Hum Genet (1998) 102: 305–313

ORIGINAL INVESTIGATION

Regina Hühn · Heike Stoermer · Beate Klingele
Elke Bausch · Alberto Fois · Mariangela Farnetani
Maja Di Rocco · Joelle Boué · Jean M. Kirk
Rosalind Coleman · Gerd Scherer

Novel and recurrent tyrosine aminotransferase gene mutations in tyrosinemia type II

Received: 8 October 1997 / Accepted: 29 October 1997

Abstract Tyrosinemia type II (Richner-Hanhart syndrome, RHS) is a disorder of autosomal recessive inheritance characterized by keratitis, palmoplantar hyperkeratosis, mental retardation, and elevated blood tyrosine levels. The disease results from deficiency in hepatic tyrosine aminotransferase (TAT). We have previously described one deletion and six different point mutations in four RHS patients. We have now analyzed the *TAT* genes in a further seven unrelated RHS families from Italy, France, the United Kingdom, and the United States. We have established PCR conditions for the amplification of all twelve *TAT* exons and have screened the products for mutations by direct sequence analysis or by first performing single-strand conformation polymorphism analysis. We have thus identified the presumably pathological mutations in eight RHS alleles, including two nonsense mutations (R57X, E411X) and four amino acid substitutions (R119W, L201R, R433Q, R433W). Only the R57X mutation, which was found in one Scottish and two Italian families, has been previously reported in another Italian family. Haplotype analysis indicates that this mutation, which involves a CpG dinucleotide hot spot, has a common origin in the three Italian families but arose independently in the Scottish family. Two polymorphisms have also been detected, viz., a protein polymorphism, P15S, and a silent substitution S103S (TCG→TCA). Expression of R433Q and R433W demonstrate reduced activity of the mutant proteins. In all, twelve different *TAT* gene mutations have now been identified in tyrosinemia type II.

R. Hühn · H. Stoermer · B. Klingele · E. Bausch · G. Scherer (✉)
Institut für Humangenetik und Anthropologie der Universität,
Breisacher Strasse 33, D-79106 Freiburg i. Br., Germany
Tel.: +49-761-270-7030; Fax: +49-761-270-7041;
e-mail: scherer@sun2.ruf.uni-freiburg.de

A. Fois · M. Farnetani
Istituto Clinica Pediatrica, Università di Siena, Via Bracci 16,
I-53100 Siena, Italy

M. Di Rocco
2A Divisione Medicina Pediatria, Istituto Gaslini,
I-16148 Genova, Italy

J. Boué
Centres d'Etudes de Biologie Prénatale, Château de Longchamp,
Bois de Boulogne, F-75016 Paris, France

J. M. Kirk
Royal Hospital for Sick Children, Sciennes Road,
Edinburgh, EH9 1LF, UK

R. Coleman
Department of Pediatrics, University of North Carolina
at Chapel Hill, CB 7220 Clinical Sciences Building,
Chapel Hill, NC 27599–7220, USA

Introduction

Tyrosinemia type II, also known as Richner-Hanhart syndrome (RHS), is an inborn error of aromatic amino acid metabolism with an autosomal recessive mode of inheritance. It results from a block in the transamination reaction converting tyrosine to p-hydroxyphenylpyruvate, a step catalyzed by the hepatic cytosolic enzyme tyrosine aminotransferase (TAT; E.C.2.6.1.5). RHS patients suffer from keratitis and palmar and plantar hyperkeratosis, accompanied by drastically elevated serum and urine levels of tyrosine and its metabolites. Mild or severe mental retardation may also be present. The condition improves rapidly on a tyrosine- and phenylalanine-restricted diet (for reviews, see Buist et al. 1985; Mitchell et al. 1995).

We have isolated cDNA and genomic clones for human *TAT* and have shown that the gene extends over 10.9 kb and consists of 12 exons, encoding a protein of 454 amino acid residues (Rettenmeier et al. 1990). The *TAT* locus was originally assigned to the q22-q24 region of chromosome 16 by somatic cell hybrid analysis and in situ hybridization (Natt et al. 1986; Barton et al. 1986). This assignment could be refined by the analysis of an RHS patient with a deletion of both *TAT* alleles, one a de novo interstitial cytogenetic deletion of the 16q22 region only, the other a submicroscopic inherited deletion that removed the entire *TAT* gene except for the 3' part of the 12th exon (Natt et al. 1987). In addition to this deletion case, we have previously identified causative point mutations in five

TAT alleles cloned from three RHS patients from Italy, France, and Japan (Natt et al. 1992).

To gain further insights into the molecular pathology of tyrosinemia type II, we have now analyzed the *TAT* genes in seven additional RHS families from various countries, including four families from Italy. This study has also been aimed at answering the question of whether RHS cases from Italy share a common *TAT* gene mutation. Because the incidence of tyrosinemia type II is higher in Italy than in other countries, with almost half of the 50 cases reported worldwide originating from this country (Buist et al. 1985; Fois et al. 1986; Mitchell et al. 1995), a founder effect could be a possible explanation for this observation.

Materials and methods

Patients

The molecular analysis of one of the Italian families, RHS3 from Lombardy, has been performed previously (Natt et al. 1992). The two affected sibs had untreated plasma tyrosine levels of 1.20 mM and 1.05 mM (normal range: 0.03–0.1 mM).

Three tyrosinemic individuals from a further large Italian kindred (RHS5; Garibaldi et al. 1979) from Calabria with two consanguineous marriages in two generations were available for study. PG (IV-9 in the pedigree) showed photophobia at the age of 4 months, when plasma tyrosine levels were 1.15 mM and a low-tyrosine diet was initiated. Subsequent development was normal but for slight mental retardation (IQ of 84). RM (IV-6) was diagnosed at the age of 4 years, because of plasma tyrosine levels of 1.90 mM. Cutaneous and ocular symptoms had begun at 5 months of age. Skin and ocular lesions disappeared after initiation of the special diet, but psychomotor development is impaired (IQ of 73). MF (III-9), mother of PG and aunt of RM, showed no clinical symptoms when first studied at 20 years of age but had a plasma tyrosine concentration of 1.0 mM.

Patient AC and his younger sister LC (family RHS6; Fois et al. 1986) were born to unrelated Italian parents from Tuscany. Age at onset of eye and skin lesions were at 1 months and later during infancy, respectively, for both sibs, whose psychomotor development was normal. Untreated plasma tyrosine levels were 0.80 mM for AC and 0.85 mM for LC, diagnosed at the age of 29 years and 27 years, respectively.

