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A Novel Acyl-CoA Synthetase, ACS5, Expressed in Intestinal Epithelial Cells and Proliferating Preadipocytes[1]

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We report here the identification, characterization, and expression of a novel rat acyl-CoA synthetase (ACS) designated as ACS5. ACS5 consists of 683 amino acids and is approximately 60% identical to the previously characterized ACS1 and ACS2. ACS5 was overproduced in *Escherichia coli* cells and then purified to near homogeneity. The purified enzyme utilized a wide range of saturated fatty acids similar to those utilized by ACS1 and ACS2, but differed in its preference for C16-C18 unsaturated fatty acids. Northern blot analysis revealed that ACS5 mRNA is present most abundantly in the small intestine, and to a much lesser extent in the lung, liver, adrenal gland, adipose tissue, and kidney. *In situ* hybridization of rat ileum revealed abundant accumulation of ACS5 transcripts in foveolar epithelial cells. The hepatic level of ACS5 mRNA was significantly increased by refeeding a fat-free high sucrose diet and reduced by fasting or refeeding a high cholesterol diet, whereas that in the small intestine was not significantly altered by various dietary conditions. In contrast to the absence of ACS1 mRNA in undifferentiated 3T3-L1 preadipocytes, ACS5 mRNA was present in proliferating 3T3-L1 preadipocytes and its level remained unaltered during differentiation, suggesting that ACS5 may provide the acyl-CoA utilized for the synthesis of cellular lipids in proliferating preadipocytes.

Key words: acyl-CoA synthetase, dietary regulation, intestinal epithelial cell, lipogenesis, proliferation.

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The ligation of fatty acids with coenzyme A (CoA) to produce acyl-CoA is a key reaction in mammalian fatty acid metabolism. This reaction, catalyzed by acyl-CoA synthetase (ACS, EC 6.2.1.3), is a crucial step in mammalian fatty acid metabolism, since mammalian fatty acid synthase contains a specific thioesterase to produce a free fatty acid as the final reaction product (*1, 2*). Therefore both *de novo* synthesized and dietary derived fatty acids cannot be metabolized without ACS in mammals. Acyl-CoA, the product of ACS, is utilized in various metabolic pathways including membrane biogenesis, energy production and fat deposition. Consistent with the multiple utilization of acyl-CoA, there are several ACSs in mammals.

In previous studies, we characterized four rat ACSs, designated as ACS1-4 (*3-7*). Although the four enzymes exhibit a structural architecture common to ACSs from various organisms, they can be classified into two subfamilies based on their structures and fatty acid preferences: one consists of ACS1 (*3*) and ACS2 (*4*), and the other of ACS3 (*5*) and ACS4 (*6*). Rat ACS1 and ACS2 share approximately 65% of their amino acids (*4*), and exhibit broad fatty acid specificities (*7*), but their tissue distributions are completely different: ACS1 is abundant in the liver, heart, and adipose tissue (*3*), whereas ACS2 is predominant in the brain (*4*). Between rat ACS3 and ACS4, approximately 68% of the amino acids are identical but they show poor amino acid identity with ACS1 and ACS2 (*6*). ACS3 utilizes laurate, myristate, arachidonate, and eicosapentaenoate most preferentially (*5*), whereas ACS4 prefers a narrow range of fatty acids including arachidonate, and eicosapentaenoate (*6*). ACS3 mRNA is expressed highly in the brain, and to a much lesser extent in the lung, adrenal gland, kidney, small intestine, and adipose tissue, but is not detected in the heart or liver (*5*). In contrast, the mRNA for ACS4 is expressed in steroidogenic tissues including the adrenal gland, ovary, and testis (*6*).

Although these ACSs exhibit different tissue distribution, none of them is preferentially expressed in the small intestine, where dietary lipids are hydrolyzed to free fatty acids in the lumen and absorbed by intestinal epithelial

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Abbreviations: ACS, acyl-CoA synthetase; SREBP, sterol regulatory element binding protein.

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cells. The absorbed free fatty acids are then converted to acyl-CoA for the synthesis of triacylglycerol and cholesterol esters in these cells, which enter the circulation as chylomicrons.

