

Idiopathic Low Molecular Weight Proteinuria Associated with Hypercalciuric Nephrocalcinosis in Japanese Children Is due to Mutations of the Renal Chloride Channel (CLCN5)

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Abstract

The annual urinary screening of Japanese children above 3 yr of age has identified a progressive proximal renal tubular disorder characterized by low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis. The disorder, which has a familial predisposition and occurs predominantly in males, has similarities to three X-linked proximal renal tubular disorders that are due to mutations in the renal chloride channel gene, CLCN5. We have investigated four unrelated Japanese kindreds with this tubulopathy and have identified four different CLCN5 mutations (two nonsense, one missense, and one frameshift). These are predicted to lead to a loss of chloride channel function, and heterologous expression of the missense CLCN5 mutation in *Xenopus* oocytes demonstrated a 70% reduction in channel activity when compared with the wild-type. In addition, single-stranded conformation polymorphism (SSCP) analysis was found to be a sensitive and specific mutational screening method that detected > 75% of CLCN5 mutations. Thus, the results of our study expand the spectrum of clinical phenotypes associated with CLCN5 mutations to include this proximal renal tubular disorder of Japanese children. In addition, the mutational screening of CLCN5 by SSCP will help to supplement the clinical evaluation of the annual urinary screening program for this disorder. (*J. Clin. Invest.* 1997. 99:967–974.) Key words: proteinuria • hypercalciuria • chloride channel mutations

Introduction

The annual urinary screening program of Japanese children above 3 yr of age has identified a progressive proximal renal tubular disorder characterized by low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis (1). In addition, hematuria, glycosuria, aminoaciduria, an impaired urinary

concentrating ability, and a mild decrease in creatinine clearance have been observed in some children (1–4). The disease, which occurs predominantly in males, has been reported to have a familial predisposition, although the mode of inheritance has not been established. This renal proximal tubulopathy in Japanese children has similarities to that of Dent's disease in British families, which is X-linked and is characterized by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, rickets, and eventual renal failure (5, 6). On the basis of these similarities, it has been proposed that the two renal proximal tubular disorders are the same (1), although there are notable differences in that the Japanese children do not suffer from rickets or renal failure. This situation is analogous to that observed between Dent's disease and the other two X-linked renal proximal tubular disorders associated with hypercalciuric nephrolithiasis in North American and Italian families, which have been referred to as X-linked recessive nephrolithiasis (XRN)¹ (7) and X-linked recessive hypophosphatemic rickets (XLRH) (8), respectively. Thus, XRN and XLRH have proximal renal tubular defects in common with Dent's disease, but rickets is absent in XRN, and nephrocalcinosis and moderate renal failure are more notable in XLRH. These three disorders of hypercalciuric nephrolithiasis (Dent's disease, XRN, and XLRH) have all been mapped to chromosome Xp11.22 (5, 8, 9) and all three have been established to share a common genetic etiology by demonstrating mutations in the renal chloride channel gene, CLCN5 (10).

CLCN5 belongs to a family of voltage-gated chloride channel genes (CLCN1 to CLCN7, and CLCNKa and Kb) which encode proteins (CLC-1 to CLC-7, and CLC-Ka and Kb) that have ~ 12 transmembrane domains and that were originally identified by cloning the *Torpedo marmorata* chloride channel, CLC-0 (11–13) (Fig. 1). These chloride channels are important for the control of membrane excitability, transepithelial transport, and possibly regulation of cell volume (11). Human CLCN5 is predominantly expressed in the kidney and studies of rat microdissected tubules using RT-PCR have demonstrated the presence of CLCN5 mRNA in large portions of the nephron (13, 14). Heterologous expression of wild-type CLCN5 in *Xenopus* oocytes has revealed that the channel, CLC-5, conducts chloride currents that are outwardly rectifying and essentially time-independent (10, 14). In addition, ion substitution experiments showed that there is a chloride > iodide conductance sequence, that is consistent with that reported for the

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1. Abbreviations used in this paper: SNUPE, single nucleotide primer extension; SSCP, single-stranded conformational polymorphism; XLRH, X-linked recessive hypophosphatemic rickets; XRN, X-linked recessive nephrolithiasis.

