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Intestinal folate transport: identification of a cDNA involved in folate transport and the functional expression and distribution of its mRNA

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Abstract

Although the mechanism of folate intestinal transport has been the subject of intensive studies, very little is known about the molecular identity of the transport system(s) involved. In this investigation, we screened a mouse intestinal cDNA library using as probe the cDNA clone of a reduced folate carrier (*RFC1*) of mouse leukemia L1210 cells, and identified a positive clone, *IFC1(RFC1)*. The cloned cDNA consisted of 2274 base pairs with an open reading frame that encodes a putative polypeptide of 512 amino acids with a predicted molecular mass of 58 112 daltons and 12 putative transmembrane domains. The polypeptide appears to carry a net positive charge (p*I* = 8.6) which may be important for its interaction with the negatively charged substrate. Functional identity of the *IFC1(RFC1)* clone was established by expression in *Xenopus* oocytes. An 11-fold increase in 5-methyltetrahydrofolate (5-MTHF) uptake was observed in oocytes injected with 10 ng *IFC1(RFC1)* cRNA compared to water-injected controls. The expressed folate uptake in the cRNA injected oocyte was: (1) 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)-sensitive; and (2) saturable with an apparent K_m of 1.99 ± 0.32 μM, and a V_{max} of 3782 ± 188 fmol/oocyte per h. The distribution of mRNA species complementary to *IFC1(RFC1)* in different mouse tissues was examined by Northern blot analysis. In addition to the small intestine, expression of such mRNA species were also found in the kidney, large intestine, brain, heart and liver. Furthermore, mRNA species complementary to *IFC1(RFC1)* were also detected by Northern blot analysis in the small intestine of human and other animal species (rat and rabbit). Expression of mRNA complementary to *IFC1(RFC1)* was markedly higher in rat intestinal villus cells than in crypt cells. These results represent the first identification of a folate transporter in mammalian intestine.

Keywords: Intestinal folate transport; Folate transport; cDNA library; DNA sequencing; (*Xenopus* oocyte)

1. Introduction

The coenzyme derivatives of folic acid play a central role in one-carbon metabolism, and are necessary for the synthesis of purine and pyrimidine precursors of nucleic acids, for the metabolism of certain amino acids, and for the initiation of protein synthesis in mitochondria [1–4]. Folate deficiency is the most prevalent vitamin deficiency in the Western hemisphere, with a significant number of cases resulting from impairment of intestinal absorption of the vitamin [5,6]. Malabsorption of folate is common in various diseases of the small intestine [5,7–9] and has been associated with the use of certain pharmacological agents (e.g., sulfasalazine, phenytoin) [9–12]. Malabsorption of folate is also caused by congenital defects in the intestinal absorption process of the vitamin [13–18]. Folate deficiency leads to a variety of clinical abnormalities including megaloblastic anemia, growth retardation and derangement of one-carbon metabolism [1,4,5].

Mammals cannot synthesize folate but instead must obtain it from exogenous sources through intestinal absorption. Dietary folates exist mainly in polyglutamate forms which are enzymatically hydrolyzed by folate conjugase. The resulting monoglutamate forms are then absorbed by the enterocyte predominantly in the proximal small intes-

Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; 5-MTHF, (6*S*)-5-methyltetrahydrofolic acid.

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tine. The mechanism of folate absorption in the small intestine has been the subject of intense investigation, with specialized carrier-mediated transport systems having been identified at both the brush-border membrane (BBM) and the basolateral membrane (BLM) domains of the absorptive cells. Despite the large volume of published works on intestinal folate absorption (which is believed to exceed the number of published work on intestinal absorption of all other vitamins; Refs. [6,19]), nothing is known about the molecular identity of the intestinal folate transporter(s) and their cellular regulation. This information is necessary for a thorough understanding both of the folate absorption process under normal physiological conditions and of the causes of derangement that occur in the absorption process under certain pathophysiological conditions.

In the present study, we used a cDNA probe (*RFC1*) of a recently cloned reduced folate carrier of mouse leukemia L1210 cells [20] to screen a mouse intestinal cDNA library and have identified and sequenced a positive clone, *IFC1(RFC1)*, that may be involved in normal folate intestinal absorption.

2. Materials and methods

[3′,5,7,9-^{3}H]-(6*S*)-5-Methyltetrahydrofolic acid (^{3}H-5-MTHF) (i.e., the L-form; spec. act. 21.7 Ci/mmol; radiochemical purity 98%) was the generous gift of Dr. Barton Kamen, Univ. Texas, Southwestern Medical Center. The intestinal epithelial cell line IEC-6 was obtained from American Tissue Type Culture (Rockville, MD). All other chemicals, reagents and kits were obtained from commercial sources.