In the course of cDNA cloning of these four ACSs, we have isolated a cDNA encoding a fifth ACS. We describe here the primary structure, fatty acid preference, tissue expression, and regulation of this newly identified ACS, designated as ACS5.

EXPERIMENTAL PROCEDURES

Molecular Characterization of Rat ACS5—Standard molecular biology techniques were performed as described by Sambrook *et al.* (*8*). A rat liver cDNA library was constructed in the λ ZapII vector (Stratagene), using poly-(A) RNA from rat liver, and screened with a 1.9 kb *Eco*RI/*Eco*RV fragment of rat ACS1 cDNA (*3*) as a probe under reduced hybridization conditions. On the screening of 1×10^6 clones, we obtained four positive clones, and one representative clone containing the largest cDNA insert (pACS5) was further characterized. The nucleotide sequences of cDNA fragments were determined by the dideoxy chain termination method (*9*) using M13 primers, T3 and T7, or specific internal primers. Sequence reactions were carried out using *Taq* DNA polymerase with fluorescently labeled nucleotides and an Applied Biosystems model 373A DNA sequencer.

For Northern blotting, total RNA prepared with acid-guanidinium thiocyanate-phenol-chloroform (*10*) was denatured with 1 M glyoxal and 50% dimethyl sulfoxide, electrophoresed on a 1.5% agarose gel, and then transferred to a nylon membrane (Zeta-Probe membrane; Bio-Rad). For normalization as to RNA loading, a 467 bp fragment of rat cyclophilin cDNA (*11*) was generated by reverse transcription-polymerase chain reaction (*12*) with nucleotide primers, 5′-TCAACCCCACCGTGTTCTTCGACAT-3′ and 5′-GGTGATCTTCTTGCTGGTCTTGCCA-3′, and used as a probe.

Construction of an ACS5 Expression Plasmid—Overproduction of ACS5 in *Escherichia coli* cells was carried out using a bacterial expression vector, pTV118N. To connect the second codon of the rat ACS5 cDNA (pACS5) adjacent to the initiator ATG of pTV118N, pACS5 was amplified by polymerase chain reaction using primer 1 (5′-CTTTTTATTTTTAACTTGTT-3′) and primer 2 (5′-AATACGACTCACTATAG-3′). The 2.3 kb PCR product was then digested with *Bam*HI, and the resulting 1.8 kb fragment was inserted into the *Nco*I (blunted)/*Bam*HI site of pTV118N. This intermediate plasmid was then digested with *Fba*I and *Pst*I, and ligated with a 2.1 kb *Fba*I/*Pst*I fragment of pACS5. The resulting expression plasmid (designated as pTV-ACS5) contains a *lac* promoter, an SD sequence and the entire coding region of ACS5 cDNA, and was used to transform *E. coli* cells, XL1-Blue.

Induction of the ACS Enzyme in E. coli—*E. coli* cells transformed with the ACS5 expression plasmid were grown in 1 liter of Terrific broth (1.2% Bacto Tryptone, 2.4% yeast extract, 0.4% glycerol, 90 mM potassium phosphate, pH 7.8) (*8*) supplemented with ampicillin (100 μg/ml) and tetracycline (25 μg/ml) at 30°C, and then induced by adding isopropyl-β-D-thio-galactopyranoside (IPTG) as described (*7*). After 12 h induction, the cells were harvested and resuspended in 100 ml of buffer A [50 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, and 10% (w/v) glycerol] containing 1 mM phenylmethylsulfonyl fluoride. The resuspended cells were lysed by sonication and then centrifuged at $10,000\times g$ for 20 min at 4°C. The supernatant was taken as the crude extract and stored at −80°C until use.

Assay for ACS Activity—ACS activity was measured by either the isotopic method (*13*) or the spectrophotometric method (*13*): the latter was used only for the purified enzyme. Protein concentrations were determined by the Lowry method (*14*) with bovine serum albumin as a standard.

Purification of ACS5—A typical purification is described. All steps were carried out at 4°C. During the purification, the enzymes were monitored as to ACS activity.

