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# Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis

**(fatty acid/cloning/expressed sequence tag/glycerolipid)**

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**ABSTRACT** **Triacylglycerols are quantitatively the most important storage form of energy for eukaryotic cells. Acyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the terminal and only committed step in triacylglycerol synthesis, by using diacylglycerol and fatty acyl CoA as substrates. DGAT plays a fundamental role in the metabolism of cellular diacylglycerol and is important in higher eukaryotes for physiologic processes involving triacylglycerol metabolism such as intestinal fat absorption, lipoprotein assembly, adipose tissue formation, and lactation. DGAT is an integral membrane protein that has never been purified to homogeneity, nor has its gene been cloned. We identified an expressed sequence tag clone that shared regions of similarity with acyl CoA:cholesterol acyltransferase, an enzyme that also uses fatty acyl CoA as a substrate. Expression of a mouse cDNA for this expressed sequence tag in insect cells resulted in high levels of DGAT activity in cell membranes. No other acyltransferase activity was detected when a variety of substrates, including cholesterol, were used as acyl acceptors. The gene was expressed in all tissues examined; during differentiation of NIH 3T3-L1 cells into adipocytes, its expression increased markedly in parallel with increases in DGAT activity. The identification of this cDNA encoding a DGAT will greatly facilitate studies of cellular glycerolipid metabolism and its regulation.**

Acyl CoA:diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) is a microsomal enzyme that plays a central role in the metabolism of cellular glycerolipids (for reviews, see refs. 1 and 2). DGAT catalyzes the only committed step in triacylglycerol synthesis by using diacylglycerol (DAG) and fatty acyl CoAs as its substrates. DAG used in the DGAT reaction can be derived from the hydrolysis of phosphatidic acid produced by the *de novo* synthesis pathway from glycerol-3-phosphate (Fig. 1). Alternatively, DAG can be derived from the esterification of monoacylglycerol (MAG), a pathway of importance in intestinal fat absorption (3), and from the hydrolysis of triacylglycerol or phospholipids. Inasmuch as DAG is a precursor for phospholipid synthesis and is an important signaling molecule that activates protein kinase C (4), DGAT activity potentially could regulate these cellular processes. Because of its role in triacylglycerol synthesis and energy storage, DGAT also may be involved in intestinal fat absorption (3), lipoprotein assembly and the regulation of plasma triacylglycerol concentrations (1, 5), fat storage in adipocytes (6), energy metabolism in muscle (7), milk production (1), and egg production, including mammalian oocytes (8). In plants, DGAT has an important function in the generation of seed oils (9). DGAT activity also has been found in *Mycobacteria* (10) and *Streptomyces* (11), and in the lipid bodies of fungi (12) and insects (13).

Although it has been partially purified (14, 15), DGAT has been difficult to isolate because it is an intrinsic membrane protein. Through homology searches of the expressed sequence tag (EST) databases by using coding sequences from acyl CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26), an acyltransferase that synthesizes cholesterol esters from cholesterol and fatty acyl CoA substrates (16), we identified an EST clone that shares homology with sequences in the ACAT C terminus. In this study, we demonstrate that this recently identified cDNA does not encode another ACAT, but in fact encodes a DGAT.‡‡ The expression of a mouse cDNA for this EST in insect cells resulted in high levels of a membrane-associated acyltransferase activity specific for DAG. This gene and its encoded activity then were characterized in detail.

## MATERIALS AND METHODS

**Cloning of DGAT cDNA.** ESTs [accession nos. R07932 (human) and W10786 (mouse)] with sequence similarity to ACAT were identified from BLAST database searches. The 5′ end of the DGAT cDNA was obtained by using 5′ rapid amplification of cDNA ends (RACE) and a mouse spleen Marathon Ready cDNA library (CLONTECH). Sequences have been deposited in GenBank (accession no. AF078752).

**Insect Cell Expression Studies.** DGAT coding sequences with or without an N-terminal FLAG epitope (IBI/Kodak, New Haven, CT) (MGDYKDDDDG-, epitope underlined) were subcloned into pVL1392 (PharMingen). High titers of recombinant baculoviruses were obtained by cotransfection of baculovirus transfer vectors with viral BaculoGold DNA (PharMingen), followed by plaque purification and amplification in Sf9 cells [cultured in Grace's medium (Life Technologies, Grand Island, NY) and 10% fetal bovine serum]. H5 insect cells [cultured in serum-free Express-Five medium (Life

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; DAG, diacylglycerol; DGAT, acyl CoA:diacylglycerol acyltransferase; EST, expressed sequence tag; MAG, monoacylglycerol; MOI, multiplicity of infection.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF078752).

