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Molecular cloning, developmental pattern and tissue expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase of the cockroach *Blattella germanica*

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In insects, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) synthesizes mevalonate for the production of nonsterol isoprenoids, which are essential for growth and differentiation. To understand the regulation and developmental role of HMG-CoA reductase, we have cloned a full-length HMG-CoA reductase cDNA from the cockroach *Blattella germanica*. This cDNA clone was isolated using as a probe a partial cDNA of *B. germanica* HMG-CoA reductase, amplified using the polymerase chain reaction. The composite 3433-bp cDNA sequence contains an open reading frame encoding a polypeptide of 856 amino acids (M_r 93165). The C-terminal region is more similar to hamster HMG-CoA reductase than is the *Drosophila melanogaster* enzyme (79% and 69% conserved residues, respectively), and the potential transmembrane domains at the N-terminal region are structurally conservative with both enzymes. The C-terminal region of the *B. germanica* protein has been expressed as a fusion protein in *Escherichia coli* and exhibits HMG-CoA reductase activity. Analysis of *B. germanica* HMG-CoA reductase mRNA levels, reveals a 3.6-kb transcript, that is overexpressed in 4-day-old embryos. Northern-blot analysis of RNA samples from different adult female tissues shows high HMG-CoA reductase mRNA levels in the ovary and lower levels in brain and muscle.

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) catalyzes the transformation of HMG-CoA to mevalonate, the common precursor to sterols, dolichol, ubiquinone, isopentenyl adenosine and isoprenylated proteins [1, 2]. The eukaryotic enzyme is a transmembrane glycoprotein located mainly in the endoplasmic reticulum [3]. Modeling studies of the deduced primary sequence of HMG-CoA reductase from a variety of organisms suggest that it consists of a N-terminal transmembrane region and a C-terminal region joined by a linker region. The C-terminal domain projects into the cytosol and contains the catalytic site [4].

Earlier molecular studies of insect HMG-CoA reductase have been carried out by Gertler et al. [5] who report the characterization of cDNA encoding the HMG-CoA reductase from *Drosophila melanogaster*. The study of this enzyme in insects is important since it represents a model system for several reasons as follows: insects cannot synthesize cholesterol *de novo* [6] because they lack the enzymes squalene synthetase and lanosterol synthase [7]; HMG-CoA reductase from these organisms does not seem to be regulated by sterols [8]; insects produce juvenile hormones, specific isoprenoid derivatives which have an important role in development and reproduction [9].

Vertebrate sterol biosynthesis and juvenile hormone biosynthesis in insect *corpora allata* share a common pathway up to farnesyl pyrophosphate synthesis [10]. Since HMG-CoA reductase is generally referred to as the key regulatory enzyme in cholesterol synthesis [1], it has been suggested that HMG-CoA reductase could also be the rate-limiting enzyme of the juvenile-hormone-biosynthesis pathway. In apparent agreement with this hypothesis, studies of different insect species have shown that juvenile hormone biosynthesis in the *corpora allata* can be blocked by HMG-CoA reductase inhibitors [10]. This is also the case for the cockroach *Blattella germanica*, in which the fungal metabolite, compactin, a potent inhibitor of HMG-CoA reductase, inhibits juvenile hormone biosynthesis by *in-vitro* incubated *corpora allata* [11], whereas mevalonate restores normal *corpora allata* behavior [12].

Considering the modern theories of metabolic control, some authors have argued against the rate-limiting role for HMG-CoA reductase in juvenile hormone biosynthesis [12, 13]. However, it seems clear that the contribution of the enzyme to the regulation of this pathway is highly significant. In addition, besides the role of HMG-CoA reductase in ju-

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Abbreviations. HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PCR, polymerase chain reaction; ORS, reductase oligonucleotides set; IPTG, isopropyl β-thiogalactopyranoside.

Enzymes. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34); squalene synthetase (farnesyl-diphosphate farnesyltransferase; EC 2.5.1.21); lanosterol synthase (2,3-epoxysqualene lanosterol-cyclase; EC 5.4.99.7).

Note. The novel amino acid sequence data published here have been submitted to the EMBL sequence data bank and shall be available under accession number X70034.

venile hormone biosynthesis, isoprenoid derivatives [8, 14] and isoprenyl-modified proteins [15, 16] play diverse and essential roles in insect development.

All these considerations prompted us to isolate and characterize a cDNA encoding *B. germanica* HMG-CoA reductase and determine the expression pattern throughout its developmental stages and in different tissues, as a contribution to the knowledge of the role that this enzyme plays in insects isoprenoid biosynthesis pathway.

MATERIALS AND METHODS

Insects

Experimental specimens of *B. germanica* were taken from a colony reared in the dark at 30±1°C and 60−70% relative humidity. The tissues chosen to study mRNA levels by blot analysis were carefully explanted from 5−6-day-old virgin females. To study mRNA levels during development, RNA was isolated from whole specimens of both sexes in each larval instar. In the case of embryos, RNA was isolated from 4, 12 and 17-day-old oothecae from mated females, in which the presence of spermatozoids in the spermatecae had been assessed.

Oligonucleotide primers

Two degenerated sets of oligonucleotides, derived from highly conserved amino acid sequences of the HMG-CoA reductase catalytic domain, were obtained from Operon Technologies. Reductase oligonucleotides set 1 (ORS_1; a sense strand composed of 20 nucleotides, with 512-fold degeneracy) corresponds to amino acid residues -PMATTEG- (positions 553−559 in hamster protein). Reductase oligonucleotides set 2 (ORS_2; an antisense 24-nucleotide strand, with 256-fold degeneracy) corresponds to amino acid sequence -GDAMGMNM- (positions 651−658 in hamster protein).

ORS_1, 5′-CC(N)ATGGC(N)AC(N)AC(N)GA(R)GG-3′; ORS_2, 5′-CAT(R)TTCAT(N)CCCAT(N)GC(R)TC(N)CC-3′. (Degenerate positions are indicated in parentheses in the following code. R, either A or G; N, A, C, G or T.)

cDNA synthesis and polymerase-chain-reaction conditions

Poly(A)-rich RNA from 6-day-old adult females was used to generate oligo(dT)-primed double-stranded cDNA as described [17]. The extension was carried out with Moloney-murine leukaemia virus reverse transcriptase (Pharmacia). An aliquot of cDNA pool (≈25 ng) and 25 pmol of each set of primers (ORS_1 and ORS_2) was used for polymerase-chain-reaction (PCR) amplification. PCR was performed with a Geneamp Kit (Cetus) in a final volume of 50 µl, following the manufacturer's recommendations. The DNA thermal cycler (Perkin Elmer-Cetus) was programmed for 40 cycles using the following procedure: denaturation, 94°C for 40 s; annealing, 50−60°C for 2 min; polymerization, 72°C for 1 min.

