

MAMMALIAN WAX BIOSYNTHESIS II: EXPRESSION CLONING OF WAX SYNTHASE
cDNAs ENCODING A MEMBER OF THE ACYLTRANSFERASE ENZYME FAMILY

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Running Title: Mammalian Wax Monoester Synthase

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SUMMARY

Wax monoesters are synthesized by the esterification of fatty alcohols and fatty acids. The mammalian enzyme that catalyzes this reaction has not been isolated. We used expression cloning to identify cDNAs encoding a wax synthase in the mouse preputial gland. The wax synthase gene is located on the X-chromosome and encodes a member of the acyltransferase family of enzymes that synthesize neutral lipids. Expression of wax synthase in cultured cells led to the formation of wax monoesters from straight chain saturated, unsaturated, and polyunsaturated fatty alcohols and acids. Polyisoprenols also were incorporated into wax monoesters by the enzyme. The wax synthase had little or no ability to synthesize cholesteryl esters, diacylglycerols, or triacylglycerols, whereas other acyltransferases, including the acyl-CoA:monoacylglycerol acyltransferase 1 and 2 enzymes, and the acyl-CoA:diacylglycerol acyltransferase 1 and 2 enzymes, exhibited modest wax monoester synthesis activities. Confocal light microscopy indicated that the wax synthase was localized in membranes of the endoplasmic reticulum. Wax synthase mRNA was abundant in tissues rich in sebaceous glands such as the preputial gland and eyelid, and was present at lower levels in other tissues. Co-expression of cDNAs specifying fatty acyl-CoA reductase 1 and wax synthase led to the synthesis of wax monoesters. The data suggest that wax monoester synthesis in mammals involves a two step biosynthetic pathway catalyzed by fatty acyl-CoA reductase and wax synthase enzymes.

INTRODUCTION

The sebaceous glands produce a lipid-rich secretion termed sebum that is exuded onto the surface of the skin. Sebaceous glands are found in the dermis of a wide variety of animals but the chemical composition of sebum is distinct in each species, and in some cases, even within a species (1). For example, adult human sebum is principally composed of wax monoesters (25% of total lipids), triglycerides (41%), free fatty acids (16%), and squalene (12%), whereas the composition of mouse fur sebum is wax monoesters (5%), wax diesters (65%), triglycerides (6%), and free and esterified sterols (23%). Within the mouse, the sebum elaborated by the preputial glands, which are specialized sebaceous glands involved in pheromone production, has a much higher content of wax monoesters (48%) than fur sebum (1). While much is known concerning the lipid content of sebum, the biosynthesis and functions of this secretion remain largely unfamiliar. Sebum may lubricate the skin and/or contribute to the development of the water barrier. These functions are implied by the role of the meibomian glands, which are modified sebaceous glands in the margin of the eyelid that secrete meibum. Like sebum, meibum is lipid-rich and contains a high percentage of wax monoesters (~35%) (2,3). Meibum forms the outer layer of the tear film and prevents the evaporation and spreading of this film (4).

Wax monoesters are a major component of mammalian sebum and meibum. Members of this class of neutral lipids are widely distributed among different organisms and fulfill various biological functions including the prevention of desiccation in insects and plants, sound transmission and/or buoyancy regulation in sperm whales, and energy storage in algae and plankton (5). Wax monoesters are synthesized by wax synthase enzymes, which conjugate a long chain fatty alcohol to a fatty acyl-CoA via an ester linkage. Wax synthase enzymes and

encoding genes are known from the jojoba plant (6) and the bacterium *Acinetobacter calcoaceticus* (7). The plant wax synthase is hydrophobic and is predicted to span the membrane seven to nine times. The protein shares sequence identity with seven *Arabidopsis* genes but does not have an obvious mammalian orthologue. Co-expression of the jojoba wax synthase gene with a fatty acid elongase and a bacterial fatty acyl-CoA reductase leads to the synthesis of large quantities of wax in the seeds of transgenic *Arabidopsis* plants (6). The bacterial wax synthase is unrelated to the jojoba wax synthase or to other mammalian proteins in the database but does share sequence identity with proteins specified by several microbial genes (7). It is a bifunctional enzyme, exhibiting wax synthase activity and the ability to form triacylglycerols from diacylglycerol substrates and fatty acyl-CoAs (acyl-CoA:diacylglycerol acyltransferase (DGAT) activity).

In addition to wax monoesters, mice and other species elaborate more complex wax diesters and triesters in fur sebum that are composed of three and four fatty acids/alcohols in ester linkage (8,9). The biosynthetic enzymes for these lipids are as yet undefined; however, mice deficient in the genes encoding DGAT1 or stearoyl-CoA desaturase 1 have reduced wax diesters in their fur (10-12). These animals also exhibit sebaceous gland atrophy, thus it is not clear whether the reduction in wax diesters in the mutant mice is due to decreased synthesis or secretion of lipid from the deteriorated gland.

In the current study, an expression cloning approach was taken to identify mouse cDNAs encoding wax monoester synthase activity. A cDNA that specifies a member of the DGAT2/acyl CoA:monoacylglycerol acyltransferase (MGAT) enzyme family with previously unknown function (13) was identified in a library constructed from preputial glands. Recombinant wax synthase, DGAT, and MGAT proteins were expressed in mammalian and

insect cells and their enzyme activities compared. The subcellular localization of the mammalian wax synthase was characterized and its tissue distribution determined by analysis of mRNA by real time polymerase chain reactions. Co-expression in cultured cells of the identified wax synthase with either of the fatty acyl CoA reductase enzymes reported in the accompanying paper (14) resulted in the synthesis of wax monoesters.

EXPERIMENTAL PROCEDURES

Expression Cloning - A cDNA library was constructed from 5 µg of poly(A)⁺-enriched mRNA isolated from the preputial glands of male mice (mixed strain, C57Bl/6J;129S6/SvEv) using the SuperScript™ Plasmid System for cDNA Synthesis and Cloning (Invitrogen, Carlsbad, CA). cDNA inserts were ligated into the pCMV•SPORT6 vector that had been cleaved with the restriction enzymes *NotI* and *SalI*, and the resulting DNAs were electroporated into *Escherichia coli* ElectroMAX™ DH10B™ Competent Cells (Invitrogen). A library of approximately 6.8 x 10⁶ independent clones was produced in which 95% of the plasmids harbored cDNA inserts with average lengths of ~1.8 kilobases (kb). Aliquots of the ligation mix containing approximately 200 clones were introduced into *E. coli* and plasmid DNA was isolated using the Wizard® Plus Miniprep DNA Purification System (Promega, Madison, WI). Human embryonic kidney (HEK) 293 cells (American Type Culture Collection CRL number 1573) were transfected with pools of cDNAs using the FuGENE6 reagent (Roche, Indianapolis, IN) and assayed for wax synthase activity as described below in the section entitled “*Wax Synthase Enzyme Assay in Transfected Cells*”.

Mammalian Expression Plasmids – A pCMV•SPORT6 vector containing the mouse wax synthase cDNA (Genbank™/EBI Data Bank Accession numbers AY611031 and AY611032) was isolated by expression cloning as described above. A human wax synthase cDNA (Genbank™/EBI Data Bank Accession number AY605053) was amplified by the polymerase chain reaction (PCR) from adult human skin Gene Pool™ cDNA (Invitrogen, catalog no. D8115-

01) using the following oligonucleotide primers: 5'-GCCTATGTCGACGGCACAATGCTCTTGCCCTCTAAG-3' and 5'-CCGTTAGGATCCTGAAGGCTACTGGGGATGTCTGTC-3'. The amplified DNA product was digested with the restriction enzymes *SalI* and *BamHI* and ligated into the pCMV6 vector (Genbank™/EBI Data Bank accession number AF239250).

A mouse MGAT1 cDNA (nucleotides 1 –1008 of Genbank™/EBI Data Bank accession number AF384162) was amplified by the PCR from random hexamer primed mouse kidney cDNA using the following primers:

5'-GCCTATGTCGACCAGTACCACAAATCCTGCGAAAGG-3' and 5'-ATTATGCGGCCGCGGAGGAAGGAGGTTATTTAAATACC-3'. The amplified DNA product was digested with the restriction enzymes *SalI* and *NotI* and ligated into the pCMV•SPORT6 vector (Invitrogen) to produce the pCMV•SPORT6-mMGAT1 expression plasmid.

A mouse MGAT2 cDNA (nucleotides 1 – 1017 of Genbank™/EBI Data Bank accession number AY157609) was amplified by the PCR from random hexamer primed mouse kidney cDNA using the following primers: 5'-GCCTATGTCGACAATCCGCAAAGCCCGTGTGTAGAG-3' and 5'-ATTATGCGGCCGCTGGAGACACTTAGCAGAACTCCAG-3'. The amplified DNA product was digested with the restriction enzymes *SalI* and *NotI* and ligated into the pCMV•SPORT6 vector generating the pCMV•SPORT6-mMGAT2 expression plasmid.

A mouse DGAT2 cDNA (nucleotides 51-1227 of Genbank™/EBI Data Bank Accession number AF384160) was amplified by the PCR from random hexamer primed mouse liver cDNA using the following primers: 5'-GCCTATGTCGACGGCTTCAGCATGAAGACCCTCATC-3'

and 5'-ATTATGCGGCCGCGTCAGTTCACCTCCAGCACCTCAG-3'. The amplified DNA product was digested with the restriction enzymes *SalI* and *NotI* and ligated into the pCMV•SPORT6 vector (Invitrogen) to make the pCMV•SPORT6-mDGAT2 expression plasmid.