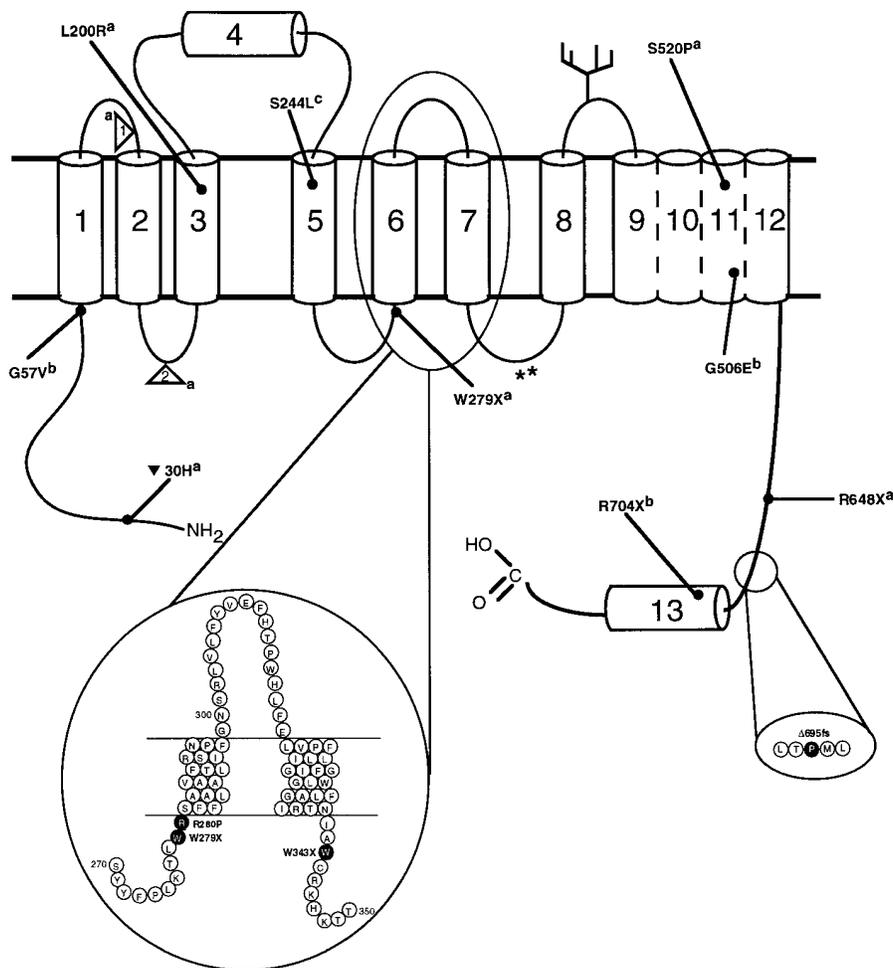


Figure 1. Schematic representation of a predicted topology of CLC-5, which consists of 746 amino acids (10, 13). The correct topology of the CLC channels is unknown and the predicted topology of the CLC-5 putative transmembrane domains (D1-D12) is based upon a model (10, 11, 15) that places D4 extracellularly, and in which the hydrophobic core of the D9-D12 region crosses the membrane three or five times and contains a hydrophilic region, the precise location of which remains unknown. The consensus phosphorylation and glycosylation sites are indicated by the asterisks and branch signs, respectively. The three nonsense (W279X, R648X, and R704X), five missense (G57V, L200R, S244L, S520P, and G506E), and one insertional (∇ 30H) mutation together with the deleted region (between arrows 1 and 2) of D2, due to donor splice site mutations at the exon5-intron5 boundary, that were reported previously (10, 16) to be associated with Dent's disease(a), XRN(b), and XLRH(c) are indicated. The four mutations (two nonsense [W279X and W343X], one missense [R280P], and one deletion [Δ C] at codon 695 which resulted in a frameshift [fs]) associated with low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis in the four Japanese kindreds (Table I) are illustrated in detail within the insets.

other chloride channels, CLC-0, CLC-1, and CLC-2, of this family (15, 17, 18). Expression of the CLC-5 mutants (Fig. 1) associated with Dent's disease, XRN, and XLRH either abolished or markedly reduced these currents (10). Thus, loss of function mutations of CLC-5 are associated with the X-linked renal proximal tubular disorders of Dent's disease, XRN, and XLRH; therefore, we undertook studies to investigate Japanese patients with idiopathic low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis for mutations in the X-linked CLCN5 gene.

Methods

Patients. 12 members of four Japanese families were clinically and biochemically assessed for idiopathic low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis, as described previously (1). All the urinary measurements were performed on early morning samples obtained with the patient fasting and after overnight water deprivation. Urinary calcium to urinary creatinine ratios (Ca/Cr) of > 0.25 mg/mg and of > 0.20 mg/mg in children and adults, respectively, were taken to be elevated (19–22). The details of the 10 affected members from these four families, designated 11.1/95, 11.2/95, 13.2/95, and 14/95, are summarized in Table I. The clinical and biochemical details that were obtained 2–4 yr earlier from the four affected males II.1 (family 11.2/95), II.1 (family 11.1/95), II.2 (family 11.1/95), and II.1 (family 13.2/95) have been reported previously (1)

as patients 2, 4, 5, and 6, respectively. Venous blood samples were obtained from 8 of the 10 affected members and 2 of the unaffected members and used for mutational analysis of the CLCN5 gene (10).

DNA sequence analysis of the CLCN5 gene. RNA was extracted from an Epstein-Barr virus transformed lymphoblastoid cell line obtained from the peripheral blood cells of an affected individual from each family, using methods described previously (10, 23). RT-PCR was performed using pairs of nested CLCN5-specific primers as described (10). The PCR products were then gel purified and the DNA sequences of both strands were determined by *Taq* polymerase cycle sequencing and a semiautomated detection system (ABI 373A sequencer; Applied Biosystems, Foster City, CA) (24). DNA sequence abnormalities were confirmed either by restriction endonuclease analysis (10) of genomic PCR products obtained by use of the appropriate primers (Fig. 2, Table II) or by single nucleotide primer extension (SNUPE) analysis (26, 27). In addition, the DNA sequence abnormalities were demonstrated to cosegregate with the disorder and to be absent in the DNA obtained from 55 unrelated normal Japanese females and 75 unrelated normal European individuals (40 males and 35 females). SNUPE analysis was used to confirm a DNA sequence abnormality detected in exon 11, using the primers 11F and 11R (Table II) as follows. PCR products from the normal individuals were pooled into groups of 10 for the SNUPE reactions. Samples were subjected to one cycle consisting of 2 min of denaturation at 94°C, 2 min of annealing at 60°C, and 2 min of primer extension at 72°C using the oligonucleotide primer CACTGTGACTGACCTTACACC (5'→3') and with either [α^{32} P]deoxy-ATP or [α^{32} P]deoxy-CTP. 5 μ l of sample was mixed with 5 μ l of formamide loading buffer, dena-

Table I. Biochemical Characteristics of Japanese Kindreds with Idiopathic Low Molecular Weight Proteinuria

