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Murine Prenatal Expression of Cholecystokinin in Neural Crest, Enteric Neurons, and Enteroendocrine Cells

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ABSTRACT **Cholecystokinin (CCK) is a regulatory peptide that is primarily expressed in two adult cell types: endocrine cells of the intestine and neurons of the central nervous system. To determine the ontogeny of CCK expression during intestinal organogenesis, we created a mouse strain in which the CCK gene was replaced by a *lacZ* reporter cassette using homologous recombination in embryonic stem cells. Initially, CCK expression in the developing intestine was limited to the myenteric plexus of the enteric nervous system. This expression pattern was widespread, extending from the proximal stomach into the colon, yet transient, being detected soon after gut tube closure [embryonic day 10.5 (E10.5)] through E15.5. Since enteric neurons are derived from the neural crest, we examined earlier (E8.5–9.5) embryos and concluded that *lacZ* was expressed in subpopulations of neural tube and neural crest cells. Endocrine cell expression in the intestinal epithelium occurred later, beginning at E15.5 as enteric neuronal expression was dwindling. This expression persisted to yield the adult pattern of scattered single endocrine cells in the upper small intestine. The data show that CCK is a very early marker of both neuronal and endocrine cell lineages in the developing gastrointestinal tract. Furthermore, reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that CCK receptor transcripts were detected in embryos as early as E10.5, suggesting that CCK signaling is established early in mouse development. *Dev Dyn 1999;216:190–200*.** © 1999 Wiley-Liss, Inc.

Key words: mouse embryogenesis; gene targeting; β-galactosidase; neural crest; enteric nervous system; enteroendocrine cells

INTRODUCTION

Cholecystokinin (CCK) is a regulatory peptide that is produced in both the gastrointestinal tract and nervous system. CCK secreted from endocrine cells in the proximal small intestine acts as a hormone to stimulate gallbladder contraction and pancreatic enzyme secretion to regulate the flow of digestive juices into the duodenum (Crawley and Corwin, 1994). Synthesis of CCK also occurs in neurons of the central and peripheral nervous systems, where it functions as a neurotransmitter or neuromodulator (Beinfeld et al., 1981; Schultzberg et al., 1980). Dual expression in neurons and gastrointestinal endocrine cells is a feature common to several regulatory peptides, including somatostatin, glucagon-like-peptide 1, and neurotensin. CCK expression provides a paradigm to characterize the molecular basis for the dual expression pattern in these distinct cell lineages.

In the adult small intestine, CCK is produced in endocrine cells within the mucosa and in a subset of neurons in the enteric nervous system. These endocrine cells and neurons are thought to arise from different embryonic origins. Stem cells residing in the intestinal crypts produce endocrine cells as well as the three other lineages of the intestinal epithelium: enterocytes, goblet cells, and paneth cells (Cheng and Leblond, 1974; Ponder et al., 1985). The intestinal stem cells are believed to be of endodermal origin, however the means by which the four cell lineages are specified is not well understood. Moreover, there are numerous types of endocrine cells defined by the hormone product they secrete and the morphology of their secretory granules. Little is known about the differentiation pathways or lineage relationships between these different types of intestinal endocrine cells. Two separate lineages of colonic endocrine cells have been proposed (Roth et al., 1992; Upchurch et al., 1996). One branch includes peptide YY (PYY)-, glucagon-, neurotensin-, and CCK-producing cells, and the other includes serotonin- and substance P-producing cells. Recent data from mouse mutants deficient in the basic helix loop helix transcription factor BETA2/NeuroD suggested that distinct endocrine lineages may also exist in the small intestine (Naya et al., 1997). Both CCK-producing and secretin-producing cells were absent in the BETA2/NeuroD deficient mice, while other enteroendocrine cell populations (glucagon, PYY, neurotensin, substance P, gastric

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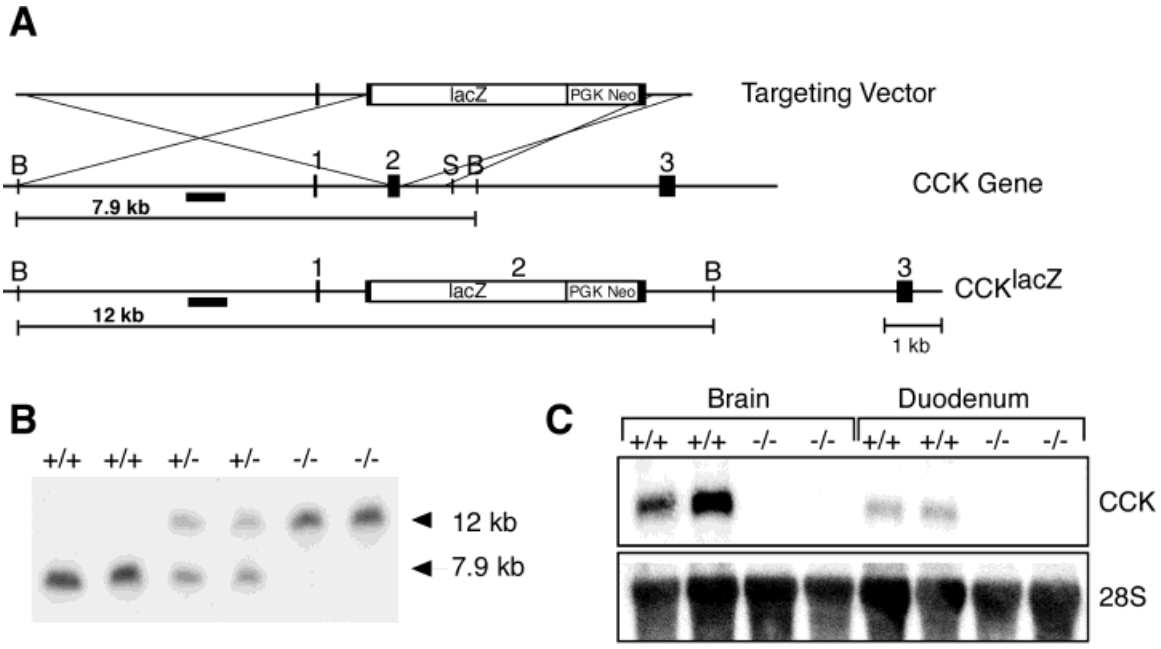

Fig. 1. Targeted insertion of the *lacZ* reporter gene into the mouse CCK gene. (**A**) Schematic diagram of the targeting vector (top), the mouse CCK gene (middle), and the mutant allele generated after homologous recombination (bottom). The mouse CCK gene contains 3 exons, the first a non-coding exon. A replacement-type targeting vector was constructed containing the bacterial *lacZ* reporter gene fused to the CCK gene at the translational start site in exon 2. B = *Bam*HI; S = *Sal*I. (**B**) Genomic Southern analysis on DNAs isolated from wild type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice. The probe and predicted *Bam*HI fragments of the wild type (7.9 kb) and mutant (12 kb) alleles are indicated in (A). (**C**) CCK transcripts are absent in tissues from homozygous mutant mice. Northern blot analysis of total RNA (20 μg) isolated from brain or duodenum from wild-type (+/+) or homozygous mutant (−/−) mice. The same blot was hybridized sequentially with a CCK cDNA probe and a 28S ribosomal probe to control for loading.

inhibitory peptide, and somatostatin) were relatively unaffected, suggesting that there may be a close lineage relationship between CCK- and secretin-producing cells.

