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# Differential expression of the mouse cholecystokinin gene during brain and gut development

**(brain–gut peptides/tissue-specific gene expression/developmentally regulated gene expression)**

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**ABSTRACT** **Cholecystokinin (CCK) is a neuropeptide found in brain and intestine. In this report, we have isolated a cDNA clone that encodes CCK from a mouse brain cDNA library. This cDNA clone has extensive homology to CCK precursors that have been sequenced previously. Southern blots of genomic DNA probed with this cDNA clone revealed single bands for each of eight different restriction enzymes, all of which could be accounted for by a single genomic clone, suggesting that the CCK gene is present as a single-copy gene in mice. RNA blots, primer extensions, and S1 nuclease protection assays have suggested that the same RNA start site is utilized in brain and in gut. Finally, we have shown, by using RNA blots and a radioimmunoassay specific for CCK, that CCK is expressed at maximum adult levels in intestine at birth but that adult concentrations of CCK and its mRNA are not reached in brain until much later in development.**

Differentiation, viewed at the cellular level, is characterized by the production of tissue-specific proteins and mRNAs in specialized cells (1). Often, these specialized proteins are unique to a particular cell type (2). Tissue specific proteins and the genes that encode them can also be expressed in some, but not all, tissues (2–4). In such a case, one of several different regulatory mechanisms might be operative. Both tissues could utilize the same RNA start site on the same gene (5). Both tissues could express different mRNAs transcribed from the same gene by differentially processing the same primary transcript or by using different promoters (4, 6, 7). Finally, both tissues could express different members of a multigene family, in which each member encodes the same or a similiar protein (3, 8). Thus, a full understanding of how a tissue-specific protein can be expressed at a restricted number of anatomic sites requires determination of the number of genes encoding that protein and characterization of the mRNAs from each of the expressing tissues.

The growing family of brain–gut peptides provides an example of proteins that are expressed in a subset of tissues. We have begun to analyze the peptide cholecystokinin (CCK) with the aim of understanding how the gene encoding it is regulated in brain and in intestine during development.

CCK is a brain–gut peptide that functions as a hormone in plasma and, in all likelihood, as a neurotransmitter in brain (9–13). Proteins with immunoreactivity to antibodies specific for CCK have been sequenced from dog and pig intestine and, recently, from dog brain (12, 14, 15). These peptides are 59, 39, 33, and 8 amino acids long and all share the same octapeptide carboxyl terminus. In intestine, the majority of CCK immunoreactivity is present as CCK-33 (33-amino acid CCK) (12). In brain, the amount of CCK immunoreactive material exceeds that found in intestine and is almost exclusively CCK-8 (13). It has been recently demonstrated, however, that in pigs and dogs, the CCK protein precursors in brain and gut are identical to each other (15, 16). To define precisely the intact precursor of CCK in mouse brain and to study the expression of the CCK gene during brain and gut development, we have used a chemically synthesized oligonucleotide to isolate a cDNA clone encoding CCK from a mouse brain cDNA library. The sequence of this cDNA clone differs from that of the previously sequenced pig and dog CCK in only two positions, and it differs from CCK derived from a rat medullary carcinoma of the thyroid in only one position (16, 17). We have used this clone to define the genomic organization of CCK and to quantify the levels of CCK mRNA in brain and intestine during development.

## MATERIALS AND METHODS

Poly(A)$^+$ RNA was prepared from adult mouse brain by the guanidium thiocyanate method followed by passage over an oligo(dT) cellulose column as described (18, 19). A cDNA library was constructed from this mRNA by the method of Land *et al.* (20). The library was screened by using the colony lift procedure described by Hanahan and Meselson (21). The filters generated from colony lifts were hybridized to a mixed oligonucleotide probe of the sequence 5′ TA(C/T)ATGGG-(A/G/C/T)TGGATGGA 3′, which is predicted by amino acids 2–7 of CCK-8. The oligomer was synthesized by using solid-phase phosphoramidite chemistry and was labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase. This probe would be expected to differ from the coding sequence of gastrin, a related peptide, in 7 of 17 positions. Hybridizations were performed on duplicate filters as described (22). Clones giving autoradiographic signals on duplicate filters after washing at 48°C were replated and rescreened.