Screening of λgt-10 cDNA libraries

The amplification cDNA (pRB-315) was used as a probe in a screen of 500000 plaques from a λgt-10 cDNA library prepared from 6-day-old adult females mRNA. The conditions for screening λgt-10 bacteriophage were as described [17].

DNA sequencing

PCR-amplification products and cDNA clones were subcloned into pBluescript vectors (Stratagene) and sequenced by using the dideoxy chain termination method [18] with-modified T7 DNA polymerase (Sequenase, United States Biochemical).

DNA blot analysis

The amplified PCR products were electrophoresed in a 2% agarose gel and transferred to Z-probe nylon membranes (Bio-Rad). Filters were prehybridized and hybridized as described [17], using as a probe an 111-bp *Hae*III/*Rsa*I fragment from pRed-227, a clone containing Chinese hamster HMG-CoA reductase cDNA (obtained from J. L. Goldstein and M. S. Brown, University of Texas, Health Science Center, Dallas, USA). Washes were carried out at 40°C with 0.30 M NaCl/0.030 M sodium citrate, pH 7.0, and 0.2% SDS.

Genomic blot analysis was carried out according to [17]. *B. germanica* genomic DNA was digested with *Bam*HI, *Eco*RI and *Hin*dIII restriction enzymes. DNA fragments were electrophoretically fractionated and bound to a Hybond™ C-Extra membrane (Amersham). Filters were hybridized, using pRB-315 insert as a probe.

RNA blot analysis

Total RNA from *B. germanica* was isolated by the guanidinium chloride/cesium chloride method [17] with minor modifications. Poly(A)-rich RNA was purified by oligo(dT)-cellulose chromatography, according to [17]. RNA samples were fractionated in 1% agarose/formaldehyde gels, transferred to Nytran-N membranes (Schleicher & Schuell) and cross-linked with ultraviolet radiation. Hybridization and washes were as described [17]. λBgRD-12 insert was used as a probe.

Expression of *B. germanica* HMG-CoA reductase in *Escherichia coli*

The λBgRD-18 cDNA insert, which encompassed part of the linker region and the entire catalytic domain of *B. germanica* HMG-CoA reductase (residues 380−856), was subcloned in the *Eco*RI site of the prokaryotic expression vector pT7-7 [19]. The new plasmid (pRBEx18) was used to transform the *E. coli* strain BL21(DE3) in which the expression occurred. After induction by isopropyl β-thiogalactopyranoside (IPTG), the newly synthesized proteins were radiolabeled with [^{35}S]methionine. An analogous experiment was carried out by adding rifampicin to the medium 30 min after induction, to minimize radiolabeling of the newly synthesized endogenous proteins. Cells were collected by centrifugation and lysed by sonication. Total proteins were measured [20] and the lysate was then used either for enzyme assays or protein fractionation by SDS/PAGE [21]. To detect radiolabeled proteins, the gel was subjected to fluorography and exposed overnight to an X-ray autoradiographic film with intensifying screens.

Assay of HMG-CoA reductase activity

HMG CoA reductase activity was determined by the radiometric method described by Goldstein et al. [22]. 1 U

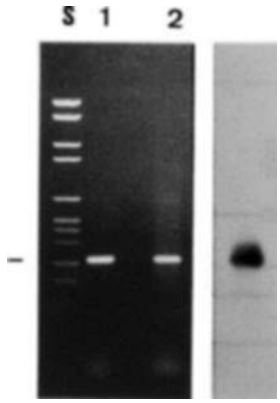


Fig. 1. Gel electrophoresis and Southern-blot analysis of amplified PCR products. After 40 cycles of amplification using ORS_1 and ORS_2 as primers, PCR products were run in a 2% agarose gel. Left, ethidium-bromide staining of the gel. Lane 1, amplified products using double-stranded cDNA pool from adult females *B. germanica* as template; lane 2 (control), amplified product using pRed-227 as template. Right, Southern blotting of lane 1 using, as probe, a 111-bp *Hae*III/*Rsa*I fragment of pRed-227 (nucleotides 1816–1927) [23]. Marker at left points to an ≈318-bp DNA fragments amplified in the PCR reactions.

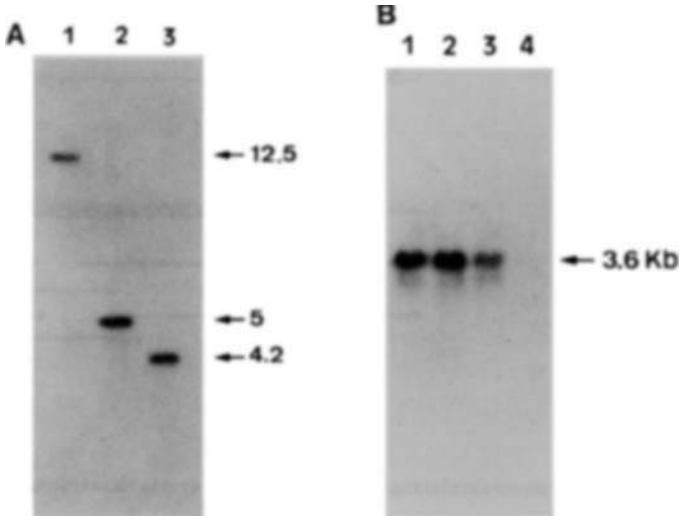


Fig. 2. Southern-blot and Northern-blot analyses. (A) *B. germanica* DNA (5 µg/lane) was digested with *Bam*HI (lane 1), *Eco*RI (lane 2) and *Hin*dIII (lane 3). Fragments were electrophoretically fractionated, bound to a Hybond™ C-Extra filter and hybridized to the radiolabeled pRB-315 insert. DNA fragment sizes are indicated on the right (kb). (B) Poly(A)-rich RNA (5 µg/lane) from 6th instar larvae (lane 1) and from whole bodies and heads of 6-day-old adult females (lanes 2 and 3) was run in a 1% agarose/formaldehyde gel, transferred to a Nytran-N membrane (Schleicher & Schüll) and hybridized to the radiolabeled pRB-315 insert. Lane 4 (control), 5 µg poly(A)-depleted RNA markers (Promega) were used to estimate size.

HMG CoA reductase is defined as the amount of enzyme which converts 1 nmol HMG-CoA into mevalonate in 1 min at 37°C.