The neurons and glia of the enteric nervous system are derived from migratory neural crest cells that colonize the gut (Le Douarin and Teillet, 1973; Le Douarin, 1986; Pomeranz et al., 1991). During mouse development, neural crest cells detach from the dorsal portion of the neural folds and migrate along predetermined routes to establish a number of different cell types, including melanocytes, adrenal medullary cells, skeletal and connective tissue components of the head, and the neurons and glia of the peripheral nervous system. Enteric ganglia are derived primarily from the vagal neural crest that originates from the level of somites 1–7 (Le Douarin and Teillet, 1973), but some cells arise from the sacral crest (caudal to somite 28) (Pomeranz et al., 1991; Serbedzija et al., 1991). When the neural crest cells reach their target tissue, they differentiate into a number of neuronal and non-neuronal cell types. In the adult enteric nervous system, CCK neurons are most abundant in the colon, with lower numbers found in the small intestine (Larsson and Rehfeld, 1979; Schultzberg et al., 1980). CCK neurons are found in both the myenteric plexus and submucosal plexus. Although CCK is detectable in the enteric nervous system, most of the CCK in the adult gastrointestinal tract is produced by endocrine cells.

While the pattern of CCK expression in neurons and endocrine cells of the adult intestine has been well characterized, the ontogeny of this expression is less clear. In this study, we have replaced part of the mouse CCK gene (*Cck*) with a bacterial β-galactosidase reporter cassette by homologous recombination in embryonic stem (ES) cells. We analyzed *lacZ* expression in heterozygous fetuses collected from timed matings to study the timing and sites of appearance of CCK-expressing cells during development. We asked when during intestinal morphogenesis CCK-expressing endocrine cells and neurons are first formed.

RESULTS

Generation of Cck^{lacZ} Mice

Homologous recombination in embryonic stem (ES) cells was used to construct a mutant mouse that expressed the bacterial *lacZ* reporter gene under the control of the CCK promoter (Fig. 1). The targeting vector was engineered such that the *lacZ* gene product, β-galactosidase, initiated at the normal CCK translational start site in exon 2. The CCK gene was further disabled by a 168-bp deletion at the site of the *lacZ* insertion. After electroporation of the targeting vector into ES cells, correctly targeted clones were identified by polymerase chain reaction (PCR) and Southern blot analysis. The *Cck^{lacZ}* mutation was subsequently trans-

ferred to the mouse germline by microinjection of targeted ES cell clones into blastocysts to generate chimeric mice, which were bred to transmit the engineered mutation to heterozygous progeny. Intercrossing heterozygous $Cck^{lacZ}/+$ mice produced the expected 1 : 2 : 1 ratio of wild-type : heterozygous: homozygous mutant mice. Southern blot analysis of genomic DNA isolated from mice of these three genotypes confirmed the allele structures (Fig. 1B). Lack of CCK mRNA in homozygous mutant mice was demonstrated by northern blot analysis of brain and intestinal RNA (Fig. 1C), and loss of CCK peptide was established by immunohistochemistry with an anti-CCK antibody (Fig. 2). The absence of CCK mRNA and protein confirms that Cck^{lacZ} is a null allele.

CCK-deficient male and female mice were indistinguishable from their wild-type littermates at birth, developed and gained weight at the same rate as wild-type controls, and reached the same adult body weight as wild-type controls (Lacourse et al., 1999). In addition, both male and female CCK-deficient mice were fertile, and females were able to bear and rear their young. Thus, CCK is not required for viability or fertility.

Reporter Gene Expression in Intestine and Brain

In the adult small intestine, CCK is primarily produced in a subset of endocrine cells known as I cells. These cells exist as scattered single cells in the epithelium, most densely localized in the upper small intestine. We confirmed accurate expression of the *lacZ* reporter gene in I cells of Cck^{lacZ} mice by demonstrating co-localization of CCK immunoreactivity and X-gal staining in adult intestinal sections. In heterozygous mice *lacZ* expression was only detected in CCK-immunoreactive cells (Fig. 2A and B). In homozygous mutant mice CCK immunoreactivity was not detected; *lacZ* expressing cells were observed, with no obvious difference in position or number in comparison to heterozygotes (Fig. 2C and D).

CCK is expressed in many regions of the brain, including olfactory bulb, cortex, thalamus, and hippocampus. We confirmed that the *lacZ* reporter gene was expressed in the brains of adult Cck^{lacZ} mice by comparing X-gal stained sections from heterozygous (Fig. 2E) or homozygous (not shown) brains to wild-type sections on which in situ hybridization had been performed to detect CCK transcripts (Fig. 2F). This analysis was limited to coronal sections in one region of the brain. The X-gal staining and in situ hybridization patterns in this region of the brain were similar, with expression seen in cortex, hippocampus, and thalamus.

Two Patterns of CCK Expression During Gastrointestinal Tract Development

To characterize the ontogeny of CCK-expressing cells during intestinal organogenesis we determined the

