Plasma lipids are maintained within a narrow physiologic range and exhibit circadian rhythmicity. Plasma triglyceride and cholesterol levels were high in the night due to changes in apolipoprotein B-lipoproteins in *ad libitum* fed rats and mice maintained in a 12-h photoperiod. Absorption of [3H]triolein or [3H]cholesterol was higher at 2400 h than at 1200 h, indicating that intestinal lipoprotein production shows diurnal variation. Moreover, intestinal microsomal triglyceride transfer protein (MTP) activity, protein, mRNA, and gene transcription showed diurnal variations and were high at 2400 h. Similar to the small intestine, hepatic MTP activity, protein, and mRNA levels also changed significantly within a day. MTP was induced in fasted animals soon after refeeding. When mice were subjected to restricted feeding, MTP expression was high at the expected time of food availability. In contrast, extended exposures to light and dark completely abolished rhythmicity in MTP expression and plasma lipid levels. These studies show that MTP expression and plasma lipid undergo diurnal regulation and exhibit peaks and nadirs at similar times and suggest that diurnal modulation of MTP is a major determinant of daily changes in plasma lipids. Furthermore, environmental factors, such as food and light, play an important role in MTP regulation.

Circadian rhythm is a repetitive occurrence of physiological processes with an approximate interval of 24 h. Several biochemical, physiological, and pathological processes exhibit circadian rhythms. Plasma lipid concentrations are maintained within a narrow range and exhibit circadian rhythmicity in humans and rodents (1–4). Plasma lipid homeostasis is maintained by balancing lipoprotein production and catabolism. Lipoprotein production is critically dependent on apolipoprotein B (apoB) and microsomal triglyceride transfer protein (MTP) (5–8). ApoB is a structural protein required for the assembly of lipoproteins, whereas MTP is an essential chaperone for their assembly (5, 6, 9). MTP is a heterodimeric protein of 97- and 55-kDa polypeptides mainly present in the endoplasmic reticulum of hepatocytes and enterocytes (5, 6, 8, 10). It is crucial for the first step of lipoprotein assembly, because it transfers lipids and binds to apoB (5, 11). Via these mechanisms, MTP is believed to avoid improper folding and premature degradation of apoB. A vital role of MTP in the formation and secretion of apoB is supported by the fact that patients with abetalipoproteinemia, a disorder caused by mutations in the *mttp* gene that codes for the 97-kDa subunit of MTP, have no apoB in the plasma (12, 13). Molecular identification of MTP as a key chaperone for lipoprotein assembly provided a novel opportunity to determine the mechanisms that control its regulation. Intestinal and liver MTP is regulated by dietary fats (14, 15), hormones such as insulin and leptin (16), transcription factors such as HNF4 (17–19), peroxisome proliferator-activated receptor α (20), and development (21–23). In addition, genetic variants of MTP have been associated with the serum concentration of low density lipoprotein-cholesterol and predisposition to coronary heart disease (24). Thus, understanding various physiologic factors that regulate MTP would be useful in devising methods to combat hyperlipidemias.

Several *in vitro* and *in vivo* studies show that cholesterol synthesis exhibits circadian rhythm in the liver and intestine (25–30). In addition, several proteins involved in lipid metabolism, such as hepatic cytochrome P450 cholesterol 7α-hydroxylase, CYP7 (31), hydroxymethylglutaryl-CoA reductase (28), lipolytic enzymes (29, 32), apolipoprotein AIV (33), LDL receptor (26, 34), and peroxisome proliferator-activated receptor α (35), exhibit diurnal variations in both humans and rodents. However, none of these changes have been directly correlated with plasma lipids. Thus, specific mechanisms responsible for the observed daily shifts in lipid levels have not been identified. In the present study, we studied diurnal variations in plasma lipids and in the expression of MTP and propose that diurnal changes in MTP might be a key determinant controlling plasma lipid levels.

**EXPERIMENTAL PROCEDURES**

**Materials**—[35S]Methionine/cysteine labeling mixture, [3H]triolein, [3H]cholesterol, [3H]cholesterol, and Solvable were from PerkinElmer Life Sciences. Rabbit anti-PDI (catalog number SPA-890; Stressgen Bioreagents), rabbit polyclonal to GAPDH (catalog number ab9485; Abcam Inc.), purified mouse anti-MTP (catalog number 612022; BD Transduction Laboratories), apoB (catalog number K23300R; Biodesign), and apoAI (catalog number 178463; Calbiochem) were used for Western blot analysis.