Seventeen separate hybridizing colonies were isolated in this fashion. Two of the clones were sequenced by the methods of Maxam and Gilbert (23) and Sanger *et al.* (24). Southern blots and RNA blots were prepared as described (2, 25, 26). Nick-translations were performed as described by Rigby *et al.* (27). Genomic clones were isolated by screening a mouse genomic library (kindly provided by S. Tonegawa) with the previously isolated cDNA by using the method of Benton and Davis (28). The primer-extension assay was performed by hybridizing a single-stranded end-labeled *Nae* I/*Pst* I fragment (purified from an acrylamide gel) to poly(A)$^+$ RNA from brain and intestine followed by addition of reverse transcriptase and deoxynucleotides (29). The *Nae* I/*Pst* I fragment was treated with bovine alkaline phosphatase and end-labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase.

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Abbreviations: CCK, cholecystokinin; kb, kilobase(s); bp, base pair(s).

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The primer used in this experiment is underlined in Fig. 4*A*. The primer extension products were electrophoresed on an acrylamide/3.5% urea gel. The S1 nuclease protection assay was performed as described (30). A 1755-base-pair (bp) *Hin*fI fragment of the clone was treated with BAP, radiolabeled with T4 polynucleoide kinase and [$\gamma$-$^{32}$P]ATP, and purified from an agarose gel. The purified fragment was hybridized to 50 $\mu$g of total RNA from brain and intestine at 59°C for 12 hr in 40 mM Pipes, pH 6.4/1 mM EDTA, pH 8.0/0.4 M NaCl/80% formamide. The resulting hybrids were digested with 1000 units of S1 nuclease for 60 min at 37°C and were resolved on an acrylamide/5% urea gel.

To monitor the concentration of CCK in brain and intestine during ontogenetic development, whole brain and upper small intestine were dissected from fetal, neonatal, and adult mice at defined time points. Boiling water extracts of these tissues were analyzed for CCK immunoreactivity by using a COOH-terminal radioimmunoassay as described (31).

## RESULTS

A cDNA library of 40,000 members was constructed from adult mouse brain poly(A)$^+$ RNA, using the double-tailing method (20). The 17-nucleotide 8-fold degenerate probe predicted by amino acids 2–7 of CCK octapeptide described above was end-labeled with T4 polynucleotide kinase and used to screen the brain cDNA library (21, 22).

Seventeen separate bacterial colonies that hybridized to the probe were selected, and the two clones with the largest inserts were grown in quantity and sequenced. Both chemical and dideoxy sequencing were used to sequence the longest of the cDNA clones (23, 24). The sequence is shown in Fig. 1; amino acids shown above the line represent mismatches between the mouse brain precursor and the previously sequenced pig intestinal precursor, and amino acids below the line represent mismatches between the mouse brain and previously sequenced dog intestine (12, 14). The amino acids are numbered from 1 to 49 beginning at the carboxyl terminus of the CCK octapeptide common to all molecular forms of this hormone. Thus, the amidated carboxyl-terminal phenylalanine, which is numbered 1 in Fig. 1, is found at the COOH terminus of all the CCK peptides. The sequence shown here is identical to that found in rat medullary thyroid carcinoma, with the exception of a Ser-Gly substitution at amino acid 19 relative to CCK-8 (17).

The sequence data presented here suggest that either the same or a very similar CCK precursor is present in brain and intestine. The data do not resolve whether the same or different genes are active in these two tissues. As a first step toward defining the genomic organization of this hormone, we prepared Southern blots on mouse genomic DNA by using a variety of different restriction enzymes. Single bands of single-copy intensity were seen for a number of different restriction enzymes including *Bam*HI, *Bgl* II, *Eco*RI, *Hin*dIII, *Xba* I, *Pst* I, *Alu* I, and *Taq* I. Data for several of these enzymes are shown in Fig. 2*A*.

