

# Gene chip analyses reveal differential genetic responses to iron deficiency in rat duodenum and jejunum

JAMES F COLLINS

Departments of Pediatrics and Nutritional Sciences, Steele Children's Research Center, University of Arizona, Tucson, Arizona, USA

## ABSTRACT

Previous studies revealed novel genetic changes in the duodenal mucosa of iron-deprived rats during post-natal development. These observations are now extended to compare the genetic response to iron deficiency in the duodenum versus jejunum of 12-wk-old rats. cRNA samples were prepared from the duodenal and jejunal mucosa of three groups each of control and iron-deficient rats and hybridized with RAE 230A and 230B gene chips (Affymetrix). Stringent data reduction strategies were employed. Results showed that several genes were similarly induced in both gut segments, including DMT1, Dcytb, transferrin receptor 1, heme oxygenase 1, metallothionein, the Menkes copper ATPase (ATP7A), tripartite motif protein 27, and the sodium-dependent vitamin C transporter. However, a subset of genes showed regulation in only one or the other gut segment. In duodenum only, gastrokine 1, trefoil factor 1 and claudin 2 were induced by iron-deficiency. Other genes previously identified were only regulated in the duodenum. Overall, these studies demonstrate similarities *and* distinct differences in the genetic response to iron deprivation in the duodenum versus jejunum and provide evidence that more distal gut segments also may play a role in increasing iron absorption in iron-deficiency anemia.

**Key terms:** ATP7A, copper transport, iron-deficiency anemia, microarray

## INTRODUCTION

Iron is a critical element for many metabolic processes, including its participation as a cofactor for cytochromes and enzymes that transfer electrons (3). However, excess iron stores can lead to oxidative damage, as free iron readily participates in redox reactions within cells. Thus, body iron levels must be tightly controlled. Body iron stores normally are regulated at the level of absorption in the proximal small intestine (5), with the greatest overall absorption rates and adaptive responses to iron deficiency being observed in the duodenum (27). Several physiological effectors are known to modulate dietary iron absorption in mammals (13), including hepatic stores, erythroid, hypoxia, and inflammatory

mediators, which act directly on the intestinal epithelium to control iron absorption and, thus, body iron levels. Hepcidin, a recently identified antimicrobial peptide, is currently recognized as the hormone responsible for regulating intestinal iron absorption. Hepcidin is synthesized in the liver and secreted into the circulation, and has been shown to decrease intestinal iron transport in mice (17). It has also been demonstrated recently that hepcidin expression is decreased in physiological situations where iron absorption is increased (e.g., hypoxia, iron deficiency, and anemia), whereas hepcidin production is upregulated under conditions in which intestinal iron transport is decreased (e.g., iron-overload such as occurs in hemochromatosis and inflammation) (21-23, 26). Additionally,

hepcidin gene expression is related to body iron stores in normal humans (11) and iron feeding of rats results in a rapid increase in hepcidin levels, which decreases iron absorption (10).

Several proteins involved in duodenal iron transport recently have been identified. Duodenal cytochrome b (Dcytb) is a brush-border membrane ferric reductase that reduces dietary ferric iron to ferrous iron (19), which can then be transported into epithelial cells by the divalent metal transporter 1 [DMT1; also called DCT1 (12) and nRAMP2 (24)]. Within enterocytes, iron is either complexed with ferritin or is trafficked to the basolateral membrane for export into the circulation by the coordinated action of the basolateral iron transport protein Iron Regulated Gene 1 [IREG1 (20); also called MTP1 (1) and ferroportin (7)] and hephaestin, which oxidizes iron for binding to transferrin and distribution throughout the body (25). However, a complete understanding of the molecular events associated with intestinal iron absorption has not yet been achieved. For example, microcytic anemia mice (9) and Belgrade rats (8) are able to absorb substantial amounts of dietary iron, despite the lack of normal DMT1 protein. Furthermore, sex-linked anemia (*sla*) mice, which have a deletion in the hephaestin gene, have substantial accumulation of iron within enterocytes and moderate-to-severe hypochromic, microcytic anemia (25). These mice, nevertheless, have the ability to absorb some dietary iron despite the possible mistargeting and subsequent degradation of the hephaestin protein (16).

We previously performed extensive gene chip analyses to characterize the genetic response to iron-deprivation in the duodenum of rats throughout post-natal development (6). These observations are now extended to compare the genetic response to iron-deficiency in the duodenum versus jejunum of 12-wk-old rats. Current results demonstrate that several genes were similarly induced in both gut segments, including known iron-responsive genes and novel genes previously identified. However, a subset of genes showed regulation in only one or the

other gut segment. Overall, these studies demonstrate similarities *and* distinct differences in the genetic response to iron deprivation in the duodenum versus jejunum, and provide evidence that more distal gut segments may also play a role in increasing iron absorption in conditions of iron deficiency.

## MATERIALS AND METHODS

### *Experimental animals*

Sprague Dawley rats were obtained from Harlan (Madison, WI) and were housed in the University of Arizona Animal Care facility. Modified AIN-93G rodent diets were obtained from Dyets Inc. (Bethlehem, PA), which contained either 198 ppm Fe (DYET# 115135; control diet with the same Fe content as standard rat chow) or 3 ppm Fe (DYET# 115102; called Low Fe diet). The diets were identical except for the addition of pure ferric citrate to the control diet. Tap water was tested initially for iron content and did not show any detectable iron. Rats were supplied with food and tap water *ad libitum*. To produce 12-week-old, iron-deficient rats, three-week-old weanling rats were placed on control or low Fe diets for 9 weeks and then sacrificed. For all studies, only male rats were used, and groups of 4-5 animals were considered as one group (n=1). The experiments were repeated three times with samples derived from different groups of control or iron-deficient rats. Rats were anesthetized by CO<sub>2</sub> narcosis, and blood was obtained by cardiac puncture. Blood was sent to the University of Arizona Animal Care Pathology Services laboratory for CBC with differential analysis of blood samples. Rats were then sacrificed by cervical dislocation and the first ~15 cm of the gut was removed just distal to the pyloric sphincter and designated duodenum. The remaining proximal one half of the small bowel was removed and designated jejunum. The jejunal segments were ~3 times longer than the duodenal segments. The intestinal segments were flushed with PBS, opened lengthwise, and light mucosal

scrapes were taken. Approximately equal amounts of mucosal tissue were mixed in the same tube from all the rats in that group, with each individual sample being immediately frozen in liquid nitrogen. Snap-frozen mucosal scrapings were stored at -80° C until use. The University of Arizona Institutional Animal Care and Use Committee approved all animal procedures.