The crude extract of the *E. coli* strain carrying pTV-ACS5 was further centrifuged at $105,000\times g$ for 90 min and the resulting precipitate was suspended in 40 ml of buffer B [buffer A containing 1% (w/v) Triton X-100] with 10 strokes of a Dounce homogenizer. The suspension was gently stirred for 1 h and then centrifuged at $105,000\times g$ for 1 h. The resulting supernatant was applied to a DEAE-Sephacel (Pharmacia) column (2.5×10 cm) equilibrated with buffer B. The column was washed with three column volumes of the same buffer, and then eluted with a linear concentration gradient formed from four column volumes of buffer C [buffer A containing 0.1% (w/v) Triton X-100] and the same volume of buffer C containing 0.5 M NaCl. The pooled active fractions were concentrated with polyethylene glycol (PEG6000) and then dialyzed against buffer C.

In Situ Hybridization—Ileum specimens were obtained from 3-month-old male rats ($n=3$). The specimens were immediately fixed with 4% paraformaldehyde containing 0.5% glutalaldehyde for 18 h at 4°C and then embedded in paraffin wax. *In situ* hybridization was performed by use of a manual capillary action system (MicroProbe staining system; Fisher Scientific, Pittsburgh, PA), with modification of the reported methods (*15, 16*). The sequence of the 30-base ACS5 oligonucleotide probe used for *in situ* hybridization analysis was as follows: 5′-AGGTAAGACTGGCTGAGGTCTGTTGATCAG-3′, corresponding to 90 to 120 of ACS5. A sense oligonucleotide probe was used as a negative control. The probes were synthesized with a 3′ biotinylated tail (Brigati tail) (5′-probe-biotin-biotin-biotin-TAG-TAG-biotin-biotin-biotin-3′) as previously reported (*17*). Tissue sections (3 μm, applied to Probe On Plus slides; Fisher Scientific) were hybridized with the anti-sense or sense oligonucleotide at 45°C for 1 h, washed twice with 2×SSC at 45°C (3 min per wash), and then incubated with alkaline phosphatase-conjugated streptavidin. After washing twice in AP Chromogen buffer™ (Research Genetics, Huntsville, AL) at room temperature, the hybridization products were visualized using Fast Red salt. The slides were counterstained with hematoxylin and air dried for light microscopic examination.

Animal Treatment—Male Wistar strain rats (six/group, caged together) weighing 200-300 g were used in the experiments. Control rats were fed on the commercial stock diet. Fasted rats were deprived of food for 48 h. Refed rats were fasted for 48 h followed by free access to a high

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fat diet (10% soybean oil and 90% stock diet), a high cholesterol diet (2% cholesterol, 0.5% cholic acid, 10% soybean oil, and 87.5% stock diet) or a fat-free high sucrose diet (69% sucrose, 20% casein, 4% mineral mixture, 2% vitamin mixture, and 5% cellulose powder) for 72 h.

Cell Culture—3T3-L1 mouse preadipocytes (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, sodium ascorbate (0.2 mM), penicillin (50 u/ml), and streptomycin (50 μg/ml), with a change of the medium every 2-3 days. Differentiation of the preadipocytes was induced by shifting the cells, after confluence had been attained, to DMEM containing with 10% fetal bovine serum, 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM methylisobutylxanthine. After 48 h, the medium was replaced with DMEM supplemented with insulin (5 μg/ml) and 10% fetal bovine serum.

RESULTS

A cDNA encoding a new type of ACS, designated as ACS5, was isolated from a rat liver cDNA library using rat ACS1 cDNA as a probe. Figure 1A shows the nucleotide and deduced amino acids sequences of the cDNA, which has an open reading frame of 2,049 bp corresponding of 683 amino acids (M_r 76,403). The putative initial methionine was preceded by an in-frame termination codon 81 nucleotides upstream.

ACS5, like other mammalian ACSs, consists of five regions: an N-terminal region, luciferase-like regions 1 and 2, a linker connecting the two luciferase-like regions, and a C-terminal region (Fig. 1B). Comparison of the amino acids in each of the five regions revealed that ACS5 belongs to the subfamily comprising ACS1 and ACS2: approximately 60% of the amino acids are identical to those in ACS1 and ACS2.

