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MOLECULAR CLONING OF THE HUMAN GENE ENCODING LANOSTEROL SYNTHASE FROM A LIVER cDNA LIBRARY

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SUMMARY: Lanosterol synthase [(*S*)-2,3-epoxysqualene mutase (cyclizing, lanosterol forming), EC 5.4.99.7] catalyzes the cyclization of (*S*)-2,3-oxidosqualene to lanosterol in the reaction that forms the sterol nucleus. We report herein the cloning and characterization of the human gene (*OSC*) encoding lanosterol synthase, a predicted 83 kDa protein of 732 amino acids. The deduced amino acid sequence is 36-40% identical to known yeast and plant homologues and 83% identical to *Rattus norvegicus* lanosterol synthase. The new gene was shown to encode lanosterol synthase. The yeast lanosterol synthase deficient mutant SMY8 was complemented by the human gene, and a cell-free homogenate of SMY8 expressing the human gene was shown to convert 2,3-oxidosqualene to lanosterol.

The enzymic conversion of (*S*)-2,3-oxidosqualene to lanosterol (**Figure 1**), a key step in the biosynthesis of cholesterol and the other steroids (1-5), is remarkable for a number of reasons. Although the reaction involves a conformationally flexible substrate and very reactive carbocation intermediates, it is efficiently channeled through a sequence of four specific cyclizations and four 1,2-group rearrangements to lanosterol with complete structural and stereochemical control (**Figure 1**). Other enzymes transform the same substrate, 2,3-oxidosqualene, to a wide variety of naturally occurring polycyclic triterpenoids, with similar structural specificity, indicating that a precise molecular interaction mechanism must exist for very fine control of the conformation of this hydrocarbon-like substrate by the catalytic protein. There is no information at present regarding the structural aspects of such control. Because of this fact and the importance of understanding this and other enzymes in sterol biosynthesis, we have been engaged for some time in the study of lanosterol synthase [(*S*)-2,3-oxidosqualene mutase (cyclizing, lanosterol forming), EC 5.4.99.7] using chemical and molecular biological approaches. Reported herein is the cloning of the human gene encoding this protein from a *Homo sapiens* liver cDNA library using hybridization techniques. This work extends our knowledge of the critical elements of the lanosterol synthase sequence when combined with information recently obtained for the cyclase genes from *Candida albicans* (6, 7), *Arabidopsis thaliana* (8), *Saccharomyces cerevisiae* (9, 10), and *Rattus norvegicus* (11).

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Abbreviations: bp, base pair; BSA, bovine serum albumin; ORF, open reading frame; LB, Luria broth; OSC, 2,3-oxidosqualene-lanosterol cyclase; SSC, sodium chloride-sodium citrate; TE, 10 mM Tris•HCl (pH 8.0) / 1 mM EDTA; TLC, thin layer chromatography.

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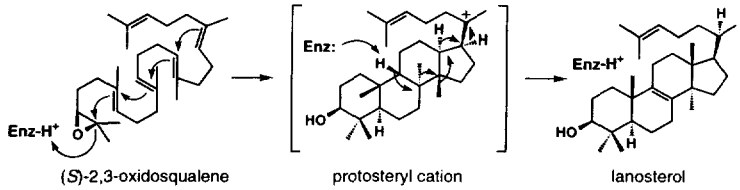

Figure 1. Lanosterol synthase cyclizes (*S*)-2,3-oxidosqualene to lanosterol via the protosteryl cation. Lanosterol is further metabolized to cholesterol and thence to steroid hormones and bile salts.

MATERIALS AND METHODS

Materials. The human liver cDNA library (Catalog No. HL1115a) in λgt10 was obtained in phage form from Clontech (Palo Alto, CA). DNA was isolated from bacteria using the alkaline lysis method in conjunction with Qiagen anion exchange chromatography (Chatsworth, CA). QIAEX gel extraction kit was purchased from QIAGEN. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) or GIBCO BRL (Gaithersburg, MD). T4 DNA ligase was from GIBCO BRL or Promega (Madison, WI). Gene Screen Plus hybridization transfer membrane and radioisotopes were purchased from New England Nuclear (Boston, MA).