#### *RNA purification*

RNA was purified from mucosal tissue with Trizol reagent (Invitrogen) as previously described (6, 14). Total RNA (100 µg) was further purified utilizing the RNeasy Mini Kit (Qiagen) according to the manufacturer's suggested protocol. The RNA was eluted at the final step twice with the same 30 µl of RNase-free water, quantified by UV spectrophotometry, and visualized by denaturing agarose gel electrophoresis. RNA concentrations were then adjusted by densitometry of the gel. Only high-quality RNA, as judged by intactness of the ribosomal bands, was utilized for gene chip analyses.

#### *Preparation of samples for gene chip analyses*

cRNA was produced from duodenal mucosa RNA samples essentially according to the manufacturer's instructions (Affymetrix; Expression Analysis Technical Manual). Experimental repetitions done in triplicate were performed at the same time with cRNA samples derived from different groups of control or iron-deficient, experimental rats. RNA was purified from all six groups from each gut segment (3 control and 3 iron-deficient) simultaneously followed by cRNA production, and then 1 µl of each cRNA sample was analyzed by denaturing, agarose gel electrophoresis. Subsequently, densitometry of the gel was performed, and the most concentrated cRNA sample was quantified by UV spectrophotometry. Then, the relative concentration of all other cRNA samples from that age group was calculated according to optical density of the most concentrated sample. Only cRNA samples

that showed a smear of material from high to low molecular weight (e.g., significantly above and below the ribosomal RNA bands) were utilized for gene chip analyses. By these procedures, we ensured that equal amounts of high-quality cRNA were hybridized with the gene chips.

#### *Gene chip data analysis*

cRNA was fractionated and hybridization cocktails were prepared, and then rat genome RAE230A and RAE230B chips were hybridized with 10 µg of cRNA as previously described (6). Hyb cocktails were hybridized to only one chip and were then discarded. Chips were immediately washed and stained with the GeneChip Fluidics Station 400 (Affymetrix), utilizing the EukGE-WS2v4 fluidics protocol. After chips had been washed and stained, they were scanned twice with the Agilent Gene Array Scanner (Affymetrix). Resulting data were analyzed with high stringency parameters as previously described (6), with 9 total comparisons being performed between each control and iron-deficient group.

Final microarray data are presented in tables that show the following: 1) previously described differentially expressed genes (6) that increased or decreased in both gut segments (Tables I-IV); 2) unique genes that were induced or repressed in only one gut segment (Table V and VII); and 3) unique genes that were increased or decreased in both gut segments (Table VI). Shown are gene name, gene symbol, GenBank accession # for the Affymetrix target sequence, and biological function and any known aliases. If the gene symbol is not known, "???" was placed in the table at that position. If the gene name is listed as "similar" to a gene, this is the name assigned by Affymetrix for that individual probe set. For some genes, the cDNA has not been cloned from rats, and if this is the case, percent homology to known mouse or human cDNA clones is shown. Further, in some cases, homology was only found to mouse or human chromosomal regions, so 10-20 kb of these regions were searched against DNA sequence databases

to see what gene(s) was present in this region. If a single known gene was present there, we listed these genes as "on the same chromosomal region." Other tables show gene name, gene symbol, GenBank accession #, average fold increase from the 9 comparisons, and the average expression levels from the 3 control and the 3 low Fe groups for each gut segment. Gene chip data have been deposited in the GEO repository under accession #GSE1892 (duodenum) and #GSE2269 (jejunum) (<http://www.ncbi.nlm.nih.gov/geo>).

## RESULTS

### *Rat health status*

Blood analysis of the rats revealed that iron-deprived rats at 12 weeks of age showed signs of hypochromic, microcytic anemia (6). These signs included decreased red blood cell counts, hemoglobin levels, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin. Further, red blood cell distribution width was increased significantly in iron-deficient rats at 12 weeks of age. Rats were weighed before sacrifice, and data were averaged from each dietary group. Iron-deprived, 12-week-old rats were not different in weight from controls, despite being highly iron deficient (6).

### *Gene chip control parameters*

The key quality control parameters for the gene chip experiments presented in this manuscript are background, raw Q, scale factor,  $\beta$ -actin 3'/5' ratio, GAPDH 3'/5' ratio, and percent present calls (Affymetrix). Data from the RAE230A and RAE230B gene chips, which were hybridized with the 12-week-old duodenal samples, were reported previously (6), and all were within acceptable ranges. Data from three 230A and three 230B chips from the jejunum for either control or low Fe diets were averaged, and the means  $\pm$  standard deviation were determined. Average backgrounds were all below the threshold of 100, and average raw Q values were all below the 3.5 threshold (with the exception of two low-iron groups

that were hybridized with RAE 230A gene chips; however, all other control parameters were within acceptable ranges for these samples). Scale factor was less than two-fold different between data sets that were compared to one another, and this parameter also was within the manufacturer's suggested guidelines. And finally,  $\beta$ -actin and GAPDH 3'/5' ratios, were significantly below the 3.0 threshold (with the exception of one low-iron sample that was hybridized with a RAE 230B gene chip; however, all other quality-control parameters were within acceptable ranges for this sample). Furthermore, present/absent calls were very similar between experimental repetitions. Thus, all data obtained from these experiments were considered valid.

