

## Abundance of mRNA of Apolipoprotein B<sub>100</sub>, Apolipoprotein E, and Microsomal Triglyceride Transfer Protein in Liver from Periparturient Dairy Cows\*

U. Bernabucci,<sup>1</sup> B. Ronchi,<sup>1</sup> L. Basiricò,<sup>1</sup> D. Pirazzi,<sup>1</sup> F. Rueca,<sup>2</sup>  
N. Lacetera,<sup>1</sup> and A. Nardone<sup>1</sup>

<sup>1</sup>Dipartimento di Produzioni Animali,  
Università della Tuscia-Viterbo, Italy

<sup>2</sup>Sezione di Medicina Veterinaria Interna,  
Università di Perugia, Italy

### ABSTRACT

Limited secretion of very low density lipoproteins (VLDL) in dairy cows is strongly related to fatty liver and other metabolic disorders in the early postpartum. Currently, there is limited information on which roles apolipoprotein B<sub>100</sub> (ApoB<sub>100</sub>), apolipoprotein E (ApoE), and microsomal triglyceride transfer protein (MTP) play in that VLDL limitation. To our knowledge, no studies have simultaneously measured ApoB<sub>100</sub>, ApoE, and MTP mRNA in periparturient dairy cows. Therefore, a trial was conducted to assess liver gene expression of these proteins in transition dairy cows and to evaluate the relationships between their expression and metabolic status. Eight multiparous Holstein cows were monitored during the transition period. To evaluate metabolic and nutritional status, body condition score was registered, and plasma indexes of energy metabolism and VLDL were determined from 35 d before to 35 d after calving. Liver biopsies were performed on d -35, 3, and 35 relative to day of calving, and gene expression of ApoB<sub>100</sub>, ApoE, and MTP were determined on liver tissue. Body condition, plasma glucose and VLDL decreased, and plasma NEFA and BHBA increased after calving. Compared with values of d -35, on d 3 after calving the ApoB<sub>100</sub> mRNA synthesis was lower, whereas MTP and ApoE mRNA abundance were higher. Negative correlation ( $r = -0.57$ ) between plasma NEFA concentration and ApoB<sub>100</sub> mRNA abundance, and positive correlation between ApoB<sub>100</sub> mRNA abundance and plasma cholesterol ( $r = 0.65$ ) and plasma albumins ( $r = 0.52$ ) were detected at 3 d postpartum. Data on changes of gene expression of the 3 main proteins involved in the regulation of synthesis and secre-

tion of VLDL in the liver suggest that decreased mRNA for ApoB<sub>100</sub> may be consistent with decreased synthesis and/or secretion of VLDL from liver during the periparturient period.

**(Key words:** apolipoprotein B, apolipoprotein E, mRNA, liver)

**Abbreviation key:** ApoB<sub>100</sub> = apolipoprotein B<sub>100</sub>, ApoE = apolipoprotein E, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, HDL = high density lipoprotein, LDL = low density lipoprotein, MTP = microsomal triglyceride transfer protein, RT-PCR = reverse transcription-polymerase chain reaction, TG = triglyceride, VLDL = very low-density lipoprotein.

### INTRODUCTION

The peripartum period is the most important phase of the production cycle of dairy cows. Most health problems occur during this phase. During the last phase of pregnancy, energy intake is reduced and nutrient demands by the fetus and mammary gland growth are increased (Bell, 1995). These conditions, together with changes in hormonal equilibrium, lead to an increase in fat mobilization with consequent increased plasma NEFA and rate of fatty acids uptake from the liver (Grummer, 1995). When the rate of hepatic triglycerides (TG) synthesis exceeds the rate of TG disappearance, accumulation of TG and cholesterol esters in hepatocytes takes place with high risk for fatty liver. Triglycerides may disappear through hydrolysis or secretion via very low density lipoproteins (VLDL) synthesis. Ruminants have the same rate of TG synthesis in the liver compared with nonruminants but have a very slow rate of hepatic VLDL secretion relative to nonruminants (Pullen et al., 1990). The regulation and coordination of lipid metabolism among several tissues (liver, adipose, gut, mammary gland) are key components of the adaptation to lactation (Drackley, 1999). The synthesis of VLDL requires TG, phospholipids, cholesterol, cholesterol esters, apolipoproteins (B<sub>100</sub>, C, and

Received January 12, 2004.

Accepted March 3, 2004.

Corresponding author: U. Bernabucci; e-mail: bernad@unitus.it.

\*Research was financially supported by University of Tuscia and PRIN-MIUR 2003.

**Table 1.** Ingredients and nutrients composition of diets fed during the experimental period (DM basis).

	Far-off dry cows	Close-up dry cows	Lactation cows
Ingredients:			
Corn silage, %	14.4	20.0	28.0
Triticale-grass silage, %	2.9	4.0	5.6
Alfalfa hay, %	5.2	7.3	10.2
Rye-grass hay, %	51.8	33.0	6.1
Corn ground, %	6.2	8.6	12.2
Oat ground, %	7.2	10.0	14.1
Soybean meal, %	5.5	7.5	10.7
Cotton seed, %	3.4	4.8	6.5
Dry beet pulp, %	2.6	3.6	5.1
Buffer, <sup>1</sup> %	0.5	0.7	0.9
Vitamin-mineral premix, <sup>2</sup> %	0.3	0.5	0.6
Nutrient composition			
NE <sub>L</sub> , Mcal/kg	1.29	1.41	1.62
CP, %	12.70	13.70	15.60
NDF, %	43.10	40.00	36.40

<sup>1</sup>A mixture of 33.3% CaCO<sub>3</sub>, 31.7% Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, 16.7% MgO, 16.6% NaHCO<sub>3</sub>, and 1.7% ZnSO<sub>4</sub>.

<sup>2</sup>Contained: 6,000,000 IU of vitamin A, 600,000 IU of vitamin D<sub>3</sub>, 7,000 mg of vitamin E, 5,000 mg of vitamin PP, 300 mg of vitamin B<sub>1</sub>, 100 mg of vitamin B<sub>2</sub>, 10,000 mg of choline chloride, 2 mg of vitamin B<sub>12</sub>, 10,000 mg of Fe, 2,500 mg of Cu, 20,000 mg of Mn, 100 mg of Mo, 100 mg of Co, 800 mg of I, 50,000 mg of Zn, and 100 mg of Se per kilogram.

