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3-Hydroxy-3-methylglutaryl-coenzyme-A synthase from *Blattella germanica*

Cloning, expression, developmental pattern and tissue expression

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Insects do not synthezise cholesterol; the 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) produced by HMG-CoA synthase is transformed to mevalonate by HMG-CoA reductase for the production of non-sterol isoprenoids, which are essential for growth and differentiation. To understand the regulation and developmental role of HMG-CoA synthase, we have cloned a 1658 bp cDNA that encompasses the entire transcription unit of the HMG-CoA synthase gene from the cockroach *Blattella germanica*. This cDNA clone was isolated using as a probe a partial cDNA of *B. germanica* HMG-CoA synthase, amplified using the polymerase chain reaction. Analysis of the nucleotide sequence reveals that the cDNA encodes a polypeptide of 453 amino acids (M_r 50338) that is similar to vertebrate HMG-CoA synthase (74–76% conserved residues). The *B. germanica* cDNA has been expressed as a fusion protein in *Escherichia coli* and exhibits HMG-CoA synthase activity. The HMG-CoA synthase transcript was differentially expressed throughout *B. germanica* development. Analysis of RNA samples from different adult female tissues shows high HMG-CoA synthase mRNA levels in the ovary and lower levels in brain and muscle.

3-Hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) synthase catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to yield HMG-CoA. In mammals, two forms of HMG-CoA synthase have been detected, a mitochondrial form and a cytoplasmic form [1, 2], which are encoded by two different genes [3]. The HMG-CoA produced by the mitochondrial enzyme is largely converted by HMG-CoA lyase to ketone bodies (acetoacetate and 2-hydroxybutyrate). The HMG-CoA produced by the cytoplasmic enzyme acts as a substrate of HMG-CoA reductase, with ultimate conversion to cholesterol and other isoprenoid compounds such as dolichol, ubiquinone, isopentenyl adenosine and isoprenylated proteins [4, 5]. These compounds participate in processes ranging from growth control to development [6, 7].

The study of this enzyme in insects is important since it represents a model system for several reasons: insects cannot synthesize cholesterol *de novo* [8] because they lack the enzymes squalene synthetase (farnesyl-diphosphate farnesyltransferase) and lanosterol synthase [9]; HMG-CoA synthase activity appears to be limited to the cytosolic fraction [10, 11]; HMG-CoA synthase of these organisms is insensitive to sterol regulation [12]; insects produce juvenile hormones, specific isoprenoid derivatives which have an important role in development and reproduction [13, 14].

Vertebrate sterol biosynthesis and juvenile hormone biosynthesis in insect corpora allata share a common pathway to farnesyl diphosphate [14]. Since HMG-CoA synthase and HMG-CoA reductase are generally considered as the key regulatory enzymes in cholesterol synthesis [4, 5] it has been suggested that HMG-CoA synthase could also be a rate-limiting enzyme of the juvenile-hormone-biosynthesis pathway [15].

All these considerations and the fact that no report has been published on the structural characterization of HMG-CoA synthase in insects prompted us to isolate and characterize a cDNA encoding *Blattella germanica* HMG-CoA synthase, and to determine the expression pattern throughout its developmental stages and in different tissues, as a contribution to the knowledge of the role of this enzyme in the insects isoprenoid-biosynthesis pathway.

MATERIALS AND METHODS

Materials

The pT7-7 prokaryotic vector and the *Escherichia coli* strains for expression experiments were a gift from F. W. Studier (Biology Department, Brookhaven National Laboratory, Upton NY). The p53K-312 plasmid, which contains a full-length cDNA for the cytosolic HMG-CoA synthase from

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Abbreviations. HMG-CoA, 3-Hydroxy-3-methylglutaryl-coenzyme-A; PCR, polymerase chain reaction; OST, synthase oligonucleotides set; G protein, guanine-nucleotide-binding regulatory protein.

Enzymes. 3-Hydroxy-3-methylglutaryl-coenzyme-A synthase (EC 4.1.3.5); 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (EC 1.1.1.34); 3-hydroxy-3-methylglutaryl-coenzyme-A lyase (EC 4.1.3.4); farnesyl-diphosphate farnesyltransferase (EC 2.5.1.21); lanosterol synthase (EC 5.4.99.7).

Note. The novel nucleotide sequence data published here have been deposited with the GenBank/EMBL sequence data banks and are available under the accession number X73679.

hamster [16], was obtained from American Type Culture Collection. The oligonucleotides used in the polymerase-chain-reaction (PCR) experiments were synthesized by Operon Technologies. Radioactive compounds were obtained from Amersham. Clone pST-359 contains a HMG-CoA synthase cDNA from *B. germanica* obtained by PCR using the degenerate oligonucleotides described below. Clone λBgST11 contains the longest HMG-CoA synthase cDNA obtained by screening of the cDNA library from *B. germanica*.

Insects

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 for study of mRNA levels by blot analysis, were carefully explanted from 5−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.

PCR conditions

Two sets of degenerate oligonucleotides (OST_1 and OST_2), derived from highly conserved amino acid sequences of the N-terminal region of HMG-CoA synthase, were used in PCR experiments. Synthase oligonucleotides set 1 (OST_1, a sense-strand 20-residue oligonucleotide, with 256-fold degeneracy) corresponds to the amino acid residues -WPSDVGI- (positions 13−19 in hamster cytosolic HMG-CoA synthase). Synthase oligonucleotides set 2 (OST_2, an antisense-strand 20-residue oligonucleotide, with 1024-fold degeneracy) corresponds to amino acid sequence -NAC-YGGT- (positions 128−134 in hamster protein).

OST_1, 5′-TGGCC(N)AA(R)GA(Y)GT(N)GG(N)AT-3′

OST_2, 5′-GC(N)GT(N)CC(N)CC(R)TA(R)CA(N)GC-3′

Degenerate positions are indicated in parentheses where R is either A or G, Y is either T or C and N is A, C, G or T.

An aliquot of cDNA pool (≈ 25 ng) and 25 pmol of each set of primers were used for 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 30 s; annealing, 50−55°C for 1 min; polymerization, 72°C for 1 min.

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 a 261-bp *Bgl*II−*Pvu*II fragment from p53K-312 [16]. Washes were performed 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 pST-359 insert as a probe.

RNA blot analysis

Total RNA from *B. germanica* was isolated by the guanidinium/cesium chloride method [18] with minor modifications. Poly(A)-rich RNA was purified by oligo(dT)-cellulose chromatography, according to [19]. RNA samples were fractionated in 1% agarose/formaldehyde gels, transferred to Nytran-N membranes (Schleicher & Schuell) and cross-linked by means of ultraviolet radiation. Hybridization and washes were as described [17]. pST-359 or λBgST11 inserts were used as a probe. The levels of mRNA were measured by densitometric scanning of the autoradiograms measured with a Molecular Dynamics computing densitometer. Densitometry values were corrected by using mouse cyclophilin [20] as a constitutive probe, which was used as control. Filters were dehybridized in water for 10 min at 100°C then rehybridized.

