shown).

Analysis of the amino acid sequence

The hydrophobicity plot of the protein deduced from the cDNA (Fig. 3) reveals the classical organization of an animal-type HMG-CoA reductase, with an N-terminal region containing the potential membrane-spanning domains, followed by a short hydrophilic linker which connects with the C-terminal region containing the catalytic domain. Eight potential membrane-spanning domains can be recognized in the hydrophobicity plot (Fig. 3), based on the algorithm of Kyte & Doolittle (1982). This number is in general agreement with the typical number in other animal HMG-CoA reductase sequences (Olender & Simon, 1992). The 'dense alignment surface' (DAS) method (Cserzo *et al.*, 1997) also predicts eight transmembrane domains, although the 'topology prediction of membrane proteins' (TopPred II) (von Heijne, 1992) was not able to recognize the number 4. The catalytic domain in the C-terminal region includes the His809, which is conserved in all HMG-CoA reductase sequences characterized to date (Bochar *et al.*, 1999).

Comparative analysis with other species

The protein sequence of *A. ipsilon* is between 50 and 65% similar to that of other animal species (Fig. 4). Not surprisingly, maximal similarity was found with the HMG-CoA reductase sequence from the fruit fly, *D. melanogaster*, the closest relative from a phylogenetic point of view, with 65% similarity and 55% identity. These percentages are even higher when comparing the catalytic domain in the C-terminal region (80% of similarity and 71% identity).

The PILEUP alignment of the HMG-CoA reductase sequence of *A. ipsilon* with other eukaryote species (not shown), which have the class I enzyme (Bochar *et al.*, 1999), reveals a high degree of conservation, especially in the catalytic domain. For example, the boundaries of the motifs known as HMG-CoA reductase signature 1 (residues 590–604), signature 2 (residues 746–753) and signature 3 (residues 799–812), align well with the homologous regions of these HMG-CoA reductase sequences. A parsimony analysis carried out with the PILEUP alignment produced a tree (Fig. 5) with a topology congruent with the conventional phylogenetic schemes. In general, plant species are clustered in a phylogenetically logical manner, for example separating monocotyledonous (*Zea*, *Oryza*) from dicotyledonous (the remaining species), showing nodes that lead to plant family levels (Solanaceae: *Solanum*, *Lycopersicon*, *Nicotiana*, *Capsicum*; Brassicaceae: *Raphanus* 3 and 8, *Arabidopsis* 1). Although the representation is more limited, the species of fungi, protists and animals also generally cluster in phylogenetically coherent groups, with the fungi

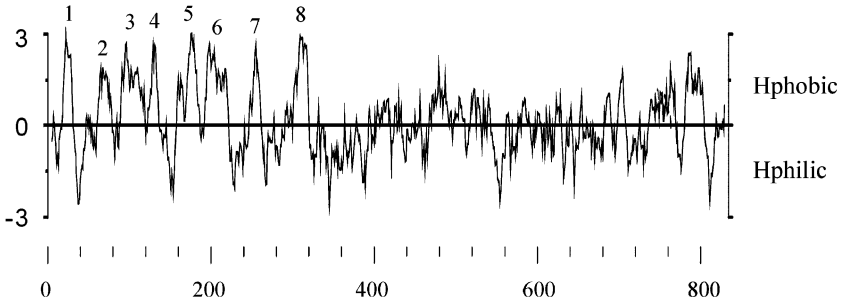


Figure 3. Hydrophobicity plot of the amino acid sequence predicted from *A. ipsilon* HMG-CoA reductase cDNA. The PEPPLOT program from the GCG package was used. numbers 1–8 indicate the potential transmembrane domains.