Patient MM is the first child of unrelated Italian parents from Tuscany (family RHS7; Fois et al. 1986). Keratitis was noted at the age of 3 months, and hyperkeratosis during infancy. He suffered from repeated convulsions. Untreated plasma tyrosine concentrations of 1.40 mM were diagnosed at 20 years of age. He has an unaffected sister.

Patient MV is the daughter of related parents from Basilicata (family RHS8; Fois et al. 1986), the parents being first cousins. Age at onset of keratitis was at 2 months. Skin lesions were absent, and psychomotor development was normal. Untreated plasma tyrosine levels of 1.10 mM were diagnosed at the age of 24 years. She has an unaffected brother.

Patient AN is the first child from a consanguineous marriage (family RHS9), the French parents being first cousins. She suffered from severe photophobia in the neonatal period. Bilateral keratitis was diagnosed at 2 months of age. Palmoplantar keratoses were absent, and psychomotor development was normal. Untreated plasma tyrosine levels were 1.30 mM. She has an unaffected sib.

Patient IR, the first child of unrelated Scottish parents (family RHS10; Barr et al. 1991), failed to thrive during the first few months of life. A plasma tyrosine concentration of 1.30 mM and an increased urinary excretion of tyrosine were the only abnormalities found. At 8 months of age, a low tyrosine diet was instituted. His younger sister KR, who presented with plasma tyrosine levels of 0.70 mM and 1.34 mM at 4 days and at 18 days of age, respectively, was subsequently put on a low tyrosine diet. Apart from repeated episodes of photophobia in the early years, both children, now in their teens, performed very well with respect to oculocutaneous sequelae, growth, and psychomotor development.

Patient DC from the USA is the only child of unrelated parents (family RHS12). He was referred at 11 months, because of photophobia, bilateral dendritic keratitis, and hyperkeratotic lesions on the tips of his fingers and toes. Psychomotor development was normal. Untreated plasma tyrosine levels were 1.50 mM.

Polymerase chain reaction and sequence analysis

The primers used for amplification of the *TAT* exons together with their exon/intron boundaries are presented in Table 1. Polymerase chain reactions (PCR) were generally carried out in a Perkin Elmer 9600 Thermal Cycler in a total volume of 50 µl with 10 pmol each primer, 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 150 µM each dNTP, and 0.2 U Super *Taq* polymerase (Stehelin, Basel). The cycling regime consisted of 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. For the primer pair Ki-5–2 and Le-3–1, the $MgCl_2$ concentration was 3 mM, and annealing was at 51°C. For half-nested PCR, 1/10 of the first round was used as the template for the second round. PCR fragments were purified by agarose gel electrophoresis, recovered from the gel with Jetsorb (Genomed), and sequenced directly by using one of the amplification primers as sequencing primer together with [α-^{35}S] dATP and Sequence kit version 2.0 (United States Biochemicals). For sequencing of the exon L mutation in RHS10, PCR products generated with primers Ki-5–2 and Le-3–1 were first cloned as *Eco*RI/*Xba*I fragments into *Eco*RI/*Xba*I-cut M13mp18, making use of an *Eco*RI site added to the 5' end of primer Le-3–1 and of an *Xba*I site within intron 11.

Single-strand conformation polymorphism analysis

Single-strand conformation polymorphism (SSCP) analysis was essentially carried out as described previously (Schmitt-Ney et al. 1995), except that radioactivity was omitted, and bands were visualized by silver staining (Herring et al. 1982). Electrophoresis was performed at 4°C for exons A–C, E+F, and G, and at room temperature in the presence of 10% glycerol for exon D.

Haplotype analysis

The 1.5-kb human cDNA clone hapto-6 (Raugei et al. 1983) was used to type for the haptoglobin alles *Hp1* and *Hp2* by probing *Pst*I Southern blots (Oliviero et al. 1985). For subtyping of *Hp1* alleles as *Hp1F* or *Hp1S*, the same cDNA clone was hybridized to *Xba*I Southern blots (Maeda et al. 1984). The probes and enzymes used to type the three biallelic restriction fragment length polymorphisms (RFLPs) B1 and B2, M1 and M2, and H1 and H2 at the *TAT* locus defining four major haplotypes were as described previously (Westphal et al. 1988; Wullich et al. 1989). The two *Taq*I RFLPs A1 and A2, and B1 and B2 that define four haplotypes at the *CTRB* locus were typed as described (Westphal et al. 1987).

Expression of mutant TAT proteins

To generate a clone that encompasses the entire coding region of human *TAT*, two partial cDNA clones were used, viz., clone M13mp18-L6 (unpublished), which contains a reverse transcription/PCR product extending from nucleotide 36 to 326 and which thus spans the ATG start codon at position 97, and clone phcTAT2-16 (Rettenmeier et al. 1990), which extends from nucleotide 249 to 2703 and which spans the termination codon at position 1459 (nucleotide numbering according to Rettenmeier et al. 1990). By using these two cDNA clones as templates and a forward primer with the ATG start codon at its 5' end and two different reverse primers in two rounds of PCR

Table 1 PCR primers used for amplification of *TAT* exons

Exon	Primer	Sequence[a]	Position[a]	Product in bp	Restriction fragments used for SSCP
A	Ap-5-1	GAGCTGGCTGCTGAGTTGTC	(–103)	284	
	Ai-3-1	GCCCTAGCTGGAAAAAGAAA	(+ 97)		
B	Bi-5-1	CTGTCCCCCAGCGCTATGT	(– 54)	387	AluI: 238, 149
	Bi-3-1	CCCCAACTGCCATCTTCTC	(+ 86)		
C	Ci-5-1	CTGAACATGGGAGAATAACC	(– 55)	258	
	Ci-3-1	CATCAGGAAAGTGAAGAGGA	(+ 98)		
D	Di-5-1	TGTGTCACTGCACTCCAAGTC	(– 88)	210	
	Di-3-1	AAGGGATAGTTTGCCCTAAAT	(+ 54)		
E+F	Ei-5-1	CCCCTAATGAAATTAACCTTT	(– 35)	600[c]	AluI: 400[c], 128, 66
	Fi-3-1	gaaTTCACACAACAGCTGAAG	(+ 62)		DdeI: 250[c], 200[c], 156
G	Gi-5-1	AAGCAATTCACATTAAAACTG	(– 44)	199	
	Gi-3-1	CTGTAATGAAACGATTAGCAG	(+102)		
H+I[b]	Hi-5-1	actgcAGACAAGAAAACAACCATAAC	(– 37)	670[c]	NT[d]
	Hi-5-2	ACAACCATAACAAGTCCCTC	(– 27)		
	Ii-3-1	CGCATCTCTGACTCCCAAAC	(+ 49)		
H+I+J[b]	Hi-5-1	actgcAGACAAGAAAACAACCATAAC	(– 37)	1130[c]	NT[d]
	Hi-5-2	ACAACCATAACAAGTCCCTC	(– 27)		
	Ji-3-1	agaATTCTGCTGAATGACTGCCC	(+ 69)		
K+L[b]	Ki-5-1	actgCAGTGATCAGTAGCACAG	(–123)	800[b]	NT[d]
	Ki-5-2	AAGTAGCCCCTACCTTAA	(– 88)		
	Le-3-1	aagaaTTCCCTAGATGGGACACA	(+ 41)		

