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Molecular Cloning of the Human UMP Synthase Gene and Characterization of Point Mutations in Two Hereditary Orotic Aciduria Families

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Summary

Uridine monophosphate (UMP) synthase is a bifunctional enzyme catalyzing the last two steps of de novo pyrimidine biosynthesis, orotate phosphoribosyltransferase (OPRT) and orotidine-5′-monophosphate decarboxylase (ODC). Loss of either enzymatic activity results in hereditary orotic aciduria, a rare autosomal recessive disorder characterized by retarded growth, anemia, and excessive urinary excretion of orotic acid. We have isolated the UMP synthase chromosomal gene from a λEMBL-3 human genomic library and report a single-copy gene spanning ~15 kb. The UMP synthase genomic structure encodes six exons ranging in size from 115 bp to 672 bp, and all splicing junctions adhere to the canonical GT/AG rule. Cognate promoter elements implicated in glucocorticoid- and cAMP-mediated regulation as well as in liver-, myeloid-, and lymphocyte-specific expression are located within the 5′ flanking sequence. Molecular investigation of UMP synthase deficiency in a Japanese orotic aciduria patient revealed mutations R96G (A-to-G transition; nt 286) and G429R (G-to-C transversion; nt 1285) in one allele and V109G (T-to-G transversion; nt 326) in the other allele. Expression of human UMP synthase cDNAs containing these mutations in pyrimidine auxotrophic *Escherichia coli* and in recombinant baculovirus–infected *Sf*21 cells demonstrates impaired activity presumably associated with the urinary orotic acid substrate accumulations observed in vivo. We further establish the identity of two polymorphisms, G213A (v = .26) and 440Gpoly (v = .27) located in exons 3 and 6, respectively, which did not significantly compromise either OPRT or ODC function.

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Introduction

Hereditary orotic aciduria is a rare, autosomal recessive disorder of pyrimidine metabolism characterized by elevated orotic acid secretion in the urine, megaloblastic anemia, and failure to thrive (Webster et al. 1995). The condition results from low activity in one or both final steps of de novo pyrimidine biosynthesis, orotate phosphoribosyltransferase (OPRT; EC 2.4.2.10), which converts orotic acid to orotidine-5′-monophosphate (OMP), and OMP decarboxylase (ODC; EC 4.1.1.23), the terminal enzymatic reaction that decarboxylates OMP to uridine monophosphate (UMP). Except for a single description of orotic aciduria with ODC deficiency and concomitant OPRT elevation (McKusick 258920), all subsequent cases exhibit combined reductions in these enzymatic activities, a status termed "type I hereditary orotic aciduria" (McKusick 258900).

The concerted loss of OPRT and ODC activities initially suggested a defect in a regulatory gene shared by each enzyme (Krooth 1964; Worthy et al. 1974). However, the recent copurification and the cloning of a cDNA identified a bifunctional protein, labeled "UMP synthase" (UMPS), encoded by a single mRNA (Suchi 1988; Suttle et al. 1988). In contrast with OPRT and ODC of lower eukaryotes and bacteria, which exist as independent polypeptides transcribed from distant loci (Begueret et al. 1984; Poulsen et al. 1984; Turnbough et al. 1987), human UMP synthase was mapped to a single chromosomal locus within 3q13 by in situ hybridization (Qumsiyeh et al. 1989).

We previously carried out northern blot analyses of a cell line established from a type I orotic aciduria patient (Morishita et al. 1986). No alteration in size or quantity of UMP synthase mRNA was evident (Suchi et al. 1989). Southern blot hybridization of genomic DNA from another type I patient also did not detect gross rearrangements in the UMP synthase gene (Suttle et al. 1988). Moreover, the presence of normal mRNA size and quantity was independently confirmed by northern blot and S1 nuclease analyses (Winkler and Suttle 1988). Consideration of these and related data argues that UMP synthase, as expressed in type I patients, may contain amino

acid substitutions impairing steady-state levels (e.g., altered stability, folding, or proteolytic resistance), substrate binding, or catalytic efficiency. Indeed, isolated patient UMP synthase has demonstrated increased thermolability and altered molecular charges (Worthy et al. 1974; Perry and Jones 1989).

To clarify the molecular basis of hereditary orotic aciduria and to further explore UMP synthase deficiency, we have isolated the human UMP synthase gene. Also described are two polymorphisms and three disease-related point mutations occurring in two type I orotic aciduria families. Preliminary investigation of the mutant proteins, by expression in pyrimidine auxotrophic *Escherichia coli* and in *Sf*21 insect cells, illustrates functional deficits suggestive of a causal relationship with the increased urinary orotic acid deposition observed in vivo (Fox et al. 1973). Our data convey the first report of the complete human UMP synthase genomic structure and of mutations in the coding sequence. Knowledge of the gene, its promoter sequence, and the disease-related mutations presented will enable rapid identification of other genetic lesions and provide for significant advances toward understanding UMP synthase deficiency in hereditary orotic aciduria.

Subjects and Methods

Cloning and Characterization of the Human UMP Synthase Gene

A λEMBL-3 (European Molecular Biology Laboratory) human genomic DNA library (Tomatsu et al. 1989) was obtained from the Japanese Cancer Research Resources Bank. Plasmids, containing the entire coding region of the human UMP synthase cDNA (Suchi et al. 1989), were used to screen ~6×10^6 phage clones. *Eco*RI-digested cDNA fragments from pHMS18 and pHMS28 were labeled with [α-^{32}P]-dCTP (Amersham) by the random-primer technique (Feinberg and Vogelstein 1983). Phage plaques were transferred to Magna nylon membranes (Micron Separations) and hybridized with 5×10^5 counts/min (cpm) of probe per filter according to standard techniques (Sambrook et al. 1989). Restriction digests (*Eco*RI and *Hin*dIII; TaKaRa Shuzo, Japan) of phage DNA isolated from positive clones were separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with selected [^{32}P]-labeled human UMP synthase cDNA segments, i.e., the *Eco*RI-*Hinf*I fragment from pHMS18 and the *Hinf*I-*Bam*HI, *Bam*HI-*Bam*HI, *Bam*HI-*Sac*I, *Sac*I-*Pvu*II, and *Pvu*II-*Eco*RI fragments from pHMS28.

Exon-containing fragments were subcloned into pBluescript SK(+) (Stratagene) and labeled "pSK-hUMPS2E4," "pSK-hUMPS1E3," "pSK-hUMPS4H5," and "pSK-hUMPS4H3." One region, determined by PCR (Saiki et al. 1988), was amplified using primer sequence information from within pSK-hUMPS2E4 and pSK-hUMPS1E3, i.e., 5′ACGCTTTCTGGCACTGAAGC3′ and 5′CCATGACCATAAGTCTCTAGA3′. Thermocycling commenced with initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min, followed by a terminal 7-min extension at 72°C. The amplified genomic segment was cloned into pCRII vector (Invitrogen) and designated "pCR-hUMPS18-33."

Intron-exon junction and 5′ flanking sequence information was obtained by the dideoxy chain-termination method (Sanger et al. 1977) using synthetic oligonucleotide primers and Sequenase 2.0 (United States Biochemical). All custom DNAs were purchased from a commercial source (Rikaken). Intron sizes were determined by restriction mapping and/or by PCR, where 1 ng of plasmid DNAs served as templates. Amplification conditions were 20 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 3 min. Oligonucleotides used for intron size determination by PCR are provided in table A1 of the appendix. 5′ flanking sequence information was searched for relevant transcription factor binding sites using MacPattern version 3.4 (Fuchs 1994), Transcription Factor Database release 7.4 (Ghosh 1992), and the published transcription factor compilation by Faisst and Meyer (1992).