	Age (yr)	Sex	Serum						Urine						Nephrocalcinosis	
			TP	alb	BUN	Cr	Ca	P	TP	Ca/Cr	β 2-m	Osmolality	Tm P/GFR	CCr		
			g/dl		mg/dl		mg/dl		mg/dl	mg/ml	μ g/liter	mosM/kg	ml/min/1.73 m ²			
Normal ranges																
Adult			6.2–7.9	3.9–4.8	8–18	0.4–1.1	8.6–10.5	2.7–4.4	2–18	< 0.20	< 240	> 850	2.5–4.3	108–152	–	
Children			6.0–7.7	3.4–4.4	6–19	0.1–0.8	9.0–10.6	3.8–5.6	4–22	< 0.25	< 320	> 800	2.7–5.6	97–165	–	
Family*																
11.1/95																
	I.2	35	F	7.0	4.4	13	0.5	10.1	3.7	5	0.18	4700	ND	ND	ND	ND
	II.1	9	M	7.4	4.9	12	0.4	10.0	5.1	42	0.56	32000	807	4.5	121	+
	II.2	7	M	7.2	4.7	15	0.4	10.1	4.8	28	0.43	35000	840	4.3	117	+
11.2/95																
	I.2	44	F	6.8	4.3	14	0.5	10.0	3.5	5	0.19	4360	ND	ND	ND	ND
	II.1	14	M	7.6	4.7	15	0.8	9.7	4.2	160	0.26	435800	727	3.7	114	+
13.2/95																
	I.2	45	F	7.0	4.2	11	0.6	10.4	3.8	4	0.17	3319	ND	ND	ND	ND
	II.1 [‡]	13	M	7.2	4.6	16	0.9	9.9	4.9	144	0.45	165000	745	4.3	118	+
14/95																
	I.2 [‡]	33	F	6.9	4.3	12	0.6	10.2	3.8	4	0.20	3610	ND	ND	ND	ND
	II.1	5	M	7.4	4.5	12	0.3	9.6	5.4	78	0.67	156000	752	3.8	138	+
	II.2	3	M	7.6	5.4	10	0.2	10.1	5.3	88	0.47	243200	760	4.8	102	+

TP, total protein; alb, albumin; BUN, blood urea nitrogen; Cr, creatinine; Ca, calcium; P, phosphorus; β 2-m, β 2-microglobulin; Tm P/GFR, threshold phosphate concentration; CCr, creatinine clearance; all values refer to those obtained on early morning samples after an overnight fast. Glycosuria and aminoaciduria were absent in all the individuals with the exception of individual II.1 (family 11.2/95) who had both glycosuria and aminoaciduria, and individual II.1 (family 13.2/95) who had aminoaciduria. Nephrocalcinosis was detected by ultrasonography or computerized tomography scanning. +, present; –, absent; ND, not done; F, female; M, male. *The family designation is shown, and each individual is indicated by a generation (I or II) and sibship number (1 or 2); the affected mothers are all I.2, and their affected sons are either II.1 or II.2. [‡]Member not available for CLCN-5 mutational analysis.

tured for 2 min at 90°C and 3 μ l was loaded onto a 12% polyacrylamide gel containing 6 M urea. Gels were run at 50 V for 1–2 h, dried, and visualized by autoradiography.

Single-stranded conformational polymorphism (SSCP) analysis. Genomic DNA from the affected family members and 10 unrelated

normal individuals was used with appropriate primers (Table III) for PCR amplification, as described above (Tables II and III), and the PCR products were analyzed for SSCP using the Phast electrophoresis system (Pharmacia LKB, Uppsala, Sweden) (24) as follows. A 1- μ l aliquot of the PCR product was diluted with 2 μ l of 66% formamide,