[a] Primer sequences are from published (Rettenmeier et al. 1990) and unpublished sequences and are written 5'→ 3'. Nucleotides in *lower* case were included to create restriction sites. Numbers in *parentheses* give position of 5' nucleotide within intron preceding (–) or following (+) the corresponding exon; for Ap-5-1 and Le-3-1, the positions preceding the translation start site and following the termination codon are given, respectively

[b] Half-nested PCR, using primers 5-1 in first and 5-2 in second round

[c] Approximate size

[d] Not tested in SSCP

with *Pfu* polymerase, a 500-bp fragment extending from position 97 to 596 was generated. Following digestion with *Pst*I, a subfragment extending from position 97 to 529 was ligated to a 933-bp subfragment from phcTAT2-16 that extends from the *Pst*I site at position 530 to a *Stu*I site at position 1462 immediately downstream of the termination codon. The resulting 1366-bp cDNA fragment was blunt-end-ligated, during the same reaction, into the *Sma*I site of the glutathione S-transferase (GST) expression vector pGEX-3X (Pharmacia; Smith and Johnson 1988), resulting in clone GST-TAT. In-frame fusion with the coding region of the GST gene and the correct sequence of the *TAT* cDNA sequence amplified during PCR was confirmed by DNA sequence analysis (details of the cloning protocol can be obtained on request).

To introduce the exon L point mutations R433Q and R433W, PCR was performed with *Pfu* polymerase and clone GST-TAT as template with either forward primer M1A 5'-TGCAGCC*A*GATCC AGGAG-3' (nucleotide position 1387–1404, mutant nucleotide in italics) for R433Q or primer M1B 5'-TGCAGC*T*GGATCCAGGAG-3' for R433W together with reverse primer RP2 5'-CAGTCA CGA*GAATTC*CCCCTA-3', which spans an *Eco*RI site (italics) in the multiple cloning site of GST-TAT adjacent to the *Sma*I site used for cloning above. The resulting PCR fragments were used as reverse primers in a second PCR with GST-TAT as template and forward primer FP1 5'-GCGCATTTTGGACCCCTGTA-3' (position 1038 –1057). The PCR products thus obtained were cut with *Sma*I (position 1100) and *Eco*RI generating 395-bp fragments that were exchanged for the corresponding *Sma*I/*Eco*RI wildtype fragment within GST-TAT, resulting in mutant clones GST-TATR433Q and GST-TATR433W. The presence of the desired mutations and the absence of undesired mutations were verified by DNA sequencing.

Bacterial cultures (HB101) transformed with the wildtype or mutant constructs were grown in LB medium at 37°C to an OD600 of 0.6 and induced with 0.1 mM IPTG for 0, 30, 60, 120, and 240 min. Induction and correct size of the GST-TAT fusion proteins were confirmed by electrophoresis in 0.1% SDS/10% polyacrylamide gels after staining with Coomassie blue. For enzyme measurements, sonic extracts of cells induced for 60 min were used either directly or after heat treatment for 2 min at 72°C, employing the marked heat resistance of mammalian TAT (Hargrove et al. 1989). The TAT enzyme assay was according to Diamondstone (1966).

Results

PCR and SSCP analysis

The primers used to amplify the twelve *TAT* exons A–L, individually or in groups of two or three, by PCR are listed in Table 1, together with the sizes of the resulting PCR products. PCR conditions were optimized to produce unique PCR fragments (not shown; Hühn 1995). For exons H–L, this could only be achieved by employing half-nested PCR, whereas for exons A–G, a single round of PCR sufficed.

For six of the seven patients (one patient sample per family), exons A–G were screened for mutations by SSCP analysis. Patient RHS5 was not included, because the homozygous nonsense mutation E411X in exon L (see below) had previously been identified earlier in this study. For more efficient mutation detection, PCR-generated fragments larger than 300 bp were first cut with an appropriate restriction enzyme to generate smaller subfragments (Table 1). Abnormal migration patterns were detected in exon A for RHS patients 6 and 7 (not shown), in exon B for patients 6, 7 and 10, in exon C for patient 12, in exon D for patient 8, and in exon E or F for patient 9 (Fig. 1). This latter bandshift was more prominent in the 400-bp *Alu*I subfragment than in the smaller-sized *Dde*I subfragment (Table 1; not shown). No altered migration pattern was detected for exon G. A polymorphism in exon B was also detected by SSCP analysis (see below).

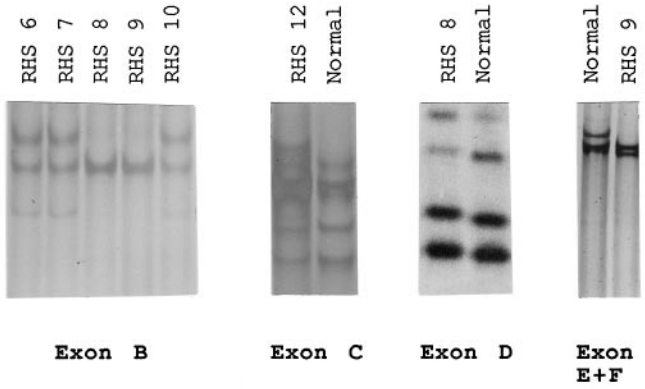

Fig. 1 SSCP analysis of PCR products from normal and mutant *TAT* alleles. PCR products from exons C and D were analyzed directly, those from exons B and E+F after digestion with *Alu*I. Only the upper part of the gels showing the bands derived from the larger AluI subfragments are shown for exons B and E+F. Denatured fragments were loaded on 6% native polyacrylamide gels and electrophoresed at 4°C, except for exon D, which was run at room temperature in the presence of 10% glycerol. DNA sequences of RHS PCR products with altered band patterns are presented in Fig. 2

Mutations

Fragments revealing altered migration behavior on SSCP gels were sequenced directly, by using the PCR primers as sequencing primers. In addition, exons H–L were sequenced for all seven RHS patients without a prior SSCP screen. Apart from the exon B polymorphism (see below), six different and three indentical point mutations were thus identified; these are documented by the autoradiographs shown in Fig. 2 and summarized in Fig. 5. When more than one affected individual was present in an RHS family, the *TAT* mutations that were first identified in one patient were subsequently confirmed in the other affected sibs.