Subjects

Patient YF is the first reported Japanese diagnosed with hereditary orotic aciduria (Morishita et al. 1986). A case report detailing the clinical course and immunologic investigation of this family (Family 1) is described elsewhere (Yazaki et al. 1987). The patient is under a physician-supervised regimen of oral uridine supplementation (300 mg/kg/d) and is presently doing well. In the course of a screening program to identify purine and pyrimidine metabolism disorders among institutionalized mentally disabled patients, we encountered a second type I family (Family 2; fig. 1). A 3-year-old boy (YK) and his mother were identified who excreted high levels of urinary orotic acid (93.0 and 38.1 μmol/g creatinine, respectively; normal range is 13.7 ± 3.9 μMD/g at age 3 years and 4.9 ± 1.8 μMD/g for adults). No hematologic manifestations were evident, yet both OPRT and ODC activities in red blood cells were reduced to levels commonly observed among heterozygous individuals for hereditary orotic aciduria (Imaeda et al. 1995). On the basis of these and other findings, YK and his mother were diagnosed as heterozygotes for the metabolic error.

RNA Extraction, Reverse Transcription, and cDNA Amplification

Transformed B-cell lines (LCLs) from individuals in Family 1 were established from peripheral lymphocytes using B95-8–derived Epstein-Barr virus. LCLs were cultured in RPMI1640 medium and 10% heat-inactivated FCS (Gibco-BRL); patient YF's LCLs required an addi-

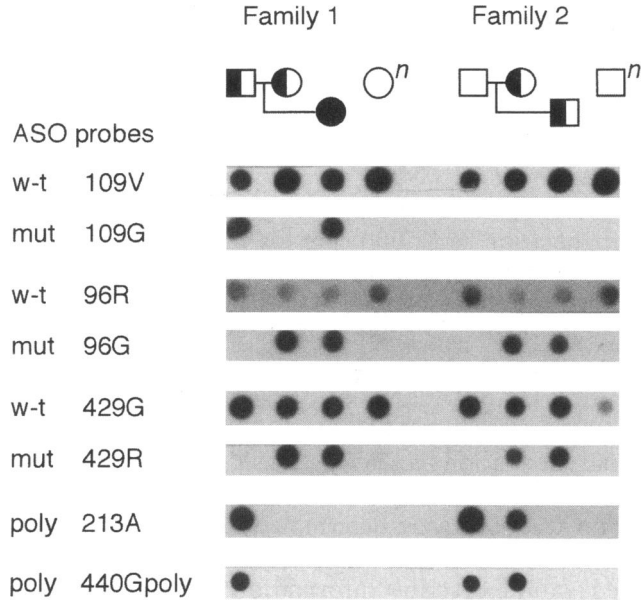

Figure 1 ASO hybridization analysis of PCR-amplified genomic DNA from selected members of Families 1 and 2. Amplicons spanning exons 2, 3, and 6 of the human UMP synthase gene were hybridized with ^{32}P-labeled oligonucleotides complementary to either wild-type ("w-t"; 96R, 109V, and 429G), mutant ("mut"; 96G, 109G, and 429R), or polymorphic ("poly"; 213A and 440Gpoly) allele sequence. "*n*" denotes unaffected and unrelated controls.

tional 10 μM uridine (Wako Pure Chemicals) for growth. Approximately 5×10^7 cells from each cell line were harvested, and total RNAs were isolated by the guanidine isothiocyanate method (Chirgwin et al. 1979). Ultracentrifugation was subsequently performed in a TLS-55 rotor (Beckman) at 40,000 rpm for 4 h, each tube containing 1.5 ml of cell lysate and 0.5 ml of a 5.7 M cesium chloride cushion. As specified by Werner et al. (1984), poly(A)+ RNA was prepared using messenger-activated paper (Orgenetics).

First-strand cDNA synthesis was carried out according to the manufacturer-recommended protocol with oligo-$(dT)_{12\text{-}18mer}$ or random hexamers and Super-Script II (Gibco-BRL). One set of oligonucleotide primers (5′AAACAGGCAGCGCGCGAC3′ and 5′GGAGGACCACATATCTTC3′) was designed to amplify the entire protein coding region of the human UMP synthase cDNA by PCR. Thermocycling conditions were 30 cycles of 94°C for 1 min, 55°C for 40 s, and 72°C for 1 min 30 s.

Cloning and Sequencing of Amplified cDNA

PCR-amplified products were phenol:chloroform extracted (1:1), ethanol precipitated, resuspended in 10 μl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and subcloned into pCRII vector. Nucleotide sequence information from the pCRII-UMP synthase cDNA subclones of patient YF and her parents was determined by dideoxy chain termination using UMP synthase cDNA–specific oligonucleotide primers. For each PCR amplification, at least six subclones were sequenced across the entire UMP synthase coding region.

Genomic DNA Preparation, PCR Amplification, and Dot Blot Hybridization

Peripheral leukocyte genomic DNA was isolated as described by Baas et al. (1984) from selected members of Families 1 and 2 (fig. 1) and from 50 unrelated, healthy Japanese individuals following informed consent agreement. The prevalence of mutations R96G, V109G, and G429R found in YF's cDNA, as well as of base changes associated with polymorphisms G213A and 440Gpoly detected in a cDNA from YF's father, was assessed by dot-blot hybridization. Four regions within the UMP synthase gene, containing the putative mutant or polymorphic sites, were amplified by PCR. Thermocycling conditions were 30 cycles of 94°C for 40 s, 54°C (57°C, 52°C) for 40 s, and 72°C for 40 s. Oligonucleotides used in these amplifications are provided in table A2 of the appendix.

PCR products (10 μl) were mixed with an equal volume of 4× SSC and heat denatured at 95°C for 5 min. The denatured samples were blotted to Hybond-N+ nucleic acid transfer membrane (Amersham) via a Bio-Dot apparatus (BioRad). Filters were hybridized with allele-specific oligonucleotides (ASO) labeled with [γ-^{32}P]-ATP (>5,000 Ci/ml; Amersham) and T4 polynucleotide kinase. The ASOs were 5′TGCTTATTAGAAGGAAA3′ and 5′TGCTTATTGGAAGGAAA3′ for 96R and 96G, respectively; 5′GCGTCTTGTAGAAGGAA3′ and 5′GCGTCTTGGAGAAGGAA3′ for 109V and 109G, respectively; 5′CATAATGGTTCTCCCCT3′ and 5′CATAATGCTTCTCCCCT3′ for 213G and 213A, respectively; 5′ATAATCTTGGCCAACAG3′ and 5′ATAATCTTCGCCAACAG3′ for 429G and 429R, respectively; and 5′GTTATTGGCAAACGAGG3′ and 5′GTTATTGGTAAACGAGG3′ for 440G and 440Gpoly ("440Gpoly" polymorphism), respectively. Hybridizations, performed in 5× saline sodium phosphate EDTA, 5× Denhardt's solution, and 0.5% SDS for 14 h at suitable temperatures, are summarized in table A3 of the appendix. Following hybridization, the blots were washed at room temperature for 30 min in 6× SSC containing 0.1% SDS and then twice for 30 min in the appropriate final wash solutions and temperatures. Probe hybridization was visualized by autoradiographic exposure of New RX Medical X-ray film (Fuji).

