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Cloning, cellular distribution and functional expression of small intestinal epithelium guinea pig ClC-5 chloride channel

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Abstract

We report the cloning of a guinea pig ClC-5 chloride channel (gpClC-5) from distal small intestinal epithelial cells by RT-PCR and RACE. The transcript is shown to be present in duodenum, jejunum and ileum epithelium by RT-PCR and Northern analysis. This is confirmed by in situ hybridisation which also shows the transcript to be homogeneously distributed in the crypt and villus regions. Expression of gpClC-5 in HEK-293 cells generated markedly outwardly rectified chloride currents with a perm-selectivity sequence of $NO_3^- > Cl^- > Br^- > I^- > F^- >$ gluconate$^-$. The possible role of gpClC-5 in this epithelial location is discussed.

Keywords: Small intestine; Epithelial transport; Chloride channel; Guinea pig cloride channel protein-5; Patch clamp

The most numerous family of chloride channel proteins discovered so far is termed ClC and consists of nine different mammalian members. Sequence identity between different ClC proteins varies between 30 and 90% but they share the characteristic of being voltage-gated and some selectivity properties [1]. The importance of the ClC Cl^- channel family is highlighted by the association of certain human inheritable diseases with mutation of several of its members. ClC-1 mutations are responsible for myotonia whilst altered ClC-Kb and ClC-5 account for two renal diseases, Bartter syndrome type III and Dent's disease [2–5].

The absence of the gene or the occurrence of inactivating mutations in ClC-5, a chloride channel mainly expressed in the kidney, are responsible for Dent's disease, a syndrome characterised by urinary loss of low molecular weight protein, calcium and phosphate and nephrolithiasis [3,6,7]. ClC-5 has been demonstrated to be located in early acidic endosomes of the proximal tubule cells where it is thought to fulfil a role in the endocytic salvage of low molecular weight proteins from the glomerular filtrate. A mechanism to explain the proteinuria has been hypothesised as follows: the loss of function of ClC-5 prevents the dissipation of the positive membrane potential that would otherwise be generated during acidification of the endosomes by the vacuolar proton ATPase. The impaired acidification of this intracellular compartment that would take place in the absence of a parallel chloride conductance, as when ClC-5 function is defective, could in turn affect

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endocytic processes, thus causing defective proximal tubular reabsorption of proteins [3,8,9]. This model gains strong support from the recent detailed functional study of a ClC-5 knockout mouse [10]. The mechanisms for hypercalciuria, however, are less well understood. One possibility is that all the alterations in calcium homeostasis are secondary to a defective apical endocytosis of parathyroid hormone (PTH) and 25-hydroxyvitamin D_3 ($25(OH)D_3$), as proposed from work on the ClC-5 knockout model [10]. On the other hand it has been suggested that the hypercalciuria could be attributable to intestinal hyperabsorption of calcium secondary to elevated $1,25(OH)_2D_3$ levels, as has been demonstrated in Dent's disease patients [11]. This raises the intriguing possibility that the role of ClC-5 might be to regulate the production of $1,25(OH)_2D_3$ in the renal proximal tubule. Alternatively, if ClC-5 were also expressed in the intestinal epithelium, it may directly be involved in the expression of calcium transport proteins responsible for transepithelial absorption across the intestinal mucosa [12].

Four types of mechanism have been recognised for the intestinal uptake of peptides and proteins. Three of these involve endocytosis: receptor-mediated, adsorption-mediated and fluid phase endocytosis [13]. On the other hand, intestinal calcium absorption has been proposed to occur by facilitated diffusion or by vesicular transport. In this last model calcium enters the cell, either through a calcium channel or by endocytosis, and is localised into lysosomes and then extruded from the cells by exocytosis [14]. Therefore, both protein and calcium intestinal transport would involve endocytosis. By analogy with the proximal tubule, we speculate that ClC-5 in the intestine could be related to these endocytic mediated transport processes.

In this work we describe the cloning of ClC-5 from the epithelium guinea pig duodenum (GenBank accession No. AF326968), the distribution of ClC-5 transcript along the small intestine epithelia and its functional expression in HEK-293 cells.

