Mutations of the Microsomal Triglyceride-Transfer–Protein Gene in Abetalipoproteinemia

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Summary

Elevated plasma levels of apolipoprotein B (apoB)–containing lipoproteins constitute a major risk factor for the development of coronary heart disease. In the rare recessively inherited disorder abetalipoproteinemia (ABL) the production of apoB-containing lipoproteins is abolished, despite no abnormality of the apoB gene. In the current study we have characterized the gene encoding a microsomal triglyceride-transfer protein (MTP), localized to chromosome 4q22-24, and have identified a mutation of the MTP gene in both alleles of all individuals in a cohort of eight patients with classical ABL. Each mutant allele is predicted to encode a truncated form of MTP with a variable number of aberrant amino acids at its C-terminal end. Expression of genetically engineered forms of MTP in Cos-1 cells indicates that the C-terminal portion of MTP is necessary for triglyceride-transfer activity. Deletion of 20 amino acids from the carboxyl terminus of the 894-amino-acid protein and a missense mutation of cysteine 878 to serine both abolished activity. These results establish that defects of the MTP gene are the predominant, if not sole, cause of hereditary ABL and that an intact carboxyl terminus is necessary for activity.

Introduction

Elevated plasma levels of LDL, the product of VLDL catabolism, are a major risk factor for the development of atherosclerotic vascular disease (Cholesterol Consensus 1992). LDL is the main cholesterol-carrying particle in blood. Apolipoprotein B (apoB) is the major protein component of LDL and the ligand that mediates the clearance of LDL-cholesterol by the LDL-receptor pathway (Brown and Goldstein 1983). ApoB in addition nucleates the assembly of triglyceride-rich lipoproteins, VLDL, and chylomicrons in the secretory pathways of the liver and intestine, respectively. ApoB that does not acquire sufficient lipid to attain a native structure undergoes rapid presecretory degradation (Boren et al. 1990; Davis et al. 1990). The balance between degradation and lipoprotein assembly determines the proportion of apoB that is secreted (Pullinger et al. 1989; Dixon et al. 1991; White et al. 1992; Sakata et al. 1993).

Abetalipoproteinemia (ABL) is a rare recessively inherited disorder characterized by the selective absence of apoB-containing lipoproteins from plasma (Kane and Havel 1989). Its presentation is clinically heterogeneous. It may present in infancy with failure to thrive but can present in young adults as a spinocerebellar degenerative disorder with peripheral neuropathy and a pigmented retinopathy. ABL can be caused by defects of the gene encoding a microsomal triglyceride-transfer protein (MTP) (Wetterau et al. 1992; Sharp et al. 1993; Shoulders et al. 1993). In vivo MTP forms a heterodimer with protein disulfide isomerase, a ubiquitous endoplasmic reticulum (ER) resident protein, and functions in the loading of apoB with lipid (Wetterau et al. 1990). However, it remains to be determined if mutations of the MTP gene are the sole, or even main, cause of ABL. Differences in presentation may reflect locus heterogeneity. Indeed, defective translocation of apoB into the secretory pathway of the liver and intestine has been suggested as another cause of the ABL phenotype (Thrift et al. 1992).

In the present study we have investigated a cohort of eight individuals with classically inherited ABL, for defects of the MTP gene localized on chromosome 4q22-24 (Sharp et al. 1994). MTP gene mutations that specified a truncation of the encoded product were identified in both alleles of all individuals. The analysis of naturally occurring and genetically engineered mutants of MTP indicates that lipid-transfer activity requires the presence of a cysteine residue at position 878, 17 amino acids short from the carboxyl terminus. We propose that

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Table 1

Plasma Lipid, apoB, and Vitamin E Concentrations in Patients with ABL and in Their First-Degree Relatives

Individual	Age at Diagnosis (years)	Plasma Lipid Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	apoB (mg/dl)	Vitamin E[a] (µmol/liter)	Previous Report(s)
Patient 1	1.6	26.6	19.3	21.9	.1	4.6	Muller et al. 1983 (patient 6)
Mother	...	150.8	99.0	63.7	15.0	40.0	
Father	...	163.7	102.5	46.5	27.5	29.0	
Patient 2	25.0	43.0	<8.8	39.1	<1.0	.8	Dullaart et al. 1986
Mother	...	320.0	227.7	...	120.0	...	
Patient 3	.4	28.5	16.6	...	.2	4.6	Muller et al. 1983 (patient 3)
Father	...	190.6	68.3	34.0	25.0	19.4	
Patient 4	.9	27.3	19.3	27.7	.2	3.0	Muller et al. 1983 (patient 4)
Mother	...	264.1	119.1	60.5	100.0	...	
Patient 5	...[b]	29.3	2.6	27.3	.3	3.4	Talmud et al. 1988
Sister	1.7	35.5	2.6	31.3	.6	4.5	
Mother	...	132.8	61.3	43.0	61.0	23.0	
Father	...	148.4	175.2	35.2	84.0	25.1	
Patient 6	.7	36.7	14.9	31.6	.2	3.5	...
Mother	...	160.9	78.0	42.2	37.5	23.3	
Father	...	194.5	95.5	27.7	92.5	26.8	
Patient 7	1.1	34.4	16.6	27.7	.1	6.9	Muller et al. 1983 (patient 5)
Mother	...	204.7	77.1	68.0	41.5	27.8	
Father	...	193.0	184.0	56.6	44.5	27.3	
Patient 8	.1	23.4	<8.8	17.6	.1	.6	Muller et al. 1983 (patients 1 and 2), Shoulders et al. 1993
Father	...	257.8	122.6	45.3	112.0	23.6	
Mother	...	187.5	52.6	39.1	74.0	24.3	
Sister	...	136.7	96.4	...	83.0	17.4	
Sister	...	164.1	87.6	...	80.0	...	
Brother	.2	35.2	<8.8	31.3	.2	1.5	

[a] Maximum level after supplementation was received. At diagnosis, vitamin E was undetectable in individuals who presented early.
[b] Patient diagnosed on the basis of cord blood.

this cysteine forms a disulfide linkage that is necessary for the integrity of the protein.

Methodology

Clinical Evaluation

The diagnosis of ABL in eight apparently unrelated individuals was based on clinical examination and on the lipid profiles of the patients and their parents (table 1). Seven of our patients were referred to the Hospitals for Sick Children, Great Ormond Street, and presented with fat malabsorption in infancy. On examination, neurology was normal except in the case of patient 1, who had absent knee and ankle jerks and a decreased vibration sense, and patient 7, who, in addition to absent knee and ankle jerks, was hypotonic (table 1). The patients were managed by restricting dietary intake of long-chain fats and administering supplements of the fat-soluble vitamins, A, E, and K. This regime has prevented the development of neurological complications (Muller et al. 1983). Patient 2 was diagnosed at 25 years of age (Dullaart et al. 1986). Her childhood was uneventful, although she showed a notable intolerance for fatty foods. At age 14 years, symptoms of spinocerebellar degeneration were apparent, and by the age of 25 years her gait had become ataxic, visual acuity was significantly diminished, and a pigmentary retinopathy was present.