The three identical point mutations generate a stop codon in place of an arginine codon at codon position 57 in exon B of alleles RHS6A (not shown), RHS7A, and RHS10B (Fig. 2A). This R57X mutation is present in heterozygous form in all three cases. Another homozygous stop codon mutation, viz., E411X in exon L of RHS5, is the result of a single T insertion that creates a frameshift. This immediately results in a TGA stop codon in place of the GAG glutamic acid codon 411 (Fig. 2E).

Four point mutations create amino acid substitutions. Two occur in homozygous form, one leading to the replacement of an arginine by a tryptophan residue (R119W) in exon D of RHS8 (Fig. 2C), the other to replacement of a leucine by an arginine residue (L201R) in exon F of RHS9 (Fig. 2D). The other two are heterozygous mutations affecting the same codon 433 in exon L, resulting in the substitution of the wildtype arginine residue by a glutamine (R433Q) or a tryptophan residue (R433W) in alleles RHS10A (Fig. 2F) and RHS12A (Fig. 2G), respectively.

A further heterozygous point mutation, in exon C of RHS12, is a silent substitution changing a TCG to a TCA codon, both encoding serine at position 103 (S103S; Fig. 2B). Because this silent substitution represents a polymorphism (see below), the second pathological *TAT* mutation for RHS12 remained unidentified. Moreover, no second *TAT* mutations were identified for RHS6 and RHS7.

Haplotype analyses

The R57X mutation found in heterozygous form in patients RHS6 and RHS7 from Italy and in patient RHS10 from Scotland has previously been found in patient RHS3, also from Italy, as a homozygous mutation (Natt et al. 1992). To determine whether this mutation was of common origin in the Italian cases, haplotype analyses were performed in the respective families, and also in the Scottish family. For these analyses, RFLPs at the *TAT* locus were employed, as were RFLPs at the haptoglobin (*HP*) and at the chymotrypsinogen B (*CTRB)* loci, which have been shown to be proximal and distal flanking loci for *TAT* at distances of 7 cM and 9 cM, respectively (Westphal et al. 1987).

As shown in Fig. 3, the two affected children from the RHS3 family, both homozygous for the R57X mutation, inherited the indentical extended haplotype from their parents, who are not known to be closely related. This allows for an unambiguous assignment of this particular haplotype to the R57X mutation in this family. The same haplotype is found as one of the two haplotypes in the two af-

