

## Human Adenine Phosphoribosyltransferase Deficiency

### Demonstration of a Single Mutant Allele Common to the Japanese

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#### Abstract

Complete adenine phosphoribosyltransferase (APRT) deficiency causes 2,8-dihydroxyadenine urolithiasis. In previous reports, analysis of the kinetic properties of APRT from APRT-deficient Japanese subjects revealed strikingly similar abnormalities suggesting a distinct "Japanese-type" mutation. In this paper, we report studies of 11 APRT-deficient lymphoblast cell lines. Nucleotide sequence analysis of APRT genomic DNA from WR2, a Japanese-type homozygote, identified a T to C substitution in exon 5, giving rise to the substitution of threonine for methionine at position 136. RNase mapping analysis confirmed this mutation in WR2 and revealed that six other Japanese-type homozygotes carry the same mutation on at least one allele. The remaining Japanese subject, who does not express the Japanese-type phenotype, did not demonstrate this mutation. Southern blot analysis showed that all seven Japanese-type subjects were confined to one TaqI restriction fragment length polymorphism (RFLP) haplotype. These studies provide direct evidence for the nature of the mutation in the Japanese-type APRT deficiency.

#### Introduction

Adenine phosphoribosyltransferase (APRT)<sup>1</sup> catalyzes the formation of AMP from adenine and phosphoribosylpyrophosphate (PRPP). Deficiency of this enzyme in humans, which is inherited in an autosomal recessive manner, results in the accumulation of adenine and its major oxidation product, 2,8-dihydroxyadenine (2,8-DHA). Since 2,8-DHA is relatively insoluble in urine, subjects with complete APRT deficiency often present with 2,8-DHA nephrolithiasis (1).

Since Kelley and his colleagues described the first case of

partial APRT deficiency in a heterozygote (2), more than 50 affected families that include 49 homozygotes have been reported throughout the world. 13 homozygotes, from 12 families, are Caucasians while the other 36 homozygotes are Japanese.

Human APRT has been extensively studied. The amino acid sequence of erythrocyte APRT has been determined (3). The APRT gene is located on chromosome 16 (4). It consists of five exons and four introns, which span 2.6 kilobases (kb) and encodes a protein of 180 amino acid residues (5, 6). The nucleotide sequence of the normal human APRT gene has been determined (7, 8).

The frequency of heterozygosity at the APRT locus has been estimated to be 0.4–1.1% from three surveys of erythrocyte APRT activity in Caucasian populations (9–11). Analysis of urinary stones in Japan indicates that the incidence of heterozygotes in the Japanese population is > 1.2% (12). Thus, APRT deficiency is a common genetic disorder occurring at a frequency comparable to sickle cell anemia, thalassemia, hemophilia, and phenylketonuria (1). It is likely that many patients with APRT deficiency remain undiagnosed.

Assays of erythrocyte APRT activity from 11 Caucasian homozygotes with 2,8-DHA nephrolithiasis showed < 3% of normal activity (1, 13, 14). In contrast, 79% of Japanese subjects with APRT deficiency, i.e., "Japanese-type" mutants, have a mutant form of the enzyme with an altered  $K_m$  for PRPP (12). When cellular lysates from these subjects are assayed in vitro in the presence of saturating concentrations of PRPP, APRT activity is detectable. However, APRT activity is virtually absent in vivo in the presence of physiological concentrations of substrate. These subjects are thus phenotypically completely deficient. Cultured cells from these subjects remain viable in medium containing the adenine analogue, 6-methylpurine. This compound is toxic to cells that have APRT activity in vivo. This selective medium thus permits identification of functionally deficient cells. This finding suggests a distinct Japanese-type mutation possibly derived from a common progenitor with a single mutated APRT gene (12, 15).

