Vitamin B<sub>12</sub> (cobalamin, cbl, B<sub>12</sub>) binds to 3 proteins in mammals: intrinsic factor (IF), transcobalamin (TC), and haptocorrin (Hc).<sup>1</sup> A deficiency of either IF or TC leads to hematologic or neurologic disorders. IF is essential for the intestinal uptake of B<sub>12</sub>, whereas TC is the critical plasma transporter that delivers B<sub>12</sub> to peripheral cells.<sup>2-4</sup> The main features of TC have been described in recent reviews.<sup>1,5,6</sup> The full-length complementary DNA (cDNA) contains an open reading frame of 1,284 nucleotides encoding a nonglycosylated protein of 409 amino acids with a molecular weight of 45 kd. The TC gene, composed of 9 exons and 8 introns, has been assigned to human chromosome 22. TC binds to vitamin B<sub>12</sub> with high affinity and is rapidly cleared from plasma. The cellular uptake involves a specific receptor-mediated endocytosis.

An early study revealed that TC in human sera has 4 common phenotypes, named X, S, M, and F, according to their isoelectric points (pI).<sup>5,8</sup> Subsequently, DNA sequencing of TC expressed in HT-29 cells trans- fected with Caco-2 TC complementary DNA and in 159 healthy Caucasians. We found that codon 259-R is buried and, thus, the genetic polymorphism provides no explanation why the TCs from HT-29 and Caco-2 cells have different isoelectric points in nondenaturing isoelectric focusing (IEF). The newly translated TC in HT-29 cells from the Caco-2 complementary DNA recombinant plasmid had the same isoelectric point as the TC constitutively expressed in HT-29 cells, suggesting that TC phenotypic variability involves a specific cell folding of the protein. The codon 259 polymorphism was found to have a biallelic distribution: homozygotes P = 34.6%, heterozygotes R/P = 47.8%, and homozygotes R = 17.6%. In heterozygous samples, the IEF showed that the TC 259-P/TC 259-R ratio = 1.6. The blood apo-TC concentration of 259-P < 259-R exhibited unequal concentrations, and have different pIs measured at 6.4 in native IEF and a pI at 6.7 in denaturing IEF. In nondenaturing IEF, HT-29 cells only show one phenotype with a pI at 6.4. The 2 TC variants expressed in HT-29 cells have a pI at 6.6 in native IEF and a pI at 6.7 in denaturing IEF. In nondenaturing IEF, HT-29 cells only show one phenotype with a pI at 6.4. The 2 TC variants expressed in HT-29 cells are only observed in denaturing IEF, exhibit unequal concentrations, and have different pIs measured at 6.6 and 6.8. This result is consistent with the hypothesis that codon 259 residue is buried and thus only creates a change in the net charge if the protein is unfolded after the effect of urea. This hypothesis implies that the codon 259 polymorphism does not generate TC phenotypic variability in nondenaturing conditions.

Introduction

Transcobalamin (TC) is the plasma transporter that delivers vitamin B<sub>12</sub> to cells. We have already reported that HT-29 and Caco-2 cells secrete different TC variants. HT-29 secretes 2 TC isoproteins (codon 259-Pro/Arg [259-P/R]), exhibiting unequal concentrations (TC 259-P > TC 259-R), and Caco-2 cells only secrete the phenotype 259-R. We investigated the relation between phenotypic and genetic TC polymorphism in HT-29 cells transfected with Caco-2 TC complementary DNA and in 159 healthy Caucasians. We found that codon 259-R is buried and, thus, the genetic polymorphism provides no explanation why the TCs from HT-29 and Caco-2 cells have different isoelectric points in nondenaturing isoelectric focusing (IEF). The newly translated TC in HT-29 cells from the Caco-2 complementary DNA recombinant plasmid had the same isoelectric point as the TC constitutively expressed in HT-29 cells, suggesting that TC phenotypic variability involves a specific cell folding of the protein. The codon 259 polymorphism was found to have a biallelic distribution: homozygotes P = 34.6%, heterozygotes R/P = 47.8%, and homozygotes R = 17.6%. In heterozygous samples, the IEF showed that the TC 259-P/TC 259-R ratio = 1.6. The blood apo-TC concentration of 259-P < 259-R exhibited unequal concentrations, and have different pIs measured at 6.4 in native IEF and a pI at 6.7 in denaturing IEF. In nondenaturing IEF, HT-29 cells only show one phenotype with a pI at 6.4. The 2 TC variants expressed in HT-29 cells are only observed in denaturing IEF, exhibit unequal concentrations, and have different pIs measured at 6.6 and 6.8. This result is consistent with the hypothesis that codon 259 residue is buried and thus only creates a change in the net charge if the protein is unfolded after the effect of urea. This hypothesis implies that the codon 259 polymorphism does not generate TC phenotypic variability in nondenaturing conditions.

Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood

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and hence cannot explain why HT-29 and Caco-2 cells display 2 different phenotypes, with a pI at 6.4 and 6.6, respectively. Therefore, an additional factor other than the codon 259 polymorphism is involved in generating TC phenotypic variability. This factor may be the existence of a differential signal peptide cleavage or a different protein folding in each cell line.

The aim of this work was to investigate the relation between the TC genetic polymorphism and the phenotypic pattern in Caco-2 cells, in HT-29 cells transfected (or not) with TC-cDNA expressed in Caco-2 cells, and in healthy Caucasians. The development of an amplification-refractory mutation system (ARMS) enabled us to genotype TC and determine the allelic distribution in a Caucasian population rapidly.

### Material and methods

#### Cell culture and blood collection

HT-29 and Caco-2 cells were cultured at 37°C in a 10% CO2 atmosphere. The medium was Dulbecco’s modified Eagle’s medium with 1% nonessential amino acids and decompemented fetal calf serum (10% for HT-29 and 20% for Caco-2 cells) (Life Technologies, Cergy Pontoise, France). The cells were used on day 15 after seeding.

Blood samples were collected on ethylenediaminetetraacetic acid from a group of healthy Caucasians originating from northern France (n = 159, mean age 59.1 ± 12.6 years, male/female ratio = 3). All tests were performed after informed consent and approval by the ethical committee of the university hospital.

#### Extraction of RNA and genomic DNA from HT-29, Caco-2 cells, and blood

Total RNA was extracted from the cell lines grown until day 15, using ultracentrifugation through cesium chloride gradients, and genomic DNA was prepared by the QIAamp spin column procedure (Quiagen, Hilden, Germany).

#### Preparation and cloning of the full-length TC cDNA expressed in Caco-2 cells

RNA (4 μg) was primed with an antisense-specific oligonucleotide AS5 (5′-1330 AAGCCTAGGGAGTGTGCGAGG-3′) located in the 3′ untranslated region and reverse transcribed to cDNA after 1 hour of incubation at 42°C in a 20 μL reaction volume containing 10 mM dithiothreitol, 500 μM deoxyribonucleoside triphosphate (dNTP), and 200 units Moloney murine leukemia virus reverse transcriptase (Life Technologies). Amplification was performed in a reaction volume composed of 10% of the reverse transcription (RT) solution, 200 μM dNTP, 10 pmol of a forward primer JLO1 (5′-15 CAGTGAA TTCCCACCA TGAGGCACCTTTG GGGCCCTTC21 3′) containing a Kozack sequence inserted between an GGGGCCTTC21 3′ allele specificity. The matched primers and the amplified fragment lengths are listed in Table 1. All amplifications were started in a 50 μL reaction volume containing 2 mM MgCl2, 200 μM dNTP, 10 pmol of each primer, and 1.25 units Taq polymerase (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ) and performed in the GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). The amplification program consisted of a 2-minute denaturation step at 94°C, followed by 30 cycles, each including 20 seconds at 94°C, 20 seconds at 58°C, 25 seconds at 72°C, and ending with a 10-minute step at 72°C. ARMS was first tested in HT-29 and Caco-2 cells, and specificity was demonstrated by comparing the size and number of the amplification products to the known DNA sequence. These 2 cell lines provided a reliable control for assessing codon 259 genotyping because HT-29 cells express the TC variant 259-R/P and Caco-2 cells only express the 259-R variant. After the reaction proved to be reproducible, it was tested on the Caucasian genomic DNA. The sequencing of 50 random samples gave identical results to the genotyping; thus, the specificity of the ARMS reaction was consequently demonstrated by the band pattern observed for each codon on 1.5% agarose gel.