Guinea pig intestinal epithelium was obtained from ketamine-anaesthetised animals that were then sacrificed by excess anaesthetic according to institutional bioethical guidelines. Total RNA was prepared from mixed crypts and villi isolated from the ileum as described [15]. cDNA was prepared by SuperScript system (Gibco BRL kit), using random and oligo(dT) primers in the presence of RNase inhibitors (RNasin, Promega). PCR was done using a sense primer designed from a previously identified fragment of gpClC-5 (GenBank accession No. AF133215). The sequence of this specific primer, termed here P1, is 5′-CATTCATTCTGTTGGGCATATTTGG-3′, and corresponds to a location within segment D7. The antisense (P2) primer, 5′-ATATCCACTAC(T/A/G)AT(C/G)TCCAT-3′, overlaps partially with D13 and was derived from conserved amino acid sequences. The obtained amplicon was subcloned in the pGEM-T vector (Promega) and sequenced, giving a 1144 bp fragment. RACE (Marathon cDNA Amplification kit, Clontech) was performed to obtain the 5′- and 3′-ends of the sequence. Finally, to obtain the totality of the coding region, cDNA was prepared from duodenum epithelia and amplified using primers designed from the untranslated 5′- and 3′-regions (sense, 5′-GAGGAATAGCTTCTTCAAATAGG-3′ and antisense, 5′-GTTTTATATCCAGAAGAATGGGG-3′). The obtained amplicon was subcloned into the pGEM-T vector and sequenced giving the nucleotide sequence in Fig. 1. The continuous cDNA obtained contains a 2238 bp open reading frame. The start codon is located at bp number 25 and is within a Kozak consensus sequence for translation initiation [16]. The predicted amino acid sequence for gpClC-5 is also given in Fig. 1. This yields a 746 amino acid protein with an estimated molecular mass of 83 kDa. The putative transmembrane domains have been assigned after a Kyte–Doolittle analysis and analogy with previously reported assignation [8]. There are two potential *N*-glycosylation sites but only one of them, located between D8 and D9, faces the extracellular milieu. There are two potential protein kinase A-mediated phosphorylation sites, located between D7 and D8. Eight potential protein kinase C phosphorylation sites are present but only four of them, one located in the N-terminus and three in the C-terminus, are predicted to be intracellular. The amino acid sequence of gpClC-5 was found to be 96% identical to rat (GenBank accession No. Z56277) and human (GenBank accession No. NM 000084) ClC-5. The degree of identity with the amino acid sequence of gpClC-3 (GenBank accession No. AF133214) was found to be 75% whilst the equivalent figure for