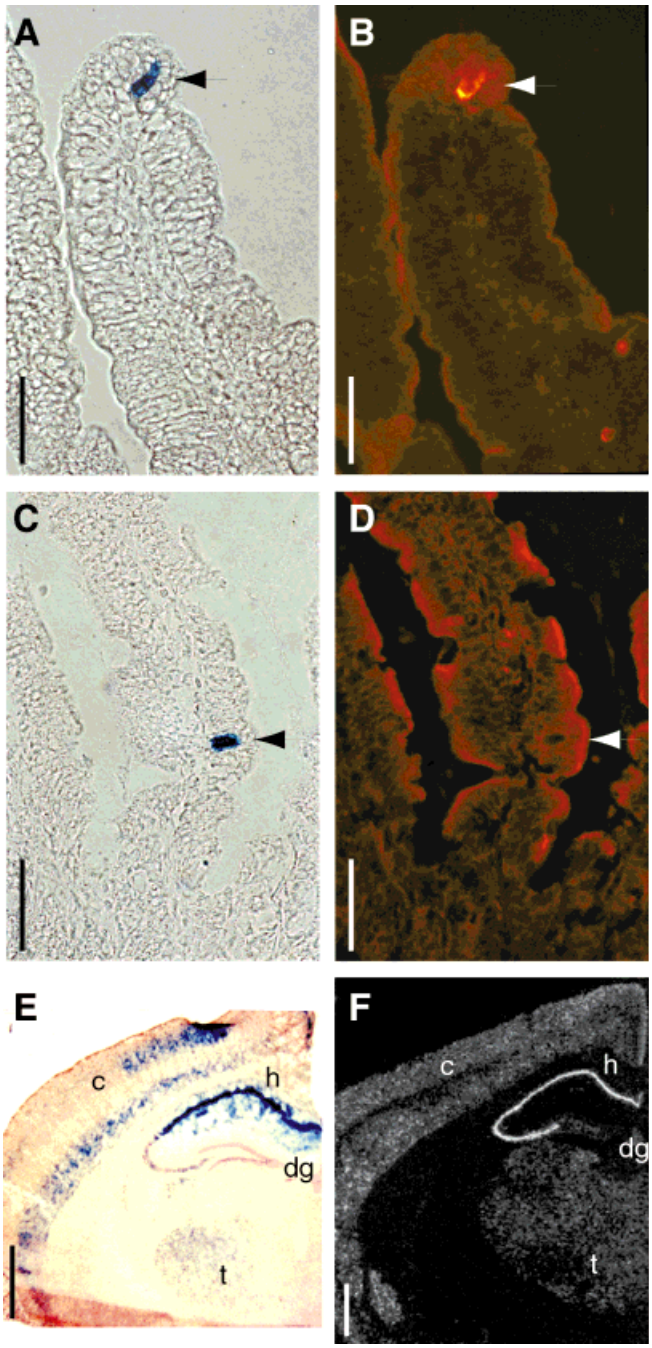

Fig. 2. Verification of *lacZ* expression in CCK-expressing cells in adult mice. (**A–D**) Sections of small intestine from heterozygous (A and B) and homozygous (C and D) Cck^{lacZ} mice were co-stained with X-gal to detect *lacZ* expression (A and C), and with an antibody to CCK (B and D). The arrowheads indicate a single stained CCK-endocrine cell in each longitudinal villus section. Scale bars represent 50 μm. (**E–F**) Sections of brain from Cck^{lacZ} heterozygous (E) or wild-type (F) mice were either stained with X-gal (E), or in situ hybridization was performed to detect *Cck* transcripts using an ^{35}S-labeled riboprobe (F). Expression is seen in both preparations in the cortex (c), hippocampus (h), and thalamus (t), but not in the dentate gyrus (dg). Scale bars = 1 mm.

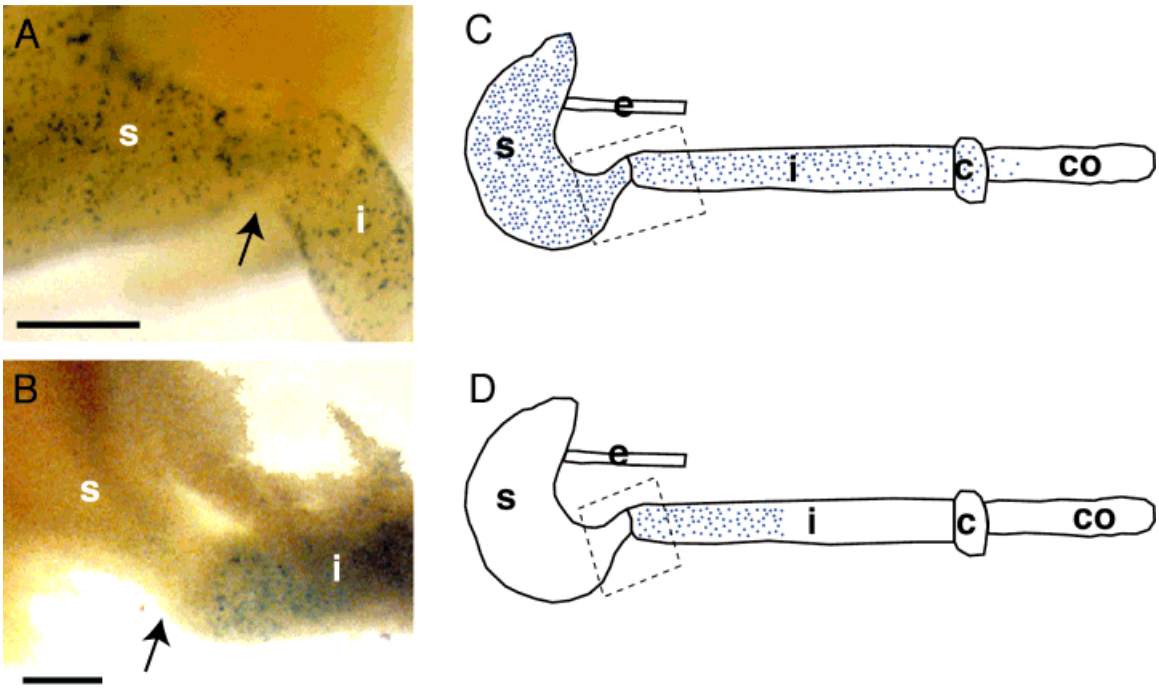

Fig. 3. *Cck*^{lacZ} expression in embryonic gastrointestinal tract. Gastrointestinal tracts from E14.5 (**A,C**) or E16.5 (**B,D**) were dissected and stained as whole-mounts with X-gal. (A) A view of the stomach/intestinal junction (arrow) at E14.5 shows X-gal stained blue cells extending throughout the stomach and intestine. (B) A similar view at E16.5 shows that the blue-stained cells are restricted to the intestine. (C,D) Schematic diagrams of the X-gal staining pattern of gastrointestinal tracts of E14.5 (C) or E16.5 (D) embryos. The dashed boxes indicate the general regions shown in the high powered views of tissues (A,B). Labels: e, esophagus; s, stomach; i, intestine; c, cecum; co, colon. Scale bars = 170 μm.

pattern of *lacZ* expression in heterozygous (*Cck*^{lacZ}/+) fetuses from timed matings. Whole-mount X-gal staining of gastrointestinal tracts showed extensive staining in scattered single cells at both E14.5 and E16.5 (Fig. 3), while no staining was observed in control (+/+) fetuses (not shown). However, the localization of X-gal positive cells along the tracts differed significantly at these two developmental time points. At E14.5, positive cells were found along the majority of the length of the tract, with staining extending from the esophageal/stomach border into the proximal colon. This pattern is in contrast to the more restricted pattern of staining at E16.5, with X-gal positive cells concentrated in the upper small intestine and no staining in the proximal gastrointestinal tract, including esophagus and stomach. Figure 3 summarizes the staining pattern along the length of the gastrointestinal tract and shows a high-power view of the stomach/duodenum border. At E14.5 X-gal stained cells are distributed evenly across this border (Fig 3A), while at E16.5 stained cells were observed in the small intestine but not the stomach (Fig. 3B).

