Enteroendocrine cell expression of a cholecystokinin gene construct in transgenic mice and cultured cells

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Submitted 6 August 2004; accepted in final form 8 October 2004

CCK, which stimulates the entry of bile and pancreatic juice into the intestine, is produced in one subtype of enteroendocrine cell termed the I cell (33). CCK, as is seen with several other gastrointestinal hormones, is also widely expressed in the nervous system, with high levels of CCK found in the cortex, hippocampus, and thalamus of the central nervous system (2). Cellular expression of CCK in the intestine varies during development. Although in adults, the primary site of expression is in enteroendocrine cells of the proximal small intestine, during fetal development, CCK gene expression occurs in the enteric nervous system (ENS) as it develops from migratory neural crest (20). Widespread ENS expression is transient, extinguishing at approximately embryonic day 15 (E15) in the mouse. Endocrine cell expression is activated at the time that ENS expression is diminishing, which corresponds to the morphological transformation from a stratified undifferentiated epithelium to a columnar epithelium when the fingerlike villi start to emerge. Epithelial cell expression persists into the adult, where renewal of the epithelium from intestinal stem cells leads to the continual development of CCK-expressing endocrine cells throughout the lifespan of the organism.

Recent studies (31, 32) with genetically engineered mice suggest that the Notch signaling pathway plays an important role in lineage determination in the intestine. Not surprisingly, some of the key signaling determinants for neuronal differentiation appear to play a role in enteroendocrine cell differentiation. The basic helix-loop-helix (bHLH) transcription factor neurogenin 3 (Ngn3) is required for formation of both pancreatic and intestinal endocrine cells, as well as a subset of endocrine cells in the stomach (6, 17, 21). Mice with a complete loss of Ngn3 as a result of targeted gene disruption do not form intestinal endocrine cells, although the other cell types in the intestine appear to develop normally (17). Another bHLH transcription factor, NeuroD/BETA2, which is activated by Ngn3 (15), has been implicated in activation of Cck expression or I cell development, because mice with a targeted gene disruption of NeuroD/BETA2 do not produce intestinal CCK (26).

Although candidate transcription factors governing development of I cells and/or activation of CCK expression in enteroendocrine cells are starting to emerge from these and other studies, little is known about the cis-acting CCK gene sequences required for enteroendocrine cell expression. Transfection studies in cultured cells have identified general transcriptional enhancer sequences in the rat or human CCK
promoter that do not appear to determine tissue or cell specificity (4, 7, 10–12, 24, 27, 29). The aim of the present study is to define a CCK genomic region capable of directing gene expression in intestinal I cells. Our approach involved the use of transgenic mice to analyze the tissue and cell specificity of reporter gene expression driven by a mouse CCK genomic fragment. In addition, expression in the enteroendocrine cell line STC-1 was examined to define an in vitro system to analyze endocrine-specific CCK expression.

METHODS

Transgene cloning. The CCKL1 transgene construct contained 6.4 kb of mouse CCK sequences, including 5.2 kb of 5′-flank downstream of a BamHI site and the first exon and intron, which were joined to the lacZ reporter at the translational start in the second CCK exon (Fig. 1). The construct was prepared by subcloning sequences from the CCK-lacZ targeting vector, which contained a small deletion in intron 1 (146 bp downstream of the BseI site) that had previously been shown not to be important for correct expression in intestine (20). The lacZ in the targeting vector was derived from pCH110 (Pharmacia), which contains an SV40 poly(A) cassette. Fusion of lacZ and Cck used a PCR strategy, with the transition at the ATG site for both genes.

Production of transgenic mice. The 10-kb CCKL1 transgene was isolated from the vector by BamHI/kpnI digestion and purification from an agarose gel using the SpinBind DNA Recovery System (FMC Bioproducts). Purified transgene DNA was microinjected into (C57BL/6 X SJL)F2 zygotes by the University of Michigan Transgenic Animal Model Core (www.med.umich.edu/tamc/). In one set of seven founders, transgenic lines were formed by breeding to strain C57BL/6J. After initial screening for expression in intestine by RT-PCR, lines J11, J12, and J14 were maintained for detailed analysis by continued breeding to C57BL/6J. Progeny of the N3 and later generations were used for analysis. Transgene expression was analyzed in intestinal I cells of a transgenic line (20). Nontransgenic littermates were used as negative controls in this study. Arrowheads indicate the “knock-in” allele were used as positive controls. CCKL1 transgene-derived PCR product than the transgene band.

