Mammalian Wax Biosynthesis

II. EXPRESSION CLONING OF WAX SYNTHASE cDNAs ENCODING A MEMBER OF THE ACYLTRANSFERASE ENZYME FAMILY

Wax monoesters are synthesized by the esterification of fatty alcohols and fatty acids. A 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 triglycerols, 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 monooester synthetase activities. Whole cell 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. Coexpression of cDNAs specifying fatty acyl-CoA reductase 1 and wax synthase led to the synthesis of wax monoesters. The data suggest that wax monooester synthesis in mammals involves a two step biosynthetic pathway catalyzed by fatty acyl-CoA reductase and wax synthase enzymes.

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 composed principally of wax monoesters (25% of total lipids), triglycerides (41%), free fatty acids (16%), and squalene (12%), whereas the composition of mouse fur sebum is much higher content of wax monoesters (48%) than fur sebum (1). Although 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 distributed widely 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 ortholog. Coexpression 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 data base 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/esters 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 the result of decreased lipid synthesis or secretion from the deteriorated gland.

In the current study, an expression cloning approach was taken to identify mouse cDNAs encoding wax monooester synthase activity. A cDNA that specifies a member of the DGAT2/acyl-CoA:monoacylglycerol acyltransferase (MGAT) enzyme family with previously unknown function (15) was identified in a subgroup of pools from preputial glands. Recombinant protein, 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 PCRs. Coexpression 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 monooesters.

**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). cDNA inserts were ligated into the pCMVSPORT6 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 ~6.8 × 10⁶ independent clones was produced in which 95% of the plasmids harbored cDNA inserts with average lengths of >1.9 kb. Aliquots of the ligation mix containing ~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) were transfected with pools of cDNAs using FuGENE 6 reagent (Roche Applied Science) and assayed for wax synthase enzyme activity. A cDNA insert from a pool of transfectants was selected based on the enzyme activity as described below under 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 Y09050/5) was amplified by PCR from adult human skin Gene Pool cDNA (Invitrogen) using the oligonucleotide primers 5'-GCTATCTGTCGACCCACCATGGATTACAGGATGACGACGATAAGAG-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. The encoded MGAT1 protein has the FLAG epitope at the amino terminus.

A mouse MGAT1 cDNA (nucleotides 1–1008 of GenBank™/EBI Data Bank accession number AF384162) was amplified by the PCR from random hexamer-primer-mouse kidney cDNA using the primers 5'-GCTATCTGTCGACCCACCATGGATTACAGGATGACGACGATAAGAG-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 generating the pCMV-SPORT6-mMGAT1 expression plasmid.

A mouse MGAT2 cDNA (nucleotides 1–1017 of GenBank™/EBI Data Bank accession number AT157609) was amplified by the PCR from random hexamer-primer-mouse kidney cDNA using the primers 5'-GCTATCTGTCGACCCACCATGGATTACAGGATGACGACGATAAGAG-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 produce the pCMV-SPORT6-mMGAT1 expression plasmid.

A mouse DGAT1 cDNA (nucleotides 1–1008 of GenBank™/EBI Data Bank accession number AF384160) was amplified by the PCR from random hexamer-primer-mouse liver cDNA using the primers 5'-GCTATCTGTCGACCCACCATGGATTACAGGATGACGACGATAAGAG-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 generating the pCMV-SPORT6-mDGAT1 expression plasmid.

A mouse DGAT2 cDNA (nucleotides 1–1017 of GenBank™/EBI Data Bank accession number AF384162) was amplified by the PCR from random hexamer-primer-mouse liver cDNA using the primers 5'-GCTATCTGTCGACCCACCATGGATTACAGGATGACGACGATAAGAG-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 produce the pCMV-SPORT6-mDGAT2 expression plasmid.