E) and microsomal triglyceride transfer protein (**MTP**) (Mensenkamp et al., 2001; Shelness and Sellers, 2001; Fisher and Ginsberg, 2002). The limiting step of VLDL synthesis in ruminants is unknown; Bauchart (1993) hypothesized that it is probably represented by the availability of VLDL constituents. Microsomal triglyceride transfer protein transfers neutral lipids from outside the endoplasmic reticulum to the lumen and plays a fundamental role in coordinating the assembly of VLDL (Hussain et al., 2003). The MTP appears to be involved in the first step of lipidation of the newly synthesized apolipoprotein B<sub>100</sub> (**ApoB<sub>100</sub>**) molecule (MTP facilitates the association of cholesteryl ester, triglyceride, and phospholipid with ApoB<sub>100</sub>) resulting in the formation of nascent lipoprotein particles (Watterau et al., 1997). Apolipoprotein B<sub>100</sub> is the TG-binding protein in VLDL, it is the most important structural component of VLDL and it is necessary for stabilizing the VLDL particles (Gibbons, 1990). Recent studies revealed the role of apolipoprotein E (**ApoE**) in the control of intracellular lipid metabolism and VLDL production by the hepatocytes (Mensenkamp et al., 2001). Nascent VLDL particles acquire the ApoE during the assembly/secretion cascade (Fazio et al., 1992; Fazio and Yao, 1995), and animal studies and in vitro experiments demonstrated the role of ApoE in the regulation of VLDL secretion by the liver (Mensenkamp et al., 2001). To our knowledge, no studies investigated ApoE gene expression in periparturient dairy cows.

Regulation of tissue-specific gene expression is of great interest. Its study is possible using RNA as a parameter of gene expression (Farrell, 1998). Messenger-RNA biogenesis, maturity, and function are influenced transcriptionally and posttranscriptionally and together constitute myriad regulation control points within the cell.

Therefore, a trial was conducted to assess liver gene expression of ApoB<sub>100</sub>, ApoE, and MTP, the 3 main proteins involved in the VLDL assembly and secretion in liver, and to evaluate the relationship between their expression and metabolic status in periparturient dairy cows to get a better understanding of what may be limiting VLDL secretion.

## MATERIALS AND METHODS

### Management of Cows

The trial was carried out in a commercial dairy herd. The average milk yield per lactation (305 DIM) of the herd was about 9000 kg per cow, and fat and protein contents of the milk were 3.7 and 3.3%, respectively. Eight multiparous Holstein cows due to calve in June were monitored from 35 d prepartum to 35 d postpartum. Cows were fed diets for dry cows and lactating cows: Table 1, consisting of a base-ration fed as a TMR given daily at 0930 h and offered ad libitum to achieve 5 to 10% refusals. The close-up diet was offered starting 10 d before the expected calving.

### Measurements and Samplings

Feeds were sampled and analyzed. Dry matter was determined by forced air oven drying at 65°C to constant weight. Crude protein was determined by macro-Kjeldhal method (AOAC, 1984). Ether extract and ash were determined according to AOAC methods (AOAC, 1984). The NDF was analyzed according to the method described by Goering and Van Soest (1970).

During the experimental period body condition of cows was scored by one person weekly following the method of ADAS (1986). Blood samples were taken at 0800 h from the jugular vein at -35, -27, -20, -12, and -4 prepartum (before expected calving) and at 3, 10, 17, 24, 30, and 35 postpartum. Blood samples were collected in Vacutainer tubes containing Li-heparin as anticoagulant agent and put in ice. Blood was centrifuged at 2700 × g for 10 min at 4°C. Aliquots of plasma were stored at -20°C until analyzed for glucose, cholesterol, and albumins (kits from Instrumentation Laboratory, Lexington, MA), BHBA (Barnouin et al., 1986) and NEFA (NEFA-C kit; Wako Fine Chemical Industries USA, Inc., Dallas TX). On d -35, 3, and 35 relative to day of calving blood samples were collected in Vacu-

tainer tubes without anticoagulants. Serum was obtained by centrifuging blood at  $2700 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Very low density, low density (LDL), and high density (HDL) lipoproteins were separated on agarose gel (Hydragel Lipo+Lp(a), Sebia Electrophoresis, Norcross, GA) and the relative quantification was done by densitometric scanning (Contois et al., 1999). The assay is based on the separation of HDL, LDL, and VLDL on agarose gels, followed by enzymatic cholesterol staining (Contois et al., 1999).

Liver biopsies (Vazquez-Anon et al., 1994) were performed on d -35, 3, and 35 relative to day of calving after first locating the liver by ultrasound. The biopsy samples were obtained from the right lobe of the liver inserting the needle through the 10th or 11th intercostal space.

Immediately after collection, liver samples were rinsed in RNase-free water (diethyl-pyrocyanate-treated water), frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until the ribonuclease protection assay of ApoB<sub>100</sub>, ApoE, and MTP mRNA (Ilian et al., 1999).

#### Quantification of ApoB<sub>100</sub>, ApoE, and MTP mRNA by Ribonuclease Protection Assay

**Isolation of total RNA from liver tissue.** Total RNA was isolated by homogenizing biopsies (50 to 100 mg of liver tissue) in 1 mL of TRI reagent solution containing phenol and guanidinium thiocyanate (Sigma-Aldrich, Milan, Italy) following the procedure described by the manufacturer. The homogenate was incubated in 100  $\mu\text{L}$  of 2 M Na-acetate (pH 4) and 200  $\mu\text{L}$  of chloroform:isoamyl-alcohol, incubated in ice for 15 min. After incubation, the mixture was centrifuged at  $14,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . Following centrifugation, to precipitate RNA, the colorless aqueous phase was transferred to a fresh tube and an equal volume of ice cold isopropanol was added. The mixture was incubated at  $-20^{\circ}\text{C}$  for 1 h. After incubation, the mixture was centrifuged at  $14,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ , the pellet was washed twice with 75% ethanol by centrifugation at  $14,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The pellet was redissolved in RNase-free water and incubated for 10 min at  $55^{\circ}\text{C}$ . Total RNA was quantified by measuring its absorbance at 260 nm. To verify integrity, cellular RNA (10  $\mu\text{g}$ ) was electrophoresed on 1.2% agarose—2.2% formaldehyde denaturing gel in  $1\times$  MOPS [3-(*n*-morpholino)propanesulfonic acid] and stained with ethidium bromide. Cellular RNA that had intact 28S and 18S ribosomal bands were used in subsequent analyses. Isolated RNA was stored at  $-80^{\circ}\text{C}$  until the ribonuclease protection assay.