To overproduce ACS5 in *E. coli* cells, a bacterial expression plasmid containing a *lac* promoter and the entire coding region of the ACS5 cDNA was generated. The enzyme in the *E. coli* cells transformed with the expression plasmid was induced with isopropyl-β-D-thio-galactopyranoside and the resulting enzyme was purified to near homogeneity. The purification procedure involved solubilization of the enzyme with Triton X-100 and chromatography on DEAE-Sephacel (Table I). The overall purification was 39-fold, with a yield of 21.8%. The specific activity of the purified ACS5 was 5.1 μmol/min/mg when assayed with palmitate as a substrate. As shown on SDS-polyacrylamide gel electrophoresis (Fig. 2), the purified enzyme was nearly homogenous and had an apparent molecular mass of

TABLE I. **Summary of the purification of recombinant ACS5.** One liter of a culture of *E. coli* cells carrying pTV-ACS5 was used to prepare the crude extract. Enzyme activity was measured by the isotopic method using palmitate as a substrate.

Step	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Recovery (%)
Crude extract	2,842	369	0.13	100
140,000 × *g* pellet	184	221	1.2	59.9
Triton X-100 extract	145	174	1.2	47.2
DEAE-Sephacel	15.8	80.6	5.1	21.8

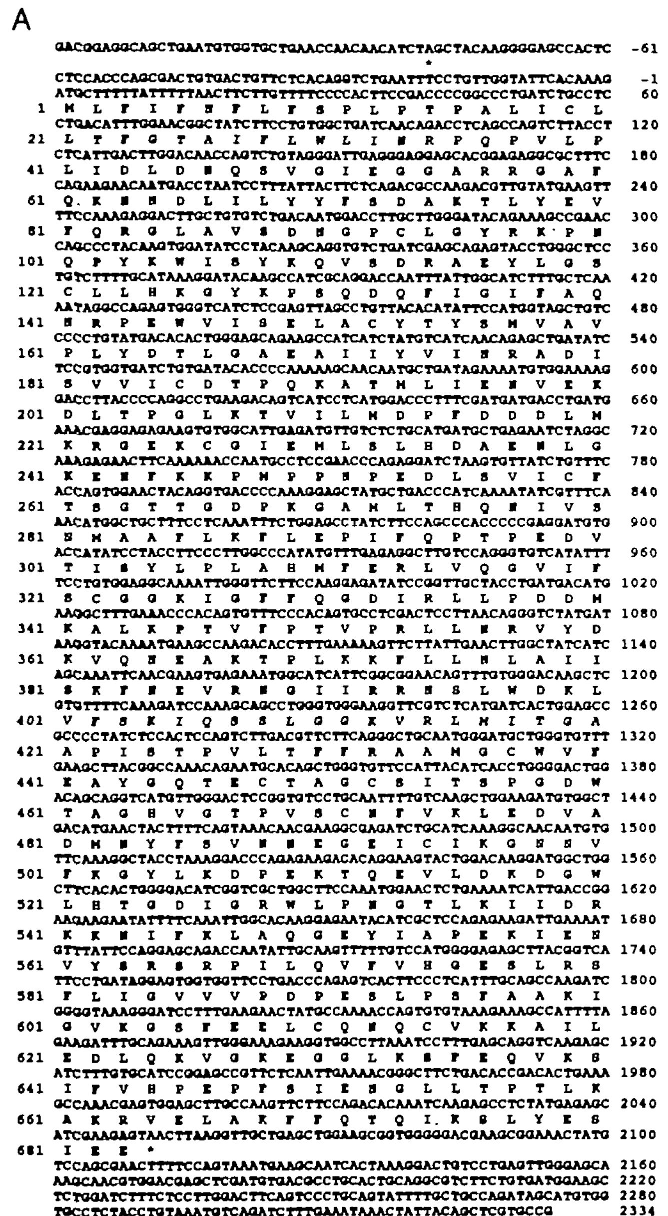

Fig. 1. **Structure of rat ACS5 cDNA.** (A) Nucleotide and deduced amino acid sequences of rat ACS5 cDNA. Nucleotide residues are numbered on the right and amino acids are numbered on the left. Nucleotide 1 is the A of the initiator AUG codon. Negative numbers refer to the 5′-untranslated region. Two in-frame translation termination codons, at −81 and 2050, are indicated by asterisks. A potential polyadenylation signal is underlined. (B) Comparison of the amino acids in rat ACS5 with those in the other four known rat ACSs. The five common regions in rat ACSs are shown in the upper portion: N-terminal region (N-term), luciferase-like region 1 (LR1), linker region (linker), luciferase-like region 2 (LR2), and C-terminal region (C-term). Figures indicate the percentage amino acid identities within each region of rat ACS1-4 compared with ACS5.