2.1. Cloning of an intestinal folate carrier

A directional cDNA library was constructed using a modified lambda vector (ZAP Express, Stratagene, La Jolla, CA) and doubly selected poly(A)$^+$ RNA isolated by the method of Chirgwin et al. [21] from an adult female DBA2 mouse jejunum (Jackson Laboratories, Bar Harbor, ME) by cesium chloride density gradient centrifugation [22]. Three hundred thousand clones were screened at high stringency (0.2 × SSC, 0.1% SDS, 55°C) using randomly labeled *Bam*HI-*Kpn*I 2.3 kb fragment from plasmid pRFC1 [20]. After four rounds of plaque purification, twelve positive clones were identified. Plasmids containing the corresponding cDNA were obtained by in vivo excision according to ZAP Express instructions. Nine of these contained overlapping inserts as determined by restriction mapping and terminal sequencing. Four of those nine contained identical 2.3 kb inserts which corresponded with the size of the hybridizing mRNA species of mouse small intestine on Northern blot (Fig. 4). One of the four plasmids was selected for further study and was named pIFC1 (intestinal folate carrier). The remaining three had different inserts and were saved for future analysis.

2.2. cDNA sequence determination and analysis

The cDNA insert of pIFC1 was sequenced on both strands in its entirety by Sanger's method of dideoxy chain termination [23] using the Sequenase kit version 2.0 (United States Biochemical, Cleveland, OH) following instructions provided by the manufacturer. The nucleotide sequence was analyzed using computer programs DNA Strider 1.2 and MacPattern 3.2 with database Prosite release 12 [24,25] to obtain the deduced amino acid sequence of the major open reading from (ORF) and potential regulatory sites of the putative polypeptide.

2.3. Preparation of IFC1(RFC1) cRNA

cRNA was prepared from pIFC1 linearized with *Xho*I by in vitro transcription using T3 mMessage mMachine (Ambion, Texas). The resulting cRNA was then phenol extracted, ethanol precipitated, resuspended in water and micro-injected into oocytes as described in Ref. [22].

2.4. Expression studies in Xenopus oocytes

Oocytes were obtained from healthy *Xenopus laevis* purchased from Xenopus I (Ann Arbor, MI). Stage V–VI oocytes were defolliculated and micro-injected with 50 nl in vitro transcribed cRNA (50 ng) or with water (control). Oocytes were incubated at 18°C for 3 to 8 days and assayed for folate uptake by incubating 5–8 oocytes in 200 μl of Ringer solution (in mM: 120 NaCl, 2 KCl, 1.8 $CaCl_2$, and 20 Mes-Tris; pH 5.5–8) containing 0.25 μM of labeled and different concentrations of unlabeled 5-MTHF and/or other compounds at room temperature. Incubation was terminated by the addition of 5 ml of ice-cold Ringer solution followed by four successive washes with the same buffer. Cells were then transferred individually to scintillation vials, dissolved in 250 μl of 10% sodium dodecyl sulfate; scintillation fluid was added and radioactivity was counted.

As control for oocyte quality, 10 ng of in vitro transcribed *SGLT-1* cRNA (the plasmid containing the intestinal sodium-dependent glucose transporter *SGLT-1* cDNA was a generous gift from Dr. M.A. Hediger, Harvard Medical School, Boston, MA) was micro-injected into *Xenopus* oocytes, which were incubated for 5 days and assayed for methyl α-D-[U-^{14}C]glucopyranoside (3.5 μM) uptake. Generally, there was approximately a 65-fold increase in uptake activity over water-injected controls (2400 ± 400 vs. 40 ± 10 fmol/oocyte per h, respectively). Oocytes from donors demonstrating a good expression of SGLT-1 were used for studies of *IFC1(RFC1)* expression.

The metabolic form(s) of the radioactivity taken up by

cRNA-injected oocytes following 1 h incubation with ^{3}H-5-MTHF was determined by thin-layer chromatography [26]. After incubation, oocytes were washed, then homogenized in 70% ethanol containing 5% β-mercaptoethanol. The supernatant was then applied onto a non-ionic cellulose pre-coated thin-layer chromatography plate and chromatographed with 0.1 M phosphate buffer, 5% β-mercaptoethanol, pH 7.0.

2.5. Northern analysis

Total RNA and poly(A)$^+$ RNA were prepared from different mouse tissues and from the small intestine of human and different animal species as described [22]. Samples (5 μg) of mRNA containing ethidium bromide were electrophoresed through RNA-denaturing gels, capillary blotted onto Magna 0.22 μm nylon membranes (Micron Separations, Westport, MA) [27], and UV-cross-linked. Hybridization was done using randomly labeled *Eco*RI-*Xho*I 2.3 kb fragment from plasmid pIFC1 [20]. After washing at high stringency (0.2 × SSC, 0.1% SDS, 55°C, 30 min), the blots were autoradiographed for 1 to 10 days at −80°C with intensifying screens. The hybridization signal was normalized to cyclophilin expression by scanning densitometry (LKB, Bromma, Switzerland).

2.6. Cell culture and uptake studies

The rat-derived intestinal epithelial cell line IEC-6 was used between passage 16 and 20. Cell culturing, maintenance and use in uptake studies were performed as described in Ref. [28]. Protein contents of cell digests were measured in parallel wells by the method of Lowry et al. [29]. Uptake data presented in this paper are means ± S.E. of multiple separate monolayers performed on two separate occasions and are expressed in pmol/mg protein per unit time.