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2 The abbreviations used are: apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; LDL, low density lipoprotein(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high density lipoprotein(s); PBS, phosphate-buffered saline.
Animals—Animal experiments were performed in accordance with the institutional animal care and use committee guidelines. Male Sprague-Dawley rats (10–12 weeks) and C57BL/6J mice (about 10 weeks) were purchased and acclimatized for at least 7 days in an air-conditioned room at 22 ± 0.5 °C with a 12-h lighting schedule (700–1900 h) prior to experiments. All animals had free access to water and standard laboratory chow. For experiments, animals (n = 6, each time point) were retrieved at the indicated times and anesthetized, and blood was collected from the heart. Liver was collected, washed in ice-cold PBS, cut into small pieces, and frozen for further analysis. Small intestines were obtained from the duodenum, washed, and cut open. They were divided into 4–5-cm pieces starting from the duodenum. Mucosa was scraped from these segments and snap frozen. The first segment was used for lipid analyses, the second for MTP activity and protein measurements, and the third segment for mRNA quantifications. For fasting followed by feeding experiments, two groups of animals were fasted for 24 h starting at 1200 and 2400 h. Subsequently, these animals had free access to food for 0, 2, 6, or 12 h. For restricted feeding experiments, mice had access to food only from 900 to 1500 h for 10 days, and then they were used to measure MTP at the indicated times. To study the effect of light and dark, animals were kept in these conditions for 5 days and used for circadian experiments.

Plasma Lipid Analysis—Plasma high density lipoproteins (HDL) were isolated after the precipitation of apoB-lipoproteins with phosphotungstate/MgCl₂ reagent (HDL-cholesterol; FIGURE 1. Diurnal variations in rat plasma lipids and lipoproteins. Plasma was obtained from ad libitum fed male Sprague-Dawley rats (10 weeks old) at the indicated times. Time reflects clock hours, with the light on at 700 and off at 1900 h. Triglyceride (A–C) and cholesterol (D–F) levels in the plasma, non-HDL apoB-lipoproteins, and HDL were measured as described under "Experimental Procedures." Each time point represents the mean ± S.D., n = 5–6. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with the 1200 h time point."

Animals—Animal experiments were performed in accordance with the institutional animal care and use committee guidelines. Male Sprague-Dawley rats (10–12 weeks) and
Sigma). Total and HDL triglycerides and cholesterol were measured using commercial enzymatic assays adapted for micromethods (36). Non-HDL apoB-lipoprotein triglyceride and cholesterol were determined by subtracting HDL lipid values from totals.

In Vivo Absorption of Lipids—Mice were fed a mixture of 2 μCi of [14C]cholesterol, 1 μCi of [3H]triolein, and 2 g/liter cholesterol (Sigma) in 50 μl of olive oil. Blood was collected from the tail, and plasma was used for liquid scintillation counting. Four hours later, mice were subjected to euthanasia, and intestines were removed, flushed with phosphate-buffered saline (PBS), and cut in four equal parts. The mucosa was scraped and rapidly frozen in liquid nitrogen. Liver and intestinal tissues were digested by incubating with Solvable, and radioactivity was determined after adding scintillation mixture. In some experiments, to avoid clearance of lipoproteins, plasma lipoprotein lipase was inhibited by the injection of 100 μl of Triton WR1339/PBS (1:7, v/v) (37).

In Vivo Loop Technique—Small intestines were opened by making two small incisions at both ends and flushed with PBS. A loop (20 cm) was made by tying with strings (38, 39). PBS (1.2 ml) containing [3H]triolein or [3H]cholesterol (2.5 μCi/ml) and cholesterol (0.2 mg/ml) was introduced into the loop with a microsyringe. After 2 h, entire loops were collected. In addition, the rest of the intestines until the distal incision were collected and divided into four segments. Total counts in plasma, HDL, and non-HDL apoB-lipoproteins were measured as described previously (36). In addition, counts in the intestinal loop and various segments as well as the liver were measured.