Construction and screening of λgt-10 cDNA libraries

Poly(A)-rich RNA from 6th-instar larvae of *B. germanica* was used to generate oligo(dT)-primed double-stranded cDNA according to Gubler and Hoffman [21]. First-strand cDNA was synthesized with Moloney-Murine Leukemia Virus reverse transcriptase (Pharmacia). This step was followed by RNase H and DNA polymerase I reactions for second-strand synthesis. The double-stranded cDNA was made blunt-ended with T4 DNA polymerase (Pharmacia) and ligated to *Eco*RI adapters. cDNA longer than 600 bp were selected on a Sepharose CL-4B column and ligated to λgt-10 arms. The ligated DNA was packaged with Gigapack II gold packaging extracts (Stratagene). 600000 plaque-forming units of the library were screened using the cDNA amplified by PCR as a probe (see above). The conditions for screening λgt-10 bacteriophage were as described [17]. The positive phages were purified through two additional rounds of plaque screening and were then amplified.

DNA sequencing

PCR amplification products and cDNA clones were subcloned into pBluescript vectors (Stratagene) and sequenced by the dideoxynucleotide chain-termination method [22] with modified T7 DNA polymerase (Sequenase, United States Biochemical).

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

The insert of clone λBgST4 of *B. germanica* HMG-CoA synthase, which contains the complete coding region with only two nucleotides uptream of the initiator methionine, was used in the expression experiments. A 1422-bp *Sma*I−*Hin*dIII fragment isolated from the pBgST4 (pBluescript which contains the insert of λBgST4) was cloned into the *Hin*dIII/*Bam*HI(fill-in) sites of the prokaryotic expression vector pT7-7 [23]. The new plasmid, called pSBEx4, was used to transform *E. coli* strain BL21(DE3), in which expression occurred. Expression was induced with isopropyl-thio-β-D-galactopyranoside, and the newly-synthesized proteins were labelled with [^{35}S]methionine added at the time of induction. An analogous experiment was carried out by adding rifampicine to the medium 30 min after induction, to minimize

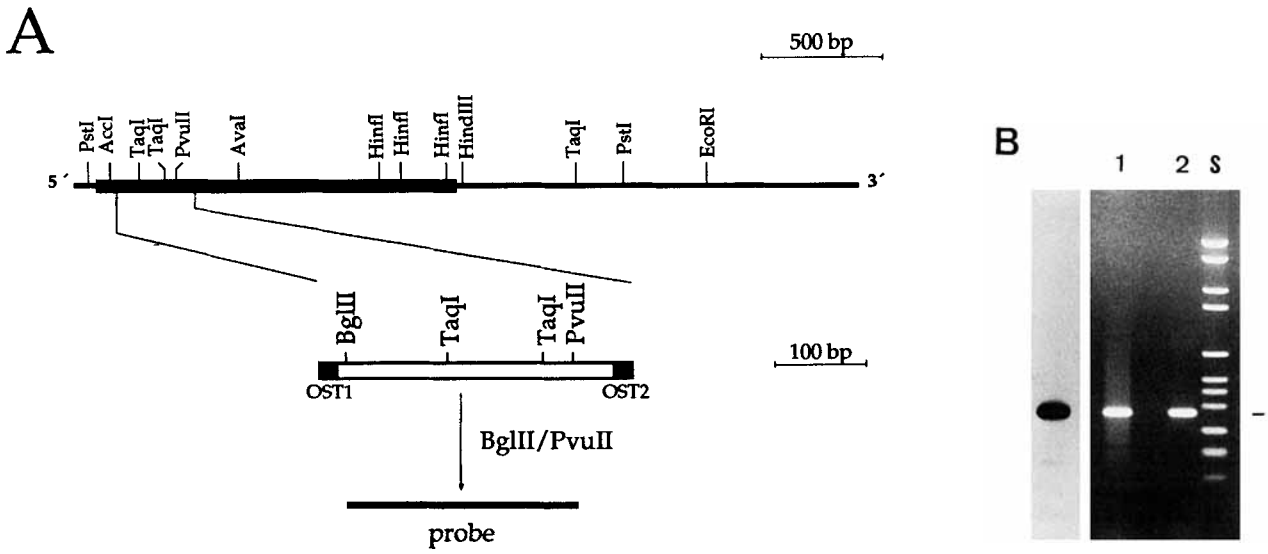


Fig. 1. Gel electrophoresis and Southern-blot analysis of amplified PCR products. (A) Restriction-endonuclease map of the clone p53K-312, which contains a full-length cDNA for the cytosolic HMG-CoA synthase from hamster [16], used as control in PCR experiments and fragment of this clone amplified by PCR using OST_1 and OST_2 as primers. The probe used in the Southern blot is shown below. (B) After 40 cycles of amplification using OST_1 and OST_2 as primers, PCR products were run in a 2% agarose gel. On the right, ethidium bromide staining of the gel is shown. Lane 1, amplified products using a double-stranded cDNA pool from adult females of *B. germanica* as template; lane 2 (control), product amplified using p53K-312 as template. Left, Southern blot of lane 1 using as probe a 261 bp *Bgl*II–*Pvu*II fragment (nucleotide +69 to +330) of p53K-312. Marker at right points to an ≈ 359-bp DNA fragment amplified in the PCR reactions.

radiolabelling of the newly synthesized endogenous proteins. Cells were collected by centrifugation and lysed by sonication. Total proteins were measured [24] and the lysate was then used either for enzyme assays or protein fractionation by SDS/PAGE [25]. To detect the radiolabelled proteins, the gel was subjected to fluorography and placed in contact with X-ray autoradiographic film with intensifying screens overnight.

Assay of HMG-CoA synthase activity

HMG-CoA synthase activity was determined by the radiometric method described by Clinkenbeard et al. [26], as modified by Gil et al. [16]. 1 U HMG-CoA synthase is defined as the amount of enzyme which catalyzes the formation of 1 μmol HMG-CoA in 1 min at 37°C.