2.7. Isolation of rat villus and crypt intestinal epithelial cells

Villus and crypt intestinal cells were isolated as described in Ref. [30]. Fractions 1 and 6, containing mostly villus tip and crypt cells, respectively, were saved for further study. Na^+-dependent D-glucose uptake and alkaline phosphatase activity were assayed as markers of villus cells. Na^+-dependent D-glucose uptake was 23-fold higher in fraction 1 (villus cells) than in fraction 6 (crypt cells) (7.98 ± 1.60 vs. 0.35 ± 0.09 pmol/mg protein per 15 min, respectively). Also, alkaline phosphatase activity was enriched 4.8-fold in fraction 1 over fraction 6 (0.451 ± 0.05 vs. 0.094 ± 0.01 μmol/mg protein per min, respectively). These results are in good agreement with published findings [30,31]. Thymidine incorporation, an index of thymidine kinase activity, itself a marker of crypt cells, was also determined as described in Refs. [31–33]. Isolated cells were incubated in oxygenated buffer A containing 40 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) at 37°C for 2 h and then centrifuged at 500 × *g* for 5 min. Pellets were washed twice with 0.9% NaCl, and the trichloroacetic acid (TCA)-precipitable radioactivity was determined as described. Thymidine kinase activity, was 0.3 ± 0.03 fmol/mg TCA-precipitable material per 2 h in fraction 1 (villus), and 2.0 ± 0.18 fmol/mg TCA-precipitable material per 2 h in fraction 6 (crypt), indicating a successful separation of villus from crypt cells. Poly(A)$^+$ RNA was isolated from the villus tip and crypt cells and used in Northern blot analysis to determine the relative distribution of mRNA complementary to *IFC1(RFC1)*.

3. Results

3.1. Sequence analysis of IFC1(RFC1)

The cDNA insert of pIFC1 clone was sequenced on both strands in its entirety and found to consist of 2274 nucleotide pairs, including a tail of 19 adenylate residues (GenBank accession no. L36539) (Fig. 1). The cDNA insert was divided into three regions: (1) nucleotides 1–96 comprise the 5′-untranslated region (5′-UTR); (2) nucleotides 97–1635 comprise the major open reading frame (ORF); and (3) nucleotides 1636–2274 comprise the 3′-untranslated region (3′-UTR). The major ORF has an initiation site that surrounds the initiator methionine (5′-GGCAA CATGG-3′) matching 8 out of 10 nucleotides of the Kozak consensus sequence (5′-GCCRCCATGG-3′; R = purine) [34]. The ORF encoded a putative polypeptide of 512 amino acids, with a predicted molecular mass of 58 112 daltons. Examination of the predicted amino acid sequence of the ORF revealed two potential *N*-glycosylation sites at Asn-211 and Asn-277; one cAMP- and/or cGMP-dependent protein kinase phosphorylation site at Thr-58; five protein kinase C phosphorylation sites at Ser-24, Thr-236, Thr-245, Ser-324 and Ser-412; three casein kinase II phosphorylation sites at Thr-245, Ser-467 and Ser-477; four N-myristoylation sites at Gly-161, Gly-218, Gly-303 and Gly-447; and a microbodies C-terminal targeting signal at Ala-510. IFC1 was predicted to contain 37 acidic amino acid residues and 55 basic amino residues (p*I* = 8.6) that gave an average of 11.3 positive charges at neutral pH. Using the Kyte and Doolittle algorithm [35] with a window of 11 amino acids, twelve potential membrane spanning segments were predicted at residues 27–42, 71–88, 93–115, 122–142, 156–178, 181–201, 272–292, 307–328, 333–352, 362–384, 388–411, and 429–448. In the 3′-UTR a match of 5 out of 6 (5′-ATTAAA-3′) to a polyadenylation signal sequence (5′-AATAAA-3′) at nucleotides 2238–2243 was detected.

