

THE MOLECULAR BASIS FOR DENT'S DISEASE: AN X-LINKED HYPERCALCIURIC NEPHROLITHIASIS DISORDER*

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SUMMARY

Renal stone disease, which affects 12% of males and 5% of females by the seventh decade, occurs as an inherited disorder in 45% of patients when it is most commonly associated with hypercalciuria. The biochemical basis for hereditary nephrolithiasis and hypercalciuria is unknown, and this has therefore been investigated by a 'positional cloning' approach. As a first step in this approach, the chromosomal location of Dent's disease, which is an unusual form of the renal Fanconi syndrome was determined. An X-linked inheritance for Dent's disease, which is characterised by a low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis and renal failure, was indicated by the observation of a greater disease severity in males and an absence of male to male transmission in five British families. X-linked polymorphic genetic markers were used in linkage studies of these families and the gene was mapped to Xp11. In addition, in one family with Dent's disease, a microdeletion involving the DNA probe M27 β was identified. This microdeletion was further characterised by using yeast artificial chromosomes (YACs) and its size was estimated to be 515 Kb. A search for renal expressed genes from this region identified a novel gene encoding a chloride channel (CLCN5) with similarities to a family of voltage-gated chloride channels. Molecular genetic studies of CLCN5 demonstrated that mutations, which resulted in a functional loss, were associated with Dent's disease. Similar mutations of CLCN5 have also been identified in other hypercalciuric nephrolithiasis disorders, referred to as X-linked recessive nephrolithiasis (XRN), X-linked recessive hypophosphataemic rickets (XLRH) and the idiopathic low molecular weight proteinuria of Japanese children (JILMWP) that have been reported in families from North America, Italy and Japan, respectively. Thus, four hereditary disorders of nephrolithiasis are due to mutations of the novel chloride channel, CLCN5.

Renal stone disease (nephrolithiasis and nephrocalcinosis), which affects 12% of males and 5% of females by the age of 70 years,^{8,30,36} occurs as an inherited disorder in 10% to 45% of patients^{11,30} and is most commonly associated with hypercalciuria.⁶ The inheritance of nephrolithiasis and hypercalciuria has been established to be either autosomal dominant or X-linked in some families.^{2,11,14,15,27,35} The biochemical basis for hereditary nephrolithiasis and hypercalciuria is unknown, and this has therefore been investigated by a 'positional cloning' approach.⁷ The first step in this approach is represented by determining the chromosomal locations of hereditary nephrolithiasis disorders, and then isolating the genes from the critical region. This approach has defined the molecular basis for four diseases of hereditary

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nephrolithiasis, referred to as Dent's disease, X-linked recessive nephrolithiasis (XRN), and X-linked recessive hypophosphataemic rickets (XLRH), and a disorder identified in Japanese children who suffer from idiopathic low molecular weight proteinuria and hypercalciuria (JILMWP). All these four disorders, which represent unusual forms of the renal Fanconi syndrome,¹ are characterised by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis and eventual renal failure.^{2,14,27,35} The clinical features of these disorders together with the molecular genetic studies which demonstrated the aetiological role of a novel renal chloride channel, CLCN5, will be discussed.

DENT'S DISEASE

Clinical features

Dent's disease is a renal tubular disorder characterised by a low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis and eventual renal failure. Dent's disease is also associated with the other multiple proximal tubular defects of the renal Fanconi syndrome which include aminoaciduria, phosphaturia, glycosuria, kaliuresis, uricosuria and impaired urinary acidification, and thus, Dent's disease may be classified as a form of the Fanconi syndrome.³⁵ However, the common occurrences of hypercalciuria, nephrocalcinosis and kidney stones in Dent's disease and the unusual or rare association of these with the Fanconi syndrome are important differences between the two disorders. Dent's disease has been reported in 23 patients (14 males, 9 females), and 20 (11 males, 9 females) of these patients were from five unrelated, non-consanguineous British families and the remaining three patients were males in whom a familial basis for the disorder could not be unequivocally established.^{27,35} Two of the male patients were originally described by Dent and Friedman in 1964⁹ as suffering from the syndrome of 'hypercalciuric rickets' and this disorder has been reported in nine other males;^{4,10,16,28,34} six of these males were also found to suffer from nephrocalcinosis or nephrolithiasis^{10,28,34} and a familial basis for the disorder was established in three patients.^{4,34} An X-linked inheritance for Dent's disease was postulated on the basis of a greater disease severity in males and an absence of male to male transmission in the families.