Gene Organization and Chromosomal Localization

Positions of intron/exon junctions were determined as described elsewhere (Shoulders et al. 1994). Intron sizes

were determined by DNA sequencing and size fractionation of appropriate PCR products. Rapid amplification of 5′ and 3′ cDNA ends (RACE) was performed with liver poly $(A)^+$ RNA and RACE kits obtained from Gibco-BRL and Clonetech, respectively. The 5′ end of MTP mRNA was obtained by use of oligonucleotide cDNA1 (5′GGATCAACTGGTCATCATCACCATCAGGA3′), in the first-strand cDNA reaction, and oligonucleotide cDNA2 (5′ACGCTGTCTTGCAGTTTTCCTT3′), in the reverse transcriptase–PCR reaction. Amplification of the 3′ end of MTP mRNA was performed with oligonucleotide cDNA3 (5′TGACTAAGTACTTGCTCTCTG3′), in the first-round PCR, and oligonucleotide cDNA4 (5′CTGCACAATAGCTAGGATGAC3′), in the second round. Assignment of the MTP gene to chromosome 4 was performed by PCR analysis of a panel of rodent × human somatic cell hybrids (Wong et al. 1987). In 15 hybrid cell lines, the human-specific PCR-amplification product was completely concordant with chromosome 4, 8 hybrids being positive and 7 being negative. FISH was performed as described elsewhere (Pinkel et al. 1986; Gharib et al. 1993), by 0.2 μg of biotinylated total DNA from a Bluescript pKS subclone that contained ~16 kbp of the MTP gene. Localization was determined by simultaneous examination of fluorescent signals and R-banding.

PCR Amplification and Mutation Screening

The promoter, 189 bp of 5′ flanking sequences, and all 18 exons and their associated splice sites were amplified from patient genomic DNA by PCR and were sequenced. The conditions of PCR and the DNA sequences of the oligonucleotides used to amplify and sequence the individual exons are available on request from one of the authors (C.C.S). PCR products were bound directly to streptavidin-coated magnetic beads (Hultman et al. 1989), as recommended by the manufacturer (Dynal), at 48°C, were denatured, and were sequenced with an appropriate primer by an Applied Biosystems PRISM Sequenase Terminator Single-Stranded DNA Sequencing Kit. Unincorporated nucleotides were removed by ammonium acetate precipitation.

Polymorphic Sequence Analysis and Allele-Specific Oligonucleotide (ASO) Hybridization

Oligonucleotides MTP/CA5′ (5′CCACAGGATTCATAACCTCTG3′) and MTP/CA3′ (5′TCTTTCCTTCTATTGGGAGCC3′) were used to amplify a 170–190-bp region of DNA spanning a dinucleotide-repeat polymorphism within intron 10 of the MTP gene (Sharp et al. 1994). PCR amplifications were carried out in 25-μl volumes each containing 20–100 ng of genomic DNA and 12.5 ng of each primer. Each reaction contained 1–2×10^5 cpm of one ^{32}P 5′-labeled primer and 1.25 units of *Taq* polymerase (BRL). The PCR buffer consisted of 10 mM Tris pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, and 200 μM each of dATP, dCTP, dGTP, and dTTP. The products of the PCR were size-fractionated on 6% denaturing polyacrylamide gels. To ensure correct identification of alleles, DNA samples of individuals with defined genotypes were run on each gel. Alleles are numbered in ascending order of size. Oligonucleotides for the amplification of additional polymorphic regions of the gene are given in table 2. PCR products were size-fractionated, blotted onto Hybond N+ (Amersham), and hybridized at 30°C to ^{32}P radiolabeled ASOs (table 2). Blots were washed for 5 min in 6 × SSC, 0.1% SDS at the temperature specified in table 2.

Construction of Mutant MTP cDNAs

Deletion mutagenesis was performed by a PCR-based strategy. The template was the plasmid pSV7D/MTP, which contains a cDNA sequence encompassing the entire coding region of MTP in the mammalian expression vector pSV7D (Leiper et al. 1994). In each of the mutagenesis reactions the forward primer was oligonucleotide MTPM1 5′, which spans nucleotides 1403–1422 of MTP cDNA. Sequences of the reverse oligonucleotides were 5′GTTTCGTCGACCTCAGCTGGAAGTACTATC3′ (C-3), 5′TGGAGTCGACTCACGGCTGAGGGGCAAACAC3′ (C-8), 5′TATCGTCGACTCAGGCAAACACCACTTTGCACAT3′ (C-11), and 5′CCACTTTGGTCGACTCTTAGTTCTCTTG3′ (C-20). Each contained an in-frame terminator codon and a recognition site for *Sal*I. The PCR products were restricted with *Aat*II and *Sal*I and were cloned back into the plasmid pSV7D/MTP, replacing the corresponding wild-type sequence fragment with the mutant sequence. Site-directed mutagenesis was performed with an Altered Sites in vitro mutagenesis kit obtained from Promega. The sequences of the M877V and C878S mutant oligonucleotides were 5′GAGAACTCAGAGGTGTGCAAAGTGGTGT3′ and 5′GAGAACTCAGAGATGTCCAAAGTGGTGTTT3′, respectively. Genetically engineered constructs were sequenced with appropriate primers by a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems).