RESULTS

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

Comparison of the primary structure of mitochondrial and cytosolic HMG-CoA synthases from the rat [3, 27] revealed several highly conserved sequences in the N-terminal region, which contains 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 (359 bp) was obtained. To check the specificity of the process, the cDNA amplification products were analyzed by Southern blot, using the 261-bp *Bgl*II–*Pvu*II fragment of p53K-312 [16] as a probe, which did not contain any of the primer sequences (Fig. 1A). The result of this analysis showed that only the major PCR-amplified product hybridized (Fig. 1B). Sequence analysis of this major amplification product (pST-359) showed an open reading frame coding for 119 amino acid residues with high identity to other HMG-CoA synthase sequences (79% identity to cytosolic HMG-CoA synthase from Chinese hamster or rat).

A genomic Southern analysis using pST-359 as a probe yielded a hybridization pattern showing that the putative HMG-CoA synthase gene is present as a single copy in the *B. germanica* genome (Fig. 2A). Analysis of *B. germanica* mRNA by Northern blot using this cDNA as a probe showed that the size of the transcript corresponding to this gene is 1.7 kb (Fig. 2B). This analysis also showed that mRNA levels were higher in 6th-instar larvae than in whole body or head from 6-day-old adult females.

In a screening of a λgt-10cDNA library from 6th-instar larvae, several positive clones were obtained and characterized by restriction enzyme mapping. Clone λBgST11, 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 λBgST11 insert spans 1658 bp, which closely agrees with the mRNA size of 1.7 kb estimated by RNA blot analysis (see above). This sequence revealed an open reading frame of 1359 bp coding for a polypeptide of 453 amino acids, with a molecular mass of 50338 Da, and a deduced isoelectric point of 6.08. The coding region is flanked by 5′ and 3′ untranslated sequences, of 76 bp and 223 bp, respectively (Fig. 3B). The position of the initiator methionine was assigned from the observation of a stop codon in the 5′ upstream region (position −42), and by comparison with other HMG-CoA synthase sequences. A canonical polyadenylation site (AAUAAA) 13 bp upstream of the poly(A)-rich region was found (nucleotide 1558, Fig. 3B).

B. germanica HMG-CoA synthase shows two potential asparagine-linked glycosylation sites (residues 30 and 386). Near the C-terminal end, the protein presents two Pro-Glu-Ser-Thr (PEST) sequences (residues 400–411, and 419–

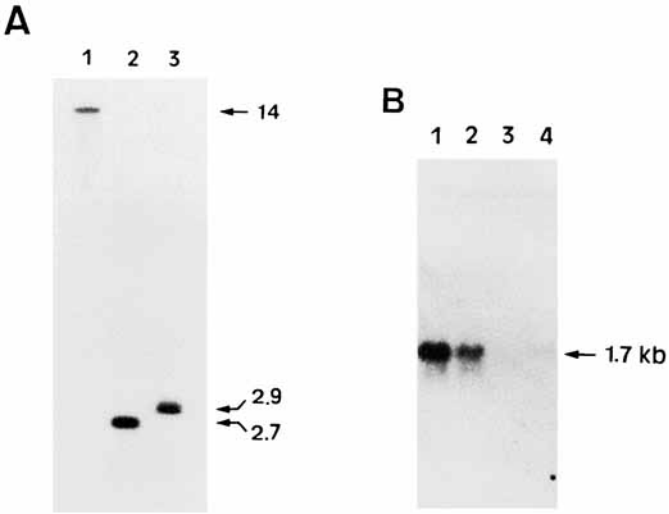


Fig. 2. Southern-blot and Northern-blot analyses. (A) *B. germanica* DNA (5 μg/lane) was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3) restriction enzymes. Fragments were electrophoretically fractionated, bound to a Hybond C-Extra filter and hybridized to the radiolabelled pST-359 insert. DNA fragment sizes are indicated, in kb, at the right. (B) Poly(A)-rich RNA (5 μg/lane) from 6th-instar larvae (lane 1), and from whole bodies and heads of the 6-day-old adult females (lanes 2 and 4) was run in a 1% agarose/formaldehyde gel, transferred to a Nytran-N membrane (Schleider & Schuell) and hybridized to the radiolabelled pST-359 insert. Lane 3 (control) 5 μg of poly(A)-depleted RNA. RNA markers (Promega) were used to estimate size.

444), which are characteristic of many proteins with rapid turnover rates [28].

λBgST11 cDNA corresponds to HMG-CoA synthase

At present, the primary structure of HMG-CoA synthase is known from hamster [16], rat [27] and chicken [29]. Comparison of *B. germanica* HMG-CoA synthase with these reveals extensive conservation, especially in the N-terminal region, where the catalytic site is located [30].

Alignments between *B. germanica*, rat and chicken cytoplasmic HMG-CoA synthase sequences are shown in Fig. 4. Alignments were obtained using the CLUSTAL program [31]. *B. germanica* protein shows 64% identity with both proteins, which is extended to 76% and 74%, respectively, when conserved residues are considered. The amino acid sequence of the putative catalytic site [30], spanning positions 103–121 of the sequence, is highly similar (with a positional identity of 84%) to that of mammalian and avian HMG-CoA synthase sequences. However, a gap of two amino acids has been detected in this sequence (corresponding to Ser115 and Gly116 in hamster protein). Since the expression product of the *B. germanica* cDNA in *E. coli* exhibits HMG-CoA synthase activity, these amino acids do not appear to be necessary for the catalytic function of the protein. The *B. germanica* enzyme is shorter than vertebrate cytosolic HMG-CoA synthase proteins since it lacks two sequences of 11 and 49–50 amino acids at the N-terminal and C-terminal ends, respectively. In the amino acid sequence corresponding to OST_1 there is also a mismatch between *B. germanica* and both mammalian enzymes (Fig. 4).

To test whether the protein of *B. germanica* shows HMG-CoA synthase activity, a construction containing the complete coding region of the cDNA in the correct reading frame was made, and the protein was then expressed in *E. coli* (see Materials and Methods). The isopropyl-thio-β-D-galactopyranoside-induced protein had an apparent molecular mass of 52 kDa by SDS/PAGE, which is in agreement with the molecular mass expected for the fusion protein (Fig. 5). HMG-CoA synthase activity was measured in a lysate of cell cultures harboring pSBEx4. The specific activity levels ranged from 0.075 mU/mg (at initial time) to 13.5 mU/mg (3 h after isopropyl-thio-β-D-galactopyranoside induction). No activity was found in bacteria lacking plasmids or in cells containing pT7-7 without an insert. Acetoacetyl-CoA was a potent inhibitor of HMG-CoA synthesis at concentrations exceeding 30 μM, as occurs in mammalian systems [32]. Like cytosolic synthases, the *B. germanica* enzyme was activated by Mg^{2+} (120% activation with 20 mM $MgCl_2$) [1] (data not shown).