We have previously defined the mutations on both alleles from a Caucasian subject who was completely deficient in APRT activity. This subject was a compound heterozygote (16). To define the spectrum of the mutations and specifically examine the possibility of a Japanese-type mutation, we studied the mutant APRT genes and mRNA from 11 lymphoblast cell lines derived from Caucasian and Japanese patients with APRT deficiency.

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1. Abbreviations used in this paper: APRT, adenine phosphoribosyltransferase; 2,8-DHA, 2,8-dihydroxyadenine; PRPP, phosphoribosylpyrophosphate.

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## Methods

**Materials.** Nucleotide reagents, M13 sequencing primer, mp18 and mp19 plasmids and Klenow fragment were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Restriction endonucleases and T4 DNA ligase were from New England Biolabs, New Haven, CT.  $\lambda$ gt10 arms and packaging extracts were from Vector Cloning Systems, San Diego, CA. pGEM-3blue plasmid, SP6 RNA polymerase, DNase I, and ribonuclease inhibitor were from Promega Biotech, Madison, WI. RNase A and RNase T1 were from Sigma Chemical Co., St. Louis, MO. [ $\alpha$ - $^{32}$ P]dCTP, [ $\alpha$ - $^{32}$ P]GTP, [ $\alpha$ - $^{35}$ S]dATP, and  $^{125}$ I-protein A were from New England Nuclear, Boston, MA. Nytran paper was from Schleicher & Schuell, Keene, NH. Ampholytes were from LKB Instruments, Inc., Houston, TX.

**Cell lines.** All cell lines were established by Epstein-Barr virus transformation of peripheral B lymphocytes from humans (16). We examined four classes of lymphoblast cell lines derived from APRT deficient subjects (Table I): (i) Japanese-type homozygotes WR2, WR4, WR5, WR12, WR20 (17), WR63, and WR77, derived from unrelated families in Japan; (ii) Japanese-type heterozygote WR64, derived from a member of the family of WR63; (iii) non-Japanese-type homozygotes 904, derived from a Caucasian (16), and WR11 derived from a Japanese (17); (iv) non-Japanese-type heterozygote WR10, derived from a member of the family of WR11 in Japan (17). Cell line WR2 was derived from the Japanese subject homozygous for APRT deficiency, previously described by Mitsuno et al. (18). The subject was the offspring of a consanguineous mating (the parents were cousins). The lymphoblast cell line, WIL2, with normal APRT activity was used as control.

All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Radioisotopic assays of lymphoblast cell extracts were performed as described previously (16). Resistance of the lymphoblast cell lines to a toxic adenine analogue was confirmed by culture in medium containing 100  $\mu$ M of 6-methylpurine. APRT<sup>-</sup> cells, including the  $K_m$  mutants, are viable in this medium whereas APRT<sup>+</sup> cells are not.

**Isoelectric focusing.** Isoelectric focusing of lymphoblast extracts (50  $\mu$ g) was performed in a polyacrylamide slab gel containing 5% ampholytes (pH 3.5–10.0), as described by Wilson et al. (19). The proteins in the gel were transferred electrophoretically to nitrocellulose paper in 0.7% acetic acid at 25 V for 1 h by using Trans-Blot Cell (Bio-Rad Laboratories, Richmond, CA). The nitrocellulose paper was incubated

with rabbit anti-human APRT at room temperature. After washing, the paper was incubated with  $4 \times 10^5$  cpm of  $^{125}$ I-labeled protein A and autoradiographed (20).

**Northern blot analysis.** Total cytoplasmic RNA was prepared from cultured lymphoblasts according to the modified method of Chirgwin et al. (21). Total cellular RNA (20  $\mu$ g) was electrophoresed in a 0.8% agarose-formaldehyde gel and blot transferred to a Nytran paper (22). The paper was probed with a 0.54-kb Bam HI fragment of the normal APRT cDNA, pT05, labeled by random oligonucleotide priming using [ $\alpha$ - $^{32}$ P]dCTP (23).