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70 kDa, which is close to the molecular mass calculated from the deduced amino acid sequence of the cDNA.

Figure 3 compares the fatty acid preference of the purified recombinant ACS5 with that of ACS1 (7), as determined by the spectrophotometric method, using various fatty acids. Both the purified enzymes efficiently utilize saturated fatty acids with 12-18 carbon atoms and unsaturated fatty acids with 16-20 carbon atoms. Among these fatty acids, the best substrates are palmitic, palmitoleic, oleic, linoleic, and linolenic acids for ACS5, and palmitic acid for ACS1.

Northern blotting of RNA from various rat tissues revealed hybridization to a major transcript of 2.6 kb long, corresponding to pACS5 (Fig. 4). ACS5 transcripts are

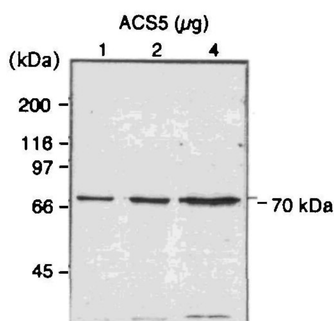

Fig. 2. **SDS-polyacrylamide gel electrophoresis of the purified recombinant ACS5.** 1, 2, and 4 μg of the purified recombinant ACS5 were subjected to electrophoresis on an 8% SDS-polyacrylamide gel, and then staining with Coomassie Brilliant Blue R-250. Molecular size markers are indicated on the left.

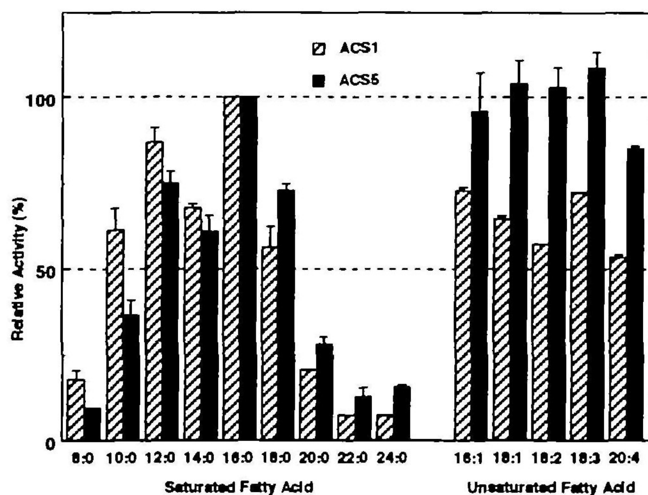

Fig. 3. **Fatty acid specificity of the purified recombinant ACS5.** Enzyme activity was determined by the spectrophotometric method with 1 μg of the purified enzyme and the standard reaction mixture (*13*), except that various saturated and unsaturated fatty acids (final concentration, 0.1 mM) were used. Enzyme activity is expressed as a percentage of that obtained with palmitate as a substrate (5.1 μmol/min/mg). The data represent the means ± SD for triplicate determinations. Saturated fatty acids: 8:0, octanoic acid; 10:0, decanoic acid; 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 20:0, arachidic acid; 22:0, docosanoic acid; 24:0, tetracosanoic acid. Unsaturated fatty acids: 16:1, palmitoleic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid.