### *Gene chip data*

Genes expressed differentially in control versus iron-deficient rats were examined and were classified as either increased or decreased. We found that some genes increased or decreased in both gut segments and also that some other genes increased or decreased in one or the other gut segment. The following cutoffs were used to prepare tables for this report: 1) for genes previously shown to be regulated at 4 or 5 different post-natal ages from sucklings to adults (6), a 1.5-fold cutoff was implemented; 2) for novel genes that were regulated in both duodenum and jejunum, a 2.0-fold cutoff was used; and 3) for novel genes that were regulated in only one gut segment, a 3.0-fold cutoff was used. Table I shows genes that were reported previously to be induced at several post-natal ages in the duodenum that are now shown also to be responsive in the jejunum. These included DMT1, Dcytb, transferrin receptor 1, metallothionein, tripartite motif protein 27, the Menkes copper ATPase (ATP7A), glycerol-3-phosphate acyltransferase, factor-responsive smooth muscle protein, phosphoglucomutase-related protein, acidic calponin 3, glutathione peroxidase 2, integrin alpha 6, early growth response 1, sodium-dependent vitamin C transporter, selective LIM binding factor, small nuclear RNA activating complex, polypeptide 1, amyotrophic lateral sclerosis 2, laminin

TABLE I

Genes that increased in duodenum *and* jejunum of 12-wk-old, iron-deficient rats

Gene Name	Symbol	Acc. #	Biological Function/ Aliases
Transferrin Receptor	TFRC	M58040	Iron Homeostasis; Transferrin-Iron Endocytosis
Farnesyl Transferase Beta Subunit Farnesylation of Proteins	FNTB	M69056	Catalytic Activity; Post-Translational
Cytotoxic T Lymphocyte-Associated Protein 2 Beta Precursor (85% to mouse)	CTLA2B	AI230591	Immunity
Sepiapterin Reductase	SPR	M36410	Metabolism
Prolyl 4-Hydroxylase Alpha Subunit	P4HA1	BI274401	Procollagen-Proline 4-Dioxygenase Activity: HIF 1- $\alpha$ regulation
Neural Precursor Cell Expressed Developmentally Downregulated 9	NEDD9	BF555968	Cell Adhesion Molecule Activity; Regulation of Cell Growth
Heme Oxygenase 1	HMOX1	NM_012580	Heme Oxidation
Peripheral Myelin Protein 22 Similar to Laminin Gamma 2 Chain Precursor	PMP22	AA943163	Cell Cycle Arrest
	LAMC2	BM385282	Inflammatory Response Pathway; Cell Adhesion
Tripartite Motif Protein 27 (95% similar to mouse)	TRIM27	NM_009054	Oncogenesis; Formation of Cellular Compartments
Amyotrophic Lateral Sclerosis 2 (Juvenile) Chr. Reg., Cand. 13	ALS2CR13	AA945854	Unknown
Small Nuclear RNA Activating Complex Polypeptide 1 (84% similar to mouse)	SNAPC1	BI294596	Regulation of Transcription, DNA-dependent
Sodium-Dependent Vitamin C Transporter (97% similar to mouse)	SLC23A2	BI275077	Vitamin C Transport
Selective LIM Binding Factor, Rat Homolog	SLB	NM_053792	Modulation of Activity of Specific LIM Transcription Factors
Divalent Metal Ion Transporter 1, all +/-IRE transcripts	DMT1	AF029757	Divalent Metal Ion Transport
Duodenal Cytochrome B (91% similar)	CYBRD1	BF419070	Rat Sproutin; Ferric-Reductase
Duodenal Cytochrome B (on same chromosomal region)	CYBRD1	AI010267	Ferric-Reductase
Transferrin Receptor	TFRC	BF417032	Iron Ion Homeostasis; Transferrin-Iron Endocytosis
Metallothionein 1 and 2	MT1/2	BM383531	Metal Ion Binding; Copper Binding
Tripartite Motif Protein 27 (86% similar)	TRIM27	BI294862	Oncogenesis; Formation of Cellular Compartments
ATPase, Cu <sup>++</sup> Transporting, Alpha Polypeptide	ATP7A	AI136839	Copper Ion Efflux from Enterocytes
Divalent Metal Ion Transporter, +IRE transcripts only	DMT1	NM_013173	Divalent Metal Ion Transport
Metallothionein	MT1/2	AF411318	Metal Ion Binding
Glycerol-3-Phosphate Acyltransferase (87% similar)	GPAM	BG666882	Glycerolipid Synthesis
Factor-Responsive Smooth Muscle Protein	SM20	NM_019371	Apoptosis; Protein Metabolism; Oxidoreductase
Phosphoglucomutase-Related Protein (on same chr. region)	PGM-RP	AI412174	Cell Adhesion; Carbohydrate Metabolism/ Aciculin
Glutathione Peroxidase 2 (Gastrointestinal)	GPX2	AA800587	Defense Against Oxidative Stress
Integrin, Alpha 6	ITGA6	BE110753	Cell Adhesion; Cell Surface Mediated Signaling
Early Growth Response 1	EGR1	NM_012551	Regulation of Transcription, DNA-Dependent
Calponin 3, Acidic	CNN3	BI274457	Actin/Calmodulin Binding



gamma 2 chain precursor, peripheral myelin protein 22, heme oxygenase 1, neural precursor cell expressed developmentally downregulated 9 (NEDD9), prolyl 4-hydroxylase alpha subunit, sepiapterin reductase, cytotoxic T lymphocyte-associated protein beta 2 precursor, and farnesyl transferase beta subunit.

Table II presents genes that were shown previously to be decreased at 4 or 5 different post-natal ages, which are now shown also to be decreased in the jejunum. Tables III and IV show average expression levels and fold changes of genes listed in Tables I and II. Table V shows fold changes of unique genes that are induced in the duodenum only. Some of these genes are as previously described (6) and also may be presented in Table I and III; however, they represent novel probe sets for these specific genes. Finally, Tables VI and VII show fold changes of unique genes that were increased in both gut segments (Table VI) or decreased (Table VII) in only one or the other gut segment.