#### Transfection of HT-29 cells with the TC cDNA expressed in Caco-2 cells containing plasmid

HT-29 cells were seeded at a density of 1 × 106 cells/cm² in a 75-cm² flask, allowed to grow for 1 day, and then transfected with 35 μg recombinant pcDNA3 using the calcium phosphate method. Two days after transfection, the supernatant was collected and tested for the presence of TC isoproteins. TC reaches peak levels in HT-29 and Caco-2 cells between day 10 and 15 in culture. Two cell populations were used to assess transfection yield: (1) nontransfected HT-29 cells grown for 3 days and (2) HT-29 cells transfected with a nonrecombinant plasmid.

#### TC genotyping by amplification-refractory mutation system

TC genotyping by ARMS was developed to detect the previously described TC substitutions at codons 198, 219, 234, 259, and 376 rapidly. For each codon, the forward primers used are specific to the single base change and the reward primer is common. The point mutation of interest is located at the last base of the forward primer’s 3′ end. In some of the forward primers an additional mismatch is introduced 3 bases from the 3′ end to ensure allele specificity. The matched primers and the amplified fragment lengths are listed in Table 1. All amplifications were started in a 50 μL reaction volume containing 2 mM MgCl2, 200 μM dNTP, 10 pmol of each primer, and 1.25 units Taq polymerase (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ) and performed in the GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). The amplification program consisted of a 2-minute denaturation step at 94°C, followed by 30 cycles, each including 20 seconds at 94°C, 20 seconds at 58°C, 25 seconds at 72°C, and ending with a 10-minute step at 72°C. ARMS was first tested in HT-29 and Caco-2 cells, and specificity was demonstrated by comparing the size and number of the amplification products to the known DNA sequence. These 2 cell lines provided a reliable control for assessing codon 259 genotyping because HT-29 cells express the TC variant 259-R/P and Caco-2 cells only express the 259-R variant. After the reaction proved to be reproducible, it was tested on the Caucasian genomic DNA. The sequencing of 50 random samples gave identical results to the genotyping; thus, the specificity of the ARMS reaction was consequently demonstrated by the band pattern observed for each codon on 1.5% agarose gel.

#### Relative evaluation of the concentration of the TC transcripts expressed in HT-29 cells

TC cDNA was obtained after an identical RT as above, except that the RNA was primed with 2 μL of a hexanucleotide mix 10 × (Boehringer Mannheim, Meylan, France). A cDNA fragment (176 base pairs) encoding the TC transcript, the cycle number corresponding to 50% of the maximal optical density intensity was determined.

#### Automatic sequencing

DNA was directly subjected to double-strand sequence analysis with AmpliTaq FS by the Dye deoxy terminator cycle sequencing method on the ABI 373 DNA sequencer (PE Applied Biosystems, Foster City, CA). The primers used were specific to the TC gene and spanned the nucleotide triplet corresponding to the target codon. The reaction volume was 20 μL containing 12 ng DNA and 3.2 pmol of specific primer. Thermal cycling was carried out for 25 cycles, each consisting of a 10-second step at 96°C, a 5-second step at 50°C, and a 4-minute step at 60°C. The amplification product was then ethanol-precipitated, loaded for overnight migration on a 6% polyacrylamide gel, and analyzed by the software.
CHOC: the cent homocysteine adduct is then isocratically separated by a C18 aminosulfonyl-7-fluorobenzo-2-oxa-1,3-diazole (ABD-F) for 5 minutes at liquid chromatography method (Bio-Rad, Ivry-sur-Seine, France). This Plasma homocysteine was determined by the Bio-Rad high-performance Homocysteine determination method. The tracer bound to HC was measured by centrifugation at 2000 \( \times g \) for 15 minutes at 10°C. The tracer bound to HC was measured by aspiration after 15 minutes at 10°C. The tracer bound to TC was measured by radioisotope assay with the SimulTrac-SNB kit (ICN Pharmaceuticals, Costa Mesa, CA).23 Determination of TC-[\( ^{57} \)Co]Cbl was performed as previously described,24,25 using a silica matrice (QUSO) to adsorb TC. Briefly, 3.75 g QUSO was suspended in 25 mL distilled water, and 100 \( \mu \)L stirred suspension was added to 500 \( \mu \)L plasma, incubated with \( [^{57} \)Co]Cbl, and centrifuged at 2000 \( \times g \) for 15 minutes at 10°C. The tracer bound to TC was measured by \( \gamma \)-counting in the supernatant. The tracer bound to TC was calculated from the difference between whole plasma and superna-
tant values.