Transfection of Cos-1 Cells, and Harvesting and Partial Purification of Expressed MTP

Cos-1 cells were grown to 70% confluence, were harvested by trypsinization, and were resuspended, at 10^7 cells/ml, in PBS. Twenty-five micrograms of MTP plasmid and 25 μg of β-galactosidase control plasmid in 1 ml of cells were electroporated on a Bio-Rad gene pulser (960 microfarads at 250 V) before being plated out in Dulbecco's modified Eagle medium supplemented with 10% FCS. Approximately 36 h posttransfection, Cos-1 cells (2×10^7) were harvested in PBS containing 2 mM EDTA, were washed five times in 10 mM phosphate

Table 2

Conditions for Detection of PCR-Based MTP Gene Polymorphisms

MTP Gene	Primer Sequence (5′–3′)	ASO (5′–3′)	Washing Temperature (°C)
−130	GCTCCAACCTCATACAGTTTCA	TTCACCCAATGAGGAAA	49
(T/C)	TCAACTCAGCCATGCTTTCAC	TTTCCTCACTGGGTGAA	47
Exon 3	GAAGTAGCACCATGTTTCAATC	TTTCCATGGATTAGATGA	46
(I128T)	GGGAACTCAGGGCATTGATTC	TCATCTAACCCATGGAAA	48
Exon 3	CAGAGATACTCTGATGAAGACA	AATCAGCAGAGAGGAGA	48
(Q97H)	GGGAACTCAGGGCATTGATTC	AATCAGCACAGAGGAGA	48
Exon 4	CGACTTCACTACACAAGTCAC	AGAGAGGTCTGGCTAG	47
(T/C)	CAGATTTGGAGTGTCAGATCTC	AGAGAGGCCTGGCTAG	49
Exon 6	TCCCCTATGGCCTATTAGAGA	GAATTTCCTACAAACCATTA	46
(Q244E)	CCCATTGTTAAGCAATGCATGA	GAATTTCCTAGAAACCATTA	46
Exon 7	GAGCTTTCAGTTACCTCTGGAG	CAGAGCCACTGTAAAGG	51
(H297Q)	CTCTGTGGTAGCTGGTAGTTC	CAGAGCCAGTGTAAAGG	51
(CA repeat)	CCACAGGATTCATAACCTCTG	NA	NA
Intron 10	TCTTTCCTTCTATTGGGAGCC	NA	NA
Exon 18	TGGTACAGTCAGAATAGTTATTC	ATGACAGGTCCTTATTTT	48
(C/G)	AACTGTGTATGTGTCTAGGCTC	ATGACAGGTGCTTATTTT	48

NOTE.—NA = not applicable.

buffer pH 6.8, and were resuspended in 1 ml of 10 mM phosphate buffer pH 6.8 containing 25 μg of a trypsin inhibitor. Transfection efficiencies were compared by a β-galactosidase enzyme assay system obtained from Promega. Total RNA was extracted from transfected cells by a kit obtained from Stratagene and was analyzed by northern blotting.

Microsomal contents were released on ice by probe sonication and were cleared of cellular debris by one round of ultracentrifugation at 100,000 *g* for 60 min at 4°C. Supernatants were applied to 2 ml of preequilibrated (10 mM phosphate buffer pH 6.8) DEAE cellulose columns and were washed with 2 ml of 10 mM phosphate pH 6.8/30 mM NaCl buffer. MTP was eluted with 3.0 ml of 10 mM phosphate buffer pH 6.8/220 mM NaCl and was concentrated, in a Centricon 30 cartridge, to ~200 μl.

Analysis of Expression of MTP in Cos-1 Cells

Cos-1 cell extracts containing partially purified MTP protein were assayed for total protein, MTP protein, and MTP activity. Protein estimation was performed by a Bradford reagent obtained from Bio-Rad. An aliquot containing 25% of the partially purified extract was resolved by 8% SDS-PAGE and was blotted onto pretreated polyvinylidene difluoride membrane, as described elsewhere (Harlow and Lane 1988). Prehybridization was with 5% nonfat milk powder in 10 mM Tris pH 7.4, 1 M NaCl, 0.5% Tween for 2 h at room temperature (RT). Hybridization was for 2 h at RT, with rabbit anti-MTP peptide antisera, diluted 1:500 in the prehybridization buffer (Brett et al. 1995). Immunoreactive products were visualized with a goat anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (Bio-Rad), followed by enhanced chemiluminescence (Amersham).

MTP activity was measured by a triglyceride-transfer assay adapted from Wetterau et al. (1990) and Leiper et al. (1994). Vesicles were prepared by probe sonication in 2.5 ml of 15:35 buffer (15 mM Tris pH7.4, 35 mM NaCl, 0.5% BSA, 0.02% NaAzide + 1 mM EDTA). Donor vesicles comprised 1.7 mg of phosphatidyl choline, 3 μg of $[^3H]$glycerol trioleate, 235 μg of cardiolipin, and 0.01% butylated hydroxytoluene. Acceptor vesicles comprised 10.4 mg of phosphatidyl choline, 25 μg of triolein, 0.01% butylated hydroxytoluene, and 7.2 μg of ^{14}C dipalmitoyl phosphatidyl choline. Partially purified MTP (1–10 μg) was added to 5 μl of each of the vesicles, in a final volume of 100 μl in 15:35 buffer, and incubated at 37°C for 1 h. Reactions were quenched by 400 μl ice-cold 15:35 buffer. Donor vesicles were absorbed onto 0.5 ml of preequilibrated DEAE (Whatman DE-52) in 15:35 buffer pH 8.0, without BSA. DEAE-bound vesicles were pelletized by centrifugation at 13,000 rpm for 10 min. To remove remaining traces of DEAE-bound vesicles, 500 μl of the supernatant was respun prior to liquid scintillation counting. Lipid-transfer activity is expressed as picomoles of $[^3H]$ triglyceride transferred from donor to acceptor vesicles, per milligrams of protein per hour, under conditions where it was verified that the assay was linear with extract concentration.

Results

Lipid and apoB levels for the patients and their first-degree relatives are shown in table 1. Plasma cho-

lesterol, triglyceride, and apoB levels in the patients were markedly lower than those observed in their parents and unaffected siblings and in a normal control population. In comparison, levels of HDL-cholesterol were within the normal range in all but two of the patients.

In order to facilitate the screening of mutations in subjects with ABL, the organization of the MTP gene was established (fig. 1*A*). This gene was localized to chromosome 4q22-24 (fig. 1*B*), which confirms the assignment reported (Sharp et al. 1994) while this work was in progress. Genomic DNA from the ABL individuals (table 1) was screened for mutations and polymorphisms within the MTP gene by sequencing products of PCR-amplified DNA. The products spanned the promoter, all exons, and intron-exon junctions of the gene (fig. 1*A*). Seven novel mutations and seven polymorphic nucleotides were identified.