## DISCUSSION

Early studies suggested that only the proximal region of the mammalian small intestine is involved in iron transport and in the adaptive response to iron-deficiency (27). In fact, known iron-responsive genes such as DMT1 have been described to be induced in only the proximal portion of the gut (4). However, the current data clearly show that DMT1 and duodenal cytochrome b, genes known to be involved in transepithelial iron transport, also are strongly induced at the gene level in more distal intestinal segments of iron-deficient rats. This is a significant finding, as induction of mRNA expression for both of these genes is known to translate into increased protein levels. Many other novel genes induced by iron-deficiency also were regulated similarly in both gut segments, and some of these genes may play unidentified roles in intestinal iron and metal ion homeostasis. Overall, the current data demonstrate that induction of iron transport-related genes was equivalent or greater in jejunum as compared to duodenum.

Our previous studies indicated that the gene chips are highly reliable and accurate, as real-time PCR confirmed changes in expression of many of the genes that are now shown to also be regulated in more distal portions of the small intestine (6). Furthermore, in the current investigation, gene chip data were analyzed utilizing very strict reduction strategies to minimize the possibility of reporting false positives. Additionally, these presented data include many genes that have been reported to be regulated by dietary iron intake levels by other investigators using different techniques, such as Northern and Western blots and immuno-histochemical methods. Thus, the current presented data have a very high probability of being accurate.

We previously found that the brush-border membrane iron transport-related genes (DMT1 and Dcytb) were more strongly and consistently induced with iron deprivation than the genes encoding the basolateral membrane proteins, hephaestin and IREG1 (6). This observation is now extended to show the same trend in the jejunum of 12-week-old, iron-deficient rats. We further demonstrated that a host of other genes also was induced in the duodenum of iron-deprived rats, and we now show that many of these genes are also induced in the jejunum of 12-week-old, iron-deficient rats. These genes include the Menkes copper ATPase (APT7A) and metallothionein. This may suggest that under iron-deficient conditions, DMT1 functions to transport copper into enterocytes, which leads to induction of ATP7A and metallothionein. We have demonstrated that DMT1 and ATP7A are also strongly induced at the protein level, and that ATP7A is present in brush-border and basolateral membrane domains in the duodenum of iron-deficient rats (Ravia et al., 2005). Additionally, a brush-border membrane ferric reductase has been reported to reduce dietary copper (15), and DMT1 has been shown to transport reduced (e.g., cuprous) copper (2). These observations thus may explain why iron-deficient rats have significantly increased liver copper levels. Moreover, the fact that these genetic responses are conserved throughout the duodenum *and* jejunum

strongly suggests a functional coupling between iron and copper transport-related genes in the iron-deficient state.

Other novel genes also were induced by iron, deprivation in both the duodenum and jejunum of 12-week-old rats. Some of these genes encode proteins with known roles in intestinal iron homeostasis, including transferrin receptor 1, heme oxygenase 1, and prolyl 4-hydroxylase. Additionally, another strongly induced gene in both gut segments was the sodium-dependent vitamin C transporter, which may increase vitamin C absorption, from the interstitial fluids and which, in turn, could enhance the reducing capacity of a brush-border membrane ferric

reductase (15). Other genes with unknown roles in iron homeostasis also were induced in duodenum and jejunum, including tripartite motif protein 27 and integrin alpha 6. Tripartite motif protein 27 is one of the most consistently induced genes in our previous and the current studies, along with DMT1, Dcytb and APT7A, suggesting potential physiological relevance. Integrin alpha 6 is very strongly expressed and also very consistently induced by iron, deprivation, and we have noticed that for highly expressed genes, the gene chips tend to underestimate changes in expression. Thus, tripartite motif protein 27 and integrin alpha 6 are of particular interest for further study.

TABLE II

Genes that decreased in duodenum *and* jejunum of 12-wk-old, iron-deficient rats

Gene Name	Symbol	Acc. #	Biological Function/ Aliases
Monoamine Oxidase A	MAOA	D00688	Oxidation of Monoamine Neurotransmitters and Hormones
Solute Carrier Family 34, Member 2	SLC34A2	NM_053380	Sodium-Dependent Phosphate Cotransporter; P <sub>i</sub> Absorption/NaPi-IIb
3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase 2	HMGCS2	M33648	Acetyl-CoA Metabolism; Cholesterol Biosynthesis
Glucose-6-Phosphatase, Catalytic	G6PC	U07993	Glycogen Biosynthesis; Glycolysis and Gluconeogenesis
Phosphoenolpyruvate Carboxykinase, Cytosolic (GTP)	PCK1	BI277460	Gluconeogenesis; Lipid Metabolism
Glucose-6-Phosphatase, Catalytic	G6PC	NM_013098	Glycogen Biosynthesis; Glycolysis and Gluconeogenesis
UDP-Glucuronosyltransferase	UGT2B12	U27518	Glucuronidation of Endobiotics and Xenobiotics
Cyclin-Dependent Kinase Inhibitor 1C, p57	CDKN1C	AI013919	Tumor Repressor Activity; Regulation of Cell Proliferation, Differentiation
UDP-Glucuronosyltransferase	UGT2B12	NM_031980	Glucuronidation of Endobiotics and Xenobiotics
Solute Carrier Family 2, Member 5	SLC2A5	NM_031741	Facilitated Glucose, Fructose Transporter/ GLUT 5
UDP-Glucuronosyltransferase 2B3 Precursor, Microsomal	UDPGT	M31109	Glucuronidation of Endobiotics and Xenobiotics
Non-Oncogenic Rho GTPase-Specific GTP Exchange Factor	AKAP13	AI407536	Cell Growth and Maintenance/A kinase (PRKA) Anchor Protein 13
Hypoxia Inducible Factor 1, Alpha Subunit	HIF1A	NM_024359	Regulation of Transcription, DNA-Dependent; Response to Hypoxia

