CHARACTERIZATION OF THE HUMAN MOTILIN GENE

by

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A DISSERTATION

Presented to the Department of Biochemistry
and Molecular Biology
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

June 1990

APPROVED:

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(Professor in Charge of Thesis)

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(Chairman, Graduate Council)

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Dedication

I dedicate this thesis to my Father, who provided me with the desire to understand and the example to follow.

ACKNOWLEDGEMENTS

My graduate student experience has been enhanced and I have benefited greatly as a result of assistance from and associations with many individuals. First I would like to acknowledge and thank Jim Douglass for all of his advice, support and time in seeing me through this endeavor. He honored a committment during a difficult time to take me on as a graduate student and has been a very helpful and supportive advisor. John Adelman got me started working on motilin and provided me ample opportunity to learn to screen lambda libraries. He has been very generous in letting me work in his laboratory and and a model of enthusiasm for science. I would like to acknowledge and give much thanks to Gaj Nilaver, who also opened his lab for me and who provided me with much support and experimental help with this project. I have also relied upon and appreciated the advice of Richard Jones throughout my graduate years.

I have appreciated the help and collegiality of the many people working in these labs and the Vollum Institute. In particular, Mike Collard, Richard Allen, Jim Garrett, Chris Bond, Bill Wolfgang, and Ron Sauter were of significant help at the bench. Mitch and Cyn have provided good scientific discussions and even better friendship - thanks. Additionally, I would like to acknowledge help with cutting tissue sections from Susan Rosario and Eva Marie Shannon, and the work of June Shiigi, Vicki Robertson and Ken Kroneiss on the graphics and photography in this thesis.

Finally a special thanks to my wife Wendy Lou who has been patient through all the long hours -- and helped me get through the references!

ABSTRACT

The mammalian gastrointestinal system consists of several anatomically and functionally related organs which work in a coordinated manner to allow the animal to absorb virtually all of its required nutrients and to excrete many waste products. These functions are accomplished by a diversity of cell types that is without parallel in the rest of the organism and which are highly regulated by complex, endogenous neural and endocrine systems. The gastrointestinal tract itself consists of a series of motile tissues which provide for the propulsion of contents through the tract; the function of the system as a whole is dependent upon this motility. Correspondingly, there are a number of diseases described in humans resulting from disorders of gastrointestinal motility.

Motilin is a small peptide purified from the duodenum in 1974 and named for its ability to stimulate contractions of gastric smooth muscle. Several physiologic studies suggest that motilin plays a major role in the control of motility in the small bowel. The structure of the promotilin precursor was deduced from the porcine and human motilin cDNAs cloned in 1987. However, there had been no description of a motilin gene from any species. The first objective of this thesis, therefore, was to isolate and characterize the human motilin gene. To this end, I have isolated a human gene encoding prepromotilin and determined that the precursor molecule is encoded on five exons. DNA sequence analysis revealed that the gene has a highly unusual exon/intron architecture, with the 22 amino acid motilin peptide encoded by

two distinct exon domains. Data also presented indicates that there is a single motilin gene in the human genome.

Cloning of the human motilin gene provides a tool with which to study its expression in humans and nonhuman primates. The second objective of this thesis, therefore, was to use a fragment of the human gene as a probe to examine the tissue-specific expression of the motilin gene in the monkey as a model for motilin gene expression in human and other nonhuman primates. Northern blot hybridization and Polymerase Chain Reaction techniques were used to demonstrate that while motilin mRNA is expressed at highest levels in the small intestine, low levels are found throughout the gastrointestinal tract as well as some extra-gastrointestinal tissues. The results of these experiments also suggest that another gene which is highly related to, yet distinct from motilin, is expressed in the gastrointestinal system. In addition, *in situ* hybridization analysis was used to demonstrate the site of motilin mRNA synthesis at the cellular level.

The final major objective of this thesis was to examine the relation of motilin synthesis in the small intestine to that of other peptide hormones produced in the region. In particular, much evidence suggests that diversity in function of signalling cells, whether neuronal, neurohormonal, or hormonal, can result from synthesis and secretion of more than one kind of signalling molecule. Therefore, immunohistochemical techniques were employed to determine if motilin is colocalized with other gastrointestinal peptide hormones. Evidence

presented here suggests that a subset of motilin-producing cells also contain Substance P.

In addition to these major objectives, Northern blot hybridization analysis was used to compare motilin mRNA from different species and the resulting data indicate that there is not a high degree of conservation of motilin mRNA sequence in rodents compared with higher mammals.