To understand the cellular basis for the changing pattern of expression in the developing gastrointestinal tract, we examined cryosectioned embryos from E10.5 to birth (Fig. 4). Similar to the results described above for whole-mount stained tissue, the pattern of staining changed between E14.5 and E16.5. In the early period (E10.5 to E14.5), expression in the gastrointestinal tract was limited to cells in the mesenchyme surrounding the epithelium, while in the later time period (E15.5 to birth), staining was observed in epithelial cells. In the early embryo, soon after the gut tube closes (E10.5), a few positive cells appeared to be migrating into the mesenchyme surrounding the tube (Fig 4A). Subsequently this staining was organized in a concentric ring around the gut tube (Fig. 4B–E). Because this early expression had not been previously described for CCK, we verified the expression pattern by in situ hybridization of E12.5 embryo sections with a CCK antisense riboprobe (Fig. 4C).

At E15.5, the submucosal staining was still present, however X-gal positive cells were now apparent in the intestinal epithelium on the nascent villi (Fig. 4F and G). By E16.5, the submucosal staining had diminished, but the epithelial staining on the developing villi persisted. Immunohistochemical analysis of E16.5 embryos with a CCK antibody demonstrated that the X-gal positive epithelial cells synthesize CCK protein (Fig. 4J). The epithelial expression pattern was maintained during intestinal organogenesis as villi and crypts are formed, to become the characteristic I cell expression pattern observed in the adult (compare Fig. 4I with Fig. 2A).

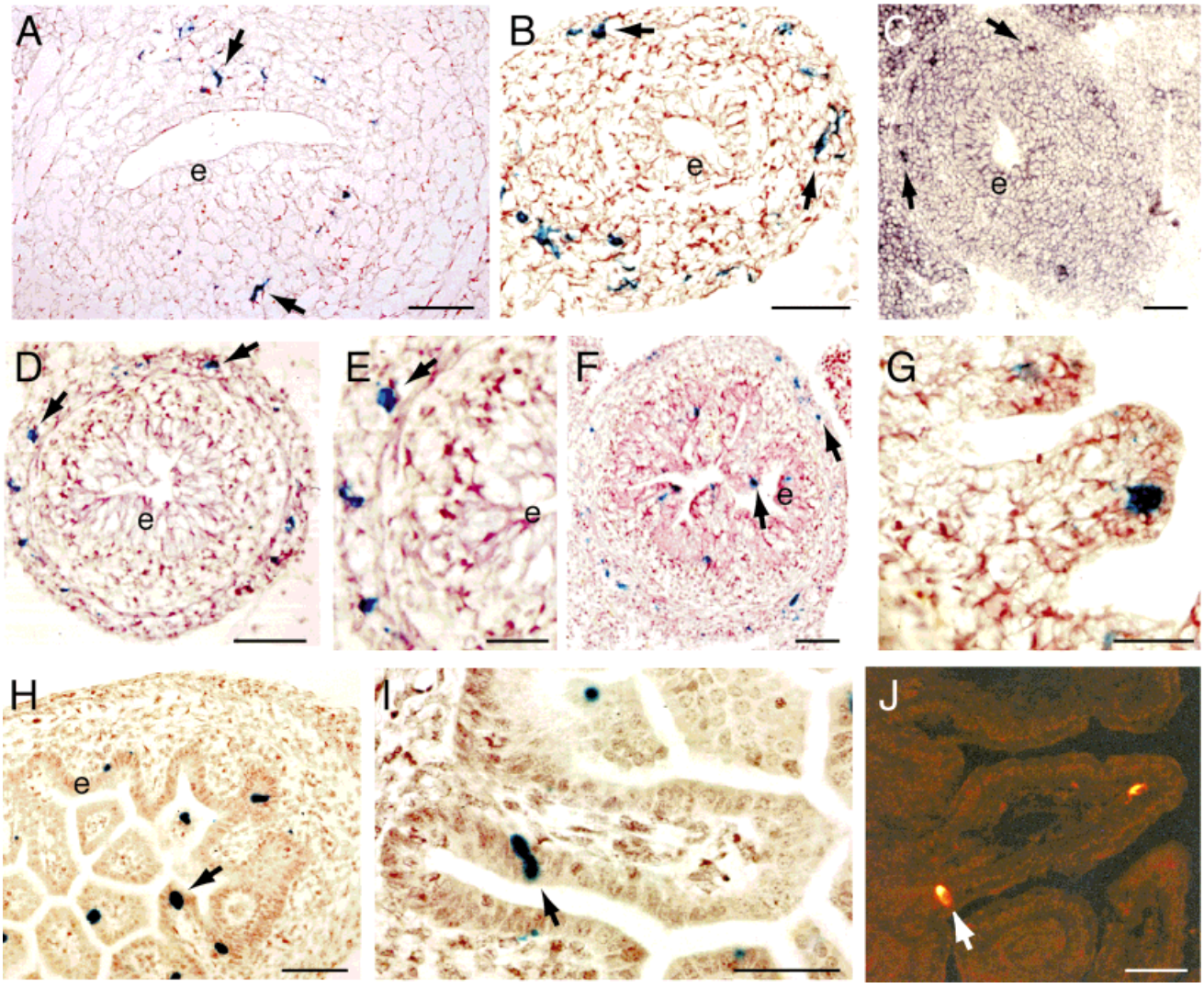

Fig. 4. Localization of *lacZ* expressing cells within the prenatal intestine. Expression was first seen in the mesenchyme surrounding the gut tube (A–F), and later in enteroendocrine cells located in the epithelium (e) (F–J). Arrows indicate some of the positive cells. (**A**) E10.5 heterozygous embryo. (B–C) E12.5. A heterozygous (**B**) embryo shows staining in the developing enteric nervous system. In situ hybridization with a digoxigenin-labeled antisense CCK riboprobe on a wild type embryo (**C**) detects the same pattern of staining. There was no hybridization observed with control experiments using a sense riboprobe (not shown). (D–E) E14.5 heterozygous embryo at lower (**D**) and higher (**E**) magnification. (F–G) E15.5 heterozygous embryos. Staining is apparent in both enteric nervous system and developing endocrine cells. (**G**) shows a higher magnification view of the endocrine expression on developing villi. (H–J) E16.5 heterozygous embryos. Lower magnification (**H**) or higher magnification (**I**) views show that expression is restricted to the intestinal epithelium. A wild-type section (**J**) stained with an anti-CCK antibody shows that peptide is detected in the same pattern as X-gal staining. Scale bars = 70 μm (A, B, C, D, F and H) or 30 μm (E, G, I, J).