Molecular genetic studies

Twenty-two X-linked polymorphic genetic markers were used in linkage studies (Fig 1) of two families with Dent's disease. These studies²⁷ established linkage between Dent's disease and the pericentromeric markers DXS426, ARAF1, DXS255 and DXS988, which have been previously mapped to the short arm of the X chromosome, band Xp11.22. In addition, a specific molecular genetic abnormality was identified²⁷ in one family, designated 12/89, with Dent's disease (Fig 2). This abnormality consisted of a microdeletion involving the DNA probe M27 β , which defines the locus DXS255. This microdeletion in Dent's disease was further characterised using yeast artificial chromosomes (YACs), which contained 100 to 350 kilobase (Kb) fragments of human DNA from the region of M27 β (Fig 3). Initially a YAC of 185 Kb size containing M27 β was isolated.¹² The DNA sequence from the telomeric end of this YAC was demonstrated in the Dent's disease patient with the microdeletion, whereas the DNA sequence from the centromeric end was demonstrated to be absent. Thus, the direction and region from which further YACs needed to be isolated to establish a YAC contig i.e. a series of overlapping clones, covering the microdeletion was defined. Isolation of further YACs and deletion

mapping studies revealed that the microdeletion was approximately 515 Kb in size (Fig 3), and a search for renal expressed genes from this region was undertaken using YACs as hybridisation probes to screen a renal cDNA library. These studies^{12,13} lead to the isolation of a cDNA that encoded a protein of 746 amino acids (Fig 4) with homologies to the voltage-gated chloride channel (CLC) gene family, and this novel channel is now referred to as CLC-5 and the gene as CLCN5.