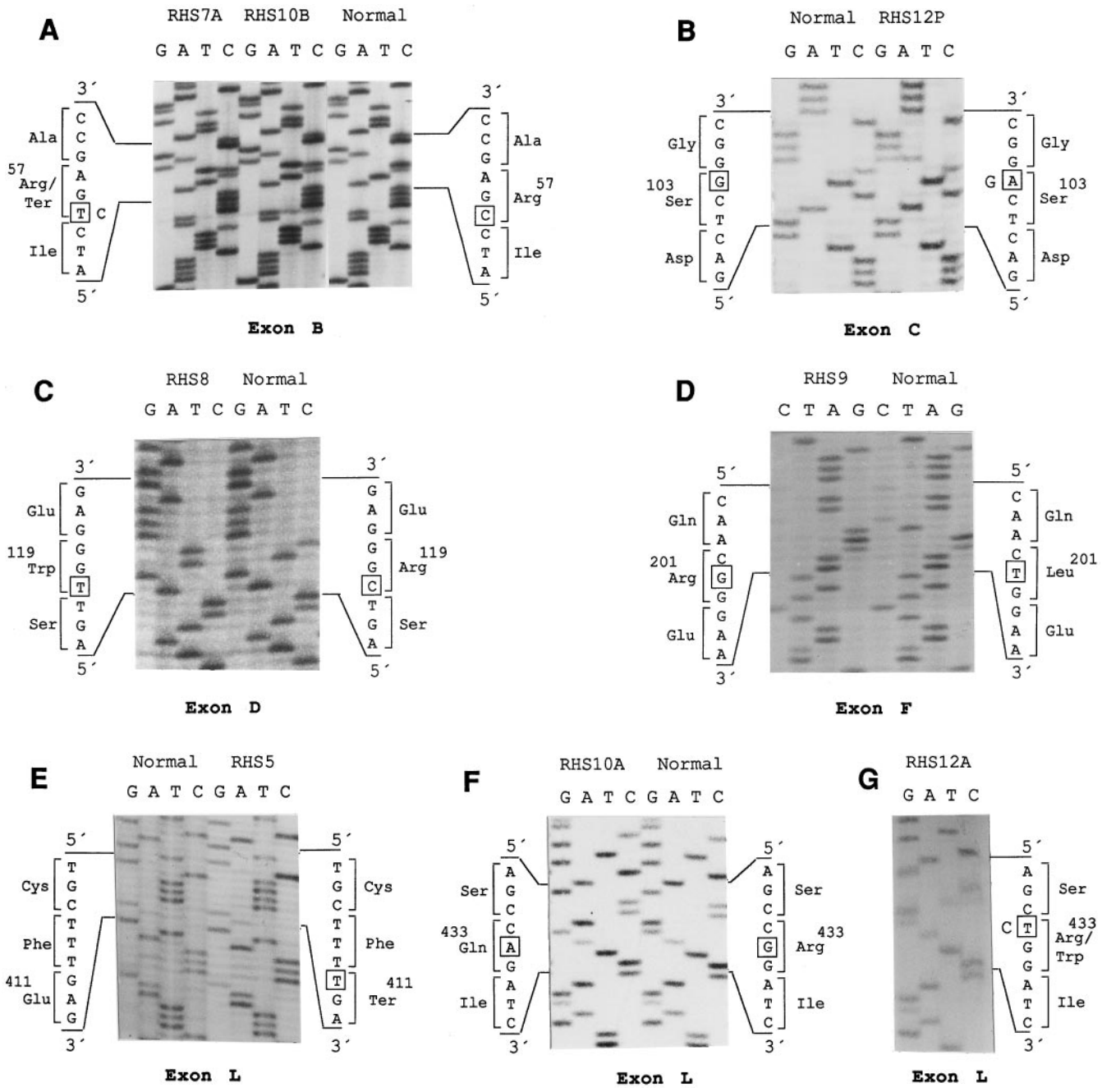

Fig. 2A–D *TAT* mutations identified in RHS patients. Autoradiographs are from 6% polyacrylamide sequencing gels, with ^{35}S-labeled sequencing products from normal and mutant *TAT* alleles run side by side. Fragments carrying suspected mutations were identified by SSCP analysis (Fig. 1) and were sequenced directly (**A–D**). Exon L mutations were identified by sequencing all RHS PCR products, without prior SSCP analysis; sequences shown in **E** and **G** are from uncloned PCR products, those shown in **F** are from a cloned normal (1 of 2 clones) and a cloned mutant allele (1 of 2 clones). The sequence read from the autoradiographs is of RNA-like polarity, if read from *bottom* to *top* (**A–C**) or from *top* to *bottom* (**D–G**). The reading frame is indicated by *brackets*. Nucleotides affected by mutations are *boxed*, and the resulting amino acid substitutions or stop codons (*Ter*) are indicated. Note that the silent substitution in exon C in RHS12 (**B**) is a polymorphism, and that a single T insertion created the stop codon in exon L of RHS5 (**E**). The mutations are summarized in Fig. 5

fected sibs in family RHS6 and in the affected son in family RHS7, who are heterozygous for the R57X mutation. Their second haplotypes are different and specific for the individual pedigree. Although it is not possible clearly to assign the R57X mutation to one or the other haplotype in the RHS6 and RHS7 pedigrees, it seems likely that this mutation is associated with the haplotype common to families RHS3, RHS6, and RHS7. Because the two haplotypes

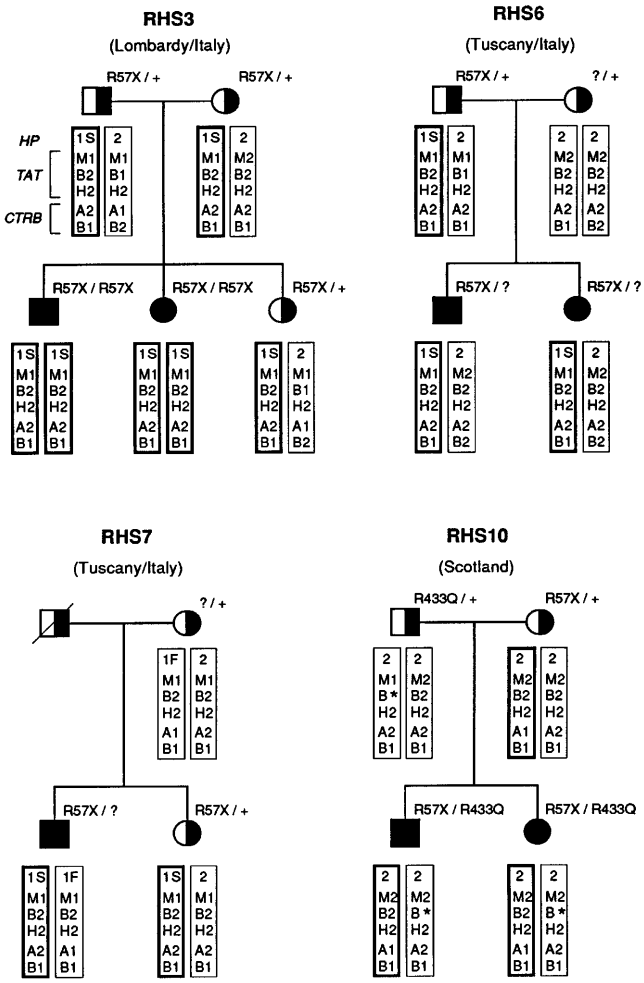

Fig. 3 Haplotype analyses in RHS families segregating for the R57X mutation. The *TAT* mutant genotype is indicated for each family member; unidentified *TAT* mutations in the RHS6 and RHS7 pedigrees are denoted by a *question mark*. Two RFLPs at the *HP* locus, three RFLPs at the *TAT* locus, and two RFLPs at the *CTRB* locus were used to generate the haplotypes. *B** in pedigree RHS10 denotes a new *Bam*HI fragment generated by and diagnostic for the R433Q mutation affecting a *Bam*HI site. Haplotypes associated with (RHS3, RHS10) or possibly associated with (RHS6, RHS7) the R57X mutations are *framed* in *thicker outline*

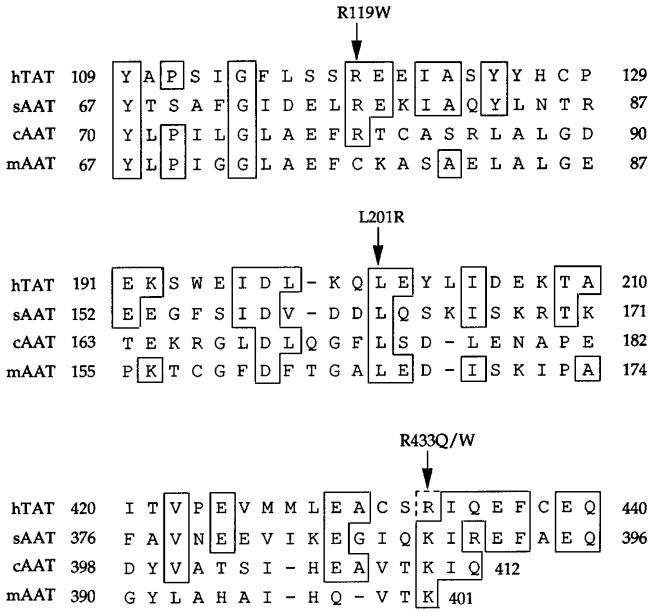

Fig. 4 Amino acid substitutions R119W, L201R, and R433Q/W affect residues conserved in other aminotransferases. Amino acid sequences in single-letter code from human TAT (hTAT; Rettenmeier et al. 1990) are compared with sequences from aspartate aminotransferases (AATs) from the archaebacterium *S. solfataricus* (sAAT; Cubellis et al. 1989) and from the cytoplasmic (cAAT) and mitochondrial (mAAT) AAT isoenzymes of pig (Mehta et al. 1989). Numbering of residues is indicated. Residues in human TAT conserved in the other transaminases are *boxed*. *Dashes* indicate gaps introduced to maximize alignments. The alignment is according to Cubellis et al. (1989), with modifications

found in the affected sibs of the Scottish family RHS10 differ from the Italian haplotypes, the R57X mutation found on allele RHS10A is clearly of independent origin.