TABLE III

Changes in expression levels of genes that increased in duodenum *and* jejunum

Gene Name	Symbol	Acc. #	Duodenum			Jejunum		
			Fold Inc.	Control	Low Fe	Fold Inc.	Control	Low Fe
Transferrin Receptor	TFRC	M58040	<b>6.1</b>	144	670	<b>5.1</b>	153	484
Transferrin Receptor	TFRC	BF417032	<b>6.1</b>	642	4241	<b>7.3</b>	602	4114
Metallothionein 1 and 2	MT1/2	BM383531	<b>4.6</b>	1093	3432	<b>2.9</b>	648	1667
Tripartite Motif Protein 27 (86% similar)	TRIM27	BI294862	<b>4.3</b>	122	447	<b>2.8</b>	87	355
Metallothionein	MT1/2	AF411318	<b>4.3</b>	2114	8166	<b>3.1</b>	1854	6169
Divalent Metal Ion Transporter 1, all +/-IRE transcripts	DMT1	AF029757	<b>4.1</b>	1892	7236	<b>3.8</b>	1088	3641
Cytotoxic T Lymphocyte-Assoc. Protein 2 Beta Precursor (85% similar to mouse)	CTLA2B	AI230591	<b>4</b>	797	2334	<b>1.8</b>	533	941
Farnesyltransferase Beta Subunit	FNTB	M69056	<b>3.7</b>	715	2848	<b>1.7</b>	360	679
Glycerol-3-Phosphate Acyltransferase (87% similar to mouse)	GPAM	BG666882	<b>3.4</b>	2114	8166	<b>1.6</b>	316	505
Heme Oxygenase 1	HMOX1	NM_012580	<b>3.2</b>	730	2602	<b>2.4</b>	582	1449
Amyotrophic Lateral Sclerosis 2 (Juvenile) Chr. Reg., Cand. 13	ALS2CR13	AA945854	<b>3.2</b>	975	2975	<b>2</b>	905	1545
ATPase, Cu <sup>++</sup> Transporting, Alpha Polypeptide	ATP7A	AI136839	<b>3.1</b>	4891	15697	<b>2.5</b>	2467	6615
Sepiapterin Reductase	SPR	M36410	<b>3</b>	452	1575	<b>1.9</b>	410	859
Proliferating Cell Nuclear Antigen	PCNA	NM_022381	<b>3</b>	1978	6746	<b>1.9</b>	1038	2113
Neural Precursor Cell Expressed Developmentally Downregulated 9	NEDD9	BF555968	<b>2.9</b>	2032	6373	<b>1.5</b>	2371	3527
Early Growth Response 1	EGR1	NM_012551	<b>2.9</b>	559	1759	<b>1.6</b>	821	1268
Prolyl 4-Hydroxylase Alpha Subunit	P4HA1	BI274401	<b>2.8</b>	147	384	<b>2.7</b>	172	415
Tripartite Motif Protein 27 (95% similar to mouse)	TRIM27	NM_009054	<b>2.7</b>	591	1292	<b>2.1</b>	551	938
Small Nuclear RNA Activating Complex, Polypeptide 1 (84% to mouse)	SNAPC1	BI294596	<b>2.5</b>	394	1130	<b>2.3</b>	213	512
Duodenal Cytochrome B (on same chromosomal region)	CYBRD1	AI010267	<b>2.5</b>	2817	5602	<b>1.9</b>	90	1968
Similar to Laminin Gamma 2 Chain Precursor	LAMC2	BM385282	<b>2.4</b>	1254	3642	<b>1.7</b>	1753	3203
Phosphoglucomutase-Related Protein (on same chr. region)	PGM-RP	AI412174	<b>2.3</b>	868	1675	<b>1.6</b>	1116	1717
Peripheral Myelin Protein 22	PMP22	AA943163	<b>2.2</b>	1585	3549	<b>2.4</b>	2065	4764
Factor-Responsive Smooth Muscle Protein	SM20	NM_019371	<b>2.2</b>	1035	2241	<b>2</b>	1075	2138
Glutathione Peroxidase 2 (Gastrointestinal)	GPX2	AA800587	<b>2</b>	4566	9172	<b>1.9</b>	2167	4015
Sodium-Dependent Vitamin C Transporter (88% similar to mouse)	SLC23A1/2	BI275077	<b>1.8</b>	1265	1926	<b>2.1</b>	667	1244
Duodenal Cytochrome B (91% similar)	CYBRD1	BF419070	<b>1.7</b>	6470	10246	<b>21.7</b>	303	4981
Divalent Metal Ion Transporter, +IRE transcripts only	DMT1	NM_013173	<b>1.6</b>	7452	13341	<b>4.2</b>	2815	12758
Integrin, Alpha 6	ITGA6	BE110753	<b>1.6</b>	12335	20379	<b>1.6</b>	7234	14678
Selective LIM Binding Factor, Rat Homolog	SLB	NM_053792	<b>1.5</b>	1542	2502	<b>1.6</b>	1267	2227
Calponin 3, Acidic	CNN3	BI274457	<b>3.2</b>	561	2043	<b>2</b>	207	379