Since plasmid pIFC1 was obtained through hybridization screening using as probe the mouse leukemia L1210 reduced folate carrier *RFC1* cDNA insert of plasmid

```
IFC1(RFC1)    1                                                        GGCA
IFC1(RFC1)    5 CGAGGCAATCTGCTGTGAGCTGAGACCCGGTCAAGCGCTGGACGGGTGGA

IFC1(RFC1)   55 GTGTCATCTTGGCCCGCCAGTTCCCATGAGGACCTGGGCAACATGGTGCC
                ||||||||||||||||||||||||||||| ||||||||||||||||||||
      RFC1    1 GTGTCATCTTGGCCCGCCAGTTCCCATGA-GACCTGGGCAACATGGTGCC
                                                          M  V  P
                  ... (homology continues) ...

IFC1(RFC1) 2005 GTGTCGGGCTCAACTATGCCCACCAGGCCATTCTGCCTTTTTTGCTCTGG
                ||||||||||||||||||
      RFC1 1950 GTGTCGGGCTCAACTATGCC 1969

IFC1(RFC1) 2055 GAAGCTCTGGGCAAATCATGTCTCGCCCTTAGGGCTAGTGAGGTAGCCCA
IFC1(RFC1) 2105 GATGCCAGCCAAGGTCCGCTGAGCCTGCAGGGTGCCAGTTGCCTGCCCTA
IFC1(RFC1) 2155 GGCTCCTGGCTATGCTGCCATACATCCTTCCACTATCGGAGGTGCTTGTT
IFC1(RFC1) 2205 TAAATGGTTTTCTGATTTCTTTTTTAACTTGACATTAAAATGGAGAACTA
IFC1(RFC1) 2255 CAAAAAAAAAAAAAAAAAAA 2274
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Fig. 1. Comparison between *IFC1(RFC1)* and *RFC1* nucleotide sequences. The complete *IFC1(RFC1)* nucleotide sequence without the 3′-terminal poly(A)$^+$ has been submitted to GenBank (accession No. L36539). Shown here are *IFC1(RFC1)* sequences that represent additional information at the 5′- and 3′-termini in comparison to the published *RFC1* sequence [20]. There is complete homology between *IFC1(RFC1)* nucleotides 55–2024 and *RFC1* nucleotides 1–1969, except for *IFC1(RFC1)* nucleotide 84, which represents an insertion of a G/C base pair. Underlined nucleotides around the methionine initiation codon of the *IFC1(RFC1)* open reading frame (ORF) denote matching nucleotides with the Kozak consensus sequence (5′-GCCRCCATGG-3′, R = purine). Underlined nucleotides near the 3′-end of the *IFC1(RFC1)* sequence denote matching nucleotides with the polyadenylation signal sequence (5′-AATAAA-3′).

pRFC1 [20], we are interested in comparing the nucleotide sequence of the cDNA insert of plasmid pIFC1 to that of the published *RFC1* sequence. The comparison shows that nucleotides 55–2024 of the cDNA insert in plasmid pIFC1 are identical to the published 1969 nucleotides of *RFC1*, with the exception of nucleotide 84 of the cDNA insert in plasmid pIFC1 which represents an G/C insertion in the *RFC1* sequence. In other words, the cDNA insert in plasmid pIFC1 contains 54 additional nucleotides at the 5′-end, an additional G/C base pair at position 84 and 250 additional nucleotides at the 3′-end, in comparison to the published *RFC1* sequence. It should be noted that the major ORF of the cDNA insert in plasmid pIFC1 is identical to that of the *RFC1* sequence. Thus the designation *IFC1(RFC1)* will be used to refer to the cDNA insert of plasmid pIFC1 to denote the fact that the cDNA insert of plasmid pRFC1 is contained within the cDNA insert of plasmid pIFC1 and the designation *RFC1* will be used to refer to the cDNA insert of plasmid pRFC1.

3.2. Functional expression of IFC1(RFC1) in Xenopus oocytes

In order to establish the functional identity of the cloned intestinal folate carrier *IFC1(RFC1)*, we injected in vitro transcribed *IFC1(RFC1)* cRNA into oocytes. Control oocytes were injected with an equal amount of water. Oocytes injected with 10 ng of *IFC1(RFC1)* cRNA showed approximately a 11-fold increase in uptake of ^{3}H-5-MTHF (0.25 μM) after three days of incubation, compared to water-injected controls (73.0 ± 5.7 vs. 6.7 ± 0.6 fmol/oocyte per h, respectively). By eight days after injection the expression of the folate transport system was approximately 16-fold greater than water-injected controls (110 ± 6.4 vs. 6.9 ± 0.6 fmol/oocyte per h). In preliminary studies, we found the uptake of 5-MTHF by *IFC1(RFC1)* cRNA-injected oocytes to be linear with time for 1 h of incubation ($Y = 1.41X + 48.5$; $r = 0.99$). Furthermore, the majority (94.4%) of the ^{3}H radioactivity taken up by oocytes following incubation with ^{3}H-MTHF (1.25 μM) was found to be in the form of intact 5-MTHF. Furthermore, transport of 5-MTHF (0.25 μM) by the expressed carrier was completely inhibited by the addition to the incubation medium of 1 mM DIDS, an inhibitor of anionic transport systems and of folate transport in native intestinal preparations [36,37] (4.9 ± 0.1 vs. 110 ± 7.2 fmol/oocyte per h in cRNA-injected cells incubated with and without DIDS, respectively) (the K_i for DIDS inhibition was determined in a separate study and found to be