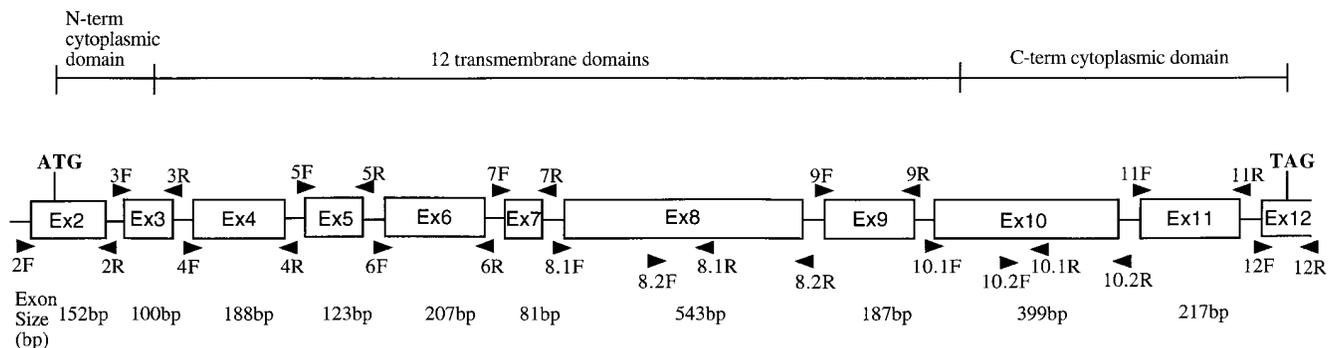


Figure 2. Schematic representation of the genomic structure and position of PCR primers in the CLCN5 gene. The human CLCN5 gene consists of 12 exons that span 25–30 kb of genomic DNA (21) and encodes a 746-amino acid protein. The coding region is organized into 11 exons (Ex2 to Ex12) and 10 introns (indicated by a line but not to scale). The gene encodes a putative chloride channel with \sim 12 transmembrane domains (Fig. 1). Exon 2 and part of exon 3 encode the 57 amino acids of the NH₂-terminal cytoplasmic domain; the 3' end of exon 3 and exons 4 to part of exon 10 encode the 491 amino acids of the transmembrane domains and loops; and the 3' end of exon 10, exon 11, and part of exon 12 encode the 198 amino acids of the COOH-terminal cytoplasmic domain. The start (ATG) and stop (TAG) sites in exons 2 and 12, respectively, are indicated. The full size of exon 12 has not been determined (25), and primers 12F and 12R (Table II) were used to amplify the 251-bp segment that contains the translated region and the stop codon. The locations of the 13 pairs of primers (shown by arrows, F, forward; R, reverse primer), which were designed (Table II) to amplify the exons, and exon-intron boundaries are shown.

Table II. Primers Used for Genomic PCR of CLCN5

Exon	Sequence (5'-3')	Product size bp
2F	TCATCTGATAGTTTAAGGGCCCG	245
2R	ATTTCTAACACTTACCCATGTGC	
3F	GGTCTCTATTCTCCAGTGATTG	271
3R	ACTTACGGGAGAGACATACCAC	
4F	CCGAGATTCAAGTTAACTTTGGC	300
4R	TCCGCAATTGCTGTGAGCACAG	
5F	TGTCTCACTGAAGCTGTGTAGGC	309
5R	CTTAGATGGCCTCAGGATCTG	
6F	TAGCCTTGTGACTTCCTTAGTC	294
6R	CAAAAAGAGACTATTAAGGCCATTC	
7F	GTTCAAGTAACACTAACATTTCTTC	221
7R	CACAAAGCATGCACATGCTGTAC	
8.1F	GACTGAGTTTGTCTTCTCACCTTC	427
8.1R	CTTGGAGGAGTCCAGAAGGCC	
8.2F	CAGCCATCACTGCCATCCTG	321
8.2R	ATACCTGGGCTCTGCCCTCC	
9F	TCACTAACCATCTATTGGTTTCTC	271
9R	TCTTCTGTTGTATGTCACCTGGG	
10.1F	GAGAACTCTGAATCGTCTCCATTG	329
10.1R	ACGGATGTGGGCATCATAGATG	
10.2F	CATGACAAGCAAGTGGGTGGC	350
10.2R	GTTCCACAGTGGATTCCACTATC	
11F	TCCATCTTCAATTTGTTTTTCTTC	304
11R	GCATCCTCTTTCTCCACAATTC	
12F	TGAAAAGGACTGAGGAGGACAAG	251
12R	GGTACCAGTTAATACAACATATCC	

All PCRs were carried out in 1 mM MgCl₂ at an annealing temperature of 58°C. The locations of these primers in relation to the CLCN5 gene are shown in Fig. 2.

denatured at 99°C for 10 min, cooled on ice for 2 min, and 0.3 µl was loaded onto a precast 12.5 or 20% polyacrylamide gel (Table III) with native buffer strips (Pharmacia LKB), which had been precooled to the required electrophoresis temperature. Gel electrophoresis was performed at either 10, 15, or 20°C and at 200–400 Vh depending upon the size of the fragment. The gel was fixed in 10% acetic acid and 50% ethanol for 10 min; 5% glutaraldehyde for 5 min, 5% acetic acid and 10% ethanol for 10 min. Silver staining with 0.025 M aqueous silver nitrate for 10 min revealed the SSCPs.

Functional expression in *Xenopus* oocytes. After engineering an NcoI site at the initiator methionine, a cDNA encoding the human CLC-5 protein was inserted into the NcoI site of the expression vector, PTLN, which contains *Xenopus* globin untranslated sequences to boost expression in the oocyte (28). Mutations were introduced by recombinant PCR and verified by sequencing. Capped cRNA was transcribed using SP6 RNA polymerase from the linearized construct. About 10 ng of cRNA was injected into *Xenopus* oocytes prepared and handled as described (29). After incubating for 2–3 d at 16°C, oocyte conductance was measured at room temperature by standard two-electrode voltage-clamp technique (10) using a Turbo TEC-05 amplifier and pCLAMP5.5 software. Standard extracellular solution was ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM Hepes, pH 7.4). Four different batches of cRNAs and eight different batches of oocytes for the wild-type and mutant constructs were used and similar results were obtained. The results were expressed as mean values ± SEM.

Results

Mutations in the Japanese families. DNA sequence analysis of the entire 2,238-bp coding region of the CLCN-5 gene from one affected member of each of the four Japanese families investigated with low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis (Table I) revealed the presence of four mutations which consisted of two nonsense, one missense, and one 1-bp deletion resulting in a frameshift mutation (Fig. 1). Three of these mutations (W279X, R280P, and W343X) from families 14/95, 11.2/95, and 11.1/95 (Table I), respectively, occurred in exon 8 and the other (Δ695fs→699Stop) from family