**Synthesis of RNA probes of human ApoB<sub>100</sub>, bovine ApoE, MTP, and glyceraldehyde-3-phosphate**

**dehydrogenase (GAPDH).** Specific antisense ribonucleotide probes were generated using cDNA of ApoB<sub>100</sub>, ApoE, MTP, and GAPDH, which was used as internal control, that were produced from bovine liver RNA by reverse transcription-polymerase chain reaction (RT-PCR).

The MTP RNA probe-oligonucleotide primers of MTP used for RT-PCR (forward: 5'-AGACTGAAGCCAGGA AAGCA-3'; reverse: 5'-TAATACGACTCACTATAGGG AGTGA GCAGAGGTGACAGCATC-3') were designed from the bovine MTP cDNA. The reverse transcription reaction mixture (Superscript, II, GIBCO-BRL, Life Technologies, Gaithersburg, MD), and 200 U of Superscript II RNase H- reverse transcriptase (GIBCO-BRL, Life Technologies), were incubated at  $70^{\circ}\text{C}$  for 10 min. The subsequent reverse transcription reaction was carried out at  $42^{\circ}\text{C}$  for 50 min. Polymerase chain reaction was used to amplify a 305-bp MTP cDNA fragment. The total PCR reaction mixture of 100  $\mu\text{L}$  contained 5 U of Taq DNA polymerase (GIBCO-BRL, Life Technologies). To check for specificity, PCR products were analyzed by 1% agarose gel electrophoresis, TBE buffer (0.89 M Tris, 0.89 M boric acid, 20 mM EDTA, pH 8.2) and 0.5  $\mu\text{g}/\text{mL}$  of ethidium bromide.

The ApoB<sub>100</sub> RNA probe, a 378-bp ApoB<sub>100</sub> cDNA, was produced by using procedures similar to those described for MTP. Oligonucleotide primers of ApoB<sub>100</sub> used for RT-PCR (forward: 5'-GCAAGGAGCAACACCTCTTC-3'; reverse: 5'-TAATACGACTCACTATAGGGAGGTC C AACTGAACCAAGGTC-3') were designed from the human ApoB<sub>100</sub> cDNA.

The ApoE RNA probe, a 265-bp ApoE cDNA, was synthesized as described above for MTP. Oligonucleotide primers of ApoE used for RT PCR (forward: 5'-AT GAAGGTTCTGTGGGTTGC-3'; reverse: 5'-TAATACG ACTCACTATAGG GAGATGAAGGTTCTGTGCGGTTG C-3') were designed from the bovine ApoE cDNA.

The GAPDH RNA probe, a 177-bp GAPDH cDNA, was amplified by using 2 synthetic oligonucleotide primers (forward: 5'-TCATCCCTGCTTCTACTGGC-3'; reverse: 5'-TAATACGACTCACTATAGGGAGC CT GCTTACCACCT TCTTG-3') from the bovine GAPDH cDNA.

The PCR products, containing sequence of the T7 promoter at the 5' end, were transcribed in vitro directly using a Maxiscript transcription Kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The reaction mixture contained: 1  $\mu\text{g}$  of template DNA, 2  $\mu\text{L}$  of  $10\times$  transcription buffer, 1  $\mu\text{L}$  of 10 mM ATP, CTP, GTP, UTP, and finally 2  $\mu\text{L}$  of T7 RNA polymerase. Transcription was carried out at  $37^{\circ}\text{C}$  for 60 min. The DNA template was digested with DNase I for 15 min at  $37^{\circ}\text{C}$ . After transcription, all riboprobes were purified by electrophoresis on a 5% TBE-8 M urea acryl-

amide gel. The product bands were visualized by staining in 2  $\mu\text{g/mL}$  acridine orange in nuclease-free water for 15 min, excised from the gel and eluted passively from gel slices in 0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS overnight. Following EtOH precipitation, the RNA probes were resuspended in H<sub>2</sub>O and quantitated by checking the absorbance reading at 260 nm.

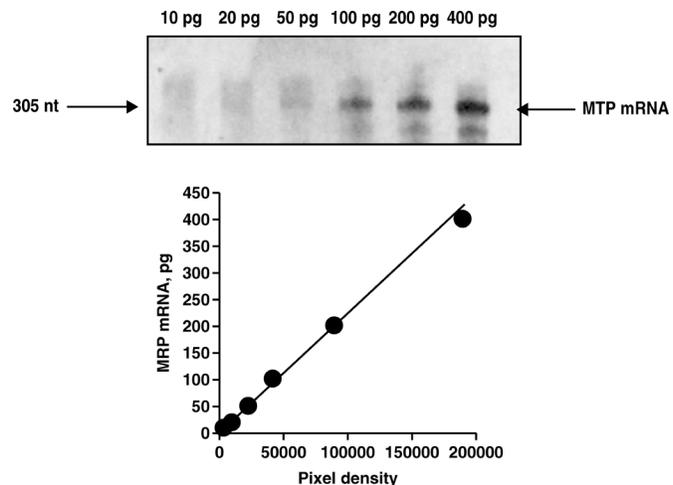
The purified riboprobes were labeled with biotin using Brightstar Psoralen-Biotin Kit (Ambion, Inc.) according to the manufacturer's instructions. The labeling was carried out by mixing the riboprobes with the Psoralen-Biotin reagent in a microtiter plate and exposing to long wavelength (365 nm) UV light. The Psoralen-Biotin reagent became covalently linked to the riboprobes in 45 min. Any excess Psoralen-Biotin reagent was removed by butanol extraction, and the probe was stored at  $-80^{\circ}\text{C}$  until use.