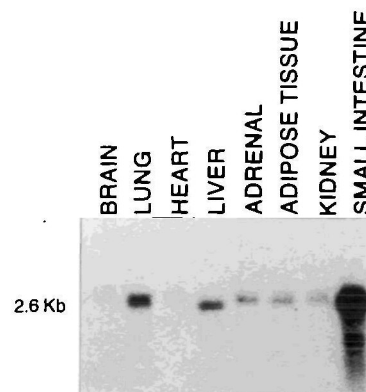

Fig. 4. **Northern blot analysis of ACS5 mRNA in various rat tissues.** Total RNA (15 μg) prepared from the indicated rat tissues was subjected to electrophoresis on a 1.5% agarose gel, blotted onto a nylon membrane, and then hybridized with the ^{32}P-labeled 2.4 kb *Eco*RI/*Eco*RI fragment of pACS5. The filter was washed in 0.1 × SSC containing 0.1% (w/v) SDS at 65°C for 30 min and then exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 48 h. RNA loading was consistent among the lanes, as judged on ethidium bromide staining (data not shown). The autoradiograph shown is representative of four independent experiments which gave essentially identical results.

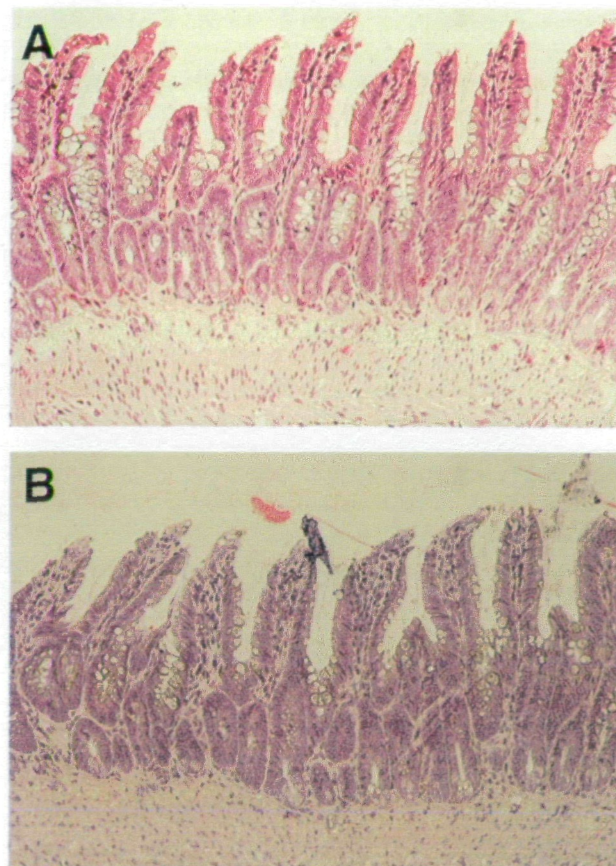

Fig. 5. ***In situ* hybridization analysis of ACS5 transcripts in the rat ileum.** Hybridization signals were visualized in red, as a result of the Fast Red salt, in the foveolar epithelial cells but not in the interstitial cells (A). The negative control with the sense oligonucleotide exhibited no accumulation of mRNA hybridization signals (B). Nuclei were made visible by counterstaining with hematoxylin. Magnification: 121×, A and B.

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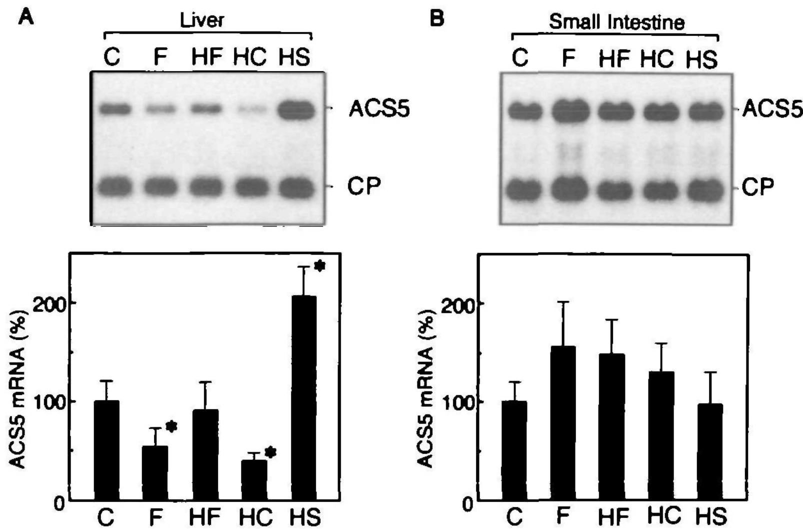