Protein Polymorphisms of MTP

Four polymorphisms resulting in amino acid changes were identified (fig. 1*A*). None represented a missense mutation, and these differences from the wild-type allele were in addition to the ABL-causative mutations. The first was identified in patient 4 and involved a G→C substitution at position 285 of MTP cDNA and changed amino acid 95 of MTP from glutamine to histidine. The second, a T→C transition at base 383, was identified in patients 2, 4, and 5 and changed amino acid 128 from isoleucine to threonine. The third, a C→G change at position 730, was identified in patient 5 and his mother who was homozygous for this allele. It changed amino acid 244 from glutamine to glutamic acid. The fourth, a C→G change at position 891, was identified in patients 2, 4, 5, and 7. It changed amino acid 297 from histidine to glutamine. In a healthy adult Caucasian population resident in England, the frequencies of the minor alleles H95, T128, E244, and Q297 were .054, .21, .01, and .44, respectively, compared with frequencies of .062, .25, .062, and .375 in our cohort of patients with ABL. The low frequency of the E244 allele in our healthy population sample appears not to be due to the error rate of *Taq* polymerase (Keohavong and Thilly 1989), since this allele was also observed in patient 5 and his mother. It was absent in the father. Moreover, previous studies (Keohavong and Thilly 1989) have indicated that the predominant mutations introduced by *Taq* polymerase during PCR-amplification of a template do not involve C→G substitutions.

Frameshift Mutations

Sequence analysis of PCR products from three patients (1, 4, and 6) identified a single adenine insertion in exon 4, between positions 419 and 420 of MTP cDNA (fig. 2). This insertion is located within a run of four adenines, suggesting that the mutation may have arisen by DNA polymerase slippage (Schlotterer and Tautz 1992). The mutation causes a frameshift after the codon for amino acid 140 and generates a premature termination codon (UGA) one amino acid into the new reading frame (table 3). Patient 1 was homozygous for the insertion whereas patients 4 and 6 were heterozygous. As predicted by the autosomal recessive transmission of the disorder, both unaffected parents of patient 1 carried one mutant allele and one wild-type allele. In patient 4 the mutant allele had been maternally transmitted, whereas in patient 6 it had been derived from the father. The second mutation in patient 4 was also found to be an adenine inserted within a run of six adenines (fig. 2). Sequence analysis of PCR products from the mother of patient 4 confirmed that this mutation is likely to have been derived from the father of patient 4, who was unavailable for investigation. This mutation is located in exon 11, between nucleotides 1401 and 1402 of MTP cDNA (Sharp et al. 1993; Shoulders et al. 1993), and generates a new reading frame after the codon for amino acid 467 (table 3).

Two of the patients, 2 and 7, were found to have a homozygous deletion of a single nucleotide. Patient 2, who previously had been reported to be the only child of consanguineous Dutch parents (Dullaart et al. 1986), was found to have a deletion of an adenine within a run of four adenines, nucleotides 1144–1147 of MTP cDNA (fig. 2). It generates a new reading frame after the codon for amino acid 382 of MTP (table 3). This sequence difference was shown to be present in one allele of the mother's DNA; but it could not be shown to be present in the DNA of the patient's father, since he was unavailable. Patient 7 was found to have a deletion of a thymidine, between nucleotides 2211 and 2213 of MTP cDNA (exon 15) (fig. 2). This sequence difference was shown to be present in one allele of both parents' DNA. It results in the generation of a new reading frame after amino acid 737 of MTP (table 3).

Splice-Site Mutations of the MTP Gene in ABL

Sequence analysis of the PCR products amplified from DNA of patient 3 revealed that he was homozygous at each of the polymorphic sites shown in table 4 and that the only sequence abnormality present involved a microdeletion at positions 5–11 of intron 10 (fig. 2). This sequence difference was shown to be present in one allele of the father's DNA; but it could not be shown to be present in the DNA of the patient's mother, since she was unavailable. Thus it is possible that one allele of patient 3 and his mother lacks the entire MTP gene, because of a deletion of >50 kb. However, since both parents originated from the same region of China, it seems likely that the mother of patient 3 also carries the intron 10 microdeletion. The microdeletion alters the