Measuring MTP Activity—Assays were performed in triplicate in black 96-well microtiter plates as described (40, 41). A final reaction mixture (100 μl) contained 5 μl of vesicles and 95 μl of buffer (blank) or sample containing 100 μg of microsomal proteins (40, 41). The microtiter plates were incubated at 37 °C, and at predetermined time points, samples were excited at 485 nm, and fluorescence was measured at 550 nm. To determine percentage of lipid transfer, fluorescence values obtained from control assays containing no MTP (blanks) were subtracted from sample values and then divided by the total fluorescence present in the vesicles reduced by blanks. To obtain total fluorescence, donor vesicles (5 μl) were incubated with 95 μl of isopropyl alcohol for 5 min (40).

Western Blot Analysis—Under anesthesia, the small intestine and liver were removed at different times. The small intestine was flushed with ice-cold PBS, and the mucosa was scraped. Portions of the mucosa and liver slices were rapidly frozen in liquid nitrogen for later preparation of microsomes and isolation of total RNA. Microsomal proteins from rat and mice small intestine and liver were isolated as described previously (40–42), separated (20 μg/lane) by SDS-PAGE, and analyzed by Western blot analyses as reported previously (38, 39). The relative densities of the bands in each reaction were determined using a densitometer.
Isolation of Primary Enterocytes—Primary enterocytes were isolated at 1200 and 2400 h as described before (36, 43). Briefly, mice were anesthetized, and intestinal contents were removed and washed with 117 mM NaCl, 5.4 mM KCl, 0.96 mM NaH₂PO₄, 26.19 mM NaHCO₃, and 5.5 mM glucose and then filled with 67.5 mM NaCl, 1.5 mM KCl, 0.96 mM NaH₂PO₄, 26.19 mM NaHCO₃, 27 mM sodium citrate, and 5.5 mM glucose (buffer A). Intestines were then bathed in oxygenated saline at 37 °C for 10 min. The buffer was discarded, and intestinal lumen were refilled with buffer A containing 1.5 mM EDTA and 0.5 mM dithiothreitol and incubated in 0.9% sodium chloride solution at 37 °C for 10 min. Luminal contents were collected and centrifuged at 1,500 rpm for 5 min. All buffers were adjusted to pH 7.4, gassed with 95% O₂, 5% CO₂ for 20 min, and maintained at 37 °C prior to use.

Metabolic Labeling of Enterocytes and Immunoprecipitation of Proteins—Isolated mouse primary enterocytes were preincubated in methionine-free Dulbecco’s modified Eagle’s medium for 30 min and pulsed with [³⁵S]methionine (100 µCi/ml). At various times, cells were lysed in solubilization buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mmol/liter EDTA, 1 mmol/liter EGTA, 2 mmol/liter phenylmethylsulfonyl fluoride, 100 kallikrein-inactivating units/ml of aprotinin, 0.1 mmol/liter leupeptin, 5 µmol/liter N-acetyl-leucyl-leucyl-norleucinal. The lysates were centrifuged for 10 min in a microcentrifuge (13,000 rpm), and the supernatants were collected. To immunoprecipitate either MTP or GAPDH, specific antibodies were added to the supernatants at a final dilution of 1:100. Tubes were rotated for 2 h at 4 °C, 20 µl of a 10% solution of protein A-Sepharose was added, and incubation continued for an additional 2 h at 4 °C. Protein A-anti-MTP complexes were collected by centrifugation at 6,000 rpm for 10 min. Pellets were washed three times in 10 mM Tris-HCl, pH 7.5, buffer containing 0.10 M NaCl and 1% Triton X-100. Antibody-antigen complexes were disrupted by adding 200 µl of buffer containing 10 mM Tris-glycine, pH 8.3, 8 mM urea, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and heating to 95 °C for 5 min. After centrifugation to remove insoluble material (1,000 rpm, 1 min), supernatants were applied to SDS-polyacrylamide gels, and proteins were separated by electrophoresis. Gels were stained, treated with Autofluor, dried, and exposed to phosphorimaging screens at room temperature for 1–3 days. The signal was quantified with an Amersham Biosciences PhosphorImager model 51. Radiolabeled proteins visualized on the fluorographs were quantified by cutting the corresponding bands from the gels, adding scintillation mixture, and counting in a liquid scintillation counter (44).