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‡‡Portions of this work were presented at the Experimental Biology meeting (San Francisco, 1998) and have been published in abstract form (34).

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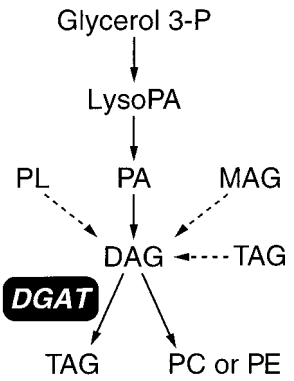


FIG. 1. Role of DGAT in glycerolipid metabolism. DAG used by DGAT potentially originates from hydrolysis of phosphatidic acid (PA), from the esterification of 2-monoacylglycerol (MAG), or from triacylglycerol (TAG) or phospholipid (PL) hydrolysis. The MAG pathway is thought to be especially important in enterocytes of the small intestine (3). P, phosphate; LysoPA, lysophosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

Technologies)] were plated on day 0 ($8.5 \times 10^6$ cells/100-mm dish) and infected on day 1 with high titers of virus at a multiplicity of infection (MOI) that was empirically determined. On day 3, cells were collected by centrifugation and washed twice with PBS. Cell pellets were homogenized by 10 passages through a 27-gauge needle in 0.1 M sucrose, 50 mM KCl, 40 mM $KH_2PO_4$, and 30 mM EDTA (pH 7.2). Total membrane fractions ($100{,}000 \times g$ pellet) were resuspended in the homogenization buffer and frozen (−80°C). Immunoblots of membrane proteins (75 $\mu$g) were performed with the anti-FLAG M2 mAb (IBI/Kodak).

For metabolic labeling, H5 insect cells were plated on day 0 ($2.9 \times 10^6$ cells/60-mm dish) and infected on day 1 with high titers of viruses. On day 3, cells were washed and incubated in methionine- and cysteine-free medium (SF900 II, Life Technologies) for 2 h, followed by incubation in the same medium containing 715 $\mu$Ci of [$^{35}$S]methionine and [$^{35}$S]cysteine (ProMix; Amersham; 1 Ci = 37 GBq) for 1 h. Cells were washed twice with PBS and collected by low-speed centrifugation. The cell pellet was resuspended in 0.5 ml of 50 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 (pH 7.4) and sonicated. Cellular proteins (100 $\mu$g) were analyzed by SDS/PAGE and autoradiography.

For ACAT assays, cell-membrane proteins (100 $\mu$g) were assayed by using [1-$^{14}$C]oleoyl CoA (51 mCi/mmol, Amersham) and cholesterol/egg phosphatidylcholine (PC) liposomes (molar ratio = 0.7) as described (17). In some assays, other acyl acceptors were substituted for cholesterol in the liposomes at a molar ratio of 0.2 (acceptor/egg PC). Incorporation of the [$^{14}$C]oleoyl group into products was assessed by TLC, followed by autoradiography. DGAT assays were based on assays optimized for rat liver (15, 18) (S.K.E., K. Pella, and S.R.L., unpublished data). The incorporation of [$^{14}$C]oleoyl CoA into triacylglycerol was measured under apparent $V_{MAX}$ conditions by using exogenous DAG provided as DAG/egg PC liposomes (molar ratio ≈0.16). Cell-membrane proteins (20–25 $\mu$g) were assayed in 0.25 M sucrose, 1 mM EDTA, 150 mM $MgCl_2$, and 100 mM Tris·HCl (pH 7.5) containing 250 $\mu$g of BSA and 20 $\mu$g of DAG in liposomes and 5 nmol [$^{14}$C]oleoyl CoA (40,000 dpm/nmol) (final volume, 0.2 ml). Reactions were carried out for 5 min, and the products were analyzed as described (19). Similar assays were performed with 1-stearoyl-2-[1-$^{14}$C]arachidonyl-*sn*-glycerol (53 mCi/mmol, Amersham) diluted to a final activity of 38,000 dpm/nmol with unlabeled 1,2-diacyl-*sn*-glycerol and unlabeled oleoyl CoA.