**Ribonuclease protection assay.** Hybridization of total RNA with riboprobes was performed using the protocol and reagents supplied in the RPA III kit (Ambion, Inc.) as described for the standard procedure. Target RNA samples (10  $\mu\text{g}$ ) and riboprobes (approximately 1 ng per riboprobe) were coprecipitated with ammonium acetate (0.5 M) and ethanol. Yeast RNA from the RPA III kit (10  $\mu\text{g}/2 \mu\text{L}$ ) was used as negative control. The RNA samples and riboprobes were subsequently processed following the procedure described by the manufacturer (Ambion, Inc.). The samples were dissolved in hybridization buffer and incubated overnight at  $56^{\circ}\text{C}$ . Unhybridized probes and RNA were digested by RNase A/T1 mixture in digestion buffer for 30 min at  $37^{\circ}\text{C}$ . The digestion was stopped by adding cold inactivation/precipitation solution. The mixture was then incubated at  $-20^{\circ}\text{C}$  for 1 h and centrifuged at  $14,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ .

The samples were dissolved in gel loading buffer, boiled for 3 min, placed in ice, and loaded on 5% acrylamide gel containing 8 M urea in  $1 \times$  TBE. Gel electrophoresis was performed at 250 V for 1 h in a Hoefer model SE 600 gel apparatus (Hoefer Amersham Pharmacia Biotech, UK). The mRNA was then electrophoretically transferred to a positively charged nylon membrane (BrightStar-Plus) in a TE42 Transphor transfer unit (Hoefer Amersham Pharmacia Biotech) with  $0.1 \times$  TBE for 45 min at 100 mA.

For the quantitative analysis of MTP, ApoB<sub>100</sub>, and ApoE mRNA known amounts of in vitro synthesized MTP, ApoB<sub>100</sub>, ApoE sense RNA were hybridized with an excess of labeled antisense probes to construct the standard curves. Figure 1 reports the standard curve constructed for MTP.

**Chemiluminescent detection.** The mRNA was cross-linked to the wet membrane after the transfer by heating in an oven at  $80^{\circ}\text{C}$  for 15 min. The nonisotopic



**Figure 1.** Example of a standard concentration curve constructed using known amounts of in vitro synthesized 'sense strand RNA' hybridized with an excess of labeled antisense probe. Ribonuclease protection assay were performed on 10, 20, 50, 100, 200 and 400 pg samples of MTP sense RNA ( $y = 0.0022x + 3.9037$ ,  $R^2 = 0.9988$ ). The reaction products were resolved on a denaturing 5% polyacrylamide gel and then quantified with the Kodak EDAS-290 densitometer and ID Image Analysis software (Eastman Kodak Company, Rochester, NY).

detection of the probe fragments protected was performed using BrightStar and BioDetect kits (Ambion, Inc.) following the procedure described by the manufacturer. The membrane was washed twice in wash buffer and incubated in a blocking solution for 30 min, then with streptavidin-alkaline phosphatase conjugate for 30 min. Then blot was rinsed in wash buffer, followed by incubation in CDP-Star at room temperature for 5 min, excess liquid was shaken off, and finally membrane was exposed to Kodak BioMax Light film (Eastman Kodak Co., Rochester, NY).

**Densitometry.** Chemiluminescent films were analyzed with the Kodak EDAS-290 densitometer and ID Image Analysis software (Eastman Kodak Co.). Samples were analyzed in conjunction with the standard curve and the intensity of the probe fragments protected by specific mRNA from the target samples was compared to the standard curve to determine the absolute amounts of ApoB<sub>100</sub>, MTP, and ApoE mRNA.

### Statistical Analysis

Data were analyzed as repeated measures using the MIXED procedure of SAS (SAS, 1999) according to the following model:

$$Y_{ij} = \mu + D_i + e_{ij}$$

where

**Table 2.** Least square means and standard error for BCS and plasma parameters during the transition period.

	Days from calving										
	-35	-27	-20	-12	-4	3	10	17	24	30	35
BCS	3.3 <sup>a</sup>	3.4 <sup>b</sup>	3.4 <sup>b</sup>	3.4 <sup>b</sup>	3.4 <sup>b</sup>	3.3 <sup>b</sup>	3.1 <sup>b</sup>	2.5 <sup>a</sup>	2.3 <sup>a</sup>	2.1 <sup>a</sup>	2.0 <sup>a</sup>
Glucose, mmol/L	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
NEFA, mmol/L	3.93 <sup>b</sup>	3.71 <sup>b</sup>	3.96 <sup>b</sup>	4.18 <sup>b</sup>	4.09 <sup>b</sup>	3.80 <sup>ab</sup>	2.99 <sup>a</sup>	2.99 <sup>a</sup>	3.02 <sup>a</sup>	3.00 <sup>a</sup>	3.51 <sup>ab</sup>
NEFA, mmol/L	0.16	0.16	0.14	0.15	0.16	0.21	0.16	0.15	0.16	0.19	0.19
BHBA, mmol/L	0.183 <sup>a</sup>	0.246 <sup>a</sup>	0.252 <sup>a</sup>	0.240 <sup>a</sup>	0.427 <sup>ab</sup>	0.864 <sup>c</sup>	0.651 <sup>cb</sup>	0.562 <sup>b</sup>	0.269 <sup>a</sup>	0.463 <sup>ab</sup>	0.377 <sup>ab</sup>
BHBA, mmol/L	0.081	0.086	0.078	0.081	0.086	0.081	0.081	0.081	0.086	0.092	0.092
BHBA, mmol/L	0.441 <sup>a</sup>	0.364 <sup>a</sup>	0.352 <sup>a</sup>	0.403 <sup>a</sup>	0.461 <sup>a</sup>	0.782 <sup>b</sup>	1.059 <sup>cb</sup>	1.433 <sup>c</sup>	1.266 <sup>c</sup>	1.017 <sup>cb</sup>	0.765 <sup>b</sup>
Albumin g/L	0.141	0.141	0.128	0.133	0.141	0.133	0.141	0.164	0.151	0.207	0.151
Albumin g/L	37.1 <sup>b</sup>	37.0 <sup>b</sup>	37.0 <sup>b</sup>	36.9 <sup>b</sup>	37.3 <sup>b</sup>	35.9 <sup>ab</sup>	35.0 <sup>a</sup>	34.2 <sup>a</sup>	34.7 <sup>a</sup>	35.8 <sup>ab</sup>	36.6 <sup>b</sup>
Cholesterol, mmol/L	0.59	0.59	0.53	0.56	0.59	0.56	0.56	0.56	0.59	0.69	0.69
Cholesterol, mmol/L	3.65 <sup>b</sup>	3.44 <sup>b</sup>	3.33 <sup>b</sup>	3.04 <sup>a</sup>	2.82 <sup>a</sup>	2.56 <sup>a</sup>	3.53 <sup>b</sup>	3.64 <sup>b</sup>	4.25 <sup>b</sup>	5.00 <sup>c</sup>	5.44 <sup>c</sup>
Cholesterol, mmol/L	0.20	0.20	0.18	0.19	0.20	0.19	0.19	0.19	0.20	0.21	0.21

<sup>a,b,c</sup>*P* < 0.05.