We have also screened four genomic equivalents of a mouse genomic library that is known to be complete by using the cDNA clone as a probe, and we have isolated a single genomic clone, $\lambda$C5, that has a 15-kilobase (kb) insert that accounts for all the hybridizing bands seen on the Southern blots (Fig. 2*B*). Of the eight separate genomic clones that were isolated, four had the same restriction map as $\lambda$C5. Four other identical isolates, $\lambda$C4, shared several restriction sites with $\lambda$C5 and were shown to overlap C4 at the 3′ end on the basis of its restriction map and heteroduplex analysis demonstrating a 10-kb overlap without any single-stranded regions. These data argue strongly that CCK is present as a single copy in the mouse genome.

We next compared the mRNAs from these tissues by using the RNA blot procedure. Equal amounts of poly(A)$^+$ RNA from brain, stomach, duodenum, and kidney were electrophoresed on agarose gels, transferred to nitrocellulose, and hybridized to the nick-translated cDNA clone. The resulting autoradiographs revealed a broad band of hybridization in the lanes for brain and intestine (Fig. 3). The mRNAs from brain and gut were of indistinguishable size, and it appeared that there might be two separate bands in the experiment shown. A small amount of mRNA also appeared to be present in mouse kidney (lane e). To define precisely the lengths of the brain and gut mRNAs, a primer-extension assay was performed. An end-labeled single-stranded probe was prepared by digesting the plasmid containing the cDNA clone with *Pst* I and *Nae* I, phosphatase treatment of the resulting fragments

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49  48  47  46  45  44   43  42  41  40  39  38  37  36  35
                                                     ala
arg ala arg leu gly ala leu leu ala arg tyr ile gln gln val
CGG GCG CGC CTG GGC GCA CTG CTA GCG CGA TAC ATC CAG CAG GTC
        his                                          ala
 34  33  32  31  30  29  28  27  26  25  24  23  22  21  20
                         val     met
arg lys ala pro ser gly arg met ser val leu lys asn leu gln
CGC AAA GCT CCT TCT GGC CGC ATG TCC GTT CTT AAG AAC CTG CAG
                                 ile

 19  18  17  16  15  14  13  12  11  10   9   8   7   6   5
ser leu asp pro ser his arg ile ser asp arg|asp tyr met gly
AGC CTG GAC CCC AGC CAT AGA ATA AGT GAC CGG GAC TAC ATG GGC

  4   3   2   1
trp met asp phe|gly arg arg ser ala glu asp tyr glu tyr pro
TGG ATG GAT TTT GGC CGG CGC AGT GCC GAG GAC TAC GAA TAC CCA


ser AM
TCG TAG TGGGCCAGCGTCTTGGCCCTGCTTGGAGGAGGTGGAATGAGGAAACAACCACAC

ATACGACCCCTCGCCTCTAATGTCTGACGTTTTGAGTATCTATTTATTAAGTCCCAATGT

GAAATCTGTCCAAAGTGCAATGCAGCCACATCTCAGCCTAGCTGTGTGGTCGGAAGG
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FIG. 1. DNA sequence of a mouse brain cDNA clone encoding CCK. A cDNA clone from mouse brain encoding CCK was isolated by probing a mouse brain cDNA library with an oligonucleotide primer specific for the CCK octapeptide (21, 22). The cDNA insert was sequenced by the methods of Maxam and Gilbert (23) and Sanger *et al.* (24). The amino acid sequence predicted by the DNA sequences are shown. Amino acids above the line are those that differ between pig intestine and mouse brain, and those that are shown below the line are those that differ between dog intestine and mouse brain (15–17). The cleavage sites used in protein processing are underlined. The CCK-8 octapeptide sequence is bordered by vertical lines. The amino acids are numbered beginning at the carboxyl-terminal phenylalanine of the CCK octapeptide and advancing toward the amino terminus.