INTRODUCTION

Background

Certainly one of the central discoveries in biology during the first half of the 20th century was that "chemical messengers" carry information about the physiologic state of the organism or the external environment from one organ to another, and that these molecules are often peptides (1). The first hormone discovered was that of the intestinal hormone secretin by Bayliss and Starling in 1902, followed soon after by that of a gastric hormonal activity which was called gastrin (2). Thus the term "gastrointestinal hormone" came into general use and a number of hormonal activities were identified based on physiologic studies. It was not, however, until the 1950's that secretin and glucagon were purified and their primary structure determined. Since then an increasing number of gastrointestinal hormones have been identified and over 20 purified; most of these have also been cloned.

The Enteroendocrine and Enteric Nervous Systems

The gastrointestinal (GI) system consists of a group of tissues which provide for nutrient absorption, biosynthesis, and waste excretion, as well as an endocrine system to provide for the regulation of these processes and coordination with other bodily functions. The system includes the gastrointestinal tract -- comprised of the mouth and associated secretory glands, esophagus, stomach, small intestine (divided histologically and

functionally into the duodenum, jejunum and ileum), and colon -- and accessory organs including the exocrine pancreas, gallbladder and bile duct, and liver. Each segment of the GI tract is further divided into subregions which are somewhat distinct histologically and functionally. A diagram of the human GI system is shown in Figure 1.

The entire GI tract contains muscle tissue to provide for mechanical digestion and transport of ingested food. Although there is sympathetic and parasympathetic input to these muscles from the peripheral nervous system, their contraction is predominantly controlled by an endogenous nervous system which can be viewed as a third division of the mammalian nervous system. This enteric nervous system is made up of nerves and ganglia which form two dense plexuses within the muscular walls of the GI tract. The myenteric, or Auerbach's plexus lies between the outer circular and inner longitudinal muscle layers; with the submucous, or Meissner's plexus located between the muscle layers and the inner mucosal layer of the gut wall. The cellular and chemical anatomy of the enteric nervous system is best described in the guinea pig, largely through the work of Furness and Costa (3); a schematic diagram of the projections of the enteric neurons within these plexuses in the guinea pig ileum in shown in Figure 2.

The gastrointestinal system also contains an endogenous endocrine system, or enteroendocrine system, which functions to coordinate the many separate GI functions. For example, secretin is secreted by the stomach to stimulate

Figure 1. The Human Gastrointestinal System

Schematic representation of the human gastrointestinal system, including accessory organs. The gastrointestinal system depicted here is essentially identical in all primate species. Adapted from Jacob ,S., and Fancone, C., Structure and Function in Man, Saunders, Philidelphia, 1965, p. 381.