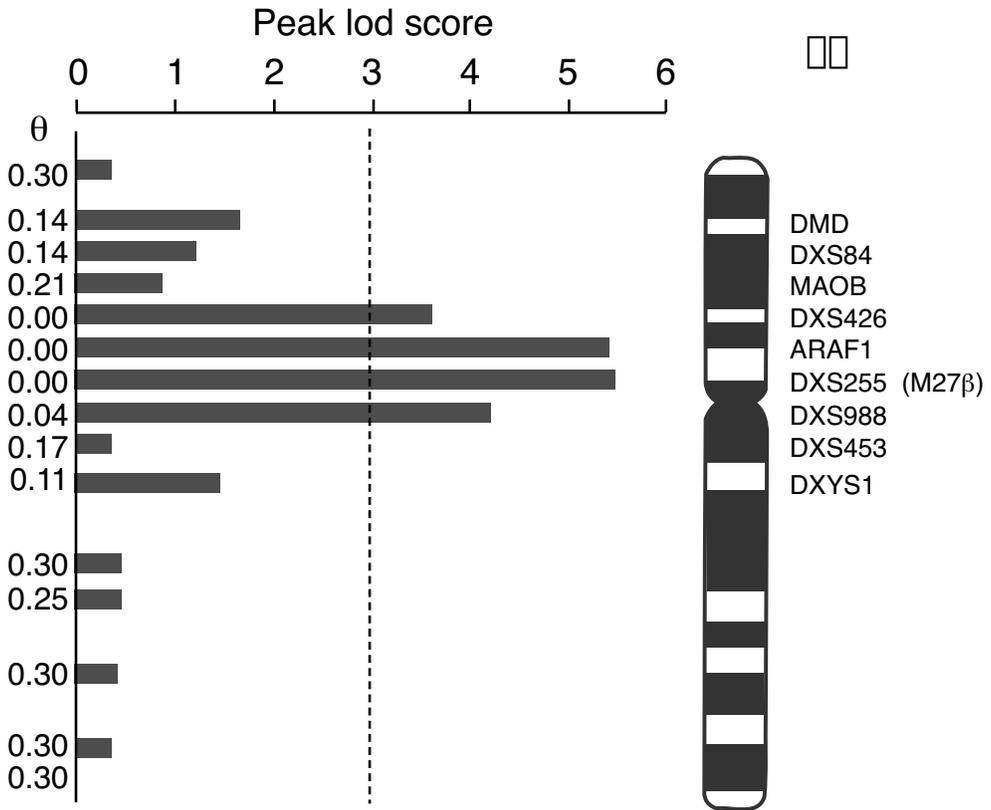


FIGURE 1

Results of linkage analysis of two Dent's disease families with 15 X-linked genetic markers. The bar represents the peak lod score (lod score = \log_{10} odds favouring linkage/odds favouring non-linkage) between Dent's disease and the genetic marker, whose position is shown on the X chromosome, while θ is the recombination fraction at which the peak lod score was obtained. The results revealed significant ($> + 3$) peak lod scores (Z_{\max}) between Dent's disease and the Xp11 loci, DXS426 ($Z_{\max} = 3.60$, $\theta = 0.000$), ARAFI ($Z_{\max} = 5.42$, $\theta = 0.000$), DSX255 ($Z_{\max} = 5.48$, $\theta = 0.000$) and DXS988 ($Z_{\max} = 4.25$, $\theta = 0.045$). Thus, Dent's disease was mapped to the pericentromeric region of the short arm of the X chromosome. (Reproduced with permission from Thakker 1997, 5th International Bone Forum, Editors J. T. Potts and E. Ogata, Yokohama, Japan, in press).³²