A mouse DGAT2 cDNA (nucleotides 1–1008 of GenBank™/EBI Data Bank accession number AF384162) was amplified by the PCR from random hexamer-primer-mouse liver cDNA using the primers 5'-GCTATCTGTCGACCCACCATGGATTACAGGATGACGACGATAAGAG-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 produce the pCMV-SPORT6-mDGAT2 expression plasmid.
from the cell lysates by centrifugation at 1,000 × g 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 TLS120.2 rotor in a TL-100 instrument at 130,000 × g 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 mixture tablet/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, and 100 μM fatty alcohol. The fatty alcohol substrates decane (C10:0), 11-eicosanol (C20:1), erucyl alcohol (C22:1), nervonyl alcohol (C24:1), and 18,20-docosanediol (C20:2) 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 of 250 μl of HEK 293 cell lysate and 100 ng of HEK 293 cell membrane protein were added to 1.5 ml of PBS to each tube, lipids were extracted into 6 ml of chloroform:methanol (2:1, v/v), and the portion of the autoradiogram containing radiolabeled cholesteryl esters were shown. 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 above.

To determine acyl-CoA preference, the above assay conditions were used except 93 μM hexadecanol, 7 μM [1-14C]hexadecanol (PerkinElmer Life Sciences), and 100 μM 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 monohydrate (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, n-9), and linolenyl-CoA monohydrate (C18:3) 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 of 250 μl of HEK 293 cell lysate and 100 ng of HEK 293 cell membrane protein were added to 1.5 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.

**Baculovirus Expression Vectors—**Baculovirus recombinant donor plasmids with wax synthase and acyl-CoA transferase CDNs were produced in pFastBac HT expression vectors (Invitrogen), which encode a hexahistidine (His₆) 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 HTC vector. To construct plasmids for the accompanying paper (14), the amplified DNA product of infectious dsRNA (lane 1) and several DNA bands (lanes 2–4) were excised from agarose gels and ligated into the pFastBac HTA vector using T4 DNA ligase. The final CDNAs were released by digestion with SalI and NotI and ligated into the pFastBac HTA vector. The amplified CDNAs were then ligated into the pFastBac HT expression vector. The amplified DNA product of infectious dsRNA (lane 1) and several DNA bands (lanes 2–4) were excised from agarose gels and ligated into the pFastBac HT expression vector. The amplified DNA product of infectious dsRNA (lane 1) and several DNA bands (lanes 2–4) were excised from agarose gels and ligated into the pFastBac HT expression vector.

**Acyltransferase Enzyme Assay in Baculovirus-infected Cells**—The amplified CDNAs were then ligated into the pFastBac HT expression vector. The amplified DNA product of infectious dsRNA (lane 1) and several DNA bands (lanes 2–4) were excised from agarose gels and ligated into the pFastBac HT expression vector. The amplified DNA product of infectious dsRNA (lane 1) and several DNA bands (lanes 2–4) were excised from agarose gels and ligated into the pFastBac HT expression vector. The amplified DNA product of infectious dsRNA (lane 1) and several DNA bands (lanes 2–4) were excised from agarose gels and ligated into the pFastBac HT expression vector.

**Immunoblotting—**Cell membranes containing FLAG epitope-tagged proteins were produced from duplicate plates of transfected HEK 293 cells. Cell extracts containing His₆-tagged enzymes were harvested from 6-well plates infected with the indicated baculovirus expression vectors. SDS-PAGE was carried out under standard conditions. Resolved proteins were transferred by electroblotting to Hybond C Extra nitrocellulose membranes (Amersham Biosciences). FLAG epitope-tagged proteins were detected by incubation with a 1:1,000 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 1:3,750 dilution of secondary donkey anti-mouse horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories). Visualization of the peroxidase was performed with enhanced chemiluminescence reagents (Amersham Biosciences). His₆-epitope-tagged proteins were detected following the manufacturer’s protocol for the Tetra-His Horseradish Peroxidase Conjugate Kit (Qiagen, Valencia, CA).