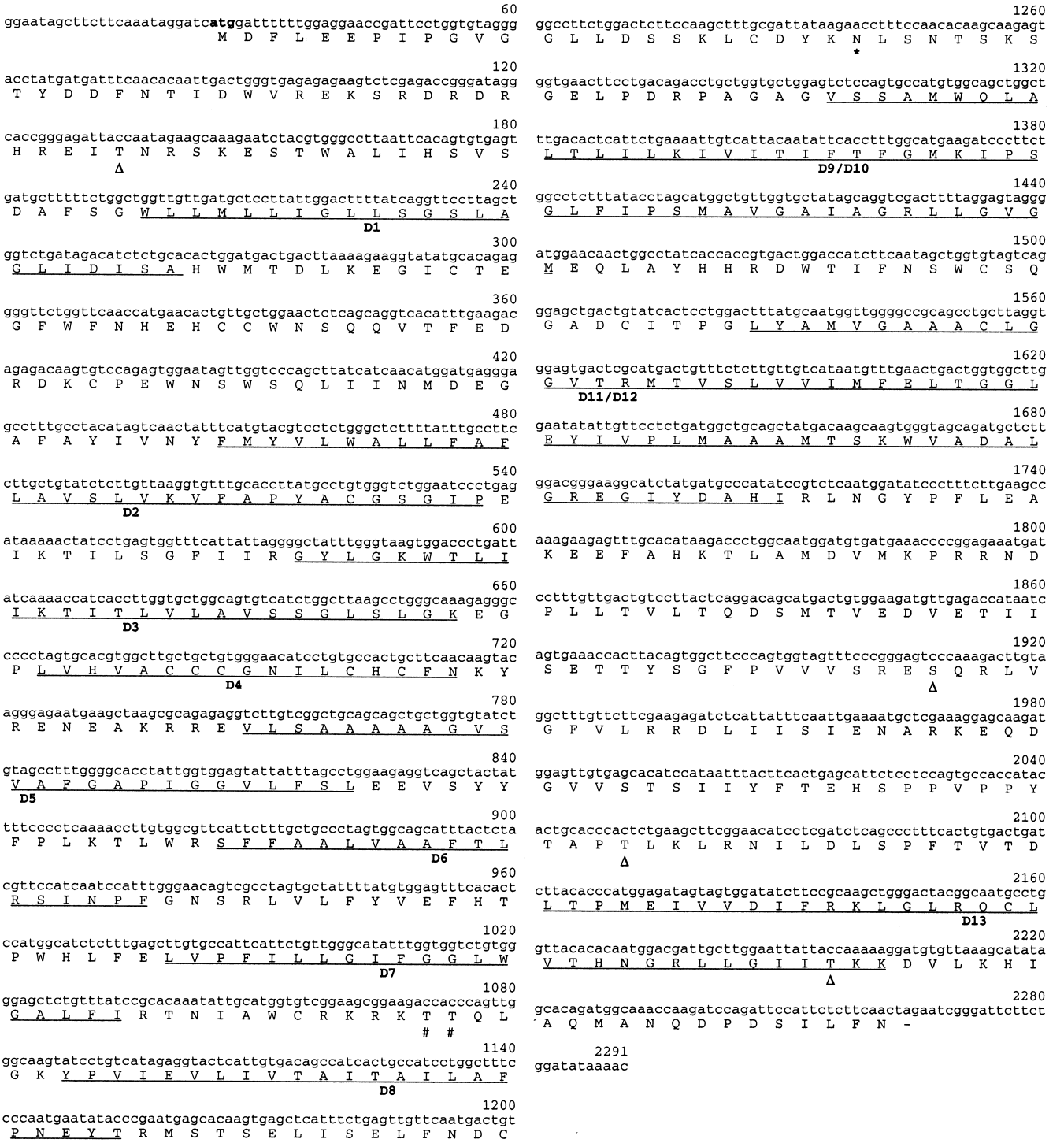

Fig. 1. Nucleotide and deduced amino acid sequence of gpClC-5. Putative transmembrane-spanning domains are underlined (*D1–D12*). *, potential *N*-glycosylation site; Δ, potential PKC-phosphorylation sites; #, potential PKA-phosphorylation sites.