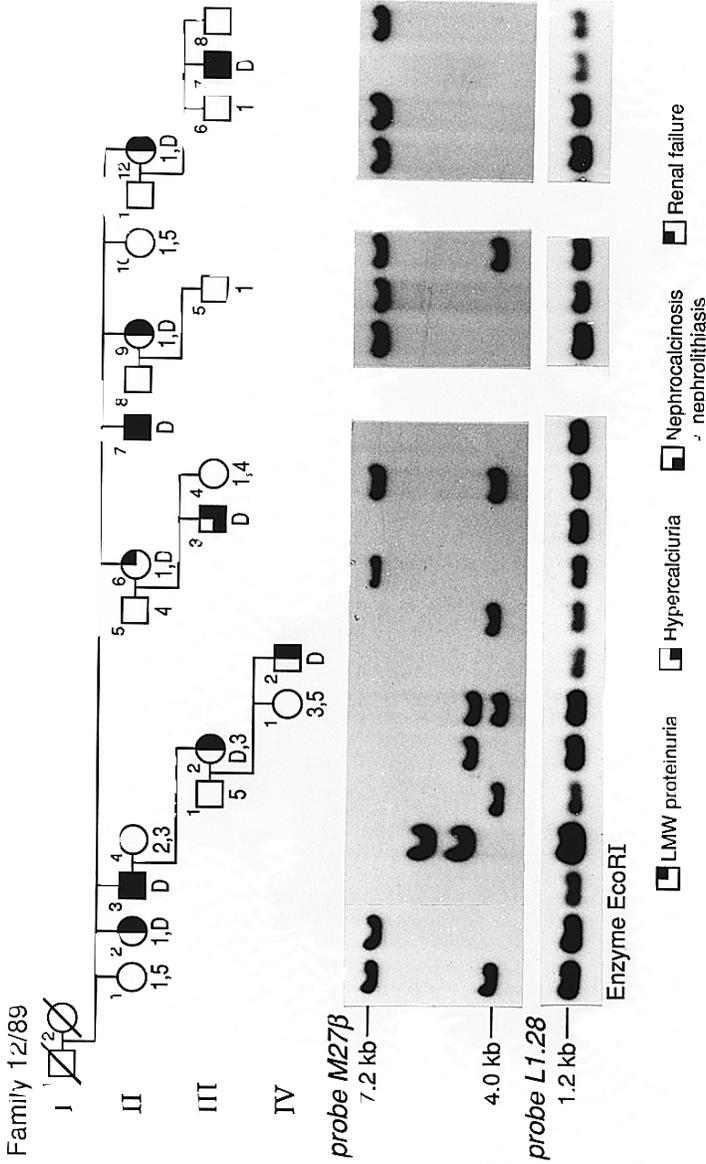


FIGURE 2

Segregation of Dent's disease with a microdeletion detected by M27 β in family 12/89 (upper panel). Probe M27 β , which defines the locus DXS255 and has been localised to Xp11.22 hybridises to EcoRI fragments in the range 3 to 7 kb in normal individuals, and heterozygosity in females exceeds 90 per cent.²⁷ Hybridisation of the Southern blot (lower panel) from family 12/89 with probe M27 β demonstrated an absence of signals in all the affected males (II.3, II.7, III.3, III.7 and IV.2) and only one fragment indicating hemizyosity was detected in the affected females (II.2, II.6, II.9, II.12 and III.2). A control hybridisation of this Southern blot with the probe L1.28, which defines the locus DXS7, yielded signals from all the lanes and demonstrated the presence of DNA in each lane. Thus, a microdeletion involving M27 β is associated with Dent's disease in family 12/89, and this maps Dent's disease to Xp11.22. (Reproduced with permission from Pook *et al* 1993, *Human Molecular Genetics* 2: 2129-2134).²⁷

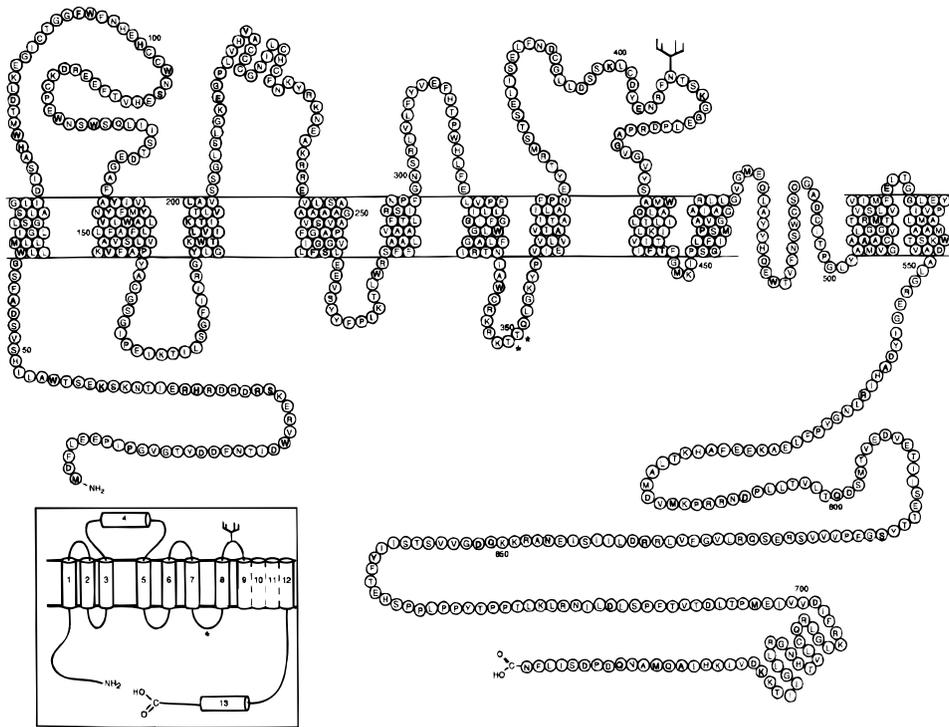


FIGURE 4

Schematic representation of a predicted topology of CLC-5,²² based on the reported DNA sequence.^{12,13} Every 50th amino acid of the 746-amino acid CLC-5 protein and the consensus phosphorylation (asterisk) and glycosylation (branch) sites at codons 349, 350 and 408, respectively, are indicated. The correct topology for the CLC channels is unknown and the predicted topology of the CLC-5 putative transmembrane domains (D1-D13) has been revised¹³ and is based upon a mode^{19,22} (inset) that places D4 extracellularly, and in which the hydrophobic core of the D9-D12 region crosses the membrane 3 or 5 times and contains a hydrophilic region (codons 481-502), the precise location of which remains unknown.