**Immunocytochemistry—**Transfection, antibody staining, and imaging were performed as described in the accompanying paper (14). Chinese hamster ovary-K1 cells were transiently transfected with either the pCMV6 or pCMV6-FLAG-mWS plasmid and then incubated with primary antibodies (anti-calnexin carboxyl terminus rabbit polyclonal antibody (Stressgen, San Diego) 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 antimouse IgG (Molecular Probes) at a 1:500 dilution.

**Real Time PCR—**cDNA synthesis and real time PCR were performed as described in Ref. 14. Male human adult skin total RNA was obtained from Stratagene. Oligonucleotide primers used to amplify the mouse wax synthase CDNAs were 5’-AGTTTTTGTCTTTTTCCCCTGA-3’ and 5’-TTCTGCTGTTATGGTCTGATTGTC-3’. Oligonucleotide primers used to amplify the human wax synthase CDNAs were 5’-TCTTGGACGTAGTCTGGAGATAGTC-3’ and 5’-TGTGCTATGGTTTCTTCACAGTGC-3’.
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 [1-14C]palmitate to the wax monoester cetylpalmitate (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 was designed thereafter. Poly(A)-enriched RNA was isolated from preputial glands, and a library consisting of 6.8 × 10^6 independent cDNA clones was made. Plasmid DNA was prepared from pools of 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 Data Bank accession number HxX_11826_33.52 for human gene and MmX_78942_32 for mouse gene) encoded the same amino acid sequence as found in the mouse wax synthase and other acyltransferase enzymes. The percent sequence identities were deduced by pairwise 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 under “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 10 carbons and 0 double bonds; 18:3 is 9,12,15 linoleyl alcohol, which has 18 carbons and 3 double bonds). B, fatty acyl-CoA substrates. Fatty acyl groups are designated by chain length and number of unsaturated bonds. C, isoprenol substrates. C5, geranyl; C10, farnesyl; C15, geranylgeranyl; C20, geranyldodecanol control. The results shown in 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.

accession number AY611031) contained a 272-nucleotide insert at the 3′ terminus that was not present in the second cDNA and which presumably reflected an alternate polyadenylation site in the gene. The second cDNA (GenBankTM/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, data base 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. 1B) 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. 2A. 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. 2B). 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. 2B).

Data base 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 ACAT1 and ACAT2, DGAT1 and DGAT2, and MGAT1 and MGAT2 (16). Sequence identities between the wax synthase and other members of the acyltransferase family are shown in Fig. 2C. 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 and 8, magnesium concentrations between 0 and 3 mM, and ionic strengths between 0 and 200 mM KCl. At pH values greater than 8, substantial nonenzymatic 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 [1-14C]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]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).

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 substrate preferences for fatty alcohols, isoprenoid alcohols, and acyl-CoAs similar to those of the recombinant mouse enzyme.

We next tested whether the MGAT and DGAT members of the acyltransferase family exhibited wax synthase 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 ~5-fold higher than background levels detected in mock transfected cells, whereas the DGAT2 activity was ~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 His6 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 5-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 5β-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 5β-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), whereas 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 S9H cells (Fig. 5, lower panel).

The subcellular localization of the mouse wax synthase protein was determined by immunocytochemistry (Fig. 6). In these experiments, Chinese hamster ovary-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 colocalization of the enzyme with calnexin (Fig. 6F), an integral membrane protein of the endoplasmic reticulum (Fig. 6, A, C, and 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 130,000 × g 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 ~1.4 kb 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 (Ct) 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 coexpressing cDNAs encoding mouse fatty acyl-CoA reductase 1 (FAR1) (14) and wax synthase. When lysates from cotransfected cells were incubated with BSA-conjugated [14C]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), whereas 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 because of 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. Coexpression 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, which produce cholesteryl esters; MGAT1 and MGAT2, which produce diacylglycerols; and DGAT1 and DGAT2, which 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). Although 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 A. calcoaceticus, which has both wax synthase and DGAT activity (7), the mammalian counterpart appears to be largely a wax synthase. These results suggest that after 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 is utilized as a substrate by the mouse enzyme in vitro (Fig. 3, A and B) and presumably 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