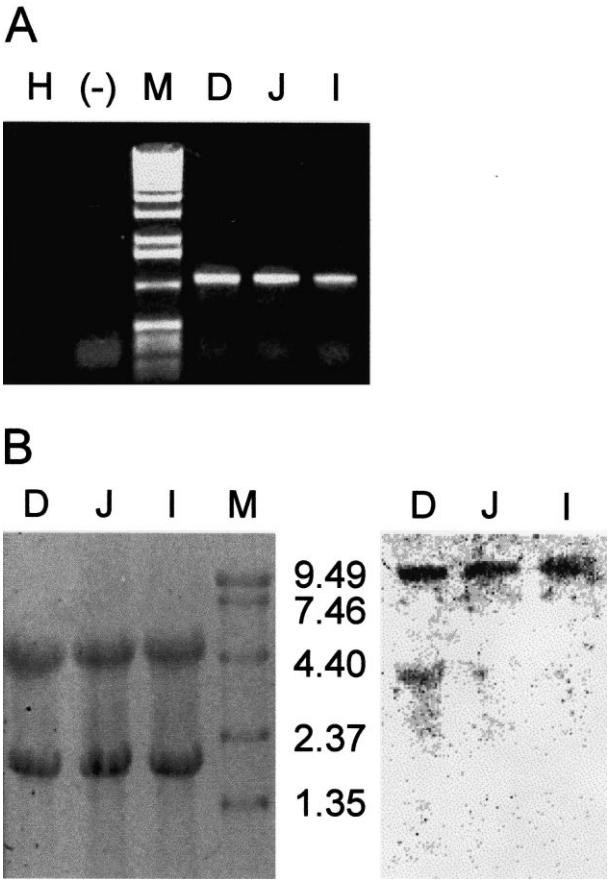

Fig. 2. Expression of gpClC-5 transcript along the small intestine. (A) Agarose gel electrophoresis of PCR products amplified with P1 (sense) and P2 (antisense) primers using epithelial cDNA prepared from guinea pig duodenum (D), jejunum (J) or ileum (I). H corresponds to amplification without DNA template and (−) corresponds to a reaction without reverse transcriptase. A lane with 1 kb molecular weight markers (M) is also shown. (B) On the left an agarose gel electrophoresis stained with ethidium bromide of 30 μg of total RNA of guinea pig duodenum (D), jejunum (J) or ileum (I). M is an RNA molecular marker ladder for which the numbers (kb) on the right apply. On the right a Northern blot hybridised with the gpClC-5 digoxigenin-labelled probe is shown.

rClC-4 (GenBank accession No. Z36944) was 78%. The degree of identity to the amino acid sequence of other mammalian ClC members falls to around 30%.

The distribution of gpClC-5 along the small intestine was studied by RT-PCR and Northern analysis. As shown in Fig. 2A, single amplicons were obtained using primers P1 and P2 (see above) with cDNA obtained from duodenum, jejunum or ileum suggesting the presence of transcript in all three small intestinal regions. The size of the PCR products was consistent with the expected size (1144 bp) and no amplification was observed in the controls without template (H) or reverse transcriptase (−).

Further confirmation of the presence of gpClC-5 in epithelium from all segments of the small bowel was obtained by high stringency Northern analysis using a 662 bp digoxigenin-labelled antisense riboprobe. Sense and antisense riboprobes were synthesised by in vitro transcription with T7 and SP6 RNA polymerase using as a template a gpClC-5 cDNA that covers nucleotides 986–1647, corresponding to amino acids P321 to A541. Given the high degree of homology between members of the branch of the ClC family represented by ClC-3, -4 and -5, control experiments were conducted to ascertain the specificity of the gpClC-5 riboprobe used. This was done by high stringency Southern analysis in which the cDNA corresponding to the full coding region of gpClC-3 (2286 bp), hClC-4 (2331 bp) and hClC-5 (2309 bp) did not hybridise with the antisense riboprobe (not shown). The integrity of the RNA used in the Northern analysis is shown in the left-hand side of Fig. 2B together with the molecular mass markers. On the right-hand side a blot obtained with the digoxigenin-labelled riboprobe reveals main bands of around 9.5 kb in size in duodenum, jejunum and ileum. A second band of approx. 4 kb is seen only in duodenum; we do not have an interpretation for this, but it is unlikely to correspond to detection of