Voltage-gated chloride channel (CLC) gene family and CLCN5

The CLC channels, which are structurally unrelated to other ion channels and form the only known large family (Table 1) of Cl⁻ channels, consist of about 12 transmembrane domains, (Fig 4).¹⁹ The first member, designated CLC-0, was cloned in 1990 from the electric organ of *Torpedo marmorata*,²⁰ and nine different CLCs (CLC-1 to CLC-7, and CLC-Ka and CLC-Kb) encoded respectively by genes CLCN1 to CLCN7, and CLCNKa and CLCNKb, have been identified in mammals.^{3,12,19} These chloride channels are important for the control of membrane excitability, transepithelial transport and possibly regulation of cell volume.¹⁹ CLC channels are known to function as multimeric complexes and recent studies have revealed that CLC-0 is a homodimer with two largely independent pores.^{24,26} The CLC genes are expressed in a variety of tissues (Table 1) and only CLCN1 and CLCN5 are known to have disease associated mutations with the myotonia disorders of Thomsen and Becker,^{21,25,31} and with the hereditary nephrolithiasis disorders, e.g. Dent's disease, respectively.²² The human CLCN5 gene consists of 12 exons that span 25 to 30 Kb of genomic DNA,¹³ and Northern blot hybridisation analysis has identified a 9.5 Kb mRNA transcript that is predominantly expressed in the kidney

and to a lesser extent in placenta and skeletal muscle.¹² The *CLCN5* coding region, which consists of 2238bp, is organised into 11 exons (exons 2 to 12) with 10 introns. Exon 2 and part of exon 3 encode the 57 amino acids of the N-terminal cytoplasmic domain (Fig 4); 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 carboxy-terminal cytoplasmic domain. *CLCN5* is highly conserved in primates, marsupials, rodents, reptiles and birds. 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.²² In addition, ion substitution experiments showed that there was a chloride >iodide conductance sequence, that is consistent with that reported for the other chloride channels, CLC-0, CLC-1 and CLC-2, of this family.

TABLE 1
THE VOLTAGE GATED CHLORIDE CHANNEL (CLC)
GENES AND DISEASE ASSOCIATIONS

Chloride channel	Function	Disease association	Animal	Tissue distribution
CLC-0	Voltage stabilisation	-	<i>Torpedo marmorata</i>	Electric organ, skeletal muscle, brain
CLC-1	Voltage stabilisation	Thomsen's myotonia Becker myotonia arrested development of righting (ADR) in mouse	Mammals (man, mouse)	Skeletal muscle
CLC-2	Cell volume regulation	-	Mammals (rat)	Ubiquitous
CLC-3	?	-	Mammals (rat)	Multiple
CLC-4	?	-	Mammals (man)	Muscle, brain, heart
CLC-5	Chloride reabsorption?	Dent's disease (nephrolithiasis)	Mammals (man, mouse, rat)	Kidney (predominantly)
CLC-6	?	-	Mammals (man, rat)	Multiple (e.g. brain, testes, muscle, kidney)
CLC-7	?	-	Mammals (man, rat)	Multiple (e.g. brain, testes, muscle, kidney)
CLC-Ka	Chloride reabsorption?	-	Mammals (man)	Kidney
CLC-Kb	Chloride reabsorption?	-	Mammals (man)	Kidney

CLCN5 mutations in Dent's disease

An analysis of 8 families with Dent's disease has identified different genetic abnormalities²² which consist of: 2 non-sense mutations; 2 missense mutations; 2 donor splice site mutations; 1 intragenic deletion; and 1 deletion encompassing the

entire gene (Table 2). These CLCN5 abnormalities co-segregate with the disease in each family and are not common sequence polymorphisms. In addition, heterologous expression of the CLC-5 mutants in *Xenopus* oocytes resulted in either an abolishment or a marked reduction in the chloride channels, thereby demonstrating their functional importance.²²

X-linked recessive nephrolithiasis (XRN)

Hereditary nephrolithiasis with renal failure has been reported to occur as an X-linked recessive disorder in one large kindred from northern New York.¹⁴ The disease occurred in males only, who suffered from nephrolithiasis, low molecular weight proteinuria and renal tubular dysfunction in childhood, and from nephrocalcinosis and renal failure in early adulthood. The renal tubular dysfunction had similarities to the Fanconi syndrome and was characterised by kaliuresis, phosphaturia, hypercalciuria, uricosuria, glycosuria, aminoaciduria and an impaired ability to concentrate urine. Linkage studies of 102 members from this one family using X-linked genetic markers helped to determine the map location of XRN to Xp11.22.²⁹ Mutational analysis studies of CLCN5 in this family with XRN demonstrated the Gly506Glu mutation (Table 2) and heterologous expression of this CLC-5 mutant demonstrated a marked reduction in Cl⁻ currents.²² Similar analysis of another XRN family revealed the non-sense mutation Arg704Stop.