Quantification of β-galactosidase activity. Extracts were prepared by homogenizing tissues in 100 mM potassium phosphate (pH 7.8) and 0.2% Triton X-100 (10 ml/g tissue), followed by three freeze (dry ice/ethanol)-thaw (37°C) cycles and clearing by centrifugation (10,000 g) at 4°C. β-galactosidase activity was determined on triplicate samples for each extract using a chemiluminescence assay (Galacto-Light Kit from Tropix), normalized to protein measured using a Bradford protein assay (Bio-Rad), and expressed as fold increase over wild-type background measurements. Significance was determined with an unpaired Student’s t-test, with P < 0.05 considered significant (n = 3 mice/group).

X-gal staining of sections. Cryosections of adult tissues (12 μm, intestine; 20 μm, brain) were thaw-mounted onto poly-l-lysine-coated slides. The slides were fixed at room temperature for 5 min in 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) containing 5 mM EGTA and 4 mM MgCl2, rinsed, and then stained overnight at 30°C in 0.1 M sodium phosphate buffer (pH 7.3), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.02% Nonidet P-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 and dehydrated, and coverslips were added using Permount (Fisher Scientific). Photographs were taken on a Leitz Aristoplan microscope.

Fig. 1. CCKL1 transgene structure and identification of transgenic mice. A: schematic diagrams of the mouse CCK gene, the CCKL1 transgene construct, and the CcklacZ “knock-in” allele. CCKL1 contains 6.4 kb of mouse genomic sequence, including 5.2 kb of 5′-flank. Mice containing the lacZ “knock-in” allele were used as positive controls in this study. Arrowheads indicate the genotyping primers. B: BamHI. B: PCR screen to identify transgenic mice. Samples of tail DNA from each of the 3 transgenic lines (J11, J12, and J14), Ccklox2, and nontransgenic (Ntg) mice were used in a multiplex PCR assay to amplify Cck-lacZ (lacZ) and endogenous Cck sequences. The higher transgene copy number in lines J11 and J12 leads to much stronger amplification of the transgene-derived PCR product than the product resulting from the endogenous gene.

AJP-Gastrointest Liver Physiol • VOL 288 • FEBRUARY 2005 • www.ajpgi.org
Heterozygous embryos were obtained from matings between transgenic males and wild-type (C57BL/6J) females. Noon of the day of presence of a vaginal plug was taken to be E0.5. Genomic DNA was prepared from tissue samples (tail or leg) and genotyped as described above. For X-gal-stained sections, gastrointestinal tracts were dissected from embryos and fixed for 1 h in 3% paraformaldehyde in PBS (8 mM Na₂HPO₄·7H₂O, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4) on ice before rinsing in PBS and embedding in OCT (Tissue-Tek). Cryosections (14 μm) were thaw mounted onto Superfrost/Plus slides (Fisher Scientific) and X-gal stained as described above but without glutaraldehyde fixation.

**Immunohistochemistry.** Cryosections were fixed for 5 min in 4% paraformaldehyde and X-gal stained as described above. After being blocked with 10% nonimmune serum, X-gal-stained slides were incubated with primary antibody at 4°C overnight, followed by detection with a Cy3-conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories). The primary antibodies used were rabbit anti-peripherin (1:1,000; Chemicon #AB1530) and rabbit anti-CCK (1:4,000; Chemicon #AB1972). Control slides on which the primary antibody was omitted showed no staining. Photographs were taken on a Leitz Aristoplan fluorescence microscope.