Construction of UMP Synthase Expression Plasmids

For transformation of pyrimidine auxotrophic *E. coli* strains, five UMP synthase cDNA subclones from Family 1 were selected for expression vector construction. Those selected were TA-Pat7, a clone of the "normal" paternal cDNA encoding two polymorphisms at nucleo-

tide positions 638 and 1320 (G213A and 440Gpoly) relative to the translation $(A)_{+1}TG$ start site; TA-Pat9, a clone of the paternal disease-related transcript containing the V109G mutation; TA-Mat34, encoding the cDNA of the maternal disease-related allele (R96G+G429R); TA-UMPS/G429R, a cDNA clone encoding the maternal G429R mutation; and TA-Mat36, a clone of the normal maternal cDNA. Several PCR errors were noted during confirmation among the constructs. Extra modifying steps were necessary for cassette removal and repair of segments containing mutations. An *Xho*I site centered at nt 549 of the UMP synthase cDNA and an *Xho*I site in the pCRII vector were utilized for this purpose.

To align the UMP synthase cDNAs in the sense orientation and in frame with respect to the β-galactosidase transcriptional unit, the resulting modified plasmids, TA-Pat7-36, TA-Pat9-36, and TA-Mat34-36, together with TA-Pat7 and TA-Mat36, were digested with *Eco*RI and religated into *Eco*RI-digested pCRII vector. Insert orientations were confirmed by *Bam*HI restriction analysis. The final prokaryotic expression constructs were dubbed: TA-UMPS, TA-UMPS/rev, TA-mock, TA-UMPS/poly, TA-UMPS/V109G, and TA-UMPS/R96G for constructs containing normal UMP synthase, UMP synthase in the reverse orientation, vector with no insert, and UMP synthase mutations G213A with 440Gpoly, V109G, and R96G mutations, respectively. A construct containing both maternal mutations (R96G and G429R; TA-UMPS/R96G+G429R) was made from TA-UMPS/R96G and TA-UMPS/G429R utilizing the aforementioned *Xho*I sites.

Expression of UMP Synthase in Uridine Auxotrophic Bacteria

Pyrimidine auxotrophic *E. coli,* AT722 and SK1105, were supplied by the National Institute of Genetics, Genetic Stocks Research Center, Japan. AT722 is a *pyr*E strain without detectable OPRT activity, and SK1105 is a *pyr*F strain lacking ODC activity; both strains require media supplementation of 100 μg/ml uridine for growth. Bacterial expression constructs TA-UMPS, TA-UMPS/rev, TA-mock, TA-UMPS/poly, TA-UMPS/V109G, TA-UMPS/R96G, TA-UMPS/R96G+G429R, and TA-UMPS/G429R were transformed into competent AT722 and SK1105 cells, prepared by the $CaCl_2$ method (Sambrook et al. 1989). Ampicillin-resistant colonies were selected and examined for proper-size inserts by restriction analysis. Selected colonies were assayed for OPRT or ODC expression by enzymatic activity and by growth without added uridine on M9 solid medium supplemented with 0.2% casamino acids, 40 μg/ml of L-tryptophan, 0.4% glucose, and 300 μg/ml thiamine hydrochloride.

For activity studies, the transformed pyrimidine auxotrophs were grown in Luria-Bertani (LB) liquid culture under ampicillin selection (50 μg/ml) and harvested in late log-phase by centrifugation. Pellets were resuspended in extraction buffer (Lin and Suttle 1993), and cellular disruption was achieved by 10 sonications of 2-s duration with an Astrason™ Ultrasonic Processor model W-385 (Heat Systems-Ultrasonics). Lysate debridement immediately followed via microcentrifugation at 12,000 rpm for 15 min. OPRT- and ODC-specific activities (nmol/h/mg) were determined by $^{14}CO_2$ release from either [carboxyl-^{14}C]-orotic acid or [carboxyl-^{14}C]-orotidine-5′-monophosphate (New England Nuclear), respectively. For accurate quantitation of OPRT assays, excess yeast ODC (0.05 units; Sigma) in 0.1 M EDTA was added following initial incubation to scavenge residual OMP to UMP (Worthy et al. 1974). Protein concentrations of supernatant fractions were determined with a BCA protein assay kit (Pierce) and supplied BSA standard. Calculated values describe information in nanomoles of UMP formed at 37°C/h/mg lysate protein. Statistical methods applied for data analyses included *F*-test variance analyses and two-sample *t*-test comparisons for equal and unequal variance (per *F*-test). Satterthwaite's freedom approximations were employed in cases of the latter (Armitage and Berry 1994).

Expression of UMP Synthase in the Baculovirus-Sf21 System

*Spodoptera frugiperda Sf*21 cells, pVL1392 transfer vector, and linearized baculovirus DNA were purchased from Pharmingen. Plasmids TA-UMPS, TA-UMPS/rev, TA-UMPS/poly, TA-UMPS/V109G, TA-UMPS/R96G, TA-UMPS/R96G+G429R, and TA-UMPS/G429R were *Eco*RI digested, and purified inserts were cloned into the *Eco*RI site of pVL1392. Orientation distinctions were achieved by *Bam*HI restriction analysis. pVL1392UMPS transfer constructs, encoding sense and antisense normal UMP synthase, as well as mutations V109G, R96G, G213A with 440Gpoly, G429R, and R96G with G429R, were sequence confirmed and designated pVL1392UMPS, pVL1392UMPSrev, pVL1392UMPS/V109G, pVL1392UMPS/R96G, pVL1392UMPS/poly, pVL1392UMPS/G429R, and pVL1392UMPS/R96G+G429R, respectively.

Plasmids were cotransfected with modified, lethal deletion–containing baculovirus into *Sf*21 cells using a BaculoGold transfection kit (Pharmingen). Recombinant virus amplification and infection were conducted per the manufacturer's instructions. The infected *Sf*21 cells were maintained at 27°C in TNM-FH media supplemented with 10% FCS as described by Summers and Smith (1987).

Four days postinfection, $\sim 7 \times 10^6$ *Sf*21 cells/infection were harvested, washed in Delbecco's PBS (10 mM $NaPO_4$, 0.9% NaCl, pH 7.5), and precipitated by centrifugation. Cellular pellets were resuspended in 0.5 ml

Table 1

Intron-Exon Boundaries of the Human UMP Synthase Gene

No.	Exon (bp)	Intron (kb)	Donor	Acceptor
1	174 (200)[a]	4.3	AGTCAGgtgctggcct	tttcttacagGTTGCA
2	154	2.2	ATTATGgtaaaataaa	tcttttctagGAACTA
3	672	1.7	ATGAAGgtaagtgtat	ttcttcttagGAGGTA
4	176	2.0	GCAGCGgtaagtggtg	ttccatttagGTTAGA
5	115	1.5	CAGGAGgtaaatctgg	tttcttgcagGAGATA
6	378	...	...	...

[a] Exon 1 size(s) based on the reported transcription initiation at positions −18 and −44 nt (Webster et al. 1995). cDNA nucleotide positions corresponding to exon-intron juctions may be calculated by summation of exonic lengths minus the reported 5′ untranslated sequence (−18 or −44 nt).

of PBS and membrane disrupted with five serial freeze-thaw cycles. Immediately prior to supernatant-fraction enzyme assays, chilled lysates were cleared by centrifugation at 12,000 rpm for 15 min.