Early Embryonic Expression in Developing Enteric Nervous System

The positioning of the CCK-expressing cells in the early embryo in a concentric ring around the gut tube suggested that these cells may be a component of the developing nervous system in the wall of the gastrointestinal tract. To test this we co-stained E14.5 embryo sections with X-gal and an antibody to the neurofilament protein peripherin. The results showed that the X-gal positive cells were positioned within the neuronal processes (Fig 5A–C). Co-staining with X-gal and rhodamine-phalloidin, to identify muscle, showed that the *lacZ*-expressing cells were just exterior to the muscle layer (Fig. 5D–F). Positioning in the neuronal network just exterior to the muscle indicates that the CCK-expressing cells in the early embryo are in the developing myenteric plexus of the enteric nervous system.

Expression in Neural Crest

The enteric nervous system is derived from neural crest cells that migrate from the dorsal region of the neural tube into the gut. We examined E8.5 and E9.5

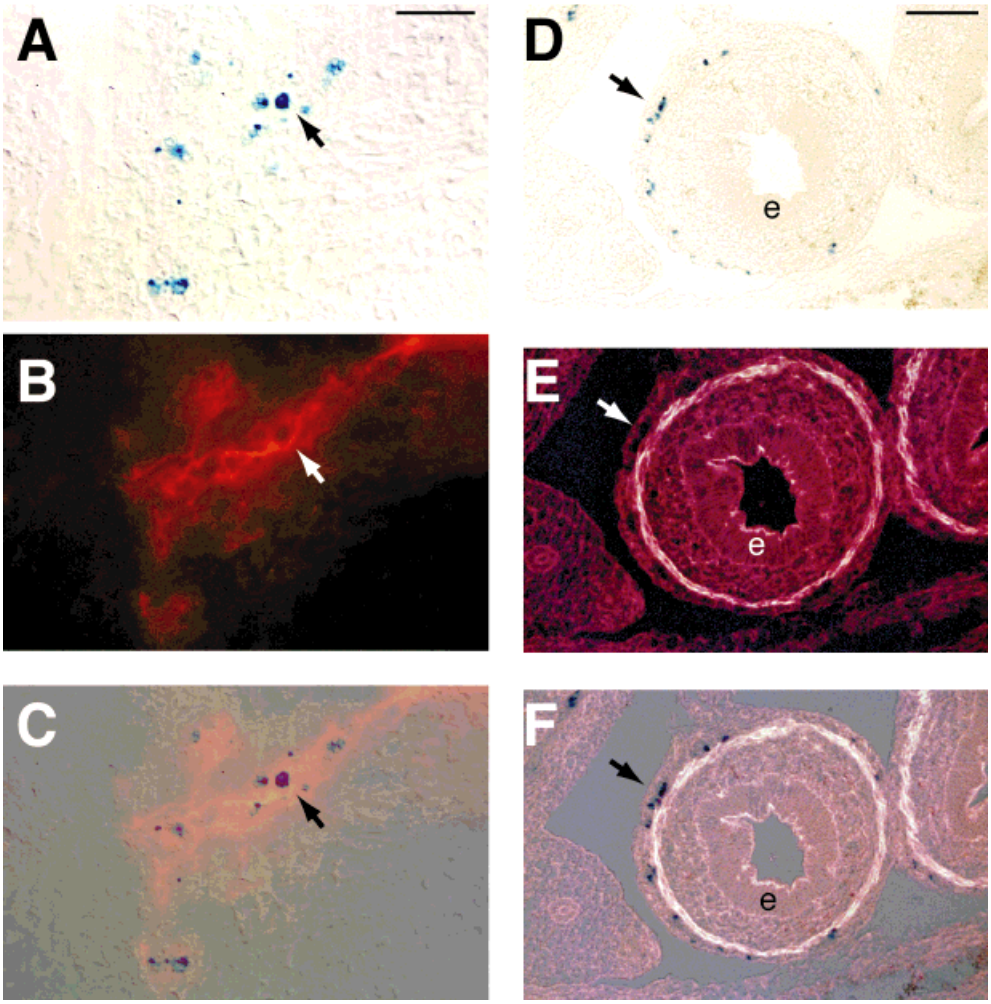

Fig. 5. *Cck*^{lacZ} is expressed in the enteric nervous system of E14.5 embryos. (**A–C**) High magnification view of a section co-stained with X-gal (A, C) and a peripherin antibody (B, C). An overlay (C) of photos A and B shows colocalization of X-gal and peripherin staining. (**D–F**). A section co-stained with X-gal (D, F) and rhodamine-phalloidin (E, F). An overlay (F) of photos D and E demonstrates that the X-gal stained blue cells are in the myenteric plexus, external to the phalloidin-stained smooth muscle layer. The gut epithelium is indicated by (e); arrows indicate some of the positive cells. Scale bars = 35 μm (A) or 100 μm (D).

embryos to determine whether there was *lacZ* expression in neural tube and migratory neural crest. Whole-mount X-gal staining revealed extensive expression in early embryos (Fig. 6). At E8.5, X-gal staining was observed in the neural tube, brain, and heart. Sections showed the neural tube staining to be in the dorsal region of the neural folds, consistent with the position of the early neural crest (Fig. 6B). At E9.5 staining in the neural tube is quite extensive, extending caudally down the neural tube as development continues (Fig. 6C). Sections of E9.5 embryos also showed that the neural tube staining is restricted to the dorsal region, from which neural crest cells detach and migrate to various destinations in the body (Fig. 6D). Other regions of staining consistent with neural crest include the dermamyotome and sympathetic chain ganglia. However, expression of *Cck*^{lacZ} was not detected in all neural crest cells or derivatives; for example, staining of dorsal root ganglia was not observed. Moreover, the neural crest/neural tube staining extended beyond the region from which the progenitors of the enteric nervous system originate.

Ontogeny of CCK-Family Ligands and Receptors

The patterns of expression of the endogenous CCK gene and its two receptors were examined by reverse transcriptase PCR (RT-PCR) analysis of total RNA isolated from embryos aged E8.5 to E17.5. In agreement with the X-gal staining results, CCK transcripts were detected at all stages examined (Fig. 7). Analysis of RNA from individual tissues of E17.5 embryos demonstrated that CCK transcripts were present in pancreas, intestine, and head, but not liver. To determine if CCK receptor transcripts were also present during mouse embryogenesis, we used primers specific for CCK-A receptor and CCK-B/gastrin receptor transcripts. Both receptor transcripts were detected as early as E10.5, and showed the same tissue specificity at E17.5 as CCK, with expression detected in pancreas, intestine, and head, but not liver. Similar to the timing of the receptors, transcripts for gastrin, the other CCK-family