**Southern blot analysis.** Genomic DNA was extracted from cultured lymphoblasts (24). After digestion with restriction endonucleases, the DNA fragments were electrophoresed in agarose gels, transferred to Nytran paper and probed with a  $^{32}$ P-labeled 2.2-kb Bam HI fragment of normal APRT genomic DNA (24).

**Genomic DNA cloning and sequencing.** The genomic DNA from lymphoblasts was cloned into  $\lambda$ gt10 (25) as previously described (16). Approximately 100,000 plaques were obtained and screened by a standard procedure using the normal APRT cDNA as probe (24). The genomic DNA inserts from positive clones were recloned into pUC18 and subcloned into M13 for sequencing. The nucleotide sequence was determined by the method of Sanger et al. (26) using [ $\alpha$ - $^{35}$ S]dATP.

**RNase mapping analysis.** For the synthesis of anti-mRNA probes, two plasmids, pG3R2 and pGS1, were constructed. pG3R2 was obtained by ligation of pGEM-3blue with the 199-base pair (bp) Rsa I fragment of normal APRT cDNA (nucleotide positions 314–512) that consists of part of exon 4 and part of exon 5. pGS1 was constructed by ligation of pGEM-3blue and the 777-bp Sma I fragment of the normal APRT genomic DNA consisting of the 5' flanking region, exon 1, and part of intron 1. The anti-mRNA probes were synthesized from pG3R2 and pGS1 as previously described (16). The RNA was hybridized to the probe and digested with RNase A and RNase T1 as previously described (16). The digested RNA was then electrophoresed in a 6% polyacrylamide gel containing 8 M urea and autoradiographed.

## Results

APRT protein of the lymphoblast cells derived from Japanese-type homozygotes (WR2, WR4, WR5, WR12, WR20, WR63, and WR77) demonstrated an isoelectric focusing point of pH 5.5, which was identical to that of the normal lymphoblast cell, WIL2 (data not shown).

Table I. APRT Deficient Subjects Examined

Category	Cell line	Zygosity	Phenotype	Race	Reference
i	WR2	Homozygote	Japanese-type	Japanese	(17)
	WR4	Homozygote	Japanese-type	Japanese	(17)
	WR5	Homozygote	Japanese-type	Japanese	(17)
	WR12	Homozygote	Japanese-type	Japanese	(17)
	WR20	Homozygote	Japanese-type	Japanese	(17)
	WR63*	Homozygote	Japanese-type	Japanese	Not published
	WR77‡	Homozygote	Japanese-type	Japanese	Not published
ii	WR64§	Heterozygote	Japanese-type	Japanese	Not published
iii	904	Homozygote	non-Japanese-type	Caucasian	(16)
	WR11	Homozygote	non-Japanese-type	Japanese	(17)
iv	WR10	Heterozygote	non-Japanese-type	Japanese	(17)

The lymphoblast cell lines derived from 11 subjects with APRT deficiency were examined. These cell lines are classified to four categories: (i) Japanese-type homozygotes, derived from unrelated families in Japan; (ii) Japanese-type heterozygote WR64, derived from a member of the family of WR63; (iii) non-Japanese-type homozygotes 904, derived from a Caucasian and WR11, derived from a Japanese; (iv) non-Japanese-type heterozygote WR10, derived from a member of the family of WR11 in Japan. \* % APRT activity in an in vitro assay was 40%.

‡ % APRT activity in an in vitro assay was 57%. § % APRT activity in an in vitro assay was 89%.

Northern blot analysis revealed that the size and amount of APRT mRNAs were grossly normal in all cell lines except for WR11, which had no detectable mRNA (data not shown). The amount of APRT mRNA from WR10, a heterozygote from the family of WR11, was normal.

The Bam HI fragment of the APRT genomic DNA from WR2 was cloned into  $\lambda$ gt10. Four positive clones were obtained, recloned into pUC18, and designated p2B2, p2B7, p2B13, and p2B15. The nucleotide sequences of these four clones were determined and compared to that of the normal APRT gene previously determined in our laboratory (8).