Further support for a common origin of the R57X mutation in the Italian RHS families stems from the observation that the deletion of a CT dinucleotide at positions –8 and –7 in the promoter region of RHS3 (Natt et al. 1992) was also found in RHS6 and RHS7 but not in RHS10 (data not shown; Hühn 1995).

Polymorphisms

SSCP analysis of *Alu*I-restricted 387-bp PCR fragments from exon B not only revealed the band shifts in the larger 238-bp *Alu*I subfragments caused by the R57X mutation (Figs. 1, 2A), but also identified a polymorphic band pattern derived from the smaller 149-bp *Alu*I subfragments (not shown; Hühn 1995). DNA sequencing revealed that the slower migrating polymorphic band corresponds to a TCC codon at position 15 (encoding serine), whereas the faster migrating polymorphic band corresponds to a CCC codon at this position (encoding proline). The polymorphic SSCP banding pattern was used to assess the frequency of this P15S polymorphism in a sample of 30 unrelated individuals (60 chromosomes). The frequencies of the C (proline) and T (serine) alleles were 0.78 (47/60) and 0.22 (13/60), respectively. The silent substitution S103S (TCG→TCA) in exon C detected in patient RHS12 (Fig. 2B) destroys an *Ava*I and creates a *Dde*I restriction enzyme site. These diagnostic enzymes were used to analyze exon C PCR products from 40 unrelated individuals (80 chromosomes), giving frequencies for the TCG and TCA alleles of 0.85 (68/80) and 0.15 (12/80), respectively.

Expression of mutant TAT proteins

The four amino acid substitutions R119W, L201R, R433Q, and R433W detected in mutant TAT proteins affect resi-

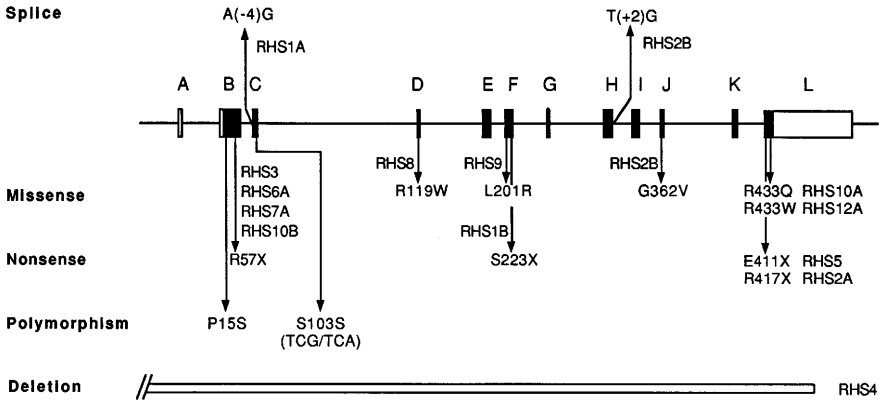

Fig. 5 Summary of *TAT* mutations identified in RHS patients. The positions of the six different mutations and of the polymorphisms identified in this study are given with respect to the exon/intron map of the *TAT* gene (Rettenmeier et al. 1990); coding sequences are indicated by *black boxes*, and 5' and 3' untranslated regions by *white boxes*. Mutations identified previously in RHS alleles 1A, 1B, 2A, 2B and 3 (Natt et al. 1992) and in RHS4 (Natt et al. 1987) are also included. Numbers indicate amino acid residues, given in single letter code. The nature of the mutations is indicated *left*. Note that the E411X nonsense mutation is caused by a frameshift; all other mutations are point mutations, except for the deletion. The RHS alleles on which the individual mutations were found are indicated. The countries of origin of the RHS patients are: Japan (RHS1), France (RHS2 and RHS9), Italy (RHS3, RHS5, RHS6, RHS7, and RHS8), USA (RHS4 and RHS12) and Scotland (RHS10)

dues conserved in rat TAT (Rettenmeier et al. 1990) and in other more distantly related transaminases, such as the cytoplasmic and mitochondrial aspartate aminotransferases from other mammals (Mehta et al. 1989) and the aspartate aminotransferase from the thermophilic archaebacterium *Sulfolobus solfataricus* which shares 25% sequence identity with human and rat TAT (Cubellis et al. 1989; Fig. 4). This high conservation over evolutionary time indicates an important functional or structural role for R119, L201, and R433 in human TAT. To determine whether the substitution of the arginine residue 433 by glutamine or tryptophan in RHS alleles 10A and 12A, respectively, has an effect on TAT enzyme activity or stability, recombinant TAT proteins harboring these mutant residues were expressed as fusion proteins with GST in *E. coli* strain HB101. Both GST-TATR433Q and GST-TATR433W showed drastically reduced TAT activity values when compared with the GST-TAT wildtype construct, close to the background levels seen in the untransformed HB101 strain (Table 2), establishing the pathological significance of the identified amino acid substitutions. Because both the wildtype and the mutant GST-TAT proteins are produced in equivalent amounts in *E. coli* (not shown), the observed reduction in enzyme activity probably results from an effect of the mutations on catalytic function rather than on enzyme stability.

Table 2 Expression of R433Q and R433W in *E. coli*

Strain	Specific TAT activity[a] (U/mg protein)	
	–	+
HB101	0.07	0.01
HB101 + GST-TAT	3.83	3.68
HB101 + GST-TATR433Q	0.13	0.09
HB101 + GST-TATR433W	0.21	0.04

[a] Activity determined according to Diamondstone (1996), without (–) or with (+) heat treatment of total bacterial sonic extract

Discussion

With the eight *TAT* mutations identified in this study and the seven *TAT* mutations identified previously (Natt et al. 1986, 1992), a total of 15 *TAT* mutations have now been described in 11 RHS patients and families from various countries, as summarized in Fig. 5. Whereas the R57X mutation was found repeatedly in four different RHS cases, all other *TAT* mutations were unique. Four patients, RHS3, RHS5, RHS8, and RHS9, are homozygous for their respective mutations, the remaining patients are compound heterozygotes. The probable pathological *TAT* mutations on both RHS alleles have been identified for three of these latter patients, viz., for RHS1 and RHS2 (who carries a "complex" RHS allele with two mutations) in previous work (Natt et al. 1992), and for RHS10 in the present report. We were unsuccessful in detecting the second *TAT* mutation in patients RHS6 and RHS7, who must be compound heterozygotes from the heterozygous state of the R57X mutation and from the observation that they have two different extended haplotypes in the *TAT* gene region (Fig. 3). Likewise, only a polymorphism but not the second pathological *TAT* mutation was identified in RHS12.