Strains and media. *E. coli* strain DH5α (12) was used for all DNA manipulations, and strain C600-*Hfl* was used as the host for λ derivatives. The human lanosterol synthase was expressed in *S. cerevisiae* strain SMY8 (*hem1Δ erg7Δ ura3-52 leu2-3,112 ade2*) (13). SMY8 transformants were selected at 30 °C on synthetic complete medium lacking uracil, supplemented with hemin chloride (13 mg/L), ergosterol (20 mg/L), and Tween 80 (0.5%) (14). Complementation analysis was performed on 1% yeast extract, 2% peptone, 2% galactose, and 2% agar, supplemented with hemin chloride (13 mg/L) and Tween 80 (0.5%). Yeast cells for the activity assay were grown in the corresponding liquid medium.

Screening of library. The library was plated on eight LB agar + 10 mM $MgSO_4$ plates (24.5 cm x 24.5 cm). Saturated culture of *E. coli* C600-*Hfl* in LB + 10 mM $MgSO_4$ + 0.2% maltose media (2 mL) was placed in eight 50 mL tubes. The library stock (titered at 5.7 x 10^9 pfu/mL) was diluted 500-fold into 1x λ dilution buffer (1 mL). This dilution was again diluted 1:8 to make a dilution with a titer of 1.4 x 10^6 pfu/mL. The resulting phage solution (107 μL) was placed in each of the eight tubes with host bacteria. The tubes were incubated at 37 °C for 15 minutes. LB top agarose + 10 mM $MgSO_4$ (30 mL) at 45 °C was added to each tube, and the mixture was poured onto a pre-warmed (*ca.* 37 °C) LB agar + 10 mM $MgSO_4$ plate. After the top agarose had hardened, the plates were incubated at 37 °C for 6-8 hours until small, non-overlapping plaques had formed. The plates were chilled for two hours at 4 °C to allow the top agarose to harden before lifting.

The plaques were lifted onto Gene Screen Plus hybridization transfer membrane (22 cm x 22 cm). The filter was placed on the plate and marked with pin holes for alignment. After 1 minute, the filter was lifted carefully off the plate and placed phage side up onto a filter paper soaked in DNA denaturing solution (1.5 M NaCl/0.5 N NaOH) for 30 seconds. The filter was then immersed in DNA denaturing solution for 1 minute, neutralized in a bath of 1.5 M NaCl/0.5 M Tris•HCl (pH 8.0) for 5 minutes, rinsed in 3xSSC, and finally dried. All eight plates were lifted in this manner. A second lift of each plate was performed in the same manner except that the filters were left on each plate for 3 minutes. The dried filters were stored at 4 °C.

A 600 bp *Rsa*I-*Hin*dIII fragment from the pEMBL-derived vector purified from the American Type Culture Collection clone 85347 (15) which had been gel purified using QIAEX was labeled with [α-^{32}P]-dATP using the Prime-It II Random Primer Labeling Kit (Stratagene). A STE Select-D, G-50 spin column (5 Prime -> 3 Prime) was used to purify the unincorporated nucleotides away from the probe. After purification, TE (50 μL) was added to each tube to bring the total volume to 100 μL. To the probe was added sonicated, boiled salmon sperm DNA (Stratagene) (1 mL, 2 mg/mL). The DNA solutions were boiled for 10 minutes and then immediately cooled on ice. Hybridization solution (2 mL) was added to each probe. The probe was then added to the filters, which had been pre-hybridized in an excess of hybridization solution (1% crystalline BSA/1 mM EDTA/0.5 M NaPi (pH 7.2)/7% SDS) for two hours at 65 °C with agitation, and the filters were hybridized overnight at 65 °C with agitation.