Results

Structural Organization and 5′ Flanking Region of the Human UMP Synthase Gene

Screening of a λEMBL-3 human genomic library using the human UMP synthase cDNA yielded six positive clones. Restriction analysis of three clones, which overlap one another and form the longest continuous sequence, describe a single copy gene encompassing six exons with lengths ranging from 115 to 672 bp. Intron 1 is the most extensive, comprising 4.3 kb. Nucleotide sequence data of intron/exon boundaries (table 1) show that all introns begin with 5′GT dinucleotides and conclude with 3′AG termini. The human UMP synthase gene spans ~15 kb and is graphically illustrated in figure 2.

Comparisons of the human UMP synthase genomic and cDNA sequences revealed two altered bases (G638-to-C and C1320-to-T; first coding ATG assigned +1) located in exons 3 and 6, respectively (data not shown). The point mutations were first identified in one of the paternal Family 1 alleles and subsequently in alleles common to both Family 2 parents. It is notable that neither parent transmitted a polymorphic allele (G213A+440Gpoly) to YK. The transversion within exon 3 encodes a conservative Gly (GGT)-to-Ala (GCT) substitution at codon 213 (G213A), while the latter transition is a silent polymorphism at codon 440 (440Gpoly). Dot blot hybridizations of 100 alleles from 50 unrelated normal Japanese identifies approximate relative frequencies of .26 and .27 for the G213A and the 440Gpoly mutations, respectively (fig. 1 and data not shown). OPRT and ODC activities of expressed protein containing these polymorphisms are described below.

The 5′ flanking sequence of human UMP synthase gene is shown to be −1150 bp upstream of the encoded translation start site (fig. 3, closed circle). No consensus TATA or CAAT boxes are found in the vicinity of the transcription initiation sites (−18 and −44) described by Webster et al. (1995). However, the sequence immediately 5′ and adjacent to this location is highly GC rich (70.1%, nt −167:-1) and contains an Sp1-binding site (Briggs et al. 1986) in addi-

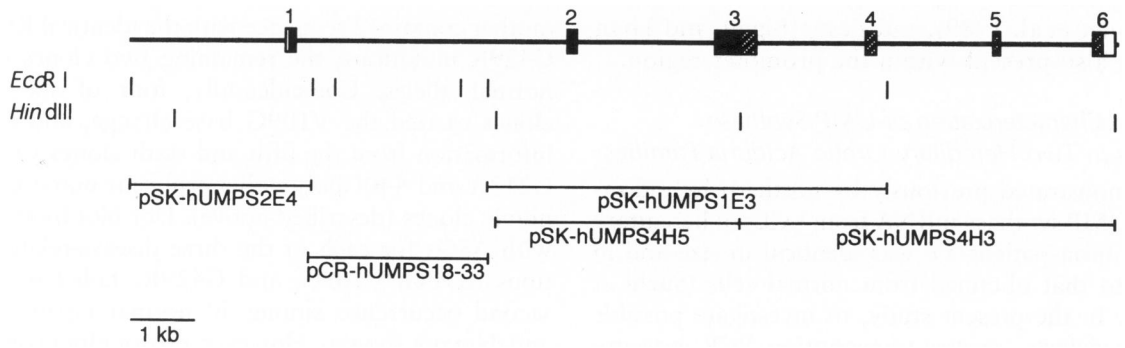

Figure 2 Structure of the human UMP synthase gene. White, black, and striped boxes identify untranslated, OPRT-coding, and ODC-coding regions, respectively. As utilized in genomic clone characterization, *Eco*RI and *Hin*dIII restriction endonuclease cleavage sites are depicted. Plasmid subclones with designations and relative positions are shown.

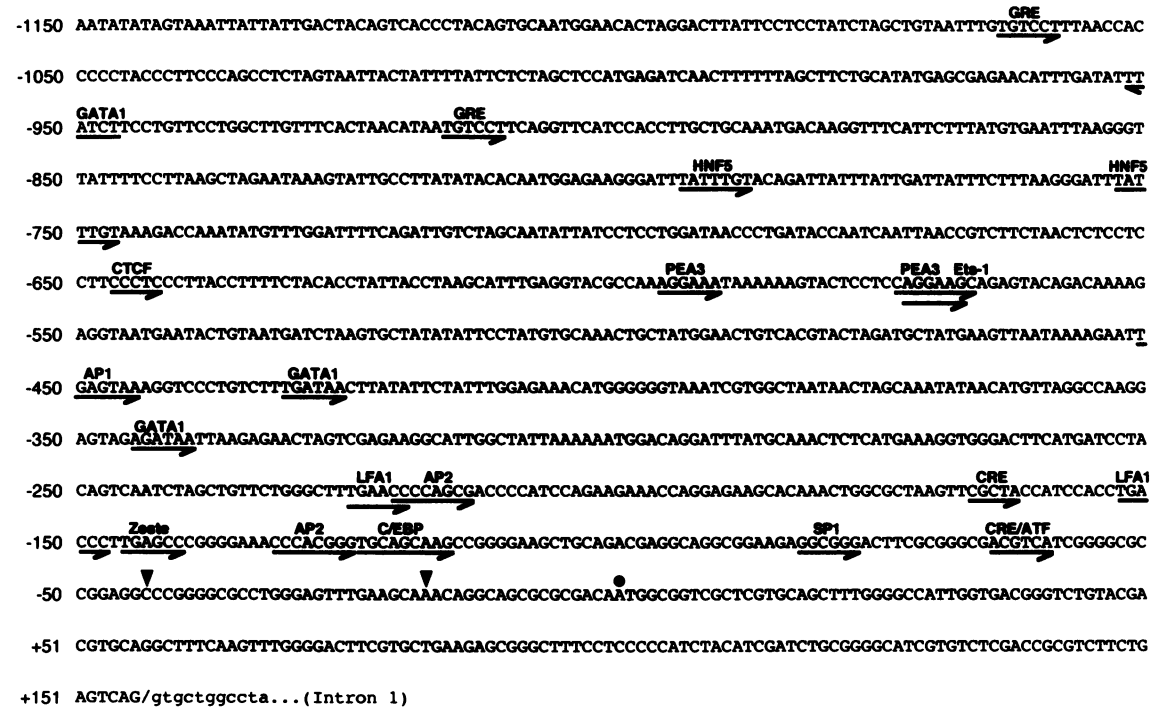

Figure 3 Nucleotide sequence of the human UMP synthase 5′ flanking region. The closed circle represents the ATG (assigned +1) translation start site. Underlined *cis*-elements delineate cognate binding motifs for the transcription factors indicated. Filled arrowheads, located at −18 and −44 nt, signify the reported transcription initiation sites (Webster et al. 1995).

tion to numerous regulatory sites known to be cAMP responsive, i.e., AP-1 (Bernstein and Colburn 1989), AP-2 (Mitchell et al. 1987), and CRE/ATF (Johnson and Krasnow 1992). Sequences homologous to GATA1 (Evans and Felsenfeld 1989) and glucocorticoid-responsive elements (GREs) (Jantzen et al. 1987) are found between positions −1064 to −911 and −431 to −232. Liver-specific transcription factors HNF5 (Grange et al. 1991), LF-A1 (Hardon et al. 1988), and C/EBP (Landschulz et al. 1988) are noted in the region spanning nt −794 to −747 and −225 to −147, respectively. Although not an exhaustive list, additional *cis*-elements corresponding to CTCF (Lobanenkov et al. 1990), PEA3 (Martin et al. 1988), Ets-1 (Prognonec et al. 1989), and Zeste (Biggin and Tjian 1988) are also present within the promoter region.