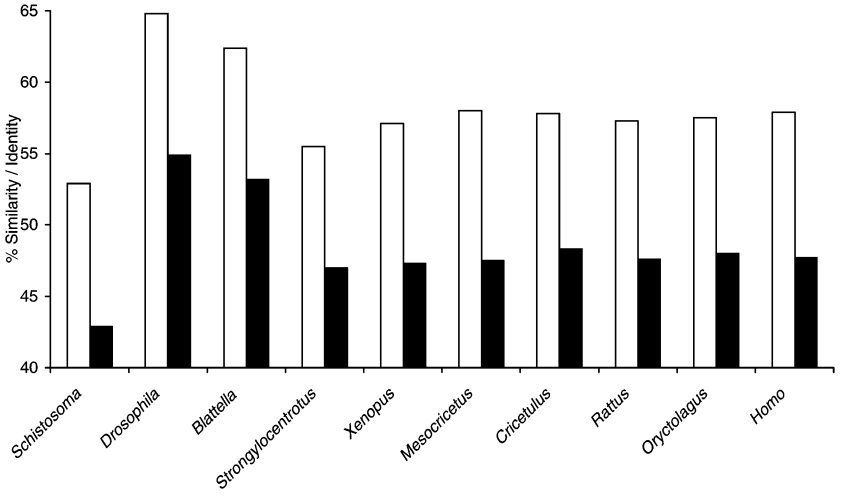


Figure 4. Percentage of similarity (white columns) and identity (black columns) of the HMG-CoA reductase sequence of *Agrotis ipsilon* with respect to other animal species: *Schistosoma mansoni*, *Drosophila melanogaster*, *Blattella germanica*, *Strongylocentrotus purpuratus*, *Xenopus laevis*, *Mesocricetus auratus*, *Cricetulus griseus*, *Rattus norvegicus*, *Oryctolagus cuniculus* and *Homo sapiens*. GenBank accession numbers have been indicated in Experimental procedures. The BESTFIT program from the GCG package was used.