TABLE 2
SOME CLCN5 MUTATIONS IN DISORDERS ASSOCIATED
WITH HYPERCALCIURIC NEPHROLITHIASIS

Disorder	Mutation (codon)	CLC-5 location	Predicted effect
Dent's	2kb deletion	D2 to D4	Loss of amino acids 132 to 241 from D2 to D4
Dent's	Donor splice sites of intron 5	D2 + loops D1 to D2 and D2 to D3	Loss of D2 + adjacent loops
Dent's	Leu200Arg	D3	Disruption of charge distribution within D3
XLRH	Ser244Leu	D5	Disruption of helix in D5
Dent's, JILMWP	Trp279Stop	Loop between D5 and D6	Loss of 469 amino acids from D6 to C-terminus
JILMWP	Arg280Pro		Disruption of helix in D6
JILMWP	Trp343Stop		Loss of 403 amino acids from D7 to C-terminus
XRN	Gly506Glu	D11	Disruption of charge distribution within D11
Dent's	Ser520Pro		Disruption of helix in D11
Dent's	Arg648Stop	C-terminal cytoplasmic domain	Loss of 98 amino acids from cytoplasmic domain
XRN	Arg704Stop		Loss of 42 amino acids from cytoplasmic domain
Dent's	515 kb deletion	N-terminal to C-terminal	Absence of CLC-5 protein

X-linked recessive hypophosphataemic rickets (XLRH)

A family with this form of hypophosphataemic rickets was identified in Italy and linkage studies mapped the XLRH locus to Xp11.22.² Interestingly, some affected members from this family also suffered from hypercalciuria, nephrocalcinosis, proteinuria and moderate renal failure. Mutational analysis revealed the Ser244Leu mutation of CLCN5 in this family (Table 2) and expression in *Xenopus* oocytes of this CLC-5 mutant demonstrated a loss of Cl⁻ currents.²²

Hypercalciuria and nephrocalcinosis in patients with idiopathic low molecular weight proteinuria in Japan (JILMWP)

The annual urinary screening programme of Japanese children above 3 years of age has identified a progressive renal tubular disorder characterised by low molecular weight proteinuria, hypercalciuria and nephrocalcinosis.¹⁸ In addition, haematuria, glycosuria, aminoaciduria, an impaired urinary concentrating ability and a mild decrease in creatinine clearance have been observed in some children. The disorder, which has a familial predisposition and occurs predominantly in males, has similarities to the X-linked proximal renal tubular disorders referred to as Dent's disease, XRN and XLRH and that are associated with mutations in the renal chloride channel gene, CLCN5. Mutational analysis of CLCN5 in some Japanese patients with this disorder has revealed non-sense and missense mutations (Table 2).²³

DISCUSSION

These results indicate that CLCN5 is a chloride channel whose functional loss results in a generalised proximal renal tubular defect, i.e. Fanconi syndrome, which is associated with the hypercalciuria and nephrolithiasis of Dent's disease, XRN, XLRH and JILMWP. However, the mechanisms whereby a loss of this renal chloride channel leads to hypercalciuria and the proximal tubular defects remain to be elucidated. The reabsorption of filtered protein occurs in the proximal tubule, whereas that of calcium occurs in the proximal tubule, thick ascending limb of Henle's, and the distal tubule; 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.¹⁷ 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.⁵ A loss of chloride channel function 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. However, these possibilities need to be explored, and the identification of the specific segments of the nephron that express CLCN5 will represent an important step in this pathway towards understanding further the role and function of CLC-5 in the aetiology of hypercalciuria and renal stones.

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