Molecular Characterization of UMP Synthase Mutations in Two Hereditary Orotic Aciduria Families

We demonstrated previously by northern hybridization that UMP synthase mRNA from LCLs of hereditary orotic aciduria patient YF was identical in size and in quantity to that obtained from normal cells (Suchi et al. 1989). In the present study, to investigate possible molecular defects, reverse-transcription PCR was applied to leukocyte RNA isolated from members of Family 1. Amplified cDNAs encompassing the UMP synthase coding region were cloned into a commercial "T-vector" (pCR II) and sequenced. To rule out polymerase errors, two to four clones per allele were sequenced simultaneously. Lanes for a given dideoxynucleotide were grouped and run in adjacent lanes to facilitate mutation identification. Autoradiograms of sequencing gels demonstrating these findings are shown in figure 4.

Three altered bases were identified in cDNA subclones of patient YF. One allele presented with an A-to-G transition in nt 286 and a G-to-C transversion at nt 1285. The mutations encode Arg-to-Gly (R96G) and Gly-to-Arg (G429R) substitutions in codons 96 and 429, respectively. YF's second allele contained a nt 326 T-to-G mutation, producing a Gly-for-Val substitution of codon 109 (V109G). Four of six clones derived from YF's mother contained sequence with the identical R96G and G429R mutations; the remaining two clones encoded normal alleles. Coincidentally, four of six paternal clones carried the V109G base change, and sequence information from the fifth and sixth clones yielded the G213A and 440Gpoly polymorphisms noted in the genomic clones (described above). Dot blot hybridization with ASOs for each of the three disease-related mutations (R96G, V109G, and G429R) failed to reveal a second occurrence among 50 normal Japanese (fig. 1 and data not shown). However, on dot blot investigation of genomic DNA from members of Family 2, we found that both the mother and YK shared R96G+G429R alleles.

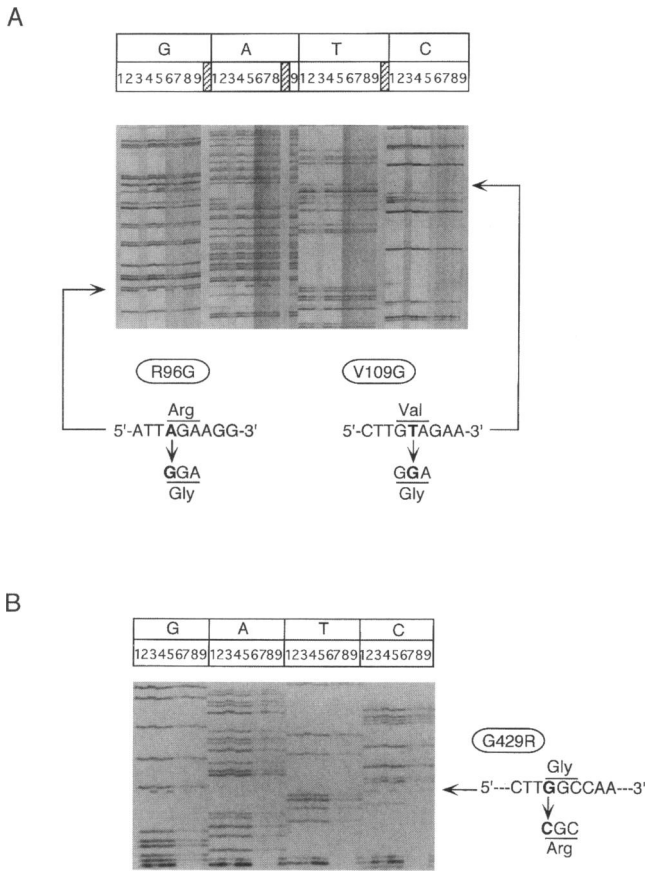

Figure 4 *A*, Sequence data illustrating R96G and V109G mutations in hereditary orotic aciduria patient YF. By use of an oligonucleotide primer spanning nt +52 to +71, the sense strand sequence information from nine clones is shown. *B*, Sequence analysis demonstrating the G429R mutation of YF. A sense oligonucleotide, extending from nt +1196 to +1215, was employed to generate the sequence information depicted.

Unsuccessful Rescue and Activity of Pyr Auxotrophic Transformants

Complementation of the *pyr* phenotypes of *E. coli* AT722 and SK1105, indicated by growth on minimal solid media without uridine supplement, was observed following transformation with the normal UMP synthase construct TA-UMPS (fig. *5A, 5B*). In addition to normal protein expression, the potential of constructs containing altered sequence to rescue the pyrimidine auxotrophs was assessed. Disease allele clones TA-UMPS/V109G, TA-UMPS/R96G, and TA-UMPS/R96G+G429R did not yield AT722 growth without added uridine. Interestingly, construct TA-UMPS/G429R containing the G429R mutation, without the corresponding and syntenic R96G mutation identified in four individuals of this study, was able to rescue AT722 on minimal solid media. In *pyr*F complementation studies (SK1105; fig. 5), all except the parental strain and the TA-mock (pCRII vector)–transformed cells grew without exogenous uridine.

OPRT activity was measured in extracts of AT722 (OPRT-) cells transformed by plasmids containing bifunctional UMP synthase with or without relevant mutations (table 2). Cells transformed with mutant constructs TA-UMPS/V109G, TA-UMPS/R96G, and TA-UMPS/R96G+G429R demonstrated significantly reduced OPRT activity ($P < .001$). Indeed, one TA-UMPS/V109G and two TA-UMPS/R96G transformants did not exceed background AT722 activity levels. On the other hand, cells transformed by TA-UMPS/poly and TA-UMPS/G429R achieved OPRT activities above that of reference transformants (TA-UMPS).

Table 3 illustrates ODC expression data as determined in transformed SK1105 (ODC-) cells. The ODC activity derived from the normal TA-UMPS construct reached levels intermediate to those measured in non-*pyr*F *E. coli* (AT722). Similar, or, in some clones, significantly higher than for TA-UMPS, ODC activity was observed with all constructs except TA-UMPS/R96G+G429R. Five of five independent clones transformed with this plasmid demonstrated reduced ODC activity compared to cells transformed with TA-UMPS (range: $P < .001$ to $P < .05$).

*Expression of UMP Synthase in Sf*21 Insect Cells

Full-length UMP synthase cDNA and cDNAs containing the described mutations or polymorphisms were expressed in *Sf*21 cells using a baculovirus expression system. The OPRT and ODC activities from insect cells infected with pVL1392UMPS recombinant virus or those containing relevant base changes are summarized in table 4. Only the reverse and V109G (Family 1) viral constructs exhibited the combined losses in OPRT and ODC activity (~1% and ~20% of normal; range: $P < .001$ to $P < .05$) witnessed in vivo. Other disease-related mutations maintained ODC activity statistically indistinguishable from the normal reference, pVL1392UMPS. In this system and consistent with bacterial expression data, activity deficits associated with these mutations commonly affected the more labile OPRT catalytic function (Lin and Suttle 1995). Constructs encoding the G213A substitution (TA-UMPS/poly), however, appeared to confer a significant OPRT activity increase (~150% normal; $P < .001$) without the concomitant ODC activity elevation noted in the prokaryotic studies.