The next day the filters were rinsed quickly at room temperature in low-stringency wash buffer (0.5% BSA/1 mM EDTA/40 mM NaPi (pH 7.2)/5% SDS). The filters were then washed in

low-stringency wash buffer at 65 °C for 30 minutes. The wash buffer was changed to high-stringency wash buffer (1 mM EDTA/40 mM NaPi (pH 7.2)/1% SDS), and the filters were washed for a total of 3-4 hours at 65 °C with agitation with three additional changes of the wash buffer. The filters were exposed to X-ray film for one to three days.

The autoradiographs were developed, fixed, and washed. The duplicate autoradiographs were aligned, and twenty-four out of sixty-five duplicate hits were picked from the master plates using the blunt end of a glass pipet. The cores were placed in 1x λ dilution buffer (1 mL), and the phage were allowed to diffuse into the buffer overnight at 4 °C. The plates for the secondary screen were plated in the same manner as the original library; however, 90 mm circular plates were used. After the plaques were grown, the plates were inspected to determine which dilution was best for screening. The plaques were lifted; and the filters were hybridized, washed, and exposed to film as before.

The tertiary screen was performed exactly the same as the secondary screen to assure homogeneity of each clone. Hits from the tertiary screen were used to isolate λ DNA.

Isolation of λ DNA. DNA was isolated from the thirteen clones purified by three rounds of screening. Standard procedures as described in (16) were used in isolating the λ DNA.

A portion of each isolated DNA was digested with *Eco*RI to assess the size of the insert. The digested DNA was analyzed by agarose gel electrophoresis.

The largest inserts were subcloned into the *Eco*RI site of pBluescript II, using standard procedures (16, 17). Enough DNA was then isolated for DNA sequencing using a QIAGEN tip-100 following the manufacturer's Midi-Plasmid Purification Protocol. Partial sequencing of these clones indicated that only 75% of the predicted ORF had been obtained. Therefore, the library was rescreened as before except an *Alw*NI-*Eco*RI restriction fragment from the largest insert was labeled and used as a probe. The second screen yielded four hits which were subsequently screened twice more to yield homogeneous clones. Two of the larger inserts were subcloned into pBluescript; however, it was discovered that there was an *Eco*RI site in the ORF.

The gene was spliced together at the *Eco*RI site after confirming the sequence at the junction by sequencing DNA from the original λ isolate. The λ DNA was sequenced using the *fmol* DNA sequencing system (Promega). Proper ligation of the gene was confirmed by restriction fragment analysis. Upon sequencing this new construct, it was discovered that the clone lacked the 3′-end so the 3′-end of the *OSC* gene was spliced on using an *Afl*II site common to both clones. Both strands of the DNA were then sequenced by the dideoxynucleotide method using the USB Sequenase Kit (version 2.0) (**Figure 2**), and the sequence data were analyzed using the GCG software package (18).

Expression. The ORF of human *OSC* was excised from pCHB-hOSC using *Spe*I and *Xho*I and ligated into the *Bgl*II-*Xho*I site of pDAD2 (19). The proper construction of the overexpression vector was confirmed by restriction fragment analysis.

The yeast shuttle vector, pDAD-hOSC, was used to transform the *erg7* strain SMY8 using a lithium acetate procedure (20). The transformants were selected on synthetic complete media lacking uracil, containing 2% glucose, and supplemented with ergosterol, hemin chloride, and Tween 80, at 30 °C.

SMY8 carrying pDAD-hOSC was grown aerobically in 1% yeast extract, 2% peptone, 2% galactose supplemented with heme (13 mg/L). Cells taken from logarithmic phase (1 g) were suspended in 5 mL of 100 mM sodium phosphate buffer (pH 7.0) and lysed in a French Press at 20,000 psi, and the homogenate was centrifuged at 10,000 x *g* for 20 min. Racemic 2,3-oxidosqualene solution (5 mg in 100 mL 20% Triton X-100) (21, 22) was added to the supernatant. After 12 hours at 25 °C, ethanol (10 mL) was added to terminate the reaction. The precipitate (denatured protein and salt) was removed by filtration, and the filtrate was concentrated under vacuum. The remaining residue was extracted with three volumes of ethyl ether three times, and the combined organic extracts were dried over sodium sulfate, concentrated under vacuum, and purified by silica gel chromatography (5% ethyl ether in hexane) to yield 1.4 mg (56% based on (*S*)-oxidosqualene) of a colorless solid. This material was indistinguishable from an authentic sample of lanosterol by 500 MHz ^{1}H NMR, infrared, and mass spectral analyses.

