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# Molecular cloning, expression, and hormonal regulation of the chicken microsomal triglyceride transfer protein

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ABSTRACT

During an egg-laying cycle, oviparous animals transfer massive amounts of triglycerides, the major lipid component of very low density lipoprotein (VLDL), from the liver to the developing oocytes. A major stimulus for this process is the rise in estrogen associated with the onset of an egg-laying cycle. In mammals, the microsomal triglyceride transfer protein (MTP) is required for VLDL assembly and secretion. To enable studies to determine if MTP plays a role in basal and estrogen-stimulated VLDL assembly and secretion in an oviparous vertebrate, we have cloned and sequenced the chicken MTP cDNA. This cDNA encodes a protein of 893 amino acids with an N-terminal signal sequence. The primary sequence of chicken MTP is, on average, 65% identical to that of mammalian homologs, and 23% identical to the *Drosophila melanogaster* protein. We have obtained a clone of chicken embryo fibroblast cells that stably express the avian MTP cDNA and show that these cells display MTP activity as measured by the transfer of a fluorescently labeled neutral lipid. As in mammals, chicken MTP is localized to the endoplasmic reticulum as revealed by indirect immunofluorescence and by the fact that its N-linked oligosaccharide moiety remains sensitive to endoglycosidase H. Endogenous, enzymatically active MTP is also expressed in an estrogen receptor-expressing chicken hepatoma cell line that secretes apolipoprotein B-containing lipoproteins. In this cell line and in vivo, the expression and activity of MTP are not influenced by estrogen. Therefore, up-regulation of MTP in the liver is not required for the increased VLDL assembly during egg production in the chicken. This indicates that MTP is not rate-limiting, even for the massive estrogen-induced secretion of VLDL accompanying an egg-laying cycle.



## 1. Introduction

The assembly of very low density lipoproteins (VLDL) occurs in the endoplasmic reticulum of hepatic cells. To form a lipoprotein particle competent for transport through the secretory pathway, apolipoprotein B (apoB) needs to associate with triglycerides, cholesteryl esters, free cholesterol, and phospholipids. This highly regulated process requires the activity of the microsomal triglyceride transfer protein (MTP). MTP is a heterodimer composed of the lipid-binding catalytic large subunit of approximately 97 kDa and protein disulfide isomerase (PDI) of 58 kDa (Berriot-Varoqueaux et al., 2000; Gordon and Jamil, 2000; Hussain and Bakillah, 2008; Hussain et al., 2003, 2012; Shelness and Ledford, 2005; Shelness and Sellers, 2001; Wetterau et al., 1997). The absence of MTP protein or activity due to mutations in the gene encoding the large catalytic subunit has been shown to be a cause for the rare inherited disease, abetalipoproteinemia (Sharp et al., 1993; Shoulders et al., 1993). Since this discovery, a variety of mutations in the MTP gene have been identified that lead to the abetalipoproteinemia phenotype (Berriot-Varoqueaux et al., 2000; Gordon and Jamil, 2000; Hooper et al., 2005; Narcisi et al., 1995; Rehberg et al., 1996; Ricci et al., 1995).

Detailed structural information on MTP, partly based on molecular modeling, has become available during recent years (Shoulders and Shelness, 2005). Altogether these findings led to the conclusion that the precise mechanism of MTP action(s) may vary between MTP proteins from different species, thus suggesting evolutionary adaptation (Rava et al., 2006; Shelness and Ledford, 2005). Based on sequence and structure comparisons, MTP is defined as a member of the so-called large lipid transfer protein (LLTP) gene superfamily (Avarre et al., 2007; Shelness and Ledford, 2005). MTP orthologs have been identified and their role in the biogenesis of lipoproteins has been

*Abbreviations:* ApoB, apolipoprotein B; Cef, chicken embryo fibroblast; CETP, cholesteryl ester transfer protein; ER, endoplasmic reticulum; LMH, leghorn male hepatoma; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein.