Discussion

This communication describes the characterization of the human UMP synthase genomic structure. The gene spans ~15 kb and consists of six exons (fig. 2). All positive phage clones, obtained by screening a λEMBL-3 human genomic library, were aligned within a common

A

Construct		Growth in uridine (-) media	
Designation	Illustration	AT722 (*pyrE*)	SK1105 (*pyrF*)
no plasmid	-	-	-
TA-UMPS/rev	REV	-	-
TA-Mock(pCRII only)	MOCK	n.d.	-
TA-UMPS		+	+
TA-UMPS/poly		+	+
TA-UMPS/V109G		-	+
TA-UMPS/R96G		-	+
TA-UMPS/G429R		+	+
TA-UMPS/R96G+G429R		-	+

B

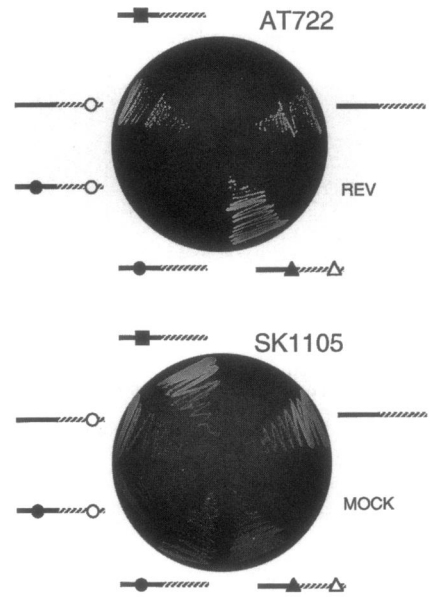

Figure 5 *A*, Schematic illustration of prokaryotic expression vector constructs and summary of transformant growth on minimal media. A filled square identifies mutation V109G; filled and open circles represent mutations R96G and G429R, respectively; and, filled and open triangles connote polymorphisms G213A and 440Gpoly, respectively. n.d. = not determined. *B*, Complementation of *pyr*E AT722 (OPRT-; upper panel) and *pyr*F SK1105 (ODC-; lower panel) with normal and mutated human UMP synthase prokaryotic expression constructs. Two AT722 and one SK1105 representative and confirmed transformants were cultured overnight in LB-ampicillin (50 mg/ml) prior to streaking on the minimal plates. Following 37°C incubation for 16–24 h, colony growth demonstrates complementation of the parental strain *pyr* auxotrophism.

restriction map, indicating that human UMP synthase exists as a single-copy gene. There were no aberrant splice-donor or -acceptor sequences; all splicing termini conform with the canonical GT/AG rule (Mount 1982).

Comparisons of human, fly, and slime-mold UMP synthase amino acid sequences disclose a high level of conservation, with >30% amino acid identity across all three proteins. However, a central region extending from amino acids 192–237 shares little homology (Suttle et al. 1988). This segment, dubbed the "interdomain bridge," spans residues linking the conserved OPRT and ODC domains. In the *rudimentary-like* gene of *Drosophila melanogaster,* encoding the UMP synthase fly homologue, a 657-bp intron divides the sequence between the conserved regions and has been provocatively suggested to represent an "evolutionary joint" wherein ancestral OPRT and ODC domains are fused (Eisenberg et al. 1993). Our findings are inconsistent with this assertion, since no such interruption of the human UMP synthase gene was found. Exon three in the human sequence encodes a segment of the OPRT domain, the interdomain bridge, and a significant portion of the ODC domain. Indeed, the third exon is 672 bp in length and encompasses nearly half of the entire coding sequence. Although beyond the scope of this study, it is of evolutionary interest how two polypeptides, catalyzing consecutive reactions in pyrimidine biosynthesis, came to be joined and encoded by a single gene.

Our molecular investigation of UMP synthase deficiency uncovered five point mutations, two with common allelic frequencies among Japanese. The two polymorphic base changes, introducing a G-to-C transversion at nt 638 ($v = .26$) and a C-to-T transition at nt 1320 ($v = .27$), were fortuitous findings detected in non–disease-related alleles. Evidence supporting the nondeleterious nature of these mutations was initially suggested by allelic-inheritance phase determination in Families 1 and 2. Subsequent expression studies in auxotrophic *E. coli* and in *Sf*21 insect cells support this contention (tables 2–4), since measurable activity reductions were not statistically evident in comparisons with reference constructs of either system. On the contrary, our data, though insufficient for a rigorous assessment, provide intriguing evidence that the G213A polymorphism may encode a variant with enhanced OPRT activity. As the first polymorphisms of the UMP synthase gene, the base changes reported herein should prove useful in family studies or in linkage analyses of neighboring genetic loci (3q13).

Three mutations of the UMP synthase coding region were identified in association with hereditary orotic aciduria. Discovery of a paternal allele containing a V109G

Table 2

OPRT Activity of Transformed Pyrimidine Auxotrophic *E. coli*

Bacterial Strain and Construct	Specific Activity[a] nmol/h/mg	SD	Significance[b]
AT722 (*pyr*E):			
None	$.03_2$	$.03_9$	$P < .001$
	$.00_1$	$.02_5$	$P < .001$
TA-UMPS/rev	$.00_6$	$.01_6$	$P < .001$
	$.01_4$	$.01_5$	$P < .001$
TA-UMPS	3.45_4	$.04_1$	...[c]
	1.40_0	$.06_6$	...[c]
TA-UMPS/poly	$10._9$	$1._7$	$.01 < P < .02$
	$18._0$	$1._7$	$.001 < P < .01$
TA-UMPS/V109G	$.041_8$	$.008_7$	$P < .001$
	$.01_8$	$.01_1$	$P < .001$
TA-UMPS/R96G	$.0039_8$	$.0008_9$	$P < .001$
	$.03_9$	$.09_7$	$P < .001$
TA-UMPS/G429R	3.8_2	$.4_1$	ns
	4.8_6	$.2_0$	$P < .001$
TA-UMPS/R96G+G429R	$.06_9$	$.03_4$	$P < .001$
	$.09_0$	$.03_2$	$P < .001$
SK1105 (*pyr*F):			
None	$41._0$	$17._1$	...[c]
	$48._7$	$7._1$	...[c]

[a] Activity data indicate mean OPRT and (1) SD values of triplicate samples. Subscripted digits convey numerically insignificant detail.

[b] *t*-Test comparisons performed with respect to TA-UMPS–transformed AT722 cells. ns = nonsignificant statistical comparisons.

[c] Comparison is not relevant.

mutation and a maternal allele containing both R96G and G429R mutations revealed the compound heterozygous status of orotic aciduria patient YF (Family 1). When expressed in transformed *pyr*E *E. coli,* the V109G UMP synthase expression construct (TA-UMPS/V109G) failed to produce distinguishable OPRT activity (table 2) or to enable *E. coli* growth on minimal medium without uridine supplementation. However, we did not observe the expected combined loss of ODC activity with expression of the V109G-containing construct in the *pyr*F strain (table 3). Val 109 falls within residues 96–155, where monofunctional *E. coli* OPRT and human UMP synthase proteins show 43% identity. Given the discrete OPRT attenuation in the prokaryotic expression data and that, at equivalent positions to the human codon 109, *Drosophila* UMP synthase and *E. coli* OPRT also encode Val residues, a necessary and conserved role for Val 109 in OPRT function may be plausible.