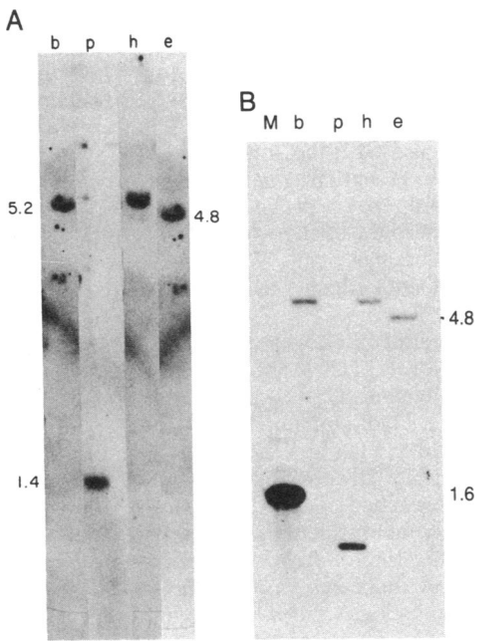


FIG. 2. (*A*) Southern blots of genomic DNA. Fifteen micrograms of genomic DNA was digested with each of several restriction enzymes. The cleaved DNA was electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to the nick-translated cDNA clone (26, 27). The filter was hybridized for 24 hr at 65°C in 5× NaCl/Cit/2× Denhardt's solution/0.2% $NaDodSO_4$/100 μg of denatured salmon sperm DNA per ml with the radioactive probe (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate; 1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone). The filter was washed in 2× NaCl/Cit/0.2% $NaDodSO_4$ at 65°C for 1 hr. Lanes: b, *Bam*HI; p, *Pst* I; h, *Hin*dIII; e, *Eco*RI. Numbers represent kb. (*B*) Southern blot of genomic clone λC5. A genomic library known to be complete was screened using the cDNA as a probe as described (28). Eight separate isolates were prepared. Southern blots of the DNA from each of the clones were prepared as described in *A*. The resulting autoradiograph for genomic clone λC5 is shown for the same restriction enzymes used on genomic DNA. Lanes: M, marker; b, *Bam*HI; p, *Pst* I; h, *Hin*dIII; e, *Eco*RI. Numbers represent kb.

with BAP, and end-labeling the fragments with [$^{32}$P]ATP and polynucleotide kinase. The primer that was used in these experiments is shown in Fig. 4*A*. The probe was annealed to

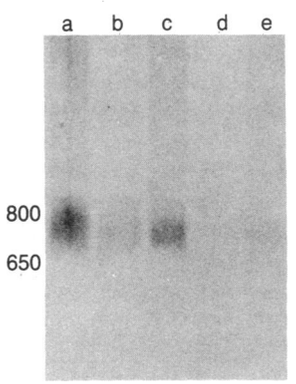


FIG. 3. RNA blots of RNA from different tissues. RNA blots of 5 mcg of mouse poly(A)$^+$ RNA derived from various tissues were hybridized at 65°C in 0.9 M NaCl/50 mM $NaPO_4$, pH 7.7/5 mM EDTA/5× Denhardt's solution (see Fig. 2 legend)/0.2% $NaDodSO_4$/100 μg of yeast tRNA per ml and nick-translated PCCK-7 (2, 25). The filters were washed in 2× NaCl/Cit (see Fig. 2 legend)/0.2% $NaDodSO_4$ at 65°C for 1 hr. Lanes: a, neonatal brain (9 days); b, adult brain; c, adult intestine; d, adult stomach; e, adult kidney. Numbers represent kb.

