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GENOMIC AND EXPRESSION ANALYSIS OF THE CONGENITAL CHLORIDE DIARRHEA GENE

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Academic dissertation

To be publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in the large lecture hall of the Haartman Institute, Haartmaninkatu 3, Helsinki, on February 9th, 2001, at 12 noon.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on five original publications that are referred to in the text by their Roman numerals.

I Höglund P, Haila S, Socha J, Tomaszewski L, Saarialho-Kere U, Karjalainen-Lindsberg M-L, Airola K, Holmberg C, de la Chapelle A, Kere J. Mutations of the Down-regulated in adenoma (DRA) gene cause congenital chloride diarrhoea. *Nat. Genet.* 14:316-319, 1996.

II Haila S, Höglund P, Scherer SW, Lee JR, Kristo P, Coyle B, Trembath R, Holmberg C, de la Chapelle A, Kere J. Genomic structure of the human congenital chloride diarrhea (CLD) gene. *Gene* 214: 87-93, 1998.

III Höglund P, Haila S, Gustavson K-H, Taipale M, Hannula K, Popinska K, Holmberg C, Socha J, de la Chapelle A, Kere J. Clustering of private mutations in the congenital chloride diarrhea/down-regulated in adenoma gene. *Hum. Mut.* 11:321-327, 1998.

IV Haila S, Saarialho-Kere U, Karjalainen-Lindsberg M-L, Lohi H, Airola K, Holmberg C, Hästbacka J, Kere J, Höglund P. The congenital chloride diarrhea gene is expressed in seminal vesicle, sweat gland, inflammatory colon epithelium, and in some dysplastic colon cells. *Histochem. Cell Biol.* 113:279-286, 2000.

V Haila S, Hästbacka J, Böhling T, Karjalainen-Lindsberg M-L, Kere J, Saarialho-Kere U. SLC26A2 (diastrophic dysplasia sulfate transporter) is expressed in developing and mature cartilage as well as in other tissues and cell types. Submitted, 2000.

Publication I also appears in the thesis of Pia Höglund (1997).
Some additional unpublished data are presented.

ABBREVIATIONS

A	adenine
ACG1B	achondrogenesis 1B
AE	anion exchanger
Alu	repetitive sequence in human genome
AO2	atelosteogenesis 2
APUD	amine precursor uptake decarboxylase
BAC	bacterial artificial chromosome
bp	base pair
C	cytosine
CCM	chemical cleavage mismatch
CD	Crohn's disease
cDNA	complementary deoxyribonucleic acid
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
Cl^-	chloride anion
CLD	congenital chloride diarrhea
CpG island	a short stretch of DNA containing unmethylated CpG dinucleotides
CU	ulcerative colitis
DAB	diaminobenzidine
DGGE	denaturing gradient gel electrophoresis
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DLD	dihydrolipoamide dehydrogenase
DNA	deoxyribonucleic acid
DTD	diastrophic dysplasia
DTDST	diastrophic dysplasia sulfate transporter
ECM	enzymatic cleavage mismatch
EST	expressed sequence tag
G	guanine
HCO_3^-	bicarbonate anion
HGNC	Human Gene Nomenclature Committee
HUGO	Human Genome Organization
IBD	inflammatory bowel disease
kb	kilobase pairs (1 kb= 1000 bp)
KCl	potassium chloride (salt)
kDa	kilodalton
LAMB1	laminin beta1
MED	multiple epiphyseal dysplasia
MIM	Mendelian Inheritance in Man
mRNA	messenger RNA
Na^+	sodium cation
NaCl	sodium chloride (salt)
NHE	Na^+/H^+ exchanger
nt	nucleotide
OH^-	hydroxide anion
p	short arm of a chromosome
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PDS	Pendred syndrome
PRKAR2B	beta2 subunit of a cAMP-dependent protein kinase
PTT	protein truncation test
q	long arm of a chromosome
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SAT-1	human sulfate anion transporter 1
Sat-1	rat sulfate anion transporter 1
SCFA	short chain fatty acid
SDS	sodium dodecyl sulfate
SLC	solute carrier
SO_4^{2-}	sulfate anion
SSCP	single-stranded conformational polymorphism
T	thymidine
TGGE	temperature gradient gel electrophoresis
UPD	uniparental disomy
V317del	deletion of a valine at codon 317
YAC	yeast artificial chromosome

TABLE OF GENE NOMENCLATURE IN THIS THESIS

Approved name by nomenclature committee	Aliases	Functional/disease name
SLC26A1	SAT-1	sulfate anion transporter-1
SLC26A2	DTDST	diastrophic dysplasia sulfate transporter
SLC26A3	CLD,	congenital chloride diarrhea
	DRA	down-regulated in adenoma
SLC26A4	PDS	the Pendred syndrome gene
Slc26a5	prestin	
SLC26A6		
SLC4A1	AE1	anion exchanger 1
SLC4A2	AE2	anion exchanger 2
SLC4A3	AE3	anion exchanger 3
SLC9A1	NHE1	sodium-hydrogen exchanger 1
SLC9A2	NHE2	sodium-hydrogen exchanger 2
SLC9A3	NHE3	sodium-hydrogen exchanger 3

Human gene symbols are in capital letters (for example DRA) while for the rodent genes the symbols are in lowercase letters (for example dra). See p. 25 for the detailed discussion and references of the nomenclature.

SUMMARY

Congenital chloride diarrhea (CLD) is a rare autosomal recessive intestinal disorder with worldwide distribution. It is particularly prevalent in Finland due to a genetic founder effect and thus belongs to the Finnish disease heritage. Other high-frequency areas include Poland, and the Arabic countries Saudi-Arabia and Kuwait. The basic defect in CLD involves the ileal and colonic Cl^-/HCO_3^- exchange leading to watery diarrhea with a high concentration of chloride.

At the beginning of this study, refined mapping had provided two positional and functional candidate genes: PRKAR2B and DRA. Direct sequencing of Finnish and Polish CLD patient samples revealed three different mutations, which confirmed the identity of the DRA gene as the CLD gene. The Finnish major mutation was shown to be a three base pair deletion V317del that was present in all CLD chromosomes studied. Another missense mutation H124L was found in heterozygous form in two unrelated Polish patients who shared an identical haplotype in one chromosome. Finally, finding a frameshift causing deletion 344delT further supported the role of DRA as the CLD gene (I).

The genomic structure of the human CLD gene was determined by direct sequencing of genomic DNA clones and genomic PCR products. Large-scale sequencing of a BAC clone containing the CLD gene was also employed. The human CLD gene spans about 30 kb of genomic DNA and consists of a total of 21 exons separated by 20 introns. In addition, the 600 bp of the 5' flanking region was analyzed by computer to reveal a putative transcription start site and transcription factor binding sites. This enabled the design of primer pairs to amplify each exon separately in the same conditions, which facilitated the screening of yet unidentified mutations and thus provided a system for the molecular diagnosis of CLD. (II)

So far, 28 different mutations in CLD patients from several ethnic backgrounds have been identified. Three of them represent founder mutations responsible for the clustering of the disorder in the high-frequency areas. A high proportion of mutations seem to concentrate into three segments, suggesting either mutational hot-spots or the functional importance of these parts of the CLD protein. (I, III, unpublished data)

In situ hybridization study using an antisense cRNA probe revealed mRNA expression of the human CLD gene in surface epithelial cells of the upper one-third of the colonic crypt. The CLD protein colocalized with mRNA. In addition to colon, CLD is expressed in seminal vesicle and sweat gland, which are not known to be affected in congenital chloride diarrhea. No significant change in CLD expression could be detected in colon samples with inflammation. Search for CLD expression in the multiple malignant colonic tissues revealed an expression that was down-regulated at a later stage than previously thought (I, IV).

In rat tissues, the northern analysis of the highly homologous transporter, the dtdst gene, had shown expression in the intestine and calvaria, which triggered us to study the in vivo expression of the DTDST gene in the colon and in various other human tissues. Immunohistochemistry revealed colonic DTDST expression that colocalizes partially with that of CLD. Expression was also observed in developing and mature cartilage corresponding to the phenotype caused by mutant alleles. In addition, some other tissues with no functional defect in DTD showed expression. Although DTDST is expressed in the colon, it is, however, obvious that it is not able to compensate for the dysfunction of the defective CLD protein as demonstrated by the phenotype of CLD patients (IV, V).

In these studies we have resolved the genetic background of CLD, which has enabled us to develop a system for extensive mutation screening. This will yield an immediate improvement for diagnosis and genetic counseling. Understanding the molecular pathology behind the CLD phenotype is also important for designing a specific and more effective treatment for CLD patients.

Finally, identifying the gene responsible for congenital chloride diarrhea also revealed the identity of a major human ileal and colonic apical Cl^-/HCO_3^- (or Cl^-/OH^-) exchanger. Our studies provide a starting point for the functional analysis of the normal CLD protein, which will lead to detailed knowledge of the basic electrolyte transport physiology of the human colon and important processes involved in the maintenance of electrolyte and pH homeostasis of man.

1. REVIEW OF THE LITERATURE

1.1. Structure and histology of the intestine

The intestinal tract is a long tubular structure starting from the stomach and ending to the rectum. It consists of two functionally and structurally related parts: the small and large intestine. The small intestine can be further divided into duodenum, jejunum, and ileum and the large intestine, or the colon, into cecum, ascending, transverse, descending, and sigmoid colon. The intestine serves a number of functions, the main function being the absorption of nutrients, water, and electrolytes. The intestinal wall consists of four layers: the mucosa, submucosa, external muscular layer, and serosa. In the small intestine, the mucosa and submucosa are modified to increase the luminal surface area by intestinal folds, villi, and microvilli, while the colon is less amplified due to absence of villi. (Guyton, 1991; Ross and Romrell 1989; Dharmsathaphorn, 1994)

The mucosa is comprised of an epithelium, a lamina propria, and a muscularis mucosae. The muscularis mucosae is a smooth muscle layer (inner circular and outer longitudinal) that constitutes the deepest part of the mucosa at the mucosal-submucosal boundary. The lamina propria is a connective tissue layer, which in addition to usual connective tissue cells, contains numerous defense system cells like lymphocytes, plasma cells, and eosinophils, which respond to microbes and other foreign substances. In addition, nodules of lymphatic tissue are found throughout the intestinal mucosa usually extending into the submucosa. Numerous simple tubular glands extend through the full thickness of the mucosa and open into the intestinal lumen. (Ross and Romrell, 1989)

The intestinal epithelium consists mainly of five different cell types: enterocytes, goblet cells, APUD (amine precursor uptake decarboxylase) cells, Paneth cells, and undifferentiated cells. In addition, lymphocytes are usually present within the epithelium. Mucus producing goblet cells are more abundant in colon than in small intestine and are mainly located in intestinal crypts and villi. Mucus serves as a lubricate allowing easy passage of increasingly solid colonic content. It also serves as a protective barrier against some intestinal contents such as bacteria and digestive enzymes. Some APUD and undifferentiated cells are also present in the epithelial crypts. However, the major cell type

is the enterocyte, which is a columnar cell lining the intestinal surface but is also found in glands. The enterocytes function as absorptive epithelial cells primarily responsible for the absorption of nutrients, electrolytes, and water in the small intestine, while in the colon they are mainly engaged in the restoration of electrolytes and water. Intestinal epithelial cells arise from progenitor cells in the base of the crypt and migrate along the crypt to become mature surface epithelial cells which are subsequently after a 3- to 6-day period shedded into intestinal lumen. Although epithelial cells are derived from the same origin, a clear functional separation between the crypt and surface epithelial cells exists. (Guyton, 1991; Ross and Romrell, 1989; Dharmsathaphorn, 1994)

One or multiple layers of epithelial cells line both the external surfaces and internal cavities of the body. In addition, epithelium covers the secretory portion of glands and their ducts, and it also forms some receptor cells of certain sensory organs. Any substance that is absorbed into the body or discharged from it has to pass through epithelial cells. The structure of the epithelium is determined by its function. In the skin, where the epithelium serves as an almost impermeable barrier, it consists of multiple cell layers, while in the intestine where epithelium is involved in absorption and secretion, it consists of only one layer of columnar cells. (Ross and Romrell, 1989)

A lipid bilayer in the epithelial cell wall constitutes a barrier for the movement of water and water-soluble substances between extra- and intracellular spaces. However, some substances can penetrate the lipid bilayer by themselves in order to enter the cell or to leave it. Large amounts of proteins are floating in the lipid bilayer thus interrupting its continuity. Many of these proteins are transport proteins, which can be divided into channel proteins and carrier proteins. Channel proteins have a watery space through them and allow the movement of certain ions or molecules, while carrier proteins bind with substances to be transported, conformational changes in the carrier moving the substances through to the other side of the membrane. (Guyton, 1991; Berne et al., 1994)

1.2. Role of Cl^-/HCO_3^- exchange in ileum and colon

The intestinal fluid absorption and secretion are secondary to the movements of electrolytes, mainly sodium and chloride, through the cell membranes. The permeability of

the intestine decreases in the distal direction and in the colon the epithelium is poorly permeable, which makes an active transport system highly necessary. The absorptive processes primarily take place in the surface/villous epithelium where the substances to be absorbed are in close contact to the large surface area, while secretory processes are localized more to the crypt epithelial cells (Welsh et al., 1982). However, also the surface epithelium is involved in secretion to some extent (Stewart and Turnberg, 1989; Kockerling and Fromm, 1993) and some absorption takes place in the colonic crypts (Singh et al., 1995). Most studies on the electroneutral NaCl absorption have been performed on rabbit ileum and rat colon. Although there are large regional (Sandle et al., 1986) and species-specific differences between various systems responsible for active transport, it is believed that the same key components exist in all species. Nevertheless, their relative contribution to overall NaCl absorption is likely to vary.

1.2.1. Coupled NaCl absorption

Multiple mechanisms are involved in NaCl absorption in the intestinal epithelium. The main component is the active Na^+-K^+-ATPase that establishes an electrochemical gradient across the intestinal mucosa by the outward movement of three Na^+ ions simultaneously with the inward movement of two K+ ions at the basolateral membrane of the epithelial cell (Charney and Donowitz, 1978; Kirk et al., 1980). This electrochemical gradient provides the driving force for sodium translocation across the brush border membrane of the epithelial cell via the Na^+ channel or the Na^+-dependent cotransporters and the exchange carriers (Will et al., 1980; Hediger et al., 1987; Orlowski and Grinstein, 1997). Intestinal chloride is absorbed either via a passive route along gradient generated by electrogenic Na^+ absorption or via an active electronegative transport system against the gradient (Davis et al., 1983).

In the human ileum and colon the active transport system appears to be responsible for the majority of NaCl absorption. Abundant research data indicate that coupled electroneutral NaCl absorption occurs via a Na^+/H^+ exchange linked to a Cl^-/HCO_3^- exchange at the apical brush border membrane of the intestinal epithelial cell (Turnberg, 1970; Knickelbein et al., 1985, 1988). While Na^+ and Cl^- are absorbed there is also a simultaneous flux of H^+ and HCO_3^- across the membrane, which is dependent on Na^+ and

Cl^- but independent of one another. In addition, Cl^- regulates the directionality of Cl^-/HCO_3^- exchange. Thus HCO_3^- can be either secreted or absorbed via Cl^-/HCO_3^- exchange but the direction does not influence Na^+/H^+ exchange activity (Feldman and Stephenson, 1990). The coupling of parallel ion exchanges is thought to occur through changes in intracellular pH. Also various substances have been shown to regulate electronegative NaCl absorption (Sundaram et al., 1991a; Sundaram, 1995). The transport proteins carrying out the Na^+/H^+ exchange function are members of the mammalian Na^+/H^+ exchanger (alias SLC9) gene family with at least six well characterized isoforms (Orlowski and Grinstein, 1997; Counillon and Pouyssegur, 2000). In many tissues, the Cl^-/HCO_3^- exchange activity has so far mainly been linked to three known anion transport proteins of the AE (from anion exchanger; alias SLC4) gene family, namely AE1, AE2, and AE3 (Alper, 1991).

Intestinal expression has been reported for all three members of the AE gene family (Alper et al., 1999; Rajendran et al., 2000). The AE2 protein has been confirmed to localize at the basolateral membrane of surface epithelial cells (Alper et al., 1999), suggesting it to be responsible for Cl^-/HCO_3^- activity there. However, at least two most likely distinct and separate processes performing the Cl^-/HCO_3^- and/or Cl^-/OH^- exchanges are present at the apical membrane of epithelial cells in rat distal colon (Vaandrager and De Jonge, 1988; Rajendran and Binder, 1993). Of these, Cl^-/HCO_3^- transport was observed in surface but not in cryptal epithelial cells, while Cl^-/OH^- exchange was demonstrated both in surface and crypt epithelial cells (Rajendran and Binder, 1999). Cl^-/HCO_3^- exchange is substantially inhibited by Na^+ depletion, which suggests Cl^-/HCO_3^- exchange to be mainly responsible for Na^+ coupled chloride absorption in rat distal colon. Thus, the Cl^-/OH^- exchanger present both in surface and cryptal epithelial cells is suggested to have a role mainly in cell volume and/or pH homeostasis (Rajendran and Binder, 1999). However, the relative contribution of these mechanisms to overall chloride absorption is likely to vary regionally and between species.

Apical Cl^-/HCO_3^- transporter is not solely responsible for ileal and colonic HCO_3^- secretion but other mechanisms participate (Sheerin and Field, 1975; Geibel et al., 2000).

Studies have failed to show the presence of the apical Cl^-/HCO_3^- exchanger in lower cryptal epithelial cells that also secrete HCO_3^-. In addition, only partial inhibition of anion inhibitor DIDS for HCO_3^- transport could be established, which suggests a role for the Cl^-/OH^- exchanger for DIDS effect (Geibel et al., 2000). Furthermore, there is a close association between Cl^- and HCO_3^- secretion. The cryptal chloride secretor cystic fibrosis transmembrane conductance regulator (CFTR) has been shown to regulate different transporters and to be linked to Cl^- independent HCO_3^- secretion in human pancreatic duct cell line, CFPAC-1 (Shumaker et al., 1999). In proximal duodenum of CFTR knockout mice HCO_3^- secretion has been noted to be absent. HCO_3^- secretion probably occurs through different mechanisms in colonic surface and cryptal epithelia (Geibel et al., 2000).

In a villus epithelial cell, Na^+/H^+ exchange activity is present in both apical and basolateral membranes but in a crypt epithelial cell Na^+/H^+ exchange is restricted only to the basolateral membrane (Knickelbein et al., 1988). So far, three of the Na^+/H^+ exchanger isoforms have been localized to ileal and colonic epithelium. NHE1 is ubiquitously expressed and localized to the basolateral membrane of rabbit ileal epithelial villus and crypt cells (Yun et al., 1995). It is believed to perform housekeeping functions and participate in intracellular pH (pH_i) and cell volume regulation. Two Na^+/H^+ exchangers, NHE2 and NHE3, are identified to be apical brush border membrane exchangers in intestinal surface or villous epithelial cells (Hoogerwerf et al., 1996; Bookstein et al., 1997). NHE3 has been proposed to be involved in Na^+ absorption due to the simultaneous stimulation of NaCl and water absorption and induction of NHE3 mRNA expression caused by glucocorticoids (Yun et al., 1993). In addition, on the basis of diarrheal phenotype occurring in mice lacking the functional Nhe3 gene (Schultheis et al., 1998b) but not in mice lacking Nhe2 (Schultheis et al., 1998a), Nhe3 is most likely mainly responsible for colonic Na^+/H^+ exchange concerning coupled NaCl absorption. However, the roles of these two apical Na^+/H^+ exchangers most likely vary among species (Maher et al., 1996; Donowitz et al., 1998; Wormmeester et al., 1998).

1.2.2. Cell volume regulation

Although most mammalian cells are located in an environment with almost constant osmolarity, especially cells in the transporting epithelia, such as intestinal epithelia, are

subject to rapid changes in cell volume. Alterations in cell volume are important events during apoptosis and differentiation, as well as during hypertrophy and atrophy. Mammalian cells utilize a wide variety of volume regulatory mechanisms, including ion transport, osmolyte accumulation, metabolism, and changes in the transcription and expression of genes. In addition, different mechanisms are responsible for determining steady-state volume than for correcting acute changes. The fastest and most efficient mechanism in responding to alterations in cell volume is the activation of ion transport across the cell membrane, which occurs within minutes and is thus most likely achieved by the redistribution of the existing transporters, not by protein synthesis (Janecki et al., 1998).