Construction of epitope-tagged expression plasmids - An expression plasmid (pCMV6-FLAG-mWS) encoding a FLAG epitope-tagged version of the mouse wax synthase protein was constructed as follows. The mouse cDNA was amplified from the pCMV•SPORT6-mWS expression plasmid described above by the PCR using the oligonucleotide primers, 5'-GTACCTGTTCGACCCACCATGGATTACAAGGATGACGACGATAAGGAGATTCTGGGCACCATGTTCTGG-3' and 5'-ATTATGCGGCCGCTCCAGTGAGGACCAGTCAAGTCAG-3'. The amplified DNA product was digested with the restriction enzymes *SalI* and *BamHI* and ligated into the pCMV6 vector. The encoded mouse protein has the FLAG epitope (amino acid sequence DYKDDDDK) linked to the amino terminus of the wax synthase enzyme.

The pCMV-Script-FLAG-hDGAT1 plasmid, composed of the human DGAT1 cDNA (nucleotides 245-1895 of Genbank™/EBI Data Bank Accession NM_012079) in the pCMV-Script vector (Stratagene, La Jolla, CA) was a generous gift of Dr. Simon M. Jackson of Tularik, Inc., South San Francisco, CA. The encoded protein has the FLAG epitope tag inserted between amino acids 2 (G) and 3 (D) at the amino terminus of the encoded protein.

An expression construct (pCMV•SPORT6-FLAG-mDGAT2) encoding a FLAG epitope-tagged version of the mouse DGAT2 protein was constructed as follows. A modified DGAT2 cDNA was amplified from the pCMV•SPORT6-mDGAT2 template described above by the PCR using the oligonucleotide primers, 5'-

GTACCTGTCGACCCACCATGGATTACAAGGATGACGACGATAAGATGAAGACCCTC
ATCGCCGCCTAC-3' and 5'-ATTATGCGGCCGCGTCAGTTCACCTCCAGCACCTCAG-3'.
The PCR-amplified DNA product was digested with the restriction enzymes *SalI* and *NotI* and
ligated into the pCMV•SPORT6 vector. The encoded DGAT2 protein has the FLAG epitope at
the amino terminus.

An expression plasmid (pCMV•SPORT6-FLAG-mMGAT1) encoding a FLAG epitope-
tagged version of the mouse MGAT1 protein was constructed as follows. The pCMV•SPORT6-
mMGAT1 template described above was used as a template with the oligonucleotide primers, 5'-
GTACCTGTCGACCCACCATGGATTACAAGGATGACGACGATAAGAGTCCTCCGGGT
AGAACCATGATG-3' and 5'-
ATTATGCGGCCGCGGAGGAAGGAGGTTATTTAAATACC-3'. The amplified DNA
product was digested with the restriction enzymes *SalI* and *NotI* and ligated into the
pCMV•SPORT6 vector. The encoded MGAT1 protein has the FLAG epitope linked to the
amino terminus.

Wax Synthase Enzyme Assay in Transfected Cells - On day 0 of an experiment, HEK 293 cells
were plated at a density of 4×10^5 cells per 60-mm dish in low glucose Dulbecco's modified
Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 100 units/ml of
penicillin, and 100 μ g/ml streptomycin sulfate. On day 2, the cells were transfected with 3.5 μ g
of a mixture of plasmids containing 0.5 μ g of pVA1 (15) and 3 μ g of mouse preputial gland
cDNA pools or 3 μ g pCMV6, pCMV•SPORT6-mWS, pCMV6-hWS, pCMV6-FLAG-mWS,
pCMV-Script-FLAG-hDGAT1, pCMV•SPORT6-FLAG-mMGAT1, or pCMV•SPORT6-FLAG-
mDGAT2 expression plasmid using the FuGENE6 reagent. After 16-20 h, medium was replaced

with fresh plating medium supplemented with 2.4 μM [$1\text{-}^{14}\text{C}$]hexadecanol (American Radiolabeled Chemicals, St. Louis, MO). After 6-18 h of incubation, cells transfected with individual expression plasmids were washed once with 2 ml phosphate buffered saline (PBS), and scraped into 2 ml of PBS with a rubber policeman. Cells transfected with mouse cDNA pools were scraped directly into the medium with a rubber policeman. Lipid metabolites were extracted from the PBS-cell slurry or the medium-cell slurry with 8 ml of chloroform:methanol (2:1, v/v) and resolved by thin layer chromatography (TLC).

TLC – Separation of fatty alcohol and wax monoester lipids was performed as described in the accompanying paper (14) using solvent system 2 (development in hexane followed by development in toluene). Separation of glycerol lipids was accomplished using solvent system 3 containing hexane:ether:acetic acid (80:20:1, v/v/v). Stock solutions (10 mM) of lipid standards purchased from Sigma Chemical Co. (St. Louis, MO) were made as follows: palmitic acid, stearic acid, hexadecanol, octadecanol, 1-oleoyl-rac-glycerol, (*S*)-1,2-diolein, and glyceryl trioleate were dissolved in ethanol; dipalmitin and glyceryl tripalmitate were dissolved in chloroform; and cholesteryl oleate, cholesteryl palmitate, cetyl palmitate, and stearyl stearate were dissolved in hexane. Aliquots (5 μl) of individual stock solutions were chromatographed on lanes adjacent to those containing radiolabeled cell lipids.

Preparation of HEK 293 cell lysates or membranes - Cells were grown and transfected as described above. Cells were harvested approximately 26-36 h after transfection by scraping into 2 ml PBS, and then pelleted by centrifugation in a desk-top centrifuge at 1000g for 5 min at 4°C. The cell pellet was washed once by resuspension in PBS and the cells collected again by

centrifugation. Cells were resuspended in hypotonic lysis buffer (10 mM Hepes-KOH, pH 7.6, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, supplemented with one mini-complete protease inhibitor cocktail tablet (Roche, Indianapolis, IN) per 10 ml) and incubated on ice for 10 min. The swollen cells were lysed by 20 passages through a 23 gauge needle. Nuclei were removed from the cell lysates by centrifugation at 1000g for 5 min at 4°C. Thereafter, 0.1 volume of 2.5 M sucrose, 1 M Tris-HCl, pH 7.4 was added to the supernatant prior to freezing in liquid N₂ and storage at -80° C. In some cases, membranes were prepared by further centrifugation of cell lysates in a Beckman TLA 120.2 rotor in a TL-100 instrument at 130,000g for 30 min at 4° C. Membrane pellets were resuspended in assay buffer (0.3 M sucrose, 0.1 M Tris-HCl, pH 7.4, 1 mM EDTA, and one mini-complete protease inhibitor cocktail tablet (Roche) per 10 ml), frozen in liquid N₂, and stored at -80° C.

Assay of Fatty Alcohol and Acyl-CoA Preferences – Wax synthase enzyme activity was determined in a volume of 500 µL of 0.3 M sucrose, 0.1 M Tris-HCl, pH 7.4, 1 mM EDTA, 2.5 mM DTT, 5 mM MgCl₂, 0.8 mg/ml BSA, one mini-complete protease inhibitor cocktail tablet (Roche) per 10 ml, 98 µM palmitoyl-CoA, 11 µM [1-¹⁴C]palmitoyl-CoA (Perkin Elmer), and 100 µM of fatty alcohol. The fatty alcohol substrates decanol (C10:0), 11-eicosenol (C20:1), erucyl alcohol (C22:1), nervonyl alcohol (C24:1), and 9,12,15-linolenyl alcohol (C18:3) were purchased from Nu-Check Prep, Inc. (Elysian, MN). The fatty and isoprenoid alcohol substrates: lauryl alcohol (C12:0), tetradecanol (C14:0), hexadecanol (C16:0), 1-octadecanol (C18:0), eicosanol (C20:0), palmitoleyl alcohol (C16:1(9)), oleyl alcohol (C18:1(9)), linoleyl alcohol (C18:2(9,12)), geraniol, farnesol, and geranylgeraniol were purchased from Sigma. Fatty alcohol and isoprenoid alcohol stock solutions were dissolved in ethanol at a concentration of 50 mM.

Before use, eicosanol was heated to 60° C to ensure complete resuspension. Aliquots (15-25 µg) of HEK 293 cell membrane protein prepared from cells transfected with either the pCMV6, pCMV•SPORT6-mWS, or pCMV6-hWS plasmids were incubated at 37° C for 25-30 min. The reaction was stopped by addition of 100 µL of 6 N HCl. Following addition of 0.9 ml of PBS to each tube, lipids were extracted into 6 ml of chloroform:methanol (2:1, v/v). TLC of resuspended lipids was performed as described above.

To determine acyl-CoA preference, the above assay conditions were used except 93 µM hexadecanol, 7 µM [1-¹⁴C]hexadecanol (Perkin Elmer), and 100 µM of acyl-CoA were used. The acyl-CoA stocks, including decanoyl-CoA monohydrate (C10:0), lauroyl-CoA lithium salt (C12:0), myristoyl-CoA lithium salt (C14:0), palmitoyl-CoA lithium salt (C16:0), stearoyl-CoA lithium salt (C18:0), arachidoyl-CoA monohydrate (C20:0), palmitoleoyl-CoA lithium salt (C16:1), oleoyl-CoA lithium salt (C18:1), linoleoyl-CoA lithium salt (C18:2), and arachidonoyl-CoA monohydrate (C20:4), were purchased from Sigma Chemical Co., and dissolved in 0.01 M sodium acetate, pH 6.0, at a concentration of 10 mM.