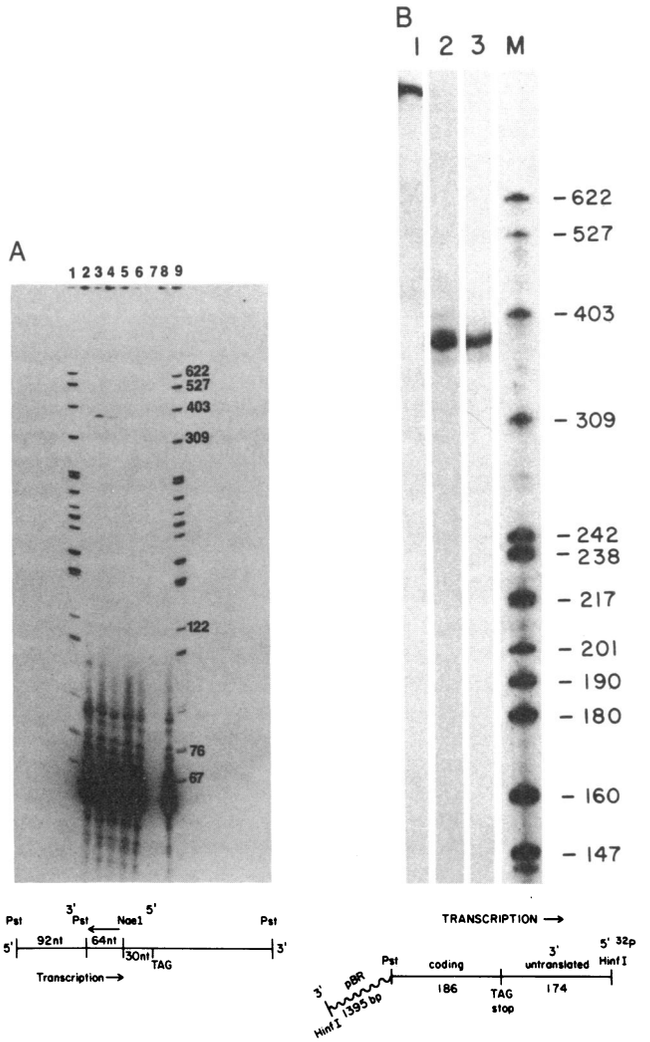


FIG. 4. (*A*) Primer extension assay. An end-labeled single-stranded probe was prepared by digesting the cDNA-containing plasmid with *Nae* I and *Pst* I, phosphatase treatment of the resulting fragments with BAP, and end-labeling the fragments with [$^{32}$P]ATP and polynucleotide kinase, followed by purification from an acrylamide gel. The *Nae* I site that was labeled is situated five nucleotides 3′ to the CCK octapeptide sequence and is 30 nucleotides upstream from a TAG stop codon. The resulting 64-nucleotide (nt) primer was hybridized to mRNA from several tissues overnight as described, and the primer was extended with reverse transcriptase and deoxyribonucleotides as described (29). The primer extension products were resolved on an acrylamide/3.5% urea gel. Lanes: 1, markers; 2, kidney; 3, intestine; 4, brain; 5, stomach; 6, liver; 7, spleen; 8, blank; 9, markers. (*B*) S1 nuclease protection assay. Mouse brain cDNA clone was digested with *Hin*fI, treated with bovine alkaline phosphatase, end-labeled with [$^{32}$P]ATP, and a 1755-bp fragment was purified from an agarose gel. This fragment was hybridized to 50 μg of total RNA from brain and intestine, treated with 1000 units of S1 nuclease, and resolved on an acrylamide/5% urea gel (30). This 1755-bp fragment includes 174 bp of 3′ untranslated region, 186 bp of coding sequence, and ≈1395 bp of pBR. As shown, both brain and intestinal RNA protect 348-bp fragments of identical length. Lanes: 1, undigested probe; 2, brain RNA; 3, intestinal RNA; M, markers. Numbers on right represent bp.

mRNAs from brain and intestine as described, and the primer was extended by using reverse transcriptase and unlabeled dNTPs. The primer-extended fragments were electrophoresed on a 3.5% acrylamide gel and the resulting autoradiograph is shown in Fig. 4*A*. This figure shows that the primer-extension products derived from brain and gut DNA are of identical length 5′ to the primer. The primer that was selected hybridizes to the 3′ end of the coding sequence so