Several nucleotide differences were identified. First, in two clones, p2B2 and p2B7, a single nucleotide substitution, A to C, was identified at position 857 in intron 2 (Fig. 1 A). In contrast, the same area of the sequence of p2B13 and p2B15 was normal. These results indicate that p2B2 and p2B7 were derived from one allele, designated allele I, and p2B13 and p2B15 were derived from the other allele, designated allele II. This base difference between the two alleles does not affect the predicted amino acid sequence.

Secondly, a C to G transversion was found at position 998 in intron 2 of all four clones (Fig. 1 B), resulting in the elimination of a TaqI site. This base substitution does not affect the

predicted amino acid sequence of the protein and represents the previously described TaqI polymorphism within the APRT gene (6). This TaqI polymorphism was confirmed by Southern blot analysis of WR2 genomic DNA.

Thirdly, a T to C substitution, was identified at position 2069 in exon 5 of the genomic DNA from all four clones (Fig. 1 C and D). Thus, both alleles had the same nucleotide change at the same position within the coding sequence. This T to C transition predicts a methionine to threonine substitution at amino acid position 136.

No other abnormality in the coding sequence was found in the APRT genomic DNA of WR2. The Bam HI genomic DNA clones lack 35 bp at the 5' end of the coding region in exon 1. To exclude another mutation within this region, RNase mapping analysis was performed. The result of the RNase mapping analysis using RNA from WR2 cells and the RNA probe containing the 5'-flanking region and part of exon 1 was identical to that obtained using normal mRNA. Therefore, the T to C transition, which is the only nucleotide change in the coding region, seems to be responsible for the abnormal APRT protein from WR2 although the possibility that this represents a polymorphism has not been definitely excluded.

Secondary structure predictions were performed by analyzing the predicted amino acid sequences of normal and WR2 APRT by the method of Chou and Fasman (27, 28). The structure of  $\beta$ -sheet: $\beta$ -turn: $\alpha$ -helix in the mutated area does not appear to be substantially changed by the amino acid substitution of threonine for methionine (data not shown).

RNase mapping analyses of cell lines with the Japanese-type phenotype (WR4, WR5, WR12, WR20, WR63, and WR77) were performed to determine if their genotypes were identical to that of WR2. The 199-nucleotide RNA probe protected from RNase should be cut into two pieces of 106 and 93 nucleotides if the T to C mutation identified in the WR2 gene is present in mRNA from these cell lines (Fig. 2 A). In the lanes containing WR2, WR4, WR5, WR12, WR20, WR63, and WR77, the expected 106- and 93-nucleotide long subbands were found, whereas the normal pattern was observed in the control, WIL2, (Fig. 2 B). These results indicated that Japanese-type mutants have the same mutation as that found in WR2 on at least one allele. Because RNase mapping analysis of WR64 mRNA showed the same subbands, the single mutant allele of this heterozygote was also of Japanese-type. RNA probe was completely digested from WR11, which is consistent with the absence of APRT mRNA in WR11 cells. WR10 (heterozygote and the mother of WR11) also did not show subbands of 106 nucleotides and 93 nucleotides, indicating that WR11 and WR10 do not have the Japanese-type mutation. Cell line 904, with an exon 4 deletion on one allele, showed a 112-nucleotide subband as predicted (16).

When the TaqI site in intron 2 is present, a 2.1-kb band appears on Southern blot analysis. Otherwise, a 2.8-kb band should appear. Thus, three polymorphic haplotypes, represented by 2.1/2.1, 2.1/2.8, 2.8/2.8, in terms of the TaqI restriction fragment length polymorphism (RFLP) are possible. Southern blot analysis using TaqI revealed that all of Japanese-type mutants had only one 2.8-kb band, indicating that all of them were confined to a single TaqI polymorphic haplotype, 2.8/2.8 (Fig. 3). The normal cell line, WIL2 and non-Japanese-type mutant, 904, had one 2.1-kb band (haplotype 2.1/2.1). WR11 showed a 2.1- and a 1.8-kb band, suggesting that the former band reflected an intact TaqI site on one allele.