Relative triacylglycerol and DAG masses were determined by total lipid extraction of membranes or cells followed by TLC, iodine vapor visualization, photography of the plates, and densitometric analysis. Triolein standards were used to estimate the mass of triacylglycerols, and DAG units were estimated relative to one another. Triacylglycerol values were normalized to 1 for wild-type virus-infected cell membranes to correct for inter-experiment variability.

**mRNA Expression.** Human Multiple Tissue Northern blots (CLONTECH) were hybridized with a $^{32}$P-labeled 1.1-kb human DGAT fragment from the human EST. For mouse tissues, total RNA was prepared with Trizol reagent (Life Technologies), and samples (10 $\mu$g) were analyzed by Northern blot with a $^{32}$P-labeled, 1-kb mouse DGAT fragment from the mouse EST. Blots were stripped and sequentially reprobed for glyceraldehyde-3-phosphate dehydrogenase and 28S RNA (20). Bands in autoradiograms from the 3T3-L1 experiments were quantified with a PhosphorImager (Fuji Medical Systems, Stamford, CT).

**NIH 3T3-L1 Differentiation.** NIH 3T3-L1 fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM L-glutamine. 3T3-L1 cell differentiation into adipocytes was induced by incubating confluent monolayers of cells in serum-containing medium supplemented with $10^{-5}$ M dexamethasone, 0.5 mM isobutylmethylxanthine, and 10 $\mu$g/ml insulin (21).

**Gene Mapping.** Primers derived from the human EST sequences were used to identify genomic clones in an arrayed bacteria artificial chromosome (BAC) library according to the manufacturer's protocol (Research Genetics, Huntsville, AL). The BAC clone was mapped to chromosome 8qter by fluorescent *in situ* hybridization (22). The clone (RMC08P051) may be requested from the website http://rmc-www.lbl.gov. Linkage analysis for mouse gene mapping was performed with a panel of 67 progeny derived from an interspecific backcross [(C57BL/6J× *Mus spretus*)$F_1$ × C57BL/6J] (23). This backcross panel has been typed for more than 400 loci throughout the genome (24). Briefly, parental strain DNAs were screened for restriction fragment-length variants by restriction enzyme digestion and hybridization with a radiolabeled, 1-kb mouse DGAT cDNA fragment as described (23). Filters were washed in 1.0× SSC/0.1% SDS at 50°C for 20 min. Autoradiograms were exposed for 3 days at −70°C. Linkage to previously typed chromosomal markers was detected by using MAP MANAGER version 2.6.5, and loci were ordered by minimizing the number of recombination events between DGAT and the markers (25).

## RESULTS

Through homology searches of the EST databases using coding sequences from ACAT, we identified an EST clone that shared homology with sequences in the ACAT C terminus. The translation of a full-length cDNA for this EST predicts an ORF encoding a 498-aa protein that is ≈20% identical to mouse ACAT (Fig. 2*A*), with the most highly conserved regions in the C terminus. The predicted protein sequence contains a potential N-linked glycosylation site and a putative tyrosine phosphorylation site. A serine residue found in ACAT that is necessary for enzyme activity (26) appears to be conserved. The protein has multiple hydrophobic domains and 6–12 possible transmembrane domains (Fig. 2*B*). Analysis by a transmembrane region prediction program (http://ulrec3.unil.ch/software/TMPRED_form.html) favors nine transmembrane domains (amino acids 96–114, 140–157, 174–198, 200–218, 293–311, 337–360, 412–434, 436–456, and 461–484).

Given the 20% sequence identity to ACAT, experiments were designed to test whether this cDNA encoded an enzyme that catalyzed cholesterol esterification. FLAG epitope tagged (at the N terminus) or untagged versions of the cDNA were expressed in H5 insect cells by using a baculovirus expression system. Cells infected with the virus containing this cDNA