Isolation of Total RNA and Quantitative Real Time Polymerase Chain Reaction—Total RNA was isolated from the intestine and liver using Trizol reagents (1 ml/100 mg of tissue) (45) according to the manufacturer’s instructions, and RNA concentrations were measured by spectrophotometry. Isolated total RNA was reverse-transcribed, and the reaction mixtures were quantified by real time PCR on the ABI Prism 7000HT Sequence Detection System (Applied Biosys-
tems) as described previously (42, 46, 47). The primer-probe sets used to measure different transcripts are summarized in Table 1.

**Nuclear Run-on Assays**—The nuclei (1–1.5 × 10⁸) were suspended in a 2× reaction buffer consisting of transcription buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.3 mM KCl), 1 mM each ATP, CTP, and GTP, and 500 μCi of α-[³²P]UTP (800 Ci/mmol, 20 mCi/ml, PerkinElmer Life Sciences) and incubated at 30 °C for 30 min. The nuclei were then digested with 20 μl of 1 mg/ml RNase-free DNase I for 10 min at 30 °C. The reaction was terminated by incubating this reaction mixture with 200 μl of 2% SDS, 20 mM Tris, pH 7.4, 1 M NaCl, and 50 μl of 10 mg/ml proteinase K and 38 μl of 10 mg/ml yeast RNA at 55 °C for 50 min. RNA was extracted by the RNA Trizol method and precipitated by adding an equal amount of cold isopropyl alcohol at −20 °C. RNA precipitates were washed with 75% ethanol. The dried radiolabeled RNA pellets were suspended in RNase-free water and used for hybridization.

For hybridization, cDNAs (10 μg) were applied to nitrocellulose membrane using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad) and cross-linked by exposing to the UV light. Drs. Stephanie Dougan and Richard S. Blumberg (Harvard Medical School, Boston, MA) kindly provided mouse MTP cDNA. GAPDH was cloned in the pZErO-2 vector (Invitrogen), and the vector alone was used as a negative control. The membranes were prehybridized (overnight, 42 °C) in Express Hyb supplemented with heat-denatured salmon sperm DNA. The assay was performed in triplicate, and each point represents the mean ± S.D. of all of the 5–6 preparations. To determine changes in MTP protein expression, intestinal tissues were obtained from rats (B) and mice (D) at different time points and homogenized, and 20 μg of proteins were separated on polyacrylamide gels, transferred to nitrocellulose, and subjected to Western blot analysis for MTP or GAPDH. A representative blot is shown. ***p < 0.001 compared with the 1200 h time point.
EDTA (Tris-NaCl-EDTA) containing 1% SDS at 42 °C for 15 min; washed three times in Tris-NaCl-EDTA buffer containing 2× SSC and 0.1% SDS at 42 °C for 15 min; and washed in the same buffer containing 0.2× SSC and 0.1% SDS at 42 °C for 30 min. The membranes were air-dried and exposed to x-ray film for 72 h at −70 °C.

**Statistical Analysis**—GraphPad Prism was used for graphing and statistical evaluations. Statistical significance was evaluated using Student’s t test. Each time point represents the mean from five or six animals. Correlations were calculated using Spearman’s two-tailed t test. Data are presented as means ± S.D. p < 0.05 was considered significant.

**RESULTS**

**Diurnal Variations in Plasma Lipids and ApoB-Lipoprotein Levels**—To determine the diurnal variations in lipids and lipoprotein levels, plasma was collected at different times. Fig. 1A shows changes in rat plasma triglyceride during 24 h. Total plasma triglyceride levels were 2-fold higher at midnight (2400 h, 114 ± 26 mg/dl) than those present at midday (1200 h, 62 ± 16 mg/dl). Triglyceride in apoB-lipoproteins (Fig. 1B) at 2400 and 1200 h were 96 ± 10 and 12 ± 2 mg/dl, respectively, indicating 6–10-fold change within a day. No significant changes were observed in HDL triglyceride (Fig. 1C). Plasma cholesterol
also showed diurnal variations (Fig. 1D); however, changes were not as prominent as those seen for triglyceride. Again, apoB-lipoprotein cholesterol (Fig. 1E) changed significantly within 24 h, but HDL cholesterol did not (Fig. 1F). We also measured changes in plasma lipid levels in mice (Fig. 2). Triglycerides in plasma (Fig. 2A) as well as in apoB-lipoprotein (Fig. 2B) were high in the night and low in the day. In contrast, changes in HDL levels (Fig. 2C) were not significantly different. Similar to triglycerides, plasma cholesterol levels were high at 2400 h in plasma (Fig. 2D) and apoB-lipoproteins (Fig. 2E) but not different in HDL (Fig. 2F). Analyses of plasma apolipoproteins showed that apoB levels were higher at midnight than at midday (Fig. 2G); however, apoA1 levels were similar (Fig. 2H). These data show that plasma lipid levels change significantly within a day mainly due to changes in apoB-lipoproteins.