TABLE IV

Changes in expression levels of genes that decreased in duodenum *and* jejunum

Gene Name	Symbol	Duodenum			Jejunum			
		Acc. #	Fold Control Dec.		Low Fe	Fold Control Dec.	Low Fe	
Solute Carrier Family 34, Member 2	SLC34A2	NM_053380	<b>18.9</b>	838	59	<b>1.7</b>	3005	1573
3-Hydroxy-3-Methylglutaryl-Coenzyme A Synthase 2	HMGCS2	M33648	<b>9.7</b>	292	32	<b>2.6</b>	839	259
Glucose-6-Phosphatase, Catalytic Phosphoenolpyruvate Carboxykinase, Cytosolic (GTP)	G6PC	U07993	<b>5.5</b>	823	227	<b>2.7</b>	1224	475
Cyclin-Dependent Kinase Inhibitor 1C, p57	PCK1	BI277460	<b>4.6</b>	2650	475	<b>1.6</b>	5659	3094
UDP-Glucuronosyltransferase 2	CDKN1C	AI013919	<b>3.9</b>	1149	363	<b>2.2</b>	1453	743
Glucose-6-Phosphatase, Catalytic	UGT2B12	U27518	<b>3.7</b>	3263	858	<b>2</b>	466	183
UDP-Glucuronosyltransferase 2	G6PC	NM_013098	<b>3.4</b>	2894	632	<b>2.1</b>	3413	1669
Solute Carrier Family 2, Member 5	UGT2B12	NM_031980	<b>3.4</b>	4538	1125	<b>2.3</b>	1586	656
UDP-Glucuronosyltransferase 2 B3 Precursor, Microsomal	SLC2A5	NM_031741	<b>2.9</b>	3974	1331	<b>1.7</b>	2820	1744
Non-Oncogenic Rho GTPase-Specific GTP Exchange Factor (93% similar to human)	UDPGT	M31109	<b>2.4</b>	916	382	<b>1.9</b>	772	396
Monoamine Oxidase A	AKAP13	AI407536	<b>2.4</b>	362	132	<b>1.6</b>	848	462
Hypoxia Inducible Factor 1, Alpha Subunit	MAOA	D00688	<b>2</b>	1756	888	<b>1.9</b>	544	308
	HIF1A	NM_024359	<b>1.9</b>	1263	643	<b>2</b>	1913	1050

A host of other genes showed induction of at least three-fold in only the duodenum, while no genes were found to be induced at least three-fold only in the jejunum. These data suggest that the effects of iron-deficiency are more profound in the proximal small intestine, as judged by the number of genes being differentially expressed. Some genes induced solely in the duodenum were reported previously (6); however, different probe sets representing these genes were identified in the current studies. These genes include aquaporin 4 and two probes sets recognizing SRY-BOX containing gene 9 (SOX9). Altogether, three distinct probe sets showed strong

induction of SOX9 in only the duodenum of 12-week-old, iron-deficient rats. Other novel genes uniquely induced in the duodenum have not been associated previously with intestinal iron transport or metal homeostasis; however, some of these genes, such as gastrokine 1, claudin 2, and trefoil factor 1, are known to play important roles in gut physiology. Their relationship to iron deficiency is currently unknown. Another group of novel genes was found to be induced in both gut segments or uniquely decreased in one or the other gut segment, but involvement of these genes in iron transport and gut homeostasis is unknown.

TABLE V

## Unique genes that increased only in duodenum in response to iron deficiency

Fold Increase	Gene Title	Symbol	Acc. #	Biological Function/ Aliases
9.1	Aquaporin 4	AQP4	NM_012825	Water Transport
7.7	Gastrokine 1	GKN1	AI639014	Role in Mucosal Protection Postulated/ Foveolin, CA11
6.7	Membrane-Spanning 4-Domains, Subfamily A, Member 7 (89% to mouse)	MS4A7	AI408286	Unknown
6.7	Unknown EST Clone	???	AI044560	Unknown
5.4	Cyclin D1	CCND1	X75207	Regulation of Cell Cycle; Wnt Signaling
5.2	Highly Sim. to Mouse Nucleolar Protein Family A, Member 1			AI579023 Unknown
5.0	SRY-Box Containing Gene 9 (97% to mouse)	SOX9	AI072788	Chondrogenesis; Testis Determining Pathway
4.6	Similar to Cryptdin-7 Precursor (95% to rat)	LOC290857	AI639089	Paneth Cell Corticostatin/ Defensin
4.4	Unknown EST Clone	???	BI298306	Unknown
4.3	RT1 Class II, Locus Db1	RT1-DB1	BI279526	MHC Class II Protein Complex; Immunity
4.3	Similar to Lrp2bp-Pending Protein	LOC361149	BI291430	Low Density Lipoprotein Receptor-Related Protein Binding Protein
4.1	Solute Carrier Family 12, Member 2	SLC12A2	NM_031798	Na <sup>+</sup> /K <sup>+</sup> /2Cl <sup>-</sup> Cotransporter
4.0	Mink-Related Peptide 2	KCNE3	NM_022235	Potassium Channel Regulator Activity
3.9	Myristoylated Alanine Rich Protein Kinase C Substrate	MARCKS	BE111706	Actin Binding; Calmodulin Binding; Kinase Activity
3.9	Trans-Acting Transcription Factor 6 (94% to mouse)	SP6	BI278449	Transcriptional Regulation of Gene Expression
3.6	Myristoylated Alanine Rich Protein Kinase C Substrate	MARCKS	BE111706	Actin Binding; Calmodulin Binding; Kinase Activity
3.5	Immunoglobulin Joining Chain (89% to mouse)	IGJ	AA817898	Immune Response
3.4	Stearoyl-Coenzyme A Desaturase 2	SCD2	BE107760	Desaturation of Saturated Fatty Acyl-CoAs
3.4	Brain Specific Protein Homolog (95% to mouse)	???	AI171466	Unknown
3.4	Similar to Phosphoinositide-3-Kinase, Class 2, Beta Polypeptide	PI3K-C2B	BE105801	Signal Transduction
3.3	Claudin 2 (93% to mouse)	CLDN2	BM392116	Tight Junction; Integral Membrane Protein
3.2	SRY-Box Containing Gene 9 (93% to mouse)	SOX9	AI548994	Chondrogenesis; Testis Determining Pathway
3.0	Putative Small Membrane Protein NID67	NID67	AF313411	Neurogenesis; Ion Channel Activity
3.0	Protein phosphatase 1, regulatory (inhibitor) subunit 1B	PPP1R1B	AA942959	Intracellular Signaling; Protein Phosphatase Inhibitor Activity
3.0	Deleted in Liver Cancer 1	DLC1	AI176713	Hepatocarcinogenesis; Oncosuppressive Activity
3.0	Similar to TG interacting factor	TFIG	BM392224	TGFB-induced factor (TALE family homeobox)
3.0	Trefoil Factor 1	TFF1	NM_057129	Maintenance of Gastrointestinal Epithelium; Growth Factor Activity
3.0	Insulin-like Growth Factor Binding Protein 3	IGFBP3	NM_012588	Regulation of Cell Growth; Insulin-like Growth Factor Binding