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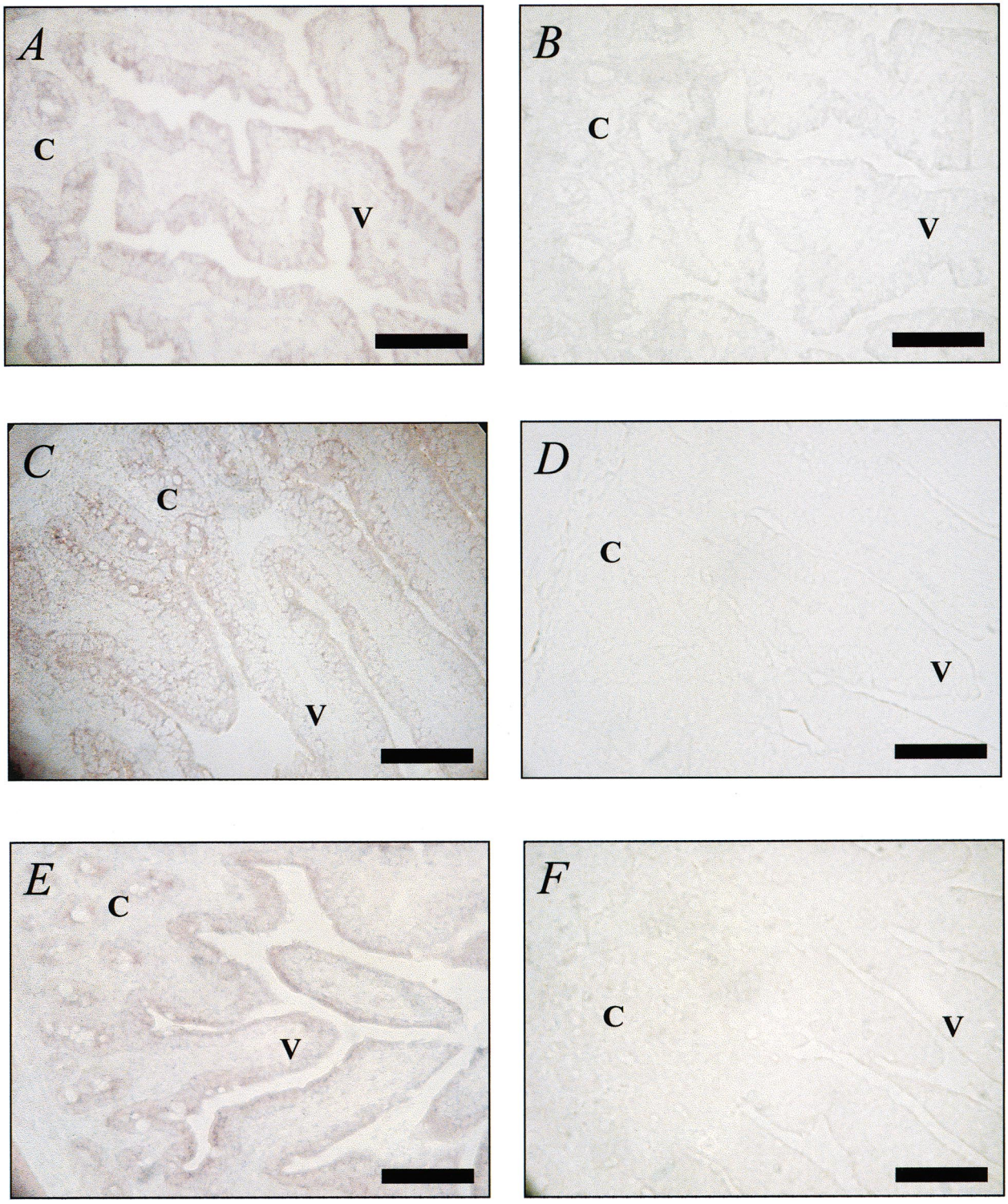
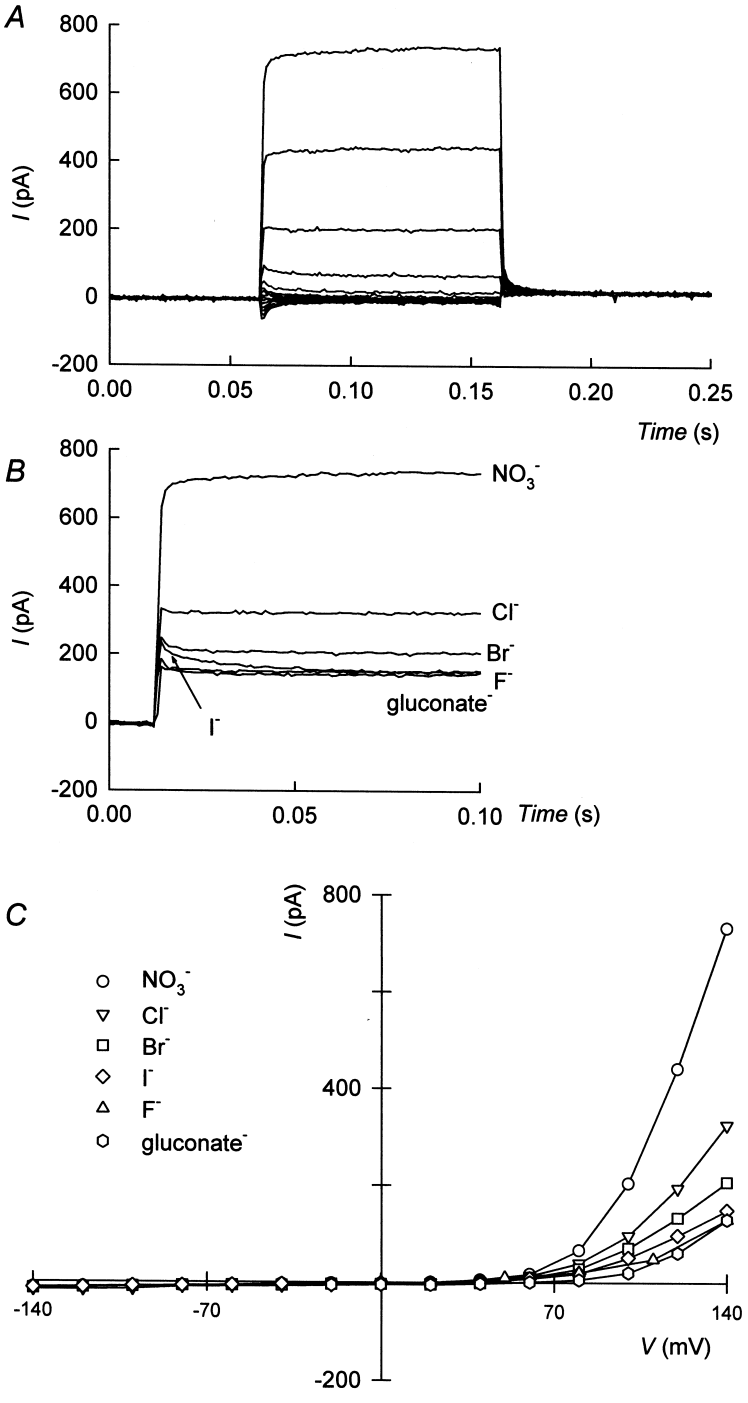
Fig. 3. Localisation of gpClC-5 transcript in small intestinal epithelium by in situ hybridisation. 7 μm thick cryosections were probed with digoxigenin-labelled antisense and sense riboprobes. The antisense probe was made with SP6 RNA polymerase from a *Nco*I-linearised plasmid DNA, and the sense probe with T7 RNA polymerase from a 662 nt *Mlu*I DNA fragment. Hybridisation was at 65°C for 16 h with either riboprobe at 10 ng/μl in a 50% formamide solution. Anti-digoxigenin-alkaline phosphatase conjugate (Boehringer, Mannheim, Germany) treatment was followed by histochemical reaction using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate to reveal gpClC-5 localisation. A and B, C and D, and E and F correspond to antisense and sense reactions for duodenum, jejunum and ileum respectively. Crypt and villus regions (c and v) are identified. Calibration bar is 100 μm.