The major ion transport systems activated following cell shrinkage are the basolateral Na^+-K^+-$2Cl^-$ cotransporter (Geck and Pfeiffer, 1985) and the Na^+/H^+ exchangers (Orlowski and Grinstein 1997). Activation of Na^+/H^+ exchange leads to intracellular alkalinization and a parallel activation of coupled $Cl^-/HCO3^-$ exchange (Ericson and Spring, 1982). The net result is intracellular accumulation of K^+, Na^+, and Cl^-, and thus cell swelling. In addition, Na^+-K^+-ATPase is activated to replace accumulated Na+ by K+. However, not much is known about the ion transport proteins participating in regulatory volume increase (RVI). Activities of Na^+/H^+ exchangers NHE1 and NHE2 are upregulated by cell shrinkage, while NHE3 is inhibited (Demaurex and Grinstein, 1994; Kapus et al., 1994). Of known anion exchangers (AEs) AE2, but not AE1, is suggested to have a role in RVI (Jiang et al., 1997). In cell swelling, mainly separate K^+ and nonselective anion channels are activated, but also electroneutral KCl cotransport. In some cells KCl exit is performed by a parallel activation of K^+/H^+ and Cl^-/HCO_3^- exchange. The anion exchanger AE1 has been suggested to participate in regulatory cell volume decrease (RVD) (Garcia-Romeu et al., 1996; Motais et al., 1997). Further studies of the regulatory proteins are likely to reveal more components.

1.2.3. pH regulation

Intracellular pH (pH_i) is tightly regulated to remain within a narrow physiological range in order to ensure a proper performance of biological processes involved in cell functioning and cell survival (Busa and Nuccitelli, 1984; Orchard and Kentish, 1990; Isfort et al.,

1993). Especially in the intestinal epithelial cells, pH_i regulation is challenged by the continuous transepithelial traffic of ions and other acid/base equivalents (Boron, 1986). Thus, a single cell utilizes multiple simultaneous pH_i regulatory mechanisms, which can be divided into the acid loaders and acid extruders. In many cell types, parallel Na^+/H^+ and Cl^-/HCO_3^- exchanges perform acid-extruding and acid-loading functions, respectively, in adjusting intracellular pH (Simchowitz and Roos, 1985; Jentsch et al., 1986; Paradiso et al., 1987; Boyarsky et al., 1988; Stuenkel et al., 1988; Sundaram et al., 1991b). Both mechanisms are pH_i-sensitive and, besides the regulation of basal pH_i, Na^+/H^+ exchange is thus activated in the recovery from acid load and $Cl^-/HCO3^-$ exchange in the recovery from alkaline load (Boyarsky et al., 1990; Sundaram et al., 1991b).

In a human intestinal epithelial cell, selective activation of apical Na^+/H^+ exchange in response to the local pH_i change generates a local pH_i microenvironment that optimizes the efficient absorptive function most likely by recycling H^+ across the membrane (Thwaites et al., 1999). In addition, tightly regulated extracellular surface pH microclimate (pH_s) is present directly adjacent to the apical membrane of the intestinal epithelial cells (Rechkemmer et al., 1986; McNeil et al., 1987; Genz et al., 1999) as well as pH microdomain in the lumen of the colonic crypt (Chu and Montrose, 1995). In a guinea-pig, bicarbonate, which is mainly secreted by apical Cl^-/HCO_3^- exchanger but also by $SCFA/HCO_3^-$ exchanger is responsible for establishing and maintaining the pH microclimate at the surface of colonic mucosa (Genz et al., 1999).

1.3. Mechanisms of diarrhea

Diarrhea is usually defined as an increase in stool mass, stool frequency, or stool fluidity. It is classified as acute or chronic according to duration. Acute diarrhea is most frequently due to different infectious agents (Binder, 1990), while the causes of chronic diarrhea are more diverse, including irritable bowel syndrome, idiopathic inflammatory bowel disease, malabsorption syndromes, chronic infections, and idiopathic secretory diarrhea. Regardless of the primary cause, the major mechanisms leading to the intestinal dysfunction resulting in diarrhea involve the abnormal amount of osmolytes in the intestinal lumen, altered intestinal motility, increased net secretion of water and

electrolytes, and a decrease in the normal absorption of solutes (Fine et al., 1989). Multiple mechanisms are often involved.

Osmotically active substances can be retained in the intestinal lumen due to generalized malabsorption or due to specific absorption defects, such as ones caused by disaccharidase deficiencies (Christopher and Bayless, 1971) or rare inherited disorders like glucose-galactose malabsorption (Wright, 1993; Martin et al., 1996). Excessive ingestion of poorly absorbable substances, such as sorbitol, lactulose or divalent ions, also causes increased luminal osmolality and osmotic diarrhea (Saunders and Wiggins, 1981; Rumessen and Gudmand-Hoyer, 1988). Enhanced intestinal motility leads to a too rapid transit time of the intestinal contents and thus disturbs absorption, while decreased motility facilitates bacterial overgrowth (Fine et al., 1989). Secretory diarrhea results from either enhanced secretion or a combination of enhanced secretion and the failure of reabsorption (Field et al., 1989). A typical feature of secretory diarrhea is the fact that no improvement is achieved by fasting. Bacterial endotoxins, hormones, and detergents are known stimulants for intestinal secretion. Decreased absorption of ions may be secondary to intestinal factors, such as decreased surface area and altered epithelial cell dynamics, or due to primary defects in intestinal ion transporters. Rare causes of diarrhea include congenital chloride diarrhea and congenital sodium diarrhea that result from inherited defects of the Cl^-/HCO_3^- exchange and the Na^+/H^+ exchange, respectively (Holmberg et al., 1977a; Booth et al., 1985; Holmberg and Perheentupa, 1985). Unabsorbed electrolytes retained in the intestinal lumen lead to diarrhea mainly by osmotic mechanisms. These rare congenital disorders with a diarrheal manifestation provide unique knowledge on the importance of the individual mechanisms for intestinal transport physiology.

1.4. Inflammatory bowel disease (IBD)

Ulcerative colitis (colitis ulcerosa, CU) and Crohn's disease (CD) are chronic intestinal inflammatory disorders that have many features in common. Their etiology is unknown, although infections, immunological changes, genetic predisposition, diet, smoking, and several other causes have been proposed to participate in their pathogenesis. Both CU and CD are most common in the Western World with prevalence approximately 1/500 in CU and 1/1500 in CD. The clinical features in both disorders are highly variable depending on the site of the disease and the severity of the inflammation, typically including diarrhea

with or without blood, abdominal pain, mild fever, and general malaise. Crohn's disease most often affects the terminal ileum and the right side of the colon, although any site of the gastrointestinal tract can be involved. CU, on the other hand, is typically limited to colon and rectum. However, in both CU and CD extraintestinal manifestations may be detected. Histologically, CD is characterized by the presence of non-caseating granulomas, transmural infiltration by lymphocytes and plasma cells, fibrosis, and hypertrophy of the muscularis mucosae. In contrast, in CU inflammation typically affects only mucosa and submucosa, and the leukocyte infiltrate consists of mononuclear cells and neutrophils as well as occasional eosinophils and mast cells. (Crawford, 1994; Souhami and Moxham 1994)

In ulcerative colitis, absorption of NaCl accompanied with water is impaired in inflamed colonic segments. The concentration of sodium and chloride in the stools of CU patients is elevated and stool pH is lower than normal (Harris et al., 1972; Hawker et al., 1980). Reduced secretion of bicarbonate coincides with high fecal loss of chloride (Caprilli et al., 1986), which alone would make it tempting to speculate the participation of apical Cl^-/HCO_3^- exchanger in the pathogenesis of diarrhea in ulcerative colitis. However, also electrogenic Na^+ transport is mainly lost in inflamed colon, and overall ionic permeability and the electrical conductance of the colonic mucosa are increased. In addition, the activity of the basolateral membrane Na^+-K^+-ATPase in epithelial cells is diminished (Sandle et al., 1990). Although electrogenic absorption of sodium and chloride has been shown to be defective in IBD, the role of coupled, electronegative NaCl absorption mechanism in the pathogenesis of diarrhea has remained unclear. Recent study using a rabbit model of chronic inflammation established the impairment of coupled electronegative NaCl absorption in villus cells to result from decreased apical Cl^-/HCO_3^- exchange activity while apical Na^+/H^+ exchange activity remained unaffected (Sundaram and West, 1997). In chronically inflamed rabbit ileum, the Cl^-/HCO_3^- exchange was inhibited through a diminished affinity for chloride whereas the number of transporters was not altered (Coon and Sundaram, 2000).

The mechanisms leading to altered epithelial ion permeability and disturbances in active transport processes in inflamed intestine are unclear. The roles of different inflammatory mediators have been studied extensively. In addition, cell turnover is enhanced in active

colitis and thus the proportion of cryptal-like enterocytes that lack the ion absorption machinery normally present in mature surface epithelial cells is increased (Allen et al., 1985).

1.5. Congenital chloride diarrhea (CLD)

Congenital chloride diarrhea (CLD, MIM No. 214700) was recognized as a new syndrome in 1945 by Gamble et al. and Darrow, who described it as "congenital alkalosis with diarrhea" (Gamble et al., 1945; Darrow, 1945). In 1971 the genetic study of 14 Finnish and 12 other families showed it to be inherited as an autosomal recessive trait and the name congenital chloride diarrhea was recommended for the disorder (Norio et al., 1971).

1.5.1. Clinical characteristics

The main clinical feature of CLD is a chronic, life-threatening, watery diarrhea (Holmberg et al., 1977a). It begins during the prenatal period and leads constantly to polyhyrdamnios often associated with premature birth. Birth weights and lengths are normal for the gestational age (Holmberg et al., 1977a). The babies have distended abdomens and lack meconium, which may lead to a suspicion of intestinal obstruction and an unnecessary laparotomy (Langer et al., 1991). Voluminous diarrhea that may be mistaken as urine causes an excessive loss of weight with severe dehydration in the first few days of life, and the babies fail to thrive. Also hypochloridemia and hyponatremia develop rapidly, followed later by hypokalemia, hyperbilirubinemia, and metabolic alkalosis (Norio et al., 1971; Holmberg et al., 1977a; Holmberg, 1986). Blood sodium levels, however, normalize after the neonatal period. In addition to a high chloride concentration, a low concentration of bicarbonate and a low pH is detected in stools, and urine is Cl^--free due to the volume depletion (Holmberg et al., 1977a). Most untreated patients die during the first months of their life. Some do survive without treatment but suffer from chronic hypochloridemic dehydration causing retarded growth and development. Furthermore, chronic hypovolemia, together with acute insults of dehydration and anuria in patients with inadequate substitution, leads to an early kidney failure which is histologically characterized by juxtaglomerular hyperplasia, hyalinized glomeruli, calcium deposits, and vascular changes similar to those in hypertension (Holmberg et al., 1977b). Adequate

treatment corrects all electrolyte and hormonal abnormalities and prevents the development of renal lesions (Holmberg, 1986). Properly treated children are therefore likely to grow and develop normally.

1.5.2. Diagnosis and treatment

A history of polyhydramnios and prematurity together with persistent watery diarrhea should lead to a suspicion of congenital chloride diarrhea. If electrolyte disturbances are corrected, a detection of high fecal chloride content (over 90 mmol/l) verifies the diagnosis (Holmberg, 1986). After the neonatal period, the fecal Cl^- concentration exceeds the sum of the Na^+ and K^+ concentrations, although Cl^- concentration may be low due to severe dehydration and disturbed electrolyte balance (Holmberg, 1978). Ultrasonographic diagnosis already during the prenatal period is possible on the basis of distended loops of the intestine (Kirkinen and Jouppila, 1984).

The aim of the treatment is to maintain a normal fluid and electrolyte balance, which is achieved simply by the continuos supplementation of the diarrheal loss of water, Cl^-, Na^+, and K^+. In the case of newborns, the supplementation is carried out intravenously, while older children and adults take balanced supplement solutions orally (Holmberg, 1986). Oral administration is based on passive diffusion of electrolytes through the more permeable small intestinal epithelium. The dosages of NaCl and KCl are adjusted to maintain normal pH in blood and Cl^- excretion into urine (Holmberg, 1986). Although diarrhea continues as long as the fluid and electrolyte balance remains normal, secondary consequences are prevented.

1.5.3. Intestinal pathophysiology

In CLD, the basic defect is the impairment or absence of an active ileal and colonic Cl^-/HCO_3^- exchange mechanism (Holmberg et al., 1975). The defect in chloride absorption causes hypochloridemia and large amounts of chloride are retained in intestinal lumen, which leads to diarrhea by osmotic mechanisms. Simultaneous bicarbonate secretion is absent, leading to intracellular alkalinity and acidification of intestinal content. Although the Na^+/H^+ exchanger itself is intact, both of these further inhibit sodium absorption through it (Holmberg, 1978). Hyponatremia and chronic hypovolemia lead to secondary

hyperaldosteronism and high renin activities that further enhance the loss and depletion of K^+, thus increasing metabolic alkalosis (Holmberg, 1978).

1.5.4. Epidemiology and genetics

Since the first description of CLD by Gamble et al. and Darrow in 1945, approximately 200 CLD cases have been reported worldwide, about 50 of them in Finland (Norio et al., 1971, 1973; Holmberg et al., 1977a; Holmberg, 1986). Other high-frequency areas include Poland, Saudi Arabia, and Kuwait (Tomaszewski et al., 1987; Lubani et al., 1989; Shaltout et al., 1989; Khan and Yaish, 1992; Kagalwalla, 1994). The highest incidences have been reported in the Arabic countries where consanguineous marriages are common. In Saudi-Arabia, CLD is estimated to occur 1 in 5,000 newborns (Kagalwalla, 1994) and in Kuwait even 1 in 3,200 (Badawi et al., 1998). In the eastern provinces of Finland, the incidence is 1 in 20,000 births, while in Poland the disorder is much more rare with an estimated incidence of 1 in 200,000 (Höglund et al., 1998). Furthermore, sporadic patients have been described in many different ethnic backgrounds and populations, including almost all European countries, the United States and Canada, Argentina, many countries in Asia and the Middle East, and Australia. Many patients are likely to remain unrecognized and die in early infancy.

The hunt for the CLD gene started by screening three candidate genes for mutations and a subsequent linkage mapping of the CLD gene close to a known chloride transporter, the cystic fibrosis transmembrane conductance regulator (CFTR) gene in chromosome 7q (Kere et al., 1993). The genetic linkage disequilibrium study in the Finnish founder population refined the localization of the CLD gene (Höglund et al., 1995), and a physical map was constructed in order to identify the gene responsible for congenital chloride diarrhea (Höglund et al., 1996). The physical map consisted of 51 YAC clones that covered 2,7 Mb around marker D7S496. Four known genes (DRA, PRKAR2B, LAMB1, and DLD) and 13 CpG islands corresponding most likely to yet unknown genes were established to that region (Höglund et al., 1996). The DRA (for down-regulated in adenoma) gene localized to the most critical region suggested by the linkage disequilibrium mapping (Höglund et al., 1995). It was initially cloned as a putative tumor suppressor gene due to its down-regulated expression in colon adenomas and carcinomas (Schweinfest et al., 1993). Furthermore, its expression was limited to intestinal epithelium

(Schweinfest et al., 1993) and it demonstrated protein homology to known sulfate transporters across a large taxonomic span. Both the DRA gene and another gene, PRKAR2B, map within 450 kb of marker D7S496. PRKAR2B encodes a regulatory subunit for protein kinase A (Solberg et al., 1992). They were both concluded to be positionally and functionally relevant candidate genes (Höglund et al., 1996), although the possible participation of the DRA gene in anion transport made it an especially attractive candidate for CLD.

1.6. Diastrophic dysplasia sulfate transporter (DTDST)

Diastrophic dysplasia (DTD, MIM No. 222600) is an autosomal recessive chondrodysplasia characterized by short-limbed dwarfism, spinal deformation, and generalized dysplasia of the joints (Walker et al., 1972). A new linkage disequilibrium approach was successfully utilized in the search for the gene responsible for DTD, and a novel gene was designated as the diastrophic dysplasia sulfate transporter, DTDST, was cloned. The northern analysis of the Finnish DTD patient samples demonstrated markedly reduced expression of the DTDST gene. Its identity as the gene responsible for diastrophic dysplasia (DTD) was confirmed by identifying point mutations in DTD patients from other populations (Hästbacka et al., 1994). Furthermore, fibroblasts from a DTD patient demonstrated a defective sulfate uptake, which gave further functional support. Since then, three more chondrodysplasias, achondrogenesis IB (ACG1B; Superti-Furga et al., 1996), atelosteogenesis type II (AO2; Hästbacka et al., 1996), and recessive multiple epipyseal dyplasia (rMED; Superti-Furga et al., 1999), have been shown to result from mutations in the DTDST gene and thus to be allelic to diastrophic dysplasia.

Surprisingly, the northern analysis showed DTDST expression in all tissues studied although clinical abnormalities in DTD are restricted to cartilage and bone (Hästbacka et al., 1994). However, later northern analysis of rat tissues has suggested a more restricted expression pattern including only calvaria and intestine (Satoh et al., 1998). As the DTDST gene was cloned, it was shown to be homologous to the known rat sulfate transporter Sat-1 (Bissig et al., 1994) and also to the recently cloned putative tumor suppressor gene DRA (Schweinfest et al., 1993). Functional study has established that both human and rat DTDST cRNA injected to Xenopus oocytes induces Na^+-independent sulfate transport that can be inhibited by extracellular chloride and bicarbonate (Satoh et

al., 1998). DTDST was suggested to function as a SO_4^{2-}/Cl^- antiporter. Similar activity has also been detected using chondrocytes. Studies using DTD patient fibroblasts and chondrocytes have demonstrated defective sulfation of proteoglycans (Rossi et al., 1996).

Sulfate is an important constituent of highly sulfated macromolecules, like mucins and proteoglycans, produced by the cells. Inorganic sulfate available for sulfation in the intracellular biosynthesis of macromolecules is supplied by SO_4^{2-} transport, but also by catabolizing sulfur-containing amino acids (Elgavish and Meezan, 1991). The ability of cells to use sulfated amino acids as the alternative sources for sulfation reactions at low extracellular concentrations of free inorganic sulfate is variable. Although Chinese hamster ovary cells and mouse 3T3 fibroblasts synthesize properly sulfated macromolecules in the absence of exogenous sulfate (Esko et al., 1986; Keller and Keller, 1987), study on human chondrocytes deficient with DTDST has shown only partial ability to compensate for the lack of inorganic sulfate in their macromolecular biosynthesis (Rossi et al., 1996), leading to severe chondrodysplasia.

1.7. Human gene nomenclature

The significance of a standardized, consistent nomenclature was generally recognized to get the most (consistency and searchability) out of the multiple databases used for different purposes. The Human Gene Nomenclature Committee (HGNC) is a subcommittee of The Human Genome Organization (HUGO) working on approving and implementing human gene names and symbols. The aim is that the approved symbols are always unique and no two genes have the same name or symbol. Some journals (for example Nature Genetics, Genomics etc.) insist on the use of approved gene symbols. The confusing disconcordance of gene names and symbols is partly due to researchers' desire to give their own name for the gene they have discovered and published. Recently gene nomenclature has been further challenged by the development of high-throughoutput sequencing techniques and data provided into electronic databases throughout the world. There has also been a need for a system for naming members of a gene family. Several systems are in use, but probably the most frequently used is the symbol stem followed by a number assignment (for example SLC26A1, SLC26A2, SLC26A3, etc.), which makes expanding easy when related genes are discovered. For the system to function the gene family has to be well established and characterized. Guidelines for Human Gene

Nomenclature (1997) was published in order to help each gene symbol to be unique and appropriate (White et al., 1997). However, scientists are responsible for a new gene symbol to be verified in the HUGO Nomenclature Committee (see http://www.gene.ucl.ac.uk/nomenclature/).

Some basic concepts according to the nomenclature guidelines (White et al., 1997) follow:

1. A gene symbol may be used to designate a clearly defined phenotype shown to be inherited as a monogenic mendelian trait (Example: CLD).
2. A gene symbol may be used to designate a cloned segment of DNA with a sufficient structural, functional, and expression data to identify it as a transcribed entry (Example: DRA).
3. Gene family members should be made recognizable in order to facilitate retrieval of related data from databases. This is often done by using the consistent gene symbol stem and a numbering system (Example: SLC26A1, SLC26A2, SLC26A3, etc.).

The suggested model for using different gene symbols for the same gene is to use the approved gene symbol alongside the alias favored by the author in the title and/or abstract, and either the approved symbol or alias can then be used in the rest of the paper without further confusion. The challenge for meaningful gene names and symbols is novel genes without sufficient characterization. Renaming also happens. It is usual for the gene symbol to change when the gene product or function responsible for the clinical disorder is identified. Often a gene symbol based on the product or function already exists and usually supersedes the symbol derived from the clinical disorder.