RESULTS

Isolation of a cDNA for HMG-CoA reductase from *B. germanica*

The comparison of the primary structure of HMG-CoA reductases previously reported [5, 23–29] revealed several highly conserved regions in the catalytic domain. Two of these motifs were selected for the synthesis of oligonucleotides to be used as primers in PCR experiments. After PCR amplification, a cDNA fragment of the expected size (315–318 bp) was obtained with annealing temperatures of 50–60°C. To check the specificity of the process, the cDNA amplification products were analyzed by Southern-blot analysis using as a probe the 111-bp *Hae*III/*Rsa*I fragment of pRed-227 [23], which did not contain any of the primer sequences and only the major product hybridized (Fig. 1). Sequence analysis of this major amplification product (pRB-315) showed an open reading frame coding for 105 amino acid residues exhibiting high identity to other HMG-CoA reductase sequences.

A genomic Southern-blot analysis, using pRB-315 as a probe, yielded a hybridization pattern showing that the putative HMG-CoA reductase gene is present as a single copy in the *B. germanica* genome (Fig. 2A). Analysis of *B. germanica* mRNA by Northern-blot analysis using this cDNA as a probe, showed that the size of the transcript corresponding to this gene is 3.6 kb (Fig. 2B).

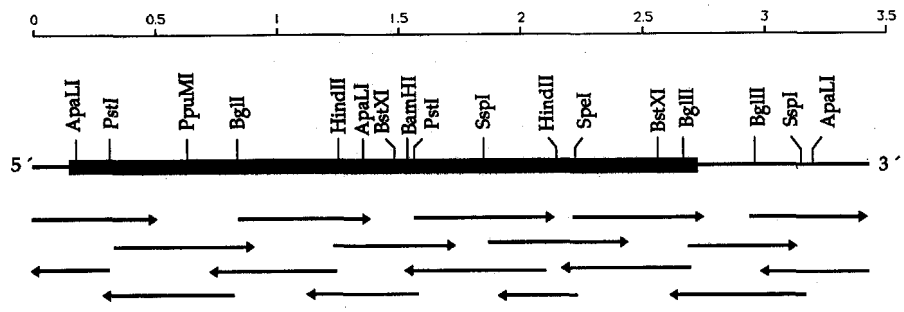
In a screening of a λgt-10 cDNA library from *B. germanica* adult females, several positive clones were obtained and characterized by restriction-enzyme mapping. Clone λBgRD12, which contained the longest insert, was subcloned for further analysis. The restriction map and sequencing strategy of this clone are shown in Fig. 3A. The sequence of λBgRD12 reveals an open reading frame of 2568 bp, coding for a polypeptide of 856 amino acid residues (M_r 93165) flanked by 5′ and 3′ untranslated regions, of 152 bp and 713 bp, respectively. The nucleotide sequence of this cDNA and the predicted amino acid sequence of the protein are shown in Fig. 3B. The position of the initiator methionine was assigned from the observation that there was no other AUG codon in frame in the 5′ upstream region and by comparison of the N-terminus with other HMG- CoA reductase sequences. The translated region of BgRD12 cDNA showed an identity of 56% and 60% to that of *D. melanogaster* and Chinese hamster HMG-CoA reductase cDNA, respectively. A canonical polyadenylation site (AAUAAA), 25 bp upstream of the poly(A)-rich region, was found (nucleotide 3238, Fig. 3B).

Analysis of the deduced amino acid sequence of *B. germanica* HMG-CoA reductase

The method of Kyte and Doolittle [30] was applied to predict the hydropathy plot from the deduced amino acid sequence of *B. germanica* HMG-CoA reductase. This analysis revealed the occurrence of the three characteristic regions of the HMG-CoA reductase [4]; a hydrophobic N-terminal domain (residues 1–364) connected by a highly hydrophilic linker region (residues 365–462) to a C-terminal domain (residues 463–856) that contains the catalytic site. The hydrophobic N-terminal domain comprises seven (or eight, see Discussion) potential transmembrane spans. The location of each of these spanning regions and their similarity to hamster and *D. melanogaster* hydropathy plots are shown in Fig. 4.

B. germanica HMG-CoA reductase presents four potential glycosylation sites (residues 326, 412, 700 and 838) and

A



B

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                                                                -152      GGCCGGTATTAATAAATTTCTATCATTTGAAA
-120  TGTTGTAAGTGTATTAAAAATTGCCATGTGGAAAGATTGAATGAAAATAGCGGAGAACGTTAAGTGGCCGATTGACGTTCTGTCTCCGCTAGCTGGTTCGAGTGAGCCGGTTAGATCATA

   1  ATGGTTGGGCGGTTGTTTCGTGCACATGGTCAATTCTGTGCCTCACATCCATGGGAAGTGATAGTTGCAACCCTCACGCTGACAGTATGCATGCTGACAGTAGATCAGCGGCCCTTAGGA
   1  MetValGlyArgLeuPheArgAlaHisGlyGlnPheCysAlaSerHisProTrpGluValIleValAlaThrLeuThrLeuThrValCysMetLeuThrValAspGlnArgProLeuGly

 121  CTTCCTCCTGGATGGGGACATAATTGTATCACACTGGAGGAGTACAATGCTGCAGACATGATAGTGATGACATTGATCCGCTGTGTGGCAGTCCTTTACAGTTACTACCAATTCTGCCAT
  41  LeuProProGlyTrpGlyHisAsnCysIleThrLeuGluGluTyrAsnAlaAlaAspMetIleValMetThrLeuIleArgCysValAlaValLeuTyrSerTyrTyrGlnPheCysHis

 241  CTCCAGAAGCTGGGCTCGAAATATATTCTTGGTATTGCTGGCCTCTTCACTGTCTTCTCCAGTTTTGTCTTCAGTTCCAGTGTCATCAACTTCCTTGGTAGTGATGTTTCTGACTTAAAA
  81  LeuGlnLysLeuGlySerLysTyrIleLeuGlyIleAlaGlyLeuPheThrValPheSerSerPheValPheSerSerSerValIleAsnPheLeuGlySerAspValSerAspLeuLys

 361  GATGCTCTCTTTTTCTTCTTACTGCTGATTGATTTATCAAAAGCAACAGTTCTGGCTCAGTTTGCCCTGAGTTCACGCAGTCAAGATGAAGTGAAGCACAACATTGCAAGAGGGATAGCC
 121  AspAlaLeuPhePhePheLeuLeuLeuIleAspLeuSerLysAlaThrValLeuAlaGlnPheAlaLeuSerSerArgSerGlnAspGluValLysHisAsnIleAlaArgGlyIleAla

 481  ATGCTGGGTCCCACTATTACCCTGGACACTGTCGTAGAGACGCTTGTCATTGGTGTAGGCATGTTATCAGGCGTGCGTCGATTGGAAGTACTGTGCTGTTTTGCCTGCATGTCAGTTATT
 161  MetLeuGlyProThrIleThrLeuAspThrValValGluThrLeuValIleGlyValGlyMetLeuSerGlyValArgArgLeuGluValLeuCysCysPheAlaCysMetSerValIle