UMP synthase cDNA expression constructs of the maternal disease-related allele (R96G+G429R) similarly did not produce OPRT activity following transformation into *pyr*E AT722 (table 2). To examine this functional deficit in more detail, constructs encoding individual mutations were assembled. OPRT activity of R96G-substituted UMP synthase paralleled the losses observed for the doubly substituted construct; however, no such reductions were associated with the G429R protein. ODC activity was not found lower for either monosubstituted protein but was moderately depressed among all five R96G+G429R constructs (table 3). These findings are consistent with the notion that the R96G mutation is responsible for OPRT deficits and, possibly, for pathogenicity associated with the R96G+G429R allele. Furthermore, it is likely that the R96G mutation does not appreciably alter protein stability or turnover, as evidenced by ODC activity comparable to reference construct levels (TA-UMPS), but rather some aspect affecting OPRT catalysis. Alternately, because we cannot control for plasmid copy number and since bifunctional UMP synthase expressed in bacteria is cleaved into two labile yet functional subunits (Lin and Suttle 1993), these observations may be artifactual and provide unreliable inference to events occurring within eukaryotic cells. The prokaryotic *pyr*E (OPRT-) growth data correlation, however, affords some reassurance in that all disease-related bacterial expression constructs, with the notable exception of TA-UMPS/G429R (without R96G), failed to complement the AT722 pyrimidine auxotrophism and allow growth on minimal solid media (fig. 5).

Table 3

ODC Activity of Transformed Pyrimidine Auxotrophic *E. coli*

Bacterial Strain and Construct	Specific Activity[a] nmol/h/mg	SD	Significance[b]
SK1105 (*pyr*F):			
None	$.00_1$	$.01_4$	$P < .001$
	$-.016_4$	$.004_0$	$.001 < P < .01$
	$.02_9$	$.03_4$	$.001 < P < .01$
TA-UMPS/mock	$.01_8$	$.01_9$	$P < .001$
	$-.00_2$	$.01_0$	$.001 < P < .01$
	$.02_1$	$.02_0$	$.001 < P < .01$
TA-UMPS	$52._0$	$2._7$	...[c]
	$54._3$	$6._4$	...[c]
	$53._2$	$5._9$	...[c]
TA-UMPS/poly	$67._6$	$2._4$	$.001 < P < .01$
	$69._6$	$2._3$	$.01 < P < .02$
	$53._0$	$3._7$	ns
TA-UMPS/V109G	$4_{8.0}$	$2_{1.0}$	ns
	$69._6$	$7._1$	$.02 < P < .05$
	$4_{1.0}$	$3_{3.0}$	ns
TA-UMPS/R96G	$58._2$	$1._0$	$.02 < P < .05$
	69.7_0	$.9_2$	ns
	$59._2$	$4._4$	ns
TA-UMPS/G429R	$3_{4.0}$	$2_{1.0}$	ns
	$3_{5.0}$	$2_{0.0}$	ns
	$4_{8.0}$	$1_{1.0}$	ns
TA-UMPS/R96G+G429R	$27._8$	$1._8$	$P < .001$
	$35._9$	$2._2$	$.001 < P < .01$
	$41._3$	$2._2$	$.02 < P < .05$
	$30._3$	$1._6$	$P < .001$
	$33._0$	$3._8$	$.01 < P < .02$
AT722 (*pyr*E):			
None	$45._8$	$3._7$	...[c]
	$62._8$	$9._5$	...[c]

[a] ODC activity data relate mean and (1) SD quantities for triplicate samples. Subscripted digits convey numerically insignificant detail.

[b] *t*-Test comparisons performed with respect to TA-UMPS–transformed SK1105 cells. ns = nonsignificant statistical comparisons.

[c] Comparison is not relevant.

To assess the data gathered from the prokaryotic system, we pursued eukaryotic expression studies utilizing a recombinant baculovirus-*Sf*21 system. The V109G-containing UMP synthase expressed in *Sf*21 cells demonstrated significantly lower OPRT and ODC activity, in agreement with patient observations. Recalling the bacterial V109G data, wherein ODC levels were essentially normal, the depressed ODC activity of the baculovirus-expressed V109G protein is discordant (~20% of control). Also in marked contrast with bacterial expression, the R96G- and R96G+G429R-substituted proteins produced marked OPRT elevations coupled with attendant reductions in ODC activity. Taken together, these results are intriguing and possibly highlight biochemical differences specific to these systems or to expression modality. UMP synthase has been demonstrated to exist in three distinct conformational states: a monomeric 3.6s protein, a 5.1s dimer, and a 5.6s "altered-dimer" (Traut and Jones 1979). The more condensed 5.6s conformation, which forms on OMP binding, is predominantly responsible for ODC activity (Traut and Payne 1980). Considering that *Sf*21 cells produce endogenous UMP synthase, which may interfere with dimerization or substrate-dependent "altered-dimer" transition, thereby preferentially lowering measured ODC:OPRT ratios, interpretation of these data must be guarded for allotropic artifacts. A comprehensive mutational analysis of the disease-related and polymorphic substitutions reported herein will be necessary to assign a particular significance for each of these residues.

Two additional occurrences of R96G and G429R mutations were noted in members of Family 2 (fig. 1). YK, having been ascertained during a screening program among mentally disabled individuals, was initially iden-

Table 4

OPRT and ODC Activities of *Sf*21 Cells Infected with Recombinant Baculovirus–UMP Synthase Constructs

Constructs	Specific Activity[a] (nmol/h/mg)				Significance[b]	
	OPRT	SD	ODC	SD	OPRT	ODC
pVL1392UMPS/rev	$18._7$	$2._7$	$57._2$	$6._8$	$P < .001$	$.01 < P < .02$
pVL1392UMPS	$1{,}61_{5\cdot0}$	$4_{0\cdot0}$	$5_{,400\cdot0}$	$1_{,300\cdot0}$	...[c]	...[c]
pVL1392UMPS/poly	$2{,}43_{0\cdot0}$	$1_{00\cdot0}$	$5{,}81_{0\cdot0}$	$6_{50\cdot0}$	$P < .001$	ns
pVL1392UMPS/V109G	$1_{0_{0\cdot0}}$	$1_{1\cdot0}$	$1{,}09_{4\cdot0}$	$2_{7\cdot0}$	$P < .001$	$.02 < P < .05$
pVL1392UMPS/R96G	$2{,}0_{10\cdot0}$	$1_{60\cdot0}$	$6{,}1_{80\cdot0}$	$8_{60\cdot0}$	$.01 < P < .02$	ns
pVL1392UMPS/G429R	$1{,}40_{0\cdot0}$	$7_{8\cdot0}$	$2{,}97_{0\cdot0}$	$6_{9\cdot0}$	$.01 < P < .02$	ns
pVL1393UMPS/R96G+G429R	$2{,}42_{7\cdot0}$	$9_{0\cdot0}$	$3{,}2_{80\cdot0}$	$4_{60\cdot0}$	$P < .001$	ns

[a] Activity data specify mean OPRT and ODC measurements with indicated (1) SD for triplicate samples. Subscripted digits convey numerically insignificant detail.