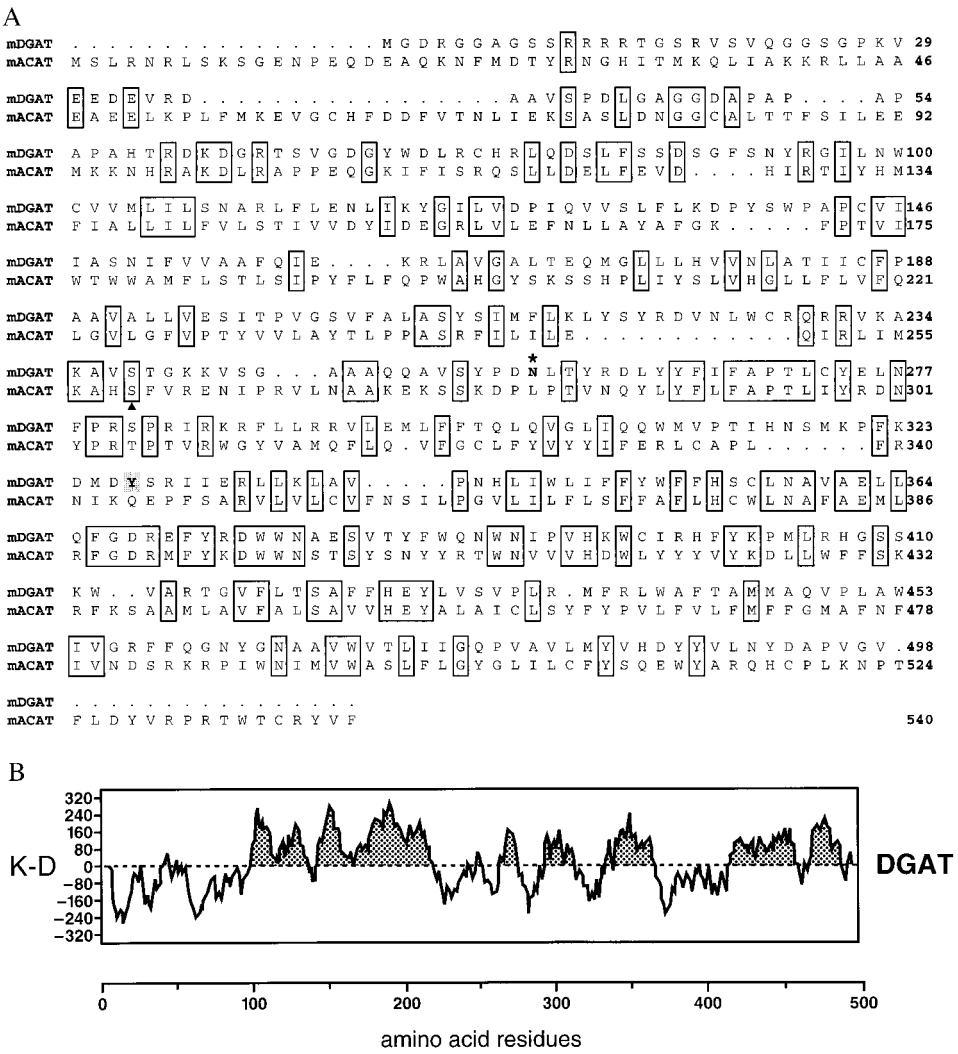


FIG. 2. The mouse DGAT protein. (*A*) Predicted amino acid sequence of the mouse DGAT cDNA. The predicted amino acid sequence of mouse DGAT (mDGAT) is shown in alignment with mouse ACAT (mACAT) (32). The two sequences are ≈20% identical (identical residues are boxed). A potential N-linked glycosylation site (asterisk) and tyrosine phosphorylation site (shaded) are indicated. A serine residue in ACAT known to be necessary for catalytic function is also indicated (triangle). (*B*) Hydrophobicity plot of DGAT as assessed by Kyte–Doolittle (K–D) analysis (33). Hydrophobic regions are shaded.

expressed an ≈47-kDa protein at high levels in the membrane fraction (Fig. 3*A*) but lacked detectable cholesterol esterification activity as compared with ACAT virus-infected cells (Fig. 3*B*). However, further analysis of TLC plates from these assays revealed that membranes from these cells had significantly increased triacylglycerol mass (as assessed by $I_2$ visualization) (data not shown) and incorporated significantly more [$^{14}$C]-oleoyl CoA into triacylglycerols than did membranes from wild-type virus-infected cells (197 vs. 55 pmol/mg protein per min). These data suggested that the identified cDNA encoded a DGAT.