The gene responsible for congenital chloride diarrhea will be called the CLD gene throughout this thesis, the oldest designation being the CLD gene for the defined recessively inherited CLD phenotype. In addition, we find that the CLD gene is the functionally correct designation for the suggested intestinal anion transporter.

1.8. Methods for genomic and expression analysis of a disease gene

1.8.1. Mutational screening

Four traditional approaches for identifying human disease genes are functional cloning, positional cloning, position-independent candidate gene strategy, and positional candidate gene strategy. Identification of the disease gene using functional cloning relies purely on the available biological information, such as the pathogenesis of the disease or the biochemical fuction of the protein. In positional cloning, the disease gene is isolated on the basis of its location in the genome (Collins 1992, 1995). The subchromosomal location of the gene may be determined by genetic linkage analysis, by loss of heterozygosity screening, or by chromosomal abnormalities. No knowledge of the subchromosomal location is needed in position-independent candidate gene approach (Collins 1995). In this method, a candidate gene is suggested by homologous genes, which code proteins in similar human phenotypes, or by homologous genes related to an animal phenotype showing similarity to a human disorder. The positional candidate gene approach combines positional cloning with a candidate gene strategy (Ballabio 1993). Due to the Human Genome project and recent advances in identification of cDNAs throughout the human genome, it is becoming the most popular method to identify disease genes.

When a candidate gene or genes are found they need to be analyzed for the presence of putative disease causing mutations. Mutation screening usually starts from the coding region of the gene, although some mutations affecting the function are located outside the coding region. After screening the coding region with a negative result, the clinical picture of the patient should be checked once more to make sure that the condition is not different from the one you are studying. Then mutation screening can be extended to the putative promoter region or to other areas containing gene regulatory elements.

Multiple methods are available for the mutational screening of the gene of interest. In a research laboratory, both availability and suitability often determine the methods to be chosen. The number of samples to be analyzed, the size and number of candidate genes, the expected nature of mutations, and both the speed and accuracy of the method will affect the choice. Much time is not wanted to be spent on optimizing the test at this stage.

Direct sequencing

A widely used method for screening novel mutations in patient samples is the direct sequencing. It has become popular after automated fluorescence sequencers have provided a fast and reliable option for more laborious manual sequencing methods (Smith et al., 1986). If genomic PCR is not possible, sequencing can be performed with RT-PCR fragments (I). However, a high quality template is needed to avoid investigating sequence artifacts. Direct sequencing detects all sequence changes, which simultaneously become fully characterized. The possible disease causing nature of the detected mutations has to be verified with further study. A major disadvantage of direct sequencing with an automated sequencer is its high cost, which makes cheaper methods more attractive, especially when scanning large genes with multiple exons for mutations. Often sequencing is used as a secondary method to confirm and characterize the mutations detected by some other methods such as SSCP, DGGE or DHPLC.

Single-stranded conformational polymorphism (SSCP) and heteroduplex analysis

The SSCP (Orita et al., 1989; Sheffield et al., 1993) and heteroduplex gel mobility are frequently used simple, inexpensive methods suitable for mutational screening. They can be performed simultaneously on a single gel. In SSCP, after denaturation amplified DNA samples are separated under non-denaturing conditions in a polyacrylamide gel. This allows single-stranded fragments to fold up and form a structure that is stabilized mostly by hydrogen bonding. The electrophoretic mobility of these structures will depend on chain length and on conformation determined by the DNA sequence. Sequence variations are then detected as mobility shifts in the gel.

Heteroduplexes are formed by denaturing the amplified DNA sample and allowing it to cool down slowly. Heteroduplexes have often altered electrophoretic mobility under nondenaturing conditions. Both SSCP and heteroduplex gel mobility are suitable for analyzing short fragments (~200 bp) and numerous control samples are needed to reveal differences from wild-type pattern. The mutation detection sensitivity of these methods is limited and they don't reveal the position or nature of the sequence change causing the mobility shift. Sequencing is needed to confirm mutations.

Other methods also rely on the altered properties of heteroduplexes. Denaturing gradient gel electrophoresis (DGGE) is based on the unique melting temperature of DNA duplex. Amplified fragments are separated in a chemical (DGGE) or temperature gradient gel (TGGE). The mobility of the fragment changes significantly when it denatures. This method requires the design of special primers containing GC extension (~40 bp) called a GC-clamp at their 5'-end. 100% sensitivity has been reported with 2D-DGGE (Dhanda et al., 1998), but even the basic version is sensitive.

Denaturing high performance liquid chromatography

Heteroduplex formation in amplified samples is utilized by denaturing high performance liquid chromatography (DHPLC) as well (Oefner and Underhill 1995; Underhill et al., 1997; Liu et al., 1998). PCR products are subjected to ion-pair reverse-phase liquid chromatography under partially denaturing conditions. Heteroduplexes display reduced column retention time relative to their homoduplex counterparts and the elution profiles for the samples containing heteroduplexes differ from those containing homozygous sequence. A maximum sensitivity is achieved with fragments of 150-550 bp, although fraqments up to 1500 bp can be analyzed (O'Donovan et al., 1998). The most advantages of DHPLC include semi-automation of the method, speed of analysis, and flexibility of fragment size. Thus, it is a highly sensitive and reliable method for mutational screening.

Chemical and enzymatic cleavage of mismatch (CCM, ECM)

A sensitive method for identifying sequence changes in larger fragments (up to 1 Kb) is the cleavage of mismatched bases. It can be performed either chemically (CCM; Cotton et al., 1988) or enzymatically (ECM; Youil et al., 1995), but enzymatic cleavage is more convenient due to the toxicity of chemicals. Its advantage lies in the fact that the sequence change can be localized on the basis of the size of the fragments generated.

Protein truncation test (PTT)

PTT is a sensitive method for the detection of protein truncating mutations. Its major advantage is that, in addition to detecting mutations, it also demonstrates their deleterious effects on the functional level. Other benefits include a low false-positive rate and

localization of the mutation. In a recent modification of PTT, an N-terminal tag has been added to the forward primer, which facilitates the detection of correctly initiated proteins only (de Koning Gans et al., 1999). Overall, PTT is sensitive and efficient in screening disease genes in which a significant proportion of mutations are known to be truncating, for example in tuberous sclerosis (TSC1, Benit et al., 1999), neurofibromatosis (NF1, Heim et al., 1995), and breast cancer (BRCA1, Lancaster et al., 1996a; BRCA2, Lancaster et al., 1996b).

Nature of the sequence variation

After a sequence change in the coding region of a gene is found its possible pathogenicity has to be verified. The nature of the sequence variation and its genomic context can give some clues to its possible pathogenicity. Whole gene deletions are certain to destroy gene function. Also small deletions as well as insertions in the coding region are likely to be pathogenic, especially if they introduce a frameshift. Nonsense and frameshift mutations cause premature termination of translation and thus truncated polypeptides, which obviously destroys or severely affects the function. Mutations within 5' or 3' consensus splice sites of introns are likely to affect the splicing and the function of the gene. If a missense mutation occurs in the region of a known structure or important function it is more likely to be pathogenic for the function. Mutations affecting evolutionary conserved residues or causing non-conservative amino acid substitutions are more prone to affect the function. The pathogenic nature of the mutation is supported by an independent detection of the mutation in an unrelated patient with the same disease and the failure to show such a mutation in a large number of normal controls. Also separate mutations in the same gene in different families affected by the disease make pathogenicity more probable. Further support is provided by the cosegregation of the mutation and disease phenotype through a family pedigree. Finally, the effect of the mutation for the function of the protein can be studied in vitro to demonstrate that a mutant protein in vitro shares the same biochemical properties and characteristics as its in vivo counterpart.

1.8.2. Testing for specified mutations

After the sequence variation in the candidate gene has been found, the verification of its pathogenicity often starts with screening a large number of normal control chromosomes

for the presence of the change. In addition, screening is necessary in studying other patients with diseases with one particular sequence change or a limited number of specific mutations, and in studying family members of affected individuals with a specified sequence change. Any of the methods suggested for mutational detection can also be used in screening the specified mutations. The optimal screening method is fast, easy to perform, sensitive, and economical.

The presence of most deletion and insertion mutations can be detected directly after gel electrophoresis due to the altered size of the designed PCR amplification fragment (I, III). When mutation generates a novel restriction enzyme recognition site or causes loss of an existing one, a simple screening method based on restriction enzyme digestion after PCR amplification can be designed (I, III). An artificial restriction site to detect mutation can also be introduced by PCR mutagenesis. PCR-OLA (oligonucleotide ligation assay; Landegren et al., 1988) is a suitable method for automated mutation detection. After PCR-amplification of the template sequence, two oligonucleotides are hybridized to adjacent sites in the target, followed by the covalent ligation of the oligonucleotides in case of a perfect match. The method has been further developed for simultaneous screening of two mutations in a single microtiter plate well (Romppanen and Mononen, 2000).

Heteroduplex instability is utilized in allele-specific oligonucleotide (ASO) hybridization. A short ASO probe will hybridize to target sequence only if base complementarity is perfect between them and even a single nucleotide substitution renders heteroduplex unstable (Conner et al., 1983; Lau and Tolan, 1999). The PCR equivalent of ASO hybridization is called allele specific amplification (ASA; Newton et al., 1989). It is based on the dependence of PCR amplification on correct base pairing at the 3'end and thus utilizes oligonucleotide primers that are designed to differ at the very 3' terminus.

A real-time PCR method, based on the continuos monitoring of samples during PCR, was developed for the analysis of PCR kinetics (Higuchi et al., 1992). The improved real-time PCR method utilizes the 5'--> 3' exonuclease activity of Taq DNA polymerase and a labelled probe (Holland et al., 1991). The probe anneals to a specific amplification product and is cleaved by the 5' nuclease activity of the polymerase as the new DNA strand is extended from the upstream primer. The cleavage releases the separate nucleotides

containing the label. Recently, the development of new fluorescent techniques for real-time PCR has provided new tools for the detection of specific sequence changes. Melting curve analysis of a PCR product provides a good system for the detection of single-nucleotide polymorphisms (Lay and Wittwer, 1997).

Solid phase minisequencing (or single-nucleotide primer extension; Syvänen et al., 1990; 1990) is a method developed for detecting single-nucleotide variations. In this method, a detection primer anneals to the target nucleic acid directly adjascent to a variable nucleotide. A DNA polymerase incorporates a labeled nucleotide complementary to the variable nucleotide to the 3' end of the detection primer. An advantage of solid phase minisequencing is its excellent ability to discriminate between heterozygous and homozygous genotypes. Recently, it has been shown to be useful in detecting a large and a small deletion in addition to single-nucleotide changes in an oligonucleotide array format (Pastinen et al., 1997).

1.8.3. From sequence to function

After the gene and mutations responsible for a certain disease or phenotype are found, a whole new field will open up for further study. The next goal is to understand the function of the protein and its physiological role in the organism as well as pathophysiology resulting from its defective function. The function of a specific gene product involves its structure, biochemical function (such as substrate specificity or cofactors), interaction with other macromolecules, cellular localization, and possible targets, as well as the pathway and the organs it affects, and the physiological role it plays in the total organism.

Sometimes the mechanisms underlying certain disease or a pathological phenotype are well characterized, which aids in directing expression and functional studies (Holmberg et al., 1986). The phenotype can present some clues to the expression sites or affected pathway. Often a good starting point for expression studies is the affected organs, where the expression of the gene and protein is probable. A novel gene can be connected to a certain pathway or complex based on the similar phenotype with other pathway members (Monreal et al., 1999). Computational methods are fast and highly useful in analyzing amino acid sequence in order to establish a possible functional linkage with an already characterized protein. Overall amino acid homology with a known human protein suggests

a resembling structure and biochemical function (Lohi et al., 2000). Although no human homology can be detected, amino acid sequence homology may be identified with some other organism like yeast, which may then help in defining the function of the human sequence (Oliver, 1996). Computational amino acid sequence analysis can reveal domains or profiles that are common with already characterized proteins and known to perform or participate in specific functions (Ezer et al., 1999; Furusawa et al., 2000). Even if no homology or functional linkage with known protein or structure can be assigned, analyzing the amino acid sequence can provide useful information of the protein. For example, the hydrophobicity profile may reveal membrane-spanning segments in the sequence. Despite extensive computational analysis, no expression pattern or biochemical function can, however, be assigned for the majority of novel proteins.

1.8.4. Gene and protein expression

The expression can be studied both at the level of mRNA and protein, the basic difference being that mRNA-based methods measure the expression intermediate and protein-based methods the final functional expression product. A major advantage of some protein-based methods is the possibility to detect post-translational modifications, cellular localization, and protein complexes. However, mRNA-based methods are often easier and provide useful information about the state of the cell and gene activity. The disadvantage of most methods used for expression studies is that they do not allow quatitation of the expression.

Northern blot analysis

One of the first expression studies performed on the gene of interest is usually northern blot hybridization. A genomic or cDNA fragment is labeled and used as a probe to study the RNA expression of the gene. The probe is hybridized against membrane, various lanes of which contain samples of RNA (total or mRNA) from a variety of sources. Sample RNA can be isolated from affected and normal individuals, which allows the detection of the effect of the mutation on RNA levels (Hästbacka et al., 1994). A frequently used form of northern blot contains RNA in different lanes, which has been isolated from a variety of normal adult tissues. Also embryonic or fetal tissues can be utilized to determine expression at different developmental stages or in different cell types. Hybridization reveals the gross tissue expression pattern of the transcript as well as its relative

abundance in different tissues. RNA separated in a gel is size-fractionated, which facilitates the estimation of the size of transcripts and allows the detection of differently sized isoforms that are visible as multiple bands in one lane (Ranta et al., 1999). Sometimes RNA dot blots are used instead of gel separated RNA blots to allow the evaluation of a broader spectrum of samples in one experiment (Gilligan et al., 1999).

Reverse transcriptase polymerase chain reaction

Conventional reverse transcriptase polymerase chain reaction (RT-PCR) is a fast and sensitive method for obtaining the gross expression pattern of the gene. It can be used to study the presence of transcript or to detect its isoforms in different tissues and cell types together with their rough relative abundancies. It is most useful when the amount of template is limited, like in preimplantation embryos (mouse model screening) or in single cell analysis. Like northern blot analysis, RT-PCR detects mRNA in tissue homogenates.

Quantitative polymerase chain reaction

A real-time polymerase chain reaction (PCR) system (see p. 31) can be utilized for quantitative analysis of a gene expression. The quantitative PCR utilizes the theoretical relationship between the amount of starting target sequence and the amount of PCR product at any given cycle. Measurements are performed in the exponential phase where reaction components are not limiting. Both DNA and RNA (RT-PCR) can be used as a starting material. Relative quantitation of a gene expression can be achieved using either the comparative C_T method or the standard curve method. In comparative C_T method, the number of the target gene copies is normalized to an endogenous reference gene. An alternative method relies on the use of a standard curve containing serial dilutions of a specific standard sample.

Tissue *in situ* hybridization

Tissue *in situ* hybridization can be used when specific nucleotide sequences are detected in morphologically preserved tissues and cells (Gall and Pardue, 1969; John et al., 1969; Willcox, 1993). A thin tissue section mounted on a microscope slide is hybridized with a suitable probe. Double-stranded cDNA, an antisense RNA, or an oligonucleotide can be used as a probe, but highest sensitivity is achieved with an antisense riboprobe which

hybridizes specifically to the sense mRNA of the gene (Cox et al., 1984). Probes are labeled using radioisotopes, fluorochromes, or chemicals. Visualization and analyzing the hybridization results depend on the label used in the probe. mRNA tissue *in situ* hybridization allows the spatial and temporal analysis of the gene expression in various physiological and pathological conditions, as well as during development. However, *in situ* hybridization allows no quantitation of RNA expression levels unless an image analysis with appropriate software is used (Jonker et al., 1997).

Immunoblotting

Immunoblotting is used to detect a certain protein in a protein mixture, such as tissue or cell culture lysates, and to estimate the size of the denatured protein. The amount of the protein can be estimated by comparing the staining intensity of an unknown protein to the staining intensity of protein standards. Proteins are separated according to their size under denaturing conditions in SDS-PAGE gels. After electrophoresis, proteins can be stained using an appropriate stain, e.g. Coomassie blue (Wilson, 1983), or silver staining (Switzer et al., 1979). However, the fractionated proteins are usually transferred from the gel to a membrane which is then either stained or exposed to a protein-specific antibody (western blotting). Ponceau S staining of the membrane is transient, which makes it possible to first stain all proteins in the membrane and then continue with exposing to a specific antibody.

Immunohistochemistry

At protein level, an equivalent to the mRNA tissue *in situ* hybridization is immunohistochemistry, which is used to localize proteins in specific cell types of different tissues or in other multicellular structures. Similarly, antibodies are also used to detect proteins in single cells (immunocytochemistry) in order to reveal subcellular localization of the protein. Instead of a nucleic acid probe, a specific mono- or polyclonal antibody or antiserum is utilized to recognize specific epitopes of the protein. Recent advances in fluorescence tehcnology have enabled the development of multicolour immunostaining to evaluate co-localization of two or more antigens in an objective manner and also dual immunohistochemical and mRNA *in situ* hybridization detection of the same tissue section (Zaidi et al., 2000). The fluorescent methods are also more suitable for quantitative computerized image analysis (Mosedale et al., 1996).

2. AIMS OF THE STUDY

The principal goal of this study was to provide basic information about the major intestinal Cl^-/HCO_3^- transporter and pathophysiology of congenital chloride diarrhea.

The specific aims were:

1. to identify the CLD gene (I)
2. to determine the genomic structure of the CLD gene (II)
3. to characterize mutations in CLD patients (I, III)
4. to establish the expression pattern of CLD in human tissues (I, IV)
5. to study the expression of closely related anion transporter DTDST in human tissues and compare its colonic expression with that of CLD (IV, V).

3. MATERIALS AND METHODS

All parts of the study were approved by the Ethics Committee of the Department of Medical Genetics, Haartman Institute, University of Helsinki. The clinical phenotype of all patients was characterized by a profuse watery diarrhea with a high chloride content typical for CLD.

3.1. Mutation detection

Total RNA for the RT-PCR was extracted from lymphoblast cell lines of CLD patients. First strand synthesis of the cDNA was performed using M-MLV reverse transcriptase and random hexamers. Two rounds of PCR were performed using either nested or the same set of primers. The products were separated by electrophoresis, excised, and purified. Sequencing reactions were done using specific primers and employing dye-terminator chemistry and an automated sequencer (ABI373A). After the identification of V317del mutation, its presence in controls was screened by detecting the 3-bp deletion in the 81-bp genomic PCR fragment using PAGE and silver staining.

Also H124L and 344delT mutations were identified by the direct sequencing of a 133-bp genomic PCR fragment. The screening of 344delT was based on the detection of a 1-bp deletion in 133-bp genomic PCR fragment using PAGE and silver staining while H124L generated a novel restriction site for enzyme HinfI (GANTC) that was utilized in the same 133-bp genomic PCR fragment. The digestion products of either the 49-bp and 84-bp or the undigested 133-bp fragment were separated in PAGE and visualized by silver staining.

Screening for T to G transversion at position 921 was based on the generation of a novel restriction site for BslI (CCNNNNNNNGG) and a loss of site for restriction enzyme Tsp45I (GTC/GAC). Genomic PCR fragments approximately 560 bp in size were digested with both restriction enzymes and separated with agarose gel electrophoresis.

3.2. SSCP analysis of mutations

PCR amplification was performed using intronic exon specific primers and genomic DNA as a template. After amplification, 5 µl of the 75 µl PCR assay was added to bromophenol-

blue and formamide containing stop solution. After denaturation, samples were separated on a non-denaturing polyacrylamide gel (FMC) and visualized by silver staining. PCR fragments demonstrating a mobility shift in SSCP analysis were purified and sequenced using an automated sequencer.

3.3. Genomic cloning

We constructed a genomic library using DNA from a CLD patient with paternal uniparental disomy who was homozygous for all loci in chromosome 7 (Höglund et al., 1994). Genomic DNA was partially digested with Sau3AI, and fragments larger than 15 kb were ligated into the phage vector EMBL3 and packaged. The library was screened by plaque lift hybridization with a 32P-labelled mixed human DRA cDNA probe. After hybridization, filters were washed and autoradiography was performed on X-ray films at +4 ^{o}C overnight.

Positive genomic phage inserts were examined by dot blot hybridization analysis using each of the five CLD cDNA probes separately. Two of the phages that were found to span most of the CLD gene were purified, partially digested with Sau3AI, and subcloned. Insert-positive subclones were identified by hybridization utilizing the same set of CLD cDNA probes and sequenced by cycle sequencing using both universal vector primers and an internal primer designed for the pUC118 cloning site. CLD-specific primers were also used to sequence from the exons into the introns using genomic phage clones as templates. With this, most of the intron-exon boundaries of the coding region of the CLD could be established.