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demonstrated in several species, including the invertebrates *Drosophila melanogaster* (Sellers et al., 2003) and *Caenorhabditis elegans* (Shibata et al., 2003). Mainly based on comparisons of MTP sequences from these and other species and, where available, their structures and biochemical properties, it was found that the early ancestors likely had phospholipid transfer activity only. The capacity of MTP to transfer neutral lipids appears to have been gained during the evolutionary transition from invertebrates to vertebrates (Rava and Hussain, 2007).

From a functional perspective, the hypothesis that MTP participates in the initial association of apoB with lipids in the endoplasmic reticulum is supported by an extensive body of data. For instance, it was demonstrated that MTP is able to confer lipid transfer activity onto cells lacking endogenous MTP. Significantly, such cells gain the capacity to secrete lipoprotein particles when apoB, or fragments thereof, are co-expressed (Gordon et al., 1994; Gretch et al., 1996; Leiper et al., 1994; Patel and Grundy, 1996; Wang et al., 1996). Regardless, without MTP, apoB does not fold properly and is degraded within the endoplasmic reticulum, preventing secretion. As expected for a protein that transfers lipids onto nascent apoB, a transient physical interaction of MTP with apoB, most prominent at early stages of lipoprotein synthesis, was demonstrated (Dashti et al., 2002; Hussain et al., 1998; Patel and Grundy, 1996; Wu et al., 1996). Consistent with this key role in lipoprotein assembly, MTP-specific inhibitors reduce the secretion rate of apoB from hepatoma cells (Jamil et al., 1996), apparently by blocking only the initial lipid loading of apoB (Gordon et al., 1996).

The formation of VLDL particles in avian species is tightly regulated by estrogen; this hormone causes a massive induction of the synthesis of hepatic apoB, as well as of other yolk precursors such as apoVLDL-II and vitellogenins I, II, and III, in the chicken (Evans et al., 1988; Kirchgessner et al., 1987). Therefore, chicken hepatocytes provide a useful model system to study the regulation of VLDL biosynthesis. Since the discovery of MTP, a key unanswered question in this field is whether or not the massive upregulation of VLDL secretion in oviparates in response to estrogen would require a commensurate upregulation of MTP. On the one hand, increases in MTP are not required to generate the modest increases in VLDL secretion observed upon fatty acid feeding in mammalian systems (Nakamuta et al., 1996; Pease and Leiper, 1996). However, treatment of hepatic cells with MTP inhibitors results in a dose-dependent decrease in apoB secretion, suggesting that MTP is rate-limiting for VLDL secretion to some extent (Jamil et al., 1998). Similar conclusions were drawn from a study with gonadectomized female and male rats treated with gonadal hormones (Ameen and Oscarsson, 2003). Another report also pointed towards a regulatory effect of estrogen on MTP mRNA and protein levels and on VLDL production in ovariectomized rats (Barsalani et al., 2010). Interestingly, mammalian MTP is controlled at the transcriptional, post-transcriptional, and post-translational levels, including regulation by hormones, such as insulin and leptin (Johansson et al., 2003) as well as growth hormone (Ameen and Oscarsson, 2003). To enable molecular investigations into the role of MTP in the process of VLDL assembly in an oviparate system and to obtain additional structural and functional insights into potentially important domains of the avian protein, the cDNA encoding chicken MTP was cloned and sequenced. Herein is described the identification and characterization of the *Gallus gallus* MTP cDNA and protein. In addition, the key question of whether MTP upregulation is required for the estrogen-induced increase in VLDL secretion is directly addressed in the estrogen-responsive LMH-2A chicken hepatoma cell line (Hermann et al., 1997).