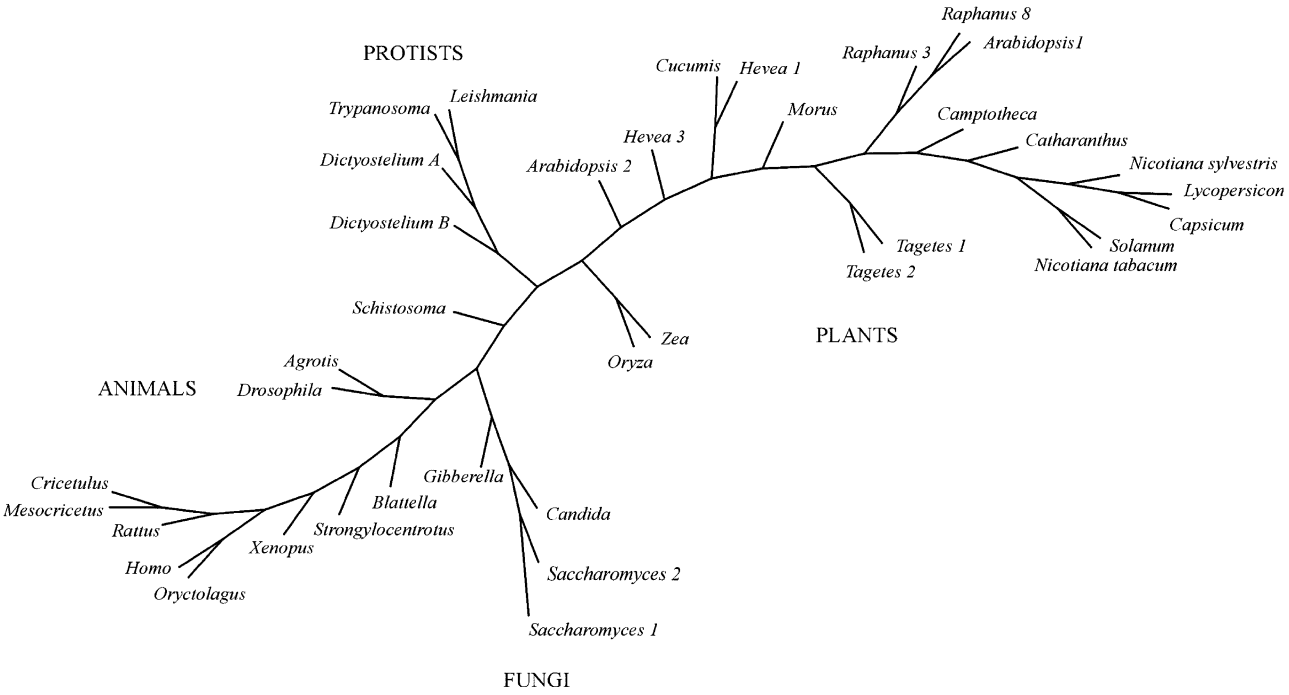


Figure 5. Parsimony analysis of the amino acid sequences of HMG-CoA reductase in eukaryotes, using the alignment generated by the PILEUP program from the GCG package (not further refined), and the PROTPARS program from PHYLIP package. Complete specific names and the corresponding GenBank accession numbers have been indicated in Experimental procedures.

appearing as the sister group of animals, as in other protein-based phylogenies recently reported (see, for example, Doolittle *et al.*, 1996).

Given the general phylogenetic congruence of the HMG-CoA reductase-based eukaryote tree (Fig. 5), a more precise analysis on animal species was carried out. Parsimony analysis based on a PILEUP alignment of animal species, using the fungi *Candida utilis* as outgroup, gave the dendrogram represented in Fig. 6. Bootstrap values were high in all nodes, and, again, the topology was coherent with the current phylogenies based in different characters (see, for example, Avise, 1994). Concerning the insect species, all three are integrated in a phylogenetically coherent manner.

Apparently, the ability of CA of *Agrotis* to synthesize both mevalonate and homomevalonate that leads to the JH-II homologue (Duportets *et al.*, 1996) is not reflected by the position of *Agrotis* in the dendogram relative to

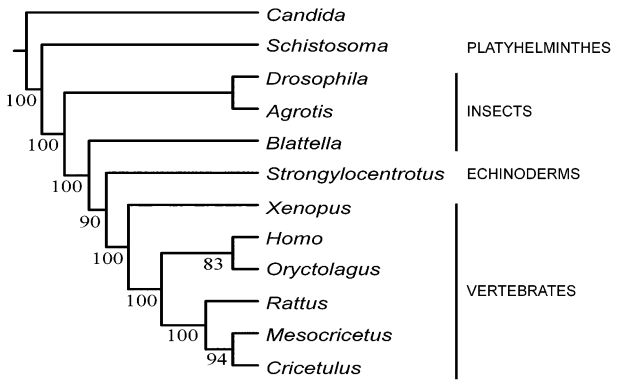


Figure 6. Parsimony analysis of the amino acid sequences of HMG-CoA reductase available in animals, using the fungi *Candida utilis* as outgroup. The analysis was carried out with the PROTPARS program from PHYLIP package, from an alignment generated by the PILEUP program from the GCG package which was 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 Experimental procedures.

Blattella and *Drosophila* which only produce mevalonate (Schooley & Baker, 1985). This is fairly consistent with data showing that CA from cockroaches can be forced to synthesize homomevalonate when supplied with propionate, thus suggesting that lepidopterans produce homomevalonate because of their ability to generate 3-hydroxy-3-ethylglutaryl-CoA from propionyl-CoA or 3-ketovaleryl-CoA. This would be associated with a weak substrate specificity of the HMG-CoA reductase and the followings enzymes in the JH biosynthetic pathway (Feyereisen & Farnsworth, 1988). Our RT-PCR experiments indicate that the mRNA sequence encoding the catalytic domain of the HMG-CoA reductase is expressed in the CA. However, the possibility that a different HMG-CoA reductase form is specifically expressed in the CA cannot be ruled out.

In summary, the topology of the dendrogam (Fig. 6) reflects the phylogeny of the insect groups included in it, as occurs in other eukaryotes (Fig. 5). It seems that during eukaryote evolution the HMG-CoA reductase diversified according to the taxonomic divergence. This suggest that the amino acid sequence of the HMG-CoA reductase appears to be phylogenetically informative, and could be a useful tool not only to study the molecular evolution of this particular enzyme, but also in phylogeny reconstruction of eukaryote groups.