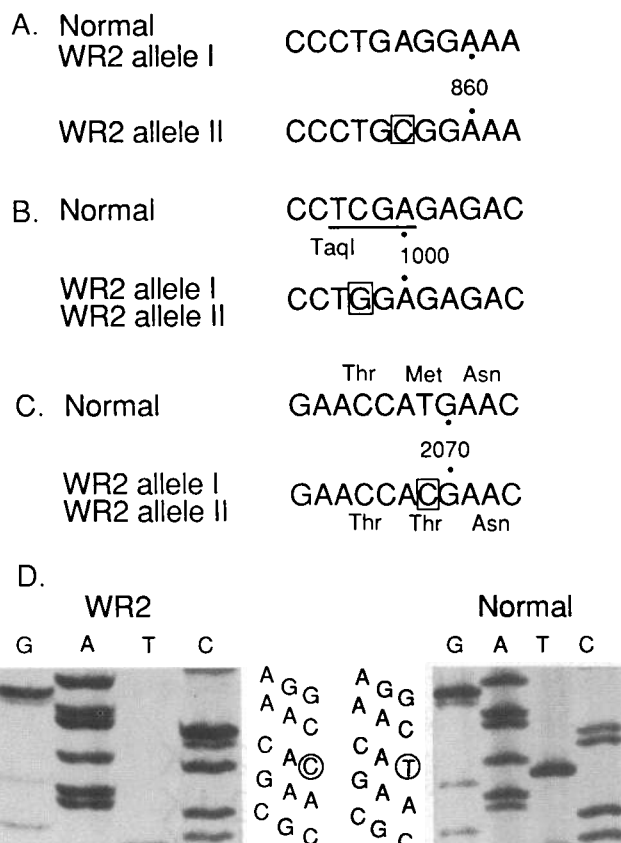
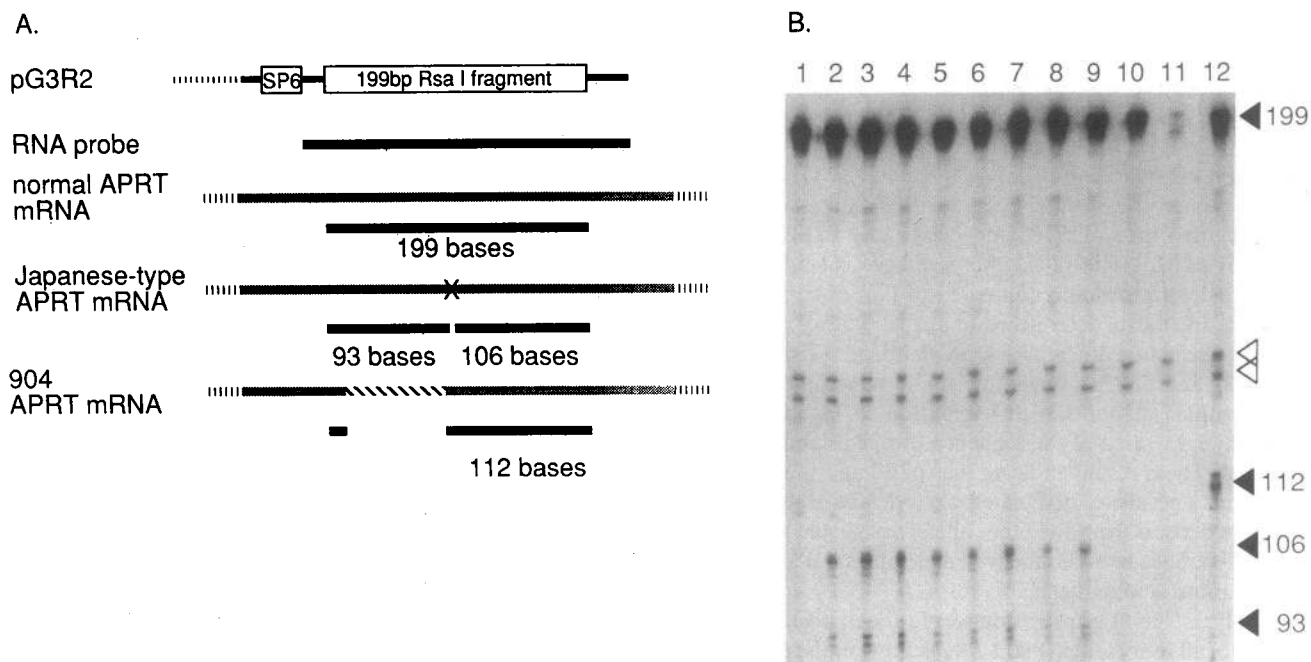