Fig. 6. **Dietary effects on the hepatic and intestinal ACS5 mRNA levels.** Total hepatic (A) and intestinal (B) RNA from rats ($n=6$) fed the control diet (C), fasted (F), and fed high fat (HF), high cholesterol (HC), and fat-free high sucrose (HS) diets were subjected to Northern blot analysis with rat ACS5 cDNA as described in the legend to Fig. 4, followed by autoradiography (exposed to XAR-5 film for 27 h). Northern blot analysis with a rat cyclophilin (CP) cDNA probe was carried out for normalization. The radioactivity in each band was quantified using a Bio-imaging Analyzer (BAS-2000, Fuji) with various exposure times and normalized as to the cyclophilin signal. The values in the lower panels are the means for six separate experiments±SD, relative to the mRNA level in control rats (set at 100). *$p<0.01$ compared to the control.

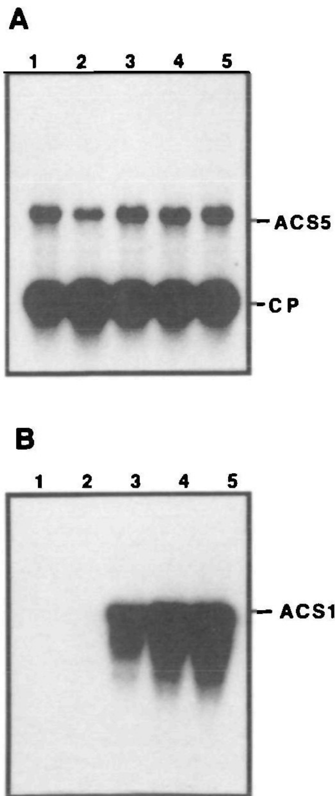

Fig. 7. **Expression of ACS5 transcripts during the differentiation of 3T3-L1 cells.** (A) 3-day postconfluent 3T3-L1 preadipocytes were harvested immediately prior to (lane 1), and 1 day (lane 2), 3 days (lane 3), 5 days (lane 4), and 7 days (lane 5) after differentiation. Total RNA (10 μg) was analyzed by Northern blotting with the rat ACS5 (ACS5) and rat cyclophilin (CP) probes as described above, followed by exposure to Kodak XAR-5 film with an intensifying screen at −80°C for 18 h. (B) The same membrane as in (A) was hybridized with a 0.96-kb *Eco*RI/*Eco*RV fragment of rat ACS1 cDNA (pRACS15) and then exposed to Kodak XAR-5 film with an intensifying screen at −80°C for 12 h. The autoradiographs shown in (A) and (B) are each representative of four independent experiments that gave essentially identical results.

present most abundantly in the small intestine and to a much lesser extent in the lung, liver, adrenal, adipose tissue, and kidney in the normal adult rat (Fig. 4).

To locate cells expressing ACS5 mRNA, *in situ* hybridization was carried out using tissue sections prepared from adult rat ileum. Hybridization signals for ACS5 transcripts, appearing red as a result of the Fast Red reaction, were detected in the foveolar epithelial cells, but not in the interstitial cells (Fig. 5A). In negative controls for mRNA *in situ* hybridization using the sense oligonucleotide probe, no significant accumulation of ACS5 mRNA hybridization signals was detected (Fig. 5B).

A high level of hepatic ACS1 mRNA was induced by refeeding a high fat diet or a fat-free high sucrose diet after 48 h fasting (*3*). To determine the dietary effects on the level of ACS5 mRNA, Northern blotting was carried out using total RNA from the livers or small intestines of adult male rats fed various diets. Whereas the intestinal level of ACS5 mRNA was not altered significantly by various dietary conditions, that in the liver was significantly changed by fasting, or by refeeding a high cholesterol or fat-free high sucrose diet (Fig. 6). Fasting and refeeding a high cholesterol diet decreased the hepatic level of mRNA by 54±17.5 and 39.2±7.8%, respectively. In contrast, refeeding a fat-free high sucrose diet increased the hepatic level of ACS5 mRNA approximately 2-fold.