Experimental procedures

Insects

Adult moths *A. ipsilon* were reared on an artificial diet and maintained at 21 ± 1 °C, 60% relative humidity and a 16L : 8D photoperiod according to Poitout & Buès (1974). Day 0 refers to the first day of the adult life. Adults were kept in plastic boxes and fed with a 20% sucrose solution.

Isolation and cloning of A. ipsilon *HMG-CoA reductase*

Twenty brain–suboesophagal ganglion complexes including the corpora allata of adult moths were dissected out together and immediately frozen in liquid nitrogen, then stored at –80 °C. Total RNA was isolated using TRIzol Reagent (Gibco BRL). An aliquot of total RNA (1 μg) was used as template for RT-PCR using the Gene Amp RNA PCR Kit (Perkin Elmer) with an oligo(dT) primer and according to the manufacturer's recommendations.

The resulting cDNAs were directly used for PCR amplification with the following final conditions: 100 μl final volume of 1× PCR buffer provided by the manufacturer, 1 μM of primers, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 U of Taq DNA polymerase (Perkin Elmer). PCR was carried out in a Perkin Elmer Gene Amp PCR System 2400 programmed for thirty-five cycles (1 min at 94 °C, 30 s at 35 °C, 1.30 min at 72 °C). Primers were two degenerate set previously designed from highly conserved amino acid sequences of the HMG-CoA reductase catalytic domain (Martinez-Gonzalez *et al.*, 1993).

Two PCR products of about 330 and 140 bp were obtained on a TAE (Tris-acetate 40 mM, EDTA 1 mM, pH 8.3), 2.5% agarose gel. The 330 bp PCR product was excised from the ethidium bromide stained gel and purified using Wizard PCR preps purification system (Promega). One-fiftieth of the purified product was used as template for a second round of PCR amplification under the same conditions. The resulting PCR product was cloned using pCR-Script Amp SK(+) Cloning Kit (Stratagene) and clone pAIHMGR-1 was sequenced by Eurogentec (Seraing, Belgium).

From pAIHMGR-1 sequence, the forward primer H1, 5′-GCCATCGGCATTCGAGGAG-3′, and the reverse primer H2, 5′-CTCTTGGAGGCGAGCAAATC-3′, were designed and used to screen a cDNA library of *A. ipsilon* brain–subesophageal ganglion complex (Duportets *et al.*, 1999). About $n = 60\,000$ pfu were plated following standard procedures and after an overnight growth sixteen equal portions of the top-agar were scraped and eluted in 500 μl of SM (NaCl 0.1 M; $HgSo_4$ 8 mM; Tris-HCl 50 mM, pH 7.5) buffer. Agar was eliminated by centrifugation (10 000 ***g***, 10 min) and supernatants were used as template for PCR amplification with the following final conditions: 1 μM of primers H1 and H2, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1 U of Taq DNA polymerase (Appligene) in a 50 μl final volume of 1× buffer provided by Appligene. PCR was programmed for 1 min 45 s at 94 °C + 35 × (15 s at 94 °C, 30 s at 48 °C) + 7 min at 72 °C. One of the fractions from the cDNA library that gave a PCR product of 183 bp was tittered, and an aliquot containing about five times the number of plaques count in the positive agar portion was replated, grown and portioned in sixteen equal parts each of which was PCR tested. The same procedure was repeated until the complete isolation of a single phage (λAIHMG-4) was achieved. Phagemid pAIHMGGR-4 was excised from λAIHMGR-4 and sequenced (Genome Express, Grenoble, France).