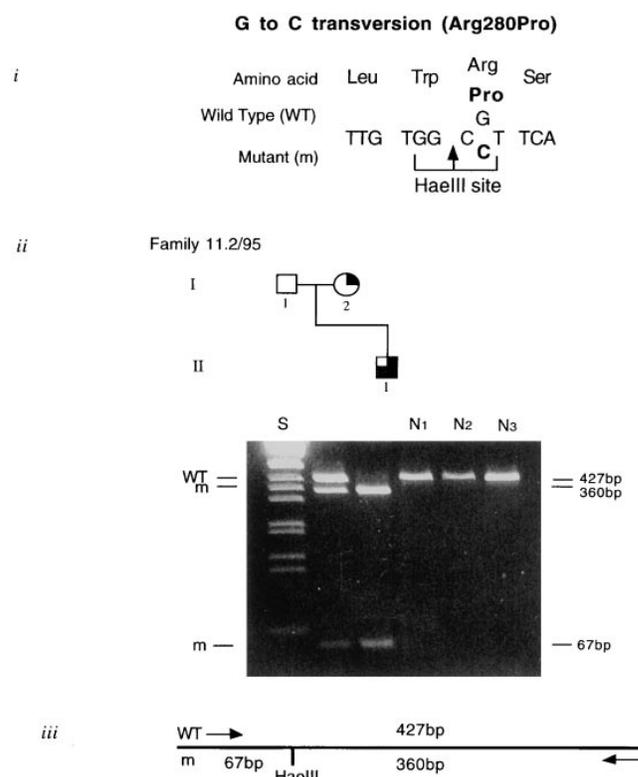


Figure 3. Detection of mutation in exon 8 in family 11.2/95 by restriction enzyme analysis. DNA sequence analysis of individual II.1 revealed a G to C transversion at codon 280 (i), thus altering the wild-type (WT) sequence. CGT, encoding an arginine (R) to the mutant (m) sequence, CCT, encoding a proline (P). This missense mutation (R280P) also resulted in the gain of an HaeIII restriction enzyme site (GG/CC). PCR amplification and HaeIII digestion (ii) would result in two products of 360 and 67 bp from the mutant sequence but only one product of 427 bp from the normal sequence as is illustrated in the restriction map in iii. Cosegregation of this R280P mutation with the disease and its heterozygosity in the affected mother I.2 was demonstrated (ii), and the absence of this R280P mutation in 220 alleles from 55 unrelated Japanese females and 75 (40 males, 35 females) unrelated Northern European individuals, N₁ to N₃ shown, indicates that it is not a common DNA sequence polymorphism. Individuals shown are: box, male; circle, female; open, unaffected; filled quadrants, upper right, low molecular weight proteinuria; lower right, hypercalciuria; lower left, nephrocalcinosis. The standard size marker (S) in the form of the 1-kb ladder is indicated. Similar restriction enzyme analysis was used to confirm and demonstrate the cosegregation of the W279X and W343X mutations (Fig. 1) of families 14/95 and 11.1/95 (Table I), respectively.

Frameshift mutation (ΔC) in a female with idiopathic LMWP

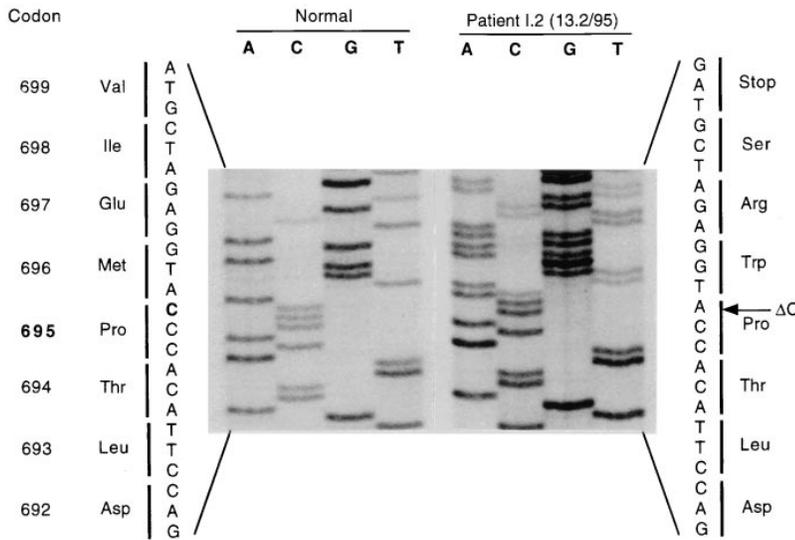


Figure 4. Detection of frameshift mutation, due to deletion (Δ) of a cytosine (C) at codon 695 in family 13.2/95. The DNA sequence autoradiograph from codons 692 to 699 of the female (I.2 from family 13.2/95) with low molecular weight proteinuria (Table I) is shown on the right hand side and is compared with one obtained from a normal individual on the left-hand side. The patient's DNA sequence reveals a 1-bp deletion of a C from codon 695 which encodes for proline (CCC), with a frameshift in subsequent codons. The predicted amino acid sequences for the normal and patient's DNA sequence are shown adjacent. The deletion does not alter the encoded proline (CCA) at codon 695 in the patient but results in a frameshift and a missense peptide at codons 696, 697, and 698, with a termination codon (*Stop*) at 699. This deletional mutation (ΔC) was confirmed and demonstrated to cosegregate with the disease by SNuPE analysis.