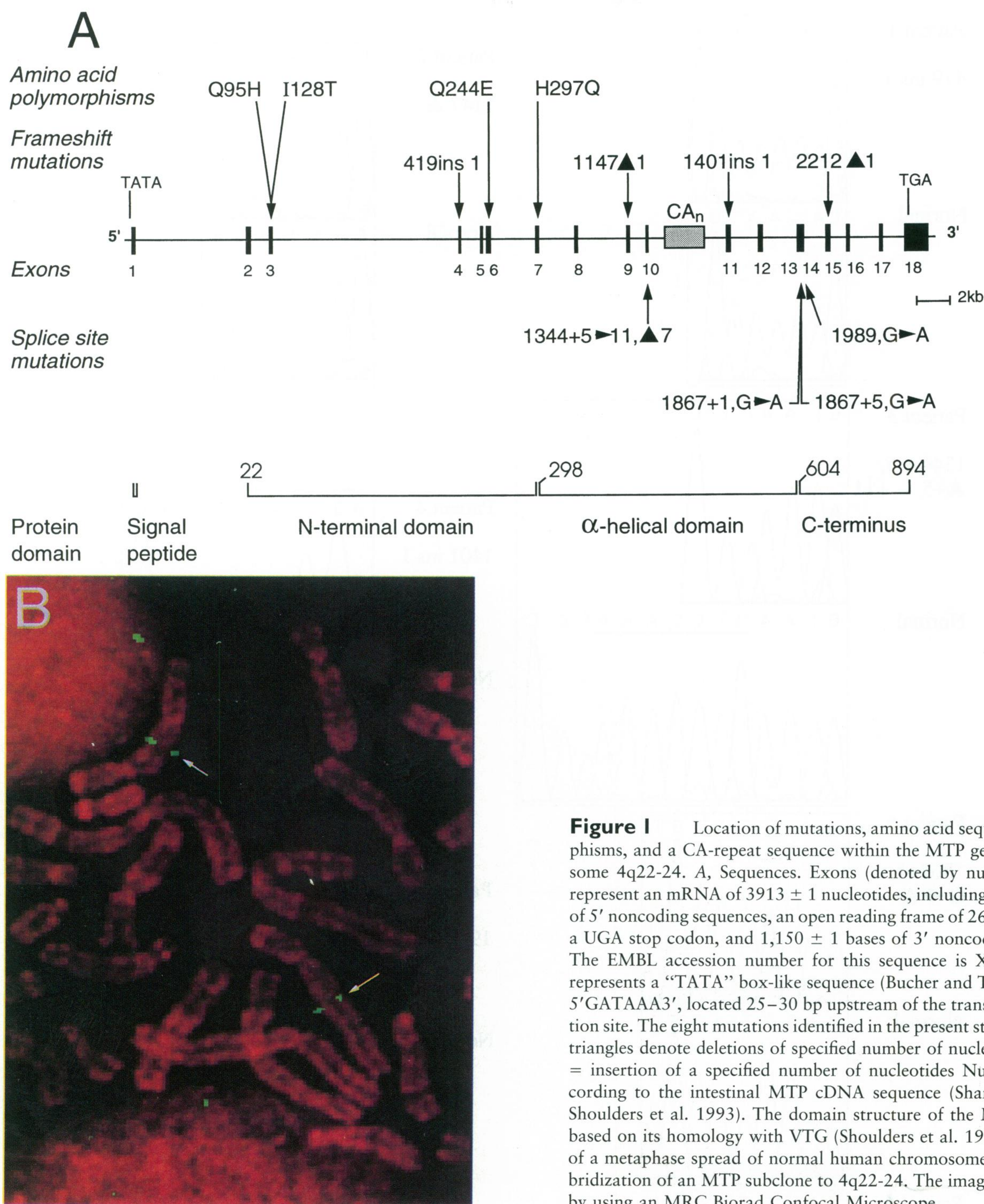


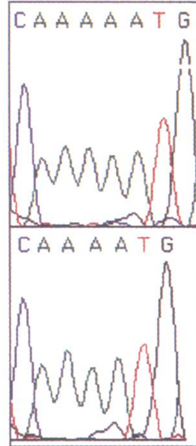
Figure 1 Location of mutations, amino acid sequence polymorphisms, and a CA-repeat sequence within the MTP gene on chromosome 4q22-24. *A*, Sequences. Exons (denoted by numbered boxes) represent an mRNA of 3913 ± 1 nucleotides, including 88 nucleotides of 5′ noncoding sequences, an open reading frame of 2682 nucleotides, a UGA stop codon, and 1,150 ± 1 bases of 3′ noncoding sequences. The EMBL accession number for this sequence is X91148. TATA represents a "TATA" box-like sequence (Bucher and Trifonov 1986), 5′GATAAA3′, located 25–30 bp upstream of the transcription-initiation site. The eight mutations identified in the present study are shown; triangles denote deletions of specified number of nucleotides; and ins = insertion of a specified number of nucleotides Numbering is according to the intestinal MTP cDNA sequence (Sharp et al. 1993; Shoulders et al. 1993). The domain structure of the MTP protein is based on its homology with VTG (Shoulders et al. 1994). *B*, Portion of a metaphase spread of normal human chromosomes, showing hybridization of an MTP subclone to 4q22-24. The image was obtained by using an MRC Biorad Confocal Microscope.

normal 5′ splice-site sequence of intron 10, from 5′AA:GTAAGT3′ to 5′AA:GTAATC3′, which differs at three positions from the 5′ splice-site consensus sequence, 5′AG:GT(A/C)AGT3′ (Shapiro and Senepathy 1987). The guanine normally occurring at the +5 position is changed to an adenine. Mutation of this guanine base to any other nucleotide is associated with aberrant splicing in a variety of human diseases, including ABL (Krawczak et al. 1992; Shoulders et al. 1993). The outcome of the microdeletion could include exon skipping, activation of a nearby cryptic splice site, or the production of an incompletely spliced mRNA (Krawczak et al. 1992). The first would give rise to an mRNA species that lacks exon 10 sequences, nucleotides 1237–1344,

Patient 1

419 ins 1

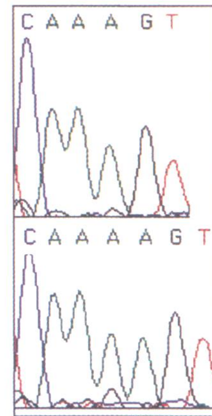
Normal



Patient 2

1147 ▲ 1

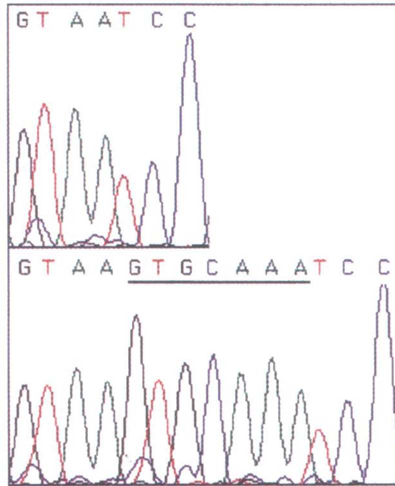
Normal



Patient 3

1344 + 5,
▲+5 – +11

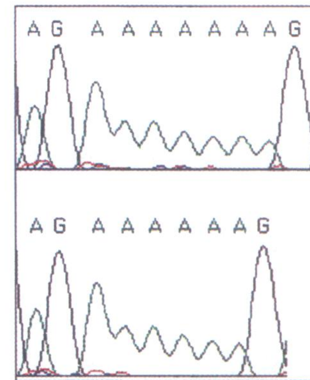
Normal



Patient 4

1401 ins 1

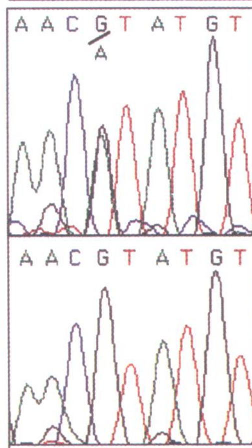
Normal



Patient 5

1867+1,
G ➔ A

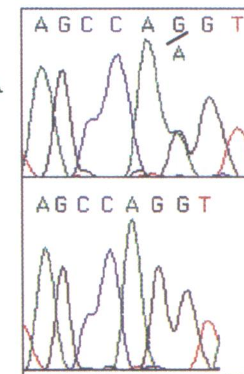
Normal



Patient 5

1989 G ➔A

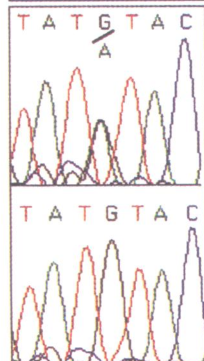
Normal



Patient 6

1867+5,
G ➔ A

Normal



Patient 7

2212 ▲ 1

Normal

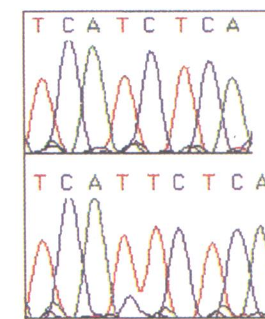


Table 3

MTP Gene Mutations in 11 Patients with ABL and the Predicted Size of the Encoded Protein(s)

ABL Case	Country of Origin	Mutation		Codon		Encoded-Protein Size (amino acids)	
		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
1	England	419 ins1	419 ins1	140	140	140	140
2	Holland	1147 del1	1147 del1	383	383	407	407
3	China	1344+5→11, del7	1344+5→11, del7	448	448[a]	463,858	463,858[b]
4	England/Ireland	419 ins1	1401 ins1	140	468	140	511
5	England	1867+1, G→A	1989, G→A	623	663[a]	653,590	680,625[b]
6	America	419 ins1	1867+5, G→A	140	623	140	653,590
7	Poland (Ashkenazi Jewish)	2212 del1	2212 del1	737	737	746	746
8	Ireland	1867+5, G→A	1867+5, G→A	623	623	653,590	653,590
9	Not stated (Sharp et al. 1993)	215 del1	215 del1	72	72	78	78
10	Not stated (Sharp et al. 1993)	1783, C→T	1783, C→T	595	595	594	594
11	Not stated (Ricci et al. 1995)	2593, G→T	2593, G→T	865	865	864	864

[a] Last codon of exon preceding splice-site mutation.
[b] Cryptic splicing may give rise to additional translation products.