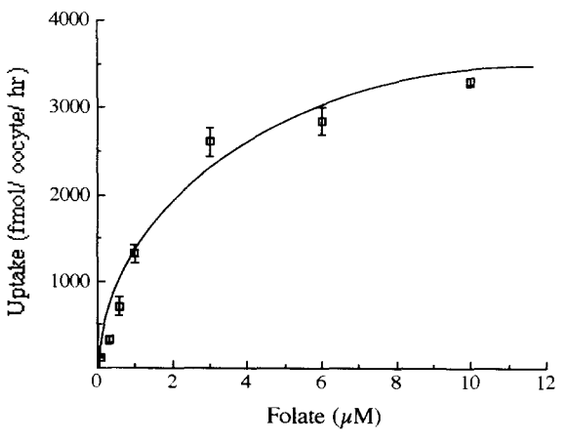

Fig. 2. Uptake of folate by *Xenopus* oocytes injected with *IFC1(RFC1)* cRNA as a function of concentration. cRNA- and water-injected oocytes were incubated at room temperature for 1 h in normal Ringer buffer containing different concentrations of labeled and unlabeled 5-MTHF. Results are means ± S.E. of 5–10 separate determinations. Kinetic parameters of the uptake process were determined as described in Section 2.

Table 1
Substrate specificity of *IFC1(RFC1)* expressed in *Xenopus* oocytes

Condition	Uptake (fmol/oocyte per h)	*P* value [a]
Control (water-injected)	5.0 ± 0.8 (8) [b]	
Control (*IFC1(RFC1)* cRNA injected)	90 ± 0.6 (8)	
5-MTHF (100 μM)	1.1 ± 0.4 (5)	< 0.001
Folinic acid (100 μM)	5.5 ± 1.0 (5)	< 0.001
Folic acid (100 μM)	35.8 ± 3.2 (8)	< 0.001

Oocytes were incubated at room temperature for 1 h in Ringer buffer in the absence (control) or presence of the compound under study and ^{3}H 5-MTHF (0.25 μM).
[a] *P* values were calculated using the Student's *t*-test; comparisons were made relative to simultaneously performed control experiments.
[b] Number of oocytes.

160 μM). In the same experiment, DIDS produced a slight inhibition of 5-MTHF uptake in water-injected controls (4.4 ± 0.2 vs. 8.5 ± 0.3 fmol/oocyte per h in oocytes incubated with and without DIDS, respectively). In another study, we examined the uptake of 5-MTHF as a function of concentration (0.1 to 10 μM) by the induced folate carrier following injection of *IFC1(RFC1)* cRNA (10 ng) into oocytes. Uptake by the induced carrier was determined by subtracting the amount of 5-MTHF uptake at each concentration in water-injected oocytes from uptake by cRNA-injected cells. The results (Fig. 2) showed a clear saturation in 5-MTHF uptake with concentration. Kinetic parameters of the induced uptake carrier were then calculated using a computerized model of the Michaelis–Menten equation as described by Wilkinson [38], and found to be 1.99 ± 0.32 μM and 3782 ± 188 fmol/oocyte per h for the apparent K_m and V_{max}, respectively. We also examined the effect of unlabeled 5-MTHF and that of the structural analogs folinic acid and folic acid on the uptake of ^{3}H-5-MTHF (0.25 μM). As shown in Table 1, the uptake of ^{3}H-5-MTHF by IFC1/RFC1 expressed in *Xenopus* oocytes was significantly inhibited by both reduced and oxidized folates. The inhibition constants (K_i) were determined in a separate study and found to be 1.87 and 28 μM for folinic acid and folic acid, respectively. In a separate study, we examined the effect of buffer pH on the uptake of 5-MTHF (0.05 μM) by the expressed folate carrier in cRNA-injected oocytes. The results showed a minor effect of pH on 5-MTHF uptake (22.8 ± 0.96, 24.4 ± 2.8 and 22.8 ± 0.9 fmol/oocyte per h at buffer pH of 5.5, 6.5, and 8.0, respectively).

3.3. Expression of mRNA species complementary to IFC1(RFC1) in different mouse tissues, in the small intestine of human and other animal species, and in different cultured intestinal epithelial cell lines

In this study, we investigated the distribution of mRNA complementary to *IFC1(RFC1)* in different mouse tissues by Northern blot analysis. The results showed that at high stringency, predominant hybridization occurred between a randomly labeled *IFC1(RFC1)* cDNA probe and a 2.3 kb mRNA species in mouse kidney, small intestine, large intestine, brain, heart and liver (Fig. 3). The degree of hybridization was highest in the kidney and lowest in the liver (data was normalized relative to the constitutively expressed gene cyclophilin).