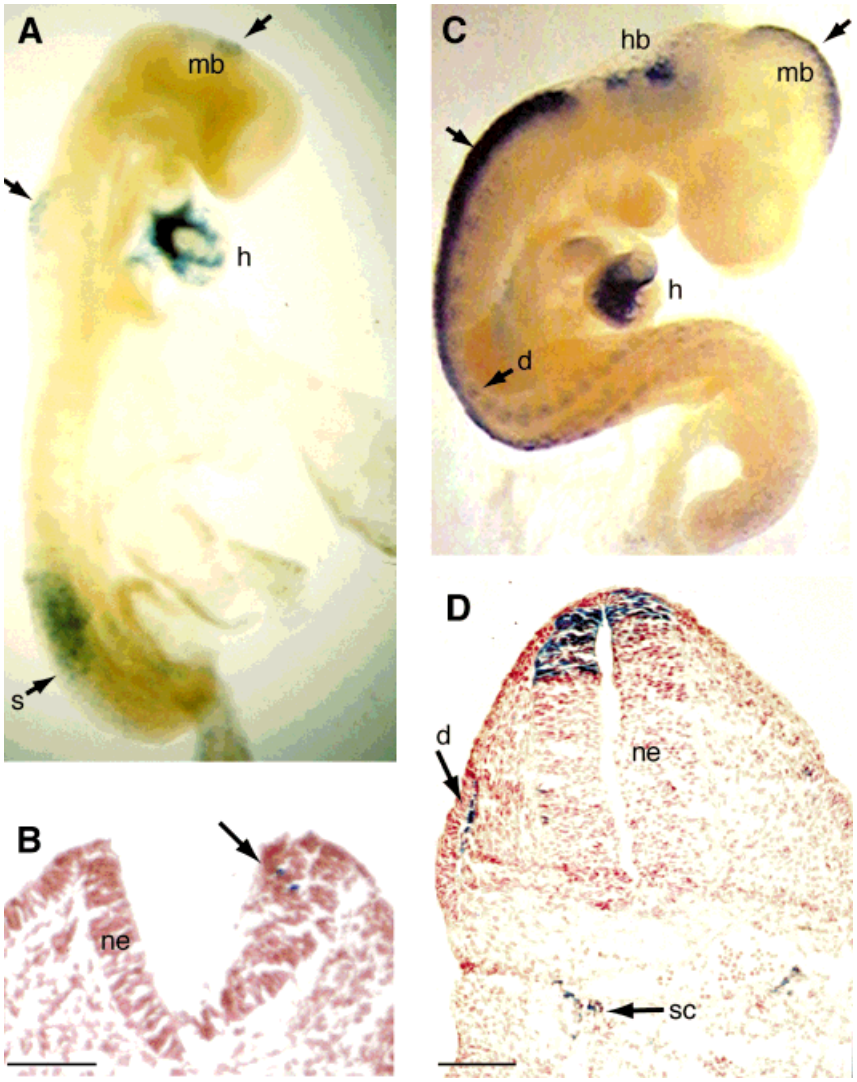

Fig. 6. *Cck*^{lacZ} expression in whole-mount X-gal stained embryos. (**A**) Staining in the neural tube and midbrain (mb) (unlabeled arrows), and heart (h) and somites (s) are seen in an E8.5 embryo. (**B**) A cryosection of an E8.5 embryo exhibits staining in neural epithelium (ne) in the dorsal region of the neural folds (arrow). (**C**) An E9.5 embryo shows more extensive neural tube staining, and expression in midbrain, hindbrain (hb), heart and dermomyotome (d). Hindbrain expression is localized to rhombomeres 2 and 4. (**D**) A cryosection of an E9.5 embryo reveals that the neural tube staining is restricted to the dorsal region of the neural epithelium where neural crest cells originate. Staining is also seen in the dermomyotome and sympathetic chain (sc). Scale bars = 100 μm.

ligand, were first detected at E10.5, with expression continuing through birth.

Since it has been suggested that CCK- and secretin-producing cells in the gut share a common lineage pathway, we also examined the ontogeny of secretin expression. RT-PCR analysis showed that secretin transcripts were present very early in development, similar to the timing of CCK gene expression in embryos as early as E8.5 (Fig. 7).

DISCUSSION

We have utilized a new mouse strain with a *lacZ* reporter cassette inserted into the CCK gene to study the spatial and temporal pattern of CCK gene expression during intestinal organogenesis. The cellular pattern of CCK expression was easily determined by histochemical staining for the *lacZ* gene product β-galactosidase. Our studies demonstrate that CCK is expressed in developing neurons and endocrine cells in a complex pattern. Expression in mouse intestine as early as E14 was previously shown by Vitale et al. (1991) by RNase protection assay. Our results show that CCK-expressing cells can be found in the intestine as early as E10.5. The early (E10.5–E14.5) gut expression is limited to developing neurons of the myenteric plexus of the enteric nervous system. This widespread expression in the developing enteric nervous system is transient, ending just after endocrine expression is first detected at E15.5. It is not clear whether the cessation of expression represents down-regulation of CCK transcription within these cells, or whether these cells are eliminated. Once activated, the endocrine expression pattern persists through intestinal morphogenesis to yield the adult pattern in scattered single cells in the intestinal epithelium. The detection of endocrine CCK several days before birth, at the time of rudimentary villus formation, indicates that CCK gene expression is

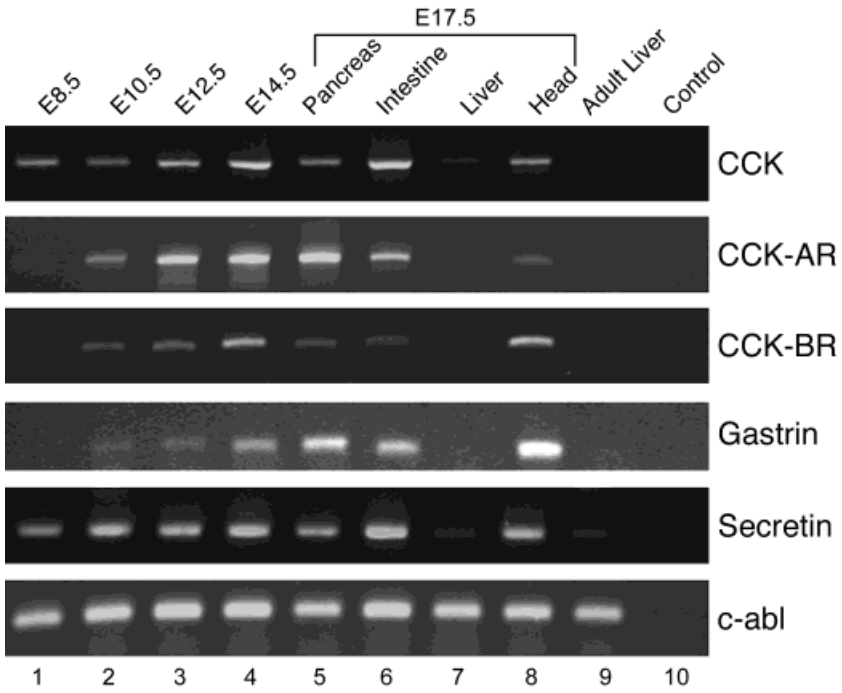

Fig. 7. Expression of CCK and related transcripts in embryogenesis. Samples of total RNA from either whole embryos (lanes 1–4), or individual tissues (lanes 5–9), or a negative water control (lane 10) were analyzed by RT-PCR. Primers were designed to detect transcripts for CCK, CCK-A receptor (CCK-AR), CCK-B receptor (CCK-BR), gastrin, secretin, and c-abl, as a positive control.

an extremely early event in enteroendocrine cell differentiation.