Baculovirus Expression Vectors - Baculovirus recombinant donor plasmids with wax synthase and acyltransferase cDNAs were produced in pFastBac HT expression vectors (Invitrogen), which encode a hexa-histidine sequence at the 5'-end of the inserted cDNAs. For the mouse wax synthase, the cDNA insert of the pCMV•SPORT6-mWS plasmid described above was released by digestion with the restriction enzymes *SalI* and *NotI* and ligated into the pFastBac HTA vector. To construct a mouse MGAT2 cDNA donor plasmid, the MGAT2 cDNA was amplified by the PCR from the pCMV•SPORT6-mMGAT2 template described above using the oligonucleotide primers: 5'- GCCTATGTCGACAGCCAGCTCAGCATGGTGGAGTTC -3'

and 5'-ATTATGCGGCCGCTGGAGACACTTAGCAGAACTCCAG-3'. The amplified DNA product was digested with *SalI* and *NotI* and ligated into the pFastBac HTC vector. A mouse DGAT2 donor plasmid was prepared by digesting the pCMV•SPORT6-mDGAT2 plasmid described above with the restriction enzymes *SalI* and *NotI* to release the cDNA, which was then ligated into pFastBac HTC. A donor plasmid containing the mouse Δ^4 -3-oxosteroid 5 β -reductase cDNA (steroid 5 β -reductase, nucleotides 48-1053 of Genbank™/EBI Data Bank accession number BC018333) was produced as described (14). Generation of infectious *Autographica californica* nuclear polyhedrosis baculovirus stocks from the various donor plasmids was done in *Spodoptera frugiperda* Sf9 cells using the Bac-to-Bac Baculovirus Expression System kit (Invitrogen).

Acyltransferase Enzyme Assay in Baculovirus-Infected Cells - Sf9 cells were plated on day 0 at a density of 9×10^5 cells in Sf-900 II SFM media (Invitrogen) in 6-well plates. After a 2-4 h attachment period, the medium was replaced with Sf-900 II SFM medium supplemented with 50 units/ml of penicillin and 50 μ g/ml streptomycin sulfate, and cells were infected for approximately 50 h with recombinant baculovirus at a multiplicity of infection (MOI) of 1-3. The medium was replaced with 2 ml of fresh plating medium supplemented with 37.5 μ M bovine serum albumin (BSA)-conjugated palmitic acid and 2.7 μ M of BSA-conjugated [1- 14 C]palmitic acid (prepared as described in (14)) or 2.7 μ M [1- 14 C]hexadecanol. Cells were incubated with fatty acids for approximately 20 min or fatty alcohols for approximately 1.5 h at room temperature, washed with 2 ml PBS, and then harvested by scraping in 2 ml PBS with a rubber policeman. Lipid metabolites from 1.5 ml of the resuspended cells were extracted into 8 ml of chloroform:methanol 2:1 (v/v) and resolved by TLC.

Immunoblotting – Cell membranes containing FLAG epitope-tagged proteins were prepared from duplicate plates of transfected HEK 293 cells. Cell extracts containing hexa-histidine-tagged enzymes were harvested from 6-well plates infected with the indicated baculovirus expression vectors. Polyacrylamide gel electrophoresis in the presence of SDS was carried out under standard conditions. Resolved proteins were transferred by electroblotting to HybondC Extra nitrocellulose membranes (Amersham, Piscataway, NJ). FLAG epitope-tagged proteins were detected by incubation with a 1:1000 dilution of the primary FLAG M2 mouse monoclonal antibody (Sigma) in a solution of 5% (w/v) powdered milk, 1% (v/v) fetal calf serum, 0.05% (v/v) Tween 20 in PBS, followed by incubation with a 1:3750 dilution of secondary donkey anti-mouse horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, catalog #715-035-150). Visualization of the peroxidase was performed with enhanced chemiluminescence reagents (Amersham). Hexa-histidine epitope-tagged proteins were detected following the manufacturer's protocol for the Tetra-His HRP Conjugate Kit (Qiagen, Valencia, CA).

Immunocytochemistry – Transfection, antibody staining, and imaging were performed as described (14). Chinese hamster ovary (CHO)-K1 cells were transiently transfected with either the pCMV6 or pCMV6-FLAG-mWS plasmids, and then incubated with primary antibodies (anti-calnexin carboxyl terminus rabbit polyclonal antibody (Stressgen, San Diego, CA) at a 1:200 dilution and/or α -FLAG M2 monoclonal antibody (Sigma) at a 1:500 dilution). Cells were incubated for 1 h with secondary antibodies (Alexa Fluor 568 goat anti-rabbit IgG

(Molecular Probes, Eugene, OR) and/or Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes) at a 1:500 dilution.

Real Time PCR – cDNA synthesis and real time PCR reactions were performed as described in (14). Male human adult skin total RNA was obtained from Stratagene (catalog #735031). Oligonucleotide primers used to amplify the mouse wax synthase cDNA were:

5'-AGTTTTTGTCTTTTTCCCCTGAGA-3' and 5'-CTTGCTGCTAATGCTTTCATGAA-3'.

Oligonucleotide primers used to amplify the human wax synthase cDNA were: 5'-TCTTTGCAGCTACTGGTGAGATAGTC-3' and 5'-TTGTCCATTGTTTTCTCAATGC-3'.

Reconstitution of Wax Biosynthetic Pathway – Enzyme activities were measured in a volume of 500 μ l of 0.3 M sucrose, 0.1 M Tris-HCl, pH 7.4, 1 mM EDTA, 2.5 mM DTT, 5 mM MgCl₂, 0.8 mg/ml BSA, one mini-complete protease inhibitor cocktail tablet (Roche) per 10 ml, 2.5 mM β -NADPH, 2.5 mM β -NADH, 1 mM ATP, 100 μ M CoA, 2.9 μ M of BSA-conjugated [1-¹⁴C]palmitic acid, and 40 μ M BSA-conjugated palmitic acid. Aliquots (500 μ g protein) of lysates from 293 cells transfected with pCMV6, pCMV•SPORT6-mFAR1, pCMV•SPORT6-mWS, or pCMV•SPORT6-mFAR1 plus pCMV•SPORT6-mWS plasmids were assayed. Alternatively, lysate (250 μ g protein) from HEK 293 cells transfected with pCMV•SPORT6-mFAR1 was mixed with the same amount of lysate from cells transfected with the pCMV•SPORT6-mWS and added to the assay mix. Membrane proteins (130 μ g) isolated from freshly dissected male mouse preputial glands as described in the legend to Fig. 8 were used as a positive control for the synthesis of fatty alcohol and wax monoester. All reactions were incubated at 37° C for 30 min and then stopped by the addition of 100 μ l of 6 N HCl. Thereafter,

0.9 ml of PBS was added to each tube and lipids were extracted with 6 ml of chloroform:methanol 2:1 (v/v). TLC on resuspended lipids was performed as described above.

RESULTS

The mouse preputial gland is a bilateral sebaceous gland that flanks the reproductive tract and is enriched in wax esters (48% of total lipid content, (1)). Membrane preparations from the gland were found to contain abundant wax synthase activity as judged by the conversion of hexadecanol and [¹⁴C]palmitate to the wax monoester cetyl palmitate (see below). This source of enzyme activity was used in preliminary experiments to identify a solvent system that resolved waxes from other classes of neutral lipids on TLC plates and to optimize in vitro conditions for wax synthesis. An expression cloning strategy to isolate cDNAs encoding the wax synthase enzyme(s) was designed thereafter. Poly(A)⁺-enriched RNA was isolated from preputial glands and a library consisting of approximately 6.8×10^6 independent cDNA clones was made. Plasmid DNA was prepared from pools of approximately 200 cDNAs, transfected into HEK 293 cells, and the formation of wax esters from [1-¹⁴C]hexadecanol was monitored in the culture medium and cells by TLC. Several positive pools of cDNAs were identified, and two of these pools were progressively subdivided by repeated rounds of bacterial transformation, cell transfection, and assay until two single cDNAs were obtained (Fig. 1A). DNA sequence analysis indicated that the two cDNAs encoded the same protein but differed from each other in the untranslated regions. One cDNA (GenBank[™]/EBI Accession Number AY611031) contained a 272 nucleotide insert at the 3'-terminus that was not present in the second cDNA and that presumably reflected an alternate polyadenylation site in the gene. The second cDNA (GenBank[™]/EBI Accession Number AY611032) extended 25 nucleotides further toward the 5'-end of the mRNA than did the first cDNA.

Humans do not have a direct counterpart to the mouse preputial gland; however, database searches indicated the presence of a skin mRNA encoding a putative human wax synthase. Based on this information, a cDNA specifying the human protein was isolated by reverse transcriptase-PCR from skin mRNA. Transfection of an expression vector containing this cDNA into HEK 293 cells followed by incubation with [1-¹⁴C]hexadecanol led to the synthesis of wax esters (Fig. 1*B*), and confirmed that the human cDNA encoded a bona fide wax synthase.

The deduced amino acid sequences of the mouse and human wax synthase enzymes are shown in Fig. 2*A*. The proteins are the same length (333 amino acids) and share 84% sequence identity. Comparison of the cDNA sequence with the genomic DNA sequence reveals that the mouse wax synthase gene is located on the X chromosome, band C3, and contains at least seven exons spanning ~10.3 kb (Fig. 2*B*). The human wax synthase gene is located in a syntenic region of the X chromosome, band q13.1, and is predicted to have an identical exon-intron structure encompassing ~8.2 kb (Fig. 2*B*).