3.4. cDNA probes

Five overlapping human DRA cDNA probes, a 501-bp fragment (nt 154-654), a 620-bp fragment (nt 601-1220), a 614-bp fragment (nt 1089-1702), a 622-bp fragment (nt 1650-2271) and a 582-bp fragment (nt 2217-2798) were prepared by PCR amplification of a human colon cDNA library. The primers were 18-24 bp in length corresponding to the ends of the amplified fragment. When preparing the 501-bp fragment, polymerase was added to the reaction during the first denaturation. PCR fragments were excised from agarose gel and used as templates for hybridization probes.

3.5. Primers, PCR assays and sequence analysis

CLD-specific primers were designed on the basis of the published human DRA cDNA sequence (GenBank number L02785). The primers were used for amplifying cDNA fragments for probes, for genomic PCR, and for sequencing. The nucleotide sequence obtained from the genomic regions adjacent to exon borders enabled us to develop intronic primers for amplifying each exon of the CLD gene separately (II). Exon specific PCR assays were either analysed in agarose and PAGE gels or purified and sequenced. The templates for sequencing included human CLD positive phage clones, YAC DNA, P1 obtained from a commercial library, and genomic DNA from a Finnish patient and controls. Genomic PCR fragments were excised from agarose gel, purified, and used together with CLD-specific sequencing primers to complete the sequence obtained from genomic clones. In this way, all the remaining intron-exon boundaries of the coding region of the CLD could be established.

All DNA sequencing was automated (using an ABI-373A), with sequence editing and analysis done by the Auto-assembler software (Perkin-Elmer) and GCG programs, respectively (Program manual for the Wisconsin package, Version 8, August 1994; Genetics Computer Group, Madison, WI). For the analysis of the putative promoter region, the compiled genomic sequences were subjected to analysis using the MatInspector Release 2.1 (Quandt et al., 1995).

3.6. Large-scale sequencing

To determine the full gene structure, the genomic DNA sequence of two bacterial artificial chromosomes (BACs), H_RG013F03 and H_RG364P16, known to encompass the gene (see http://www.genet.sickkids.on.ca/chromosome7/), were analysed. Although the sequence of these clones was not completed at the time of this study (http://genome.wustl.edu/gsc/human/chrom7.html/), it was sufficient to allow us to align the complete cDNA to the genomic sequence. This was accomplished by establishing a BLAST database containing the BAC DNA sequences using GCG and aligning CLD cDNA to the genomic DNA sequence within the database (the BLAST results were filtered with MSPcrunch).

3.7. Northern hybridization

Commercially available Multiple Tissue Northern Blot (Clontech) was hybridized with a 32P-labelled 622-bp (nucleotides 1650-2271) CLD cDNA fragment that was generated by PCR amplification using human colon cDNA library as a template. After hybridization, filters were washed and autoradiography was performed on X-ray films at +4 ºC.

3.8 Tissues

Formalin fixed, paraffin-embedded archival specimens from adult patients were obtained from the Department of Pathology, Haartman institute, University of Helsinki (adult tissues) and the Department of Pathology, University of Oulu (fetal tissues). All inflammatory intestinal samples represented an active, ulcerative phase of the diseases and were from adult patients. All fetal material originated from medical abortions.

3.9 *In situ* hybridization

A 622-bp fragment corresponding to positions 1650-2271 of the CLD cDNA (GenBank number L02785) and a 549-bp fragment corresponding to positions 1422-1970 of the DTDST cDNA (GenBank number U14528) were generated by polymerase chain reaction. These fragments were designed with a T7 RNA polymerase promoter at the 3' end and SP6 RNA polymerase promoter at the 5' end. Both sense and antisense probes were transcribed from the same PCR product. Both CLD and DTDST antisense and sense RNA probes were labeled with alpha-^{35}S-UTP. As previously described in detail (Prosser et al., 1989), deparaffinized tissue sections were digested with proteinase K and treated with acetic anhydride in triethanolamine buffer. After hybridization at 52°C overnight, the slides were washed under stringent conditions, including RNase A, and exposed to LM-1 emulsion for 21 to 50 days. The slides were developed and counterstained with hematoxylin and eosin. Normal colon samples known to be positive were used as controls in each experiment and a sense RNA probe was used as a negative control.

3.10. Immunohistochemistry

Antisera were raised in rabbits against synthetic peptides, corresponding to nucleotides 2375-2416 of the CLD cDNA sequence (GenBank number L02785) and nucleotides 199-244 and 2095-2146 of the DTDST cDNA sequence (GenBank number U14528). Peptide synthesis and antibody production were purchased from Research Genetics (Huntsville, Alabama, USA). Immunohistochemistry was performed on serial sections of those used for *in situ* hybridization.

For the detection of the CLD protein the peroxidase-antiperoxidase technique was performed using an automatic immunostaining device (Ventana Medical Systems) and Ventana kits. For the detection of the DTDST protein the peroxidase-antiperoxidase technique was performed using the Vectastain Elite ABC Kit. Diaminobenzidine (DAB) was used as the chromogenic substrate. Preimmune sera were used as a negative control for parallel sections.

3.11. Western blotting

The specificity of the antibodies was demonstrated by Western blotting using homogenized human colon epithelium (CLD) or osteosarcoma tissue (DTDST). Denatured proteins were separated in SDS-polyacrylamide gel and blotted using standard protocols. For the detection of the CLD protein, biotin conjugated anti-rabbit IgG was utilized as the secondary antibody, which was then detected using streptavidin-POD. For the detection of the DTDST protein, peroxidase conjugated anti-rabbit IgG was used as the secondary antibody. The protein bands were visualized by chemiluminescence according to standard protocols. For the CLD protein the pre-serum of the same rabbit was used as a negative control. For the DTDST protein detection, normal rabbit IgG was used as a negative control antibody.

4. RESULTS AND DISCUSSION

4.1. Mutations in the DRA gene confirm its identity as the CLD gene (I and III)

In order to verify the role of the positional and functional candidate gene DRA in the pathogenesis of congenital chloride diarrhea, we searched for mutations using RT-PCR and direct sequencing on samples from CLD patients and control individuals. As an alternative approach, genomic DNA fragments from a unipaternal disomy 7 (UPD7pat) patient with CLD were subcloned and DRA-positive clones were selected by hybridization with DRA cDNA fragments. The genomic fragments were sequenced, assembled, and analyzed.

4.1.1. Mutation analysis of the DRA gene (I)

The sequencing of samples from 32 Finnish CLD patients revealed the same mutation in all CLD chromosomes. The mutation was a homozygous in-frame three base pair deletion (GGT) at nucleotides 951-953 that caused the loss of a highly conserved valine (V317del) in the predicted amino acid sequence. All 43 parents studied as well as 23 of 32 healthy sibs were heterozygous for the deletion. Three carriers were found among 252 healthy control individuals living in Eastern Finland where the disease is more prevalent due to founder effect. No carriers were revealed among 184 healthy controls originating from Southwestern Finland. In addition, two unrelated Polish patients sharing an identical haplotype in one chromosome were heterozygous for a missense A to T transversion that resulted in the replacement of histidine by a hydrophobic leucine (H124L). Finally, another two Polish CLD patients were found to carry a deletion of T at nucleotide position 344. The one patient was homozygous while the other was heterozygous for this single base deletion, which created a frameshift at codon 115 and the termination of translation 18 codons downstream truncating 85% of the protein. Furthermore, a polymorphic T to G transversion causing substitution of tryptophan for cysteine (C307W) was found 30 bp upstream of V317del mutation at nucleotide 921 in homozygous form in all the 32 Finnish CLD patients. We concluded that DRA causes CLD when mutated and hence be called the CLD gene in the rest of the paper.

4.1.2. The age of the Finnish founder mutation (I)

The reverse application of the modified Luria-Delbrück equation was utilized to estimate the number of generations since the onset of V317del expansion in the isolated subpopulation of Eastern Finland. The estimation was based on the actual distance between the CLD gene and different markers around the CLD locus. The respective generation values (g) were calculated for each marker using the following formula:

$$p_{excess} = \alpha(1-\theta)^g, \text{ where } p_{excess} = p_{affected} - p_{normal} / (1 - p_{normal})$$

The value of α = proportion of mutations descending from the founding ancestor can be set at $\alpha = 1$ due to the presence of the V317del mutation in all the Finnish CLD chromosomes. On the basis of the rough assumption that 1cM equals 1 Mb, θ can be estimated from the high-resolution physical map. The formula is simplified by ignoring the mutation term from the original formula (de la Chapelle, 1993).

Locus	Physical distance (Mb)	θ	p_{excess}	g
D7S658	5.3	0.053	0.22	28
D7S501	1.1	0.011	0.786	20
AFMa126zc1	0.56	0.0056	0.868	25
D7S496	0.23	0.0023	0.94	27
AFMa305ye9	0.15	0.0015	0.9677	22
AFMa297yf9	0.30	0.0030	0.907	32
D7S692	1.05	0.0105	0.734	29
D7S523	5.11	0.0511	0.446	15
D7S799	2.23	0.0223	0.693	16

The utilization of additional markers and more accurate physical distances has enabled the adjustment of the estimation. The calculation now resulted in an average age of 24 generations, with a range of 15 to 32. This is still well in line with the population history (de la Chapelle, 1993) and wide linkage disequilibrium interval of 13 cM across the CLD gene region (Höglund et al., 1995). The Luria-Delbrück equation was initially designed for the estimation of mutation rate in rapidly growing bacterial cultures (Luria and Delbrück, 1943) but has since been modified and applied successfully to the mapping of Finnish disease genes (Lehesjoki et al., 1993; Hästbacka et al., 1992; Höglund et al., 1995). This

was, however, the first time that the reverse application of the formula was adapted to estimate the age of a mutation.

4.1.3. The CLD gene and protein

The CLD gene encodes a hydrophobic 764 amino acid integral membrane protein that is variably N-glycosylated and has a predicted molecular mass of 84,5 kD (Schweinfest et al., 1993; Byeon et al., 1996). Topology predictions suggest it to have 10, 12, or 14 transmembrane segments and a large carboxyl-terminus that is intracellular like amino-terminus (Hästbacka et al., 1994; Silberg et al., 1995; Bairoch and Apweiler, 1996; Byeon et al., 1996). The experimental data also support the number of transmembrane domains to be even (Byeon et al., 1996; our unpublished results). A precise topological organization needs to be determined experimentally, however.

4.1.4. Function of the CLD gene

After initial identification of the CLD gene as a putative tumor suppressor gene, two different groups surveyed its function with different expression systems. Functional studies demonstrated it to mediate a Na^+-independent transport of sulfate and oxalate that was inhibited by the anion transporter inhibitor DIDS (Silberg et al., 1995; Byeon et al., 1996). A recent in vitro expression study using Xenopus Laevis oocytes confirmed the role of CLD also in chloride transport and the identities of V317del as a functional mutation and C307W as a silent polymorphism (Moseley et al., 1999). Wild type and mutagenized CLD cRNA were injected to Xenopus oocytes and uptakes of Cl^- and SO_4^{2-} were studied. Wild-type CLD-injected oocytes demonstrated transport of both anions, while transport activity was significantly reduced in oocytes injected with the Finnish deletion mutant V317del. Some chloride uptake was, however, detected also with this mutant suggesting residual function retained by the defective protein. C307W, which had been already denoted as a functionally neutral polymorphism based on genetic studies in the Finnish population, transported both anions as efficiently as wild–type CLD. However, in this cell model, the CLD protein demonstrated features consistent with Cl^-/OH^- change and its connection with Cl-/HCO3- exchange remained somewhat unclear.

The role of CLD in Cl^-/HCO_3^- exchange was confirmed *in vitro* with HEK 293 cells stably expressing the mouse cld gene (Melvin et al., 1999). A reversed Cl^- gradient in a solution containing HCO_3^- lead to a rapid intracellular alkalinization indicating the presence of Cl^-/HCO_3^- exchange in these cells. As expected, cld-mediated Cl^-/HCO_3^- transport was Na^+-independent and insensitive to changes in the membrane potential. However, some alkalinization occurred also in the absence of an extracellular HCO_3^- reversed Cl^- gradient, although the rate was significantly lower. The authors suggested that the extracellular anion-binding site may be relatively nonspecific and thus alkalinization in the absence of extracellular HCO_3^- may be due to the transport of other anions, such as OH^- and/or gluconate.

4.1.5. The SLC26 gene family

The CLD protein shows amino acid conservation with members of the so-called sulfate transporter gene family, which currently consists of six well-characterized mammalian members, and based on the EST data, many more remain unidentified (Everett and Green, 1999; Kere et al., 1999; Lohi et al., 2000). DTDST, CLD, and PDS have been identified to be responsible for the recessive diseases if mutated, namely diastrophic dysplasia, congenital chloride diarrhea, and Pendred syndrome, respectively (I; Hästbacka et al., 1994; Everett et al., 1997). The functional characterization of Sat-1, DTDST, CLD, and PDS has verified their role as anion transporters, which was already presumed on the grounds of their homology with sulfate transporters of other species. However, they demonstrate highly variable substrate specificities with primary anions that can readily be implicated in the respective disease. The expanding gene family was recently renamed solute carrier family 26 (SLC26) by Human Gene Nomenclature Committee, CLD becoming SLC26A3 (solute carrier family 26 member 3).

SLC26A1 (also known as SAT-1) was originally isolated from a rat liver cDNA library and encodes a high-affinity SO_4^{2-}/HCO_3^- or SO_4^{2-}/oxalate transporter (Bissig et al., 1994). In situ hybridization study of brain tissue sections demonstrated sat-1 mRNA expression in the hippocampus and granular cell layer of the cerebellum (Lee et al., 1999). By western blotting and immunohistochemistry, its expression in kidney was shown to be

restricted to the basolateral membranes of proximal tubule cells (Karniski et al., 1998). The sequence of human SAT-1 has been recently published (Lohi et al., 2000).

SLC26A2 (alias DTDST) is discussed in more detail in the Review of the literature (see p. 24).

SLC26A4 (alias PDS) is very similar to the CLD gene at the gene level. Both genes are located in chromosome 7q22-31.1 in tail-to-tail orientation with only ~48 kb distance between them, and both have very similar genomic structure (II; Everett et al., 1997). Despite the similarities, their tissue expression pattern and substrates for transport are different. RNA *in situ* hybridization study of mouse Slc26a4 has demonstrated expression in several areas in the inner ear (Everett et al., 1999). In addition, northern hybridization revealed an intense signal for SLC26A4 expression in thyroid (Everett et al., 1997). The expression pattern corresponds well with the Pendred syndrome phenotype caused by the mutations in the SLC26A4 gene. Substrate spectrum for SLC26A4 includes at least chloride, iodide, and formate, but specifically not sulfate (Scott et al., 1999; Scott et al., 2000).

Slc26a5 (alias prestin) was isolated from the outer hair cell cDNA pool of a gerbil as a result in the search for the cochlear amplifier, the protein providing local mechanical amplification in the mammalian hearing organ (Zheng et al., 2000). Its expression is restricted to cochlear outer hair cells, and kidney cells transiently transfected with prestin demonstrate voltage-dependent motility, which suggests the identity of prestin as the motor protein in the cochlear outer hair cells. Substrates for its transport remain yet to be discovered.

SLC26A6 was cloned and characterized recently as a result of an effort to expand the SLC26 family (Lohi et al., 2000). Northern analysis suggested most abundant expression in kidney but expression was also detected in pancreas, placenta, and skeletal muscle. Immunohistochemistry revealed SLC26A6 protein expression in both the apical and basolateral membranes of kidney tubule cells and in the luminal brush border of pancreatic ducts. However, its functional role in kidney and/or pancreatic anion exchange as well as substrates for its transport function remain to be established.

4.2. The CLD gene consists of 21 exons and 20 introns (II)

The full coding region of the human CLD cDNA had been previously established (GenBank number L02785; Schweinfest et al., 1993), but its genomic organization was first determined in this study (II). Cloning and sequencing of the human CLD gene revealed 21 exons that span approximately 39 kb of genomic DNA. The exons and introns ranged in size from 55 to 234 bp and from 105 to 9850 bp, respectively. Initiation codon is located in exon 2 and the termination codon in exon 21. All exon intron boundaries confirmed with the consensus for the splice acceptors and donors. We observed one discrepancy between nucleotide sequences obtained by the genomic sequences and the sequence of previously published human CLD cDNA (GenBank number L02785). In the cDNA sequence there is a 23-bp inversion and a 9-bp deletion in the beginning of exon 1. We confirmed the genomic sequence from three independent sources of DNA and found an identical sequence in all. This suggested a rearrangement in the published cDNA sequence and the transcription initiation site remained uncertain. In addition, altogether 19 Alu repeats of various subclasses were identified in the introns of the CLD gene. Five of them were truncated but 14 were typical primate repeats. (II)

In addition to CLD, our analysis of the genomic sequence obtained by large-scale sequencing of bacterial artificial chromosome clone H_RG364P16 revealed the presence of 10 putative exons from a separate highly homologous gene. This gene demonstrated 58 % amino acid identity to exons 9-20 of CLD and had an almost identical exon-intron structure. During our study, this gene was shown to cause Pendred syndrome when mutated and was named PDS (Everett et al., 1997).

The CLD gene demonstrates similar exon-intron structure as PDS with 21 exons and SLC26A6 with 20 exons (Fig. 1). These three genes share 12 exons similar size with each other. However, SAT-1 and DTDST have genomic structures that differ strikingly from those of the other members (Fig. 1). SAT-1 contains three exons (Lohi H., personal communication), whereas the DTDST gene extends over 40 kb of genomic DNA and has two large coding exons and at least one 5’ UTR exon (Hästbacka et al., 1999).

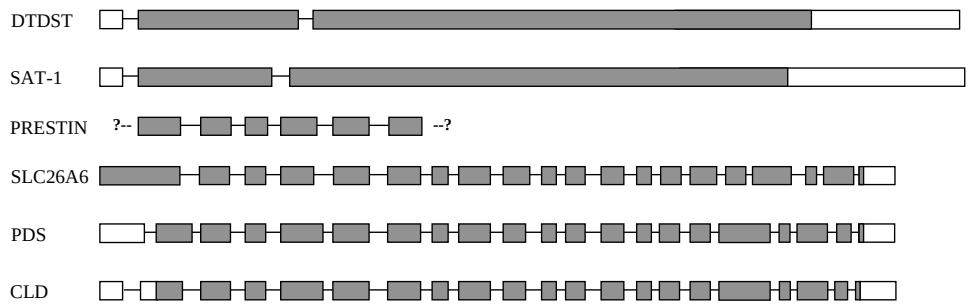

Figure 1. The genomic structures of the SLC26 gene family members. The exons in the coding region are drawn in scale and darkened, while the introns and the noncoding exons are not in scale.

A 600-bp sequence upstream from the putative transcription initiation site was analysed using different computer programs to localize potential transcription factor binding sites. Three potential transcription factor binding sites for AP-1 were found as well as three motifs for the erythroid specific transcription factor GATA-1. There were two potential CCAAT boxes and a putative TATA box located at an optimal position at nt –33 upstream of the putative transcription start site. (II)

This study enabled us to design primer pairs for amplifying each exon separately. All exon specific PCR reactions work well in the same conditions and can thus be performed simultaneously.

4.3. Mutation spectrum of the CLD gene (I, III)

The identification of the CLD gene, the determination of its genomic organization, and the design of exon specific intronic primers enabled an extensive screening of mutations in the patients with CLD originating from several different populations. The amplified samples were first screened by the SSCP analysis and fragments suggesting a mobility shift were further analyzed by sequencing to characterize sequence variations. Some samples showing no mobility shift in the SSCP screening, originating from populations with founder mutations, or the mutation hot-spot regions were sequenced directly after PCR amplification. Finally, to rule out the possibility of polymorphism, the presence of the sequence variation was studied in a set of control individuals.

Analysis of the coding region and exon-intron boundaries of the CLD gene in CLD patients has revealed a wide spectrum of different sequence alterations. Regardless of ethnic background, in all cases mutations have been detected in the CLD gene suggesting no locus heterogeneity in CLD. To date, 28 different mutations have been identified among c. 100 CLD patients, most of whom originate from the three high-frequency populations (Table 1; I, III, Etani et al., 1998; Höglund et al., 1998; Höglund et al., submitted). Approximately 34% of the mutations are single base pair substitutions, 38% small insertions or deletions, and 17% splice-site defects. The first two genomic rearrangements have been characterized recently (Höglund et al., submitted). In addition, four polymorphisms have been detected. So far, no whole gene deletions, promoter region mutations, or de novo mutations have been reported. Mutations are distributed throughout the coding sequence of the CLD gene, although some clustering can be seen, suggesting either the functional importance or mutation-prone structures of these regions (Fig. 2). The cluster nearest to the 5' end (nucleotides 344-392) is located in a region called **"**sulfate transport domain" with a strong amino acid conservation between family members even across species. Four different mutations (344delT, H124L, G120S, and P131R) lie in this cluster consisting of only 49 bp. A wider putative cluster contains 40% (12/28) of the mutations detected so far within a 326 bp area (nucleotides 1306-1631) that comprises 14% of the CLD protein.