Homocysteine determination
Plasma homocysteine was determined by the Bio-Rad high-performance liquid chromatography method (Bio-Rad, Ivry-sur-Seine, France). This technique involves trialkylphosphine reduction, derivatization with 4 aminosulfonil-7-fluorobenzo-2-oxa-1,3-diazole (ABD-F) for 5 minutes at 50°C, cooling, and deproteinization with trichloroacetic acid. The fluorescent homocysteine adduct is then isocratically separated by a C18 reversed-phase high-performance liquid chromatography cartridge and quantified by fluorescence.

Gel filtration
An aliquot of plasma or 10-fold concentrated cell supernatant (300 \( \mu \)L) was incubated with 20 \( \mu \)L (0.74 pmol) cyanol[\( ^{57} \)Co]Cbl (Amersham, Les Ulis, France), except for 3-day-old nontransfected cells incubated with 40 \( \mu \)L cyanol[\( ^{57} \)Co]Cbl. After 20 minutes shaking at room temperature, the sample was applied to a Sephaeryl S300 HR column (Pharmacia, Uppsala, Sweden) and eluted with 20 mM Tris HCl buffer, pH 7.4, containing 0.7 M NaCl, 0.01% Triton, and 0.01% Na2S at a flow rate of 0.3 mL/min. The fractions containing saturated TC (0.3 mL each) were collected for IEF.

Isoelectric focusing
IEF was performed in a 110 mL LKB column (Pharmacia, Uppsala Sweden) displaying a 5%–to-50% sucrose gradient with or without 5 M urea. Ampholines with pH 5 to 7 (Sigma, St Louis, MO) were added to a final dilution of 1:33. Focusing was run at 0.5 kV for 48 hours, and refrigeration was provided by a continuous water flow at room temperature. The pH values were measured with a combined electrode on a pH meter 535 multical microprocessor (WTW, Weilheim, Germany).

Radioisotope assays of serum folates and serum vitamin B12 and TC
Folates and unsaturated B12 binding capacity were quantified by radioisotope dilution assay with the SimulTrac-SNB kit (ICN Pharmaceuticals, Costa Mesa, CA). Determination of TC-[\( ^{57} \)Co]Cbl was performed as previously described, using a silica matrice (QUSO) to adsorb TC. Briefly, 3.75 g QUSO was suspended in 25 mL distilled water, and 100 \( \mu \)L stirred suspension was added to 500 \( \mu \)L plasma, incubated with \( [^{57} \)Co]Cbl, and centrifuged at 2000 \( \times g \) for 15 minutes at 10°C. The tracer bound to HC was measured by \( \gamma \)-counting in the supernatant. The tracer bound to TC was calculated from the difference between whole plasma and superna-
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Homocysteine determination
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Statistics
Data were expressed as the mean value \( \pm \) SD. One-way analysis of variance (ANOVA) was used to estimate the significant statistical differences between the genotypes followed by a Student \( t \) test. \( P < .05 \) was considered statistically different.

Results
TC genotypes and phenotypes in Caco-2 cells, HT-29 cells, and in HT-29 cells transfected with the plasmid-containing TC cDNA expressed in Caco-2 cells
Consistent with the previous data obtained by sequencing TC cDNA, the ARMS detected the TC codon 259 polymorphism that exists in HT-29 cells (TC 259-R/P) but not in Caco-2 cells (TC 259-R) (Figure 1). The next step consisted of investigating the phenotype of TC initially expressed in Caco-2 cells—after its encoding DNA was transfected into HT-29 cells—to determine whether the same protein had different pi values depending on the folding that occurs in the cell line in which it is expressed.