RESULTS AND DISCUSSION

Several approaches are currently under investigation which are aimed at understanding the molecular details of the cyclization/rearrangement step in sterol biosynthesis, specifically the detailed atomic interactions between lanosterol synthase and (*S*)-2,3-oxidosqualene. One of these,

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ccctttgcctactgctcatgggtgtggagactgatattctggaagactgataggcagattta 61
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Figure 2. Deduced amino acid sequence from 2199 bp ORF of human lanosterol synthase (*OSC*) (GenBank accession no. U22526).

X-ray crystallographic structure determination of the enzyme bound to the substrate-analog inhibitor 2,3-iminosqualene (23), is hampered by the insolubility and hydrophobicity of the yeast protein, which has been studied thus far. Lanosterol synthase from *S. cerevisiae* is not water-

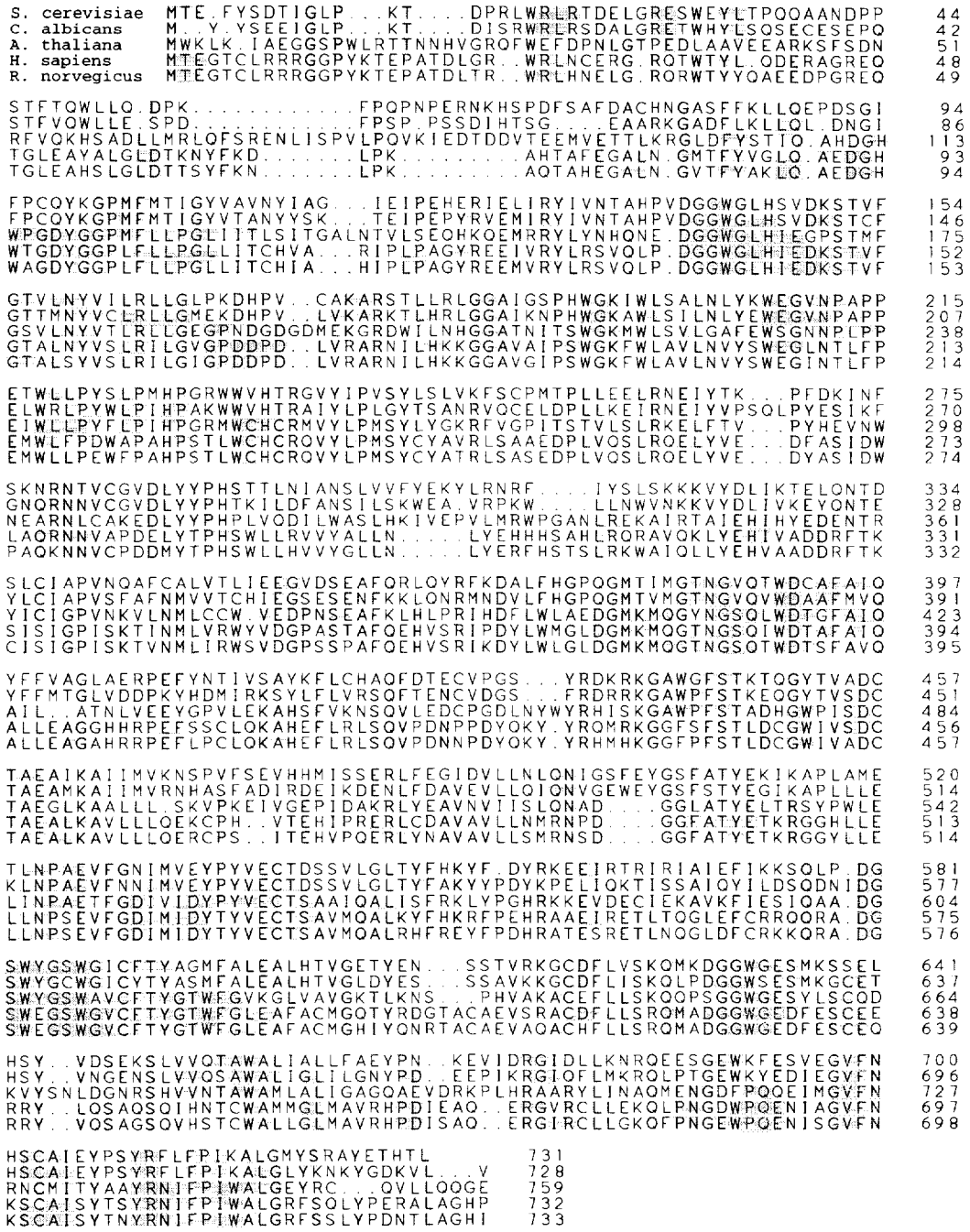

Figure 3. Alignment of predicted amino acid sequences of cyclases from *Saccharomyces cerevisiae, Candida albicans, Arabidopsis thaliana, Homo sapiens,* and *Rattus norvegicus.* Shading denotes identical amino acids in three out of the five protein sequences. Gaps have been introduced to maximize homology.

soluble unless glycerol (*ca.* 20%) and detergent (*ca.* 0.2% Triton X-100) are present. In addition the protein loses activity fairly rapidly above 0 °C. Consequently, more emphasis has been placed near term, on the use of mechanistic probes such as affinity labeling and site-directed mutagenesis-enzyme activity studies. In connection with the latter, knowledge of the structural elements of lanosterol synthase which have been conserved through evolution is critical. Partly for this reason we selected the human enzyme for study.

Out initial intent was to use conserved sequences from the *Candida albicans* lanosterol synthase (6, 7) and *Arabidopsis thaliana* cycloartenol synthase (8) to amplify a lanosterol synthase fragment from human cDNA, and probe a human cDNA library with the radiolabeled fragment. We have used this approach successfully