We next determined the level of ACS5 mRNA during the differentiation of 3T3-L1 cells. As shown in Fig. 7, ACS5 transcripts were detected in undifferentiated proliferating adipocytes and their level was not altered during differentiation. In contrast, ACS1 transcripts were undetectable in undifferentiated proliferating adipocytes and markedly induced during differentiation.

DISCUSSION

In this study, we characterized a new ACS, designated as ACS5, that is abundantly expressed in intestinal epithelial cells. Although ACS5 resembles ACS1 and ACS2 in structure and substrate preference as to saturated fatty acids, the tissue distribution and regulation of its mRNA are completely different from those of ACS1 and ACS2. These

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differences may reflect the biological roles of these three structurally similar ACSs, ACS1, ACS2, and ACS5.

In addition to the activation of fatty acids to form acyl-CoAs, ACS plays a role in the uptake of long-chain fatty acids, in cooperation with a fatty acid transporter (*18*). The abundant expression of ACS5 mRNA in intestinal epithelial cells suggests that it plays a part in the uptake of dietary derived fatty acids into these cells and their subsequent utilization. Consistent with this hypothesis, the purified recombinant ACS5 utilizes a wide range of saturated and unsaturated fatty acids.

Although the liver is not the major organ that expresses ACS5 mRNA, the regulation of the hepatic ACS5 mRNA level by various dietary conditions is noteworthy. Like that of ACS1 mRNA (*3*), the hepatic level of ACS5 mRNA was increased by refeeding a fat free high sucrose diet, but unlike that of ACS1 mRNA it was not induced by refeeding a high fat diet. It is also noteworthy that fasting and refeeding a high cholesterol diet significantly decreased the hepatic level of ACS5 mRNA. The induction of hepatic ACS5 mRNA by refeeding a fat-free high sucrose diet and reduction by fasting suggest that the plasma level of insulin may play a part in the regulation of the hepatic level of ACS5 mRNA: some of the lipogenic genes, including the acetyl-CoA carboxylase and fatty acid synthase genes, exhibit a similar pattern of dietary expression and are regulated by insulin (reviewed in Refs. *19* and *20*). In addition, the induction of hepatic ACS5 mRNA by refeeding a fat-free high sucrose diet and the down regulation by refeeding a high cholesterol diet suggests transcriptional regulation of the mRNA by sterol regulatory element-binding proteins (SREBPs) (reviewed in Ref. *21*). SREBPs are membrane-membrane bound transcription factors that mediate the synthesis of cholesterol and its uptake from low density lipoprotein in animal cells. In addition to the down regulation of genes involved in cholesterol metabolism, they also regulate the expression of genes encoding the enzymes for fatty acid metabolism (*22, 23*). Transgenic mice overproducing a truncated form of SREBP-1a exhibit massive liver enlargement due to increased synthesis of cholesterol and triglycerides (*24*). Therefore, it will be interesting to determine if hepatic transcription of the ACS5 gene is mediated by SREBPs and induced in transgenic mice overproducing a truncated form of SREBP-1a, since the metabolic fate of fatty acids is highly dependent on the activity of ACS enzymes.

The most interesting feature of ACS5 is the presence of its mRNA in proliferating preadipocytes. Although ACS1 mRNA is detected in a wide range of tissues, including liver and fat cells, it is not detected in either proliferating preadipocytes (*25*) or proliferating liver (*26*). Inhibition of ACS by specific inhibitors, triacsins, profoundly reduces the synthesis of cellular phospholipids, thereby blocking the proliferation of mammalian cells (*27*). Therefore, ACS5 may provide the acyl-CoA required for the synthesis of cellular lipids during the proliferation of preadipocytes. Further studies are required to determine the exact role of ACS5 in lipid metabolism.

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