DGAT activity was measured in membranes from H5 insect cells expressing the putative DGAT cDNA and found to be more than 5-fold higher than in membranes from wild-type virus-infected cells (Fig. 3*B*). The DGAT activity level increased proportionately with the amount of FLAG-tagged protein expressed in membranes isolated from cells harvested at different time points after infection (Fig. 3*C*). DGAT activity levels in membranes from cells expressing the cDNA were similar regardless of whether [$^{14}$C]DAG or [$^{14}$C]oleoyl CoA was used as the labeled substrate (Fig. 3*D*). In the absence of added oleoyl CoA, [$^{14}$C]DAG was not incorporated into triacylglycerols. In addition, [$^{3}$H]oleic acid was not incorporated into triacylglycerols in membranes from DGAT virus-infected cells (7 ± 6 vs. 49 ± 47 pmol triacylglycerol/mg protein per min for wild type, $n = 3$), establishing the requirement for a fatty acyl CoA. Triacylglycerol mass was increased more than 10-fold in membranes from DGAT virus-infected cells compared with membranes from wild-type virus-infected cells (11 ± 7 vs. 1 ± 0.5 pg/$\mu$g membrane protein, $P = 0.04$, $n = 5$). No change in relative DAG mass was observed (0.33 ± 0.05 vs. 0.34 ± 0.12 units for DGAT and wild type, respectively). We also tested a variety of other possible acyl acceptors, including 25-hydroxy-, 26-hydroxy-, 7$\alpha$-hydroxy- or 7$\beta$-hydroxycholesterols, 7-ketocholesterol, vitamins D2 and D3, ethanol, $\beta$-sitosterol, lanosterol, and ergosterol (shown in Fig. 3*E*), and vitamin E, retinol, and dehydroepiandrosterone (data not shown), as substrates for the expressed DGAT enzyme. Although [$^{14}$C]oleoyl CoA was consistently incorporated into triacylglycerols (by using the endogenous diacylglycerol as the acyl acceptor), it was not incorporated into esters for any other substrate tested, as assessed by autoradiography of TLC plates used to analyze reaction products (Fig. 3*E*).

DGAT mRNA expression was examined in a cultured cell model of adipocyte differentiation and in mammalian tissues. The mRNA increased markedly (≈8-fold) in parallel with DGAT activity in NIH 3T3-L1 cells during their differentiation