![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 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).](image-url)
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 coexpression 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 monooester 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 because 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 monooesters of defined chemical composition. Wax monooesters 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). Although a majority of the 3 billion pounds of wax produced each year is derived from petroleum in the form of paraffins, which are mixtures of normal and isoaalkanes, the properties of these chemically manufactured paraffins are different from those of wax monooesters. The development of cell lines that express the FAR 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.

Acknowledgments—We thank Jill Fairless and Lisa Beatty for expert assistance with tissue culture; Helen Yin, Greg Graf, Manuel Martinez, Wei-Ping Li, Mike Roth, and James Waddle for assistance and access to confocal microscopes; and Jonathan Cohen, Joel Goodman, and Jay Horton for critical review of the manuscript.

REFERENCES

1. Nikkari, T. (1974) J. Investig. Dermatol. 62, 257–267
2. Nicolaides, N., Kaitaranta, J. K., and Rowdhan, T. N. (1981) Investig. Ophthalmo-v. Vis. Sci. 20, 522–536
3. McCuller, J. P., and Shine, W. E. (2002) Adv. Exp. Med. Biol. 506, 373–378
4. Driver, P. J., and Lemp, M. A. (1996) Survey Ophthalmo-v. 40, 343–367
5. Koltuukady, P. E. (1976) Chemistry and Biochemistry of Natural Waxes, Elsevier, Amsterdam
6. Lardizabal, K. D., Metz, J. G., Sakamoto, T., Hutton, W. C., Pollard, M. R., and Las neger, M. W. (2000) Plant Physiol. 122, 645–655
7. Kalscheuer, R., and Steinbuechel, A. (2000) J. Biol. Chem. 275, 8075–8082
8. Nicolaides, N. (1965) J. Am. Oil Chem. Soc. 42, 691–702
9. Sanzone, G., and Hamilton, J. G. (1969) Liphid. 4, 435–440
10. Miyazaki, M., Man, W. C., and Ntambi, J. M. (2001) J. Nutr. 131, 2260–2268
11. Miyazaki, M., Gomez, F. E., and Ntambi, J. M. (2002) J. Lipid Res. 43, 2146–2154
12. Chen, H. C., Smith, S. J., Tow, B., Elias, P. M., and Farese, R. V., Jr. (2002) J. Clin. Invest. 109, 175–181
13. Cases, S., Stone, S. J., Zhou, P., Yen, E., Tow, B., Lardizabal, K. D., Voelker, T., and Farese, R. V., Jr. (2001) J. Biol. Chem. 276, 38870–38876
14. Cheng, J. B., and Russell, D. W. (2004) J. Biol. Chem. 279, 37789–37797
15. Schneider, R. J., and Shenk, T. (1987) Annu. Rev. Biochem. 56, 317–332
16. Buhman, K. K., Chen, H. C., and Farese, R. V., Jr. (2001) J. Biol. Chem. 276, 40369–40372
17. Brown, C. J., and Greally, J. M. (2003) Trends Genet. 19, 432–438
18. Snyder, F., and Blank, M. L. (1969) Arch. Biochem. Biophys. 130, 101–110
19. Jenks, M. A., Eigenbrode, S. D., and Lemieux, B. (2002) in The Arabidopsis Book (Somerville, C. R., and Meyerowitz, E. M., eds) doi/10.1199/tab.0016,http://www.aspb.org/publications/arabidopsis/, American Society of Plant Biologists, Rockville, MD
20. Kunst, L., and Samuels, A. L. (2003) Prog. Lipid Res. 42, 51–80
21. Thody, A. J., and Shuster, S. (1989) Physiol. Rev. 69, 393–416