These failures may result from inefficiency in the SSCP screen for exons A–G, from overlooking the mutation in the sequence screen for exons H–L and their flanking intron sequences, or from the mutations residing in regions of the *TAT* gene not analyzed here. Patient RHS4 is a unique compound heterozygote with an inherited deletion removing most of the TAT gene (Fig. 5) and a de novo cytogenetic deletion removing the second *TAT* allele (Natt et al. 1987).

As to the molecular nature of the *TAT* mutations, all but two are single-base substitutions, the exceptions being the large deletion in RHS4 and the +1 frameshift in RHS5 that creates the E411X stop codon immediately following the single T insertion. This T insertion occurs at a TTT triplet that forms a phenylalanine codon, leading to a TTTT quadruplet (Fig. 2E). Runs of identical nucleotides are known to be prone to addition or removal of nucleotides as a cause of frameshift mutations, probably by a slippage mechanism (Cooper and Krawczak 1993). Of the ten different single-base substitutions in the *TAT* gene found in RHS (excluding the polymorphisms), five are CpG to TpG or CpA transitions, a mutational hot spot mechanism caused by desamination of a methylated C residue in the CpG dinucleotide on either the coding or non-coding strand and accounting for the majority of point mutations in human disorders (Cooper and Krawczak 1993). All five transition mutations affect arginine codons (R57X, R119W, R417X, R433Q, R433W), a particularly nice example being provided by the R433Q and R433W mutations, which change the same CGG arginine codon to a CAG glutamine or a TGG tryptophan codon, respectively (Fig. 2F, G). A sixth CpG hot spot mutation is the silent substitution S103S (Fig. 2B).

Four amino acid substitutions encoded by mutant *TAT* alleles were identified in this study (R119W, L201R, R433Q, R433W) and one previously (G362V). Only the R433Q and R433W substitutions were clearly shown to cause an essentially complete loss of TAT enzyme activity when expressed as recombinant proteins in *E. coli* (Table 2). A similar functional test with chimeric genomic constructs in a transient expression assay in eukaryotic cells has provided strong but not definitive evidence that the G362V mutation is also a loss-of-function mutation (Natt et al. 1992). As discussed in the report of the G362V substitution and as shown here for the R119W and L201R substitutions (Fig. 5), the respective wildtype residues are conserved in other transaminases over long evolutionary distances. Although this strongly indicates that the identified amino acid substitutions are pathologically relevant, a precise functional test as used for the R433Q/W mutations is needed to clarify this point.

In contrast to these conserved residues, the P15S amino acid polymorphism is probably irrelevant for TAT enzymatic activity, since the amino-terminal 64 residues can be removed from rat TAT with no loss of enzyme activity (Hargrove et al. 1989). Correspondingly, the N-terminal 64 residues in human TAT differ at 18 positions from those in rat TAT, which has a serine residue at position 15, whereas the remainder of the two 454-residue proteins differ at 17 positions (Rettenmeier et al. 1990). Moreover, the N-terminal extension present in TAT is absent from related transaminases (Mehta et al. 1989; Cubellis et al. 1989).

Five Italian RHS patients and their relatives were included in this and the previous study. This Italian sample includes three homozygotes, RHS3, RHS5 and RHS8, and two compound heterozygotes, RHS6 and RHS7, and thus represents seven RHS alleles. Three of these seven RHS alleles, viz., RHS3, RHS6A and RHS7A, were found to be indentical in carrying the same R57X mutation probably on the same extended haplotype (Fig. 3); alleles RHS5 and RHS8 carried the E411X and R119W mutation, respectively, whereas the mutations on alleles RHS6B and RHS7B are still unidentified. Including these two unknown mutations, five different *TAT* gene mutations are present in this sample. Therefore, the higher incidence of tyrosinemia type II in Italy (Buist et al. 1985; Mitchell et al. 1995) cannot simply be accounted for by spreading of one major *TAT* mutation. Only the R57X mutation appears to constitute a more prevalent mutation, which according to the haplotype data traces back to one founder individual. This founder probably lived in Northern Italy as indicated by the finding that the three RHS families carrying the R57X mutation are from the Northern provinces of Lombardy and Tuscany (Fig. 3), whereas families RHS5 and RHS8 are from the Southern provinces of Calabria and Basilicata, respectively.

No clear picture emerges from the present and our previous study with respect to genotype/phenotype correlations. Patients RHS1 and RHS2 are both compound heterozygotes for different splice and nonsense mutations (Fig. 5) that have been shown to be loss-of-function mutations (Natt et al. 1992), in accordance with the absence of TAT enzyme activity in liver biopsies from both patients (Lemonnier et al. 1979; Kida et al. 1982). However, untreated plasma tyrosine levels differed markedly, reaching 1.18 mM in RHS1 with normal psychomotor development, and 2.85 mM in RHS2 with severe mental retardation. The two affected sibs from the RHS3 family with untreated plasma tyrosine levels of 1.05 mM and 1.20 mM and normal psychomotor development are homozygous for the R57X mutation, which leads to a severely truncated TAT protein. On the other hand, patients PG and RM from the RHS5 family with untreated plasma tyrosine levels of 1.15 mM and 1.90 mM, respectively, and moderate mental retardation are homozygous for the E411X mutation, which only removes the C-terminal 44 residues from the mutant TAT protein. The impairment in mental development in the latter two patients cannot therefore be correlated with a more severe *TAT* mutation but probably results from a less strict dietary regime and/or from a less stimulating sociocultural environment. It should be noted, however, that MF, the mother of PG and aunt of RM, who has the same homozygous E411X mutation and an untreated tyrosine level of 1.0 mM, is of normal intelligence, although she has never followed a tyrosine-restricted diet (Garibaldi et al. 1979). The compound heterozygotes from families RHS6, RHS7, and RHS12 cannot yet be fully evaluated, because only the R57X mutation has been iden-

tified in the former two families, and only the R433W mutation has been shown to be a pathological mutation in family RHS12. The homozygous missense mutations R119W and L201R in RHS8 and RHS9, respectively, although not tested functionally, appear to be strongly inactivating mutations, as shown by the high untreated plasma tyrosine levels of 1.10 mM in RHS8 and 1.80 mM in RHS9. Finally, the strongly inactivating mutations R57X and R433Q found in the two compound heterozygotes from family RHS10 again correlate with the high untreated plasma tyrosine levels of 1.30 mM in the affected son and up to 1.35 mM in his affected sister.