**Cell culture and transient transfection.** Chinese hamster ovary (CHO)-K1, IEC-6, and Hela cells were obtained from American Type Culture Collection, and STC-1 enteroidocrine cells were obtained from Andrew Leiter (New England Medical Center). CHO and STC-1 cells were cultured in DMEM (high glucose), IEC-6 cells were cultured in DMEM (high glucose) supplemented with 0.1 U/ml bovine insulin, and Hela cells were cultured in MEM supplemented with sodium pyruvate and nonessential amino acids; all media contained 10% fetal bovine serum and penicillin/streptomycin. CCKL1 expression was tested in cultured cells after gene transfer with Lipofectamine Plus (Invitrogen). Cells were plated in six-well plates at a density of 1–2 × 10⁵ cells/well and transfected 24 h later with 1 μg DNA/well. Cells were harvested 48 h later, and β-galactosidase activity was measured in cell lysates using the Galacto-Light Kit (Tropix) and an Optocomp II luminometer (MGM Instruments). β-galactosidase activity was measured in cell lysates using the Galacto-Light Kit (Tropix) and an Optocomp II luminometer (MGM Instruments). pCMV-SPORT-β-gal (Invitrogen) and pGEMnlacF were used as positive and negative controls, respectively. pGEMnlacF was constructed by subcloning the lacZ gene from pnlacF (Richard Palmiter, University of Washington, Seattle, WA) into the pGEM4 vector (Promega).

**RESULTS**

**Tissue-specific expression in transgenic mice.** To identify a CCK genomic fragment capable of expressing reporter genes in intestinal endocrine cells, a construct containing 6.4 kb of the mouse CCK gene driving bacterial lacZ was tested in transgenic mice (Fig. 1). On the basis of preliminary measurements of β-galactosidase expression by RT-PCR, three of seven transgenic lines were determined to express the transgene in intestine and were selected for detailed analysis (lines J11, J12, and J14). Transgene copy number was determined by genomic Southern analysis, with 35, 20, and 2 copies measured for lines J11, J12, and J14, respectively (not shown).

CCKL1 expression was determined by measuring β-galactosidase activity in tissue extracts, and levels were compared with nontransgenic mice and to a “knock-in” strain, which had the lacZ gene inserted into the endogenous CCK locus (CcklacZ; Fig. 2). Four tissues were tested, with transgene expression detected in CCK-expressing tissues (intestine and brain). Expression was not observed in nonexpressing tissues (liver and stomach), with the exception that very low β-galactosidase activity was detected in J11 liver. There was some variation in expression among the lines, with two lines (J12 and J14) showing significant expression in intestine and two (J11 and J12) expressing in brain. Transgene expression was significantly lower than expression of the endogenous CCK gene as indicated by the higher β-galactosidase activity in CcklacZ mice compared with the CCKL1 transgenics. RT-PCR analysis of RNAs isolated from transgenic tissues confirmed the β-galactosidase assay results and also showed that there was no transgene RNA produced in the pancreas or heart in the three lines (not shown).

**Cell-specific transgene expression.** To determine the cellular specificity of transgene expression we examined cryosections of adult transgenic mouse tissues that had been stained with X-gal. In agreement with the results of the β-galactosidase activity assays, X-gal-stained cells were observed in intestinal
sections from lines J12 and J14 but not line J11 (Fig. 3). Scattered single positive cells were seen in both expressing lines, although the cellular localization differed markedly between the two. Line J14 expressed the transgene in epithelial cells (Fig. 3C), similar to the normal pattern of expression in scattered single cells seen in CcklacZ (Fig. 3D). In contrast, line J12 expressed the transgene in cells located in the submucosa (Fig. 3B).

To determine whether the epithelial cell type expressing the transgene in J14 mice is CCK-expressing endocrine cells, cryosections were costained with X-gal and an antibody to CCK. The X-gal-positive cells stained with the CCK antibody, demonstrating that the transgene was expressed in the correct cell type in this transgenic line (Fig. 3F). However, only a proportion of the CCK-positive cells expressed lacZ.

The submucosal staining pattern of J12 mice suggested expression in enteric neurons. This was confirmed on costaining with X-gal and an antibody to the neuronal marker peripherin (Fig. 3G). Although expression in enteric neurons of the duodenum is not the normal pattern of CCK expression in the adult, it is characteristic of an earlier developmental pattern of gene expression in the fetus (20).