$Y_{ij}$  = dependent variable;  
 $\mu$  = overall mean of the population;  
 $D_i$  = mean effect of day of sampling ( $i = 1, \dots, 3$  for MTP, ApoB<sub>100</sub>, and ApoE;  $i = 1, \dots, 11$  for BCS, and metabolic parameters); and  
 $e_{ij}$  = unexplained residual element assumed to be independent and normally distributed.

Data were analyzed across sampling days relative to day of calving with d 0 representing the day of calving. For each analyzed variable, cow was subjected to 3 covariance structures: compound symmetric, autoregressive order one, and unstructured covariance. The covariance structure that had the largest Akaike's information criterion and Schwarz's Bayesian criterion was considered the most desirable analysis. For all parameters tested the best covariance structure was compound symmetric. Comparisons for the time factor was made only if the *F*-test was significant. Least square means were separated with the PDIFP procedure of SAS (1999). Correlation coefficients among different variables were determined by the CORR procedure of SAS (SAS, 1999). Significance was declared at *P* < 0.05 and trends at *P* < 0.10.

**RESULTS**

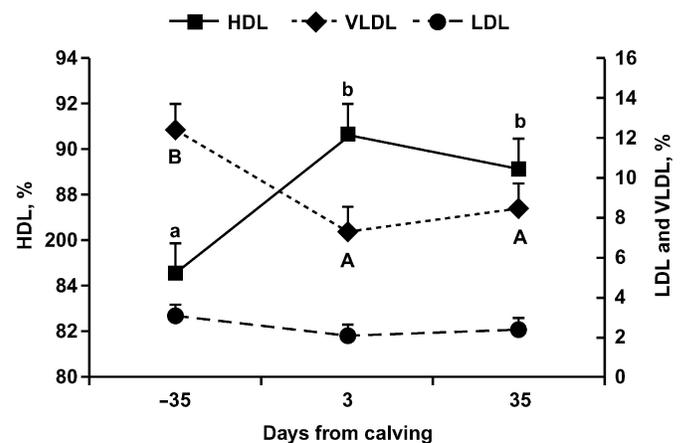
**Body Condition Score and Plasma Indexes**

Thirty-five days before calving the mean value of BCS of the 8 cows was 3.3 ± 0.2 and none of the 8 cows lost body condition approaching the calving event (Table 2). After calving BCS showed a significant (*P* < 0.05) decrease reaching the minimum values (2.0 ± 0.2) at 35 DIM. The decrease of BCS after calving was 0.9 ± 0.2 and 1.3 ± 0.2 U after 17 and 35 DIM, respectively.

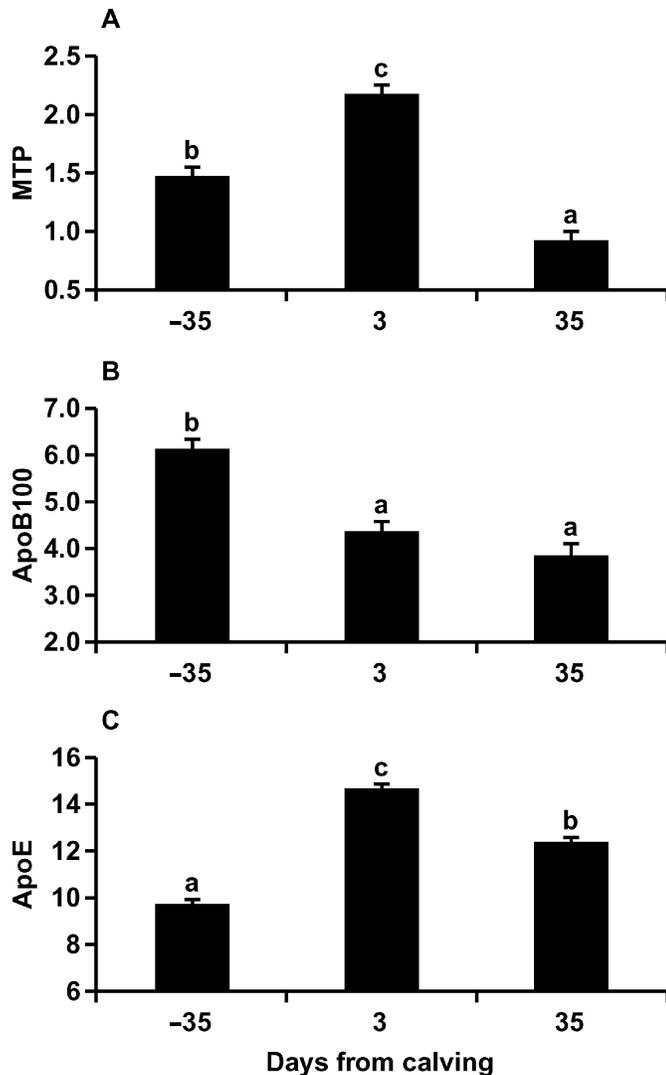
Plasma glucose concentrations were lower (*P* < 0.01) after calving and started to recover only at 35 DIM

(Table 2). The NEFA and BHBA plasma concentrations were higher (*P* < 0.01) after calving compared with levels registered before calving (Table 2). The NEFA reached the maximum level (0.864 ± 0.081 mmol/L) 3 d after calving, and BHBA reached the maximum (1.433 ± 0.164 mmol/L) at 17 DIM.