In conclusion, all the various *TAT* mutations identified in this and in the previous study have been shown to be or are likely to be strongly inactivating mutations with respect to TAT enzyme function. It is, therefore, impossible at present to correlate variations in the clinical phenotype of RHS patients with any particular *TAT* gene mutation. A larger sample of RHS patients needs to be studied to ascertain whether such correlations are possible, and whether mild RHS alleles exist in patients with classical tyrosinemia type II.

Acknowledgements We thank E. Natt for providing TAT sequence information, E.-M. Westphal for part of the RFLP analysis in the RHS3 pedigree, D. G. D. Barr for referring the RHS10 patients for biochemical investigations, J. van Hove for a fibroblast culture from case RHS12, and all RHS families for their collaboration. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Sche 194/4–5) to G.S.

References

Barr DGD, Kirk JM, Laing SC (1991) Outcome in tyrosinaemia type II. Arch Dis Child 66:1249–1250

Barton DE, Yang-Feng TL, Francke U (1986) The human tyrosine aminotransferase gene mapped to the long arm of chromosome 16 (region 16q22q24) by somatic cell hybrid analysis and in situ hybridization. Hum Genet 72:221–224

Buist NRM, Kennaway NG, Fellman JH (1985) Tyrosinemia type II: hepatic cytosol tyrosine aminotransferase deficiency (the Richner-Hanhart syndrome). In: Bickel H, Wachtel U (eds) Inherited diseases of amino acid metabolism. Thieme, Stuttgart, pp 203–235

Cooper DN, Krawczak M (1993) Human gene mutation. Bios Scientific, Oxford

Cubellis MV, Rozzo C, Nitti G, Arnone MI, Marino G, Sannia G (1989) Cloning and sequencing of the gene coding for aspartate aminotransferase from the thermoacidophilic achaebacterium *Sulfolobus solfataricus*. Eur J Biochem 186:375–381

Diamondstone TI (1966) Assay of tyrosine transaminase activity by conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde. Anal Biochem 16:395–401

Fois A, Borgogni P, Cioni M, Molinelli M, Frezzotti R, et al (1986) Presentation of the data of the Italian registry of oculocutaneous tyrosinemia (abstract). J Inherit Metab Dis 9:262

Garibaldi L, Pregliasco P, Romano C, Siliato F, Durand P (1979) La tirosinosi oculo-cutanea. Un problema italiano? Ped Med Chir (Med Surg Ped) 1:279–284

Hargrove JL, Scoble HA, Mathews WR, Baumstark BR, Biemann K (1989) The structure of tyrosine aminotransferase. Evidence for domains involved in catalysis and enzyme turnover. J Biol Chem 264:45–53

Herring AJ, Inglis NF, Ojeh CK, Snodgrass DR, Menzies JD (1982) Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. J Clin Microbiol 16:473–477

Hühn R (1995) Mutationsanalyse im Tyrosinaminotransferase-Gen bei Patienten mit Tyrosinämie Type II. PhD thesis, Albert-Ludwigs-Universität, Freiburg

Kida K, Takahashi M, Fujisawa Y, Matsuda H, Machino H, Miki Y (1982) Hepatic tyrosine aminotransferase in tyrosinaemia type - II. J Inherit Metab Dis 5:229–230

Lemonnier F, Charpentier C, Odievre M, Larregue M, Lemonnier A (1979) Tyrosine aminotransferase isoenzyme deficiency. J Pediatr 94:931–932

Maeda N, Yang F, Barnett DR, Gowman BH, Smithies O (1984) Duplication within the haptoglobin *Hp2* gene. Nature 309:131–135

Mehta PK, Hale TI, Christen P (1989) Evolutionary relationships among aminotransferases. Eur J Biochem 186:249–253

Mitchell GA, Lambert M, Tanguay RM (1995) Hypertyrosinemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 1077–1106

Natt E, Kao F-T, Rettenmeier R, Scherer G (1986) Assignment of the human tyrosine aminotransferase gene to chromosome 16. Hum Genet 72:225–228

Natt E, Westphal E-M, Toth-Fejel SE, Magenis RE, Buist NRM, Rettenmeier R, Scherer G (1987) Inherited and de novo deletion of the tyrosine aminotransferase gene locus at 16q22.1→q22.3 in a patient with tyrosinemia type II. Hum Genet 77:352–358

Natt E, Kida K, Odievre M, Di Rocco M, Scherer G (1992) Point mutations in the tyrosine aminotransferase gene in tyrosinemia type II. Proc Natl Acad Sci USA 89:9297–9301

Oliviero S, De Marchi M, Bensi G, Raugei G, Carbonara AO (1985) A new restriction fragment length polymorphism in the haptoglobin gene region. Hum Genet 70:66–70

Raugei G, Bensi G, Colantuoni V, Romano V, Santoro C, Costanzo F, Cortese R (1983) Sequence of human haptoglobin cDNA: evidence that the α and β subunits are coded by the same mRNA. Nucleic Acids Res 11:5811–5819

Rettenmeier R, Natt E, Zentgraf H, Scherer G (1990) Isolation and characterization of the human tyrosine aminotransferase gene. Nucleic Acids Res 18:3853–3861

Schmitt-Ney M, Thiele H, Kaltwaßer P, Bardoni B, Cisternino M, Scherer G (1995) Two novel SRY missense mutations reducing DNA binding identified in XY females and their mosaic fathers. Am J Hum Genet 56:862–869

Smith DB, Johnson KS (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67:31–40

Westphal E-M, Burmeister M, Wienker TF, Lehrach H, Bender K, Scherer G (1987) Tyrosine aminotransferase and chymotrypsinogen B are linked to haptoglobin on human chromosome 16q: comparison of genetic and physical distances. Genomics 1:313–319

Westphal E-M, Natt E, Grimm T, Odievre M, Scherer G (1988) The human tyrosine aminotransferase gene: characterization of restriction fragment length polymorphisms and haplotype analysis in a family with tyrosinemia type II. Hum Genet 79:260–264

Wullich B, Natt E, Wienker TF, Scherer G (1989) A *Bam*HI RFLP at the human tyrosine aminotransferase (TAT) gene locus at 16q. Nucleic Acid Res 17:3331