In another study, we examined by Northern blot analysis using a randomly labeled *IFC1(RFC1)* cDNA probe, the distribution of mRNA species complementary to *IFC1(RFC1)* in the small intestine of human and other animal species. Mouse small intestinal poly(A)$^+$ RNA was also included as a positive control. As expected, the lane containing poly(A)$^+$ RNA of mouse intestine showed strong hybridization with a 2.3 kb mRNA species (Fig. 4). The lane containing poly(A)$^+$ RNA of rat intestine showed hybridization of similar intensity but with a slightly smaller size mRNA species than 2.3 kb. On the other hand, the lane containing poly(A)$^+$ RNA of the human small intestine showed hybridization of a lesser intensity with a 3.0

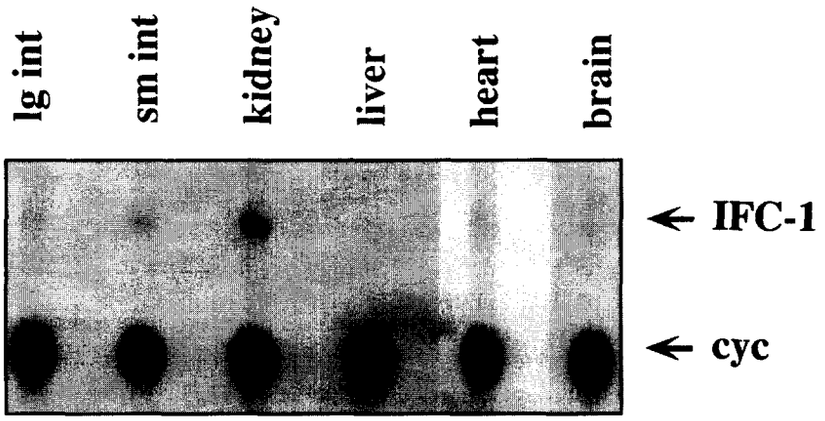

Fig. 3. Northern blot analysis for the presence of *IFC1(RFC1)* mRNA in various mouse tissues. Poly(A)$^+$ RNA (5 μg), isolated from the indicated organs (see Section 2) was electrophoresed on a 1% agarose/20% formaldehyde RNA-denaturing gel. RNA was then blotted onto a nylon membrane by capillary transfer and UV-crosslinked. Hybridization and autoradiography were done as described in Section 2.

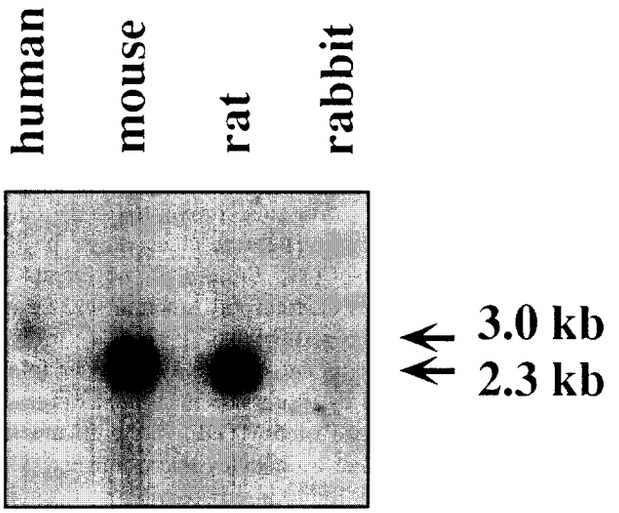

Fig. 4. Northern blot analysis for the distribution of mRNA complementary to *IFC1(RFC1)* in the intestine of different animal species. Poly(A)$^+$ RNA (5 μg) was isolated from the jejunum of different animal species and blotted and hybridized as described in Section 2 and Fig. 3.

kb mRNA species, and the lane containing poly(A)$^+$ RNA of rabbit intestine showed an even less intense hybridization with 2.1 kb mRNA species.

We also tested by Northern blot analysis whether an intestinal epithelial cell line in culture also possesses mRNA species that are complementary to *IFC1(RFC1)* cDNA. For this we used the rat-derived non-transformed IEC-6 cells. Mouse intestinal poly(A)$^+$ RNA was again included as a positive control. The results showed clear

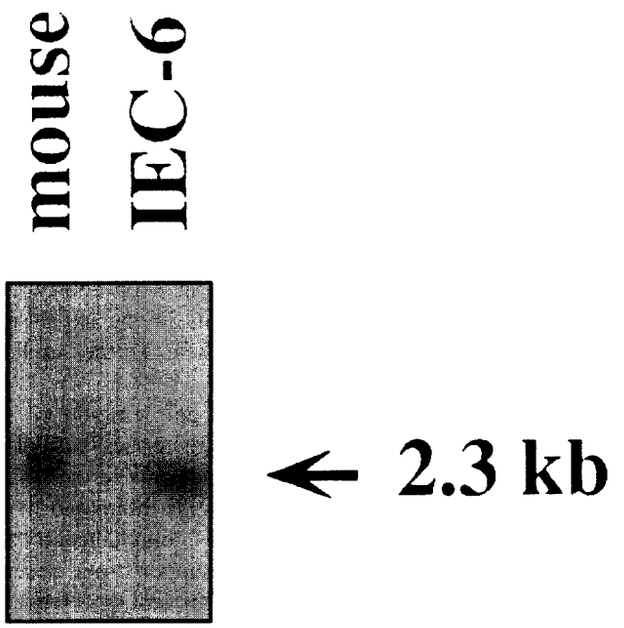

Fig. 5. Northern blot analysis for the distribution of mRNA complementary to *IFC1(RFC1)* in the intestinal cell line IEC-6. Poly(A)$^+$ RNA (5 μg) was isolated from mouse jejunum and from confluent cultures of IEC-6 cells, and blotted and hybridized as described in Section 2 and Fig. 3.

Table 2
Effect of incubation buffer pH, structural analogs and DIDS on the uptake of 5-MTHF by the rat-derived intestinal epithelial cell line IEC-6

Condition	5-MTHF uptake (pmol/mg protein per 3 min)	*P* value [a]
(A) Effect of pH		
pH 5.0	20.93 ± 0.51 (3) [b]	
pH 5.5	16.38 ± 0.24 (3)	
pH 6.0	13.30 ± 0.43 (3)	
pH 6.5	8.85 ± 1.13 (3)	
pH 7.5	7.16 ± 1.11 (3)	
pH 8.0	5.09 ± 1.43 (3)	
(B) Effect of structural analogs and DIDS [c]		
Control	12.78 ± 0.40 (6)	
Unlabeled 5-MTHF (25 μM)	3.43 ± 0.86 (6)	< 0.01
Methotrexate (25 μM)	3.03 ± 0.52 (5)	< 0.01
Folic acid (25 μM)	3.11 ± 0.54 (5)	< 0.01
DIDS (1 mM)	4.51 ± 0.66 (6)	< 0.01

Confluent IEC-6 cells were incubated in Krebs–Ringer buffer at 37°C. Incubation was performed for 3 min in the presence of 0.1 μM ^{3}H-5-MTHF. Data are means ± S.E. Notice the pH-dependence in 5-MTHF uptake, and the significant inhibition in ^{3}H-5-MTHF uptake by structural analogs and DIDS.

[a] *P* values were calculated using the Student's *t*-test; comparisons were made relative to simultaneously performed control experiments.

[b] Number of separate experiments.

[c] pH of the incubation buffer was 6.0.

hybridization of a randomly labeled *IFC1(RFC1)* cRNA with a 2.3 kb mRNA species in the mouse lane, and with a 2.2 kb mRNA species in the IEC-6 lane (Fig. 5). Since no study exists to show whether IEC-6 cells have a folate uptake system, and whether such system is similar to that of the native small intestine, we performed a number of 5-MTHF uptake experiments to address these issues. The results showed that 5-MTHF (0.1 μM) uptake to be DIDS-sensitive, pH-dependent and is inhibited by the structural analogs folic acid and methotrexate (Table 2). The inhibition constants for DIDS, folic acid, and methotrexate were determined in a separate study and found to be 97.9, 2.0, and 1.36 μM, respectively. These results demonstrate the existence of a carrier-mediated system for 5-MTHF uptake in these cells which is very similar to that of the native intestine [36,37,39].