Diurnal Changes in Lipid Absorption—Changes in plasma apoB-lipoproteins could occur due to variations in their production or catabolism. To determine whether intestinal lipoprotein production contributes to variations in plasma lipid levels, mice were injected with Triton WR1339 to inhibit lipoprotein catabolism and gavaged with [3H]triolein at 2400 and 1200 h, and the appearance of radioactive lipids in the plasma was studied over the next 4 h (Fig. 3). Triolein-derived counts increased with time in the plasma at both times (Fig. 3A). However, mice gavaged at 2400 h had higher triglyceride in their plasma, indicating greater absorption. After 4 h, we also studied the distribution of radiolabeled lipids in the intestinal segments. The [3H]triolein-derived counts were higher in the proximal intestinal segments (Fig. 3B) at 2400 h. Furthermore, absorption of radiolabeled cholesterol (Fig. 3, C and D) was very similar to that observed for triolein. Again, cholesterol absorption was higher at 2400 than at 1200 h (Fig. 3C) and the amounts of cholesterol in the proximal intestinal segments were higher at 2400 h (Fig. 3D). These data indicate that intestinal lipid absorption varies significantly within a day and is high at midnight.

Lipid Absorption from in Situ Rat Small Intestinal Loops—It is known that gastric emptying and pH changes show rhythmic behavior (48). To determine whether diurnal variations in lipid absorption were due to changes in the intestinal function, we studied the absorption of lipids from rat jejunal loops severed from gastric apposition. [3H]Triolein or [3H]cholesterol was injected into the in situ jejunal loops at different times, and the appearances of radioactive lipids in the plasma and lipoproteins were measured after 2 h. The [3H]triolein-derived counts in the plasma as well as non-HDL apoB-lipoproteins were higher at 2400 compared with 1200 h (Fig. 4A), and a similar trend was observed for [3H]cholesterol (Fig. 4B). We also measured the amounts of lipids that remained in the loop and those transported to downstream intestinal regions. Radioactivity in intestinal loops was low at 1200 h but was high in the subsequent segments (Fig. 4, C and D), indicating less absorption. This was confirmed by measuring radiolabeled lipids transported to the liver. [3H]Triolein-derived (Fig. 4E) and [3H]cholesterol-derived (Fig. 4F) counts were higher at midnight in the liver. These studies indicate that intestinal lipoprotein production exhibits diurnal variation independent of the stomach function.

Diurnal Changes in Intestinal Microsomal Triglyceride Transfer Protein—ApoB-lipoprotein production is critically dependent on MTP. We hypothesize that the rhythmic changes

Diurnal Regulation of MTP and Plasma Lipid Levels

FIGURE 8. Diurnal variations in genes involved in intestinal absorption. Intestines were harvested from mice at the indicated times (n = 6 for each time point) and used to isolate total mRNA. Quantitative reverse transcription-PCR was used to measure mRNA levels of apoB (A), apoA1 (B), DGAT1 (C), MGAT2 (D), and SR-BI (E) as described under “Experimental Procedures.” Values obtained at 400 h were normalized to 100%. Line graphs and error bars represent average ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with the 1200 h time point.
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in plasma lipids and lipid absorption are linked to changes in MTP (Fig. 5). MTP activity was high in the dark phase (20–24 h) and low in the light phase (8–12 h) in rats (Fig. 5A) as well as in mice (Fig. 5C). MTP protein also changed during the day; higher amounts were observed at 2400 than at 1200 h (Fig. 5, B and D). In contrast, GAPDH, a cytosolic protein, did not change throughout the day in these animals (Fig. 5, B and D), consistent with earlier studies (38). These studies indicate that MTP activity and protein levels show diurnal variations similar to plasma apoB-lipoproteins.