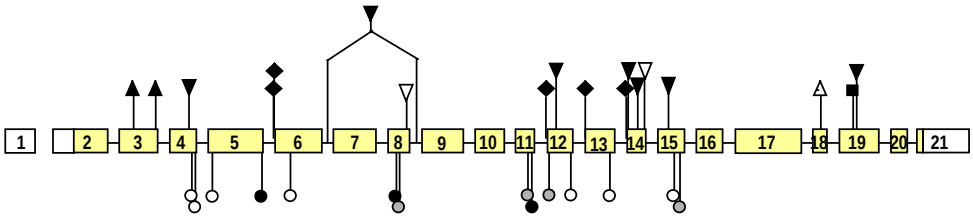

Figure 2. The genomic structure of the human CLD gene showing the distribution of the 28 mutations characterized so far. Circles indicate the point mutations, triangles deletions, inverted triangles insertions, and diamonds splice site mutations. The square indicates the replacement mutation. Open symbols indicate missense mutations, while nonsense mutations are indicated with solid symbols. Gray symbols indicate polymorphisms.

Table 1. Mutations of the human CLD gene

Mutation	Intron/Exon	Nucleotide change	Result	References
177-178insC	exon 3	insertion of C at 177-178	frameshift, truncated	III
268-269insAA	exon 3	insertion of AA at 268-269	frameshift, truncated	Höglund et al. 1998
344delT	exon 4	deletion of T at 344	frameshift at codon 155, truncated	I
G120S	exon 4	G to A at 358	glycine to serine at codon 120	III
H124L	exon 4	A to T at 371	histidine to leucune at codon 124	I
P131R	exon 5	C to G at 392	proline to arginine at codon 131	III
G187X	exon 5	G to T at 559	glycine to stop at 187, truncated	Höglund et al. 1998
IVS5-2A>G	intron 5	A to G at 571-2	destruction of the intron acceptor AG	Höglund et al. 1998
IVS5-1G>T	intron 5	G to T at 571-1	destruction of the intron acceptor AG	Höglund et al. 1998
S206P	exon 6	T to C at 616	serine to proline at codon 206	Höglund et al. submitted
exon 7-8 deletion	intron 6-intron 8	3.5 kb deletion of genomic DNA	loss of exons 7 and 8, frameshift	Höglund et al. submitted
Y305X	exon 8	C to A at 915	tyrosine to stop at 305, truncated	III
V317del	exon 8	deletion of GGT at 951-953	in-frame loss of a valine at 317	I
Q436X	exon 11	C to T at 1306	glutamine to stop at 436, truncated	Höglund et al. submitted
IVS11-1G>A	intron 11	G to A at 1312-1	destruction of the intron acceptor AG	Höglund et al. 1998
1342-1343delTT	exon 12	deletion of TT at 1342-1343	frameshift, truncated	Etani at al. 1998
D468V	exon 12	A to T at 1403	aspartic acid to valine at codon 468	Höglund et al. submitted
IVS12-1G>C	intron 12	G to C at 1408-1	destruction of the intron acceptor AG	Höglund et al. submitted
L496R	exon 13	T to G at 1487	leucine to arginine at codon 496	Höglund et al. 1998
IVS13-2delA	intron 13	deletion of A at 1515-2	destruction of the intron acceptor AG	Höglund et al. submitted
1516delC	exon 14	deletion of C at 1516	frameshift, truncated	III
1548-1551delAACC	exon 14	deletion of AACC at 1548-1551	frameshift, truncated	III
Y527del	exon 14	deletion of TTA at 1578-1580	in-frame loss of a tyrosine at 527	III
1609delA	exon 15	deletion of A at 1609	frameshift, truncated	Höglund et al. 1998
I544N	exon 15	T to A at 1631	isoleucine to asparagine at codon 544	Höglund et al. submitted
I675-676ins	exon 18	insertion of ATC at 2025-2026	in-frame addition of an isoleucine	Höglund et al. 1998
2104-2105delGGins29	exon 19	replacement of GG with 29 bp	frameshift, truncated	Höglund et al. submitted
2116delA	exon 19	deletion of A at 2116	frameshift, truncated	III
Polymorphism				
C307W	exon 8	T to G at 921	cysteine to tryptophan at 307, function	I
1299G/A	exon 11	G to A at 1299	no change at amino acid level	Höglund et al. submitted
1314C/T	exon 12	C to T at 1314	no change at amino acid level	Höglund et al. submitted
R554Q	exon 15	G to A at 1661	arginine to glutamine at 544	Höglund et al. submitted

4.3.1. Founder mutations

Although rare cases of CLD occur worldwide, the clustering of CLD cases in the certain high incidence areas suggests a founder effect and thus the presence of a single major founder mutation in each of these populations. In Finland, V317del mutation has been found in all but one disease associated chromosomes, accounting for 98% of mutations. This finding is in agreement with earlier haplotype data suggesting one major mutation to be responsible for the disease in Finnish population (Höglund et al., 1995). This also coincides with the population history and geographic isolation of Finland, which have lead to the enrichment of certain alleles and depletion of others in the Finnish population (Norio et al., 1973; de la Chapelle, 1993). Finnish V317del mutation and the C307W polymorphism were detected also in seven out of eight Swedish CLD-associated chromosomes, as was expected on the basis of haplotype

analysis (Höglund et al., 1995). This is in agreement with recent emigration from Finland to Sweden. Distinctive founder mutations have also been recognized in Saudi Arabia and Kuwait as well as in Poland (Höglund et al., 1998). In Saudi Arabia and Kuwait the high incidence of CLD most likely owes the combination of a founder effect with a high frequency of consanguinity. A nonsense point mutation G187X has been demonstrated in 94% (17 /18) of disease chromosomes (Höglund et al., 1998). In Poland the situation is somewhat more complicated. There the major insertion mutation was found only in 47% of CLD-associated chromosomes, which together with more rare mutations results in compound heterozygotes and thus the higher disease frequency (Höglund et al., 1998).

4.3.2. Small deletion and insertion mutations

Seven small deletions or insertions were found during this study and four more have been reported later (Höglund et al., 1998; Höglund et al., submitted). Of these, eight result in a frameshift and lead to the premature termination of protein translation and three are in-frame changes. The Finnish founder mutation at codon 317 causes in-frame loss of a valine which is highly conserved within human SLC26 family members (I). Its pathogenicity has recently been verified (Moseley et al. 1999). Another in-frame mutation is the loss of a tyrosine at codon 527 removing a highly conserved amino acid (III). The Polish founder mutation, an in-frame addition of an isoleucine at codon 676, is located at the long intracellular carboxyterminal tail (Höglund et al., 1998). The most proximal mutation is an insertion of a C between the nucleotides 177 and 178, leading to a nonsense change at codon 60 and stop at codon 70 (III). The most distal mutation is a deletion of a single A causing frameshift and nonsense change at codon 706 and stop at codon 711, found in heterozygous form in one Finnish patient (III). The most distal mutation leaves more than 90% of the protein intact truncating only the very end of the intracellular C-terminal end. In addition, four other frameshift mutations truncate one-third or less of the protein. Based on the phenotype, no remarkable residual activity is however retained, which suggests the C-terminal region to be of great importance for the proper function of the CLD protein.

4.3.3. Point mutations

In this study, we found three missense mutations and one nonsense point mutation. Three more missense and two nonsense mutations have been reported later (Höglund et al., 1998; Höglund et al., submitted). Most single nucleotide substitutions hit evolutionary conserved amino acid residues which can thus be expected to affect the functional domains of the protein. The only "non-Finnish" Swedish disease chromosome carried a G to A transition leading to a glycine to serine change at codon 120, which was also found in a Polish and a Norwegian patient (III; Höglund et al., submitted). Different haplotypes suggest that the mutation has occurred three times. The substituted glycine is highly conserved and invariant in all six SLC26 family members, as is also the next histidine at codon 124 that is changed to leucine due to an A to T missense mutation (I). However, proline at codon 131 that is changed to arginine by a C to G transversion (III) is found only in pendrin in addition to the CLD protein. All these three missense mutations reside in the most homologous area called the sulfate transport domain. Also a leucine to arginine change at codon 496 (Höglund et al., 1998) affects a conserved amino acid identical in five of the mammalian SLC26 family members.

After this study the first large genomic rearrangements have also been found (Höglund et al., submitted).

4.3.4. Polymorphisms

So far only four polymorphic sequence variations have been detected in the CLD gene. Two of the polymorphisms are silent single nucleotide substitutions, while two are polymorphic both at the gene and the protein level. Both the C307W and R554Q polymorphism change unconserved amino acids. The C307W change was originally found to occur in homozygous form in association with V317del mutation in Finnish CLD patients (I). However, also two healthy individuals demonstrated it in homozygous form and the carriership was detected to be more than ten-fold of that predicted for CLD. The C307W polymorphism has later been detected in different populations suggesting it to be a common polymorphism outside Finland as well. Functional testing has verified its neutral nature (Moseley et al., 1999). It is possible

that some of the missense single nucleotide changes turn out to be rare silent polymorphisms in functional testing.

4.4. CLD has a limited expression pattern and DTDST partially colocalizes with it

4.4.1. Northern analysis (I)

A multiple tissue northern blot (Clontech) was hybridized with a CLD cDNA probe in order to obtain a general overview of the expression pattern of the human CLD gene. The northern analysis suggested a restricted expression pattern for CLD, demonstrating a single 3.7-kb transcript with a strong signal in colon and prostate (I). A weak signal was observed in small intestine and an extremely weak one in testis. No signal was detected with spleen, thymus, ovary, and peripheral blood leukocyte mRNA.

4.4.2. *In situ* hybridization and immunohistochemistry (I, IV, V, unpublished data)

We performed *in situ* hybridization and immunohistochemistry in order to establish the expression pattern of the human CLD gene and protein, which aids in defining the normal physiological role of this anion transporter and also elucidates the mechanisms underlying congenital chloride diarrhea. CLD expression was studied *in vivo* in various normal human epithelia and in inflammatory and neoplastic colonic epithelia. Intestinal samples of a CLD patient were also included.

Northern blot analysis suggests the homologous anion exchanger DTDST to have an ubiquitous tissue distribution including intestine, although cartilage is the only tissue known to be affected by its defective function (Hästbacka et al., 1994; Satoh et al., 1998). The expression pattern of the human DTDST gene was also studied and compared with that of CLD in order to reveal the possible colocalization of these homologous anion transporters.

As expected, strong expression for both the CLD mRNA and protein was detected in ileal and colonic surface epithelium. The intestinal samples of the patient with CLD and the inflammatory colon samples demonstrated CLD expression similar to the normal control. In the neoplastic colonic epithelium, the expression of CLD appeared

to depend on the differentiation level: the poorer the differentiation, the lower the CLD expression. DTDST partially colocalized with CLD in colon. Of the other epithelial tissues studied, only eccrine sweat gland showed both CLD and DTDST expression. In addition, CLD was expressed in seminal vesicle, while the expression of DTDST could be detected in fetal and mature cartilage, placenta, bronchial glands, tracheal epithelium, and pancreas. (I, IV, V)

Histologically normal colon (I, IV)

In situ hybridization of normal colonic sections revealed a strong signal for CLD mRNA in the luminal surface epithelium and the upper one-third of the colonic crypt (I). The signal was less intense or absent in the base areas of the crypts. The immunostaining for the CLD protein colocalized with mRNA signal in the colon epithelium (IV). An intense CLD expression was detected both in the apical side of the nucleus in the cytoplasm and focally in the brush border of the absorptive epithelial cells.

The expression of the CLD protein in the brush borders of luminal enterocytes is consistent both with its identity as a membrane glycoprotein (Byeon et al., 1996) and with its suggested function in chloride absorption. The absorptive processes in colon are known primarily to be located at the luminal surface epithelia where substances to be absorbed are in a close contact with the epithelia. In addition, strong immunostaining for the CLD protein was detected in the cytoplasm in the apical side of nucleus, which most likely indicates a high level of synthesis and presence of the nascent CLD protein in enterocytes.

Intestinal samples of the CLD patient (I, unpublished data)

Normal or close to normal expression of CLD mRNA was detected in a colon sample from a Finnish CLD patient with V317del mutation (I). In the appendix of a CLD patient, the immunostaining of the CLD protein was found to be similar to a normal control (Fig. 3; unpublished data). However, these methods do not permit the quantitative evaluation of CLD mRNA and protein levels. Despite normal protein production, a mutation can affect its targeting, stability and function which is clearly

defective in the patients with CLD. The abolished function resulting from the Finnish founder mutation V317del has recently been established (Moseley et al., 1999).

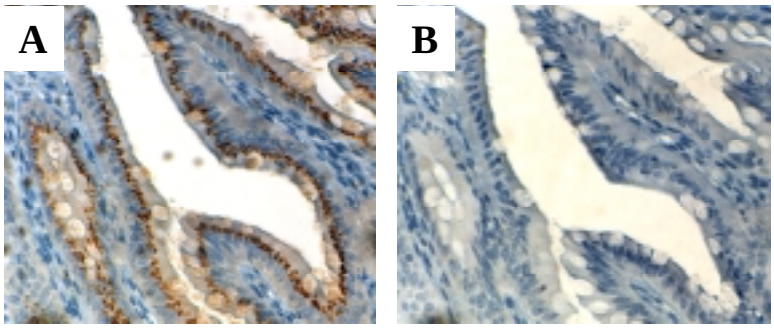

Figure 3. Expression of the human CLD protein in the appendix of a CLD patient. Anti-CLD serum demonstrates immunoreactivity in the apical side of the nucleus in the surface epithelial cells (A). In contrast, a serial section stained with preimmune serum shows no specific immunoreactivity (B).

Inflammatory bowel samples (IV)

Impaired Cl^- absorption in exchange for bicarbonate suggesting defective function of an active Cl^-/HCO_3^-exchange mechanism is detected in ulcerative colitis (Caprilli et al., 1986). However, no significant change in CLD mRNA and protein expression was detected in the samples with ulcerative, Crohn's, and ischemic colitis (IV), although both inflammation and IL-1 stimulation have previously been reported to reduce the expression of the CLD gene (Yang et al., 1998). Normal cytoplasmic immunostaining for the CLD protein was detected even in the samples with active inflammation or epithelium bordering ulcerations (IV). While brush border staining was missing in ulcerative colitis, the brush border in the samples with Crohn's or ischemic colitis demonstrated immunoreactivity similar to the normal control (IV). A chronically inflamed rabbit ileum has demonstrated inhibition in villus Cl^-/HCO_3^- exchange, which is a secondary phenomenon due to a decrease in the affinity for Cl^- without an alteration in transporter numbers (Coon and Sundaram, 2000). The specific transport protein was not, however, established.

Some evidence also suggest that different inflammatory mediators may specifically regulate different transport pathways in a chronically inflamed tissue (Sundaram et al.,

1997; 1998a; 1998b). The role of cytokines in the mucosal immune system has been studied intensively and an altered expression profile of colonic mucosal chemokines was reported in inflammatory intestinal tissues. In IBD, a disturbed balance between proinflammatory and anti-inflammatory cytokines has been suggested to play an important role in the maintaining of chronic inflammation (Casini-Raggi et al. 1995; Noguchi et al. 1998). Cytokines have also been shown to alter epithelial ion transport and permeability (McKay and Baird 1999). Despite extensive studies on inflammatory cytokines, little is known about the regulation of enterocytic protein expression by inflammatory cytokines. However, when surface epithelium was absent or severely affected, the expression of the CLD protein was found to be redistributed to the lower, more dedifferentiated, parts of the. Even the base areas near the muscularis mucosae showed positive immunostaining in some samples crypts (unpublished data). This is the first evidence of the up-regulation of CLD. Local factors associated with tissue vitality may act as regulators of the expression of CLD.

Increased proliferative activity in IBD has previously been shown in terms of expansion of the proliferative zone of the colonic crypt up to the lower two-thirds of the crypt (Serafini et al., 1981). We show that CLD is expressed also in the proliferative zone (IV), suggesting that the expression of CLD is not limited to non-proliferating cells, although in normal tissues no CLD expression is usually observed in the cells of the proliferative cell compartment.

Proliferative epithelial lesions of the colon (IV)

We studied the expression of CLD in a set of different colonic tumors, including all stages of colon tumor progression, in order to clarify the role of its down-regulation in the neoplastic transformation and progression. In these tumors, we found a variable CLD expression pattern that was dependent on the differentiation status. Adenomatous polyps with mild to moderate dysplasia demonstrated cytoplasmic immunostaining that was reduced but clearly present in all samples (IV). However, the brush border immunoreactivity normally found in the apical membrane could not be detected. In more advanced dysplasias and adenocarcinomas also the cytoplasmic staining was gradually lost, although the non-neoplastic epithelium adjacent to tumors expressed CLD normally (IV).

Initially, the CLD gene was isolated as a putative tumor suppressor gene on the basis of its down-regulated expression in colon adenomas and adenocarcinomas (Schweinfest et al., 1993). Further studies demonstrated some correlation between down-regulation and tumor progression, and down-regulation was suggested to be particularly significant in the earliest phases of the neoplastic evolution (Antalis et al., 1998). An epidemiological survey on the incidence of cancer among CLD patients with a non-functional V317del germline mutation and their parents (obligatory carriers) gave little clinical support to this hypothesis. The total cancer incidence was unchanged, although a slightly elevated risk of colon cancer was found (Hemminki et al., 1998). Since the patients homozygous for this mutation thrive well without prominent cancer predisposition, a major role of CLD in the initiation of cancer is unlikely. However, all CLD patients studied were young (all under 32) owing to the lethality of CLD in the past and further follow-up would thus be beneficial for the accurate determination of their cancer risk. Furthermore, in the expression analysis, normal or close to normal expression was revealed in the intestinal samples of a Finnish patient homozygous for V317del mutation. Thus, the localization of the CLD protein can be defective causing the dysfunction of the anion transport properties, but the protein is translated and may have retained the ability to interact with other proteins in addition to its putative function as tumor suppressor. Finally, although a recent *in vitro* study has demonstrated functional defect of V317del mutation, some retained activity could be detected (Moseley et al., 1999).

Our results indicate that CLD is intensely expressed in the normal and benign colon surface epithelium and that its down-regulation is a common but later event in the neoplastic evolution of colonic mucosa than previously thought. The significance of this finding remains open; the putative tumour-suppressor capability of CLD appears unlikely, however. These findings demonstrate that the expression of CLD is limited to a highly mature epithelium and loss of expression reflects loss of terminal differentiation.

DTDST is partially colocalized with CLD in a normal human colon (IV, V)

The colon surface epithelium demonstrated both DTDST mRNA and protein expression. A strong signal for the DTDST mRNA was detected in the upper one-third of the colonic crypt epithelium, while the signal was absent both in the luminal surface epithelium and the base areas of the crypts (IV). Immunohistochemistry confirmed a similar protein expression pattern (V). Interestingly, there was some variability in immunostaining depending on the pre-treatment used. When epitopes were unmasked with a gentle SDS incubation, the surface epithelial cells and especially apical cell membranes demonstrated strong immunoreactivity. In contrast, when epitopes were unmasked using the microwave treatment, the immunostaining was detected in the cytoplasm at the apical side of the nucleus corresponding to the area of Golgi apparatus. The heavy microwave treatment most likely unmasks epitopes hidden deeper in the cell, probably corresponding with the protein under construction and processing in Golgi apparatus, but simultaneously it destroys the apical, most likely functional, form of the protein.

Interestingly, in the colon DTDST is localized between two known chloride transporters, namely CFTR, which secretes chloride and is located in the bottom areas of the crypt (Strong et al., 1994), and CLD, which absorbs chloride in the exchange for bicarbonate and is localized in the upper one-third of the crypt and the lumenal surface epithelium (I; IV). Although DTDST partially colocalizes with CLD, it is clearly unable to compensate for the defective function of CLD as is shown by the strong diarrheal phenotype in congenital chloride diarrhea. However, the possible role of DTDST between two important transporters as a regulator or modifier remains yet to be solved. Chloride secretion into the crypts, mainly mediated by CFTR, has been suggested to regulate bicarbonate secretion of luminal enterocytes. The location of DTDST in the upper-one third of the crypt provides it with excellent possibilities to modify luminal chloride content. It is already known that CFTR induces the expression of CLD when expressed simultaneously in tracheal epithelial cells (Wheat et al., 2000).