Developmental pattern and tissue expression of *B. germanica* HMG-CoA synthase

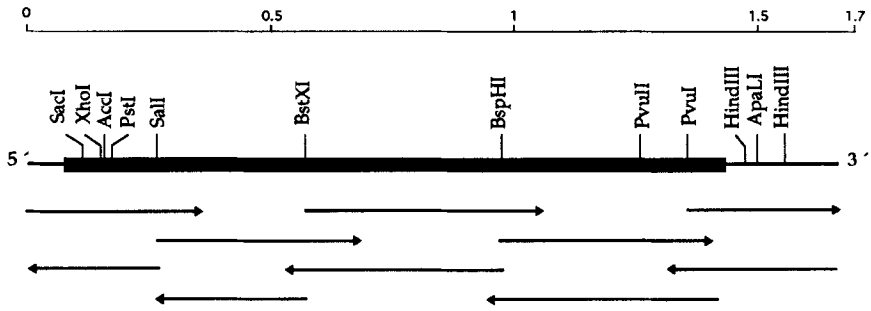
BgST11 cDNA was used as a probe to hybridize total RNA obtained at various stages of *B. germanica* development (Fig. 6A). A single transcript of approximately 1.7 kb was detected at all developmental stages. The HMG-CoA synthase mRNA levels in embryos at 4 and 17 days and in the 2nd-instar and 6th-instar larvae were 2–4-times higher than in all the other stages (compared with the cyclophilin mRNA levels, taken as an internal standard). No difference was observed in mRNA levels of HMG-CoA synthase during the gonotrophic cycle of adult females nor between mRNA levels of adult males or females (data not shown). Northern-blot analysis of different tissues from virgin females of *B. germanica* (5–6-day-old) showed that HMG-CoA synthase mRNA levels in ovary were approximately five-times those of brain and muscle, whereas they were not detectable in gut or fat-body tissues (Fig. 6B).

DISCUSSION

The strategy to isolate *B. germanica* HMG-CoA synthase cDNA was based on the assumption that the evolutionary distance between mitochondrial and cytosolic HMG-CoA synthases from the rat would not be very different from the distance between cytosolic enzymes from other mammals and insects. Accordingly, we chose fully conserved amino acid sequences between published HMG-CoA synthases, whether mitochondrial or cytosolic [3, 16, 27], and the corresponding oligonucleotides were used in PCR experiments. This approach was successful since we isolated a partial cDNA (359 bp) encompassing a fragment of *B. germanica* HMG-CoA synthase (119 amino acids). This cDNA was used as a probe to isolate a full-length cDNA for HMG-CoA synthase.

The identification of the cDNA as HMG-CoA synthase was based on several criteria: the amino acid sequence predicted from this cDNA shares extensive conservation with cytosolic HMG-CoA synthase from hamster, rat and chicken (74–76% conserved amino acids); the predicted amino acid sequence from the reported HMG-CoA synthase contains a region with a high level of identity to that of the active site of chicken HMG-CoA synthase [30]; expression of this cDNA in *E. coli* using a suitable vector produced a fusion protein (52 kDa) that exhibits HMG-CoA synthase activity. These observations strongly suggest that the cDNA-encoded protein represents *B. germanica* HMG-CoA synthase.

A



B

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-76   TGTTTCGAGGTTAGGATGTCACGCAAAGAGGGCATGACTCAAAGCTAGCAGTCTGTGCTCAGTCTGTCCTAAGACG

   1  ATGTGGCCGTCAGACGTAGGTATTGTGGCTCTGGAGCTCATATTCCCTTCGCAGTATGTGGATCAGGTGGACCTCGAGGTCTACGACAAT
       M  W  P  S  D  V  G  I  V  A  L  E  L  I  F  P  S  Q  Y  V  D  Q  V  D  L  E  V  Y  D  N
                                   10                                  20                                  30
  91  GTTTCTGCAGGAAAGTACACGGTGGGGTTGGGACAGGCTCGCATGGGGTTCTGCACGGACAGGGAGGACATCAACTCTCTGTGTCTCACC
       V  S  A  G  K  Y  T  V  G  L  G  Q  A  R  M  G  F  C  T  D  R  E  D  I  N  S  L  C  L  T
                                   40                                  50                                  60
 181  GTCGTCAGTCGACTGATGGAACGATGGAGCATCCCCTACTCGCAAATTGGGCGCCTGGAAGTAGGCACCGAGACCCTTCTGGACAAGTCG
       V  V  S  R  L  M  E  R  W  S  I  P  Y  S  Q  I  G  R  L  E  V  G  T  E  T  L  L  D  K  S
                                   70                                  80                                  90
 271  AAGAGCGTCAAGACTGTCCTGATGCAACTCTTCAAGGACAACACGGACATCGAGGGCGTGGATACCGTGAACGCCTGTTACGGGGGCACC