The parallel diurnal variations in MTP activity and plasma apoB-lipoproteins indicated that these might be correlated. To test this hypothesis, we evaluated correlation between MTP specific activity and apoB-lipoprotein triglyceride using a twotailed test (data not shown). The data were evenly scattered, indicating correlation over wide ranges of apoB-triglyceride and MTP specific activities. The Pearson r value was 0.58, and the p value was 0.0002. Similar analysis with apoB-lipoprotein cholesterol revealed segregation of data points in two clusters (data not shown). The Pearson r value was 0.39, and the p value was 0.0192. These correlation studies indicate that MTP activity is better correlated with triglyceride than cholesterol in apoB-lipoproteins. No attempts were made to measure cholesterol esters and to correlate them with MTP activity. We suggest that MTP might play an important role in determining daily variations in plasma triglyceride.

To further understand the mechanisms responsible for changes in MTP protein levels, we studied the synthesis of MTP in enterocytes isolated at 1200 and 2400 h (Fig. 6). The amounts of MTP synthesized at 2400 h after various times of pulse labeling were 2–4-fold higher than those synthesized at 1200 h (Fig. 6, A and B). In contrast, the synthesis of GAPDH remained unchanged at different times of the day (Fig. 6, A and C). These data indicate that MTP synthesis shows diurnal variation.

To assess if diurnal variations in MTP synthesis were related to its mRNA levels, we measured steady state mRNA levels at different times (Fig. 7). Rat (Fig. 7A) and mouse (Fig. 7C) intestinal MTP mRNA levels showed diurnal variations. The lowest amounts of MTP mRNA were at 1200 h, and higher amounts were at 2000–2400 h. We also measured changes in GAPDH mRNA levels during the day and found no significant changes in rats (Fig. 7B) and mice (Fig. 7D). These studies show that MTP mRNA levels change significantly within a day.

To understand mechanisms underlying the changes in MTP mRNA levels, we measured gene transcription rates in the nuclei isolated from intestinal mucosa at 1200 and 2400 h (Fig. 7, E and F). GAPDH transcription rates were also measured and used as controls. To determine if MTP transcription rates were different at 1200 and 2400 h, MTP mRNA counts were normalized to GAPDH levels (Fig. 7F). The MTP gene transcription was ~2-fold higher at midnight. In summary, MTP activity, protein, mRNA, and gene transcription show diurnal variations. We suggest that changes at the level of gene transcription contribute to variations in MTP levels.

Daily Changes in Other Genes Involved in Lipid Absorption—To determine if MTP was the only gene regulated within a day, we performed quantitative reverse transcription–PCR for a few candidate genes involved in lipid absorption (Fig. 8). ApoB mRNA levels (Fig. 8A) showed variations, and its changes roughly paralleled those of MTP mRNA levels as well as plasma lipid levels. DGAT1 mRNA levels (Fig. 8C) differed at different time points, but they were not similar to the changes observed in plasma lipids. ApoAI (Fig. 8B), MGAT2 (Fig. 8D), and SR-B1 (Fig. 8E) mRNA levels did not change significantly. These studies identify MTP and apoB as two candidate genes that show significant diurnal variations, and their changes were similar to those observed for plasma lipids.

Variations in Hepatic Microsomal Triglyceride Transfer Protein—To examine the tissue specificity of circadian rhythm, the diurnal variation in MTP activity, protein, and mRNA levels was also examined in the liver (Fig. 9). Rat hepatic MTP activity (Fig. 9A), protein (Fig. 9B), and mRNA levels (Fig. 9C) were significantly higher at 2400 h. Similar changes in the MTP activity (Fig. 9E), protein (Fig. 9F), and mRNA levels (Fig. 9G) were also observed in mice. Although the MTP protein and mRNA...
levels were low in the day compared with the night, the levels of GAPDH protein (Fig. 9, B and F) and mRNA levels (Fig. 9, D and H) were similar. These data indicate that hepatic MTP activity, protein, and mRNA levels change within a day.