 601  GTAAATTACGTGGTGTTCATGACATTTTATCCAGCATGTCTATCTCTAATACTTGAGTTGTCTCGCAGTGGTGAAAGTGGTCGGCCTGCTTGGCATGACAAATCATTGATCATAAAGGCT
 201  ValAsnTyrValValPheMetThrPheTyrProAlaCysLeuSerLeuIleLeuGluLeuSerArgSerGlyGluSerGlyArgProAlaTrpHisAspLysSerLeuIleIleLysAla

 721  CTTCATGAGGAAGACCAAAAACCAAATCCAGTTGTTCAACGTGTGAAGGTGATAATGTCAGCAGGGTTGATGCTAGTCCATGCACATAGGTGGGTAAGATGCCTATCTATTGCTCTCTGG
 241  LeuHisGluGluAspGlnLysProAsnProValValGlnArgValLysValIleMetSerAlaGlyLeuMetLeuValHisAlaHisArgTrpValArgCysLeuSerIleAlaLeuTrp

 841  CCTGACCTGACCTCGCTCCGCTATTTCTGTACACATTGTGATACTGGTGTTTCTTACAGTCGCTGGTCTTTTGCAAGCGAAGGTGAGGAATTACCCACTGTGAAACTTGTAACTGGCGAC
 281  ProAspLeuThrSerLeuArgTyrPheCysThrHisCysAspThrGlyValSerTyrSerArgTrpSerPheAlaSerGluGlyGluGluLeuProThrValLysLeuValThrGlyAsp

 961  TCTGTGGTAAATTCAAACAGTACAGATGATGCACAGCTTCATTATTATATTATGAGATGGCTGACAGTGAGTGCAGACCACATTGTTATCCTTATTTTGCTGCTGGCACTAGCTGTGAAG
 321  SerValValAsnSerAsnSerThrAspAspAlaGlnLeuHisTyrTyrIleMetArgTrpLeuThrValSerAlaAspHisIleValIleLeuIleLeuLeuLeuAlaLeuAlaValLys

1081  TTTGTATTCTTTGAAACAAGAGACGAGTTGACAACCACAAGGGGCATGGATGGCTGGGTGGAAGTGAGCAGCCCAGTGGAACACAAGTATGTTCAAACAGAACAACCCTCATGCAGTGCA
 361  PheValPhePheGluThrArgAspGluLeuThrThrThrArgGlyMetAspGlyTrpValGluValSerSerProValGluHisLysTyrValGlnThrGluGlnProSerCysSerAla

1201  CCAGAGCAGCCTCTTGAGGAACCTCCTGCTTCCAACAGGAGCATTGATGAATGTCTCTCAGTCTGCAAATCTGATGTTGGAGCACAAGCACTGAGTGATTGTGAAGTGATGGCACTCGTA
 401  ProGluGlnProLeuGluGluProProAlaSerAsnArgSerIleAspGluCysLeuSerValCysLysSerAspValGlyAlaGlnAlaLeuSerAspCysGluValMetAlaLeuVal

1321  ACAAGTGGCCATATTGCTGGCTATCAACTTGAAAAGGTTGTGAGAAACCCTGAGCGCGGTGTGGGGATCCGGCGACAGATACTTACCAAAACTGCAGATTTAAAGGATGCCCTAGATAAT
 441  ThrSerGlyHisIleAlaGlyTyrGlnLeuGluLysValValArgAsnProGluArgGlyValGlyIleArgArgGlnIleLeuThrLysThrAlaAspLeuLysAspAlaLeuAspAsn

1441  TTGCCTTACAAAAATTATGACTATTTGAAAGTAATGGGAGCTTGTTGTGAGAATGTGATAGGATACATGCCTGTGCCTGTAGGAGTGGCAGGACCACTCAATTTAGATGGACGCCTGGTG
 481  LeuProTyrLysAsnTyrAspTyrLeuLysValMetGlyAlaCysCysGluAsnValIleGlyTyrMetProValProValGlyValAlaGlyProLeuAsnLeuAspGlyArgLeuVal

1561  CATGTGCCACTTGCAACAACTGAGGGCTGTCTAGTGGCAAGCACCAACAGAGGCATGCGAGCTTTAATGCGATGTGGTGTCACCTCTCGTATTGTAGCTGATGGAATGACTCGTGGACCG
 521  HisValProLeuAlaThrThrGluGlyCysLeuValAlaSerThrAsnArgGlyMetArgAlaLeuMetArgCysGlyValThrSerArgIleValAlaAspGlyMetThrArgGlyPro

1681  GTTGTGCGATTCCCCAATATTGACAGAGCAAGTGAAGCCATGTTGTGGATGCAAGTGCCATACAATTTTGAACAAATTAAGAAGAACTTTGACTCTACCAGTCGTTTTGCACGTCTTTCC
 561  ValValArgPheProAsnIleAspArgAlaSerGluAlaMetLeuTrpMetGlnValProTyrAsnPheGluGlnIleLysLysAsnPheAspSerThrSerArgPheAlaArgLeuSer

1801  AAAATCCACATAAGAGTAGCTGGTCGTCATTTGTTTATACGCTTCATTGCTACAACTGGAGATGCAATGGGAATGAACATGCTCTCTAAGGGTACAGAAGTGGCTTTGGCATATGTGCAG
 601  LysIleHisIleArgValAlaGlyArgHisLeuPheIleArgPheIleAlaThrThrGlyAspAlaMetGlyMetAsnMetLeuSerLysGlyThrGluValAlaLeuAlaTyrValGln

1921  CAAGTGTACCCAGACATGGAGATCCTCAGTTTAAGCGGCAACTTTTGCACTGATAAGAAACCAGCAGCTGTCAACTGGATTGAAGGTCGAGGAAAGTCTGTTGTCTGTGAAGCCATTGTG
 641  GlnValTyrProAspMetGluIleLeuSerLeuSerGlyAsnPheCysThrAspLysLysProAlaAlaValAsnTrpIleGluGlyArgGlyLysSerValValCysGluAlaIleVal

2041  CCAGCTGACATTATAAAGTCTGTTCTGAAAACAAGTGTTCAAGCACTAGTGGATGTGAACATTACAAAGAATCTGATTGGCTCAGCAGTGGCAGGAAGCATTGGAGGTTTCAATGCGCAT
 681  ProAlaAspIleIleLysSerValLeuLysThrSerValGlnAlaLeuValAspValAsnIleThrLysAsnLeuIleGlySerAlaValAlaGlySerIleGlyGlyPheAsnAlaHis