The colon is a site of major macromolecular sulfation due to the large number of cells producing protective mucus. Colonic mucus is composed of protein and oligosaccharides that are heavily sulfated in contrast to mucin from other parts of the gastrointestinal tract (Goso and Hotta, 1993). Sulfate anion concentrations in colonic lumen vary conciderably in different diets (Florin et al., 1991), but many species of bacteria in the colon are able to degrade the highly sulfated macromolecules, releasing free sulfate which would then become available for uptake to epithelial cells. In addition, in vivo studies have indicated sulfate absorption from the gut (Krijgsheld et al., 1979). As a sulfate transporter with colonic expression, SLC26A2 might well have a role in an inorganic sulfate uptake for the abundant synthesis of highly sulfated macromolecules in colon epithelial cells.

Eccrine sweat glands demonstrate both CLD and DTDST expression (IV, V)

The luminal side and intercellular canaliculi of the epithelial cells in the coiled secretory part of eccrine sweat gland demonstrated abundant immunoreactivity for the CLD protein (IV). Also both DTDST protein and mRNA expression were detected in the same structures (V). The main electrolytes of primary sweat are sodium, chloride, potassium, and bicarbonate (Bijman, 1987). Sweat also contains some proteins. The presence of proteolytic enzymes as well as mucin has been established (Seutter et al., 1970). It is, however, controversial if these sulfated macromolecules are true derivatives of sweat rather than contaminants from the skin surface. The mechanisms responsible for primary sweat formation are not clear but a complex co-ordination of multiple ion transporters has been suggested (Bijman, 1987). At least during cholinergic sweat secretion Cl^-/HCO_3^- and Na^+/H^+ exchangers have been suggested to participate in the control of sweat composition, although the main role has been assigned to Na^+-K^+-$2Cl^-$ cotransporters (Sato et al. 1989). The localization of transporters like CLD and DTDST to the secretory part of eccrine sweat glands is not surprising, since a need for especially chloride, but also sulfate transport, has been established. More studies are needed to reveal their possible involvement in the primary sweat formation.

CLD is expressed in seminal vesicle (IV)

In addition to colon, northern analysis suggested strong expression of CLD mRNA in the prostate. At the histological level, however, both the stromal and epithelial tissues of the prostate itself remained negative for CLD mRNA and protein but expression was found in intra- and extraprostatic seminal vesicles (IV). Microvilli of the secretory columnar cells of ductal epithelium, in particular, demonstrated strong immunoreactivity. The seminal vesicle fluid contains a high concentration of HCO_3^- (Okamura et al., 1985) and CLD might well be involved in this process, since its intestinal manifestation includes a defect in Cl^-/HCO_3^- exchange (Holmberg et al., 1975). At ejaculation, the alkalic seminal vesicle fluid mixes with stored sperm activating the motility of the primarily quiescent sperm. Defects in the neutralization process result in acidity of the luminal fluid, causing hypomotility of the sperm and infertility/subfertility (Breton et al. 1996). It is important to re-evaluate the clinical significance of the seminal vesicular expression of the CLD gene in male CLD patients.

DTDST is expressed in fetal and mature cartilage (V)

In *in situ* hybridization, developing diaphysic cartilage of fetal limb long bone at gestational week 12 demonstrated strong signal for DTDST mRNA (V). The only adult cartilage showing DTDST mRNA expression was bronchial cartilage (V). Although both DTDST protein and mRNA levels in adult cartilage were almost undetectable, a strong signal for DTDST mRNA was observed in developing fetal cartilage. The proteoglycan composition of human cartilage does not remain constant throughout life. In the fetal period, chondroitin sulfate predominates and structural changes of cartilage proteoglycans are small. There is, however, considerable variation in both the degree and position of sulfation of chondroitin sulfate (Roughley et al., 1987). In in situ hybridization, the fetal DTDST expression was detectable in most mature hypertrophic chondrocytes in diaphysis of developing bone at gestational week 12. At the same time 6-sulfation, which is more sensitive to extracellular inorganic sulfate depletion (Ito et al., 1982; Rossi et al., 1996), predominates in chondoitin sulfate chains (Roughley et al., 1987). Concurrently vasculature is only starting to penetrate developing bone. 6-sulfation has also been demonstrated to be more essential for the growth response of the cells to fibroblast growth factor (Pye et al., 1998), which has already been shown to

be dependent on overall sulfation of matrix proteoglycans (Satoh et al., 1998). Therefore, although chondrocytes, together with other cells, are to some extent able to utilize alternative sulfate sources in proteoglycan biosynthesis, the availability of sulfated aminoacids is not adequate to meet the enhanced demand in a practically avascular tissue like cartilage.

DTDST is expressed also in other epithelial tissues (V)

Placental syncytiotrophoblasts function as an absorptive epithelium providing essential nutrients, including sulfate, for the fetus. The syncytiotrophoblast apical surface as well as some cytoplasmic granules have been reported to contain at least chondroitin sulfate, a highly sulfated glycoconjugate (Parmley et al., 1984). In addition, the presence of an active, Na^+-independent, and an anion transport inhibitor (DIDS) sensitive sulfate transport mechanism in human placental tissue has been reported (Cole and Rastogi, 1991). The localization of DTDST in human placental sycytiotrophoblasts makes it an excellent candidate for participating in maternofetal sulfate transport in addition to providing inorganic sulfate for macromolecule biosynthesis.

Histologically, submucosal seromucous bronchial glands and tracheal epithelium demonstrated strong DTDST mRNA and protein expression. In bronchial sections, DTDST was abundant in cells of airway submucosal glands and in tracheal epithelium. Airway mucus is primarily produced in mucus-producing cells of submucosal glands and is known to be extremely highly sulfated. The presence of a SO_4^{2-}/Cl^- exchanger in apical membrane of tracheal epithelial cells has been proposed (Elgavish et al., 1987). ^{35}S-sulfate incorporation studies have shown an uptake into secretory cells both in the tracheal epithelium and submucosal glands (Svitacheva et al., 1998). The localization of DTDST in submucosal glands as well as tracheal epithelium suggests that it is an excellent candidate to be responsible for sulfate uptake in these structures.

Chloride secretion has been postulated to regulate HCO_3^- secretion by pancreatic duct cells. CFTR is responsible for most chloride transport, but other Cl^--channels participate (Nguyen et al., 1997). There is also evidence for a SO_4^{2-}/Cl^- exchange mechanism (Elgavish and Meezan, 1992). In addition, a protective layer of sulfated mucin covers pancreatic ducts. It is also interesting to observe that material in the

acinar lumen stained strongly with anti-DTDST serum. This might well indicate that the DTDST protein is ultimately degraded and extruded from the cell cytoplasm to acinar lumen.

In bronchi, sweat glands, and pancreas, DTDST colocalizes with the chloride secretor CFTR (Trezise and Buchwald, 1991; Engelhardt et al., 1992; Sato and Sato, 2000). Interestingly, mucus of cystic fibrosis patients is reported to contain oversulfated glycoconjugates at least in the respiratory tract and in the intestine (Zhang et al., 1995; Hill et al., 1997). Mutations in the CFTR gene cause defective chloride secretion. DTDST has an ability to secrete chloride in exchange for sulfate and might be trying to compensate for the chloride transport defect of CFTR, thus resulting in the transport of extra sulfate into the cells in exchange for chloride. Abundant sulfate might then be conjugated to sulfated proteoglycans in order to excrete it and to maintain intracellular electrolyte balance.

CONCLUSIONS AND FUTURE PROSPECTS

In this study the gene for congenital chloride diarrhea (CLD) was identified and its genomic structure was determined, making extensive mutation screening possible. The in vivo expression of CLD and highly homologous diastrophic dysplasia sulfate transporter (DTDST) was studied in different tissues and cell types and the expression of DTDST was compared to that of CLD.

Summarizing the results, strong evidence suggests CLD to be the major Cl^-/HCO_3^- transporter responsible for the electronegative Na^+ coupled Cl^- absorption in ileal and colonic epithelial cells. First, intestinal perfusion studies in CLD patients demonstrate defect in apical Cl^-/HCO_3^- exchange in distal ileum and colon (Holmberg et al., 1975). Second, the DRA gene causes CLD, if mutated (I; III). Third, CLD is located in the brush border of colonic surface epithelial cells (I, IV; Byeon et al., 1996). Fourth, *in vitro* functional data show CLD-mediated Cl^-/HCO_3^- exchange activity (Melvin et al., 1999; Moseley et al., 1999).

The presence of a major founder mutation in Finland and the Arabic countries covering over 98% and 94% of disease chromosomes, respectively, facilitates the diagnostic possibilities of CLD in these high-frequency areas. It is now possible to offer fast and reliable pre- and postnatal diagnostics as DNA-based testing is available. Also carrier testing is facilitated and genetic counseling will be improved. Early diagnosis allows the people involved, both family members and health care personnel, to be prepared for possible prematurity. At present, treatment involves the replacement of continuous fluid and electrolyte losses. Appropriate treatment from birth will have positive influence on the patients' entire life; correctly treated patients are likely to develop normally and survive without kidney failure.

The mutations identified in the CLD gene show a wide spectrum of DNA variations including missense and nonsense point mutations, splice-site mutations, as well as insertions and deletions. They are distributed throughout the CLD protein, although some clustering can be observed. Studies using mutagenized constructs will clarify the effect of different mutations on the structure and function of the CLD protein. The

protein structure, however, remains to be experimentally determined in order to reveal the protein domains affected by the mutations.

Multiple truncating mutations are located in the long C-terminal most likely intracellular tail of the CLD protein. On the basis of the CLD phenotype, even most C-terminal mutations abolish the transport function, which suggests these areas to be of great importance for the proper function of the CLD protein. Some intestinal transport proteins, such as cystic fibrosis conductance regulator (CFTR) and sodium-hydrogen exchanger 3 (NHE3), are regulated by distinct domains in the C-terminal end of the protein. The presence of specific regulatory domains and their potential role for functional regulation of the CLD protein need to be verified.

During this study, a growing family of anion transporters with members across a large taxonomic span was recognized. To date, the gene family consists of six well-characterized mammalian members, while further studies are likely to reveal new ones (Lohi et al., 2000). Interestingly, besides CLD, two other genes associated with the distinct genetic diseases, namely PDS and DTDST, reside within the same gene family. All the family members are structurally well conserved but still play central roles in very distinct physiological functions including gut absorption, cartilage growth, and hearing. This mostly results from different tissue expression profiles in addition to distinct substrate specificity. The expansion of the family with the novel members and their functional characterization is likely to provide important knowledge on anion transport physiology in different parts of the human body.

Immunohistochemistry demonstrated strong CLD expression at the brush-border of ileal and colonic epithelial cells, but also cytoplasmic immunostaining on the apical side of the nucleus was detected. The development of CLD-specific antibodies enhances the studies of the intracellular processing, transport, and localization of CLD in the mammalian cell. The expression of the homologous anion transporter DTDST was partially overlapping with the CLD expression. Based on the CLD phenotype, it is obvious that DTDST is not able to compensate for the defective function of CLD.

Intestinal electroneutral NaCl absorption and thus also the mechanisms responsible for it are tightly regulated and it is most likely that different mechanisms are involved at different stages of the digestive process. Further in vivo and in vitro studies are needed to reveal the relative contribution of CLD to the basal and stimulated Cl^- absorption in ileum and colon. Its role in other physiological functions like intracellular pH and cell volume regulation is also subject to further investigation.

Although a lot of information has accumulated on the human CLD gene, we are just beginning to understand the molecular background of congenital chloride diarrhea. It is obvious that much work has to be done to define the cellular and molecular mechanisms underlying the function of the CLD protein. Its exact biochemical and physiological role and its association with ileal and colonic Cl^-/HCO_3^- exchange are not fully understood. Many basic properties affecting its function, such as the cell-specific regulation, the molecular mechanism of coupling, and the way the transport is energized, are subjects for further study. We understand neither how its substrate selectivity is determined nor how the ions are moved across the membrane. The investigation of the biosynthesis, trafficking, and degradation of the CLD protein would provide knowledge of the transporter at cellular level.

Identification of the CLD gene will, thus, start a new era in studies on an intestinal anion transport physiology and on the molecular pathophysiology of congenital chloride diarrhea.

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REFERENCES

Allen, D.C., Biggart, J.D., Orchin, J.C. and Foster, H.: An immunoperoxidase study of epithelial marker antigens in ulcerative colitis with dysplasia and carcinoma. J Clin Pathol 38 (1985) 18-29.

Alper, S.L., Rossmann, H., Wilhelm, S., Stuart-Tilley, A.K., Shmukler, B.E. and Seidler, U.: Expression of AE2 anion exchanger in mouse intestine. Am J Physiol 277 (1999) G321-32.

Alper, S.L.: The band 3-related anion exchanger (AE) gene family. Annu Rev Physiol 53 (1991) 549-64.

Antalis, T.M., Reeder, J.A., Gotley, D.C., Byeon, M.K., Walsh, M.D., Henderson, K.W., Papas, T.S. and Schweinfest, C.W.: Down-regulation of the down-regulated in adenoma (DRA) gene correlates with colon tumor progression. Clin. Cancer Res. 4 (1998) 1857-63.

Badawi, M.H., Zaki, M., Ismail, E.A. and Majid Molla, A.: Congenital chloride diarrhoea in Kuwait: a clinical reappraisal. J Trop Pediatr 44 (1998) 296-9.

Bairoch, A. and Apweiler, R.: The SWISS-PROT protein sequence data bank and its new supplement TREMBL. Nucleic Acids Res 24 (1996) 21-5.

Ballabio, A.: The rise and fall of positional cloning? Nat Genet 3 (1993) 277-9.

Benit, P., Kara-Mostefa, A., Hadj-Rabia, S., Munnich, A. and Bonnefont, J.P.: Protein truncation test for screening hamartin gene mutations and report of new disease-causing mutations. Hum Mutat 14 (1999) 428-32.

Berne, R.M. and Levy, M.N.: Intestinal absorption of water and electrolytes. In Physiology. Eds. Berne, R.M. and Levy, M.N. Mosby Book (1998) pp. 654-61.

Bijman, J.: Transport processes in the eccrine sweat gland. Kidney Int Suppl 21 (1987) S109-12.

Binder, H.J.: Pathophysiology of acute diarrhea. Am J Med 88 (1990) 2S-4S.

Bissig, M., Hagenbuch, B., Stieger, B., Koller, T. and Meier, P.J.: Functional expression cloning of the canalicular sulfate transport system of rat hepatocytes. J Biol Chem 269 (1994) 3017-21.

Bookstein, C., Xie, Y., Rabenau, K., Musch, M.W., McSwine, R.L., Rao, M.C. and Chang, E.B.: Tissue distribution of Na+/H+ exchanger isoforms NHE2 and NHE4 in rat intestine and kidney. Am J Physiol 273 (1997) C1496-505.

Booth, I.W., Stange, G., Murer, H., Fenton, T.R. and Milla, P.J.: Defective jejunal brush-border Na+/H+ exchange: a cause of congenital secretory diarrhoea. Lancet 1 (1985) 1066-9.

Boron, W.F.: Intracellular pH regulation in epithelial cells. Annu Rev Physiol 48 (1986) 377-88.

Boyarsky, G., Ganz, M.B., Cragoe, E.J., Jr. and Boron, W.F.: Intracellular-pH dependence of Na-H exchange and acid loading in quiescent and arginine vasopressin-activated mesangial cells. Proc Natl Acad Sci U S A 87 (1990) 5921-4.

Boyarsky, G., Ganz, M.B., Sterzel, R.B. and Boron, W.F.: pH regulation in single glomerular mesangial cells. II. Na+-dependent and -independent Cl(-)-HCO3- exchangers. Am J Physiol 255 (1988) C857-69.

Breton, S., Smith, P.J.S., Lui, B. and Brown, D.: Acidification of the male reproductive tract by a proton pumping (H^+)-ATPase. Nature Med. 2 (1996) 470-2.

Busa, W.B. and Nuccitelli, R.: Metabolic regulation via intracellular pH. Am J Physiol 246 (1984) R409-38.

Byeon, M.K., Westerman, M.A., Maroulakou, I.G., Henderson, K.W., Suster, S., Zhang, X.K., Papas, T.S., Vesely, J., Willingham, M.C., Green, J.E. and Schweinfest, C.W.: The down-regulated in adenoma (DRA) gene encodes an intestine-specific membrane glycoprotein. Oncogene 12 (1996) 387-96.

Caprilli, R., Frieri, G., Latella, G., Vernia, P. and Santoro, M.L.: Faecal excretion of bicarbonate in ulcerative colitis. Digestion 35 (1986) 136-42.

Casini-Raggi, V., Kam, L., Chong, Y.J.T., Fiocchi, C., Pizarro, T.T. and Cominelli, F.: Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. J Immunol 154 (1995) 2434-40.

Charney, A.N. and Donowitz M.: Functional significance of intestinal Na+-K+-ATPase: in vivo ouabain inhibition. Am J Physiol 234 (1978) E629-36.

Christopher, N.L. and Bayless, T.M.: Role of the small bowel and colon in lactose-induced diarrhea. Gastroenterology 60 (1971) 845-52.

Chu, S. and Montrose, M.H.: Extracellular pH regulation in microdomains of colonic crypts: effects of short-chain fatty acids. Proc Natl Acad Sci U S A 92 (1995) 3303-7.

Cole, D.E. and Rastogi, N.: Sulfate transport in human placenta: further evidence for a sodium-independent mechanism. Biochim Biophys Acta 1064 (1991) 287-92.

Collins, F.S.:Positional cloning moves from perditional to traditional. Nat Genet 9 (1995) 347-50.

Collins, F.S.: Positional cloning: let's not call it reverse any more. Nat Genet 1 (1992) 3-6.

Conner, B.J., Reyes, A.A., Morin, C., Itakura, K., Teplitz, R.L. and Wallace, R.B.: Detection of sickle cell beta S-globin allele by hybridization with synthetic oligonucleotides. Proc Natl Acad Sci U S A 80 (1983) 278-82.

Coon, S. and Sundaram, U.: Mechanism of glucocorticoid-mediated reversal of inhibition of Cl^-/HCO_3^- exchange during chronic ileitis. Am J Physiol 278 (2000) G570-7.

Cotton, R.G.H., Rodrigues, N.R. and Campbell, R.D.: Reactivity of cytosine and thymine in single-base-pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc Natl Acad Sci USA 85 (1988) 4397-401.

Counillon, L. and Pouyssegur, J.: The expanding family of eucaryotic Na(+)/H(+) exchangers. J Biol Chem 275 (2000) 1-4.

Cox, K.H., DeLeon, D.V., Angerer, L.M. and Angerer, R.C.: Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. Dev Biol 101 (1984) 485-502.

Crawford, J.M. The gastrointestinal tract: Idiopathic inflammatory bowel disease. In Robbins: Pathologic basis of the disease. Eds Cotran, R.S., Cumar, V. and Robbins, S.L. W.B. Saunders Company (1994) pp.800-6.

Darrow, D.C.: Congenital alkalosis with diarrhea. J Pediatr 26 (1945) 519-32.

Davis, G.R., Morawski, S.G., Santa Ana, C.A. and Fordtran JS.: Evaluation of chloride/bicarbonate. Exchange in the human colon in vivo. J Clin Invest 71 (1983) 201-7.

de la Chapelle, A.: Disease gene mapping in isolated human populations: the example of Finland. J Med Genet 30 (1993) 857-65.

de Koning Gans, P.A., Ginjaar, I., Bakker, E., Yates, J.R and den Dunnen, J.T.: A protein truncation test for Emery-Dreifuss muscular dystrophy (EMD): detection of N-terminal truncating mutations. Neuromuscul Disord 9 (1999) 247-50.

Demaurex, N. and Grinstein, S.: Na+/H+ antiport: modulation by ATP and role in cell volume regulation. J Exp Biol 196 (1994) 389-404.

Dhanda, R.K., Smith, W.M., Scott, C.B., Eng, C. and Vijg, J.: A simple system for automated two-dimensional electrophoresis: applications to genetic testing. Genet Test 2 (1998) 67-70.

Dharmsathaphorn, K.: Transport of water and electrolytes in the gastrointestinal tract: physiological mechanisms, regulation, and methods for study. In Clinical disorders of fluid and electrolyte metabolism. Eds. Maxwell, M.H., Kleeman C.R. and Narins, R.G. McGraw-Hill Book Company (1987) pp. 385-408.