3.4. Expression of mRNA complementary to IFC1(RFC1) in villus and crypt intestinal epithelial cells

In this study we examined the relative abundance of mRNA complementary to *IFC1(RFC1)* in mature absorptive cells of the villus tip and immature cells of the crypt. Rat tissue was used as a source of cells in these studies because the intestine of this rodent species was found to contain a mRNA species that is complementary to *IFC1(RFC1)* (Fig. 4), and because of practical reasons related to obtaining enough cells for isolation of poly(A)$^+$ RNA and for confirmation of cell origin. To quantitate the

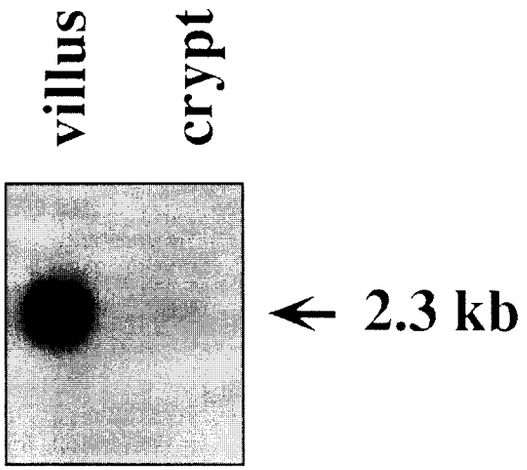

Fig. 6. Northern blot analysis for the distribution of *IFC1(RFC1)* mRNA in crypt and villus cells. Poly(A)$^+$ RNA (5 μg) was isolated from fractionated rat villus and crypt intestinal epithelial cells and blotted and hybridized as described in Section 2 and Fig. 3.

level of expression of mRNA complementary to *IFC1(RFC1)*, data was normalized relative to the constitutively expressed gene cyclophilin using a scanning densitometer (LKB; Bromma, Switzerland). The results (Fig. 6) showed an approximately 10.5-fold higher expression of *IFC1(RFC1)* mRNA in epithelial cells of the villus tip than in crypt cells.

4. Discussion

To begin addressing the issues about the molecular identity and the cellular regulation of the intestinal folate transporters, we set out to isolate a cDNA clone of the mouse intestinal folate transporter, using a cDNA probe of the recently cloned reduced folate carrier (*RFC1*) of mouse leukemia L1210 cells [20]. We constructed a cDNA library from doubly selected poly(A)$^+$ RNA isolated from the proximal part of the mouse small intestine (i.e., the jejunum), which is the preferential site of intestinal absorption of dietary folate. We identified a positive clone, *IFC1(RFC1)*, which was virtually identical to *RFC1* [20] except that *IFC1(RFC1)* contains, among other differences, 55 additional nucleotides at the 5′-end (Fig. 1). Because the 5′-region of the *IFC1(RFC1)* cDNA has not yet been completely mapped, further study will be needed to establish the regulatory significance of this sequence.

The predicted *IFC1(RFC1)* polypeptide has a molecular mass of 58 112 daltons, which is close to that of the intestinal folate transporter (56 000 daltons) identified by Reizenhuer by affinity labeling [40]. The predicted *IFC1(RFC1)* polypeptide (pI = 8.6) carries a net positive charge of 11.3 at physiological pH, which may be important in the interaction of the carrier protein with its negatively charged ligand (pK_a of folic acid are 3.5 and 4.8 for the α- and γ-carboxyl groups, respectively). Previous studies in our laboratory with group specific reagents have identified histidine residues at or near the substrate binding site of the intestinal folate carrier as functionally important [41]. It is interesting therefore to find out from the predicted amino acid sequence that there are eight such histidine residues that are predicted to be either in the extra-membrane domain or in close proximity to the outer surfaces of the membrane. These residues might be the ones identified previously [41]. Studies of the effects of site-directed-mutagenesis on transport function will be required to directly test this possibility.

The functional involvement of *IFC1(RFC1)* in folate transport was confirmed by expression in *Xenopus* oocytes and measuring the uptake of 5-MTHF. A significant increase in 5-MTHF uptake by oocytes micro-injected with *IFC1(RFC1)* cRNA was found in comparison to water-injected oocytes. This increase cannot be attributed to an increase in 5-MTHF metabolism in the cRNA-injected cells because 94.4% of the ^{3}H radioactivity taken up by these cells following incubation with ^{3}H-5-MTHF was found to be in the form of intact 5-MTHF. Furthermore, the anion transport inhibitor DIDS, which is membrane impermeable, completely inhibited the uptake of 5-MTHF by the