Table 1. Primers used in the ARMS reactions to genotype codons 198, 219, 234, 259, and 376 and the resulting amino acid in transcobalamin

<table>
<thead>
<tr>
<th>Codons</th>
<th>Forward primer containing the point mutation at the 3' end and the resulting amino acid</th>
<th>Reverse primer</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>198</td>
<td>PIS: 5’ acctgaag 581 AC ACA GCA GCA AT</td>
<td>ASP: 5’ 805 TGA GAC ATG CTG TTC CCA GTT 785 3’</td>
<td>358 base pairs (bp)</td>
</tr>
<tr>
<td></td>
<td>TACH: 5’ acctgaag 581 AC ACA GCA GCA AC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>219</td>
<td>RA: 5’ 637 CCT GGT CGG AGA CAA CAG A</td>
<td>ASP: 5’ 805 TGA GAC ATG CTG TTC CCA GTT 785 3’</td>
<td>294 bp</td>
</tr>
<tr>
<td></td>
<td>655 3’ → Ile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIN: 5’ 637 CCT GGT CGG AGA CAA CAG C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>655 3’ → Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>234</td>
<td>CIT: 5’ 682 GAG GAG ATC TTG AAG GCA CA</td>
<td>ASP: 5’ 805 TGA GAC ATG CTG TTC CCA GTT 785 3’</td>
<td>249 bp</td>
</tr>
<tr>
<td></td>
<td>701 3’ → Glu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RON: 5’ 682 GAG GAG ATC TTG AAG GCA CG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>701 3’ → Arg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>259</td>
<td>CAS: 5’ 757 CTC ATG ACT TCC CCC ATG CC</td>
<td>ERGE: 5’ 933 TGC CAG ACA GTC TGG GAA GA 914 3’</td>
<td>176 bp</td>
</tr>
<tr>
<td></td>
<td>776 3’ → Pro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIS: 5’ 757 CTC ATG ACT TCC CCC ATG CG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>776 3’ → Arg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>376</td>
<td>CHOC: 5’ 1108 TAT GAA ACA CAG GCC TCC</td>
<td>AS4: 5’ 1230 AGC AAT ACC TTG CAA CAG TGG 1210 3’</td>
<td>122 bp</td>
</tr>
<tr>
<td></td>
<td>TC 1127 3’ → Ser</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OLA: 5’ 1108 TAT GAA ACA CAG GCC TCC TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1127 3’ → Leu</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The point mutation of interest occurs at the 3’ terminus last base of the forward primers (bold). The additional mismatch introduced in some primers is underlined.
...Transfection experiment was repeated 3 times. TC secretion in cell culture medium was compared with the secretion of Hc, which is the only vitamin B12 binding protein present in fetal calf serum and is not secreted by HT-29 or Caco-2 cells. Consistent with data previously published, the supernatant gel filtration showed that HT-29 cells secreted very few TC, 3 days after the cells were plated (Figure 2A). The TC/Hc ratio, expressed as mean ± SD, was found to be 2.6 ± 0.9 and 0.45 ± 0.05 in cells transfected with the recombinant and wild-type plasmid, respectively (Figure 2B,C). This result meant that TC production in HT-29 cells transfected with the recombinant plasmid increased by 5.7-fold if compared with the HT-29 cells transfected with a nonrecombinant plasmid.

Accordingly, these cells should now contain the TC isoprotein constitutively expressed in HT-29 cells (expected to show a pI at 6.4 in native IEF) and the TC 259-R variant newly translated from the recombinant plasmid (expected to have a pI at 6.6 in native IEF). However, the supernatant focusing of HT-29 cells transfected with the recombinant plasmid showed a single isoprotein in native conditions, with a pI at 6.43 ± 0.02 expressed as the mean ± SD of triplicate experiments (Figure 3A). In denaturing IEF, the focusing between the TC isoprotein concentrations observed in HT-29 cells exists also at the transcriptional level. The separate amplifications of the truncated cDNAs encoding for a polypeptide containing either P or R at codon 259 revealed that the TC transcript 259-P variant is detected before the 259-R variant. After scanning, we observed that, for the 259-P variant, 50% of the maximal band intensity was reached at cycle 24. At this point the 259-R variant band intensity was 2.6-fold weaker than the 259-P variant (Figure 4). This result occurred in 3 independent experiments. We concluded that there were fewer TC 259-R than TC 259-P transcripts.