Donowitz, M., De La Horra, C., Calonge, M.L., Wood, I.S., Dyer, J., Gribble, S.M., De Medina, F.S., Tse, C.M., Shirazi-Beechey, S.P. and Ilundain, A.A.: In birds, NHE2 is major brush-border Na+/H+ exchanger in colon and is increased by a low-NaCl diet. Am J Physiol 274 (1998) R1659-69.

Elgavish, A. and Meezan, E.: Altered sulfate transport via anion exchange in CFPAC is corrected by retrovirus-mediated CFTR gene transfer. Am J Physiol 263 (1992) C176-86.

Elgavish, A. and Meezan, E.: Sulfation by human lung fibroblasts: SO4(2-) and sulfur-containing amino acids as sources for macromolecular sulfation. Am J Physiol 260 (1991) L450-6.

Elgavish, A., DiBona, D.R., Norton, P. and Meezan, E.: Sulfate transport in apical membrane vesicles isolated from tracheal epithelium. Am J Physiol 253 (1987) C416-25.

Engelhardt, J.F., Yankaskas, J.R., Ernst, S.A., Yang, Y., Marino, C.R., Boucher, R.C., Cohn, J.A. and Wilson, J.M.: Submucosal glands are the predominant site of CFTR expression in the human bronchus. Nat Genet 2 (1992) 240-8.

Ericson, A.C. and Spring, K.R.: Volume regulation by Necturus gallbladder: apical Na+-H+ and Cl(-)-HCO- 3 exchange. Am J Physiol 243 (1982) C146-50.

Esko, J.D., Elgavish, A., Prasthofer, T., Taylor, W.H. and Weinke, J.L.: Sulfate transport-deficient mutants of Chinese hamster ovary cells. Sulfation of glycosaminoglycans dependent on cysteine. J Biol Chem 261 (1986) 15725-33.

Etani, Y., Mushiake, S., Tajiri, H., Miki, K., Kozaiwa, K., Sawada, A., Tada, K., Ozono, K. and Okada, S.: A novel mutation of the down-regulated in adenoma gene in a Japanese case with congential chloride diarrhea. Mutations in brief no. 198. Hum Mutat 12 (1998) 362.

Everett, L.A. and Green, E.D.: A family of mammalian anion transporters and their involvement in human genetic diseases. Hum Mol Genet 8 (1999) 1883-91.

Everett, L.A., Morsli, H., Wu, D.K. and Green, E.D.: Expression pattern of the mouse ortholog of the Pendred's syndrome gene (Pds) suggests a key role for pendrin in the inner ear. Proc Natl Acad Sci U S A 96 (1999) 9727-32.

Everett, L.A., Glaser, B., Beck, J.C., Idol, J.R., Buchs, A., Heyman, M., Adawi, F., Hazani, E., Nassir, E., Baxevanis, A.D., Sheffield, V.C. and Green, E.D.: Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet 17 (1997) 411-22.

Ezer, S., Bayes, M., Elomaa, O., Schlessinger, D. and Kere, J.: Ectodysplasin is a collagenous trimeric type II membrane protein with a tumor necrosis factor-like domain and co-localizes with cytoskeletal structures at lateral and apical surfaces of cells. Hum Mol Genet 8 (1999) 2079-86.

Feldman, G.M. and Stephenson, R.L.: H+ and HCO3- flux across apical surface of rat distal colon. Am J Physiol 259 (1990) C35-40.

Field, M., Rao, M.C. and Chang, E.B.: Mechanism of disease: Intestinal electrolyte transport and diarrheal disease. First of two parts. N Engl J Med (1989) 800-6.

Fine, K.D., Krejs, G.J. and Fordtran J.S.: Diarrhea. In Gastrointestinal diesease: Pathophysiology, diagnosis, management. Philadelphia Saunders (1989) pp. 290-316.

Florin, T., Neale, G., Gibson, G.R., Christl, S.U. and Cummings, J.H.: Metabolism of dietary sulphate: absorption and excretion in humans. Gut 32 (1991) 766-73.

Furusawa, T., Ikawa, S., Yanai, N. and Obinata, M.: Isolation of a novel PDZ-containing myosin from hematopoietic supportive bone marrow stromal cell lines. Biochem Biophys Res Commun 270 (2000) 67-75.

Gall, J.G. and Pardue, M.L.: Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci U S A 63 (1969) 378-83.

Gamble, J.L., Fahey, K.R., Appleton, J. and McLachlan, E.: Congenital alkalosis with diarrhea. J Pediatr 26 (1945) 509-18.

Garcia-Romeu, F., Borgese, F., Guizouarn, H., Fievet, B. and Motais, R.: A role for the anion exchanger AE1 (band 3 protein) in cell volume regulation. Cell Mol Biol (Noisy-le-grand) 42 (1996) 985-94.

Geck, P. and Pfeiffer, B.: Na+K+2Cl cotransport in animal cells- its role in volume regulation. Ann NY Acad Sci 456 (1986) 166-82.

Geibel, J.P., Singh, S., Rajendran, V.M. and Binder, H.J.: HCO(3)(-) secretion in the rat colonic crypt is closely linked to Cl(-) secretion. Gastroenterology 118 (2000) 101-7.

Genz, A.K., v. Engelhardt, W. and Busche, R.: Maintenance and regulation of the pH microclimate at the luminal surface of the distal colon of guinea-pig. J Physiol (Lond) 517 (1999) 507-19.

Gilligan, D.M., Lozovatsky, L., Gwynn, B., Brugnara, C., Mohandas, N. and Peters L.L.: Targeted disruption of the beta-adducin gene (Add2) causes red blood cell spherocytosis in mice. Proc Natl Acad Sci USA 96 (1999) 10717-22.

Goso, Y. and Hotta, K.: Regional differences in sulfated oligosaccharides of rat gastrointestinal mucin as detected by two-dimensional chromatography. Arch Biochem Biophys 302 (1993) 212-7.

Guyton, A.C. Textbook of medical physiology. W.B. Saunders Company (1991) pp. 9-23, and pp. 687-742.

Harris, J., Archampong, E.Q. and Clark, C.G.: The effect of salazopyrin on water and electrolyte transport in the human colon measured in vivo and in vitro. Gut 13 (1972) 855.

Hawker, P.C., McKay, J.S. and Turnberg, L.A.: Electrolyte transport across colonic mucosa from patients with inflammatory bowel disease. Gastroenterology 79 (1980) 508-11.

Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M.: Expression cloning and cDNA sequencing of the Na+/glucose co- transporter. Nature 330 (1987) 379-81.

Heim, R.A., Kam-Morgan, L.N., Binnie, C.G., Corns, D.D., Cayouette, M.C., Farber, R.A., Aylsworth, A.S., Silverman, L.M. and Luce, M.C.: Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. Hum Mol Genet 4 (1995) 975-81.

Hemminki, A., Höglund, P., Pukkala, E., Salovaara, R., Järvinen, H., Norio, R. and Aaltonen, L.A.: Intestinal cancer in patients with a germline mutation in the Down-Regulated in Adenoma (DRA) gene. Oncogene 16 (1998) 681-4.

Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R.: Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y) 10 (1992) 413-7.

Hill, W.G., Harper, G.S., Rozaklis, T., Boucher, R.C. and Hopwood, J.J.: Organ-specific over-sulfation of glycosaminoglycans and altered extracellular matrix in a mouse model of cystic fibrosis. Biochem Mol Med 62 (1997) 113-22.

Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H.: Detection of specific polymerase chain reaction product by utilizing the 5'-->3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci USA 88 (1991) 7276-80.

Holmberg, C.: Congenital chloride diarrhoea. Clin Gastroenterol 15 (1986) 583-602.

Holmberg, C. and Perheentupa, J.: Congenital Na+ diarrhea: a new type of secretory diarrhea. J Pediatr 106 (1985) 56-61.

Holmberg, C.: Electrolyte economy and its hormonal regulation in congenital chloride diarrhea. Pediatr Res 12 (1978) 82-6.

Holmberg, C., Perheentupa, J., Launiala, K. and Hallman, N.: Congenital chloride diarrhoea. Clinical analysis of 21 Finnish patients. Arch Dis Child 52 (1977a) 255-67.

Holmberg, C., Perheentupa, J. and Pasternack, A.: The renal lesion in congenital chloride diarrhea. J Pediatr 91 (1977b) 738-43.

Holmberg, C., Perheentupa, J. and Launiala, K.: Colonic electrolyte transport in health and in congenital chloride diarrhea. J Clin Invest 56 (1975) 302-10.

Hoogerwerf, W.A., Tsao, S.C., Devuyst, O., Levine, S.A., Yun, C.H., Yip, J.W., Cohen, M.E., Wilson, P.D., Lazenby, A.J., Tse, C.M. and Donowitz, M.: NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. Am J Physiol 270 (1996) G29-41.

Hästbacka, J., Kerrebrock, A., Mokkala, K., Clines, G., Lovett, M., Kaitila, I., de la Chapelle, A. and Lander, E.S.: Identification of the Finnish founder mutation for diastrophic dysplasia (DTD). Eur J Hum Genet 7 (1999) 664-70.

Hästbacka, J., Superti-Furga, A., Wilcox, W.R., Rimoin, D.L., Cohn, D.H. and Lander, E.S.: Atelosteogenesis type II is caused by mutations in the diastrophic dysplasia sulfate-transporter gene (DTDST): evidence for a phenotypic series involving three chondrodysplasias. Am J Hum Genet 58 (1996) 255-62.

Hästbacka, J., de la Chapelle, A., Mahtani, M.M., Clines, G., Reeve-Daly, M.P., Daly, M., Hamilton, B.A., Kusumi, K., Trivedi, B., Weaver, A., Coloma, A, Lovett, M., Buckler, A., Kaitila, I. and Lander, E.S.: The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. Cell 78 (1994) 1073-87.

Hästbacka, J., de la Chapelle, A., Kaitila, I., Sistonen, P., Weaver, A. and Lander, E.: Linkage disequilibrium mapping in isolated founder populations: diastrophic dysplasia in Finland. Nat Genet 2 (1992) 204-11.

Höglund, P., Sormaala, M., Haila, S., Socha, J., Rajaram, U., Scheurlen, W., Sinaasappel, M., de Jonge, H., Holmberg, C., Yoshikawa, H. and Kere, J.: Identification of seven novel mutations including the first two genomic rearrangements in SLC26A3 mutated in congenital chloride diarrhea. Hum Mutat submitted.

Höglund, P., Auranen, M., Socha, J., Popinska, K., Nazer, H., Rajaram, U., Al Sanie, A., Al-Ghanim, M., Holmberg, C., de la Chapelle, A. and Kere, J.: Genetic background of congenital chloride diarrhea in high-incidence populations: Finland, Poland, and Saudi Arabia and Kuwait. Am J Hum Genet 63 (1998) 760-8.

Höglund, P., Haila, S., Scherer, S.W., Tsui, L.C., Green, E.D., Weissenbach, J., Holmberg, C., de la Chapelle, A. and Kere, J.: Positional candidate genes for congenital chloride diarrhea suggested by high-resolution physical mapping in chromosome region 7q31. Genome Res 6 (1996) 202-10.

Höglund, P., Sistonen, P., Norio, R., Holmberg, C., Dimberg, A., Gustavson, K.H., de la Chapelle, A. and Kere, J.: Fine mapping of the congenital chloride diarrhea gene by linkage disequilibrium. Am J Hum Genet 57 (1995) 95-102.

Höglund, P., Holmberg, C., de la Chapelle, A. and Kere, J.: Paternal isodisomy for chromosome 7 is compatible with normal growth and development in a patient with congenital chloride diarrhea. Am J Hum Genet 55 (1994) 747-52.

Isfort, R.J., Cody, D.B., Asquith, T.N., Ridder, G.M., Stuard, S.B. and LeBoeuf, R.A.: Induction of protein phosphorylation, protein synthesis, immediate- early-gene expression and cellular proliferation by intracellular pH modulation. Implications for the role of hydrogen ions in signal transduction. Eur J Biochem 213 (1993) 349-57.

Ito, K., Kimata, K., Sobue, M. and Suzuki, S.: Altered proteoglycan synthesis by epiphyseal cartilages in culture at low SO_4^{2-} concentration. J Biol Chem 257 (1982) 917-923.

Janecki, A.J., Montrose, M.H., Zimniak, P., Zweibaum, A., Tse, C.M., Khurana, S. and Donowitz, M.: Subcellular redistribution is involved in acute regulation of the brush border Na+/H+ exchanger isoform 3 in human colon adenocarcinoma cell line Caco-2. Protein kinase C-mediated inhibition of the exchanger. J Biol Chem 273 (1998) 8790-8.

Jentsch, T.J., Janicke, I., Sorgenfrei, D., Keller, S.K. and Wiederholt, M.: The regulation of intracellular pH in monkey kidney epithelial cells (BSC-1). Roles of Na+/H+ antiport, Na+-HCO3(-)-(NaCO3-) symport, and Cl- /HCO3- exchange. J Biol Chem 261 (1986) 12120-7.

Jiang, L., Chernova, M.N. and Alper, S.L.: Secondary regulatory volume increase conferred on Xenopus oocytes by expression of AE2 anion exchanger. Am J Physiol 272 (1997) C191-202.

John, H.A., Birnstiel, M.L. and Jones, K.W.: RNA-DNA hybrids at the cytological level. Nature 223 (1969) 582-7.

Jonker, A., de Boer, P.A., van den Hoff, M.J., Lamers, W.H. and Moorman, A.F.: Towards quantitative in situ hybridization. J Histochem Cytochem 45 (1997) 413-23.

Kagalwalla, A.F.: Congenital chloride diarrhea. A study in Arab children. J Clin Gastroenterol 19 (1994) 36-40.

Kapus, A., Grinstein, S., Wasan, S., Kandasamy, R. and Orlowski, J.: Functional characterization of three isoforms of the Na+/H+ exchanger stably expressed in Chinese hamster ovary cells. ATP dependence, osmotic sensitivity, and role in cell proliferation. J Biol Chem 269 (1994) 23544-52.

Karniski, L.P., Lotscher, M., Fucentese, M., Hilfiker, H., Biber, J. and Murer, H.: Immunolocalization of sat-1 sulfate/oxalate/bicarbonate anion exchanger in the rat kidney. Am J Physiol 275 (1998) F79-87.

Keller, J.M. and Keller, K.M.: Amino acid sulfur as a source of sulfate for sulfated proteoglycans produced by Swiss mouse 3T3 cells. Biochim Biophys Acta 926 (1987) 139-44.

Kere, J., Lohi, H. and Höglund, P.: Genetic Disorders of Membrane Transport III. Congenital chloride diarrhea. Am J Physiol 276 (1999) G7-G13.

Kere, J., Sistonen, P., Holmberg, C. and de la Chapelle, A.: The gene for congenital chloride diarrhea maps close to but is distinct from the gene for cystic fibrosis transmembrane conductance regulator. Proc Natl Acad Sci U S A 90 (1993) 10686-9.

Khan, S.N. and Yaish, H.M.: Misdiagnosis of congenital chloride-losing diarrhea. J Perinatol 12 (1992) 112-4.

Kirk, K.L., Halm, D.R. and Dawson, D.C.: Active sodium transport by turtle colon via an electrogenic Na-K exchange pump. Nature 287 (1980) 237-9.

Kirkinen, P. and Jouppila, P.: Prenatal ultrasonic findings in congenital chloride diarrhoea. Prenat Diagn 4 (1984) 457-61.

Knickelbein, R.G., Aronson, P.S. and Dobbins, J.W.: Membrane distribution of sodium-hydrogen and chloride-bicarbonate exchangers in crypt and villus cell membranes from rabbit ileum. J Clin Invest 82 (1988) 2158-63.

Knickelbein, R., Aronson, P.S., Schron, C.M., Seifter, J. and Dobbins, J.W.: Sodium and chloride transport across rabbit ileal brush border. II. Evidence for Cl-HCO3 exchange and mechanism of coupling. Am J Physiol 249 (1985) G236-45.

Kockerling, A. and Fromm, M.: Origin of cAMP-dependent Cl- secretion from both crypts and surface epithelia of rat intestine. Am J Physiol 264 (1993) C1294-301.

Krijgsheld, K.R., Frankena, H., Scholtens, E., Zweens, J. and Mulder, G.J.: Absorption, serum levels and urinary excretion of inorganic sulfate after oral administration of sodium sulfate in the conscious rat. Biochim Biophys Acta 586 (1979) 492-500.

Lancaster, J.M., Cochran, C.J., Brownlee, H.A., Evans, A.C., Berchuck, A., Futreal, P.A. and Wiseman, R.W.: Detection of BRCA1 mutations in women with early-onset ovarian cancer by use of the protein truncation test. J Natl Cancer Inst 88 (1996a) 552-4.

Lancaster, J.M., Wooster, R., Mangion, J., Phelan, C.M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., Patel, S., Hamoudi, R., Larsson, C., Wiseman, R.W., Berchuck, A., Iglehart, J.D., Marks, J.R., Ashworth, A., Stratton, M.R. and Futreal, P.A.: BRCA2 mutations in primary breast and ovarian cancers. Nat Genet 13 (1996b) 238-40.

Landegren, U., Kaiser, R., Sanders, J. and Hood, L.: A ligase-mediated gene detection technique. Science 241 (1988) 1077-80.

Langer, J.C., Winthrop, A.L., Burrows, R.F., Issenman, R.M. and Caco, C.C.: False diagnosis of intestinal obstruction in a fetus with congenital chloride diarrhea. J Pediatr Surg 26 (1991) 1282-4.

Lau, J. and Tolan, D.R.: Screening for hereditary fructose intolerance mutations by reverse dot-blot. Mol Cell Probes 13 (1999) 35-40.

Lay, M.J. and Wittwer, C.T.: Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. Clin Chem 43 (1997) 2262-7.

Lee, A., Beck, L., Brown, R.J. and Markovich, D.: Identification of a mammalian brain sulfate transporter. Biochem Biophys Res Commun 263 (1999) 123-9.

Lehesjoki, A.-E., Koskiniemi, M., Norio, R., Tirrito, S., Sistonen, P., Lander, E. and de la Chapelle. Localization of the EPM1 gene for progressive myoclonus epilepsy on chromosome 21: linkage disequilibrium allows high resolution mapping. Hum Mol Genet 2 (1993) 1229-34.

Liu, W., Smith, D.I., Rechtzigel, K.J., Thibodeau, S.N. and James, C.D.: Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. Nucleic Acids Res 26 (1998) 1396-400.

Lohi, H., Kujala, M., Kerkelä, E., Saarialho-Kere, U., Kestilä, M. and Kere, J.: Mapping of five new putative anion transporter genes in human and characterization of SLC26A6, a candidate gene for pancreatic anion exchanger. Genomics 70 (2000) 102-12.

Lubani, M.M., Doudin, K.I., Sharda, D.C., Shaltout, A.A., al-Shab, T.S., Abdul Al, Y.K., Said, M.A., Salhi, M.M. and Ahmed, S.A.: Congenital chloride diarrhoea in Kuwaiti children. Eur J Pediatr 148 (1989) 333-6.

Luria, S.E. and Delbück, M.: Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28 (1943) 491-511.

Maher, M.M., Gontarek, J.D., Jimenez, R.E., Donowitz, M. and Yeo, C.J.: Role of brush border Na+/H+ exchange in canine ileal absorption. Dig Dis Sci 41 (1996) 651-9.

Martin, M.G., Turk, E., Lostao, M.P., Kerner, C. and Wright, E.M.: Defects in Na+/glucose cotransporter (SGLT1) trafficking and function cause glucose-galactose malabsorption. Nat Genet 12 (1996) 216-20.

McKay, D.M. and Baird, A.W. Cytokine regulation of epithelial permeability and ion transport. Gut 44 (1999) 283-9.

McNeil, N.I., Ling, K.L. and Wager, J.: Mucosal surface pH of the large intestine of the rat and of normal and inflamed large intestine in man. Gut 28 (1987) 707-13.

Melvin, J.E., Park, K., Richardson, L., Schultheis, P.J. and Shull, G.E.: Mouse down-regulated in adenoma (DRA) is an intestinal Cl(-)/HCO(3)(-) exchanger and is up-regulated in colon of mice lacking the NHE3 Na(+)/H(+) exchanger. J Biol Chem 274 (1999) 22855-61.

Monreal, A.W., Ferguson, B.M., Headon, D.J., Street, S.L., Overbeek, P.A. and Zonana, J.: Mutations in the human homologue of mouse dl cause autosomal recessive and dominant hypohidrotic ectodermal dysplasia. Nat Genet 22 (1999) 366-9.

Mosedale, D.E., Metcalfe, J.C. and Grainger, D.J.: Optimization of immunofluorescence methods by quantitative image analysis. J Histochem Cytochem 44 (1996) 1043-50.