13.2/95 (Table I) occurred in exon 11 (Table III). The W279X mutation resulted from a G to A transition (TGG→TGA), the R280P mutation (Fig. 3) resulted from a G to C transversion (CGT→CCT), and the W343X mutation resulted from a G to A transition (TGG→TAG); each of these mutations resulted in an alteration of an MaeIII, HaeIII, or EcoRII restriction enzyme site, respectively, that facilitated its confirmation and cosegregation with the disease (Fig. 3) in each family. Interestingly, the W279X (TGG→TGA) mutation has been observed previously in an unrelated (confirmed by microsatellite polymorphism analysis, data not shown) British family with Dent's disease (10) and this may represent a possible mutational hot spot. The frameshift mutation, due to a deletion of C at nucleotide 2085 which involved codon 695 (Fig. 4, Table III), was not associated with an altered restriction enzyme site and was confirmed by SNuPE analysis (data not shown). The absence of each of the four DNA sequence abnormalities in 220 alleles from 130 unrelated normal individuals (55 Japanese, 75 Northern Europeans) established that these abnormalities were mutations and not functionally neutral polymorphisms which would be expected to occur in > 1% of the population. Each of the four mutations in the Japanese families predicts a structurally significant alteration to CLC-5 and is thus likely to be of importance in the etiology of the disease. The two nonsense mutations, W279X and W343X, and the frameshift mutation which results in a termination at codon 699 (Fig. 4) predict truncated CLC-5 channels that lack, respectively, the 469 amino acids from D6 to the carboxy terminus; the 404 amino acids from the loop between D7 and D8 to the carboxy terminus; and the 47 amino acids, which include D13, to the carboxy terminus. The effects upon CLC-5 function of such nonsense mutations, which have been previously assessed (10), are likely to be a loss of function.

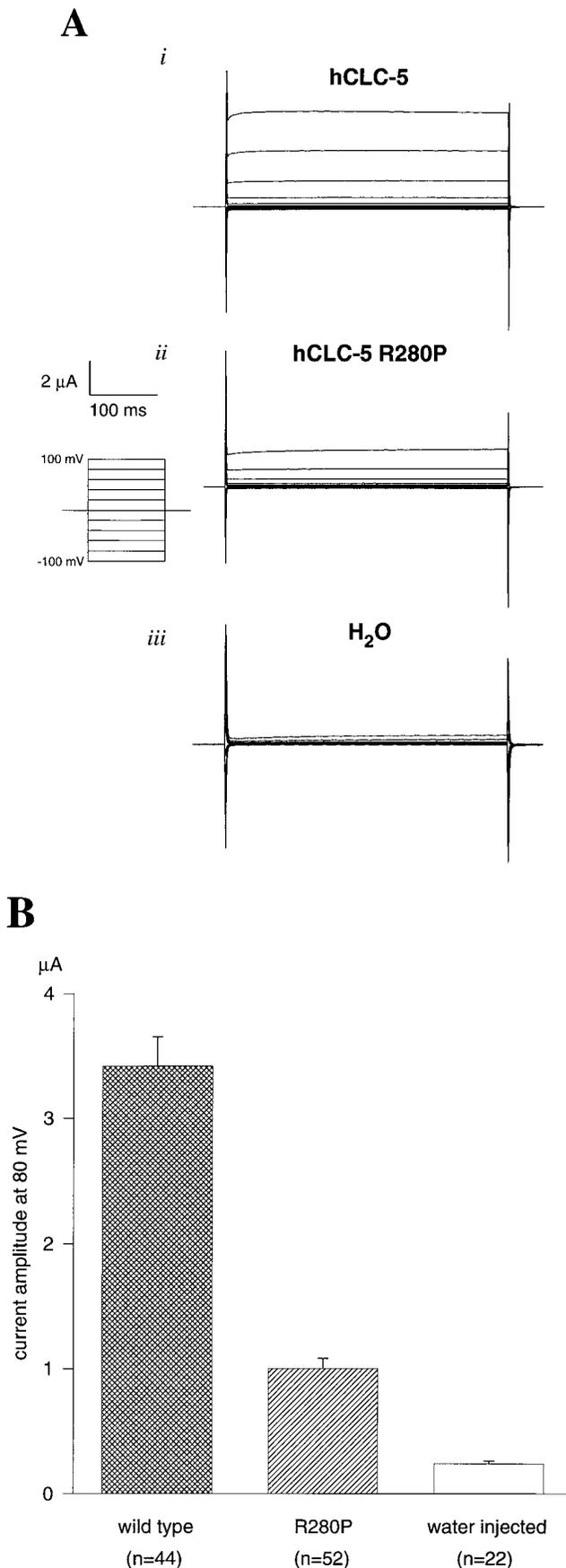
Wild-type and mutant R280P CLC-5 expression. Expression of CLC-5 nonsense mutations and some missense mutations (L200R and G506E) has been demonstrated previously to abolish CLC-5 currents (10), while some other missense mutations (S244L and S520P) have been reported to reduce CLC-5 currents (10). The nonsense mutations (W279X, W343X, and

the $\Delta 695$ fs) in the Japanese families are likely to result in a loss of function of CLC-5, as has been shown for the W279X, R648X, and R704X mutations in families with Dent's disease (10). Therefore, we chose to assess the functional significance of the R280P mutation of family 11.2/95 (Fig. 3). The results

Table III. Conditions for SSCP Analysis of the CLCN5 Gene

Exon	Primers	% gel	Temperature	Voltage-time	Mutations	Mutation detected*
			°C	Vh		
2	2F-2R	20	20	200	30H [‡]	Y
3	3F-3R	20	10	300	G57V [‡]	Y
5	5F-5R	20	10/20	200	gt→gg [§]	Y
		20	10	250	gt→at [§]	Y
6	6F-6R	12.5	10	220	L200R	Y
7	7F-7R	20	10	250	S244L	Y
8	8.1F-8.1R	12.5	20	350	W279X	Y
					R280P	Y
		12.5	10	400		
		12.5	15	400	W343X	N
9	9F-9R	12.5	20	300		
		20	20	200	G506E	Y
10	10.1F-10.1R	12.5	10/20	220	S520P	N
		20	20	200		
		20	15/20/25	250		
11	11F-11R	20	20	200	R648X	Y
		12.5	10	400		
		12.5	10	220/400	R704X	Y
		20	20	200		
		20	10/15	300		
		20	20	200		
		12.5	10	220/250	$\Delta 695$ fs	N
		12.5	15	300/400		

[§]Donor Splice site mutations; Mutations previously reported in ^{||}reference 10 and [‡]reference 16. *Y, yes; N, no.



demonstrated that the R280P mutation markedly reduced the chloride currents to $\sim 30\%$ of the wild-type CLC-5 (Fig. 5), consistent with our previous observations (10) of missense mutations of CLC-5.