Examination of CCKL1 expression in brain sections showed that J11 and J12 mice contained X-gal-stained regions (Fig. 4) as predicted from the β-galactosidase activity measurements. Transgene expression reflected a subset of the normal patterns seen in CcklacZ (Fig. 4A), with J12 expressing in cortex, hippocampus, and thalamus and J11 expression more restricted with staining observed in the cortex alone. J14 mice did not express the transgene in brain (not shown).

**Developmental expression in intestine.** CCKL1 transgenic lines J12 and J14 were evaluated during development to determine whether the timing of activation of transgene expression in intestine is similar to the timing of activation of the endogenous gene. The normal pattern of CCK gene expression in the developing gut is complex, with a change in the site of expression from neurons to endocrine cells occurring at approximately E15 in the mouse (20). We evaluated transgenic embryos at time points before (E14.5) and after (E16.5) this switch and compared the expression pattern with CcklacZ (Fig. 5). In J14, the timing of activation of expression in endocrine cells was similar to the endogenous gene, with single epithelial cells stained by E16.5 (Fig. 5F). However, this line did not exhibit the early neuronal staining pattern seen at E14.5 in CcklacZ mice (compare Fig. 5, A and C). Line J12 embryos exhibited the neuronal staining pattern at the early time point, similar to the endogenous gene (Fig. 5B). However, unlike the endogenous pattern, J12 transgene expression was not extinguished at E16.5 (Fig. 5E) but instead persisted into the adult (Fig. 3).

**Fig. 3.** Cellular localization of CCKL1 expression in transgenic intestine. Intestinal cryosections from adult mice were stained with X-gal. Transgenic lines J11 (A), J12 (B), and J14 (C) were compared with CcklacZ (D) and Ntg mice (E). Scattered single X-gal-positive cells are indicated by arrows. Costaining with X-gal and a cell-specific antibody identified the cell type expressing the transgene. A J14 section was costained with X-gal and a CCK antibody (F), and a J12 section was costained with X-gal and an antibody to the neuronal marker peripherin (G). Bright-field and fluorescence images were merged to show correspondence of staining.
Expression in transgenic founders. To determine whether the two patterns of CCKL1 expression in the gut (neuronal and endocrine) observed in the J12 and J14 transgenic lines are an accurate reflection of the activity of this transgene, another set of microinjections was done, and 15 transgenic founder mice were analyzed. Adult intestines were cryosectioned and stained with X-gal. Three founders expressed the transgene in single scattered cells in the epithelium, similar to the pattern seen for line J14, and three founders expressed in the submucosa in a pattern similar to J12 (Fig. 6). Thus the overall efficiency of expression (6/15; 40%) was similar to the first set of transgenics that were bred into lines (3/7; 43%), with one-half of the CCKL1-expressing transgenics exhibiting an epithelial-enteroendocrine pattern and the other half exhibiting a submucosal-neuronal pattern.

CCKL1 expression in cultured cells. The CCKL1 transgene was next tested in cultured cells to determine whether the enteroendocrine cell specificity observed in transgenic mice was maintained in an in vitro system. Four different cell lines were used: STC-1, which is a CCK-expressing mouse enteroendocrine cell line derived from an intestinal tumor; IEC-6, a rat intestinal epithelial line; and the two nonintestinal cell lines CHO and Hela. The results of transient CCKL1 transfection are shown in Fig. 7. The CCKL1 transgene expressed well in STC-1, with ~100-fold increased activity relative to the negative control pGEMlacF. In contrast, CCKL1 was poorly expressed in the other three lines, with activity similar to the negative control. The positive control pCMV6Sport β-gal was expressed well in all four lines demonstrating that the gene transfer was effective.

DISCUSSION

In this study, we have identified a 6.4-kb mouse Cck gene fragment capable of driving gene expression into enteroendocrine I cells. A Cck-β-galactosidase reporter gene construct was analyzed in both transgenic mice and cultured cells. In adult mice, CCK is predominantly expressed in intestinal endocrine cells and central neurons. By analyzing β-galactosidase activity, we were able to demonstrate that the transgenic

Fig. 4. CCKL1 expression in transgenic brain. Comparison of X-gal-stained coronal cryosections from CcklacZ (A) and transgenic lines J11 (B) and J12 (C). C, cortex; H, hippocampus.