Database searches with the mouse and human wax synthase sequences indicated that they were members of the acyltransferase family of enzymes involved in neutral lipid synthesis. This group of proteins includes the acyl-CoA:cholesterol acyltransferases 1 and 2 (ACAT1 and ACAT2), the DGAT1 and DGAT2 enzymes, and the MGAT1 and MGAT2 enzymes (16). Sequence identities between the wax synthase and other members of the acyltransferase family are shown in Fig. 2*C*. These data indicate that the wax synthase is most closely related to the MGAT1, MGAT2, and DGAT2 proteins, and they raise the questions of whether the acyltransferases have wax synthase enzyme activity or whether the wax synthase has acyltransferase activity.

To answer these questions, we first established the fatty acyl-CoA and fatty alcohol substrate preference of the mouse wax synthase enzyme expressed in HEK 293 cells. Membranes from transfected cells were found to contain optimum enzyme activity with hexadecanol and [1-¹⁴C]palmitoyl-CoA substrates when the assay buffer had a pH between 7-8, magnesium concentrations between 0-3 mM, and ionic strengths between 0-200 mM KCl. At pH values greater than 8, substantial non-enzymatic formation of wax esters as well as other undefined lipid metabolites was observed (data not shown). Magnesium at concentrations higher than 3 mM inhibited wax synthase activity. Standard incubation times and temperatures were fixed at 37° C and 30 min, respectively, which represented conditions where only a small percentage of input substrates were converted into product.

The alcohol preference of the mouse wax synthase was determined by incubating membrane preparations containing the enzyme with fatty alcohols of different carbon chain lengths and saturation together with [¹⁴C]palmitoyl-CoA (Fig. 3A). Of 13 fatty alcohols tested, the enzyme efficiently incorporated those containing less than 20 carbons into wax monoesters. Within a series, the wax synthase preferred monounsaturated and polyunsaturated C18:1 and C18:2 fatty alcohols over C18:0, and the 20:1 fatty alcohol over the C20:0 lipid (Fig. 3A).

The acyl-CoA substrate preference of the mouse wax synthase enzyme was examined by incubating acyl-CoAs of different carbon chain length and saturation with [1-¹⁴C]hexadecanol (Fig. 3B). Fewer acyl-CoA substrates were available than fatty alcohol substrates, but among those tested the wax synthase enzyme preferred shorter chain fatty acids (compare C10:0 to C18:0 or C20:0). As with the fatty alcohols, the enzyme did not discriminate between monounsaturated and polyunsaturated fatty acyl-CoA substrates under the conditions of the assay.

Squalene, a polyisoprenoid containing six isoprenoid units, is an abundant constituent of sebum (1), and for this reason various isoprenols were tested as wax synthase substrates. The data of Fig. 3C showed that the wax synthase incorporated 10, 15, and 20 carbon isoprenoid alcohols into wax monoesters but not as efficiently as the C16:0 fatty alcohol, hexadecanol. In experiments not shown, membranes derived from HEK 293 cells expressing the human wax synthase enzyme displayed similar substrate preferences for fatty alcohols, isoprenoid alcohols, and acyl-CoAs as those of the recombinant mouse enzyme.

We next tested whether the MGAT and DGAT members of the acyltransferase family exhibited wax synthase enzyme activity. Mouse cDNAs for the wax synthase, MGAT1, MGAT2, and DGAT2, and a human DGAT1 cDNA were engineered to encode a FLAG epitope tag at the amino terminus of each enzyme. Control transfection experiments with these cDNAs in HEK 293 cells indicated that the addition of the FLAG epitope tag reduced the acyltransferase activity of the MGAT2 enzyme but had no effects on the activities of the other four enzymes (data not shown). The wax synthase activity in intact cells expressing the three acyltransferases not affected by the FLAG tag is shown in Fig. 4A. The MGAT1 and DGAT1 wax synthase activities were approximately 5-fold higher than background levels detected in mock transfected cells, whereas the DGAT2 activity was approximately 12-fold over background. Although measurable, these activities were less than that for the wax synthase enzyme, which was >100-fold over background (Fig. 4A). Immunoblotting of cell lysates from duplicate dishes in the transfection experiment showed that the FLAG epitope-tagged MGAT1, DGAT1, DGAT2, and wax synthase proteins were expressed at similar levels (Fig. 4A).

To circumvent the inactivation of the MGAT2 enzyme by the FLAG epitope, the cDNA and a wax synthase cDNA were engineered to contain hexa-histidine epitopes and then cloned

into baculovirus expression vectors. For comparison purposes, the DGAT2 cDNA was engineered in a similar fashion. The data shown in Fig. 4B revealed that the MGAT2 enzyme possessed detectable wax synthase activity that appeared to be higher than that of the DGAT2 enzyme; however, comparison of the amount of recombinant protein in the infected cells showed that the MGAT2 enzyme is expressed at levels that are at least five-fold higher than those of DGAT2 (Fig. 4B). As with the experiment in the HEK 293 cells, the amount of wax monoester formed in cells infected with a baculovirus containing the wax synthase cDNA were much greater than either the MGAT2 or DGAT2 enzymes regardless of the amount of protein expressed in the Sf9 cells. Control experiments with uninfected cells or those infected with a virus expressing steroid 5 β -reductase (an enzyme without acyltransferase activity) showed little endogenous wax production (Fig. 4B). We concluded from the experiments shown in Fig. 4 that the rank order of wax synthase activity among the various acyltransferase enzymes was wax synthase >> DGAT2 > MGAT2 = MGAT1.

The ability of the wax synthase enzyme to produce diacylglycerols and triacylglycerols was tested in Sf9 cells to take advantage of the lower levels of endogenous acyltransferase enzyme activities in the insect cells (Fig. 5). Uninfected Sf9 cells incubated with BSA-conjugated [¹⁴C]palmitic acid produced a lipid of unknown structure and a small amount of triacylglycerol (*first lane, upper panel*), whereas those infected with a control virus (encoding steroid 5 β -reductase) incorporated the fatty acid into this same unknown product and more triacylglycerols (*second lane*). An additional product with the mobility of diacylglycerols together with an increase in triacylglycerols was observed in cells infected with an MGAT2 virus (*third lane*), while only an increase in the level of triacylglycerols was detected in cells expressing the DGAT2 enzyme (*fourth lane*). The lipid pattern observed in cells infected with

the wax synthase cDNA virus (*fifth* lane) was no different from that of cells infected with the control virus, indicating that the wax synthase had little MGAT or DGAT activity. Immunoblotting experiments showed that all four enzymes were expressed at varying levels in the infected Sf9 cells (Fig. 5, *lower* panel).

The subcellular localization of the mouse wax synthase protein was determined by immunocytochemistry (Fig. 6). In these experiments, CHO-K1 cells were transfected with either a plasmid encoding a FLAG epitope-tagged mouse wax synthase or a plasmid lacking a cDNA, and then prepared for immunofluorescence microscopy. Staining with a fluorescein-labeled secondary antiserum recognizing the anti-FLAG monoclonal antibody indicated that the wax synthase was present in the endoplasmic reticulum (Fig. 6E). This assignment was supported by co-localization of the enzyme with calnexin (Fig. 6F), an integral membrane protein of the endoplasmic reticulum (Fig. 6A, C, D). No immunofluorescent signal corresponding to the FLAG epitope was detected in mock-transfected cells (Fig. 6B). In agreement with the proposed localization of the wax synthase in the endoplasmic reticulum, wax synthase enzyme activity was present in the membrane pellet derived from centrifugation of preputial gland extracts at 130,000g for 30 min (see below).

Waxes are components of sebum and meibum, the oils secreted by sebaceous glands to coat the external surfaces of the organism. Based on this function, we anticipated that the highest levels of wax synthase mRNA in the mouse would be present in the preputial gland and eyelid. The real time PCR data of Fig. 7 show that this expected distribution was observed, and that several additional tissues contained measurable levels of wax synthase mRNA, including thymus and spleen. RNA blotting experiments showed that the major wax synthase mRNA in the preputial gland is approximately 1.4 kilobases in length and that a second minor transcript of

about 2.9 kb is also present in this tissue (data not shown). Oligonucleotide primers for the human wax synthase mRNA generated a threshold value (C_T) of 26.4 when total RNA from male skin was used as a template, indicating that the wax synthase gene is transcribed in the human dermis.

In a final series of experiments, the mammalian wax biosynthetic pathway was reconstituted in cultured HEK 293 cells by co-expressing cDNAs encoding mouse fatty acyl-CoA reductase 1 (FAR1) (14) and wax synthase. When lysates from co-transfected cells were incubated with BSA-conjugated [^{14}C]palmitate, both radiolabeled fatty alcohol and wax monoester were produced (Fig. 8, *lane 4*). Similarly, when equal amounts of lysate from cells transfected individually with the FAR1 or wax synthase cDNAs were mixed together, both the fatty alcohol and the wax were synthesized (*lane 5*). In control experiments, mock transfected cells produced little of either product (*lane 1*), while lysates from cells transfected with the FAR1 cDNA produced hexadecanol but no wax (*lane 2*), and those transfected with the wax synthase made neither product due to the absence of an endogenous fatty acyl-CoA reductase (*lane 3*). Membranes from preputial glands were used as a positive control and synthesized both hexadecanol and wax monoester (*lane 6*).