We detected extensive *lacZ* expression in the neuroepithelium of the early embryos (E8.5–E9.5), including the dorsal neural tube, consistent with neural crest expression. Expression in neural crest is significant for several reasons. First, CCK expression in the developing enteric nervous system may initiate in the neural tube in neural crest cells that continue to express CCK as they migrate to the gut. Second, the neural crest has been difficult to study in the mouse, due to a lack of reliable cellular markers. CCK gene expression may represent a marker for a certain subpopulation of neural crest cells that may be useful for future studies. Furthermore, it appears that secretin, another gastrointestinal peptide, is also expressed in embryos as early as E8.5. Since we demonstrated the presence of the secretin transcript in whole embryo RNA preparations, it remains to be determined whether the secretin gene is also transcribed in the neural tube/neural crest in a pattern similar to CCK.

Basic helix-loop-helix transcription factors play an important role in the cell-specific expression of genes and in the determination and differentiation of cells in various lineages. BETA2/NeuroD is a basic helix-loop-helix transcription factor that is expressed specifically in subsets of differentiating neurons and in endocrine cells (Lee et al., 1995; Naya et al., 1995). This transcription factor has been shown to activate secretin transcription (Mutoh et al., 1997). BETA2/NeuroD-deficient mice specifically lack both secretin and CCK intestinal endocrine lineages, while retaining others (Naya et al., 1997). We have determined that loss of CCK does not affect secretin transcript levels and that endocrine cells expressing Cck^{lacZ} do not produce secretin in place of CCK (not shown). Thus in CCK-deficient mice, both the CCK and secretin endocrine cell lineages are present. Future experiments will address whether BETA2/NeuroD directly activates CCK expression, and the role that BETA2/NeuroD may play in the differentiation of CCK-expressing endocrine cells and neurons.

In order to verify the expression pattern of the Cck^{lacZ} allele, we initially confirmed that expression in adult small intestine and brain was identical to expression of the wild-type CCK gene by immunohistochemistry and in situ hybridization. We verified the embryonic expression pattern in several ways. The presence of CCK transcripts in embryos as early as E8.5 was verified by RT-PCR. The cellular pattern of X-gal staining in developing enteric neurons and endocrine cells was confirmed by in situ hybridization and immunohistochemistry, respectively. While the Cck^{lacZ} expression and in situ hybridization represent transcription of the CCK gene, our studies did not test whether bioactive CCK peptide is produced. In order for CCK to be functionally active, the transcript must be translated and then the pro-peptide must be processed by a number of different processing enzymes. Our results do demonstrate that expression of the Cck^{lacZ} allele in neuronal lineages and intestinal endocrine cells appears to represent the true pattern of expression of the endogenous CCK gene.

Despite the widespread prenatal expression of CCK, the consequences of CCK deficiency were not apparent. The CCK-deficient male and female mice appeared to develop normally, and CCK was not required for viabil-

ity or fertility (Lacourse et al., 1999). Homozygous CCK-deficient embryos also expressed *lacZ* in the same patterns and were indistinguishable from heterozygous embryos. Thus, although CCK appears to be expressed early in developing neurons and endocrine cells, it does not appear to be necessary for their differentiation. As a secreted peptide, CCK requires receptors in order to function. We have demonstrated that transcripts for both CCK receptors are present as early as E10.5. The detection of CCK-A receptor and CCK-B/gastrin receptor transcripts suggests that fetal CCK may have previously undescribed functions that are difficult to detect phenotypically or are compensated for. While the CCK-B/gastrin receptor mediates the actions of both CCK and gastrin, CCK is the only known ligand for the CCK-A receptor. CCK-A receptor-deficient mice have been recently reported (Kopin et al., 1999). In agreement with our analysis of CCK-deficient mice, CCK-A receptor-deficient mice develop without visible abnormalities. Gastrin-deficient and CCK-B/gastrin-receptor deficient mice also develop grossly normally, although they have significant defects in the maturation of cells in the stomach involved with acid secretion (Nagata et al., 1996; Koh et al., 1997; Langhans et al., 1997; Friis-Hansen et al., 1998). Double mutant mice generated by crossing the CCK-deficient mice reported here to the gastrin-deficient mice reported in Friis-Hansen et al. (1998) also developed grossly normally (data not shown), demonstrating that although both CCK and gastrin are expressed early in embryogenesis, CCK-family peptides are not required for murine development.

EXPERIMENTAL PROCEDURES

Gene Targeting in ES Cells

The CCK targeting vector contained the bacterial *lacZ* reporter from plasmid pCH110 (Pharmacia, Gaithersburg, MD) and a neomycin selection cassette from plasmid pPNT (Tybulewicz et al., 1991) inserted into exon 2 of *Cck*. The *lacZ* reporter was introduced at the CCK translational start, at the site of a 168 bp deletion which removed the N-terminus, including the signal sequence for CCK (Fig. 1).

Mouse R1 ES cells (Nagy et al., 1993) were cultured on neo-resistant mouse embryonic fibroblast feeder cells in media supplemented with 10^3 units/ml of murine recombinant leukemia inhibitory factor (ESGRO; Life Technologies Inc., Bethesda, MD) as described (Friis-Hansen et al., 1998). After electroporation of the CCK targeting vector into R1 cells, colonies were selected in G418 (300 μg/ml) and isolated in individual wells of a 96-well culture dish. Genomic DNA was prepared (Ramirez-Solis et al., 1992) and colonies were screened using the polymerase chain reaction (PCR) with primers TS-Neo (5′CGCCTTCTATCGCCTTCTTGACGAGTTCTT) and TS-CCK (5′TTCTAGGAGACTCAACCAGGAGGTCCAGGT), to amplify a 1.5 kb product from targeted ES cells. Homologous recombination events were confirmed by Southern blotting and long-range PCR as described (Lay et al., 1998). Six correctly targeted clones were microinjected into C57BL/6J blastocysts, with three clones yielding chimeric mice that transmitted the targeted mutation through the germline. Chimeras were bred to C57BL/6J or 129/SvJ mice resulting in heterozygous CCK-deficient mice.