Moseley, R.H., Hoglund, P., Wu, G.D., Silberg, D.G., Haila, S., de la Chapelle, A., Holmberg, C. and Kere, J.: Downregulated in adenoma gene encodes a chloride transporter defective in congenital chloride diarrhea. Am J Physiol 276 (1999) G185-92.

Motais, R., Fievet, B., Borgese, F. and Garcia-Romeu, F.: Association of the band 3 protein with a volume-activated, anion and amino acid channel: a molecular approach. J Exp Biol 200 (1997) 361-7.

Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C. and Markham, A.F.: Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res 17 (1989) 2503-16.

Nguyen, T.D., Koh, D.S., Moody, M.W., Fox, N.R., Savard, C.E., Kuver, R., Hille, B. and Lee, S.P.: Characterization of two distinct chloride channels in cultured dog pancreatic duct epithelial cells. Am J Physiol 272 (1997) G172-80.

Noguchi, M., Hiwatashi, N., Liu, Z. and Toyota, T.: Secretion imbalance between tumour necrosis factor and its inhibitor in inflammatory bowel disease. Gut 43 (1998) 203-9.

Norio, R., Nevanlinna, H.R. and Perheentupa, J.: Hereditary diseases in Finland; rare flora in rare soul. Ann Clin Res 5 (1973) 109-41.

Norio, R., Perheentupa, J., Launiala, K. and Hallman, N.: Congenital chloride diarrhea, an autosomal recessive disease. Genetic study of 14 Finnish and 12 other families. Clin Genet 2 (1971) 182-92.

O'Donovan, M.C., Oefner, P.J., Roberts, S.C., Austin, J., Hoogendoorn, B., Guy, C., Speight, G., Upadhyaya, M., Sommer, S.S. and McGuffin, P.: Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. Genomics 52 (1998) 44-9.

Oefner, P.J. and Underhill, P.A.: Comparative DNA sequencing by denaturing high-performance liquid chromatography (DHPLC). Am J Hum Genet 57 (1995) A266.

Okamura, N., Tajima, Y., Soejima, A., Masuda, H. and Sugita, Y.: Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. J. Biol. Chem. 260 (1985) 9699-705.

Oliver, S.G.: From DNA sequence to biological function. Nature 379 (1996) 597-600.

Orchard, C.H. and Kentish, J.C.: Effects of changes of pH on the contractile function of cardiac muscle. Am J Physiol 258 (1990) C967-81.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T.: Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A 86 (1989) 2766-70.

Orlowski, J. and Grinstein, S.: Na+/H+ exchangers of mammalian cells. J Biol Chem 272 (1997) 22373-6.

Paradiso, A.M., Tsien, R.Y., Demarest, J.R. and Machen, T.E.: Na-H and Cl-HCO3 exchange in rabbit oxyntic cells using fluorescence microscopy. Am J Physiol 253 (1987) C30-6.

Parmley, R.T., Takagi, M. and Denys, F.R.: Ultrastructural localization of glycosaminoglycans in human term placenta. Anat Rec 210 (1984) 477-84.

Pastinen, T., Kurg, A., Metspalu, A., Peltonen, L. and Syvänen, A.C. Minisequencing: a specific tool for DNA analysis and diagnostics on oligonucleotide arrays. Genome Res 7 (1997) 606-14.

Prosser, I.W., Stenmark, K.R., Suthar, M., Crouch, E.C., Mecham, R.P. and Parks, W.C.: Regional heterogeneity of elastin and collagen gene expression in intralobar arteries in response to hypoxic pulmonary hypertension as demonstrated by in situ hybridization. Am J Pathol 135 (1989) 1073-88.

Pye, D.A., Vives, R.R., Turnbull, J.E., Hyde, P. and Gallagher, J.T.: Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity. J Biol Chem 273 (1998) 22936-42.

Quandt, K., Frech, K., Karas, H., Wingender, E. and Werner, T.: MatInd and MatInspector--New fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucl Acids Res 23 (1995) 4878-84.

Rajendran, V.M., Black, J., Ardito, T.A., Sangan, P., Alper, S.L., Schweinfest, C., Kashgarian, M. and Binder, H.J.: Regulation of DRA and AE1 in rat colon by dietary Na depletion. Am J Physiol Gastrointest Liver Physiol 279 (2000) G931-42.

Rajendran, V.M. and Binder, H.J.: Distribution and regulation of apical Cl/anion exchanges in surface and crypt cells of rat distal colon. Am J Physiol 276 (1999) G132-7.

Rajendran, V.M. and Binder, H.J.: Cl-HCO3 and Cl-OH exchanges mediate Cl uptake in apical membrane vesicles of rat distal colon. Am J Physiol 264 (1993) G874-9.

Ranta, S., Zhang, Y., Ross, B., Lonka, L., Takkunen, E., Messer, A., Sharp, J., Wheeler, R., Kusumi, K., Mole, S., Liu, W., Soares, M.B., Bonaldo, M.F., Hirvasniemi, A., de la Chapelle, A., Gilliam, T.C. and Lehesjoki, A.E.: The neuronal ceroid lipofuscinoses in human EPMR and mnd mutant mice are associated with mutations in CLN8. Nat Genet 23 (1999) 233-6.

Rechkemmer, G., Wahl, M., Kuschinsky, W. and von Engelhardt, W.: pH-microclimate at the luminal surface of the intestinal mucosa of guinea pig and rat. Pflugers Arch 407 (1986) 33-40.

Romppanen, E.L. and Mononen, I.: Detection of the Finnish-type congenital nephrotic syndrome by restriction fragment length polymorphism and dual-color oligonucleotide ligation assays. Clin Chem 46 (2000) 811-6.

Ross, M.H. and Romrell, L.J. Digestive system II: Esophagus and gastrointestinal tract. In Histology: A text and atlas. Eds. Ross, M.H. and Romrell, L.J. Williams & Wilkins (1989) pp. 421-70.

Rossi, A., Bonaventure, J., Delezoide, A.L., Cetta, G. and Superti-Furga, A.: Undersulfation of proteoglycans synthesized by chondrocytes from a patient with achondrogenesis type 1B homozygous

for an L483P substitution in the diastrophic dysplasia sulfate transporter. J Biol Chem 271 (1996) 18456-64.

Roughley, P.J., White, R.J. and Glant, T.T.: The structure and abundance of cartilage proteoglycans during early development of the human fetus. Pediatr Res 22 (1987) 409-13.

Rumessen, J.J. and Gudmand-Hoyer, E.: Functional bowel disease: malabsorption and abdominal distress after ingestion of fructose, sorbitol, and fructose-sorbitol mixtures. Gastroenterology 95 (1988) 694-700.

Sandle, G.I., Higgs, N., Crowe, P., Marsh, M.N., Venkatesan, S. and Peters, T.J.: Cellular basis for defective electrolyte transport in inflamed human colon. Gastroenterology 99 (1990) 97-105.

Sandle, G.I., Wills, N.K., Alles, W. and Binder, H.J.: Electrophysiology of the human colon: evidence of segmental heterogeneity. Gut 27 (1986) 999-1005.

Sato, F. and Sato, K.: cAMP-dependent Cl(-) channel protein (CFTR) and its mRNA are expressed in the secretory portion of human eccrine sweat gland. J Histochem Cytochem 48 (2000) 345-54.

Sato, K., Kang, W.H., Saga, K. and Sato, K.T.: Biology of sweat glands and their disorders. I. Normal sweat gland function. J. Am. Acad. Dermatol. 20 (1989) 537-63.

Satoh, H., Susaki, M., Shukunami, C., Iyama, K., Negoro, T. and Hiraki, Y.: Functional analysis of diastrophic dysplasia sulfate transporter. Its involvement in growth regulation of chondrocytes mediated by sulfated proteoglycans. J Biol Chem 273 (1998) 12307-15.

Saunders, D.R. and Wiggins, H.S.: Conservation of mannitol, lactulose, and raffinose by the human colon. Am J Physiol 241 (1981) G397-402.

Schultheis, P.J., Clarke, L.L., Meneton, P., Harline, M., Boivin, G.P., Stemmermann, G., Duffy, J.J., Doetschman, T., Miller, M.L. and Shull, G.E.: Targeted disruption of the murine Na+/H+ exchanger isoform 2 gene causes reduced viability of gastric parietal cells and loss of net acid secretion. J Clin Invest 101 (1998a) 1243-53.

Schultheis, P.J., Clarke, L.L., Meneton, P., Miller, M.L., Soleimani, M., Gawenis, L.R., Riddle, T.M., Duffy, J.J., Doetschman, T., Wang, T., Giebisch, G., Aronson, P.S., Lorenz, J.N. and Shull, G.E.: Renal and intestinal absorptive defects in mice lacking the NHE3 Na+/H+ exchanger. Nat Genet 19 (1998b) 282-5.

Schweinfest, C.W., Henderson, K.W., Suster, S., Kondoh, N. and Papas, T.S.: Identification of a colon mucosa gene that is down-regulated in colon adenomas and adenocarcinomas. Proc Natl Acad Sci U S A 90 (1993) 4166-70.

Scott, D.A., Wang, R., Kreman, T.M., Andrews, M., McDonald, J.M., Bishop, J.R., Smith, R.J., Karniski, L.P. and Sheffield, V.C.: Functional differences of the PDS gene product are associated with phenotypic variation in patients with pendred syndrome and non- syndromic hearing loss (DFNB4). Hum Mol Genet 9 (2000) 1709-15.

Scott, D.A., Wang, R., Kreman, T.M., Sheffield, V.C. and Karnishki, L.P.: The Pendred syndrome gene encodes a chloride-iodide transport protein. Nat Genet 21 (1999) 440-3.

Serafini, E.P., Kirk, A.P. and Chambers, T.J.: Rate and pattern of epithelial cell proliferation in ulcerative colitis. Gut 22 (1981) 648-52.

Seutter, E., Trijbels, J.M., Sutorius, A.H. and Urselmann, E.J.: The sweat gland as a mucous gland. Dermatologica 141 (1970) 397-408.

Shaltout, A.A., Khuffash, F.A., Hiilal, A.A. and El Ghanem, M.M.: Pattern of protracted diarrhoea among children in Kuwait. Ann Trop Paed 9 (1989) 30-2.

Sheerin, H.E. and Field, M.: Ileal HCO3 secretion: relationship to Na and Cl transport and effect of theophylline. Am J Physiol 228 (1975) 1065-74.

Sheffield, V.C., Beck, J.S., Kwitek, A.E., Sandstrom, D.W. and Stone, E.M.: The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. Genomics 16 (1993) 325-32.

Shumaker, H., Amlal, H., Frizzell, R., Ulrich, C.D., 2nd and Soleimani, M.: CFTR drives Na+-nHCO-3 cotransport in pancreatic duct cells: a basis for defective HCO-3 secretion in CF. Am J Physiol 276 (1999) C16-25.

Silberg, D.G., Wang, W., Moseley, R.H. and Traber, P.G.: The Down regulated in Adenoma (dra) gene encodes an intestine-specific membrane sulfate transport protein. J Biol Chem 270 (1995) 11897-902.

Simchowitz, L. and Roos, A.: Regulation of intracellular pH in human neutrophils. J Gen Physiol 85 (1985) 443-70.

Singh, S.K., Binder, H.J., Boron, W.F. and Geibel, J.P.: Fluid absorption in isolated perfused colonic crypts. J Clin Invest 96 (1995) 2373-9.

Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughes, P., Dodd, C., Connell, C.R., Heiner, C., Kent, S.B. and Hood, L.E.: Fluorescence detection in automated DNA sequence analysis. Nature 321 (1986) 674-9.

Solberg, R., Sistonen, P., Traskelin, A.L., Berube, D., Simard, J., Krajci, P., Jahnsen, T. and de la Chapelle, A.: Mapping of the regulatory subunits RI beta and RII beta of cAMP- dependent protein kinase genes on human chromosome 7. Genomics 14 (1992) 63-9.

Souhami, R.L. and Moxham, J.: Inflammatory bowel disease. In Textbook of Medicine. Eds. Souhami and Moxham. Churchill Livingstone (1994) pp. 591-9.

Stewart, C.P. and Turnberg, L.A.: A microelectrode study of responses to secretagogues by epithelial cells on villus and crypt of rat small intestine. Am J Physiol 257 (1989) G334-43.

Strong, T.V., Boehm, K. and Collins, F.S.: Localization of cystic fibrosis transmembrane conductance regulator mRNA in the human gastrointestinal tract by in situ hybridization. J Clin Invest 93 (1994) 347-54.

Stuenkel, E.L., Machen, T.E. and Williams, J.A.: pH regulatory mechanisms in rat pancreatic ductal cells. Am J Physiol 254 (1988) G925-30.

Sundaram, U., Wisel, S. and Fromkes, J.J.: Unique mechanism of inhibition of Na+-amino acid cotransport during chronic ileal inflammation. Am J Physiol 275 (1998a) G483-9.

Sundaram, U., Wisel, S., Stengelin, S., Kramer, W. and Rajendran, V.: Mechanism of inhibition of Na+-bile acid cotransport during chronic ileal inflammation in rabbits. Am J Physiol 275 (1998b) G1259-65.

Sundaram, U. and West, A.B.: Effect of chronic inflammation on electrolyte transport in rabbit ileal villus and crypt cells. Am J Physiol 272 (1997) G732-41.

Sundaram, U., Wisel, S., Rajendren, V.M. and West, A.B.: Mechanism of inhibition of Na+-glucose cotransport in the chronically inflamed rabbit ileum. Am J Physiol 273 (1997) G913-9.

Sundaram, U.: Mechanism of intestinal absorption. Effect of clonidine on rabbit ileal villus and crypt cells. J Clin Invest 95 (1995) 2187-94.

Sundaram, U., Knickelbein, R.G. and Dobbins, J.W.: Mechanism of intestinal secretion. Effect of serotonin on rabbit ileal crypt and villus cells. J Clin Invest 87 (1991a) 743-6.

Sundaram, U., Knickelbein, R.G. and Dobbins, J.W.: pH regulation in ileum: Na(+)-H+ and Cl(-)-HCO3- exchange in isolated crypt and villus cells. Am J Physiol 260 (1991b) G440-9.

Superti-Furga, A., Neumann, L., Riebel, T., Eich, G., Steinmann, B., Spranger, J. and Kunze, J.: Recessively inherited multiple epiphyseal dysplasia with normal stature, club foot, and double layered patella caused by a DTDST mutation. J Med Genet 36 (1999) 621-4.

Superti-Furga, A., Hastbacka, J., Wilcox, W.R., Cohn, D.H., van der Harten, H.J., Rossi, A., Blau, N., Rimoin, D.L., Steinmann, B., Lander, E.S. and Gitzelmann, R.: Achondrogenesis type IB is caused by mutations in the diastrophic dysplasia sulphate transporter gene. Nat Genet 12 (1996) 100-2.

Svitacheva, N., Hovenberg, H.W. and Davies, J.R.: Biosynthesis of mucins in bovine trachea: identification of the major radiolabelled species. Biochem J 333 (1998) 449-56.

Switzer, R.C., Merril, C.R. and Shifrin, S.: A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal Biochem 98 (1979) 231.

Syvänen, A.C., Sajantila, A. and Lukka, M.: Identification of individuals by analysis of biallelic DNA markers, using PCR and solid-phase minisequencing. Am J Hum Genet 52 (1993) 46-59.

Syvänen, A.C., Aalto-Setälä, K., Harju, L., Kontula, K. and Söderlund, H.: A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. Genomics 8 (1990) 684-92.

Thwaites, D.T., Ford, D., Glanville, M. and Simmons, N.L.: H(+)/solute-induced intracellular acidification leads to selective activation of apical Na(+)/H(+) exchange in human intestinal epithelial cells. J Clin Invest 104 (1999) 629-35.

Tomaszewski, L., Kulesza, E. and Socha, J.: Congenital chloride diarrhoea in Poland. Mater Med Pol 19 (1987) 271-7.

Toyomoto, T., Knutsen, D., Soos, G. and Sato, K.: Na-K-2Cl cotransporters are present and regulated in simian eccrine clear cells. Am. J. Physiol. 273 (1997) R270-7.

Trezise, A.E. and Buchwald, M.: In vivo cell-specific expression of the cystic fibrosis transmembrane conductance regulator. Nature 353 (1991) 434-7.

Turnberg, L.A.: Electrolyte absorption from the colon. Gut 11 (1970) 1049-54.

Underhill, P.A., Jin, L., Lin, A.A., Mehdi, S.Q., Jenkins, T., Vollrath, D., Davis, R.W., Cavalli-Sforza, L.L. and Oefner, P.J.: Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res 7 (1997) 996-1005.

Vaandrager, A.B. and De Jonge, H.R.: A sensitive technique for the determination of anion exchange activities in brush-border membrane vesicles. Evidence for two exchangers with different affinities for HCO3- and SITS in rat intestinal epithelium. Biochim Biophys Acta 939 (1988) 305-14.

Walker, B.A., Scott, C.I., Hall, J.G., Murdoch, J.L. and McKusick, V.A.: Diastrophic dwarfism. Medicine (Baltimore) 51 (1972) 41-59.

Welsh, M.J., Smith, P.L., Fromm, M. and Frizzell, R.A.: Crypts are the site of intestinal fluid and electrolyte secretion. Science 218 (1982) 1219-21.

Wheat, V.J., Shumaker, H., Burnham, C., Shull, G.E., Yankaskas, J.R. and Soleimani, M.: CFTR induces the expression of DRA along with Cl(-)/HCO(3)(-) exchange activity in tracheal epithelial cells. Am J Physiol Cell Physiol 279 (2000) C62-71.

White, J.A., McAlpine, P.J., Antonarakis, S., Cann, H., Eppig, J.T., Frazer, K., Frezal, J., Lancet, D., Nahmias, J., Pearson, P., Peters, J., Scott, A., Scott, H., Spurr, N., Talbot, C.J. and Povey, S.: Guidelines for human gene nomenclature (1997). HUGO Nomenclature Committee. Genomics 45 (1997) 468-71.

Wilcox, J.N.: Fundamental principles of in situ hybridization. J Histochem Cytochem 41 (1993) 1725-33.

Will, P.C., Lebowitz, J.L. and Hopfer, U.: Induction of amiloride-sensitive sodium transport in the rat colon by mineralocorticoids. Am J Physiol 238 (1980) F261-8.

Wilson, C.M.: Staining of proteins on gels: comparison of dyes and procedures. Methods Enzymol 91 (1983) 236-47.

Wormmeester, L., Sanchez de Medina, F., Kokke, F., Tse, C.M., Khurana, S., Bowser, J., Cohen, M.E. and Donowitz, M.: Quantitative contribution of NHE2 and NHE3 to rabbit ileal brush-border Na+/H+ exchange. Am J Physiol 274 (1998) C1261-72.

Wright, E.M.: The intestinal Na+/glucose cotransporter. Annu Rev Physiol 55 (1993) 575-89.

Yang, H., Jiang, W., Furth, E.E., Wen, X., Katz, J.P., Sellon, R.K., Silberg, D.G., Antalis, T.M., Schweinfest, C.W. and Wu, G.D.: Intestinal inflammation reduces expression of DRA, a transporter responsible for congenital chloride diarrhea. Am J Physiol 275 (1998) G1445-53.

Youil, R., Kemper, B. and Cotton, R.G.H.: Screening for mutations by enzyme cleavage using T4 endonuclease VII. Proc Natl Acad Sci USA 92 (1995) 87-91.

Yun, C.H., Tse, C.M., Nath, S.K., Levine, S.A., Brant, S.R. and Donowitz, M.: Mammalian Na+/H+ exchanger gene family: structure and function studies. Am J Physiol 269 (1995) G1-11.

Yun, C.H., Gurubhagavatula, S., Levine, S.A., Montgomery, J.L., Brant, S.R., Cohen, M.E., Cragoe, E.J., Jr., Pouyssegur, J., Tse, C.M. and Donowitz, M.: Glucocorticoid stimulation of ileal Na+ absorptive cell brush border Na+/H+ exchange and association with an increase in message for NHE-3, an epithelial Na+/H+ exchanger isoform. J Biol Chem 268 (1993) 206-11.

Zaidi, A.U., Enomoto, H., Milbrandt, J. and Roth, K.A.: Dual fluorescent in situ hybridization and immunohistochemical detection with tyramide signal amplification. J Histochem Cytochem 48 (2000)1369-75.

Zhang, Y., Doranz, B., Yankaskas, J.R. and Engelhardt, J.F.: Genotypic analysis of respiratory mucous sulfation defects in cystic fibrosis. J Clin Invest 96 (1995) 2997-3004.

Zheng, J., Shen, W., He, D.Z., Long, K.B., Madison, L.D. and Dallos, P.: Prestin is the motor protein of cochlear outer hair cells. Nature 405 (2000) 149-55.