DISCUSSION

We report the cDNA cloning and characterization of a mammalian wax synthase that catalyzes the formation of ester bonds between fatty alcohols and fatty acyl-CoAs to form wax monoesters. The wax synthase belongs to a small family of enzymes termed acyltransferases that participate in neutral lipid synthesis (16). Recombinant wax synthase utilizes a range of fatty alcohol and fatty acyl-CoA substrates and is more active in wax monoester synthesis than in cholesteryl ester, diacylglycerol, or triacylglycerol synthesis. Conversely, acyltransferase family members that share significant sequence identity with the wax synthase, including the MGAT1, MGAT2, and DGAT2 enzymes, exhibit modest wax monoester synthesis activity. When expressed in HEK 293 cells, the wax synthase localizes to the endoplasmic reticulum. Wax synthase mRNA is most abundant in tissues that are rich in sebaceous glands, including the eyelid and preputial gland, and is less plentiful in other tissues. Co-expression of fatty acyl-CoA reductase and wax synthase enzymes in naïve cells leads to the reconstitution of a wax biosynthetic pathway.

Several lines of evidence suggest that the wax synthase enzyme identified in this study plays a biologically relevant role in the synthesis of wax monoesters in mammals. First, the wax synthase is related in sequence to several members of a family of enzymes that synthesize the major classes of neutral lipids. The acyltransferase enzymes include ACAT1 and ACAT2 that produce cholesteryl esters, MGAT1 and MGAT2 that produce diacylglycerols, and DGAT1 and DGAT2 that produce triacylglycerols. Among acyltransferases, the wax synthase is most closely related to the MGAT1, MGAT2, and DGAT2 enzymes, and shares little more than random sequence identity with the ACAT1, ACAT2, and DGAT1 enzymes (Fig. 2C). While the more

closely related enzymes have measurable wax synthase activities, these are at least an order of magnitude less than that of the wax synthase enzyme (Fig. 4). Under the conditions utilized here, we were unable to demonstrate significant acyltransferase enzyme activity associated with the recombinant wax synthase (Fig. 5), nor did the wax synthase have detectable ACAT activity (e.g., Fig. 1). Thus, unlike the bacterial wax synthase enzyme from *Acinetobacter calcoaceticus*, which has both wax synthase and DGAT activity (7), the mammalian counterpart appears to be largely a wax synthase. These results suggest that following the genetic event leading to the formation of the primordial wax synthase gene in the mammalian lineage, the specificity of the encoded enzyme evolved to specialize in the formation of wax esters at the expense of other neutral lipids. This specialization may reflect the localization of the wax synthase gene on the X chromosome as genes on the sex chromosomes are subject to different selective pressures from those on autosomes (17).

A second line of support indicating the potential biological importance of the wax synthase comes from comparisons between the fatty acids and alcohols present in waxes of mouse sebum to the substrate preferences of the recombinant enzyme. The fatty acids of waxes isolated from the preputial gland are present in the following order of abundance: C16:1>C18:1>C16:0>C20:0>C14:0, and the fatty alcohol moiety is predominantly C16:0 (18). Each of these fatty acids or alcohols are utilized as substrates by the mouse enzyme in vitro (Fig. 3A, 3B), and presumably are in vivo as well. Despite this correlation, additional factors must influence the wax composition of sebum, including the substrate preferences of the fatty acyl-CoA reductases, intracellular lipid transport proteins, and fatty acid synthesis enzymes. These inputs may explain the absence of short chain fatty acids (e.g., C10:0 and C12:0) from sebum waxes even though these lipids are excellent substrates for the wax synthase in vitro.

In addition to straight chain fatty alcohols and acids, the wax synthase incorporated several polyisoprenols into waxes (Fig. 3C), and was active in the formation of retinol esters (S. Andersson, unpublished results). Polyisoprenoid-containing waxes have not been reported in sebum and meibum surveys of mammals (1,2,8,9), thus it is not clear whether these lipids are synthesized *in vivo*. Nevertheless, if they were, the recurring branched chains and unsaturation of the isoprenoid moieties would be expected to confer unique physiochemical and perhaps biochemical properties on this class of waxes. It also remains undetermined whether the formation of retinol esters by the wax synthase is biologically relevant.

The preponderance of wax synthase mRNA in the preputial gland and eyelid is a third indication that a major function of the encoded enzyme is the synthesis of wax monoesters. The preputial gland of the mouse contains large quantities of wax monoesters (1), as does the meibomian gland of the cow (2), and coincident with this lipid composition, these tissues in the mouse have the highest levels of wax synthase mRNA (Fig. 7). Similarly, human sebum is composed of ~25% wax monoesters and the wax synthase mRNA is detectable in this tissue by real time PCR. Inasmuch as mRNA levels equate with expression of the enzyme, and activity assays with membranes from the mouse preputial gland suggest this is the case (Fig. 8), these data place the wax synthase in the appropriate tissues for a role in sebaceous gland lipid synthesis. The wax synthase mRNA also is present at lower levels in several other organs, notably the thymus and spleen, which suggests that waxes may be produced in these tissues.

A fourth line of evidence that speaks to the biological role of the wax synthase is the ability to reconstitute the wax biosynthetic pathway in cells by co-expression of the fatty acyl-CoA reductase 1 and wax synthase enzymes (Fig. 8). This result demonstrates that as in plants (19,20), the minimum number of enzymes required for wax monoester synthesis in mammals is

two. Furthermore, the localization of the fatty acyl-CoA reductase enzymes in the peroxisome (14) and the wax synthase in the endoplasmic reticulum (Fig. 6), indicates the existence of transport systems that facilitate the movement of substrates and products of the pathway between these organelles. The proteins involved in this intracellular transport are presumably present in HEK 293 and Sf9 cells given the synthesis of waxes by these cell types (e.g., Fig. 4). A separate transport system may move waxes out of the cell since the products of the pathway were not secreted into the medium. The deposition of sebum in mammals is accomplished by lysis of lipid-laden sebocytes in a process termed holocrine secretion (21), whereas in plants, waxes are secreted without cell lysis (20). The proteins involved in wax secretion in plants have not been identified but it may be possible to isolate these using expression cloning in mammalian cells and an assay that screens for the presence of wax in the culture medium.

The reconstitution of the mammalian wax biosynthetic pathway in cultured cells suggests that it may be possible to assemble a bioreactor for the production of wax monoesters of defined chemical composition. Wax monoesters are an important constituent of cosmetics, polishes, and coatings, which presently are isolated by laborious methods from natural sources such as the jojoba plant (candelilla wax), Brazilian palm tree (carnauba wax), sheep wool (lanolin), or honey bee (beeswax). While a majority of the three billion pounds of wax produced each year is derived from petroleum in the form of paraffins, which are mixtures of normal and iso-alkanes, the properties of these chemically-manufactured paraffins are different from those of wax monoesters. The development of cell lines that express the fatty acyl-CoA reductase and wax synthase cDNAs, perhaps coupled with a wax transport system to allow secretion of the wax into the medium, might provide an alternate source of wax esters for commercial purposes.

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FOOTNOTES

¹The abbreviations used are: DGAT, acyl-CoA:diacylglycerol acyltransferase; MGAT, acyl-CoA:monoacylglycerol acyltransferase; kb, kilobases; HEK 293, human embryonic kidney 293; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's Medium; PBS, phosphate buffered saline; TLC, thin layer chromatography; MOI, multiplicity of infection; BSA, bovine serum albumin; CHO, Chinese hamster ovary; ACAT, acyl-CoA:cholesterol acyltransferase; FAR, fatty acyl-CoA reductase.

FIGURE LEGENDS

Fig. 1. *A*, Expression cloning of wax synthase cDNAs from mouse preputial gland. A cDNA library was prepared from male preputial gland mRNA and divided into pools of ~200 cDNAs each. Plasmid DNA from individual pools was combined with pVA1 plasmid (to increase expression from the expression plasmids, (15)), and introduced via the FuGENE6 reagent (Roche) into HEK 293 cells. [1-¹⁴C]hexadecanol was added to the medium 16-20 h later, and after a further incubation of 6-18 h, the cells were harvested by scraping into the medium. Lipid metabolites were extracted into chloroform:methanol (2:1, v/v) and resolved by TLC in solvent system 2. An autoradiogram prepared by exposure of the TLC plate to Kodak BioMax MR film for 72 h is shown. *Lane 1*, radiolabeled lipids from mock-transfected cells; *lane 2*, lipids synthesized in cells transfected with a 200-member cDNA pool encoding wax synthase activity; *lane 3*, lipids synthesized from a sub-pool of 20 cDNAs derived from the pool analyzed in lane 2; *lane 4*, lipids from cells transfected with a single positive cDNA derived from the sub-pool of lane 3. The positions to which cholesteryl esters and a wax monoester standard (cetyl palmitate) migrated to on the plate are shown on the *right*. Only the portion of the autoradiogram containing radiolabeled cholesteryl and wax esters is shown. *B*, Expression of mouse and human wax synthase enzyme activities in HEK 293 cells. Expression vectors encoding nothing (*pCMV*, *lane 1*), the human wax synthase enzyme (*hWS*, *lane 2*), or the mouse wax synthase enzyme (*mWS*, *lane 3*) were introduced into HEK 293 cells by transfection. Subsequent assay for wax ester synthesis was carried out as described in *A*.