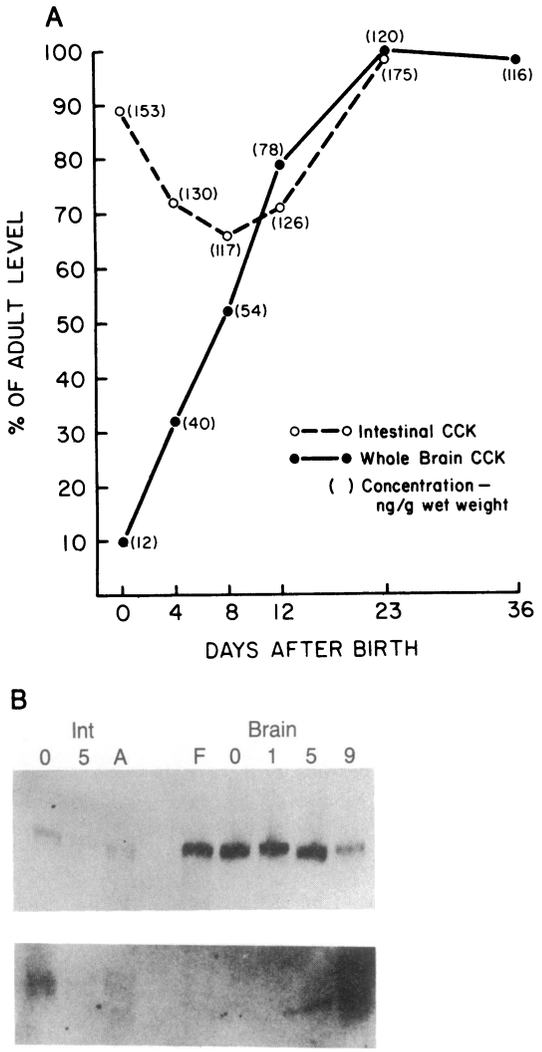


FIG. 5. (*A*) Ontogeny of CCK immunoreactivity in mouse brain and intestine. At various time points during development, boiling water extracts of duodenum and whole brain were assayed for CCK immunoreactivity by using a COOH-terminal radioimmunoassay system as described (31). The CCK concentrations are expressed as % of adult level. In parentheses are shown the average CCK concentration for four animals in ng of CCK-8 equivalents per g of tissue. (*B*) RNA blots during brain and intestinal development. DNA samples prepared from intestine (Int) and brain at various times during development were assayed by the RNA blot procedure. Intestine samples were obtained from animals at birth (lane 0), 5 days after birth (lane 5), and adulthood (lane 4). RNA was prepared from brain at 16 days of fetal life (lane F), birth (lane 0), 1 day (lane 1), 5 days (lane 5), and 9 days (lane 9) postpartum. The filters were hybridized to a nick-translated cDNA clone for tubulin and the resulting autoradiograph is shown (*Upper*). The same samples were also hybridized to the CCK cDNA clone (*Lower*).

that in this assay we scored the total length of the coding sequence and the leader sequences of RNAs from brain and gut. The fact that the two RNAs are of identical lengths in this region suggests that the same RNA start site is used in brain and gut, although definitive proof of this will await sequence analysis of full-length clones from both organs. In support of the conclusion that the RNAs from brain and gut are identical are data from an S1 nuclease protection assay (Fig. 4*B*). A 1755-bp *Hin*FI fragment was labeled at the 5′ end using BAP and T4 polynucleotide kinase, purified, and hybridized to 50 μg of total RNA from mouse brain and intestine. The resulting hybrids were digested with S1 nuclease and electrophoresed on an acrylamide/5% urea gel. This 1755-bp probe includes 174 bp of 3′ untranslated sequence, 186 bp of coding sequence, ≈1395 bp of pBR sequence. A perfect hybrid between CCK-containing RNA should protect a fragment of 360 bp. As shown in Fig. 4*B*, both brain and intestine RNAs protect identically sized fragments of this length. If the mRNA for intestinal CCK were different from that for brain CCK, the intestinal RNA should not have protected a full-length fragment, particularly considering the fact that 174 bp of the probe were from the 3′ untranslated region. These data further suggest that the same RNA is expressed in brain as in gut. We do not know, however, if the RNAs are of similar length or sequence 3′ to the S1 nuclease probe.