Mutational detection by SSCP analysis. 11 of the 14 mutations of CLC-5 (4 Japanese and 10 previously reported [10, 16] in Dent's disease, XRN, and XLRH) found by DNA sequencing of exons 2, 3, 5, 6, 7, 8, 9, 10, and 11 were correctly identified by SSCP analysis (Table III), and the detection of 5 of these is demonstrated in Fig. 6. SSCP analysis has been reported to be more reliable for the detection of mutations in PCR fragments that are smaller than 250 bp (30). However, SSCP analysis of larger CLCN5 PCR fragments, such as obtained with 8.1F and 8.1R which yielded a PCR fragment of 427 bp (Table II), detected the point mutations, thereby indicating that size of the PCR product is unlikely to be the sole determinant of reliable SSCP analysis. Our SSCP analysis of 200 individual PCR products of the CLCN5 gene did not consistently detect any other abnormal bands, thereby indicating an absence of false positives. Thus, SSCP proved to be a sensitive and reliable method in the detection of $> 75\%$ of the CLCN5 mutations.

Discussion

Our results, which have identified four mutations of the CLCN5 gene in the four Japanese families investigated with low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis, expand the spectrum of clinical phenotypes associated with CLCN5 mutations to include this proximal renal tubular disorder of Japanese children. In addition, our results establish that this proximal renal tubulopathy in some Japanese families is an X-linked disorder that has the same genetic etiology as Dent's disease in British families, as XRN in North American families, and as XLRH in Italian families. Indeed, the W279X mutation (TGG \rightarrow TGA), Fig. 1, found in the Japanese family 14/95 (Table I) is identical to the mutation which has previously been identified in an unrelated British kindred (family 7.2/94, reference 10) with Dent's disease. A comparison of the phenotypes of the affected males between the Japanese and British families with this identical W279X mutation requires cautious interpretation as the affected males from the Japanese family 14/95 are 3 and 5 yr old, whereas the affected male (individual II.1, family F, reference 6) presented with renal colic at the age of 21. Thus, the young Japanese boys (II.I

Figure 5. Electrophysiological analysis of *Xenopus* oocytes expressing human wild-type (WT) CLC-5 and the mutant R280P (Fig. 3). *A* illustrates the current traces obtained with the pulse-protocol for voltage clamping (left), in which oocytes were sequentially clamped from a holding potential of -40 mV to voltages between -100 mV and $+100$ mV for 350 ms in steps of 20 mV. The resulting current traces from an oocyte expressing WT CLC-5 (*i*) measured in ND96 (104 mM chloride), at room temperature, and from an oocyte expressing the R280P mutant CLC-5 (*ii*), and from water-injected control oocytes (*iii*) are shown. The WT CLC-5 demonstrated a strongly outwardly rectifying current that was markedly reduced by the R280P mutation. *B* shows the averaged (mean \pm SEM, n = number of oocytes) whole cell currents at $+80$ mV in the *Xenopus* oocytes injected with the WT CLC-5 (3.41 ± 0.23 μ A), the R280P mutant CLC-5 (1.00 ± 0.08 μ A), and control water (0.24 ± 0.02 μ A). The R280P mutation markedly reduced the outward CLC-5 currents.

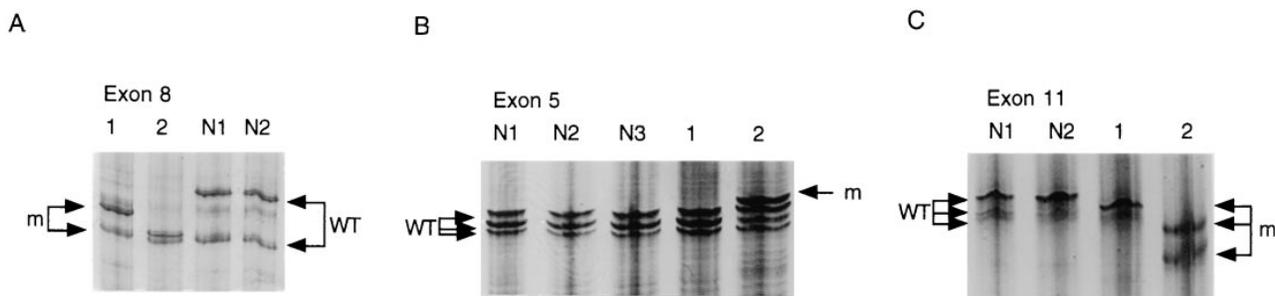


Figure 6. Detection of 5 mutations in exons 8 (A), 5 (B) and 11 (C) by SSCP. A shows the results of SSCP analysis of the W279X (lane 1) and R280P (lane 2) mutations (Table III, Fig. 1) detected in the Japanese families 14/95 and 11.2/95 (Fig. 3), respectively, together with 2 unrelated normals (N1 and N2) (12.5%, 20°C, 350 Vh). The mutant bands (*m*), lanes 1 and 2, differed from one of the wild-type (*WT*) bands. B shows the results of SSCP analysis of the previously reported (10) donor splice site mutations (*gt*→*at*, lane 1; *gt*→*gg*, lane 2) at the exon 5–intron 5 boundary (Table III, Fig. 1) in patients with Dent’s disease, together with three unrelated normals (N1 to N3) (20%, 20°C, 200 Vh). The mutation in lane 2 resulted in one mutant (*m*) band which differed from the three wild-type (*WT*) bands. The mutation in lane 1 could not be detected under these conditions but was identified at 10°C (data not shown). C shows the results of SSCP analysis of the previously reported (10) mutations R648X (lane 1) and R704X (lane 2) in patients with Dent’s disease and XRN (Fig. 1), respectively, together with two unrelated normals (N1 to N2) (20%, 20°C, 200 Vh). The mutants (lanes 1 and 2) resulted in one and two mutant bands, respectively, which differed from the three wild-type (*WT*) bands. SSCP analysis was successful in detecting > 75% of the 14 CLCN5 mutations using the conditions described in Table III.