**Variations in Other Hepatic Genes Involved in Lipid Transport**—To determine if other proteins involved in lipid transport were also regulated during the day, we measured mRNA levels for several candidate proteins at two different times (data not shown). Among the genes that are involved in triglyceride secretion, apoB, DGAT1, and ACAT2 showed significant variations, whereas DGAT2 did not. Studies related to genes involved in HDL metabolism revealed that SR-B1 levels altered significantly. In addition, small variations were observed for ABCA1. In contrast, apoAI did not differ at two time points. These data show that not only MTP but other genes involved in lipoprotein assembly and secretion also show diurnal variations.

**Changes in MTP upon Food Availability**—To study the effect of food on MTP expression, mice were fasted starting at 1200 or 2400 h. After 24 h, they were provided food ad libitum for different indicated times (Fig. 10). Within 2 h, refeeding enhanced plasma triglyceride (Fig. 10A) and cholesterol levels (Fig. 10B) mainly due to increases in non-HDL apoB-lipoproteins (Fig. 10, C and D), since changes in HDL lipids were not significantly altered (Fig. 10, E and F). Furthermore, refeeding induced MTP activity (Fig. 10, G and K), protein (Fig. 10, H and L), and mRNA (Fig. 10, I and M) levels within 2 h in the intestine and liver. No changes in GAPDH protein (Fig. 10, H and L) and mRNA (Fig. 10, J and N) levels were observed. These data indicate that MTP is induced in fasting animals after refeeding.

To study further the effect of food availability, animals were subjected to a restricted feeding regimen. Mice had daily access to food from 9 to 15 h for 10 days, because MTP expression is low at these hours in mice fed ad libitum (Figs. 5, 7, and 9). As expected, the availability of food enhanced plasma triglyceride and cholesterol levels (Fig. 11, A and B), mainly due to changes in non-HDL apoB-lipoproteins around 1200 h. MTP expression increased in the daytime in mice subjected to restricted food availability. MTP activity (Fig. 11, C and F), protein (Fig. 11, D and G), and mRNA (Fig. 11, E and H) levels were high between 800 and 1200 h. In addition, the tendency to increase MTP expression in the dark was still present. ApoB mRNA levels also increased at the time of food availability; however, the tendency to increase apoB expression in the night was lost (compare with Fig. 8A). In contrast to MTP and apoB, GAPDH protein (Fig.
and then studied daily variations in MTP (Fig. 12). These treatments completely abolished circadian variations observed in plasma lipid levels (Fig. 12, A–D), intestinal and hepatic MTP activity (Fig. 12, E and J), protein (Fig. 12, F and K), and mRNA (Fig. 12, G and L) levels. No changes in GAPDH protein (Fig. 12, F and K) and mRNA (Fig. 12, H and M) were evident. ApoB mRNAs appear to increase in the night hours when kept in constant light (Fig. 12, I and N, Light). However, when mice were kept in constant dark, no variations were observed in apoB mRNA levels (Fig. 12, I and N, Dark). These studies indicate that visual cues play an important role in the circadian variations in MTP expression.

DISCUSSION

It is known that plasma lipid concentrations are maintained within a narrow physiological range and exhibit circadian rhythmicity in humans and rodents (1, 2). However, there is no consensus regarding the mechanisms controlling diurnal variations in plasma lipid levels. Several factors, such as plasma clearance, cholesterol biosynthesis, hormonal changes, etc., have been implicated as possible reasons for plasma lipid changes. The data presented here in ad libitum fed rodents show that triglyceride in total plasma and apoB-lipoproteins exhibit diurnal variations. Exploration of physiologic mechanisms revealed that intestinal lipoprotein production contributes to diurnal variations in plasma lipid levels. On the molecular level, we identified MTP as a key molecule exhibiting diurnal variation. MTP activity, protein, mRNA, and mttp gene transcription exhibit diurnal variations. Moreover, there is a significant positive correlation between MTP activity, mRNA levels, and plasma apoB-lipoprotein triglyceride. Thus, we propose that diurnal transcriptional regulation of the mttp gene contributes to diurnal variations in plasma apoB-lipoprotein triglyceride.