Plasma albumin showed a decrease (*P* < 0.05) subsequent to calving and recovered 3 to 4 wk after calving (Table 2). Cholesterol started to decrease (*P* < 0.05) about 2 wk before calving reaching the minimum value 3 d after calving, then increased until the end of the trial (Table 2). Relative proportion of serum VLDL was lower (*P* < 0.01) and HDL was higher (*P* < 0.05) after calving. The LDL did not significantly change during the transition period (Figure 2).



**Figure 2.** Least square means and standard error for serum high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) during the transition period. Data are reported as relative percentage between the three lipoproteins. a, b = *P* < 0.05 for HDL, A, B = *P* < 0.01 for VLDL.



**Figure 3.** Least square means and standard error for microsomal triglyceride transfer protein (MTP) (A), apolipoprotein B<sub>100</sub> (ApoB<sub>100</sub>) (B) and apolipoprotein E (ApoE) (C) mRNA abundance in liver of transition dairy cows. Values are reported as pg mRNA/ $\mu$ g total RNA. a, b, c =  $P < 0.05$ .

### MTP, ApoB<sub>100</sub>, and ApoE Gene Expression

We used GAPDH gene expression as internal control. The GAPDH mRNA relative to value measured at 35 d before calving were (means  $\pm$  SD)  $1.05 \pm 0.10$  and  $0.96 \pm 0.02$  at 3 and 35 d after calving, respectively.

During transition period gene expression of all proteins tested showed significant changes (Figure 3). The MTP mRNA abundance showed the highest values ( $P < 0.05$ ) 3 d after calving and the lowest values ( $P < 0.05$ ) at 35 DIM. ApoB<sub>100</sub> mRNA showed lower values ( $P < 0.05$ ) after calving, and no significant difference was found between values observed at 3 and 35 d postpar-

**Table 3.** Pearson correlation coefficients (r) between plasma metabolic parameters and apolipoprotein B<sub>100</sub> (ApoB<sub>100</sub>) mRNA abundance at 3 d postpartum.

	ApoB <sub>100</sub> mRNA
NEFA	-0.57*
Albumin	0.52*
Cholesterol	0.68*

\* $P < 0.05$ .

tum. The gene expression of ApoE was higher after calving and showed the highest values 3 d postpartum.

Significant correlations between NEFA, cholesterol, and ApoB<sub>100</sub> mRNA abundance at 3 d postpartum were detected (Table 3).

### DISCUSSION

Changes of BCS, plasma glucose, NEFA, BHBA, cholesterol, and albumin and serum lipoproteins around parturition observed in the present study can be considered physiological and due to the negative energy balance and changes in the activity of liver which are generally observed during the transition period in dairy cows (Reid et al., 1983; Gerloff, 1987; Bertics et al., 1992; Vazquez-Anon et al., 1994; Drackley et al., 2001; Bobe et al., 2003). Total cholesterol is a constituent of several lipoproteins and its reduction could reflect the possible alteration of liver synthesis also associated with fatty liver (Herdt et al., 1983; Reid et al., 1983; Artekar and Desai, 2003). Albumin is an indicator of liver function; it decreases in the peripartum period and its reduction could be associated with hepatocellular liver diseases (Tennant, 1997) and fatty liver (Reid et al., 1983; Nehra et al., 2001).

In contrast, with data reported by Rhoads et al. (2003) we did not find any effect of the physiological phase on GAPDH gene expression during the transition period.

In the present study we found an increase of MTP mRNA abundance after calving and no relationships between MTP mRNA and metabolic parameters were detected. Bremmer et al. (2000a) reported a significant increase of MTP mRNA 2 d after calving, in agreement with our results, and no relationships between MTP activity, mass or mRNA with plasma NEFA or liver TG concentration. In a second study, Bremmer et al. (2000b) evaluated the effects of nutritional and hormonal status on gene expression, protein mass, and activity of MTP in nonlactating Holstein cows and in bovine primary cultured hepatocytes. Results of that study suggested that hepatic MTP mRNA may be affected by TG accumulation, insulin, and glucagon in vitro, but the same treatments did not affect MTP mass or activity. Data of our study and data from Bremmer

et al. (2000a) clearly demonstrated the up-regulation of MTP gene expression in the early postpartum period. In agreement with conclusion of Bremmer et al. (2000a, 2000b), MTP probably does not play a critical role in the etiology of fatty liver that occurs in dairy cows at calving.

Studies support the concept that ApoB<sub>100</sub> secretion is metabolically regulated (Adeli et al., 1995). In studies carried out on laboratory animals and on cultured hepatocytes various metabolic states such as food deprivation and carbohydrate overload and FFA or insulin alter or modulate the level of ApoB<sub>100</sub> secretion without any modification of the ApoB<sub>100</sub> mRNA level (Adeli et al., 1995; Gruffat et al., 1996). Serum ApoB<sub>100</sub> concentration was decreased in cows with fatty liver induced by ethionine (Uchida et al., 1992) and those with natural fatty liver (Marcos et al., 1990b). Katoh (2002) reviewed a decrease of serum ApoB<sub>100</sub> concentration in cows with ketosis, left displacement of abomasums, retained placenta, milk fever, and downer syndrome. This author concluded that the decrease of serum ApoB<sub>100</sub> concentration in cows with health problems are primarily attributable to fatty liver. In contrast, Mazur et al. (1992) reported that an impaired synthesis of ApoB<sub>100</sub> may result in its decreased availability for lipoprotein formation and in turn results in enhanced triglyceride accumulation.

In the lactation cycle, serum ApoB<sub>100</sub> concentration is low during early lactation (Marcos et al. 1990a; Gruffat et al., 1997). Gruffat et al. (1997) found a reduction of hepatic ApoB<sub>100</sub> concentration in lactating cows at 1, 2, and 4 wk after calving compared with pregnant nonlactating cows and cows at 12 wk in milk. In contrast, the same authors reported that hepatic concentration of ApoB<sub>100</sub> mRNA did not vary significantly during early lactation and that it was not statistically different when compared with data of pregnant nonlactating cows. Those authors hypothesized that ApoB<sub>100</sub> synthesis during early lactation is regulated posttranscriptionally by either decreased translation or increased rate of intracellular proteolytic degradation. Results obtained from laboratory animals and humans indicated that among different possible mechanisms operating in mammals, intracellular translation of ApoB<sub>100</sub> mRNA and degradation of ApoB<sub>100</sub> are more likely to be the key regulatory mechanisms controlling the acute regulation of ApoB<sub>100</sub> production by the liver (Adeli et al., 1995). In contrast, the exact mechanism for the depression in both circulating and hepatic reduction of ApoB<sub>100</sub> concentration in the early postpartum period of dairy cows is not known yet.