and II.2, family 14/95) have developed low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis to date (Table I), whereas the British man with Dent’s disease, who is now 34 yr old, suffers from low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, and renal impairment (6).

The four mutations (W279X, R280P, W343X and Δ695fs, Fig. 1) in the Japanese families predict a decrease or a complete loss in the biological activity of the CLC-5, as has been previously demonstrated for other CLC-5 mutations (10). However, loss of function of CLC-5 missense mutations has been observed previously to occur only within the transmembrane domains, whereas similar nonsense mutations were observed outside the transmembrane domains (Fig. 1) (10). Our finding of the disease-causing mutation R280P, which is predicted to be close to the border of the transmembrane domain (Fig. 1) and which markedly reduced the chloride currents (Fig. 5), is of interest in elucidating further the likely structure–function relationships of this recently cloned renal chloride channel. Thus, this mutation of codon 280 results in the replacement of a charged amino acid (arginine), which may be important for defining the position of the *trans*-membrane domain within the membrane, for a proline which is a helix breaker and thereby likely to lead to a significant structural defect. These observations of the R280P mutation in the Japanese family 11.2/95 (Fig. 3) are consistent with our observations (16) of another similarly located mutation G57V (Fig. 1), which is also at the border of a transmembrane domain and in this case the helix-breaking amino acid (glycine) is replaced by a valine. However, a correlation between the different mutations (Fig. 1), the resulting abnormal chloride currents, and the severity of the phenotypes (Table I) could not be established. Thus, the Japanese families with the nonsense mutations (families 11.1/95 and 14/95) and the deletional frameshift mutation (family 13.2/95) had a similar phenotype to that of the family with the missense mutation (family 11.2/95) (Table I), and this was consistent with our previous observations in families with Dent’s disease, XRN, and XLRH (10).

Low molecular weight proteinuria, hypercalciuria, and nephrocalcinosis have been reported to occur in > 60 patients

in Japan and their true incidence and possible genetic homogeneity have not been established (1–4). The findings of CLCN5 mutations in this renal tubular disorder will facilitate the identification of children at risk from this disease, and such mutational analysis may help to complement the annual urinary screening program of Japanese children and their families. However, the mutational diversity within the 2,238 bp of the CLCN5 gene makes mutational screening for this X-linked proximal renal tubulopathy by a direct DNA sequencing approach arduous and time-consuming. Therefore, we have explored the use of the SSCP technique for the more rapid screening of CLCN5 mutations. Our results demonstrate that SSCP was a useful, sensitive, and specific method that was successful in the detection of > 75% of the CLCN5 mutations in the Japanese, British, Italian, and North American families with this proximal renal tubular disorder. Thus, our findings indicate that mutational analysis using SSCP may be of help in supplementing the clinical and biochemical evaluation of patients with these proximal renal tubular disorders.

The mechanisms whereby a functional loss of this renal chloride channel (CLC-5) leads to a generalized proximal renal tubular defect with low molecular weight proteinuria and hypercalciuria remain to be elucidated. The intrarenal expression of CLCN5 in humans is unknown, although CLCN5 expression has been observed in the proximal and distal segments of the rat nephron (14). The reabsorption of filtered protein occurs in the proximal tubule (31), whereas that of calcium occurs in the proximal tubule, thick ascending limb of Henle, and the distal tubule (32), and several mechanisms may be postulated whereby CLCN5 mutations (Fig. 1) with a functional loss of CLC-5 may lead to proximal and some distal renal tubular dysfunction (Table I) in patients with this disorder (1, 6, 7). One possibility is that a loss of CLC-5 function in the proximal tubule may lead to a decrease in chloride reabsorption which in turn results in decreased calcium reabsorption (33). However, this does not explain the abnormal excretion of low molecular weight proteins which are specifically absorbed in the proximal tubule by endocytosis and transported in an acidic vacuolar-lysosomal system (31). A loss of CLC-5 func-

tion in this system would prevent the dissipation of the charge that is generated by the electrogenic H⁺-ATPase pump for the provision of the acidic environment. This may lead to vesicle trafficking abnormalities and a primary failure to reabsorb low molecular weight proteins and a secondary failure to reabsorb calcium and other solutes. An alternative mechanism may involve the chloride channels in the distal renal tubule that have been reported to regulate PTH- and calcitonin-mediated calcium reabsorption by increasing chloride conductance (34, 35). The PTH-dependent calcium reabsorption, which may be blocked by chloride channel inhibitors (32), is associated with epithelial cell membrane hyperpolarization, and a possible loss of CLC-5 function in these distal tubular cells may perturb the cell membrane polarization response to PTH and thereby affect calcium reabsorption. However, these possibilities need to be explored, and the identification of the specific sites in the human nephron that express CLCN5 will help in elucidating these postulated mechanisms and the role and function of CLC-5 in the etiology of hypercalciuria.

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