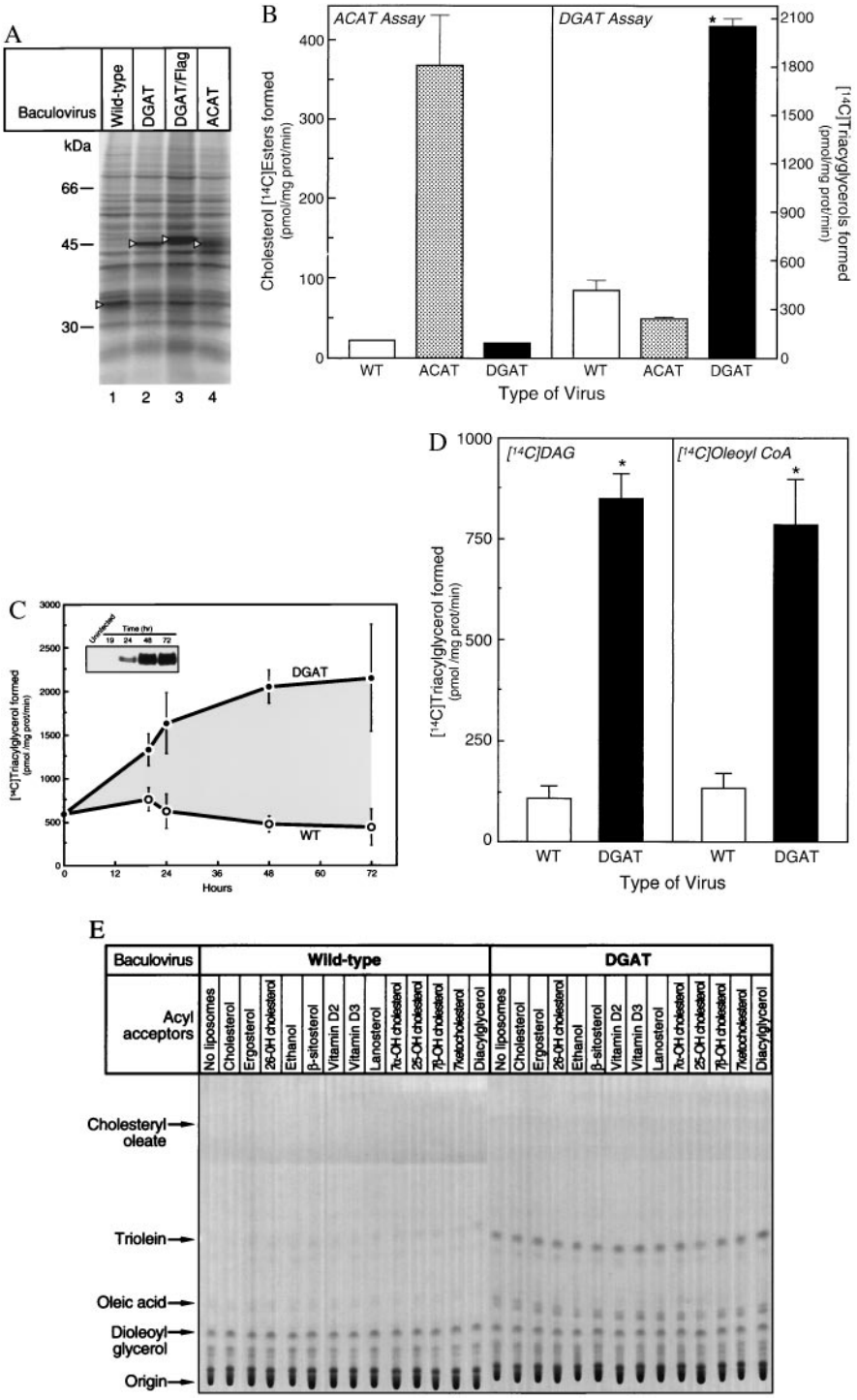


FIG. 3. Enzymatic activities in insect cell membranes expressing DGAT. Cells were infected with wild-type baculovirus (WT), mouse ACAT, or mouse DGAT recombinant baculoviruses, and membranes were assayed for enzymatic activity. (*A*) Metabolic labeling. Cell proteins, 48 h after infection, were metabolically labeled with [$^{35}$S]methionine and [$^{35}$S]cysteine, and whole-cell lysates were analyzed by SDS/PAGE and autoradiography. The expression of viral polyhedrin protein (lane 1), mouse DGAT (lane 2), FLAG-tagged mouse DGAT (lane 3), and mouse ACAT (lane 4) is indicated (triangles). (*B*) ACAT and DGAT activities. Data represent the mean (±SE) of five experiments. *, $P < 0.001$. vs. WT. (*C*) Time course of DGAT virus infection. Insect cell membranes were isolated at the indicated times after infection. Expression of the FLAG-tagged DGAT was detected by immunoblotting with an anti-FLAG antibody (*Inset*), and DGAT activity was measured. The doublet band observed in this experiment was not routinely observed, and its significance is unknown. Data represent the mean (±SE) of three experiments. (*D*) Comparison of the rate of triacylglycerol synthesis with either DAG or oleoyl CoA as the radiolabeled substrate. Assays contained the same amounts of oleoyl CoA (5 nmol) and DAG (2.5 μg) in all cases. The specific activity for DGAT virus-infected cells is less than that observed in *A* because of the reduction in DAG substrate concentration (i.e., this experiment was not performed at apparent $V_{MAX}$). Data are the mean (±SE) of five experiments. *, $P < 0.001$ vs. WT. (*E*) Acyl acceptor specificity of DGAT. Reaction products from wild-type or DGAT virus-infected membranes assayed with [$^{14}$C]oleoyl CoA and various acyl acceptor substrates were analyzed by TLC. Note that [$^{14}$C]oleoyl CoA is incorporated specifically into triacylglycerols for all reactions containing membranes expressing DGAT. Hydrolysis of the labeled oleoyl CoA to oleic acid (as shown in this experiment) was observed in some, but not all, preparation of membranes expressing DGAT; this finding was associated with membranes expressing the highest levels of DGAT activity.

into adipocytes (Fig. 4 *A* and *B*). DGAT mRNA was expressed in every human (Fig. 4*C*) and mouse (data not shown) tissue examined, with the highest expression levels in the small intestine. In addition, expression was detected in mouse adipose tissue (Fig. 4*D*). In the human Northern blots, additional hybridization signals were observed at ≈2.2 kb and ≈4.0 kb. The significance of these bands is currently unknown.

The human DGAT gene was mapped to human chromosome 8qter by fluorescent *in situ* hybridization. By using an interspecific cross, we mapped the mouse homolog for the DGAT gene (*Dgat*) to a region of chromosome 15 that exhibits homology with human chromosome 8 (Fig. 5). This region of mouse chromosome 15 has exhibited linkage with levels of plasma triacylglycerol-rich lipoproteins in several genetic crosses (24, 27, 28). In each case, the strongest linkage was observed with levels of plasma very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol, but there was also evidence of linkage with levels of plasma triacylglycerols. For example, in a cross between strains MRL and BALB/c, marker D15Mit17 exhibited a lod score of 6.7 for VLDL and LDL cholesterol and 2.7 for triacylglycerols (24).