TABLE VI

Unique genes that increased in duodenum *and* jejunum

Fold Increase Duodenum Jejunum	Gene Name	Symbol	Acc. #	Biological Function/ Aliases	
2.7	2.0	Eukaryotic Elongation Factor-2 Kinase	EEF2K	U93849	Protein Amino Acid Phosphorylation
3.1	2.3	Zinc Finger Protein 503	ZFP503	BF560938	DNA Binding: Transcriptional Regulation
2.0	2.5	Unknown EST Clone	??	AA818159	Unknown
3.2	2.0	Serologically Defined Colon Cancer Antigen 33	SDCCAG33	BE117444	Transcription Factor Activity
2.0	2.5	Unknown EST Clone	??	BF387865	Unknown
2.3	2.1	Unknown EST Clone	??	AA850472	Unknown
2.4	2.0	Similar to Retinoic Acid Inducible Protein 3	LOC312790	BI276110	Unknown
3.5	4.2	RT1 Class II, Locus Bb	RT1-BB	AI715202	MHC Class II Protein complex
2.4	2.1	RhoB gene	RHOB	NM_022542	Rho Protein Signal Transduction; Cell Growth and Maintenance
2.9	2.0	Unknown EST Clone	??	BF558827	Unknown
2.2	2.2	Pyruvate Dehydrogenase Kinase, Isoenzyme 4 (86% to mouse)	PDK4	AI175045	Muscle Metabolism
3.1	2.0	Retinol Binding Protein 7, Cellular (89% to mouse)	RBP7	BI283223	Novel Member of the Cellular Retinoid Binding Protein Family

Oligonucleotide microarray techniques have been utilized in many areas of mammalian physiology to identify novel genes involved in various metabolic processes. Surprisingly, this experimental approach had not been utilized to explore the effect of iron deprivation on the global expression of genes in the small intestinal epithelium, until our previous studies were reported in January of 2005 (6). However, Marzullo et al. (18) recently utilized differential display reverse-transcription PCR to identify differentially expressed transcripts in the intestine of iron-deficient rats. Their studies did not identify any of the genes reported in the current communication, with the exception of DMT1, whereas our previous and current studies did not identify the genes they

reported (cytochrome C oxidase [COX] subunit II mitochondrial gene, and serum and glucocorticoid-regulated kinase). These discrepancies may be due to the different experimental methods used or to differences in experimental design. It also should be noted that many genes involved in intestinal iron transport have been identified by techniques designed to identify differentially expressed transcripts [e.g., Dcytb identification by cDNA subtraction (19)] or by methods designed to detect changes in protein function [i.e., expression cloning of DMT1 in *Xenopus* oocytes (12)].

In summary, the current analysis of over 28,000 rat transcripts demonstrates that many genes are similarly regulated in the duodenum and jejunum of iron-deficient

TABLE VII

Unique genes that decreased in duodenum *or* jejunum

Duodenum				
Fold Decrease	Gene Name	Symbol	Acc. #	Biological Function/Aliases
4.5	Unknown EST Clone	???	BI281129	Unknown
4.4	Similar to Death-Associated Protein (83% to human)	LOC362136	AI716676	Unknown
4.3	Cytochrome P450, 2B19	CYP2B15	AI454613	Monoxygenase Activity
3.6	FAT mRNA	FAT	NM_031561	Transport of Long-Chain Fatty Acids/cd 36 antigen
3.4	Unknown EST Clone	???	BI279036	Unknown
3.3	Phosphatase, Orphan 1 (96% to human)	PHOSPHO1	AI112954	Putative Phosphatase Activity
3.2	Similar to RAB30	RAB30	BM392291	Oncogenesis; Member of RAS Oncogene Family
3.1	Unknown EST Clone	???	BI277131	Unknown
3.1	Unknown EST Clone	LOC308556	BI290815	Unknown
3.1	Solute Carrier Family 43, Member 2	SLC43A2	BF394311	Na <sup>+</sup> -Dependent System-L-like Amino Acid Transporter
3.0	Pancreatic Lipase-Related Protein 2	PNLIPRP2	NM_057206	Lipid Metabolism; Triacylglycerol Lipase Activity
3.0	S100 Calcium Binding Protein A9	S100A9	NM_053587	Calcium Ion Binding/ Calgranulin B
Jejunum				
Fold Decrease	Gene Name	Symbol	Acc. #	Biological Function/Aliases
4.9	Carbonic Anhydrase 3	CA3	NM_019292	One-Carbon Compound Metabolism; Zinc Ion Binding

rats. The fact that some known iron-responsive genes were strongly induced in both gut segments was unexpected, as previous studies have suggested that only the proximal intestine is responsible for iron transport. Also of particular interest is the fact that the Menkes copper ATPase and metallothionein were coordinately regulated along with DMT1 and Dcytb, suggesting a “functional coupling” of these genes. These observations further demonstrate the complex nature of intestinal iron transport and metal ion homeostasis.

## ACKNOWLEDGEMENTS

These studies were supported by NIH Grant 1 R21 DK 068349 (to JFC) and HRSA Grant (1 C76 HR 00432-01).