Relative quantitation of the TC transcripts in HT-29 cells

This experiment was carried out to determine whether the discrepancy between the TC isoprotein concentrations observed in HT-29 cells also exists at the transcriptional level. The separate amplifications of the truncated cDNAs encoding for a polypeptide containing either P or R at codon 259 revealed that the TC transcript 259-P variant is detected before the 259-R variant. After scanning, we observed that, for the 259-P variant, 50% of the maximal band intensity was reached at cycle 24. At this point the 259-R variant band intensity was 2.6-fold weaker than the 259-P variant (Figure 4). This result occurred in 3 independent experiments. We concluded that there were fewer TC 259-R than TC 259-P transcripts.

Distribution of TC genotype/phenotype in Caucasians and relation to plasma vitamin concentrations

Our purpose was to determine whether the relation observed between the codon 259 polymorphism and the TC phenotype in HT-29 and Caco-2 cells could be extended to plasma TC in healthy Caucasians. In the 159 DNA samples subjected to TC genotyping, no polymorphism was detected by ARMS at codons 198, 219, 234, and 376, where the residues present were Met, Ile, Gln, and Leu, respectively (Figure 1). However, a polymorphism was detected at codon 259 occupied either by P or R. In denaturing IEF, the homozygotes (R or P) showed only 1 isoprotein, whereas the heterozygotes showed 2 isoproteins with pIs at 6.7 and 6.9 and unequal concentrations because the TC 259-P/TC 259-R ratio was...
found to be 1.6 (Figure 3C-E). The overall codon 259 polymorphism distribution was as follows: homozygotes 259-P = 34.6%, heterozygotes 259-P/R = 47.8%, and homozygotes 259-R = 17.6%.

Serum were available from 132, 159, 135, and 150 genotyped individuals for determining plasma apo-TC, B12, folic, and homocysteine concentrations, respectively. The homozygotes 259-P, the heterozygotes 259-R/P, and the homozygotes 259-R had apo-TC median concentrations respectively at 589.9 ± 15.6, 544.4 ± 14.7, and 408.3 ± 16.5 μM. TC concentration in the homozygotes 259-P was found to be significantly higher than in the homozygotes 259-R (P < 0.0001), and TC concentration in the heterozygotes 259-R/P was significantly higher than in the homozygotes 259-R (P < 0.0006). The homozygotes 259-P had higher apo-TC concentration than the homozygotes 259-R and the heterozygotes 259-R/P taken together (P = 0.02) (Figure 5). These data indicate that the allele encoding for P at codon 259 determines a higher plasma apo-TC concentration than the allele encoding for R.

On the other hand, we found that the homozygotes 259-P, the heterozygotes 259-R/P, and the homozygotes 259-R had homocysteine median concentrations at 10.1 ± 0.4, 11.7 ± 0.4, and 10.2 ± 0.8 μM, respectively. The heterozygotes 259-R/P had homocysteine concentration significantly higher than the homozygotes 259-R and 259-P (P = 0.02 and P = 0.01, respectively) (Figure 5), but no significant difference was found between homozygotes 259-R and 259-P.

The ANOVA tests showed no significant difference concerning vitamin B12, contrary to what we reported previously in a small study.14 Neither was any significant difference noted for folates.

**Discussion**

The genetic basis for TC phenotypic variability could be explained by 2 mechanisms. The first is the splicing of the leader peptide at alternative sites. In this way, an amino peptidase cleaves after the triplet alanine-leucine-threonine, yielding a TC variant with glutamic acid as the first residue and, as an alternative to this primary processing, a second amino peptidase cleaves after the triplet threonine–glutamic acid–methionine, yielding a TC variant with cysteine as the first residue.15 The second mechanism is a genetic polymorphism consisting in the substitution of a neutral amino acid by an R residue and resulting in the modification of the pI value, thus inducing a new phenotype.11 Our results indicate that neither of these 2 mechanisms, taken separately, can plainly account for the generation of several TC isoproteins.

Indeed, DNA sequencing showed that HT-29 cells express 2 TC variants (TC 259-P or TC 259-R), whereas Caco-2 cells only express TC with R at codon 259. When the supernatants of Caco-2 and HT-29 cells are submitted to native IEF, a single TC isoprotein is observed for each cell line, with pIs measured at 6.6 and 6.4, respectively. The 2 isoproteins present in HT-29 cells are only observed in denaturing IEF, displaying pIs at 6.6 and 6.8. This result is consistent with the codon 259-R being a buried residue and thus bearing no charge in native conditions. The codon 259 genetic polymorphism does not explain the existence of different TC phenotypes detected in native IEF in HT-29 and Caco-2 cells. Considering the fact that codon 259 bears no charge in native conditions, there must be another factor than the genetic polymorphism that explains the phenotypic variability observed in native IEF between HT-29 and Caco-2 cells.