## DISCUSSION

DGAT is a microsomal enzyme that plays a central role in the biosynthesis of cellular triacylglycerols. DGAT has never been purified to homogeneity, nor has its gene been cloned. In this study, we identified a cDNA encoding a protein that possesses DGAT activity. The identification of this cDNA provides a molecular probe for studying the role of DGAT in biology.

Several findings support the hypothesis that the cDNA we identified encodes a DGAT. First, DGAT activity was more than 5-fold higher in membranes expressing the cDNA than in membranes from wild-type virus-infected cells. Although insect cells, like all eukaryotic cells, synthesize triacylglycerols (2), the ability to express large amounts of the DGAT protein enabled us to detect DGAT-specific activity at high levels, similar to those found in mammalian tissues (14) and considerably above background levels in insect cells. The acyltransferase activity depended on the presence of a fatty acyl CoA substrate and was specific for DAG; there was no activity with cholesterol or a variety of other acyl acceptor substrates. Second, its mRNA expression increased markedly in parallel with DGAT activity in NIH 3T3-L1 cells during their differentiation into adipocytes, a process known to be associated with increases in DGAT activity (29) and triacylglycerol mass accumulation (30). Third, mRNA expression was detected in every mammalian tissue examined, as expected because of the central role of DGAT in cellular glycerolipid metabolism. The highest expression levels were found in the small intestine, consistent with a proposed role for DGAT in intestinal fat