2161  GCTGCCAACATTGTGACAGCAATCTTCATTGCAACAGGGCAAGATCCAGCTCAGAATGTGGGCAGCAGTAATTGCATGACATTGATGGAACCTTGGGGGGAAGATGGCAAAGACCTTTAT
 721  AlaAlaAsnIleValThrAlaIlePheIleAlaThrGlyGlnAspProAlaGlnAsnValGlySerSerAsnCysMetThrLeuMetGluProTrpGlyGluAspGlyLysAspLeuTyr

2281  GTATCCTGCACCATGCCTTCTATAGAAATTGGTACTATTGGTGGTGGAACAGTTCTACCACCACAGGCAGCATGCCTTGACATGCTAGGAGTAAGAGGTGCAAATGAAATATGTCCAGGA
 761  ValSerCysThrMetProSerIleGluIleGlyThrIleGlyGlyGlyThrValLeuProProGlnAlaAlaCysLeuAspMetLeuGlyValArgGlyAlaAsnGluIleCysProGly

2401  GAAAATGCCAATACATTGGCACGTATTGTGTGTGGCACAGTCCTTGCAGGAGAATTATCTCTTATGTCTGCACTTGCAGCTGGGCATCTTGTCAAAAGCCATATGCGGCACAACAGATCT
 801  GluAsnAlaAsnThrLeuAlaArgIleValCysGlyThrValLeuAlaGlyGluLeuSerLeuMetSerAlaLeuAlaAlaGlyHisLeuValLysSerHisMetArgHisAsnArgSer

2521  TCTGTGAGTACATCAGGTAGCGAACCCAGCACACCTGCCTGCAAATCATGAAGTACTTTATTGGACAGATTATTCTGTTAAATTATTTAAAAAACAAACTTTAAAGTTCACATTTTCTCA
 841  SerValSerThrSerGlySerGluProSerThrProAlaCysLysSer
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2641  TATGTTTCGGTATAGTCACACATTGTGCAGGCGTGTGTGTAGTTATCATCTTCCGTGTTGTATGTTTCTGGGCCAAATGTGTGCTGATGTGATGATTTGTTGGTGCGATCATACAAGCTG
2761  CTCCGCACTGACATCCGCAGAGCTGCACAAGAGCACAAGACTTATAAGAGATCTAAAATGCTTTTATAAAACAACACACAACAGACACAATGTGGAAAGATCAACTCATTCTTTCTGATA
2881  CCATTGAAACAAACATTTAGGAATCATCCCTTATATTTGAGGCAACTAAATTTCTTCCATTCCAATTTATTGCCAATCCTATGAATTTTGATCTGTGATCACTTTGCCCCACTCTTGTTC
3001  ATTAATATTTTTTACTGTTCTTGGAGACTTGTGCACTTAAATTCATTGGATGAATGTTAGGTCTCGTCTAGTCAGTTTGCAAAATGAACTAATAGCTCTATTTGATTGAAACAGAACATT
3121  ATTTAGAAGAATTGGTTAAATTAAATAAACCAGTATTGTGAATTATTTAGCAAGAGTTCTGATGTGGACTCAAAGCTACTTTCCTTTTGCAAATAATCAACTCAATTGTATACACTAAAAT
3241  AAATTATAATATATACAAAAAT(A)19
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Fig. 3. Restriction-endonuclease map and nucleotide sequence of the cDNA encoding *B. germanica* HMG-CoA reductase. (A) Solid bar indicates the coding sequence. Scale on the top is in kilobases. Arrows indicate the start site, direction and strand sequenced. (B) Numbers to the left of the lines indicate nucleotide or amino acid residue positions. Amino acid residue 1 is the putative initiator methionine. Potential N-linked glycosylation sites (Asn-Xaa-Ser/Thr) are overlined. The potential polyadenylation signal is boxed.

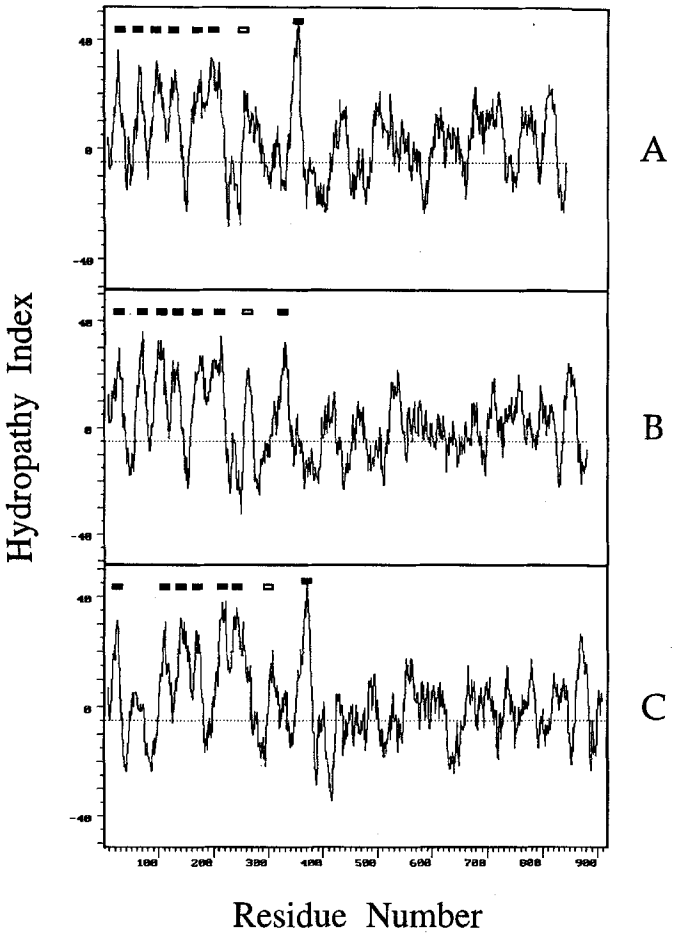

Fig. 4. Hydropathy plots of HMG-CoA reductases from *B. germanica* (A), Chinese hamster (B) and *D. melanogaster* (C). The algorithm of Kyte and Doolittle [30] was used with a window size of 15 residues. The vertical scale denotes the hydropathy index measured as the average free energy [(4.184 J/mol) $\times 10^{-1}$]. The horizontal line at the hydropathy value of -5 divides hydrophobic regions (above) from hydrophilic regions (below). The seven spanning domains are indicated by a solid bar. The additional transmembrane span found by Olender and Simoni [43] in hamster reductase is indicated by a open bar.

two PEST sequences (residues 390–412 and 840–854); these sequences are characteristic of many proteins with rapid turnover rates [31].