Tissues expression of A. ipsilon *HMG-CoA reductase*

Five-day-old mated adult male and female moths were used to dissect out the tissues, which were immediately frozen at –80 °C. Tissues sampled were fat body, ovary or muscles (≈ 10 mg in the three cases), ten brains (≈ 10 mg of tissue) and twenty pairs of CA (≈ 100 ng of tissue in the case of males and ≈ 20 ng in that of

females). Poly(A) + RNA was isolated directly from tissues using 100 µl of Dynabeads oligo (dT)25, according to the manufacturer's protocol. Dynabeads were aliquoted in ten tubes and directly used as template for RT-PCR. RT-PCR was performed using the Superscript one-step RT-PCR system (Life technologies) in a 50 µl final volume. Thermocycler was programmed for 30 min at 50 °C, 2 min at 94 °C + 40 × (30 s at 94 °C, 30 s at 45 °C, 60 s at 68 °C) + 7 min at 72 °C. Specific primers were used for both reverse transcription and PCR: a forward primer H3, 5′-TGCCTCCACAAACAGAGGG-3′, and a reverse primer H4, 5′-CGTTGCACCATCGACTCCT-3′, external to the H1/H2 primers. The expected size for PCR product was 236 bp.

To check for a possible amplification from contaminant genomic DNA, two series of samples were processed together. In one series the enzyme mix was added before placing tubes in the thermocycler. In the second, the enzyme mix was introduced during the 94 °C phase following the reverse transcription sequence and before the PCR sequence.

A nested PCR was also performed using 1 µl of the H3/H4 RT-PCR product as template for amplification using H1/H2 primers according to conditions described above for library screening.

Sequence alignments and comparisons

For comparative purposes, in addition to the sequence from *A. ipsilon* described in the present paper (GenBank accession number AJ009675), we used the previously published amino acid sequences of HMG-CoA reductase of the following eukaryote species (GenBank accession number in parentheses): *Arabidopsis thaliana* (isoform 1) (A32107), *A. thaliana* (isoform 2) (P43256), *Blattella germanica* (S30338), *Camptotheca acuminata* (U72145), *Candida utilis* (AB012603), *Capsicum annuum* (AF110383), *Catharanthus roseus* (Q03163), *Cricetulus griseus* (A93328), *Cucumis melo* (AB021862), *Dictyostelium discoideum* (isoform A) (L19349), *D. discoideum* (isoform B) (L19350), *Drosophila melanogaster* (S32572), *Gibberella fujikuroi* (Q12615), *Hevea brasiliensis* (isoform 1) (S14955), *H. brasiliensis* (isoform 3) (S22521), *Homo sapiens* (A00356), *Leishmania major* (AF155593), *Lycopersicon esculentum* (S25316), *Mesocricetus auratus* (:A23586), *Morus alba* (U43711), *Nicotiana sylvestris* (S24760), *N. tabacum* (U60452), *Oryctolagus cuniculus* (Q29512), *Oryza sativa* (AF110382), *Raphanus sativus* (isoform 3) (S29622), *R. sativus* (isoform 8) (S29623), *Rattus norvegicus* (M29249), *Saccharomyces cerevisiae* (isoform 1) (A30239), *S. cerevisiae* (isoform 2) (B30239), *Schistosoma mansoni* (A34416), *Solanum tuberosum* (S59944), *Strongylocentrotus purpuratus* (A31898), *Tagetes erecta* (isoform 1) (AF034760), *T. erecta* (isoform 2) (AF034761), *Trypanosoma cruzi* (L78791), *Xenopus laevis* (A35728) and *Zea mays* (Y09238).

Software from Genetics Computer Group (GCG, v. 9.1), University of Wisconsin (Devereux *et al.*, 1984), was used for sequence analysis. The hydrophobicity plot of the amino acid sequence was obtained with the application PEPPLOT, which is based on the algorithm of Kyte & Doolittle (1982). 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, v. 3.57c) (Felsenstein, 1995), using amino acid sequences (PROTPARS program) (Swofford & Olsen, 1990). Bootstrap analyses were carried out with the application SEQBOOT in the PHYLIP package, and the procedure was repeated 100 times.

Data deposition

The sequence reported in this paper has been deposited in EMBL/Genebank/DDBJ databases (Accession number AJ009675).

Acknowledgements

We thank Dr Christophe Gadenne for providing and dissecting insects. Financial support from the DGICYT, Spain (project No PB95–0062) to XB and the Spanish–French program PICASSO are gratefully acknowledged.

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