Fig. 5. CCKL1 expression during intestinal development. Transgenic embryos were collected at embryonic day 14.5 (E14.5) or 16.5 (E16.5), cryosectioned, and stained for β-galactosidase activity with X-gal followed by counterstaining with neutral red. The patterns of staining at E14.5 (A-C) and E16.5 (D-F) for J12 (B and E) and J14 (C and F) were compared with CcklacZ (A and D). Arrows indicate X-gal-positive cells.
lines expressed in intestine and brain. Thus this 6.4-kb mouse gene region directs appropriate tissue-specific expression. Analysis of transgenic lines and founder mice demonstrated that the CCKL1 transgene was expressed in 40% of the transgenic mice that carried the construct (3 of 7 transgenic lines and 6 of 15 founders). The level of transgene expression in the intestine did not correlate with the number of copies of the transgene incorporated into the mouse genome. For example, line J14 had the highest expression, yet it had the lowest transgene copy number (2 copies per genome).

Cellular expression of the transgene in the intestine fell into two patterns. One pattern represented by line J14 was characterized by reporter gene expression in CCK-expressing enteroendocrine cells, as demonstrated by costaining intestinal tissue sections for β-galactosidase and CCK peptide. This result suggests that the minimal cis-sequences required for CCK gene expression in intestinal I cells are contained within the 6.4-kb mouse genomic gene fragment. The other pattern of intestinal expression was represented by line J12 with reporter expression in enteric neurons, as demonstrated by costaining with a peripherin antibody; line J12 did not express in the epithelium. These two patterns of staining were also observed in transgenic founders, with one-half of the expressing transgenics following the epithelial-endocrine staining pattern and the other half following the submucosal-neuronal pattern. The variability in cell specificity and the low efficiency of expression of the transgene in I cells suggests that key transcriptional regulatory sequences such as a locus control region are missing from the 6.4-kb genomic fragment (22). Thus transgene expression could be influenced by the neighboring genomic sequences located at the unique integration site for each transgenic founder/line. Many transgenic mouse studies have observed variable expression among transgenic lines containing the same construct, which is thought to result primarily from the influence of the surrounding chromatin on transgene transcription (28). Variable levels of expression are common, and some variation in tissue specificity is also observed for many transgenes. However, the expression pattern of CCKL1 is unusual. Among the expressing lines, correct tissue specificity was observed but cell-specific expression varied between endocrine cells or neurons. The either/or expression pattern is surprising and might reflect interactions of the transgene with surrounding regulatory elements at the various insertion sites. Alternatively, variable transgene copy number could affect the expression in the transgenic lines. Transgene expression was shown to vary with copy number in one study in which reduction of a large transgene array by Cre-mediated recombination resulted in increased expression (5, 13). A mechanistic understanding of the expression pattern of CCKL1 in trans-
genic mice will not be possible until the elements regulating cell-specific expression have been identified.

The two patterns of transgene expression reflect the endocrine/neuronal expression of CCK. In the mouse, CCK expression in neurons and endocrine cells in the intestine is developmentally regulated (20). During fetal development, the CCK gene is expressed from E10.5 to E15.5 in developing enteric neurons. Transcription in enteroendocrine cells initiates at E15 and continues into the adult. The activation of CCKL1 transgene expression in line J14 between E14.5 and E16.5 suggests that the 6.4-kb genomic fragment contains the minimal information for proper developmental expression in CCK-expressing enteroendocrine cells. Line J12 followed the early activation in the developing ENS with transgene expression detected by E14.5. However, ENS expression was not extinguished appropriately and instead continued into the adult, indicating that different regulatory sequences may participate in activation and extinction of expression in enteric neurons. Moreover, activation of endocrine-cell transgene expression and extinction of ENS expression may be coordinated, because none of the transgenics expressed in both endocrine cells and neurons.