Figure 1. Partial nucleotide sequences of the normal and mutated APRT gene. (A) The nucleotide change in intron 2. (B) The nucleotide change in intron 2, which was considered to be a TaqI polymorphism. (C) The nucleotide change in exon 5, which was responsible for the mutated APRT protein. Numbers in each figure denote the nucleotide positions, starting from the first nucleotide in the start codon of the reading frame. (D) The autoradiographs of the nucleotide sequence of the mutated region are shown. The sequence of WR2 is compared to that of normal.



**Figure 2.** (A) RNase mapping analysis of the APRT mRNA from 12 lymphoblast cell lines. At the top, the recombinant plasmid, pG3R2, is shown as the bar. The SP6 promoter sequence in the pGEM-3blue plasmid and the inserted APRT cDNA fragment (199 bp) are represented as open boxes. The thick bars illustrate the antisense RNA probe that was synthesized from pG3R2 as template and was digested by RNase after hybridization. The numbers indicate the size in nucleotides of the RNA probe protected after RNase digestion. X in the WR2 mRNA indicates the location of the single nucleotide

substitution in exon 5. Oblique lines in the 904 mRNA designate the region of exon 4 deletion. (B) Autoradiograph of RNase mapping analysis of the mRNA from 12 lymphoblast cell lines. The numbers in the right side of the closed triangles indicate the length of the bands in nucleotides. The open triangles denote the nonspecific bands found in all lanes. Lanes: 1, WIL2 (normal); 2, WR2; 3, WR4; 4, WR5; 5, WR12; 6, WR20; 7, WR63; 8, WR64; 9, WR77 (lanes 2 to 9, Japanese-type); 10, WR10; 11, WR11; 12, 904 (lanes 10 to 12, non-Japanese-type).

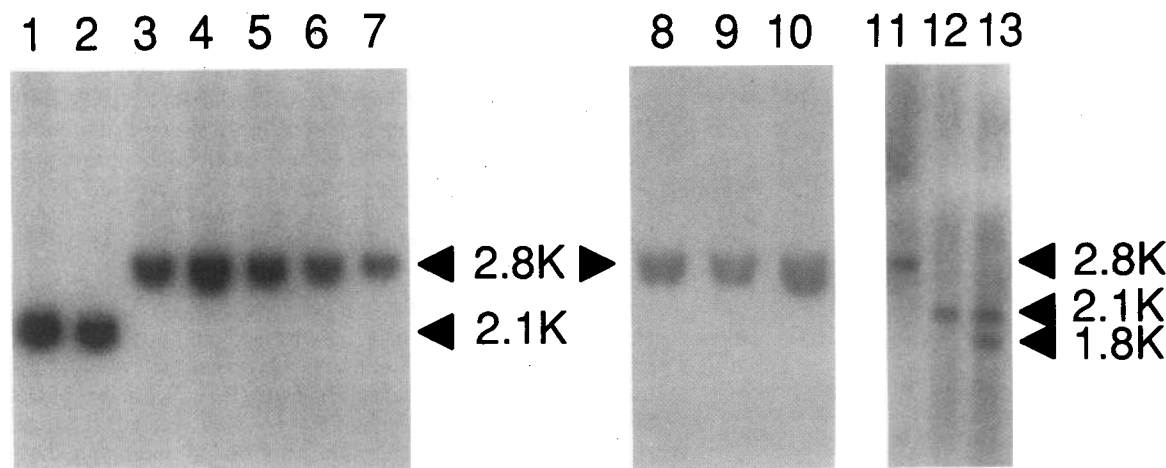
It is unclear whether the 1.8-kb band represents another TaqI polymorphism or a deletion containing the TaqI site.