In the present study, we show that HT-29 cells, transfected with a plasmid-containing TC cDNA expressed in Caco-2 cells, express TC 259-R newly translated from the recombinant plasmid. The TC 259-R variant constitutively secreted by Caco-2 cells has a pI at 6.6 in native IEF. In the transfected HT-29 cells, TC 259-R translated from the recombinant plasmid is eluted in native IEF at fraction pH 6.43 (± 0.02), which is very close to pI = 6.4, the value measured for the TC 259-R constitutively secreted by HT-29 cells. This result indicates either that TC folding is specific to the cell line in which the protein is expressed or that there is an alternative signal peptide cleavage between HT-29 and Caco-2 cells.

In denaturing conditions, the TC 259-R variant expressed in Caco-2 cells has a pI measured at 6.7. In the transfected HT-29 cells, the 259-R isoprotein newly translated from the recombinant plasmid has a pI at 6.87 (± 0.04). This value is very close to that of the TC 259-R variant constitutively secreted by HT-29 cells (pI = 6.8), though it is not identical. In the light of these data, the alternative cleavage of the signal peptide is not sufficient to completely explain the phenotypic variability observed between Caco-2 cells and the transfected HT-29 cells. Indeed, we can hypothesize that, in Caco-2 cells, the TC 259-R variant begins with glutamic acid and, in transfected HT-29 cells, the TC 259-R variant newly translated from the recombinant plasmid begins with cysteine. Glutamic acid is negatively charged and cysteine is neutral, which could explain why, in denaturing conditions, the phenotype observed in the transfected HT-29 cells has a higher pI than the one observed in Caco-2 cells. This result is in contradiction with the IEF profile obtained in native conditions, where the phenotype observed in the transfected HT-29 cells has a lower pI (6.43) than the one observed in Caco-2 cells (6.6). The existence of a differential signal peptide cleavage between HT-29 and Caco-2 cells is only consistent with our results if the NH2 terminal residue of the TC protein is buried, implying the detection of charge modification only in denaturing conditions. Therefore, even though our results cannot rule out the existence of an alternative signal peptide cleavage (between HT-29 and Caco-2 cells), they provide support concerning the contribution of the genetic polymorphism in generating TC isoproteins and show that TC phenotypic variability is a multifactor-dependent phenomenon that includes a specific cell folding of TC.

Notably, the persistent difference in vitamin B12 levels between black and white people living in the United States26 gives further evidence of the existence of a genetic mechanism explaining TC phenotype generation even though it may be combined with environmental factors. ARMS (also known as ASO-PCR) is a technique that detects nucleotidic substitutions faster than DNA sequencing.27,28 Allele-specific amplification could not be achieved for all codons using forward ASOs having the allele-specific
mismatch at the 3’ terminus only. The introduction of degeneracy by inclusion of a second nonspecific mismatch into the 3’ portion of the ASOs resulted in more efficient allelic discrimination and detection, corroborating the findings reported by others.\textsuperscript{29,30} This ARMS method was validated in HT-29 and Caco-2 cells by comparing the amplification products obtained to the known TC cDNA. It was then applied to the analysis of genomic DNA from 159 healthy Caucasians to look for putative mutations on TC cDNA. The specificity of the codon genotyping was assessed by random sequencing on 50 of the 159 samples. Contrary to previous reports,\textsuperscript{10,11} we detected no substitution at codons 198, 219, 234, and 376, and this indicated that mutations in these positions are uncommon. Codon 259 is a major determinant of TC genetic polymorphism in the Caucasian population studied and exhibits biallelic distribution.

The denaturing IEF profile of TC isoproteins from heterozygous HT-29 cells or from heterozygous Caucasians was characterized by the existence of unequal concentrations between the TC 259-P and TC 259-R variants. The results observed after the relative RT-PCR quantitation of the TC transcripts expressed in HT-29 cells showed that a transcriptional mechanism is probably involved in determining this discrepancy. RNA secondary structure prediction provided further evidence reinforcing this hypothesis when it showed that the TC 259-P transcript had an additional stem loop that did not exist in the TC 259-R transcript, potentially inducing a greater stability of one transcript versus the other.