Although little is known about the mechanisms regulating tissue-specific expression of CCK, one candidate tissue-specific transcription factor is NeuroD/BETA2, a bHLH factor that is critical for the differentiation of subsets of neurons and endocrine cells (19, 23, 26). Mice lacking NeuroD/BETA2 do not develop CCK- and secretin-expressing enteroendocrine cells in the small intestine (26). Furthermore, NeuroD/BETA2 has been shown to have a role in the terminal differentiation of secretin-expressing enteroendocrine cells (25). Because CCK and secretin have been hypothesized to have a shared lineage, NeuroD/BETA2 may also play an important role in controlling CCK gene expression and/or I cell development.

Previous studies of CCK transcriptional regulation have largely focused on the use of cultured cells to map regulatory sequences upstream of the transcriptional initiation site. Surprisingly, constructs containing as little as ~100 bp of the promoter of the rat and human CCK genes were capable of driving reporter gene activity in a variety of cell lines, including both CCK-expressing neuronal (10, 29) and enteroendocrine (1, 3) lines, and non-CCK-expressing cell lines, such as GH3 (18), COS-7 (4), and MCF-7 (12). In light of these findings, our observation that the CCKL1 construct was expressed in STC-1 but not in HeLa, CHO, or IEC-6 was unexpected. Our results support the conclusion that the mouse 6.4-kb Cck fragment contains the necessary sequences to direct expression in CCK-expressing endocrine cells. Furthermore, the restricted expression suggests that there are tissue- or cell-restrictive regulatory elements upstream of ~100 that silence expression in non-CCK-expressing cell lines. This notion is supported by transfection studies that demonstrated reduced expression of a ~778-bp rat CCK promoter fragment compared with a ~105-bp promoter construct in the MCF-7 human breast carcinoma cell line (12).

Deletion studies of the rat and human CCK promoters identified three important elements in the proximal 100 bp: an E box (bHLH-ZIP binding site), a combined cAMP- and 12-O-tetradecanoylphorbol 13-acetate-response element (CRE/TRE), and an Sp1 element. The CRE/TRE element at ~80 bp upstream of the transcription start site is of particular interest, because it integrates a number of different signals through the phosphorylation of CREB and its associated transcriptional coactivator CREB-binding protein (CBP) (9), including growth factors (10), secretagogues (1, 3), and Ca\(^{2+}\) and cAMP signals (8). The three elements described by transfection studies appear to be highly conserved, because homologous sequences are also present in the mouse and bullfrog (Rana catesbeiana) promoters (29). Whereas that may imply that this region is important for proximal promoter function, it does not address which regions regulate developmental and tissue-specific gene expression.

The transgenic mouse study reported here is the first analysis of CCK transgene expression in enteroendocrine cells in vivo. A previous study (16) of a 2.4-kb rat CCK-lacZ transgene in transgenic mice showed reporter expression in brain; however, intestine was not analyzed. Thus our observation that 6.4 kb of the mouse CCK gene is capable of driving reporter gene expression in both enteroendocrine cells and neurons is the first report that localizes the DNA sequences regulating endocrine cell expression. The identification of a mouse gene fragment that expresses in intestinal I cells can be used in future experiments to drive the expression of proteins into I cells. In addition, together with the in vitro analysis in STC-1 cells, the 6.4-kb fragment can be used as a starting point to do deletion/mutations to identify the key regulatory sequences for enteroendocrine cell expression.

ACKNOWLEDGMENTS

We thank S. Camper for helpful comments and J. Friedman (Rockefeller University) for the mouse CCK genomic clone. We also acknowledge T. Saunders and the University of Michigan Transgenic Animal Model Core and the Organogenesis Morphology Core.

GRANTS

This work was supported by the University of Michigan Gut Peptide Research Center (DK-07367), Cancer Center (CA-46592), Rheumatic Diseases Center (AR-20557), and Organogenesis Center and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-062041 (to L. C. Samuelson).

J. M. Lay was supported by the Cellular Molecular Biology Training Grant 5T32GM07315 and by the Organogenesis Training Grant HD-07505.

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