## REFERENCES

1. ABBOUD S, HAILE DJ (2000) A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J Biol Chem* 275: 19906-19912
2. ARREDONDO M, MUÑOZ P, MURA CV, NÚÑEZ MT (2003) DMT1, a physiologically relevant apical

- Cu1+ transporter of intestinal cells. *Am J Physiol Cell Physiol* 284: C1525-1530
3. BOCCIO J, SALGUEIRO J, LYSIONEK A, ZUBILLAGA M, WEILL R, GOLDMAN C, CARO R (2003) Current knowledge of iron metabolism. *Biol Trace Elem Res* 92: 189-212
  4. CANONNE-HERGAUX F, GRUENHEID S, PONKA P, GROS P (1999) Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood* 93: 4406-4417
  5. CHUNG J, WESSLING-RESNICK M (2003) Molecular mechanisms and regulation of iron transport. *Crit Rev Clin Lab Sci* 40: 151-182
  6. COLLINS JF, FRANCK CA, KOWDLEY KV, GHISHAN FK (2005) Identification of differentially expressed genes in response to dietary iron-deprivation in rat duodenum. *Am J Physiol Gastrointest Liver Physiol* 288: G964-G971
  7. DONOVAN A, BROWNLIE A, ZHOU Y, SHEPARD J, PRATT SJ, MOYNIHAN J, PAW BH, DREJER A, BARUT B, ZAPATA A, LAW TC, BRUGNARA C, LUX SE, PINKUS GS, PINKUS JL, KINGSLEY PD, PALIS J, FLEMING MD, ANDREWS NC, ZON LI (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* 403: 776-781
  8. FLEMING MD, ROMANO MA, SU MA, GARRICK LM, GARRICK MD, ANDREWS NC (1998) Nramp2 is mutated in the anemic Belgrade (b) rat: Evidence of a role for Nramp2 in endosomal iron transport. *Proc Natl Acad Sci USA* 95: 1148-1153
  9. FLEMING MD, TRENOR CC, 3RD, SU MA, FOERNZLER D, BEIER DR, DIETRICH WF, ANDREWS NC (1997) Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat Genet* 16: 383-386
  10. FRAZER DM, WILKINS SJ, BECKER EM, VULPE CD, MCKIE AT, TRINDER D, ANDERSON J (2002) Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats. *Gastroenterology* 123: 835-844
  11. GEHRKE SG, KULAKSIZ H, HERRMANN T, RIEDEL HD, BENTS K, VELTKAMP C, STREMMEL W (2003) Expression of hepcidin in hereditary hemochromatosis: Evidence for a regulation in response to the serum transferrin saturation and to non-transferrin-bound iron. *Blood* 102: 371-376
  12. GUNSHIN H, MACKENZIE B, BERGER UV, GUNSHIN Y, ROMERO MF, BORON WF, NUSSBERGER S, GOLLAN JL, HEDIGER MA (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388: 482-488
  13. HENTZE MW, MUCKENTHALER MU, ANDREWS NC (2004) Balancing acts: Molecular control of mammalian iron metabolism. *Cell* 117: 285-297
  14. HINES ER, COLLINS JF, JONES MD, SEREY SH, GHISHAN FK (2002) Glucocorticoid regulation of the murine PHEX gene. *Am J Physiol Renal Physiol* 283: F356-363
  15. KNOPFEL M, SOLIOZ M (2002) Characterization of a cytochrome b(558) ferric/cupric reductase from rabbit duodenal brush border membranes. *Biochem Biophys Res Commun* 291: 220-225
  16. KUO YM, SU T, CHEN H, ATTIEH Z, SYED BA, MCKIE AT, ANDERSON GJ, GITSCHIER J, VULPE CD (2004) Mislocalisation of hephaestin, a multicopper ferroxidase involved in basolateral intestinal iron transport, in the sex linked anaemia mouse. *Gut* 53: 201-206
  17. LAFTAH AH, RAMESH B, SIMPSON RJ, SOLANKY N, BAHRAM S, SCHUMANN K, DEBNAM ES, SRAI SK (2004) Effect of hepcidin on intestinal iron absorption in mice. *Blood* 103: 3940-3944
  18. MARZULLO L, TOSCO A, CAPONE R, ANDERSEN HS, CAPASSO A, LEONE A (2004) Identification of dietary copper- and iron-regulated genes in rat intestine. *Gene* 338: 225-233
  19. MCKIE AT, BARROW D, LATUNDE-DADA GO, ROLFS A, SAGER G, MUDALY E, MUDALY M, RICHARDSON C, BARLOW D, BOMFORD A, PETERS TJ, RAJA KB, SHIRALI S, HEDIGER MA, FARZANEH F, SIMPSON RJ (2001) An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* 291: 1755-1759
  20. MCKIE AT, MARCIANI P, ROLFS A, BRENNAN K, WEHR K, BARROW D, MIRET S, BOMFORD A, PETERS TJ, FARZANEH F, HEDIGER MA, HENTZE MW, SIMPSON RJ (2000) A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* 5: 299-309
  21. MUCKENTHALER M, ROY CN, CUSTODIO AO, MINANA B, DEGRAAF J, MONTROSS LK, ANDREWS NC, HENTZE MW (2003) Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. *Nat Genet* 34: 102-107
  22. NICOLAS G, CHAUVET C, VIATTE L, DANAN JL, BIGARD X, DEVAUX I, BEAUMONT C, KAHN A, VAULONT S (2002) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J Clin Invest* 110: 1037-1044
  23. PIGEON C, ILYIN G, COURSELAUD B, LEROYER P, TURLIN B, BRISSOT P, LOREAL O (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* 276: 7811-7819
  24. VIDAL S, BELOUCHI AM, CELLIER M, BEATTY B, GROS P (1995) Cloning and characterization of a second human NRAMP gene on chromosome 12q13. *Mamm Genome* 6: 224-230
  25. VULPE CD, KUO YM, MURPHY TL, COWLEY L, ASKWITH C, LIBINA N, GITSCHIER J, ANDERSON GJ (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat Genet* 21: 195-199
  26. WEINSTEIN DA, ROY CN, FLEMING MD, LODA MF, WOLFS DORF JI, ANDREWS NC (2002) Inappropriate expression of hepcidin is associated with iron refractory anemia: Implications for the anemia of chronic disease. *Blood* 100: 3776-3781
  27. WHEBY MS, JONES LG, CROSBY WH (1964) Studies on iron absorption. Intestinal regulatory mechanisms. *J Clin Invest* 43: 1433-1442
  28. RAVIA JJ, STEPHEN RM, GHISHAN FK, COLLINS JF (2005) Menkes Copper ATPase (Atp7a) is a novel metal-responsive gene in rat duodenum, and immunoreactive protein is present on brush-border and basolateral membrane domains. *J Biol Chem* 280: 36221-36227