ClC-3 or ClC-4 transcripts which run to 3 and 5.5 kb and 5 and 7.5 kb respectively [17,18].

The location of ClC-5 transcript small intestinal epithelium was determined by in situ hybridisation with digoxigenin-labelled riboprobes [19,20]. Fig. 3A,B shows duodenum cryosections hybridising respectively antisense and sense gpClC-5 riboprobes. Prominent staining with the antisense probe was



seen in both crypt and villus cells, whilst there was minimal staining of non-epithelial tissue. No background staining was observed in control sections hybridised with the sense probe. Similar results were obtained with jejunum and ileum cryosections. These results are illustrated in Fig. 3C,D for jejunum and Fig. 3E,F for ileum. A recent publication reports a similar distribution for rat ClC-5 protein [21].

In order to evaluate the function of the gpClC-5 the complete coding region was subcloned into the pCR3.1 vector (Invitrogen) and transfected into HEK-293 cells. Transfection induced the appearance

Fig. 4. Functional assay of gpClC-5 transfected into HEK-293 cells. HEK 293 cells at 60–80% confluence were co-transfected with 1.5 μg total expression plasmid for gpClC-5 and CD8 antigen (ratio 3:1) using Lipofectamine Plus (Life Technologies) as described [15]. After 24–48 h the cells were incubated briefly with Dynabead microspheres precoated with antibody to CD8. The experiments were performed in bead decorated cells at room temperature. Standard whole-cell patch-clamp recordings were performed as described elsewhere [24]. Patch-clamp pipettes were made from thin borosilicate (hard) glass capillary tubing with an outside diameter of 1.5 or 1.7 mm (Clark Electromedical, Reading, UK) using a David Kopf (USA) model 700C micropipette puller. The pipettes had a resistance of 3–5 MΩ. Voltage and current signals from the amplifier were recorded on a digital tape recorder (DTR-1204, Biologic, France) and digitised using a computer equipped with a Digidata 1200 (Axon Instruments, USA) AD/DA interface. The voltage pulse generator and analysis programmes were from Axon Instruments. Changes in liquid junction potentials which occurred as a result of bath solution changes during an experiment, were calculated [25] and current–voltage relations corrected accordingly. In A, a current family in response to square pulses from −140 to 140 mV in 20 mV steps is shown. The intracellular solution contained, in mM, 140 *N*-methyl-D-glucamine Cl, 2 $MgCl_2$, 2 EGTA, 10 HEPES, pH 7.4 with Tris, osmolality 273 mOsm. The bath solution was 130 $NaN0_3$, 10 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 sucrose, 10 HEPES, pH 7.4 with Tris, osmolality 288 mOsm. (B) Current responses to a 120 mV square pulse in different anions. The bath solutions were as in A, but $NaNO_3$ was replaced by equimolar Na salts of the indicated anion. (C) Current–voltage relations taken at apparent steady state for the different anions.
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of a conductance not present in either untransfected or mock-transfected cells. As shown in Fig. 4A, the current was characterised by an extremely marked outward rectification, with very small currents at negative potentials and with sizeable current only at potentials more depolarised than 60 mV. Small current was present, however, at all potentials tested and tail current analysis (not shown) suggests that a large proportion of the rectification is due to open channel rectification but with some activation at depolarised voltages. Fig. 3B shows currents elicited by depolarisation to 140 mV in normal, chloride-containing medium or when all but 16 mM chloride had been replaced by a foreign anion. Large current was observed in NO_3^- and Cl^--rich medium, whilst it was low in Br^-. Lowest currents were observed in I^-, F^- and gluconate$^-$. A current–voltage relation is shown in Fig. 3C confirming the dependence upon voltage for the different anions tested. The cord conductance was calculated at 140 mV and gave a sequence in the order $NO_3^- > Cl^- > Br^- > I^-$, F^-, gluconate$^-$. The perm-selectivity sequence, measured from the displacement in reversal potential as described previously [22], from two gpClC-5 transfected cells with extremely low leak current, was (P_X/P_{Cl} values given in parentheses, where X is the foreign anion) NO_3^- (3.2) > I^- (1.8) > Br^- (1.6) > Cl^- (1) > F^- (0.5) > gluconate$^-$ (0.2). The perm-selectivity sequence corresponds to the so-called sequence I of Eisenmann, suggesting that the pore contains weak binding sites. The discrepancies with the conductance sequence might originate from a stronger binding of I^- and Br^- making them remain longer within the channel thus reducing their mobility [23].

In conclusion, we have demonstrated the presence of ClC-5 RNA expression in villus and crypt epithelial cells from guinea pig duodenum, jejunum and ileum. The functional expression of the cloned cDNA gave currents with extremely marked outward rectification with conductance properties similar to those of rClC-5 [8]. The results of the study of perm-selectivity reported here are novel and suggest that gpClC-5 behaves unlike other ClC channels in selecting for Br^- and I^- over Cl^-. Although outside the scope of the present paper, it is tempting to speculate that in this channel the reduced conductance of these foreign anions with respect to Cl^- might be accounted for by stronger interaction with binding site(s) within the pore. The function of a chloride channel such as ClC-5 in intestinal epithelial cells is a matter of speculation. It could fulfil a role in endocytosis-mediated transport processes that could include absorption of calcium or proteins.

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