Finally, to study the developmental expression of CCK, we assayed the levels of CCK and CCK mRNA present in mouse brain and intestine at several time points during fetal and postnatal life. Radioimmunoassays were performed on extracts of brain and gut at several times pre- and postpartum. As shown in Fig. 5*A*, concentrations of CCK equal to those in adults are reached in mouse intestine by the time of birth. The relatively minor fluctuations in the concentration of this peptide in intestine during postnatal development are most likely due to increases in the amount of non-hormone-producing intestinal tissues and to differences in feeding patterns among groups of animals. In contrast, barely detectable amounts of CCK are present in brain tissue at birth. In the brain, there is a dramatic postpartum increase in the concentration of this peptide and adult levels are reached at 3–6 weeks of age. To determine whether the differential appearance of CCK is regulated at the mRNA level, we also scored CCK mRNA by probing RNA blots of poly(A)$^+$ RNA from developing brain and gut with the nick-translated CCK cDNA clone. As a control, RNA blots of RNA samples from the intestines of newborn, 5-day-old, and adult mice and from the brains of fetal, newborn, 1-day-old, 5-day-old, and 9-day-old animals were first hybridized to an α-tubulin cDNA clone in each of the samples. The autoradiographs reveal that the levels of tubulin mRNA are not substantially changed during intestinal development postpartum but that the levels of tubulin mRNA in brain decrease (Fig. 5*B*, upper panel). A similar decrease in the levels of B-tubulin mRNA has recently been reported to occur during rat brain development (32). Fig. 5*B* (*Lower*) clearly demonstrates that CCK mRNA is present in mouse intestine at birth in concentrations exceeding those found in adult animals. In brain, however, there is no detectable CCK mRNA until 5 days postpartum when a weak signal appears to be present. A dramatic increase in the levels of CCK mRNA is then seen at 9 days postpartum when the amount of CCK mRNA in brain is in fact higher than that in adult brain (Fig. 3). Thus, the differential appearance of CCK can be accounted for by differences in CCK-specific mRNA. Since we have not performed primer-extension assays on all these samples we cannot be certain however, that the same RNA start site is used in all cases.

## DISCUSSION

In this report, we have isolated a cDNA clone from mouse brain that encodes CCK, and we have used this clone to address several issues concerning the regulation of the CCK gene during development. We have shown that the CCK gene is present as a single copy in mice. Single bands are present on Southern blots of genomic DNA for each of eight restriction enzymes tested, and similar sized bands are seen for each of these enzymes when the DNA from a single genomic clone is analyzed. The conclusion that the same gene is expressed in brain and intestine is supported by data from S1 nuclease mapping experiments that show that full-length fragments are protected by RNA from brain and gut when hybridized to the end-labeled cDNA probe.

The RNA blots of poly(A)$^+$ RNA derived from brain and gut reveal that there are similar-sized CCK mRNAs present in these two tissues. We also found, by using a primer that hybridizes to the 3′ end of the coding sequence, that the primer-extension products from both tissues were of identical length. Thus, it seems most likely that the same RNA cap site is used in both tissues. Although it is possible that different promoters are used in brain and gut and are spliced so as to give identically sized messages 5′ to the primer, we consider it unlikely that in such a case identically sized primer-extension products would be generated. Nevertheless, the definitive proof of the identity of the start sites of this mRNA in brain and gut will await sequence analysis of full-length cDNA clones from these organs. Even though the same start sites are probably used, it is not clear whether the DNA sequences required for expression of this gene in each tissue are the same. It has been shown, for example, that the DNA sequences required for the responsiveness of the metallothionein gene to bacterial endotoxin are different from those required for responsiveness to heavy metals (33). Thus, the same mRNA can be regulated by different promoter elements. It would be of interest to determine whether the DNA sequences required for CCK mRNA production in brain are the same as in intestine. It should now be possible to define the DNA sequences required for CCK expression in neurons directly since we have identified a neuronal cell line that synthesizes CCK and can transfect these cells with chimeric plasmids that fuse the CCK promoter to the CAT gene, as has been done with other promoters (34, 35). Ultimately, we would like to define the DNA sequences required for CCK expression in gut as well, but we do not as yet have a CCK-producing intestinal tumor line. That this gene may be regulated differently in these two tissues is shown by the demonstration that CCK mRNA appears at different times during development in these two organs. The developmental signal responsible for the induction of CCK mRNA in brain and gut is unknown. In brain, cross-species studies have indicated that CCK first appears after most neuronal proliferation has ceased (36, 37). Similarly, the appearances of CCK and its mRNA in mouse brain seem to coincide with synapse formation. Whether synapse formation or other cell–cell contact is required for the developmental induction of this peptide remains to be determined.

In summary, the neuropeptide CCK demonstrates that the expression of a single-copy gene can undergo tissue-specific regulation at more than one level of control. Our data show that there is differential control of the concentrations of CCK mRNA in brain and gut during development. The relative contributions of transcriptional vs. post-transcriptional regulatory mechanisms remain to be assessed. In addition, the same protein precursor (pre-procholecystokinin) undergoes tissue-specific post-translational processing to yield different molecular forms of peptides sharing identical carboxyl termini: CCK-8 in brain and CCK-33 and -39 in intestine.

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