Table 4

Haplotype Arrangement of Polymorphic Sites of MTP Gene

Haplotype	5′ Region T/C	Exon 4 T/C	Exon 7 C/G (H297Q)	Intron 10 CA Repeat	3′ Region C/G	No. of Chromosomes (Frequency of Haplotype)
1	+	+	+	1	+	13 (.27)
2	+	+	+	2	+	5 (.10)
3	+	+	+	4	+	1 (.02)
4	+	−	+	4	+	2 (.04)
5	+	−	+	6	+	2 (.04)
6	+	+	−	1	−	1 (.02)
7	+	+	−	4	−	5 (.10)
8	+	+	−	5	−	1 (.02)
9	+	+	−	6	−	4 (.08)
10	+	−	−	2	−	8 (.17)
11	−	+	+	1	−	1 (.02)
12	−	+	+	2	−	1 (.02)
13	−	+	+	3	−	1 (.02)
14	−	+	+	4	−	1 (.02)
15	−	+	+	7	−	1 (.02)
16	−	−	−	1	−	1 (.02)
Total						48

Note.—+ = Presence of thymidine at positions 130 of the 5′ flanking sequence and 60 of exon 4, and presence of cytosine at positions 133 of exon 7 and 1047 of the 3′ noncoding region; and − = absence of same.

Figure 2 Sequence chromatograms showing MTP gene mutations. Symbols and numbering are as in panel *A*. Note that the chromatogram of the heterozygous 1401ins 1 mutation in patient 4 was derived by *Taq* DyeDeoxy™ Terminator Cycle Sequencing (Applied Biosystems) using cloned PCR product. Eight clones were analyzed, five of which bore the insertion. The region containing the 1989 guanine-to-adenine mutation in patient 5 was amplified by PCR, as described elsewhere (Shoulders et al. 1994), by using oligonucleotides MPCR1 and 5′ACCTCA-GCCTGATTTCTTGCAT3′. PCR products were genotyped by hybridization with ASOs—M1, 5′TGAGTTACCTGGCTACC3′, for the normal allele; and M2, 5′GGTAGCCAAGTAACTCA3′, for the mutant allele. The 1867+5, G→A mutation in patient 6 has previously been shown to be associated with aberrant splicing of MTP mRNA in patient 8 and her brother (Shoulders et al. 1994) (tables 1 and 3).

which encode amino acids 413–448 of the MTP protein. Since the exon skip would not result in a frameshift, the translation product would comprise 858 amino acids rather than the normal 894. Failure to splice intron 10 would generate an mRNA encoding 463 amino acids, 15 arising from the unspliced intron (table 3).

Sequence analysis of PCR-products from patient 5 revealed two 5′ splice-site mutations (fig. 2). One mutation substitutes guanine for adenine at the first base of the 13th intron and abolishes the invariant guanine-thymidine dinucleotide of 5′ splice junctions. It was present in the father and absent in the mother. Elsewhere we have shown (Shoulders et al. 1993) that a substitution of a guanine for an adenine at the +5 position of this particular splice junction is associated with the production of two abnormal mRNA species and no normal product. The second mutation was a guanine-to-adenine substitution at the −1 position of intron 14, which did not cause an amino acid change. It was present in the mother and absent in the father. To establish that the guanine-to-adenine change at the −1 position was not simply a polymorphism, PCR amplification products from 100 unrelated healthy White individuals, eight ABL patients, and the parents of patient 5 were hybridized with ASOs. Only the PCR product from patient 5 and his mother gave a positive signal with the M2 oligonucleotide, which bears the guanine-to-adenine change. The substitution alters the normal 5′ splice-site sequence, from 5′AG:GTAACT3′ to 5′AA:GTAACT3′, which differs, at two positions, from the 5′ splice-site consensus sequence, 5′AG:GT(A/C)AGT3′ (Shapiro and Senapathy 1987). Mutations at the −1 position are associated with aberrant splicing in a variety of human diseases, and, in every case, a guanine has been substituted by another nucleotide (Andrews and Markert 1992; Berg et al. 1992; Krawczak et al. 1992; Oshima et al. 1993; Hagiwara et al. 1994). If the intron 14 mutation caused exon skipping, it would generate a new reading frame after the codon for amino acid 622 of MTP (table 3). Failure to splice intron 14 would give rise to an mRNA encoding a translation product of 680 amino acids, 17 arising from the unspliced intron (table 3).

Haplotype Analysis of the Shared MTP Gene Mutations

Four intragenic polymorphisms and a dinucleotide-repeat sequence located within intron 10 of the MTP gene (figs. 1*A* and 3) were used to establish whether the exon 4 mutation in the families of patients 1, 4, and 6 and the intron 13 mutation (G→A, at the +5 position of the consensus splice site sequence) in the families of patients 6 and 8 had arisen as a result of independent mutations or were derived from common ancestors. Haplotype frequencies were derived from genotyping the families of 24 unrelated White, healthy subjects resident in England. Sixteen haplotypes were identified (table 4).

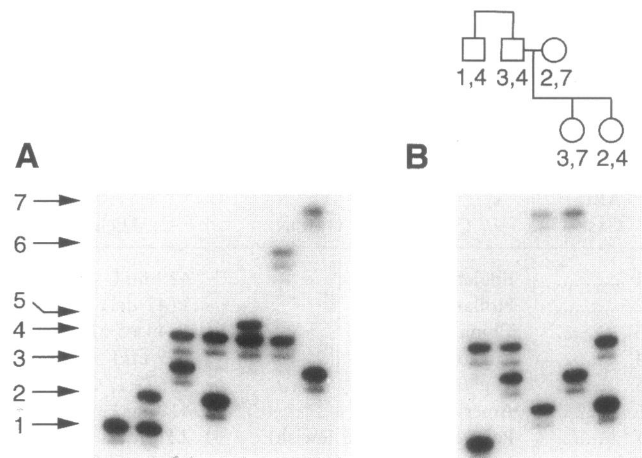


Figure 3 Autoradiographs showing genotype analysis of the CA-repeat sequence within intron 10 of the MTP gene. *A*, Seven alleles observed in 48 chromosomes. The smallest allele contains nine CA repeats. *B*, Segregation of MTP alleles in a nuclear family. Alleles 4 and 5 appear to differ by just one nucleotide; thus sequence variability, rather than the presence of an additional CA repeat in allele 5 relative to allele 4, is likely to account for the occurrence of this allele. Sequence variability in repetitive DNA is well documented (Jeffreys et al. 1991).