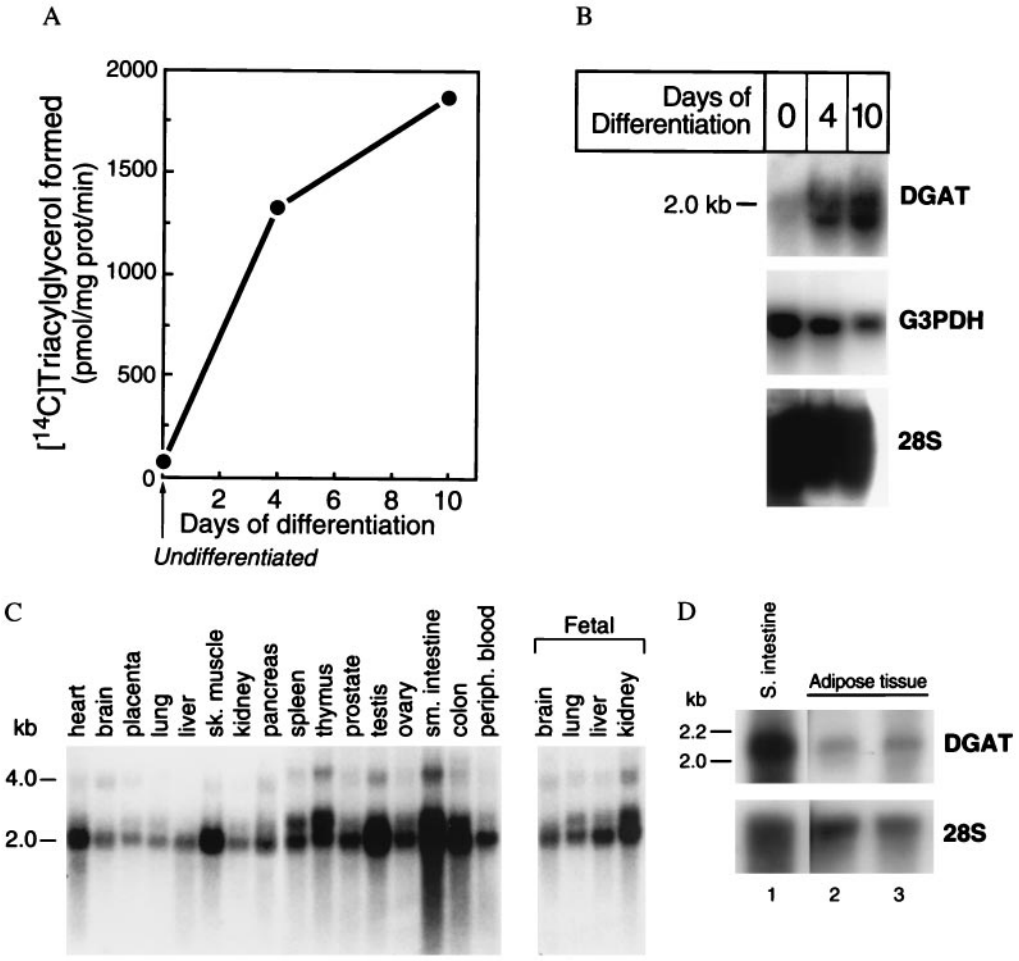


FIG. 4. Analysis of DGAT mRNA expression. (*A*) DGAT activity and (*B*) DGAT mRNA expression during differentiation of 3T3-L1 cells into adipocytes. Mouse 3T3-L1 adipocyte differentiation was induced, and RNA and membranes were isolated from undifferentiated cells or cells harvested 4 and 10 days later. Results are shown for DGAT and two controls for RNA loading [glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and 28S RNA]. Quantitation of DGAT mRNA in triplicate samples, by PhosphorImager analysis and correction for loading relative to 28S RNA (20) as an internal standard, demonstrated that DGAT levels were increased 5-fold by day 4 and 8-fold by day 10 of differentiation. The experiment was repeated three times with similar results. (*C*) DGAT expression in human tissues as assessed by Northern blot analysis. (*D*) DGAT expression in mouse small intestine and adipose tissue from two mice (lanes 2 and 3). The membrane was stripped and reprobed for 28S RNA (20).

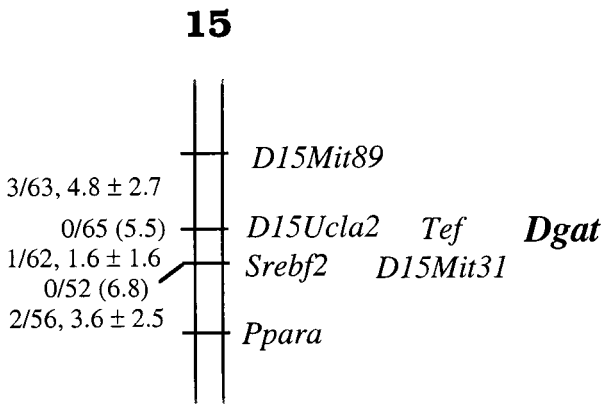


FIG. 5. Mouse chromosomal location of DGAT gene. Genetic mapping of the *Dgat* to mouse chromosome 15 was performed by linkage analysis performed with a panel of 67 progeny from an interspecific backcross [(C57BL/6J × *Mus spretus*)$F_1$ × C57BL/6J] (23). A segment of the chromosome is drawn with the centromere toward the top. The ratios of the number of recombinants to the total number of informative mice and the recombination frequencies ±SE (in cM) for each pair of loci are indicated. For pairs of loci that cosegregate, the upper 95% confidence interval is shown in parentheses. No recombination was observed between *Dgat*, D15Ucla2, and *Tef* (0/65 mice). The DGAT gene has been assigned the name *Dgat*. The data have been deposited in the Mouse Genome Database under accession number MGD-J:44983.

absorption (3, 31). In addition, the mRNA was expressed in adipose tissue, which is known to have a high level of DGAT activity (6). Interestingly, mRNA expression was relatively low in the livers of humans (Fig. 4*C*) and mice (data not shown), despite the fact that significant DGAT activity is present in the liver (14). The significance of this finding is currently unknown. One possibility is that, although the expression levels are low, the mRNA encodes a very stable protein; alternatively, it is possible that the liver expresses a second DGAT. As a final piece of evidence confirming the identity of this cDNA, we have disrupted the mouse DGAT gene in embryonic stem cells and achieved germ-line transmission of this mutation. Preliminary results indicate that DGAT activity in membranes from embryonic fibroblasts homozygous for the knockout mutation is reduced to ≈5% or less than that in wild-type fibroblast membranes (S.J.S., S.C., and R.V.F., unpublished observations).

The identification of a DGAT cDNA has significant implications for understanding the regulation of the triacylglycerol biosynthetic pathway and intracellular lipid metabolism. DGAT molecular probes will facilitate *in vivo* studies of the role(s) of DGAT and its regulation in a number of physiologic processes, such as intestinal fat absorption, lipoprotein synthesis and secretion, lactation, and adipose tissue formation. Whether DGAT regulation participates in modulating levels of cellular DAG involved in signaling also can be assessed. Ultimately, understanding DGAT at a molecular level may uncover potential approaches for treating hypertriglyceridemia or obesity in humans.

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