In contrast to the positive correlation observed between plasma triglyceride levels and diurnal regulation of MTP activities in this study, the diurnal variations in the hepatic LDL receptor and hydroxymethylglutaryl-CoA reductase show an

11, D and G) and mRNA levels (Fig. 11, E and H) did not change by food restriction. Thus, restricted feeding induces MTP expression at the anticipated time of food availability in addition to the regular rhythmic increase in the dark.

Extended Exposure to Light and Dark Abolishes Diurnal Variations in MTP—To explore other environmental factors contributing to diurnal changes in MTP, we considered the possibility that visual cues may play an important role. To test this hypothesis, we kept mice in constant light or dark for 5 days variations. Moreover, there is a significant positive correlation between MTP activity, mRNA levels, and plasma apoB-lipoprotein triglyceride. Thus, we propose that diurnal transcriptional regulation of the mttp gene contributes to diurnal variations in plasma apoB-lipoprotein triglyceride.
The LDL receptor levels peak at the onset of darkness, when the plasma LDL levels are the lowest. The maximum expression of the hepatic LDL receptors away from high plasma lipoprotein levels could maximize delivery of dietary fat to peripheral tissues and avoid accumulation of remnants in the plasma for longer periods of time.

It has been shown that the half-life of MTP protein is very long (~4 days) in HepG2 cells (49). Therefore, it was surprising to find that MTP activity and protein levels changed significantly within a day in both the liver and intestine. Changes observed during the day may or may not represent protein half-lives. The variations observed within 24 h can be achieved with a half-life of 4 days. More involved experiments are required to determine protein half-lives in vivo. Similar discordances between the half-lives of proteins measured in cultured cells and changes in tissues levels have also been reported for the LDL receptor (3). Thus, half-lives observed in cultured cells and diurnal changes observed in tissues cannot be correlated and should be interpreted cautiously.

The plasma lipid levels in rats and mice were high in the dark phase and low in the light phase. Rodents show a nocturnal feeding behavior. Thus, all of the intestinal diurnal rhythms, including the lipid transport rhythmicity, appear reasonable for the preparation of nocturnal dietary load (2, 50–52). Recent work has characterized food as zeitgeber (synchronizer) (53). Food can entrain food-anticipatory activity to impose a new feeding schedule in rodents (54–56). In fact, fasting rats for 12 h has been shown to result in plasma lipid peaks at different times (2). Our data show that restricted feeding affects intestinal and hepatic MTP expression. Moreover, there was an acute change in MTP expres-
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sion when food was provided to fasting animals. Thus, food-anticipatory activity that arises as an output of rhythms in the gastrointestinal tract and food availability play a major role in controlling MTP.

In addition to food, we observed that long term exposure to constant dark and light abolished circadian variations in plasma lipid and MTP levels. Thus, it is likely that a light-sensitive oscillator of the suprachiasmatic nucleus of the hypothalamus in the brain (54, 55, 57, 58) is a significant factor controlling MTP gene expression. More experiments are needed to understand the regulation of MTP by light.

Analyses of circadian patterns of transcription revealed tissue-specific circadian regulation in different pathways specific to individual tissues. In the suprachiasmatic nucleus, neuropeptide signaling pathways show circadian rhythms, whereas in the liver, cholesterol and xenobiotic metabolism display circadian variations (59). Here we show that MTP and apoB show diurnal variations. Shen et al. (60) have reported that apoAIV protein and mRNA levels were high in the dark compared with those present in the day. Thus, it appears that at least three genes involved in lipoprotein assembly and secretion are coordinately regulated, and we propose that lipoprotein assembly and secretion exhibit circadian variations in the liver and intestine.

Changes in MTP were due to variations in transcription rates. Assuming that other genes involved in lipoprotein production are also regulated at the transcription level, it is possible that all of these genes may share a common regulatory element. Further understanding about the signals that coordinate these genes may unravel new mechanisms controlling lipid transport pathways.

In short, MTP levels show diurnal variations due to changes in gene transcription as well as in protein and mRNA syntheses. These variations are associated with parallel changes in total lipids and apoB-lipoproteins. To our knowledge, this is the first documentation that MTP levels show diurnal variations and that they are positively correlated with plasma lipids. We propose that diurnal changes in MTP play a significant role in daily variations in plasma lipid levels.

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