## 2. Materials and methods

### 2.1. Materials

A λgt11 chicken liver cDNA library and pBluescript KS f− were obtained from Stratagene (La Jolla, CA). The plasmids pT7Blue and pCIneo were from Novagen (Madison, WI) and Promega (Madison, WI), respectively. Restriction enzymes, DNA-modifying enzymes and endoglycosidase H and the LightCycler FastStart DNA Master SYBR Green I-kit were from Roche (Mannheim, Germany). The Nucleospin RNA II kit to isolate total RNA was from Macherey-Nagel, Dueren, FRG, and Superscript II RNase $H^-$ reverse transcriptase, the TOPO TA cloning kit, and TOP10F *Escherichia coli* bacteria were purchased from Invitrogen (Paisley, UK). High Fidelity PCR DNA polymerase mix was obtained from Fermentas (St. Leon-Rot, FRG), and DNA primers for PCR were from Eurofins MWG Operon (Ebersberg, FRG). For the isolation of plasmid DNA and of DNA fragments from agarose gels the Qiagen plasmid kits and the QIAquick gel extraction kit (Qiagen, Hilden, Germany) were used. For the isolation of plasmid DNA also the FastPlasmid Mini kit from 5Prime (Hamburg, Germany) and for the isolation of DNA fragments from agarose gels the DNA gel extraction kit from GenXpress (Vienna, Austria) were used. Waymouth's medium MB 752/1 without L-glutamine, L-methionine, sodium bicarbonate and phenol red, Eagle's Minimal Essential Medium, penicillin, streptomycin sulfate, L-glutamine, lipofectin, geneticin (G418 sulfate), PBS, fetal bovine serum (FBS) and [14C]protein standards were purchased from Gibco/BRL (Gaithersburg, MD). Ampicillin, kanamycin, X-gal (5-bromo-3-indolyl β-D-galactopyranoside), gelatin, protein A, coupled with horseradish peroxidase, L-methionine, Triton X-100, PMSF, aprotinin, leupeptin, leu-leu-leu, pepstatin A and sodium deoxycholate were obtained from Sigma (Deisenhofen, Germany). Protein A-sepharose CL-4B beads were from Pharmacia (Uppsala, Sweden). Moxestrol, EN3-HANCE and X-ray films were purchased from DuPont/NEN (Brussels, Belgium). Protein standards for SDS–PAGE (Broad range markers) were obtained from Bio-Rad Laboratories (Hercules, CA). Met-label ([35S]methionine and [35S]cysteine; >1100 Ci/mmol) was purchased from ARC (St. Louis, MO). Nitrocellulose membranes and the ECL kit were obtained from Amersham (Arlington Heights, IL). Maleimide-activated KLH and Excellulose GF-5 columns were from Pierce (Rockford, IL). An anti-ER antiserum was obtained from Dr. Daniel Louvard, Paris. Texas red-conjugated affinity-purified goat anti-rabbit F(ab′)2-IgG was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY), reconstituted with 50% glycerol, and kept at −20 °C. Citifluor was obtained from Citifluor Ltd. (London, UK). Non-fat dry milk was from Maresi (Vienna, Austria). The kit for the determination of the MTP activity was purchased from Diagnescent Technologies (Yonkers, NY).

### 2.2. Construction of a full-length cDNA clone for the chicken 97 kDa MTP subunit and sequence analysis

A full-length cDNA encoding the 97 kDa subunit of chicken MTP was isolated by a combination of traditional cloning methods using a cDNA library derived from chicken liver and Polymerase Chain Reaction (PCR) technology. The nucleotide sequence of all clones and cloning intermediates was verified by automated sequencing. Details of these procedures are described in Supplementary materials. The GenBank accession number of our chicken MTP cDNA sequence is KC176805.

### 2.3. Preparation of antibodies

A peptide corresponding to the 16 carboxy-terminal amino acids of chicken MTP (CRKVFSTASDSSGSWF), termed MTP-B, was synthesized and purified by HPLC (Research Genetics, Huntsville, AL) and the peptide was coupled to activated KLH. The peptide/KLH conjugate was mixed with Freund's complete adjuvant and used to immunize an adult female New Zealand White rabbit. The antiserum, designated anti-MTP-B, was tested by Western blotting of chicken liver extracts, and IgG fractions were purified from the serum by affinity chromatography on protein A-sepharose CL-4B and adjusted to a protein concentration of approx. 5 mg/ml.