Comparison with other HMG-CoA reductase sequences

At present, the primary structure of HMG-CoA reductase is known for a variety of eukaryotic organisms, including mammals [23–25], yeast [26], sea urchin [27], plants [28, 32], *Schistosoma mansoni* [29], *Xenopus laevis* [33] and *D. melanogaster* [5]. The comparison of *B. germanica* HMG-CoA reductase with those of these species reveals extensive conservation, especially in the catalytic domain, which is located in the C-terminal region (44–66% identical amino acid residues).

Alignments between *B. germanica*, hamster and *D. melanogaster* HMG-CoA reductase sequences are shown in Fig. 5. Alignments were first obtained using the CLUSTAL program [34], then similarities were optimized by inserting gaps. Identities between N-terminal, C-terminal and linker regions of the three proteins were 33, 50 and 13%, respectively. The transmembrane spans were highly conserved (52% to 85% identical residues between both insect proteins).

The linker region in *B. germanica* appears to be much less conserved when compared with the other known reductases. Indeed, this region presents 39% and 20% identity to *D. melanogaster* and hamster HMG-CoA reductase sequences, respectively. Finally, the C-terminal domain was more similar to hamster HMG-CoA reductase than is *Drosophila* enzyme (79% and 69% conserved residues, respectively). In particular, the most conserved region among the known HMG-CoA reductases lies in the β domains [4]. Fig. 5 shows His834, which is the only position conserved in all HMG-CoA reductase characterized including the prokaryotic enzymes [35, 36].

Validation of the putative *B. germanica* HMG-CoA reductase

A truncated form of HMG-CoA reductase from *B. germanica* (residues 380–856) was overexpressed in *E. coli* cells. HMG-CoA reductase activity was measured in a lysate of cell cultures harboring pRBEx18. The activity levels ranged over 0.34 U/mg (at initial time) and 48 U/mg (3 h after IPTG induction; Fig. 6A). No activity was found in bacteria lacking plasmids or in cells containing pT7-7 without an insert. In cell harboring pRBEx18, IPTG induced two proteins with molecular masses of 51.5 kDa and 45 kDa in SDS/PAGE. The 51.5-kDa protein agrees with the expected molecular mass of the fusion protein (Fig. 6B). The 45-kDa protein could correspond to a subfragment of HMG-CoA reductase initiated at the internal ATG codon in position 437, which is preceded by a sequence that could act as a ribosome-binding site [37].

mRNA levels in development stages and in different tissues of *B. germanica*

BgRD12 cDNA was used as a probe to hybridize total RNA obtained from various stages of *B. germanica* development (Fig. 7A). A single transcript of $\approx$3.6 kb was detected at all developmental stages. The highest HMG-CoA re-

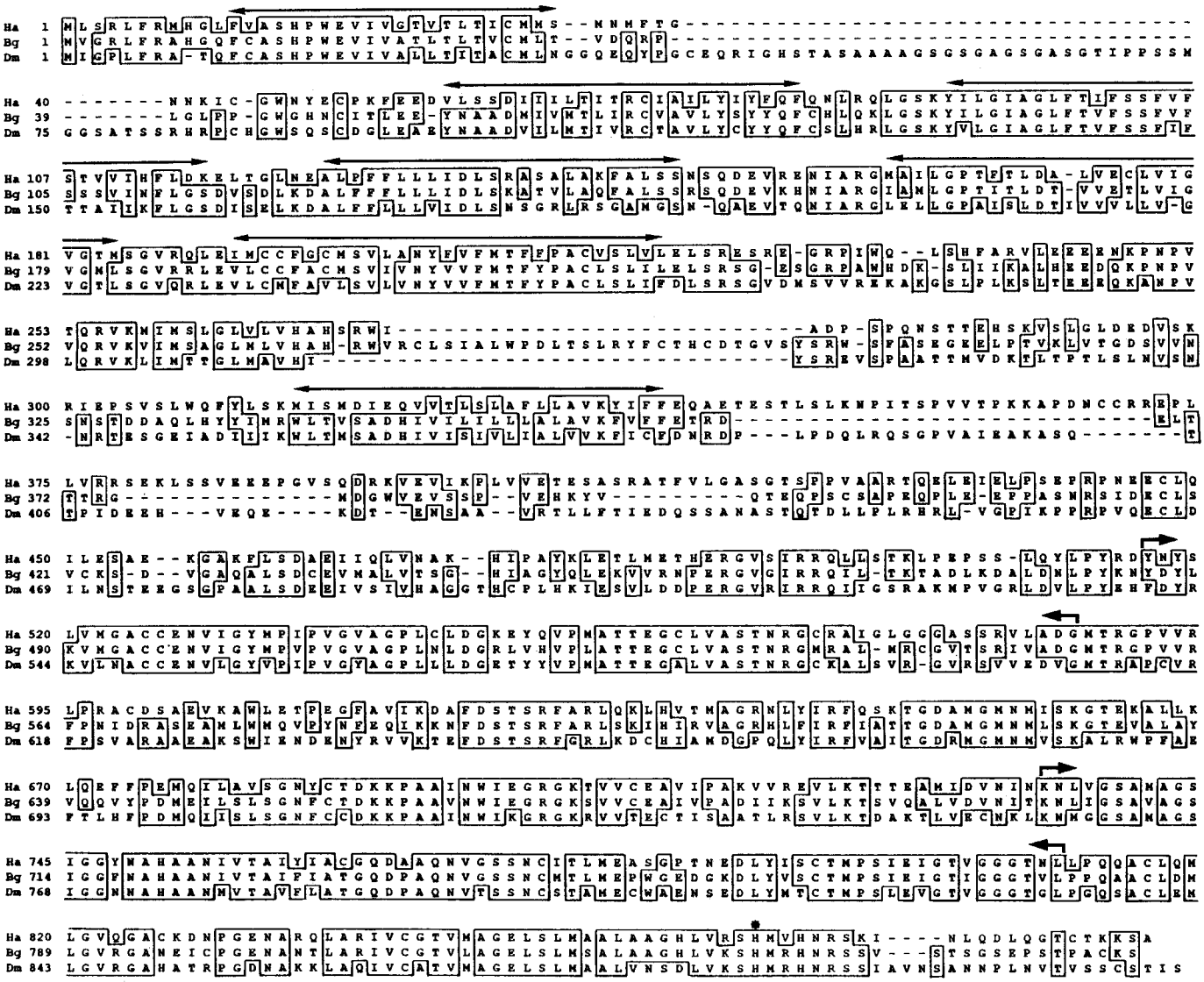

Fig. 5. Alignment of HMG-CoA reductase sequences. Amino acid sequence of *B. germanica* HMG-CoA reductase (Bg) was aligned with both Chinese hamster (Ha) and *D. melanogaster* (Dm) HMG-CoA reductases. Gaps (−) were introduced to optimize alignments. Only residues identical to those of the *B. germanica* enzyme were boxed. Amino acid residues are shown in the single-letter code. Potential membrane spanning domains of the *B. germanica* enzyme are overlined. Regions extending over β domains, that have been postulated to be part of the catalytic site of the hamster enzyme [4], correspond to residues 486−555 and 703−779 in *B. germanica*. His834 of *B. germanica* is indicated by an asterisk.