The exon 4 mutation in the families of patients 1 and 6 were found to be associated with the MTP haplotype 2 (table 4), which has a population frequency of .1. The intron 13 mutation in families 6 and 8, in contrast, was associated with the MTP haplotype 1, which has a population frequency of .27. These results suggest that the occurrence of the exon 4 mutation in the families of patients 1 and 6 is due to a common ancestor, as is the intron 13 mutation in the families of patients 6 and 8. Haplotype analysis of the MTP gene in patient 4 and her family, although incomplete, points to the same common ancestor for the exon 4 mutation.

Expression of Mutant MTP cDNAs in Cos-1 Cells

The results summarized in table 3 show that each of the mutant alleles, if translated, would encode a truncated form of MTP with a variable number of aberrant amino acids at its carboxyl terminus. The largest predicted protein encodes a polypeptide of 746 amino acids, 9 of which arise from an aberrant reading frame. Thus, the ABL phenotype in our patients could be caused by the presence of variant amino acids at the C-terminal end of MTP and/or by the lack of the normal amino acids. To evaluate this possibility, a series of deletions from the carboxyl terminus of MTP (i.e., −3, −8, −11, and −20 amino acids) were made and expressed in Cos-1 cells, which do not normally express MTP (Leiper et al. 1994).

Analysis of Cos-1 cell homogenates for MTP protein

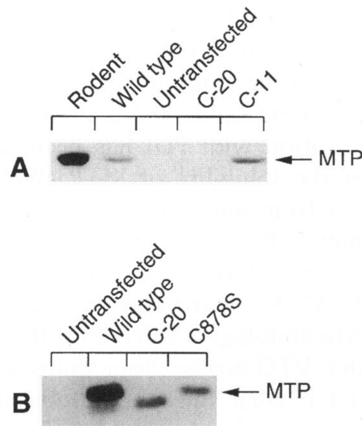


Figure 4 Western blot analysis of wild-type and mutant MTP cDNAs expressed in Cos-1 cells. Homogenates from rat intestinal extract, untransfected Cos-1 cells, and Cos-1 cells transfected with expression constructs encoding wild-type and mutant forms of MTP were size-fractionated by SDS-PAGE electrophoresis and were immunoblotted. Immunoreactive products were detected (*A*) with two rabbit anti-MTP peptide antisera, diluted 1:500, raised against amino acids 598–618 (Ab/MTP/598–618) and 841–859 (Ab/MTP/C00H) of human MTP, and (*B*) with one antibody, Ab/MTP/C00H; in *B*, overexposure of the film for the detection of wild-type MTP permitted visualization of the C-20 and C878S immunoreaction products. Panels show the results of two independent experiments.

and lipid-transfer activity showed that cells transfected with wild-type, C-3 (western-blot data not shown), C-8 (western-blot data not shown), and C-11 MTP cDNA constructs contained immunoreactive proteins of the predicted size and had comparable triglyceride-transfer activities (fig. 4*A* and table 5). In contrast, cell homogenates prepared from Cos-1 cells transfected with the C-20 construct were devoid of triglyceride-transfer activity and contained no immunoreactive product of the predicted size (fig. 4*A*), despite a level of MTP mRNA expression that was comparable to that in wild-type MTP (data not shown). Instead, a faster mobility product that migrated with an apparent molecular weight of ~88 kD, compared with 95 kD for the wild-type protein, was observed (fig. 4*B*). The level of expression of this variant protein was typically very low (<15% of wild-type MTP) but showed some variation from experiment to experiment. It was recognized by rabbit anti-MTP peptide antisera raised against amino acids 841–859 of human MTP (fig. 4*B*). This result suggests that extensive clipping of the C-terminal end of the C-20 protein does not account for its apparently smaller-than-predicted size.

Since a cysteine residue at position 878 is lost in the C-20 truncated protein, we considered that this might affect the formation of a disulfide bond essential for the tertiary structure of the protein. To examine this possibility, a missense C878S mutation was introduced into wild-type MTP and was expressed in Cos-1 cells. The resulting homogenates contained low levels of an immunoreactive product of the same size as wild-type MTP (fig. 4*B*) but had no detectable levels of triglyceride-transfer activity (table 5). By contrast, a conservative mutation of methionine 877 to valine had no effect on triglyceride-transfer activity (table 5).

Discussion

The present study and previous work (Sharp et al. 1993; Shoulders et al. 1993; Ricci et al. 1995) establish that mutations of the MTP gene, on chromosome 4q22-24 (Sharp et al. 1994), are the principal cause of classical recessive inherited ABL. This observation and the finding that the expression of MTP with apoB in Hela and Cos-1 cells is sufficient to mediate the secretion of triglyceride-rich lipoproteins (Gordon et al. 1994; Leiper et al. 1994) suggest it as a new target for the development of pharmacological agents for treating, by suppression of apoB lipoprotein production, those forms of hyperlipidemia (e.g., familial combined hyperlipidemia and hyperapobetalipoproteinemia) associated with their overproduction (Venkatesan et al. 1993).

Eleven affected probands with ABL have now been studied, and a mutation of the MTP gene has been identified in both alleles of all individuals (Sharp et al. 1993; Shoulders et al. 1993; Ricci et al. 1995; present study). Eight probands were found to be homozygous, six for a private family mutation, whereas three patients, 4–6, were compound heterozygous; patient 5 had two different private family mutations, patient 4 had a shared and a private family mutation, and patient 6 had two shared mutations. Thus, a total of 11 different mutations of the MTP gene have now been identified, and all but 2 are predicted to cause a significant truncation of the encoded

Table 5

Triglyceride-Transfer Activity in Cos-1 Cells

MTP cDNA Construct	Lipid-Transfer Activity[a] (pmol/triglyceride/mg/h)
Wild type	192
C-3	141
C-8	153
C-11	175
C-20	7
M877V	181
C878S	3
Untransfected	0

[a] Protein determinations and MTP activity were performed on partially purified extracts from transfected Cos-1 cells. Values represent the results of a single experiment; similar findings were obtained in three independent experiments. Activity of homogenates from Cos-1 cells transfected with the C-3, C-8, C-11, and M877V constructs were 70%–120% of wild-type activity.

protein (table 3). The results of our haplotype analysis suggest that the two mutations that occur in more than one family are each likely to have arisen from common ancestors. The similar ethnic background of the families is consistent with this notion. The shared intron 13 mutation was found in Irish siblings and a White "American" family of "British" ancestry, whereas the shared exon 4 mutation was found in the same "American" family, an "English," and an Anglo/Irish family. Other reports had suggested that consanguinity was present in >50% of ABL families (Herbert et al. 1982). This proposition attests to the rarity of the condition and is supported by the description of the mutations in the patients studied to date.