       K  S  V  K  T  V  L  M  Q  L  F  K  D  N  T  D  I  E  G  V  D  T  V  N  A  C  Y  G  G  T
                                  100                                 110                                 120
 361  TCGGCTCTCTTCAATGCGATTTCGTGGGTGGAGTCCAGCTCCTGGGATGGCAGGTATGCTCTTGTGGTCGCTGGGGACATTGCTGTGTAT
       S  A  L  F  N  A  I  S  W  V  E  S  S  S  W  D  G  R  Y  A  L  V  V  A  G  D  I  A  V  Y
                                  130                                 140                                 150
 451  GCTAAAGGCAGTGCGAGGCCCACCGGTGGAGCAGGGGCTGTGGCCATGCTAGTGGGCGCCAATGCTCCCCTAGTGTTCGACAGAGGAGTT
       A  K  G  S  A  R  P  T  G  G  A  G  A  V  A  M  L  V  G  A  N  A  P  L  V  F  D  R  G  V
                                  160                                 170                                 180
 541  CGTTCATCACACATGCAACATGCTTATGACTTCTACAAACCGGATCTGTCCTCGCTGTACCCCACCGTGGATGGCAAGCTGTCAATTCAA
       R  S  S  H  M  Q  H  A  Y  D  F  Y  K  P  D  L  S  S  L  Y  P  T  V  D  G  K  L  S  I  Q
                                  190                                 200                                 210
 631  TGCTATCTTAGTGCCTTAGATCATTGTTATCAACTGTACTGCTCCAAGATCCAGAAACAACTTGGAGAGAAGTTCGATATTGAGCGGCTG
       C  Y  L  S  A  L  D  H  C  Y  Q  L  Y  C  S  K  I  Q  K  Q  L  G  E  K  F  D  I  E  R  L
                                  220                                 230                                 240
 721  GATGCAGTTCTCTTCCACGCGCCTTATTGTAAGTTGGTGCAGAAGTCTCTTGCTCGCCTCGTCTTGAACGACTTTGTGCGGGCATCAGAG
       D  A  V  L  F  H  A  P  Y  C  K  L  V  Q  K  S  L  A  R  L  V  L  N  D  F  V  R  A  S  E
                                  250                                 260                                 270
 811  GAGGAGCGGACGACTAAATACTCCAGTCTGGAAGCACTAAAAGGCGTGAAGCTAGAAGATACGTACTTCGACCGAGAAGTTGAGAAAGCA
       E  E  R  T  T  K  Y  S  S  L  E  A  L  K  G  V  K  L  E  D  T  Y  F  D  R  E  V  E  K  A
                                  280                                 290                                 300
 901  GTCATGACATACAGCAAGAACATGTTTGAAGAGAAAACAAAGCCCTCGCTGTTGCTCGCCAACCAAGTCGGCAACATGTACACTCCTTCG
       V  M  T  Y  S  K  N  M  F  E  E  K  T  K  P  S  L  L  L  A  N  Q  V  G  N  M  Y  T  P  S
                                  310                                 320                                 330
 991  CTTTACGGAGGTTTGGTCTCTCTATTGGTCAGCAAGAGCGCCCAGGAGTTGGCAGGGAAGCGCGTGGCCTTGTTTTCTTACGGCTCCGGA
       L  Y  G  G  L  V  S  L  L  V  S  K  S  A  Q  E  L  A  G  K  R  V  A  L  F  S  Y  G  S  G
                                  340                                 350                                 360
1081  CTGGCCTCTTCCATGTTCTCTCTAAGAATATCATCGGACGCCAGCGCGAAATCTTCTCTGCAACGCCTCGTCTCGAATCTCTCGCACATC
       L  A  S  S  M  F  S  L  R  I  S  S  D  A  S  A  K  S  S  L  Q  R  L  V  S  N  L  S  H  I
                                  370                                 380                                 390
1171  AAGCCGCAGCTGGATCTGCGCCACAAGGTGTCACCAGAGGAGTTTGCACAAACGATGGAGACGAGGGAACACAACCACCACAAAGCTCCA
       K  P  Q  L  D  L  R  H  K  V  S  P  E  E  F  A  Q  T  M  E  T  R  E  H  N  H  H  K  A  P
                                  400                                 410                                 420
1261  TACACCCCAGAGGGCTCGATCGACGTCTTGTTTCCAGGAACTTGGTATCTGGAGAGCGTGGACAGCCTATACCGCCGCAGCTACAAGCAA
       Y  T  P  E  G  S  I  D  V  L  F  P  G  T  W  Y  L  E  S  V  D  S  L  Y  R  R  S  Y  K  Q
                                  430                                 440                                 450
1351  GTTCCTGGATGATGATGATGCCGCCACTAATGAAGTTTTACTCCTCAAGCTTTAAGTGACAGTTCCCTGTGCACTACACTCTTTGTCGGA
       V  P  G