Fig. 2. Comparison of mouse and human wax synthases. *A*, The deduced amino acid sequences of the human (*hWS*) and mouse (*mWS*) wax synthase proteins are aligned with identities between the two enzymes indicated by *black boxes*. Amino acids are numbered on the *right*. *Arrowheads* above the alignment indicate the positions of introns in the encoding genes. *Dots* indicate every tenth amino acid residue. The GenBank™/EBI Data Bank accession number for the human wax synthase cDNA sequence is AY605053 and for the mouse are AY611031 and AY611032. *B*, Structures and chromosomal locations of the human and mouse wax synthase genes. Exons are indicated by *boxes* and introns by *connecting lines*. Amino acids encoded at the beginning and end of the protein are indicated *above* the gene schematic. The structures of the gene and their chromosomal locations were deduced by comparing the sequences of the cloned cDNAs to those of genomic DNAs (GenBank™/EBI Data Bank accession number HsX_11826_33.52 for human gene and MmX_78942_32 for mouse gene). *C*, Amino acid sequence identities between mouse wax synthase and other acyltransferase enzymes. The percent sequence identities were deduced by pair wise comparisons using the BLASTP 2.2.6 program.

Fig. 3. Substrate preferences of the mouse wax synthase enzyme. Aliquots (15-25 µg) of HEK 293 membrane protein prepared from cells expressing the mouse wax synthase enzyme were incubated with the indicated radiolabeled and unlabeled lipids at 37° C for 25-30 min. Reactions were stopped by acidification and lipids extracted for TLC analysis in solvent system 2 as described in “*Experimental Procedures*”. The radiolabeled lipids used in these reactions are incorporated into multiple products by endogenous enzymes and for this reason only the portion of the autoradiogram containing wax esters is shown. *A*, Fatty alcohol substrates. Individual fatty alcohols tested are designated by chain length and number of unsaturated bonds (e.g., 10:0 is decanol, which has ten carbons and zero double bonds; 18:3 is 9,12,15 linolenyl alcohol,

which has eighteen carbons and three double bonds). *B*, Fatty acyl-CoA substrates. Fatty acyl groups are designated by chain length and number of unsaturated bonds. *C*, Isoprenol substrates. *C*₁₀, farnesol, *C*₁₅, geraniol, *C*₂₀, geranylgeraniol, 16:0, hexadecanol control. The results shown in *panels A-C* are typical of those obtained in at least two separate experiments carried out on different days using recombinant mouse and human wax synthases.

Fig. 4. Wax synthase activity of acyltransferase family members. *A*, HEK 293 cells were transfected with expression plasmids encoding the indicated acyltransferase enzymes modified to contain FLAG epitopes and then assayed for the synthesis of wax esters following addition of [¹⁴C]hexadecanol to the culture medium (*top panel*). The positions to which cholesteryl and wax esters migrated to on the TLC plate developed in solvent system 2 are indicated on the *right* of the autoradiogram. To determine relative expression levels of the acyltransferase enzymes, cell membranes were prepared from duplicate dishes and subjected to immunoblotting using a monoclonal antibody directed against the FLAG epitope (*bottom panel*). The positions to which protein standards of known molecular mass migrated to on the SDS-polyacrylamide gel are indicated on the *right* of the lumigram. *B*, Insect Sf9 cells were infected with baculovirus vectors encoding the indicated acyltransferase or control enzyme (steroid 5 β -reductase, 5 β -Red) modified to contain a hexa-histidine epitope and then assayed for the synthesis of wax esters as described in *A* (*top panel*). Endogenous levels of ACAT enzyme activity are low in Sf9 cells and thus the formation of cholesteryl esters in the infected cells was not detected in the exposure shown. Cell lysates from duplicate dishes were subjected to immunoblotting using a monoclonal antibody directed against the hexa-histidine epitope (*bottom panel*). The results shown are representative of at least two experiments performed on different days.

Fig. 5. Acyltransferase activity of wax synthase enzyme. Insect Sf9 cells were infected with baculovirus vectors encoding the mouse wax synthase (*WS*), indicated acyltransferase, or control enzyme (steroid 5 β -reductase, *5 β -Red*), each modified to contain a hexa-histidine epitope, and then assayed for the synthesis of diacylglycerols and triacylglycerols following the addition of BSA-conjugated [¹⁴C]palmitate to the culture medium (*top panel*). TLC was performed in solvent system 3. Only a product of unknown structure was detected in uninfected cells (*Cells*), whereas those infected with the control steroid 5 β -reductase virus synthesized this product and an enhanced level of triacylglycerols. Cells infected with the MGAT2 expressing virus produced elevated levels of diacylglycerols as well as triacylglycerols, and those infected with the DGAT2-expressing virus produced elevated levels of triacylglycerols. No increase in acyltransferase activity was detected in cells infected with the wax synthase expressing virus. To determine relative expression levels of the various enzymes, cell lysates from duplicate dishes were subjected to immunoblotting using a monoclonal antibody directed against the hexa-histidine epitope (*bottom panel*). The MGAT2 and wax synthase enzymes were expressed at higher levels than the steroid 5 β -reductase and DGAT2 enzymes. The results shown in panels *A* and *B* are typical of those obtained in three independent experiments.

Fig. 6. Subcellular localization of mouse wax synthase enzyme. CHO-K1 cells were transfected with the indicated expression vectors encoding nothing (*pCMV*) or a FLAG epitope-tagged wax synthase (*Wax Synthase*) enzyme. After transient expression, cells were fixed, permeabilized, and incubated with a rabbit polyclonal antiserum directed against the endoplasmic reticulum protein calnexin (*Anti-Calnexin*) and a mouse monoclonal antibody directed against the FLAG epitope (*Anti-FLAG*). Panels *A, D*: detection of rabbit polyclonal antibodies directed against calnexin with rhodamine-conjugated goat anti-rabbit antiserum. Panels *B, E*: detection of FLAG

epitope with fluorescein-conjugated goat anti-mouse antiserum. Panels *C, F*: merged images of rhodamine and fluorescein signals. Double indirect immunofluorescence microscopy was performed on a Zeiss 510 Laser Scanning Confocal microscope using a 63×1.3 NA PlanApo objective. Both the calnexin and FLAG-wax synthase enzymes are detected in endoplasmic reticulum membranes of CHO cells. These data are representative of two separate experiments.

Fig. 7. Tissue distributions of mouse wax synthase mRNA. The relative levels of wax synthase mRNA were determined by real time PCR in the tissues and cell types indicated on the *left* of the figure using cyclophilin mRNA levels as a reference standard. The data were then normalized to the threshold values (C_T) determined in the liver ($C_T = 29.2$) and then expressed on a \log_{10} scale. This experiment was repeated twice using the same preparations of tissue RNAs isolated from pools of organs or dishes of cells (macrophages).

Fig. 8. Reconstitution of mammalian wax biosynthetic pathway in cell lysates of transfected HEK 293 cells. The indicated plasmid DNAs were introduced into HEK 293 cells by transfection and allowed to express for 26 h. Cell lysates were isolated and the conversion of BSA-conjugated [14 C]palmitate into lipids was determined. TLC was performed in solvent system 2. *Lane 1*, mock-transfected cells synthesized an unknown product and triacylglycerols. *Lane 2*, cells expressing fatty acyl CoA-reductase 1 (*FARI*) produced these same two products and hexadecanol. *Lane 3*, cells expressing wax synthase (*WS*) produced only the two background lipids, indicating the near absence of fatty acyl-CoA reductase activity in HEK 293 cells. *Lane 4*, cells transfected with the fatty acyl-CoA reductase 1 and wax synthase cDNAs produced the two background lipids, hexadecanol, wax esters, and an additional lipid of unknown structure. *Lane 5*, the mixing of equal amounts of lysate derived from cells transfected individually with the fatty acyl-CoA reductase 1 and wax synthase cDNAs resulted in the

production of the background lipids plus hexadecanol and wax esters. *Lane 6*, positive control showing synthesis of hexadecanol and wax esters by membranes from mouse preputial glands. This experiment was performed in various guises many times.