Gruffat-Mouty et al. (1999) measured hepatic ApoB synthesis and VLDL secretion in ruminant and rodent liver cells and concluded that the slow rate of secretion

of VLDL in calf liver cells was not consecutive to a low rate of synthesis of ApoB but rather to a defect in VLDL assembly and/or secretion. Marcos et al. (1990b) reported that the level of liver ApoB mRNA in fatty liver cows was lower than in control cows and concluded that hepatic apolipoprotein synthesis is impeded in fatty liver cows.

Even though Gruffat et al. (1997) reported nonstatistical differences between early lactating and pregnant dry cows, ApoB<sub>100</sub> mRNA in lactating cows 1 wk after calving was 67% of that registered in pregnant dry cows. We found a significant decrease of ApoB<sub>100</sub> mRNA abundance after calving (−27%) compared with data before calving and no statistical difference was found during lactation (Figure 3). Changes of ApoB<sub>100</sub> mRNA abundance and relationships between ApoB<sub>100</sub> mRNA and plasma NEFA, cholesterol and albumins observed in the present study, would indicate a possible regulation at transcriptional level of ApoB<sub>100</sub> in the early postpartum period other than at posttranscriptional level as exclusively suggested by Gruffat et al. (1997).

To our knowledge, no data are available on changes of ApoE mRNA in periparturient dairy cows. Recent studies demonstrated that in ApoE-deficient mice VLDL-triglyceride secretion was severely impaired and mice developed fatty liver (Mensenkamp et al., 2001). Those authors have established the implication of ApoE in lipoprotein clearance and in the regulation of lipoprotein secretion by the liver, even though the exact mechanism is not clear yet. The upregulation of ApoE gene expression in the early postpartum, found in the present study, might be explained by the response of hepatocytes to the increased TG availability for VLDL synthesis. Considering our results and finding of Takahashi et al. (2003a,b), ApoE might be excluded as the possible factor implicated in the etiology of fatty liver at calving.

## CONCLUSIONS

Data on changes of gene expression of the 3 main proteins involved in the regulation of synthesis and secretion of VLDL in the liver suggest that decreased mRNA for ApoB<sub>100</sub> may be consistent with decreased synthesis and/or secretion of VLDL from liver during the periparturient period.

The mechanism by which ApoB<sub>100</sub> gene expression is downregulated and MTP and ApoE are upregulated in the early postpartum was not established.

## REFERENCES

- ADAS—Agricultural Development and Advisory Service. 1986. Condition scoring of dairy cows. Publ. 612, Agric. Dev. Advisory Serv., Min. Agric., Fisheries Food (Publ.), Lion House, Alnwick, Northumberland, UK.