1441  TGAACACACACCAAGGGACTCTGTTTTAATGAACAGTAAGCTTGTCCGCATAGTTCACACTGTAGCTGCCGATATTTTGTTGAGAGCTGC
1531  TATATATTCTGATTTATTCTTTTATTTAATAAAACAGATTCATTTAAAAAAA
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Fig. 3. Restriction-endonuclease map, and nucleotide and deduced amino acid sequence from the cDNA encoding *B. germanica* HMG-CoA synthase. (A) The solid bar indicates the coding sequence. Scale is in kilobases. Arrows indicate the start site, direction and strand sequenced. (B) Numbers to the left of the lines indicate nucleotide positions. Amino acid residue 1 is the putative initiator methionine. The potential polyadenylation signal is boxed.

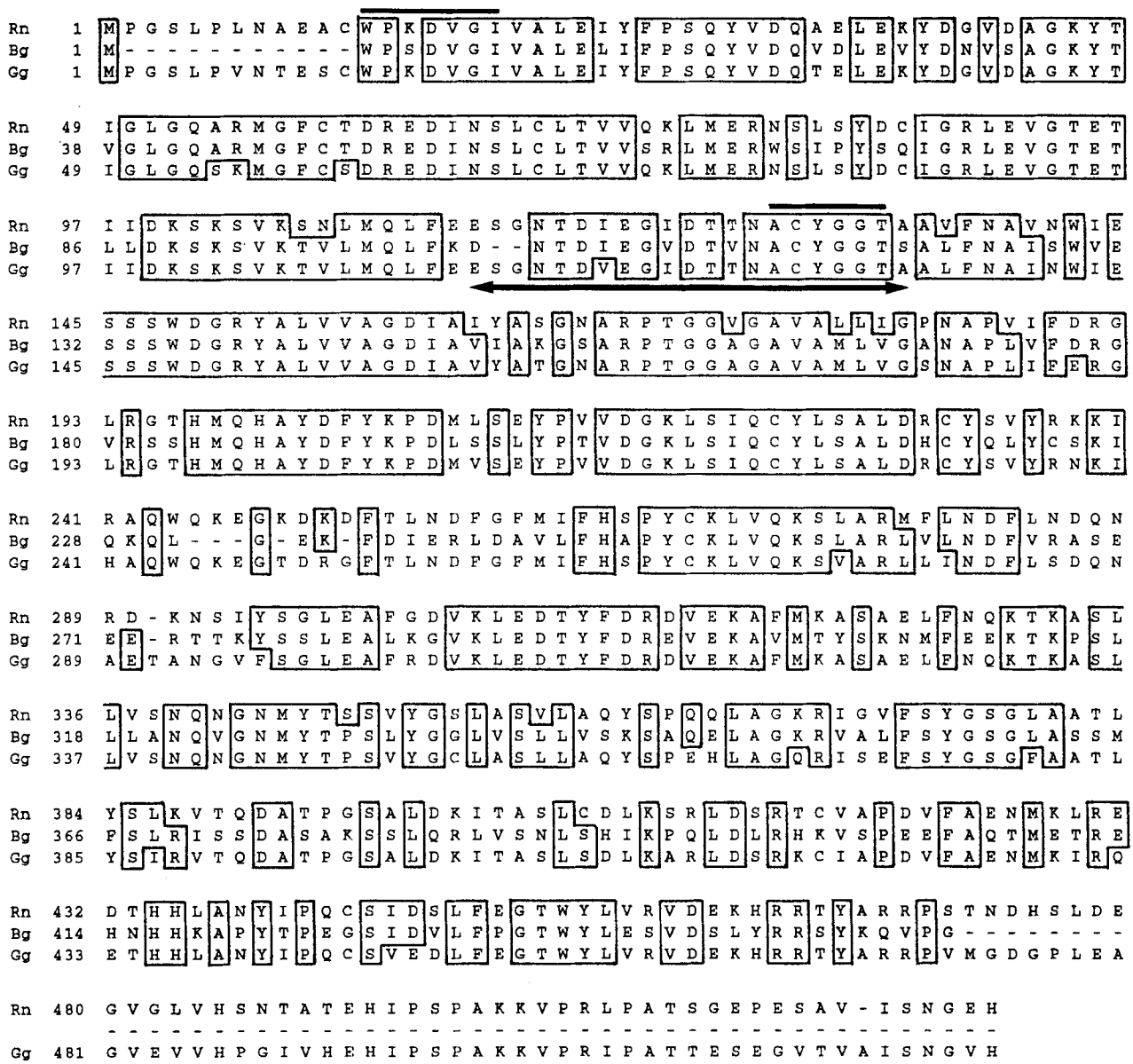

Fig. 4. Alignment of HMG-CoA synthase sequences. Amino acid sequence of *B. germanica* HMG-CoA synthase (Bg) was aligned with both rat (*Rattus norvegicus*, Rn) and chicken (*Gallus gallus*, Gg) HMG-CoA synthases. Gaps (−) were introduced to optimize alignments. Only residues identical to those of the *B. germanica* enzyme were boxed. The position corresponding to the oligonucleotides used in PCR experiments (OST_1 and OST_2) are overlined. The region that has been postulated to be part of the catalytic site of the vertebrates enzyme [29], corresponds to residues 103−121 in *B. germanica* protein (underlined).

The isolated cDNA probably corresponds to the cytosolic HMG-CoA synthase. The N-terminus does not appear to contain a leader peptide to target the protein into mitochondria. All kinds of eukaryotic organisms, from yeast to mammals, import nuclear-encoded mitochondrial proteins by using a leader peptide, rich in hydroxylated and basic amino acid residues [33]. In the analysis of several positive clones for *B. germanica* HMG-CoA synthase no such peptide was found in the N-terminal domain of the protein.

B. germanica protein and vertebrate HMG-CoA synthases are highly similar in amino acid residues, and this similarity is higher within the N-terminal region, where the catalytic site is located. Miziorko and Behnke [30] purified the mitochondrial form of the enzyme from chicken and used an active-site-specific covalent-modification reagent to identify the peptide that contained the active site. The sequence of this peptide is nearly identical in vertebrate organisms [16, 30] and closely related to *B. germanica* enzyme (Fig. 4). Thus, the sequence of the active site is conserved not only between mitochondrial and cytoplasmic enzymes from major species but also across different eukaryotic organisms. It is interesting to compare several important amino acid residues for their contribution to the structural conformation or the catalytic function of the enzyme. Glycines and prolines, which are scattered along the whole protein, appear to be highly conserved (26 out of 29, and 11 out of 16, respectively), with respect to rat cytosolic HMG-CoA synthase [27]. The level of conservation of cysteine residues is also high (6 out of 7). The importance of cysteinyl sulfhydryls to the catalytic process has been documented for avian [34], ox [35] and yeast enzymes [36]. In the avian enzyme the cysteine involved in formation of this intermediate has been iden-