### Discussion

Within the APRT gene of a subject, WR2, we identified a single nucleotide substitution, T to C, in the coding region in

exon 5. This transition is a different mutation from those we described in a previous study of an APRT-deficient Caucasian (16). The amino acid substitution predicted in this study results in a neutral amino acid change in agreement with the normal isoelectric point of WR2 APRT.

The  $K_m$  value for PRPP in APRT from WR2 was 16.3-fold greater than normal, whereas the  $K_m$  value for adenine was



**Figure 3.** Southern blot analysis of the TaqI-digested genomic DNA extracted from 12 lymphoblast cell lines. The numbers indicate the length of the bands in kilobases. This figure shows autoradiographs from three experiments. Lanes: 1, 904 (non-Japanese-type); 2, WIL2

(normal); 3, WR2; 4, WR4; 5, WR5; 6, WR12; 7, WR20; 8, WR63; 9, WR64; 10, WR77; 11, WR4 (lanes 3 to 11, Japanese-type); 12, WR10; 13, WR11 (lanes 12 and 13, non-Japanese-type).

normal (15). The predicted amino acid substitution (methionine to threonine) is within the putative PRPP binding site of the phosphoribosyltransferases (29), which may be responsible for the increased  $K_m$  for PRPP in WR2 APRT.

We examined the hypothesis that many APRT-deficient Japanese have the same mutation by performing RNase mapping analysis using an RNA probe that includes the region where the mutation occurs in WR2. Although low efficiency of cleavage of C:A mismatches in RNA:RNA hybrids have been reported (30), this method detected the nucleotide substitution in all seven Japanese-type mutants. Furthermore, the identical mutation was detected in the heterozygote WR64. Thus, RNase mapping analysis is useful for detecting the Japanese-type heterozygote that cannot be detected by conventional enzyme assay.

All seven Japanese-type mutants were shown to be homozygous for the negative TaqI RFLP (2.8/2.8). According to a previous report, the allelic frequency of the negative TaqI RFLP is 0.21 in a normal Caucasian population (6). We obtained an allelic frequency of 0.43 in the Japanese population by analysis of 16 normal Japanese subjects (data not shown). Since this frequency is relatively high, it cannot be concluded that all alleles of Japanese-type have negative TaqI RFLP.

The Japanese-type mutation appears in most of the patients with APRT deficiency in Japan whom we have studied. The occurrence of a common mutation in many unrelated families has been reported in  $\beta$ -thalassemia (31), hemophilia A (32),  $\alpha_1$ -antitrypsin deficiency (33), phenylketonuria (34), and Gaucher's disease (35). In the first two diseases, a recurrent mutation at a "hot spot" has been proposed to explain the common occurrence of the mutation (31, 32). In phenylketonuria, two particular mutations accounting for ~ 60% of the cases suggest that mutant alleles might have spread in the population by positive selection or by random genetic drift (34). Any of these mechanisms may explain the origin of the Japanese-type mutation.

The further study of the mutant alleles from subjects with APRT deficiency in different ethnic groups in Asia may elucidate the mechanism of the spread of the Japanese-type mutant allele. Additionally, survey of the Caucasian population for APRT deficiency may further delineate the spectrum of the mutations and help clarify their origins.

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