- Adeli, K., A. Mohammadi, and J. Macri. 1995. Regulation of apolipoprotein B biogenesis in human hepatocytes: Posttranscriptional control mechanisms that determine the hepatic production of apolipoprotein B-containing lipoproteins. *Clin. Biochem.* 28:123–130.
- AOAC—Association of Official Analytical Chemists. 1984. *Official Methods of Analysis*. 14th ed. AOAC, Washington, DC.
- Artekar, T. M., and H. G. Desai. 2003. Fatty liver with elevated transaminase levels due to heterozygous apolipoprotein B deficiency. *Trop. Gastroenterol.* 24:35–36.
- Barnouin, J., N. El Idilbi, Y. Chilliard, J. P. Chacornac, and R. Lefai-vre. 1986. Micro-dosage automatisé sans déprotéinisation du 3-hydroxybutyrate plasmatique chez les bovins. *Ann. Rech. Vét.* 17:129–139.
- Bauchart, D. 1993. Lipid absorption and transport in ruminants. *J. Dairy Sci.* 76:3864–3881.
- Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. *J. Anim. Sci.* 73:2804–2819.
- Bertics, S. J., R. R. Grummer, C. Codorniga-Valino, and E. E. Stoddard. 1992. Effect of prepartum dry matter intake on liver triglyceride concentration in early lactation. *J. Dairy Sci.* 75:1914–1923.
- Bobe, G., B. N. Ametaj, J. W. Young, and D. C. Beitz. 2003. Effects of exogenous glucagon on lipids in lipoproteins and liver of lactating dairy cows. *J. Dairy Sci.* 86:2895–2903.
- Bremmer, D. R., S. J. Bertics, S. A. Besong, and R. R. Grummer. 2000a. Changes in hepatic microsomal triglyceride transfer protein and triglyceride in periparturient dairy cattle. *J. Dairy Sci.* 83:2252–2260.
- Bremmer, D. R., S. L. Trower, S. J. Bertics, S. A. Besong, U. Bernabucci, and R. R. Grummer. 2000b. Etiology of fatty liver in dairy cattle: Effects of nutritional and hormonal status on hepatic microsomal triglyceride transfer protein. *J. Dairy Sci.* 83:2239–2251.
- Contois, J. H., R. G. Gillmor, R. E. Moore, L. W. Contois, J. L. Macer, and A. H. B. Wu. 1999. Quantitative determination of cholesterol in lipoprotein fraction by electrophoresis. *Clin. Chim. Acta* 282:1–14.
- Drackley, J. K. 1999. Biology of dairy cows during the transition period: The final frontier? *J. Dairy Sci.* 82:2259–2273.
- Drackley, J. K., T. R. Overton, and G. N. Douglas. 2001. Adaptation of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. *J. Dairy Sci.* 84(E. Suppl.):E100–E112.
- Farrell, R. E. Jr. 1998. *RNA Methodologies*. 2nd ed. Academic Press, Harcourt Brace & Co., San Diego, CA.
- Fazio, S., and Z. Yao. 1995. The enhanced association of apolipoprotein E with apolipoprotein B-containing lipoproteins in serum-stimulated hepatocytes occurs intracellularly. *Arterioscler. Thromb. Vasc. Biol.* 15:593–600.
- Fazio, S., Z. Yao, B. J. McCarthy, and S. C. Rall Jr. 1992. Synthesis and secretion of apolipoprotein E occurs independently of synthesis and secretion of apolipoprotein B-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* 267:6941–6945.
- Fisher, E. A., and H. N. Ginsberg. 2002. Complexity in the secretory pathway: The assembly and secretion of apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* 277:17377–17380.
- Gerloff, B. J. 1987. Body condition scoring in dairy cattle. *Agri-Practice* 6:31–36.
- Gibbons, G. F. 1990. Assembly and secretion of hepatic very-low-density lipoprotein. *Biochem. J.* 268:1–13.
- Goering, H. K., and P. J. Van Soest. 1970. *Forage Fiber Analyses (Apparatus, Reagents, Procedures, and some Applications)*. Agric. Handbook no. 379. ARS-USDA, Washington, DC.
- Gruffat, D., D. Durand, B. Graulet, and D. Bauchart. 1996. Regulation of VLDL synthesis and secretion in the liver. *Reprod. Nutr. Dev.* 36:375–389.
- Gruffat, D., D. Durand, Y. Chilliard, P. Williams, and D. Bauchart. 1997. Hepatic gene expression of apolipoprotein B<sub>100</sub> during early lactation in underfed, high producing dairy cows. *J. Dairy Sci.* 80:657–666.
- Gruffat-Mouty, D., B. Graulet, D. Durand, M. E. Samson-Bouma, and D. Bauchart. 1999. Apolipoprotein B production and very low density lipoprotein secretion by calf liver slices. *J. Biochem.* 126:188–193.
- Grummer, R. R. 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. *J. Anim. Sci.* 73:2820–2833.
- Herdt, T. H., J. Liesman, B. Gerloff, and R. Emery. 1983. Reduction of serum triacylglycerol-rich lipoprotein concentrations in cows with hepatic lipidosis. *Am. J. Vet. Res.* 44:293–296.
- Hussain, M. M., J. Shi, and P. Dreizen. 2003. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J. Lipid Res.* 44:22–32.
- Ilian, M. A., R. S. Gilmour, and R. Bickerstaffe. 1999. Quantification of ovine and bovine calpain I, calpain II, and calpastain mRNA by ribonuclease protection assay. *J. Anim. Sci.* 76:853–864.
- Katoh, N. 2002. Relevance of apolipoproteins in the development of fatty liver and fatty liver-related peripartum diseases in dairy cows. *J. Vet. Med. Sci.* 64:293–307.
- Marcos, E., A. Mazur, P. Cardot, and Y. Rayssiguier. 1990a. The effects of pregnancy and lactation on serum lipid and apolipoprotein B and A-I levels in dairy cows. *J. Anim. Physiol. Anim. Nutr.* 64:133–138.
- Marcos, E., A. Mazur, P. Cardot, and Y. Rayssiguier. 1990b. Serum apolipoprotein B and A-I and naturally occurring fatty liver in dairy cows. *Lipids* 25:575–577.
- Mazur, A., M. Ayrault-Jarrier, Y. Chilliard, and Y. Rayssiguier. 1992. Lipoprotein metabolism in fatty liver dairy cows. *Diabete Metab.* 18:145–149.
- Mensenkamp, A. R., L. M. Havekes, J. A. Romijn, and F. Kuipers. 2001. Hepatic steatosis and very low density lipoprotein secretion: The involvement of apolipoprotein E. *J. Hepatol.* 35:816–822.
- Nehra, V., P. Angulo, A. L. Buchman, and K. D. Lindor. 2001. Nutritional and metabolic considerations in the etiology of nonalcoholic steatohepatitis. *Digest. Dis. Sci.* 46:2347–2352.
- Pullen, D. L., J. S. Liesman, and R. S. Emery. 1990. A species comparison of liver slice synthesis and secretion of triacylglycerol form nonesterified fatty acids in media. *J. Anim. Sci.* 68:1395–1399.
- Reid, I. M., G. J. Rowlands, A. M. Dewes, R. A. Collins, and R. Manston. 1983. The relationship between post-parturient fatty liver and blood composition in dairy cows. *J. Agric. Sci. (Camb.)* 101:473–480.
- Rhoads, R. P., C. McManaman, K. L. Ingvarsten, and Y. R. Boisclair. 2003. The housekeeping genes GAPDH and cyclophilin are regulated by metabolic state in the liver of dairy cows. *J. Dairy Sci.* 86:3423–3429.
- SAS User's Guide: Statistics, Version 8.0 Edition. 1999. SAS Inst., Inc., Cary, NC.
- Shelness, G. S., and J. A. Sellers. 2001. Very-low lipoprotein assembly and secretion. *Curr. Opin. Lipidol.* 12:151–157.
- Takahashi, Y., F. Itho, T. Oohashi, and T. Miyamoto. 2003a. Distribution of apolipoprotein E among lipoprotein fractions in the lactating cow. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 136:905–912.
- Takahashi, Y., K. Sato, F. Itho, T. Miyamoto, T. Oohashi, and N. Katoh. 2003b. Bovine apolipoprotein E in plasma: Increase of ApoE concentration induced by fasting and distribution in lipoprotein fractions. *J. Vet. Med. Sci.* 65:199–205.
- Tennant, 1997. Hepatic function. Pages 327–366 in *Clinical Biochemistry of Domestic Animals*. J. J. Kaneko, J. W. Harvey, and M. L. Bruss, eds. Academic Press, San Diego, CA.
- Uchida, E., N. Katoh, and K. Takahashi. 1992. Induction of fatty liver in cows by ethionine administration and concomitant decrease of serum apolipoprotein B<sub>100</sub> and A-I concentrations. *Am. J. Vet. Res.* 53:2035–2042.
- Vazquez-Anon, M., S. J. Bertics, M. Luck, R. R. Grummer, and J. Pinheiro. 1994. Peripartum liver triglyceride and plasma metabolites in dairy cows. *J. Dairy Sci.* 77:1521–1528.
- Watterau, J. R., M. C. M. Lin, and H. Jamil. 1997. Microsomal triglyceride transfer protein. *Biochim. Biophys. Acta* 1345:136–150.