[b] *t*-Test comparisons performed with respect to TA-UMPS–transformed AT722 cells. ns = nonsignificant statistical comparisons.

[c] Comparison is not relevant.

tified by elevated urinary orotic acid. Subsequent enzyme assay revealed the heterozygote status of YK and his mother (Imaeda et al. 1995). Our molecular characterization demonstrated that both individuals are positive for the R96G+G429R allele by ASO hybridization. Consistent with the low OPRT and ODC activities of hereditary orotic aciduria compound heterozygote YF (2.7% and 0.4%, respectively; Morishita et al. 1986), the presence of the disease-related mutations appears responsible for the reduced activity of Family 2 members possessing the R96G+G429R allele. Although extensive pedigree inquiry did not disclose relatedness between these families, we cannot exclude the possibility. Additional data confirming the presence of this allele among Family 2 individuals was obtained by dideoxy nucleotide sequencing (R96G) and by *Eag*I digestion of PCR-amplified exonic DNA (G429R, data not shown).

UMP synthase activity is detectable in variety of tissues including liver, spleen, gut, brain (Pausch et al. 1972; Reyes and Intress 1978), skeletal muscle, erythrocytes (Brown and O'Sullivan 1977; Jones et al. 1986), and leukocytes (Sugiura et al. 1986). Evidence suggests that elevated de novo pyrimidine biosynthesis may be necessary for cells undergoing rapid proliferation, such as regenerating liver and hematopoietic cells, or during certain developmental stages including organogenesis and neural maturation (Faure et al. 1988). To investigate the regulation of UMP synthase expression, the 5′ flanking sequence was sequenced to −1165 bp from the translation start site (fig. 3). The proximal promoter region was found to be highly GC rich but contained no TATA or CAAT boxes. This feature is common among housekeeping genes (Dynan 1986; Broderick et al. 1987) and presumably reflects the basal necessity for ribosylated pyrimidines in nearly all living cells. One estimate, for example, put de novo pyrimidine biosynthetic activity of normal bone marrow as supplying 30% of the cellular pyrimidine requirement (Sugiura et al. 1986).

As shown in figure 3, two GATA1 sites were found in the promoter region between nt −431 and −340, with a third present at position −940. GATA1 is a zinc-finger *trans*-activator thought to have a pivotal role in erythroid-specific gene expression and myelogenesis (Tsai et al. 1989). Consistent with possible regulatory significance, high levels of UMP synthase are found in hematopoietic cells. Clues implicating GATA1, PEA3, and Zeste *cis*-elements in some development aspects may be meaningful as well (Martin et al. 1988; Winick et al. 1993; Judd 1995), since pyrimidine demands could intensify under particular developmental circumstances (Webster et al. 1995). Other hematopoietic regulatory sequence motifs, including sites for CTCF and Ets-1, are noted between −646 and −567. CTCF has been shown to bind its cognate motif in differentiated and undifferentiated red blood cells (Lobanenkov et al. 1990), and Ets-1 demonstrates differential promoter associations with T-cell activation (Bhat et al. 1990). Although potentially important for some aspect of lymphocyte activation, proliferation, or function, the significance of conserved γ-interferon–responsive elements (five in all, data not shown; Yang et al. 1990) remains to be elucidated.

Several liver-specific or liver-enriched transcription factor–binding sites are also apparent in the UMP synthase promoter sequence, including two HNF5 sites at −795 to −747 nt (Grange et al. 1991), two LFA1 sites at −221 and −143 nt (Ramji et al. 1991), and a C/EBP site at −120 nt (Trus et al. 1990). Three GREs (Karin et al. 1984), possibly involved in the alteration of disease manifestation on cortisone or prednisone therapy, are noted in the promoter region. However, since variation in OPRT or ODC activities following corticosteroid treatment have not been reported, the observed red cell

count improvements may stem from nonspecific effects. In addition, binding sites for the more ubiquitous cAMP-responsive transcription factors AP1 (Hope 1994), AP2 (Mitchell 1991), ATF (Hai et al. 1989), and CRE (Fink et al. 1988) reside in the proximal 5′ sequence. Combinatorial regulation of these and other *cis*-elements may conceivably modulate sophisticated and dynamic UMP synthase expression (Chen et al. 1994).

The molecular basis of hereditary orotic aciduria has enlisted the attention of researchers from many disciplines, including purine and pyrimidine metabolism, oncology, protein chemistry, and evolutionary biology. The spectrum of attraction is multifaceted. UMP synthase deficiency is the only clear example of a human auxotrophism implicated in numerous phenomena crucial for understanding cellular behavior (Pinsky and Krooth 1967), i.e., genomic stability (Qumsiyeh 1995), neoplastic potential (Weber et al. 1981; Denton et al. 1982), and developmental regulation (Reyes and Intress 1978; Rawls 1979) as well as biochemical and structure-function considerations (Ozturk et al. 1995; Yablonski et al. 1996). An enlightened understanding will emerge through advances from each of these fields. The genomic structure and the mutation analysis presented will facilitate continued investigation of UMP synthase in hereditary orotic aciduria.

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Appendix

Table A1

Oligonucleotides Used in PCR of Human UMP Synthase Introns

Intron	Primer Sequence
1	. . .[a]
2	5′ACCAATCAAATTCCAATGCT3′
	5′ATGATTAAACAGGTTTCTCC3′
3	5′TGAAGACCGGAAGTTTGCAG3′
	5′CCCAGGAAGCTATTTTAAAG3′
4	5′TGGGCAGATCTAGTAAATGC3′
	5′GTTTCATGCTTACTCGGGAG3′
5	5′GTTTTATTTCTGGCTCCCGA3′
	5′GCACTCAAACACCAAGTC3′

[a] Intron 1 determined by restriction mapping and dideoxy nucleotide sequencing.

Table A2

Oligonucleotides to PCR-Amplified UMP Synthase Point Mutations

Mutation	Primer Sequence
R96G	5′ACCAATCAAATTCCAATGCT3′
	5′GGAAGGTGACTTATAGTAAC3′
V109G	5′TAAGCAGTGTTTATAATGTG3′
	5′ATGATTAAACAGGTTTCTCC3′
G213A	5′GGAGGCAAGGACAAGTTGCA3′
	5′AGAGAAAGCAAGTCATGAAG3′
G429R and 440Gpoly	5′GGCACGTTTTTAGTTTAAAG3′
	5′GGAGGACCACATATCTTC3′

Table A3

Allele-Specific Oligonucleotide Dot Blot Hybridization Conditions

Probe	Hybridization Temperature (°C)	Final Washing Solution	Final Washing Temperature (°C)
96R	40	6× SSC, 0.1% SDS	43
96G	40	6× SSC, 0.1% SDS	43
109V	41	2× SSC, 0.1% SDS	53
109G	41	1× SSC, 0.1% SDS	57
213G	44	6× SSC, 0.1% SDS	46
213A	44	6× SSC, 0.1% SDS	46
429G	42	6× SSC, 0.1% SDS	44
429R	42	6× SSC, 0.1% SDS	44
440G	42	2× SSC, 0.1% SDS	50
440Gpoly[a]	42	2× SSC, 0.1% SDS	50

[a] Probe 440Gpoly represents the allele-specific oligonucleotide for the paternal C-to-G polymorphism noted at position +1320 nt.

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