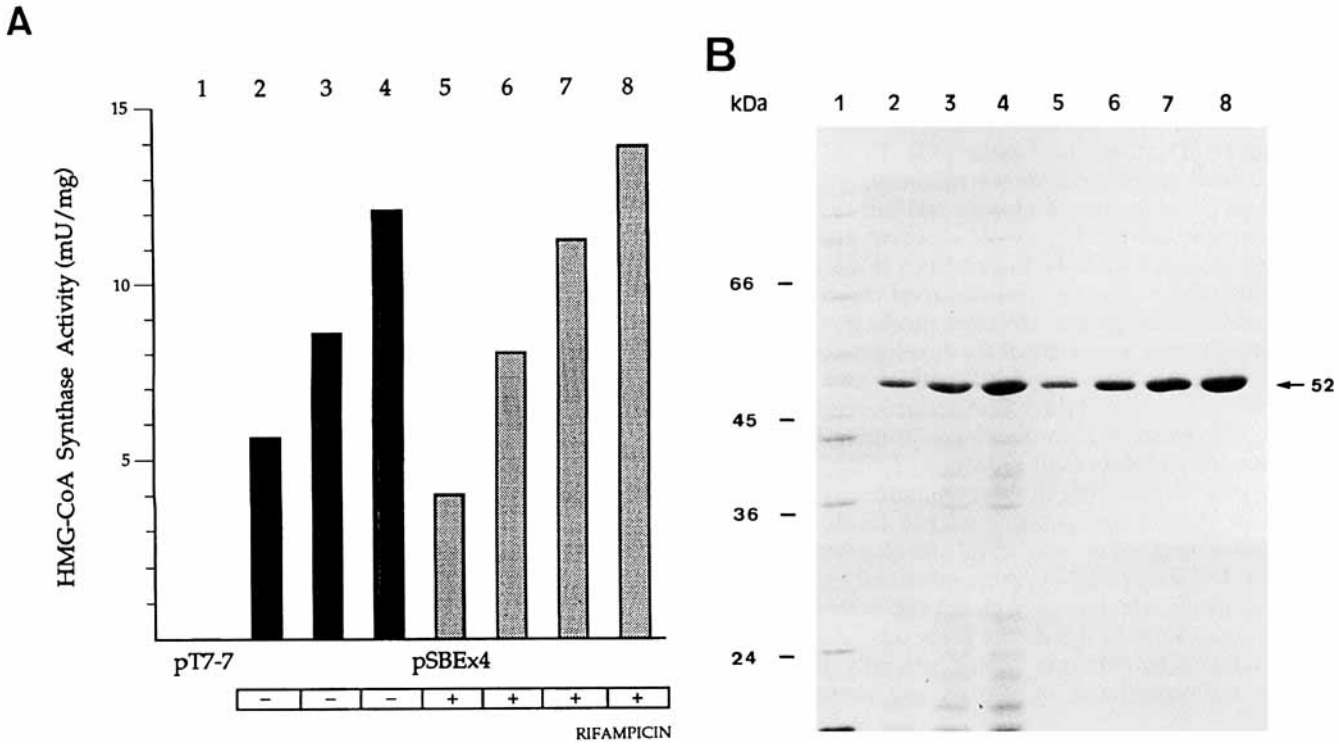

Fig. 5. Expression of *B. germanica* HMG-CoA synthase in *E. coli*. The complete coding region of the cDNA corresponding to *B. germanica* HMG-CoA synthase was placed under the control of the ϕ10 promoter (see Materials and Methods), and expressed in BL21(DE3) cells. (A) HMG-CoA synthase activity was determined in extracts of *E. coli* cells harboring pSBEx4 at different times after isopropyl-thio-β-D-galactopyranoside induction either with or without rifampicin. After 5 h, no activity was found in cells with plasmid control either with or without isopropyl-thio-β-D-galactopyranoside (lane 1); HMG-CoA synthase activity after isopropyl-thio-β-D-galactopyranoside induction at 1, 2 and 3 h (lanes 2–4, respectively); HMG-CoA synthase activity after rifampicin addition at 30 min, 1, 2 and 3 h (lanes 5–8, respectively). (B) [^{35}S]Methione radiolabelled proteins from the culture expressed were fractionated in SDS/PAGE (20 µg/lane) and subjected to fluorography. Lane 1, cells expressing the control plasmid pT7-7, 2 h after isopropyl-thio-β-D-galactopyranoside induction; lanes 2–4, cells harboring pSBEx4 1, 2 and 3 h, respectively, after induction; lanes 5–8, expression of pSBEx4 with rifampicin added 30 min after induction; samples were collected at 30 min, 1, 2 and 3 h thereafter.

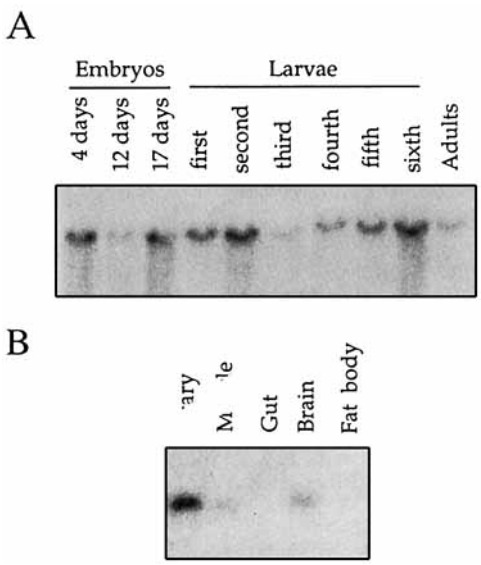

Fig. 6. Analysis of *B. germanica* HMG-CoA synthase mRNA levels during development and in different tissues. (A) A blot with total RNA (20 µg/lane) from various stages in *B. germanica* development was hybridized to the λBgST11 insert. (B) A blot with total RNA (5 µg/lane) from different tissues of *B. germanica* adult females was hybridized with the probe indicated above.

tified [37]. In the case of *B. germanica* the cysteine, which is probably involved in the formation of the acyl-*S*-enzyme intermediate, can be mapped as Cys116 in the sequence deduced from the HMG-CoA synthase cDNA. The role of other sulfhydryls that may influence enzyme activity remains to be elucidated.

Northern-blot analysis of RNA from heads and from decapitated specimens of *B. germanica* adult females indicates that HMG-CoA synthase mRNA is more abundant in the latter case (Fig. 2B). No difference was found in HMG-CoA synthase mRNA levels from females' heads on different days of the *B. germanica* gonotrophic cycle (data not shown), as occurs with HMG-CoA reductase [38]. In insects the organs that present the highest specific activity for both HMG-CoA synthase and HMG-CoA reductase enzymes are the corpora allata [39]. The production of juvenile hormone by the corpora allata is subjected to cyclic variations that have been related to the increase in either HMG-CoA synthase or HMG-CoA reductase activities [15, 40–43]. If mRNA levels of both enzymes in the corpora allata account for less than 0.025% of the head (mass percentage of corpora allata with respect to head), a hypothetical increase of 1000-fold in this fraction may not significantly affect the whole pool of their mRNA in the head (these genes are also expressed in brain [3, 38, 44]).

Developmental studies in *B. germanica* showed that the HMG-CoA synthase mRNA levels are regulated during embryonic and larval stages. In embryos, the highest HMG-CoA 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 [45]. In this stage, the embryo is well segmented, shows rudiments of all appendages and prepares for dorsal closure and full organogenesis. In developmental terms 17-day-old embryos can be considered as larvae. Therefore, the high mRNA levels observed in 17-day-old embryos and in the first larval stages can be related to larval development. Dolichol production and glycoprotein biosynthesis are essential for development and differentiation in *D. melanogaster* [46], and in this species the highest mRNA levels of HMG-CoA reductase were found in embryos [47]. In sea urchin development there is a coordinate expression of HMG-CoA reductase, dolichol production and glycoprotein biosynthesis [48]. Accordingly, analogous function for HMG-CoA synthase mRNA levels throughout *B. germanica* embryonic and larval development could be postulated. The rising mRNA levels measured from fourth to sixth larval instar, which reach a clear peak in this later instar (Fig. 6A), may be related with the processes of organogenesis preceding metamorphosis, which specially involves the formation and capacitation of primary and accessory sexual organs [49].

The comparative analysis of different adult female tissues (Fig. 6B) clearly indicates that the highest levels of mRNA HMG-CoA synthase were observed in the ovary, with lower amounts in brain and muscle, whereas expression was not detected in gut or fat-body tissues. Since we used mature ovaries in a pre-choriogenetic stage [50] it is plausible that HMG-CoA synthase could have a role in the complex process of choriogenesis, perhaps related to the glycosylation of chorion proteins. Glycosylation is a characteristic post-translational modification of certain chorion proteins [51]. Moreover, HMG-CoA synthase expression in these organs could be related to the synthesis of isoprenylated proteins, as the γ subunit of guanine-nucleotide-binding regulatory-proteins and the product of the *ras* oncogene are present mainly in the brain and ovaries of *D. melanogaster* [52, 53]. This expression pattern of HMG-CoA synthase correlates with that of HMG-CoA reductase from *B. germanica* [38], which suggests a coordinate regulation of both genes to produce isoprenoid compounds, as occurs in the mammalian sterol pathway [16, 54], presumably by interaction with a common isoprenoid-regulatory protein.

Analysis of the promoter for HMG-CoA synthase may provide useful information on transcriptional regulation by isoprenoid compounds other than cholesterol and may allow comparison with the mammalian HMG-CoA synthase promoter.