to clone lanosterol synthase from *Saccharomyces cerevisiae* (9). Fortuitously, in the Fall of 1993, a search of the genetic database Genbank using the *S. cerevisiae ERG7* gene yielded a homologous sequence from a human brain cDNA library corresponding to a part of the yeast protein. This opened the way to cloning the human lanosterol synthase gene (*OSC*) by means of hybridization techniques. A portion of the published cDNA clone was used to probe a human liver cDNA library. The largest cDNA obtained from screening over a million clones with this probe was approximately 75% of the predicted full-length sequence. A 5′-fragment of this cDNA was then used for a second screen, which provided a partial sequence homologous to the N-termini of the known homologues. The gene was reconstructed by splicing together the two halves of the ORF at an *Afl*II site in an overlapping region, since none of the isolated clones contained the entire sequence.

Sequencing of the reconstructed cDNA identified a 2199 bp ORF which encodes a 732 amino acid protein 36.1%, 36.0%, 83.3%, and 39.8% identical to cyclases from *Candida albicans*, *Saccharomyces cerevisiae*, *Rattus norvegicus*, and *Arabidopsis thaliana*, respectively. A homology alignment of these enzymes is shown in **Figure 3**.

Expression of the human gene under control of the GAL4 promoter on a 2 m plasmid complemented the *erg7* yeast strain SMY8; however, the yeast had a doubling time at least twice that of a SMY8 strain rescued with the native gene. This slow growth could be due to a number of issues including post-translational modification differences, codon bias, and lack of a "supernatant protein factor" (24-26). Proof of the expression of the human lanosterol synthase was obtained by the experimental demonstration of the catalytic conversion of 2,3-oxidosqualene to lanosterol using cell lysates from an SMY8-derived strain induced to overexpress the recombinant protein. The lanosterol product was identified by ^{1}H NMR, infrared, and mass spectral analyses.

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