ductase mRNA levels were found in young embryos (4 days old), the following embryonary stages showing lower mRNA levels, which were similar to those found in larval and adult stages. No significant differences were observed throughout the six larval instars of *B. germanica*, nor during the adult female gonotrophic cycle (data not shown). Analysis of HMG-CoA reductase mRNA from different *B. germanica* female tissues shows the highest levels in the ovary. HMG-CoA reductase mRNA was also detected in brain and muscle tissues, but not detectable in gut or fat-body tissues (Fig. 7B). Differences between mRNA levels of HMG-CoA reductase in whole adult males or females were found to be insignificant (data not shown).

DISCUSSION

Our first approach to isolate *B. germanica* HMG-CoA reductase cDNA was based on the assumption that an enzyme central to intermediary metabolism should be sufficiently conserved in evolution as to be isolated using heterologous probes. However, attempts to isolate it by cross-hybridization at reduced stringency with fragments of Chinese hamster or *D. melanogaster* HMG-CoA reductase cDNA resulted in the obtention of several artifactual clones. Therefore, we considered using the PCR as an alternative procedure to obtain a cDNA encoding for a fragment of *B. germanica* HMG-CoA reductase. PCR experiments using, as primer, oligonucleotides derived from HMG-CoA reductase amino acid sequences, highly conserved across species, led to the amplification of a cDNA encompassing a fragment of *B. germanica* HMG-CoA reductase. This cDNA was successfully used as a probe to isolate a full-length HMG-CoA reductase cDNA.

The amino acid sequence deduced from the BgRD12 cDNA clone reveals the occurrence of the three characteristic regions of the HMG-CoA reductase [4]. In addition, when

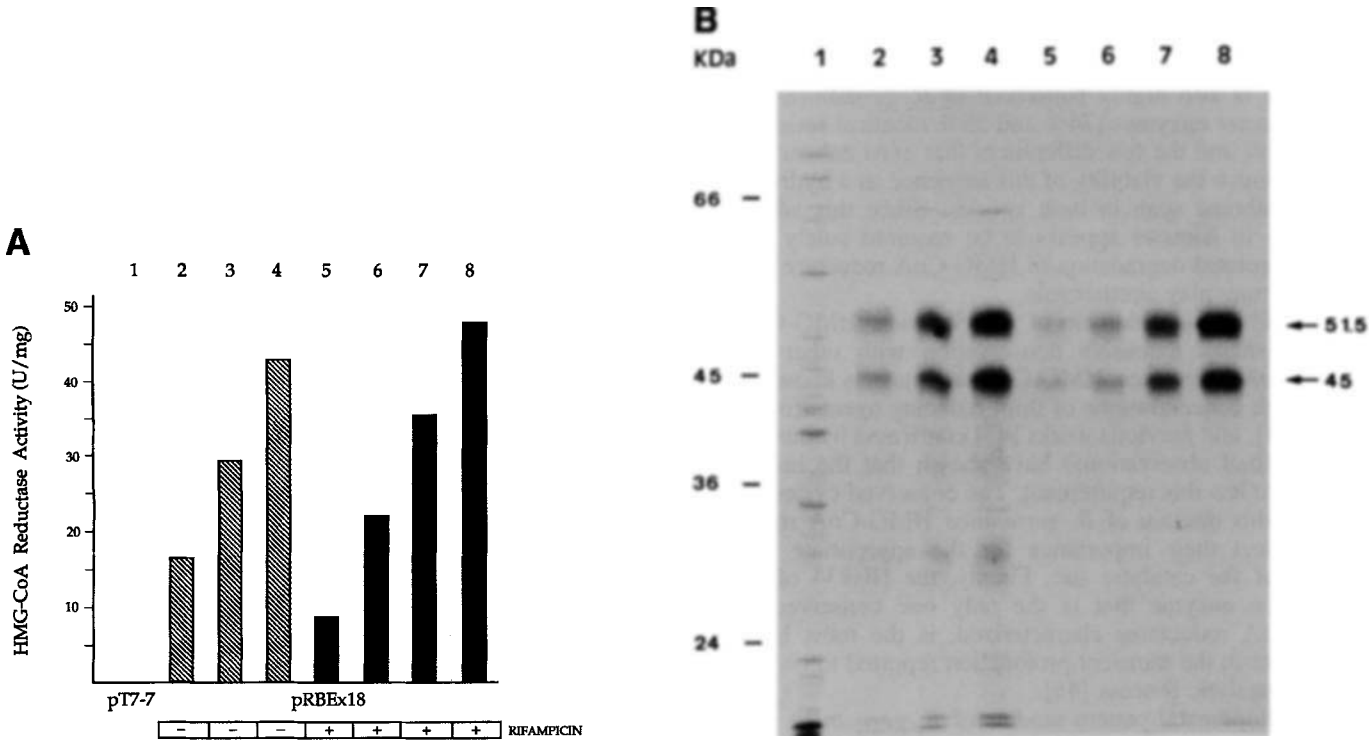

Fig. 6. Expression of *B. germanica* HMG-CoA reductase in *E. coli*. The catalytic region of *B. germanica* HMG-CoA reductase was placed under the control of the ϕ10 promoter (see Materials and Methods), and expressed in BL21(DE3) cells. (A) Effect of time induction on the level of HMG-CoA reductase activity. HMG-CoA reductase activity was determined in extracts of *E. coli* cells harboring pRBEx18 at different times after IPTG induction, either with or without rifampicin. After 5 h, no activity was found in cells with plasmid controls, either with or without IPTG (lane 1); lanes 2–4, HMG-CoA reductase activity after IPTG induction at 1, 2, and 3 h; lanes 5–8, HMG-CoA reductase activity after rifampicin addition at 30 min, 1, 2 and 3 h. (B) [^{35}S]Methione radiolabeled proteins from the expressed culture were fractionated in SDS/PAGE (20 µg/lane) and subjected to fluorography. Lane 1, cells expressing the control plasmid pT7-7, 2 h after IPTG induction; lanes 2–4, cells harboring pRBEx18 1, 2 and 3 h after induction; lanes 5–8, expression of pRBEx18 with rifampicin added 30 min after IPTG induction, then samples collected at 30 min, 1, 2 and 3 h.