The MTP complex transfers triglyceride and cholesteryl esters between donor and acceptor membranes by a ping-pong bi-bi kinetic reaction (Atzel and Wetterau 1993), suggesting a shuttle mechanism for the transfer of lipids from their sites of synthesis, in the ER membranes, to apoB as it is synthesized and translocated across the ER membranes. Thus, although previous studies have suggested that ABL might be caused by a defect of translocation of apoB into the ER (Thrift et al. 1992), the findings of the present study support the concept that failure of ApoB lipidation is the primary cause of the ABL phenotype.

Elsewhere we have suggested that the lipid-binding and -transfer domains of MTP might involve the C-terminal end of the protein (Shoulders et al. 1994). Amino acids 666–736 of MTP are homologous to amino acids 389–458 of cholesteryl-ester–transfer protein (CETP), in a region of CETP that has been implicated in cholesteryl-ester binding and transfer (Tall 1993). In addition, MTP has extensive amino acid sequence homology with vitellogenin (VTG), an ancient transport and storage lipoprotein found in egg-laying vertebrates (Wahli 1988). X-ray crystallography studies have shown that amino acids 297–607 of VTG form a double-layered, curved super-helical structure containing 17 helices, whereas amino acids 791–945 adopt a β-pleated sheet structure, which forms one side of its large-lipid storage cavity (Raag et al. 1988; Timmins et al. 1992). In MTP, conservation of the helical region (amino acids 298–603), but not of the lipid-binding cavity, is predicted (Shoulders et al. 1994). This divergence is likely to reflect the different functional roles of the two proteins. VTG binds principally phospholipid with a stoichiometry of ~35 molecules/subunit and has no lipid-transfer activity. MTP favors neutral lipids with a stoichiometry of 1–5 molecules/subunit and also functions in the kinetic transfer of lipid to apoB. In the present study, we have shown that the cysteine at position 878 of MTP is critical for its function. The mutation of this residue to serine reduced the lipid-transfer properties of MTP to undetectable levels, possibly by affecting the formation of a disulfide bond and the tertiary structure of the C-terminal domain of the protein, rather than by directly interfering with its lipid-transfer activity, since deletions up to residue 883 did not affect activity.

Since dimerization with PDI has been shown to be obligatory for the solubility of MTP (Wetterau et al. 1990), we infer from our results that mutations at the carboxyl terminus do not affect this association, since homogenates prepared from Cos-1 cells transfected with the C-20 and C878S constructs contained soluble MTP protein (see Methodology). Indeed, on the basis of the observation that VTG forms a homodimer and that helices 13, 15, and 17 form the subunit interface, we have suggested that the molecular interaction of MTP and PDI may involve the corresponding helices, amino acids 517–603 of MTP (Shoulders et al. 1994). However, while this manuscript was in preparation, Ricci et al. (1995) reported that, when a truncated form of MTP lacking 30 amino acids at its carboxyl terminus was coexpressed with PDI in the baculovirus-expression system, all of the truncated protein was insoluble. This, Ricci et al. propose, was due to its lack of interaction with PDI. This result contrasts with the observation that the C-20 protein expressed in Cos-1 cells formed, albeit at reduced levels, a soluble protein for which PDI is known to be obligatory (Wetterau et al. 1990). This difference may indicate that amino acids 864–874 of MTP are critical for mediating MTP and PDI interaction, or it simply may reflect their importance in ensuring correct folding of the C-terminal end of MTP. Previous studies have established that proteins that are incorrectly folded, or subunits of oligomeric proteins that are not correctly assembled into a protein complex, undergo degradation (Klausner and Sitia 1990). It is noteworthy that, in the study by Ricci et al. (1995), the possibility that a proportion of the C-30 protein might have formed an association with PDI before being targeted to the degradative pathway was not formally excluded.

Our sequence analysis has revealed four protein polymorphisms—Q95H, I128T, Q244E, and H297Q—within the amino terminal domain of MTP. In contrast, no polymorphic residues were identified within either the C-terminal domain, comprising amino acids 604–894, or the predicted helical domain, comprising amino acids 298–603, of MTP (Shoulders et al. 1994), possibly suggesting that the tertiary structure and/or functional requirements of the amino terminus of MTP are more flexible than those of the putative helical and C-terminal domains. Indeed, although specific functions for the putative helical and C-terminal domains have been proposed, none has been forthcoming for the amino-terminal domain (Shoulders et al. 1994). However, the finding that the plasma cholesterol level of the mother of patient 5 who was homozygous for the rare Q244E allele was

below the 5th percentile of the normal population distribution (Lipid Research Clinics 1980) suggests that the protein polymorphisms observed in this region of the molecule might influence plasma lipid levels.

Finally, all but one of our patients presented with symptoms of fat malabsorption in infancy and thereafter were placed on a low-fat diet and received supplements of the fat-soluble vitamins A, E, and K. This treatment has prevented the development of neurological complications (Muller et al. 1977, 1983). The Dutch patient, in contrast, did not present early with malabsorption and was not diagnosed until her 3d decade of life, by which time the neurological complications of the condition were well advanced (Dullaart et al. 1986). This also appears to have been the case for one of the patients studied by Wetterau and colleagues (Wetterau et al. 1992; Sharp et al. 1993). The spectrum of mutations identified to date does not help to explain the age or symptomatic differences in presentation. No missense mutations have been found. Potentially these may have encoded a protein with residual lipid-transfer activity and therefore may underlie a milder or atypical phenotype. Instead, the mutant alleles in all but one patient (patient 3), who presented early, produce mRNAs that would, if translated, encode truncated forms of MTP. These would be anticipated to be devoid of lipid-transfer activity, as was found to be the case in a patient with a homozygous nonsense mutation affecting codon 865 (Wetterau et al. 1992; Ricci et al. 1995). The identification of agents that specifically inhibit MTP-catalyzed lipid transfer might provide a new approach for the treatment of those forms of dyslipoproteinemias associated with overproduction of VLDL-apoB.

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