Figure 1

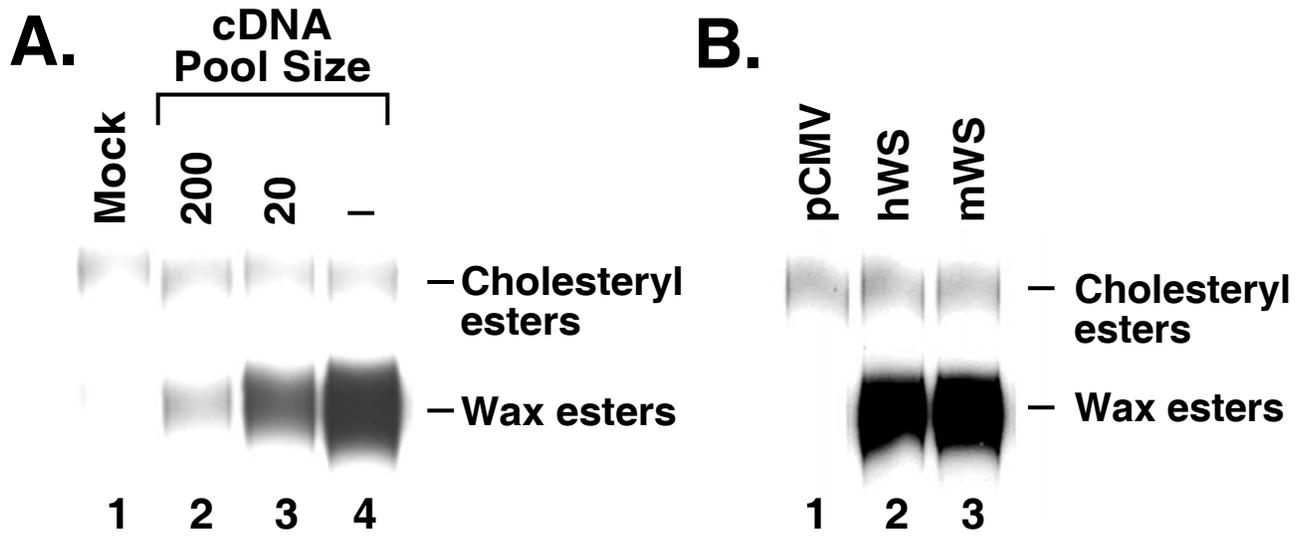
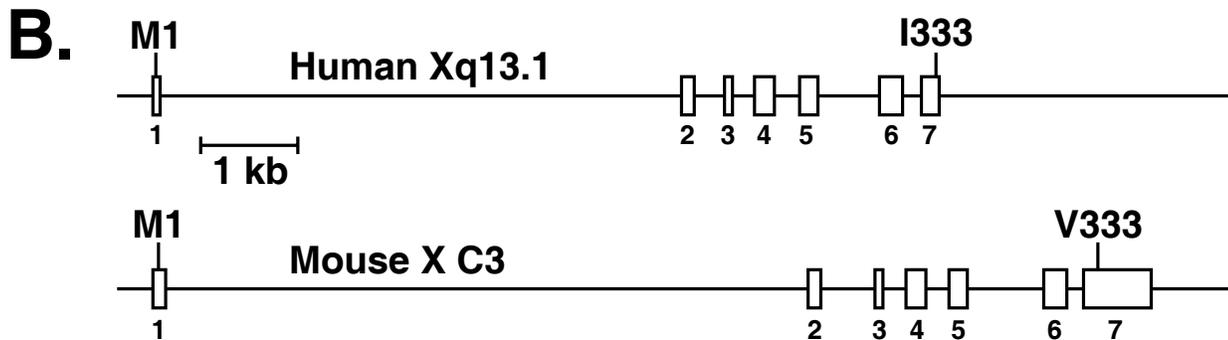
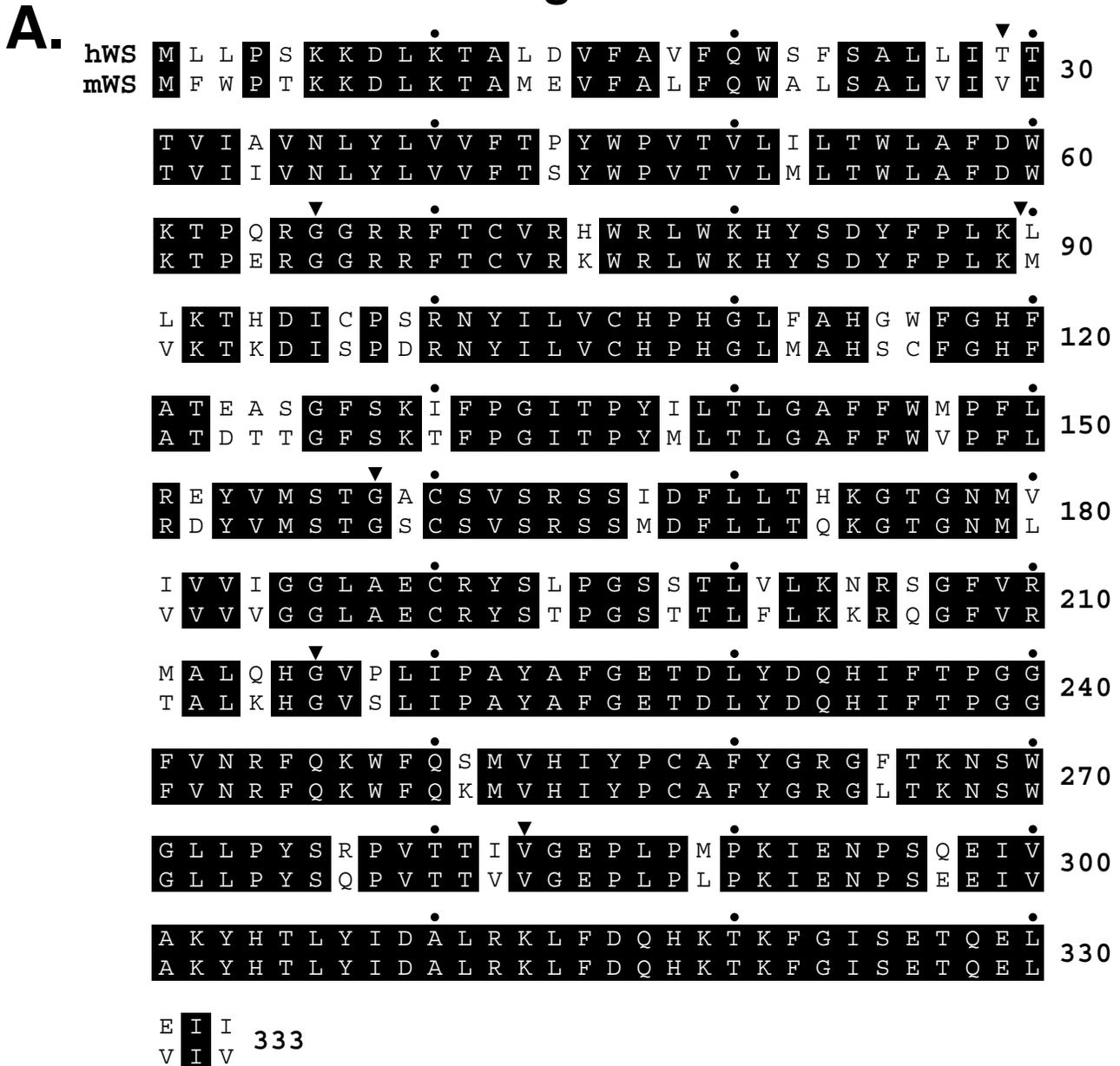


Figure 2



C.

	ACAT1	ACAT2	DGAT1	DGAT2	MGAT1	MGAT2
Wax Synthase	13.8%	15.9%	13.2%	46.5%	38.7%	40.5%

Figure 3

A.

¹⁴ C]Palmitoyl-CoA	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fatty Alcohol	-	10:0	12:0	14:0	16:0	18:0	20:0	16:1	18:1	20:1	22:1	24:1	18:2	18:3



B.

¹⁴ C]Hexadecanol	+	+	+	+	+	+	+	+	+	+	+
Fatty Acyl-CoA	-	10:0	12:0	14:0	16:0	18:0	20:0	16:1	18:1	18:2	20:4



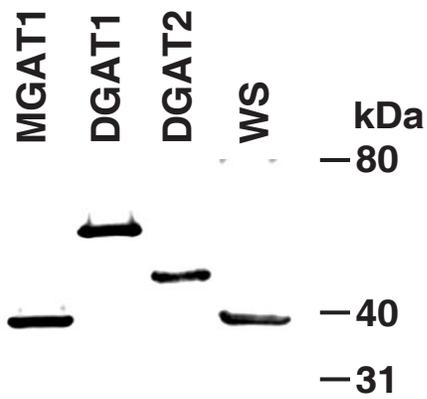
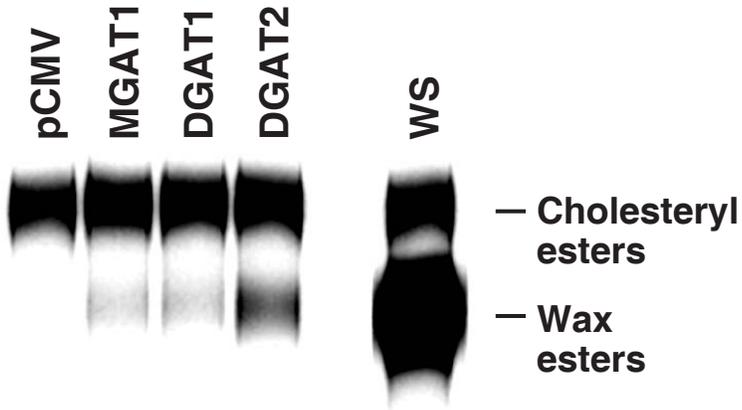
C.