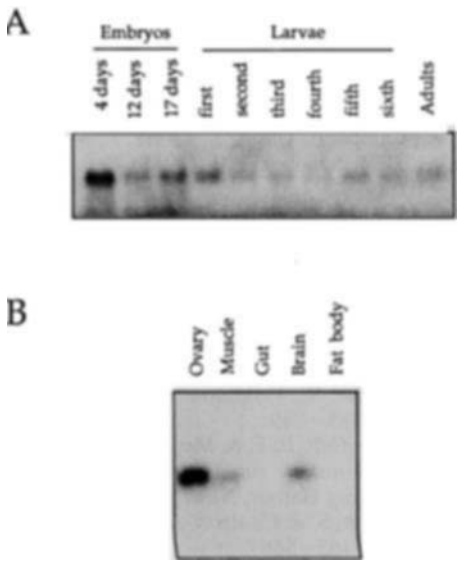

Fig. 7. Analysis of *B. germanica* HMG-CoA reductase mRNA levels during development and in differents tissues. (A) A blot with total RNA from various stages in *B. germanica* development (20 µg/lane) was hybridized to the λBgRD-12 insert. (b) A blot with total RNA from differents tissues of *B. germanica* adult females (10 µg total RNA/lane) was hybridized with the probe indicated above.

expressed as a fusion protein, the C-terminus of the *B. germanica* protein exhibits HMG-CoA reductase activity. These structural and functional data lead us to conclude that the cDNA characterized herein encodes the *B. germanica* HMG-CoA reductase.

It is interesting to note the great similarity in the hydropathy profiles of the *B. germanica*, hamster and *D. melanogaster* HMG-CoA reductases (Fig. 4), which indicates that differences in the amino acid sequences do not affect the overall protein structure. In mammals, the membrane-spanning regions modulate the sterol-triggered degradation of the enzyme [38]. In insects, however, the role of these regions is unclear, since insects cannot synthesize cholesterol [6], and it has been shown that sterols do not regulate HMG-CoA reductase [9]. Therefore, it is unlikely that this structural similarity in the transmembrane regions has been maintained only to provide sensitivity to sterol- mediated degradation. Since mevalonate affects the overall protein level, decreasing synthesis and increasing the rate of HMG-CoA reductase degradation [39, 40], it has been suggested that conservation of these domains may be essential for recognizing specific mevalonate derivatives or their binding proteins [5]. Indeed, the fundamental negative-feedback mechanism driven by an isoprenoid metabolite is present in *D. melanogaster* Kc cells [9, 41]. Recently, Simoni and coworkers showed that the spanning domains of hamster HMG-CoA reductase are responsible for targeting the protein to the endoplasmic reticulum, and are also involved in the mevalonate-triggered

and cholesterol-triggered degradation [42]. Furthermore, these authors found an additional transmembrane sequence (Pro249 to Ala275 in hamster reductase; Fig. 5) [43]. This sequence is also highly conserved in *B. germanica* and *D. melanogaster* enzymes (74% and 55% identical residues, respectively) and the few differences that exist enhance rather than diminish the viability of this sequence as a hydrophobic transmembrane span in both species. Since this additional spanning in hamster appears to be required solely for the sterol-regulated degradation of HMG-CoA reductase [42], in insect it may play another role.

The C-terminal domain of *B. germanica* HMG-CoA reductase shares extensive conservation with other known HMG-CoA reductases. HMG-CoA reductase is known to require high concentrations of thiol-reducing agents for its activity [44], and previous works [45] confirmed by our studies (unpublished observations) have shown that the insect enzyme also has this requirement. The conserved cysteine residues in this domain of *B. germanica* HMG-CoA reductase may reflect their importance for the appropriate conformation of the catalytic site. Finally, the His834 of the *B. germanica* enzyme that is the only one conserved in all HMG-CoA reductases characterized, is the most likely to participate in the transient protonation reported to occur during the catalytic process [46].

Developmental-pattern studies of *B. germanica* showed that the highest HMG-CoA reductase mRNA levels are found in 4-day-old embryos (Fig. 6A). This age corresponds to embryo stage 6, according to the classification described by Tanaka [47]. In this stage, the embryo is well segmented, shows rudiments of all appendages and prepares for dorsal closure and full organogenesis. During sea urchin embryogenesis, it has been reported that HMG-CoA reductase is developmentally regulated and the mRNA levels increase parallel to both polyisoprenoid and glycoprotein biosynthesis [48], suggesting coordinate expression of HMG-CoA reductase, dolichol production and glycoprotein biosynthesis. In addition, in *D. melanogaster* embryos, the glycoprotein biosynthesis is also essential during the process of development and differentiation [49], and in this species the highest HMG-CoA reductase mRNA levels were found in embryos [5]. Accordingly, analogous functions for HMG-CoA reductase could be postulated in the case of the *B. germanica* embryo, especially considering that embryo stage 6 precedes an extensive process of histogenesis and organogenesis.

Analysis of mRNA levels from heads and from decapitated specimens of *B. germanica* adult females indicates that HMG-CoA reductase mRNA is concentrated in the latter case. No differences were found in HMG-CoA reductase mRNA levels from whole bodies or from heads, from different days of the *B. germanica* gonotrophic cycle (data not shown), whereas during this period juvenile hormone production and HMG-CoA reductase activity in *B. germanica corpora allata* increases 28-fold [50] and 9-fold (unpublished results), respectively. If HMG-CoA reductase expression in the *corpora allata* accounted for less than 0.025% of the head (mass percentage of *corpora allata* with respect to head), then a 1000-fold induction in HMG-CoA reductase expression could still elude detection.

Conversely, the comparative study of different adult female tissues (Fig. 6B), clearly indicates that the major source of mRNA HMG-CoA reductase is the ovary. Since we used mature ovaries in a pre-choriogenetic stage [51], it is plausible that HMG-CoA reductase could play a role in the complex process of chorion formation, perhaps related to glycosylation of chorion proteins. Unfortunately, *B. germanica* chorion components are still unidentified, but glycosylation is a characteristic post-translational modification of certain chorion proteins [52]. Furthermore, the HMG-CoA reductase expression in these organs could be related to the synthesis of isoprenylated proteins, as the γ subunit of guanosine-nucleotide-binding proteins and the product of the *ras* oncogen are present mainly in the brain and ovaries of *D. melanogaster* [15, 53].

In any case, the HMG-CoA reductase overexpression in cockroach ovaries opens new research possibilities towards the elucidation of the physiological role of the enzyme in this organ. Work along these lines is currently in progress in our respective laboratories.

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