Southern Blot Analysis

Genomic DNA isolated from mouse tails was digested overnight with *Bam*HI, and Southern blotting was performed as described (Samuelson et al., 1995). The probe was a 0.7 kb *Eco*RI/*Bgl*II genomic DNA fragment from the 5′ flanking region (Fig. 1) that was ^{32}P-labeled by random oligonucleotide priming. Final wash conditions were 0.1× standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 60°C.

Genotyping of Mice or Embryos

Genotyping was done by multiplex PCR using three primers to detect the wild type (CS1: 5′ CTGGTTAGAAGAGAGATGAGCTACAAAGGC and CS2: 5′ TAGGACTGCCATCACCACGCACAGACATAC) or targeted (CS1 and LZ: 5′ TGTAGATGGGCGCATCGTAACCGTGCATCT) alleles. PCR reactions (50 μl) included approximately 100–500 ng of mouse genomic DNA, 200 μM dNTPs, 0.4 μM of primers CS1 and CS2, 0.1 μM of primer LZ, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.01% (wt/vol) bovine serum albumin and Taq polymerase. The cycle parameters were: (i) 94°C 5 min; (ii) 35 cycles of 94°C 30 sec, 65°C 30 sec, 72°C 45 sec; (iii) 72°C 5 min. Amplification products of 361 bp (wild type) and 454 bp (targeted) were detected by electrophoresis in 2% agarose with ethidium bromide staining.

RNA Preparation and Northern Blot Analysis

Total RNA was isolated from mouse tissues using either a guanidine thiocyanate homogenization/CsCl centrifugation procedure (Samuelson et al., 1988) or Trizol reagent (Life Technologies Inc.). Total RNA (20 μg) was electrophoresed in gels containing 2.2 M formaldehyde, transferred to Zeta-probe membrane (Bio-Rad) and hybridized with ^{32}P-labeled probes. Probes used were a 173 bp *Pst*I fragment of the mouse CCK cDNA (bases 142–315) and a 28S ribosomal RNA probe (Ambion). Imaging and quantitation were performed on a GS-505 Molecular Imager (Bio-Rad, Hercules, CA).

X-gal Staining

Heterozygous embryos were obtained from matings between heterozygous (Cck^{lacZ}/+) males and wild-type (CD1) females. Noon on the day of presence of a vaginal plug was taken to be embryonic day 0.5 (E0.5). Yolk sacs or tails were taken for genotyping as described above. For X-gal stained sections, embryos were removed and fixed in 4% paraformaldehyde at 4°C, frozen in OCT (Tissue-Tek), and cryosections (12 μm) were thaw mounted onto poly-L-lysine coated slides. Slides were fixed for 5 min in 0.2% glutaraldehyde at room tempera-

ture and stained overnight at 30°C in 0.1 M sodium phosphate pH 7.3, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.02% NP-40, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Boehringer Mannheim). Slides were counterstained with 0.5% neutral red, dehydrated, and coverslipped using Permount (Fisher Scientific, Fairlawn, NJ).

For X-gal stained whole-mount embryos or gastrointestinal tracts, the embryos or tissues were removed and fixed in 4% paraformaldehyde for 15–30 min, and stained 4 hr or overnight at 30°C, as described above.

Immunohistochemistry

Cryosections were fixed briefly in 4% paraformaldehyde, then X-gal stained as described above. The sections were blocked with 10% non-immune serum, then incubated with the primary antibody for either 2 hr at room temperature or at 4°C overnight. The following primary antibodies were used: rabbit anti-peripherin (1 : 1,000; AB1530, Chemicon International, Temecula, CA) and rabbit anti-CCK (1 : 4,000; AB1972, Chemicon). Primary antibodies were detected by immunofluorescent labeling with a Cy3-conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). No fluorescence was seen in controls stained with non-immune serum (not shown). The CCK antibody has been shown to be specific for CCK, by preabsorbing with CCK-33 before staining (Liddle et al., 1992). Rhodamine-phalloidin (Sigma Chemical Co., St. Louis, MO) was used with no secondary antibody for staining muscle actin. Photographs were taken using an Aristoplan fluorescence microscope (Leitz, Wetzlar, Germany).

In Situ Hybridization

Sense and antisense riboprobes were generated from a 0.7-kb *Bgl*II fragment of *Cck* containing exon 3 cloned into the pBluescript vector (Stratagene, La Jolla, CA). Riboprobes were either labeled with ^{35}S or with digoxigenin, and hybridized to cryosections overnight at 55°C. The final wash was performed in 0.1× SSC at 70°C. ^{35}S-labeled slides were dehydrated and dipped in NTB-2 emulsion (Kodak, Rochester, NY) diluted 1 : 1 with water, exposed for 1 week, developed and counterstained with cresyl violet (Sigma). Digoxigenin-labeled slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Mannheim Biochemicals, Indianapolis, IN), which was visualized using 0.2 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.2 mM nitroblue tetrazolium salt (Boehringer Mannheim).

RT-PCR

Reverse transcriptase reactions were performed with 2 μg of total RNA using the Superscript II RT Kit (Life Technologies, Inc.) with an oligo(dT) primer for the first strand synthesis. PCR reactions contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 3 mM $MgCl_2$, 0.01% bovine serum albumin, 0.2 mM dNTPs, Taq polymerase, and 0.2 μM primers. The primer pairs used were: CCK (5′AGCGGCGTATGTCTGTGCGTGGTGATG and 5′GGCTGACAGATTTCACATTGGGAC), CCK-AR (5′CATTCCTGCTACTCATCCTC and 5′CCTCAACCTTTCACCCCTGAGTA), CCK-BR (5′CTCCCTCCTCAACAGCAGTAG and 5′TGCATGCACTGCAGTATTCGA), gastrin (5′GCGCCACAACAGCCAACTATTCCCCAG and 5′CCAAAGTCCATCCATCCGTAGGCCTCTTCT), secretin (5′CACGCCGATGCTACTGCTGTTGC and 5′CATCTGGGTGGCCTGGTTGTTTCA), and c-abl (5′TTTATGGGGCAGCAGCCTGGAAAAGTACTTGGG and 5′TCACTGGGTCCAGCGAGAAGGTTTTCCTTGGAGTT). The PCR product sizes for the various genes are as follows: CCK, 470 bp (Vitale et al., 1991); CCK-AR, 1.25 kb (Lacourse et al., 1997); CCK-BR, 543 bp (GenBank Accession No. AF019371); gastrin, 318 bp (Friis-Hansen et al., 1996); secretin, 384 bp (Lan et al., 1994); and c-abl, 239 bp (Ben-Neriah et al., 1986). The RT-PCR products were detected by electrophoresis in agarose with ethidium bromide staining.

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