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REFERENCES

1. Clinkenbeard, K. D., Sugiyama, T., Reed, W. D. & Lane, M. D. (1975) *J. Biol. Chem. 250*, 3124–3135.
2. Clinkenbeard, K. D., Reed, W. D., Mooney, R. A. & Lane, M. D. (1975) *J. Biol. Chem. 250*, 3108–3116.
3. Ayté, J., Gil-Gomez, G., Haro, D., Marrero, P. F. & Hegardt, F. G. (1990) *Proc. Natl Acad. Sci. USA 87*, 3874–3878.
4. Brown, M. S. & Goldstein, J. L. (1980) *J. Lipid Res. 21*, 505–517.
5. Goldstein, J. L. & Brown, M. S. (1990) *Nature 343*, 425–430.
6. Maltese, W. A. & Sheridan, K. M. (1988) *J. Biol. Chem. 263*, 10104–10110.
7. Carson, D. D. & Lennarz, W. J. (1981) *J. Biol. Chem. 256*, 4679–4686.
8. Kircher, H. W. (1982) in *Cholesterol system in insects and animals* (Dupont, J., ed.) pp. 1–50, CRC Press, Boca Raton.
9. Beenakkers, Ad M. Th., Van Der Horst, D. J. & Van Marrewijk W. J. A. (1985) *Prog. Lipid Res. 24*, 19–67.
10. Baker, F. C. & Schooley, D. A. (1981) *Biochim. Biophys. Acta 664*, 356–372.
11. Couillaud, F. & Rossignol, F. (1991) *Arch. Biochem. Biophys. 18*, 273–283.
12. Brown, K., Havel, C. M. & Watson, J. A. (1983) *J. Biol. Chem. 258*, 8512–8515.
13. Koeppe, J. K., Fuchs, M., Chen, T. T., Hunt, L. M., Kovalick, G. E. & Briers, T. (1985) in *Comprehensive insect physiology, biochemistry and pharmacology* (Kerkut, G. A. & Gilbert, L. I., eds) vol. 8, pp. 165–203, Pergamon Press, Oxford.
14. Feyereisen, R. (1985) in *Comprehensive insect physiology, biochemistry and pharmacology* (Kerkut, G. A. & Gilbert, L. I., eds) vol. 7, pp. 391–429, Pergamon Press, Oxford.
15. Couillaud, F. (1991) *Mol. Cell. Endocrinol. 77*, 159–166.
16. Gil, G., Goldstein, J. L., Slaughter, C. A. & Brown, M. S. (1986) *J. Biol. Chem. 261*, 3710–3716.
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
18. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.
19. Aviv, H. & Leder, P. (1972) *Proc. Natl Acad. Sci. USA 69*, 1408–1412.
20. Hasel, K. W. & Sutcliffe, J. G. (1990) *Nucleic Acids Res. 18*, 4019.
21. Gubler, H. & Hoffman, B. J. (1983) *Gene (Amst.) 25*, 263–269.
22. Sanger, F., Niklen, S. & Coulson, A. R. (1977) *Proc. Natl Acad. Sci. USA 74*, 5463–5467.
23. Studier, F. W. (1990) *Methods Enzymol. 185*, 60–89.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
25. Laemmli, U. K. (1970) *Nature 227*, 680–685.
26. Clinkenbeard, K. D., Sugiyama, T. & Lane, M. D. (1975) *Methods Enzymol. 35*, 160–167.
27. Ayté, J., Gil-Gomez, G. & Hegardt, F. G. (1990) *Nucleic Acids Res. 18*, 3642.
28. Rogers, S., Wells, R. & Rechsteiner, M. (1986) *Science 234*, 364–368.
29. Kattar-Cooley, P. A., Wang, H.-H. L., Mende-Mueller, L. M. & Miziorko, H. M. (1990) *Arch. Biochem. Biophys. 283*, 523–529.
30. Miziorko, H. M. & Behnke, C. E. (1985) *J. Biol. Chem. 260*, 13513–13516.
31. Higgins, D. G. & Sharp, P. M. (1988) *Gene (Amst.) 73*, 237–244.
32. Lowe, D. M. & Tubbs, P. K. (1985) *Biochem. J. 227*, 591–599.
33. von Heijne, G. (1986) *EMBO J. 5*, 1335–1342.
34. Miziorko, H. M., Clinkenbeard, K. D., Reed, W. D. & Lane, M. D. (1975) *J. Biol. Chem. 250*, 5768–5773.
35. Lowe, D. M. & Tubbs, P. K. (1985) *Biochem. J. 232*, 37–42.
36. Middleton, B. & Tubbs, P. K. (1972) *Biochem. J. 126*, 27–34.
37. Vollmer, S. H., Mende-Mueller, L. M. & Miziorko, H. M. (1988) *Biochemistry 27*, 4288–4292.
38. Martínez-González, J., Buesa, C., Piulachs, M. D., Bellés, X. & Hegardt, F. G. (1993) *Eur. J. Biochem 213*, 233–241.
39. Monger, D. J. (1985) *Methods Enzymol. 110*, 51–58.
40. Kramer, S. J. & Law, J. H. (1980) *Insect Biochem. 10*, 569–575.
41. Bhaskaran, G., Dahm, K. H., Jones, G. L., Peck, K. & Faught, S. (1987) *Insect Biochem. 17*, 933–937.

42. Feyereisen, R. & Farnsworth, D. E. (1987) *Mol. Cell. Endocrinol. 77*, 159–166.
43. Couillaud, F. & Feyereisen, R. (1991) *Insect Biochem. 21*, 131–135.
44. Volpe, J. J. & Obert, K. A. (1981) *Arch. Biochem. Biophys. 212*, 88–97.
45. Tanaka, A. (1976) *Kontyu 44*, 512–525.
46. Gauger, A., Glicksman, M. A., Salatino, R., Condie, J. M., Schubiger, G. & Brower, D. L. (1987) *Development (Camb.) 100*, 237–244.
47. Gertler, F. B., Chiu, C., Richter-Mann, L. & Chin, D. J. (1988) *Mol. Cell. Biol. 8*, 2713–2721.
48. Woodward, H. D., Allen, J. M. C. & Lennarz, W. J. (1988) *J. Biol. Chem. 263*, 2513–2517.
49. Sehnal, F. (1985) in *Comprehensive insect physiology, biochemistry and pharmacology* (Kerkut, G. A. & Gilbert, L. I., eds) vol. 2, pp. 1–86, Pergamon Press, Oxford.
50. Pascual, N., Cerdá, X., Benito, B., Tomás, J., Piulachs, M. D. & Bellés, X. (1992) *J. Insect. Physiol. 38*, 339–348.
51. Regier, J. C. & Kafatos, F. C. (1985) in *Comprehensive insect physiology, biochemistry and pharmacology* (Kerkut, G. A. & Gilbert, L. I., eds) vol. 1, pp. 113–151, Pergamon Press, Oxford.
52. Ray, K. & Ganguly, R. (1992) *J. Biol. Chem. 267*, 6086–6092.
53. Segal, D. & Shilo, B-Z. (1986) *Mol. Cell. Biol. 6*, 2241–2248.
54. Rosser, D. S. E., Ashby, M. N., Ellis, J. L. & Edwards, P. A. (1989) *J. Biol. Chem. 264*, 12653–12656.