Another hypothesis for explaining the different TC isoprotein concentrations may be that HT-29 cells (or the heterozygous individuals) recruit the transfer RNA for synthesizing the TC 259-P variant more easily than the TC 259-R one. Indeed, P residue present in TC at codon 259 is encoded by CCU and R residue by CGU. Codon usage analysis reveals that the overall utilization frequencies of CCU and CGU by Homosapiens cells are 17.2% and 4.7%, respectively. In addition, R residue is encoded by 4 triplets, among which CGU displays the smaller utilization rate (the others being CGA 6.1%, CGC 11%, CGG 11.5%).

Among the Caucasian population, the denaturing IEF of serum from heterozygous subjects shows that the TC 259-P/TC 259-R ratio is 1.6. The radioisotopic assay shows that the apo-TC concentration ratio of homozygotes 259-P/Phomozygotes 259-R is approximately equivalent to 1.5 and subsequently is very close to the ratio observed for the 2 phenotypes in IEF. Moreover, assuming that TC has a biallelic distribution, one may wonder if the 1.6 ratio could be used to predict apo-TC serum concentration in the heterozygous group after determining, by radioisotopic assay, the apo-TC concentration in a group of homozygous subjects. In this case, we could write: predicted [c] R/P = [c] R + [c] P × 1/2 and [c] P / [c] R = 1.6 ⇒ predicted [c] R/P = \{ [c] R + [c] P \} × 1/2 = 530.4 pM, knowing that [c] R = 408 pM (see “Results”).

The predicted apo-TC serum concentration (530.4 pM) in heterozygous Caucasians is very close to the median value of the measured concentration obtained by the radioisotope assay (544.4 ± 14.7 pM), indicating that the TC 259-P/TC 259-R ratio observed in IEF is consistent with the apo-TC serum concentrations measured. This result provides additional support concerning the contribution of TC phenotypes in determining apo-TC serum concentration.

The allele encoding for P at codon 259 is responsible for determining high apo-TC concentrations as evidenced by the finding that homozygotes 259-P have higher apo-TC plasma concentrations than homozygotes 259-R (R < .0001) and homozygotes 259-R and heterozygotes 259-R/P taken together (P = .002). The lack of correlation between plasma vitamin B\textsubscript{12} and TC genotypes may be explained by the fact that the major part of plasma cobalamin is transported in blood bound to Hc\textsuperscript{31-33} although TC is the transporter responsible for the delivery of vitamin B\textsubscript{12} to peripheral cells. It is likely that plasma holo-TC, rather than apo-TC, is critical for vitamin B\textsubscript{12} bioavailability,\textsuperscript{34-36} but owing to the variations observed in measuring holo-TC, with 2 different radioisotopic tests, we were unable to analyze the relation between TC genotypes and plasma holo-TC.

We have found that heterozygotes 259 P/R have significantly higher plasma homocysteine concentration than homozygotes 259-R and 259-P (P = .02 and P = .01, respectively). However, the influence of TC genotype on homocysteine metabolism was not established by the present study because we found no significant difference in homocysteine concentration between the homozygotes P/P and R/R. If the correlation with plasma homocysteine is confirmed in larger series including different ethnic populations, the consequence would be that TC genotype affects homocysteine metabolism. The allele encoding for P at codon 259, by enhancing the intracellular vitamin B\textsubscript{12} bioavailability, would participate in lowering homocysteine levels, because the active form of vitamin B\textsubscript{12} methylcobalamin, is the coenzyme of methionine synthase, the enzyme responsible for the methylation of homocysteine to methionine.

In conclusion, the codon 259 TC polymorphism shows a biallelic distribution in a group of Caucasian individuals. TC genotype affects apo-TC plasma concentration: The TC 259-P variant yields higher concentrations than the TC 259-R variant. TC genotype may interfere on vitamin B\textsubscript{12} intracellular bioavailability and consequently on homocysteine metabolism, especially in populations with a limited vitamin B\textsubscript{12} status.

References


Transcobalamin codon 259 polymorphism in HT-29 and Caco-2 cells and in Caucasians: relation to transcobalamin and homocysteine concentration in blood

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