¹⁴ C]Palmitoyl-CoA	+	+	+	+	+
Substrate	-	C ₁₀	C ₁₅	C ₂₀	16:0



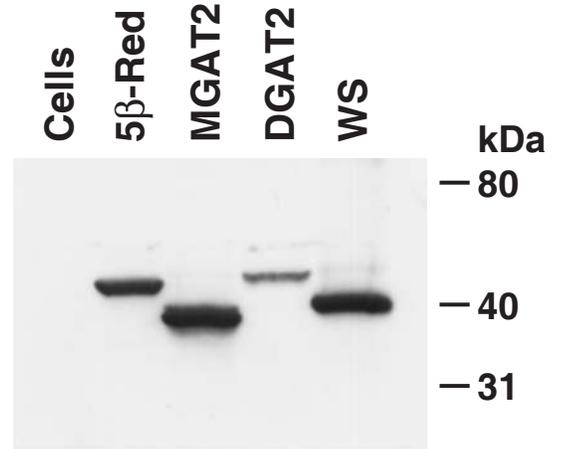
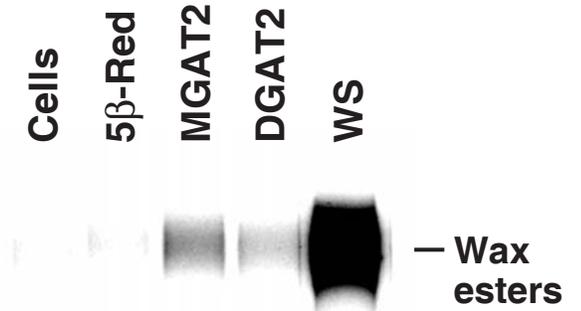
Figure 4

A. HEK 293 Cells



Anti-FLAG

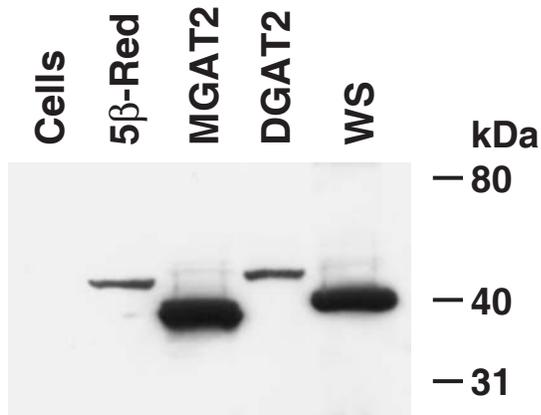
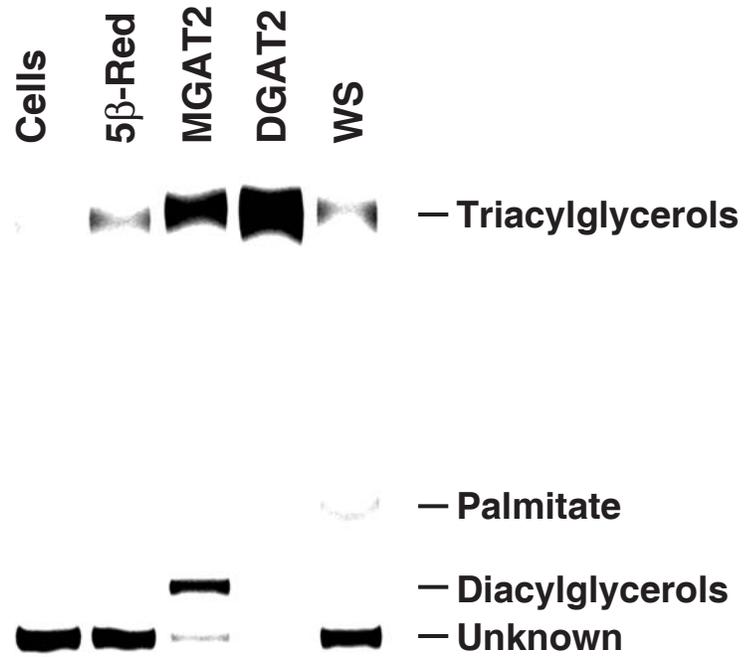
B. Sf9 Cells



Anti-His

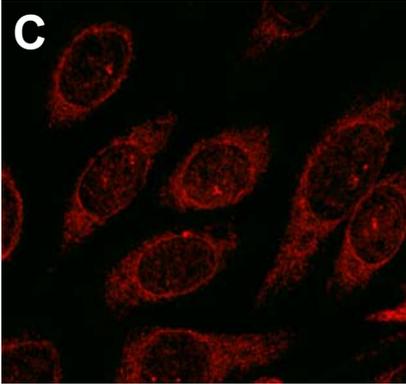
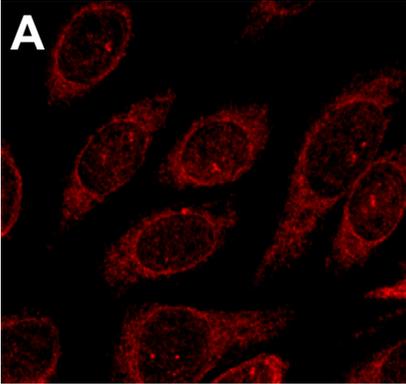
Figure 5

Sf9 Cells

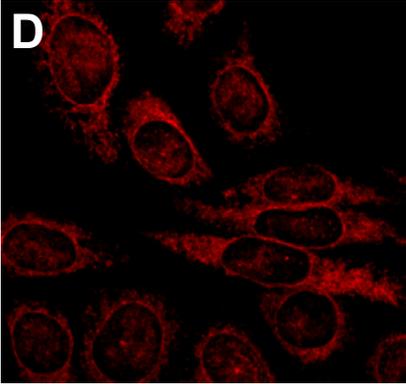


Anti-His

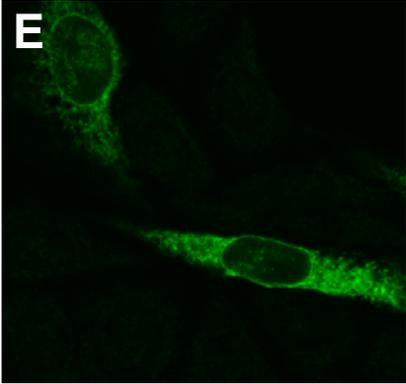
Figure 6



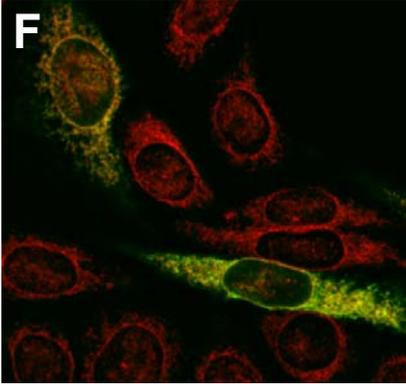
pCMV



Anti-Calnexin



Anti-FLAG



Merge

**Wax
Synthase**

Figure 7

Relative mRNA Level

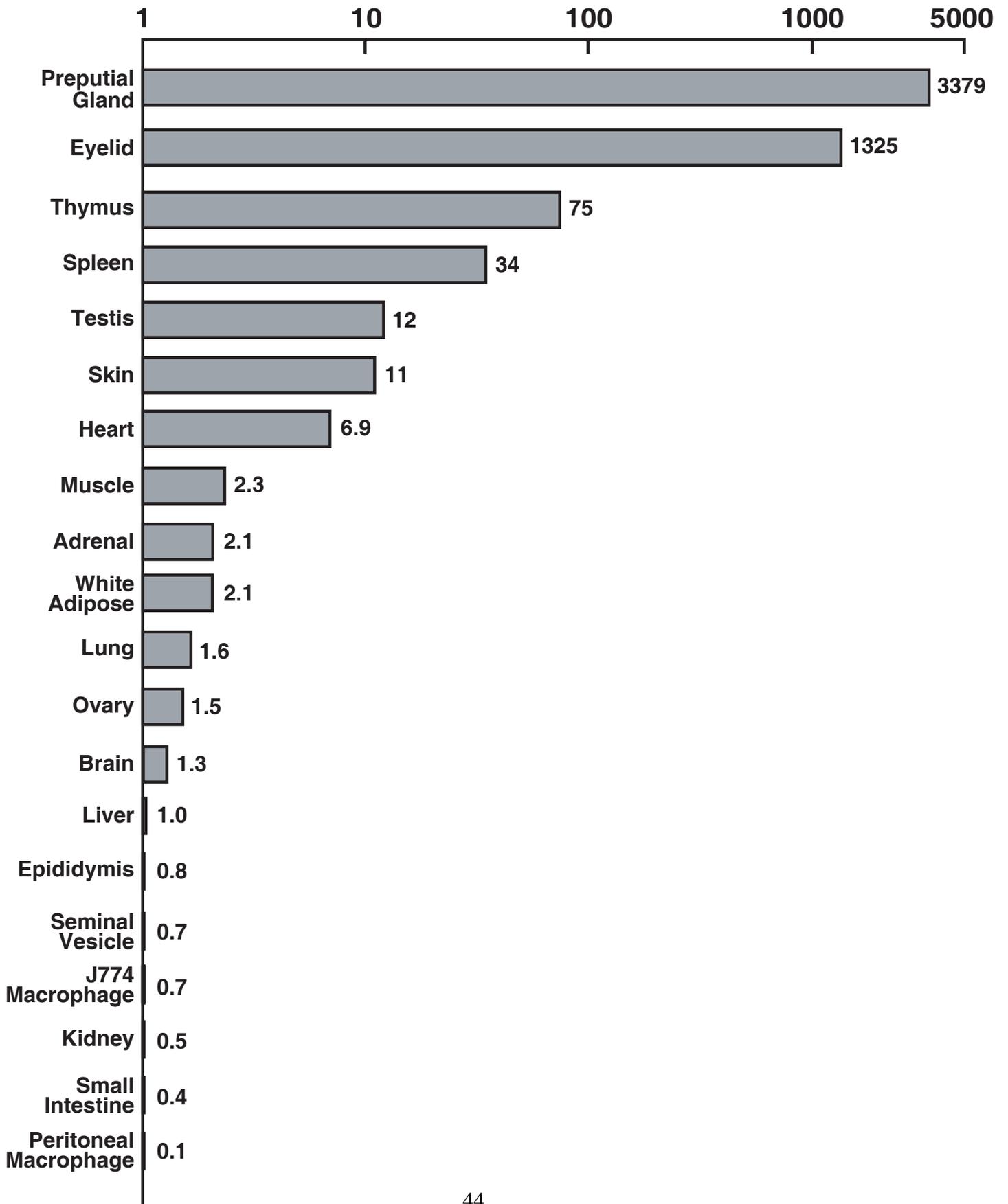


Figure 8