induced carrier in cRNA-injected oocytes. The folate uptake system expressed in *Xenopus* oocytes following micro-injection with *IFC1(RFC1)* cRNA was also found to be saturable as a function of 5-MTHF concentration, with an apparent K_m of 1.99 $\pm$ 0.32 μM and a V_{max} of 3782 $\pm$ 188 fmol/oocyte per h. In addition, structural analogs of 5-MTHF significantly inhibited the uptake of ^{3}H-5-MTHF by the induced carrier. It is notable that the properties of the induced folate carrier in cRNA-injected oocytes with regard to DIDS sensitivity and apparent K_m are very similar to those reported for folate transport in a variety of intestinal tissue preparations [36,37,39]. It is however different with regard to the pH profile. Uptake of folate in the native intestine is pH-dependent and increases with decreasing buffer pH. In contrast, the pH profile of the expressed folate carrier in *Xenopus* oocytes showed only minor response to buffer pH. Whether this effect is an indication for the existence of a tissue- (or cell-type)-specific post-translational modifications in the expressed folate carrier protein is not clear at present. Further studies are needed to address this issue.

The distribution of mRNA complementary to *IFC1(RFC1)* cDNA in different mouse tissues was also investigated. Such mRNA was found not only in the small intestine but also in kidney, large intestine, brain and liver tissue. The finding of a mRNA species complementary to *IFC1(RFC1)* in the large intestine supports our recent demonstration of the existence of an efficient, specialized, DIDS-sensitive carrier-mediated system for folate transport in purified apical colonic membrane vesicles [42]. This system may be responsible for the absorption of folate produced by the normal microflora of the large intestine [43,44]. mRNA species complementary to *IFC1(RFC1)*

cDNA was also found in mature confluent monolayers of the rat-derived cultured intestinal epithelial cell line IEC-6. It is interesting that these homogenous cells were also found to possess a specialized carrier-mediated system for folate uptake which is similar to that of native rat intestine. These findings may suggest a role for *IFC1(RFC1)* in folate uptake (see below for further discussion) and also suggest the suitability of these cells as an in vitro model system to study cellular and molecular regulation of intestinal folate transport process.

mRNA species complementary to mouse intestinal *IFC1(RFC1)* were also observed by Northern blot analysis in the small intestine of human, rat and rabbit although the size of the message and the intensity of hybridization varied among those species. This suggests that despite some similarities, differences exist among the intestinal folate transporters of different animal species. Indeed, in a recent study, we screened a human small intestinal cDNA library (using a randomly labeled *IFC1(RFC1)* probe), identified and partially sequenced a clone that induces folate transport (by expression in *Xenopus* oocytes), and that shows 70% homology to *IFC1(RFC1)* (unpublished observation). Furthermore, *IFC1(RFC1)* also appears to be different from the recently cloned cDNAs of human placenta and human lymphoblastic cell line HSC93 which appear also to be involved in folate transport [45,46].

We also performed studies to identify relative distribution of mRNA species complementary to *IFC1(RFC1)* in mature absorptive cells of the villus tip and the immature cells of the crypt. The results showed a 10-fold higher abundance of mRNA complementary to *IFC1(RFC1)* in cells of the villus tip than in crypt cells. This finding further suggests a role for *IFC1(RFC1)* in the process of intestinal folate absorption. Further studies, however, are clearly required to test this possibility.

In summary, the present study has identified a cDNA cloned from mouse small intestine that encodes a protein that is capable of transporting folate across cell membrane. This is the first demonstration of cloning of a folate transporter from mammalian intestine.

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