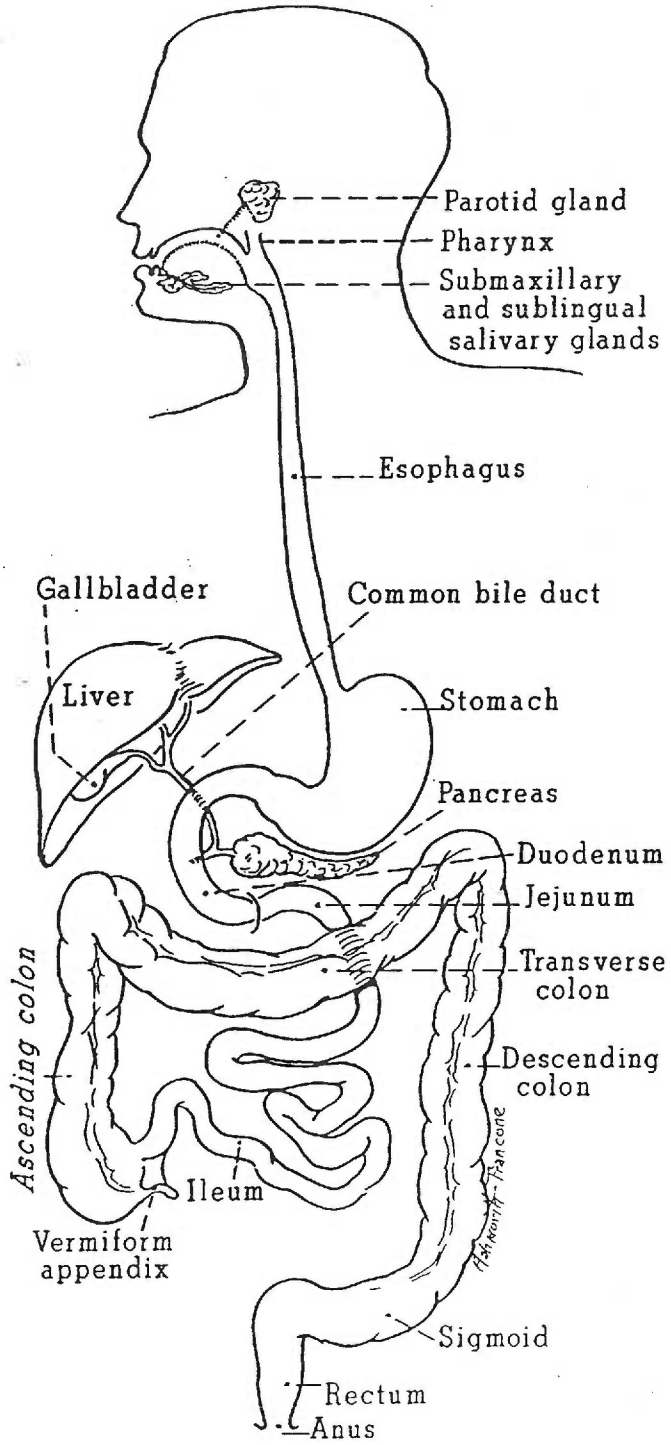
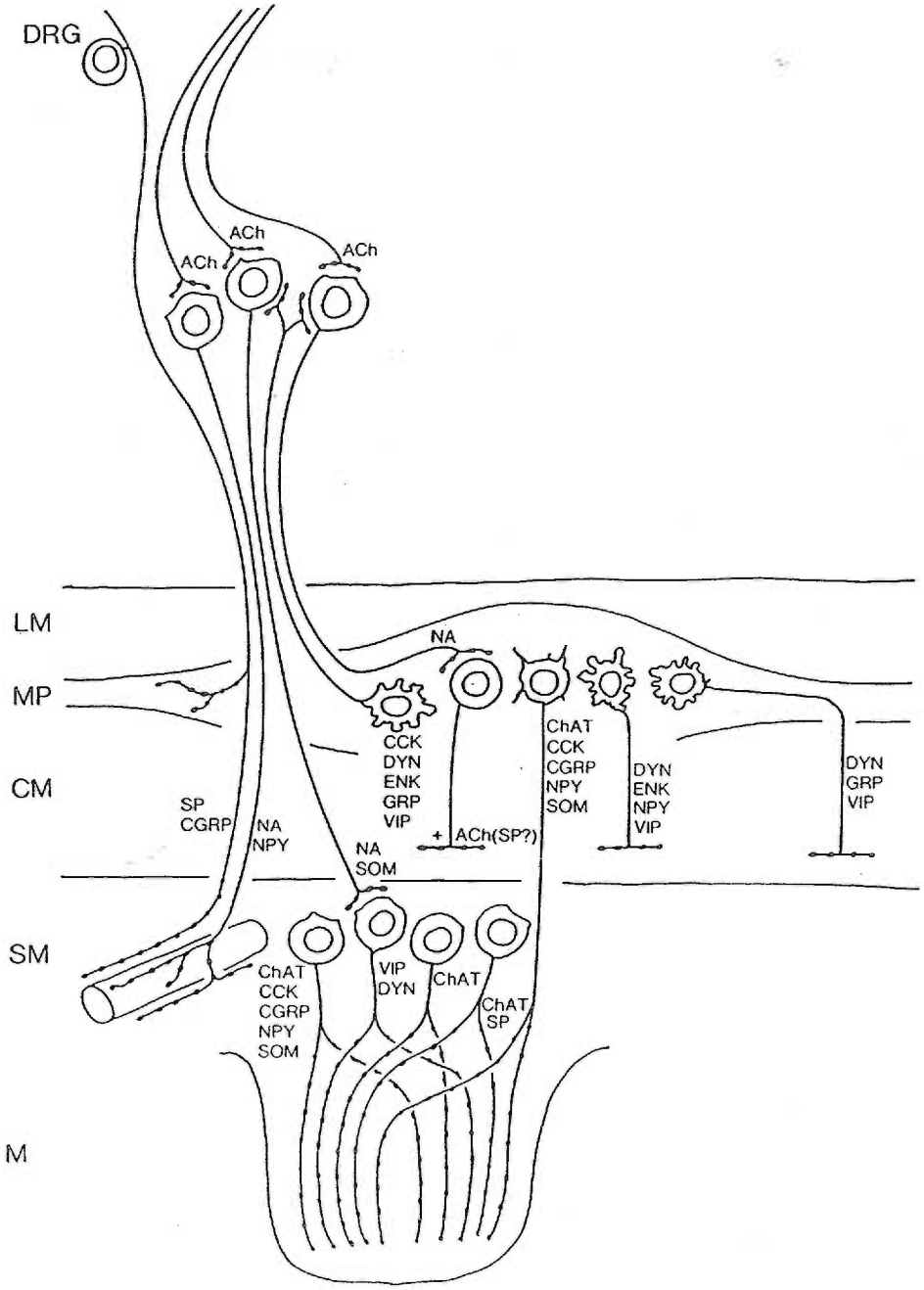

Parotid gland
Pharynx
Submaxillary and sublingual salivary glands
Esophagus
Gallbladder
Common bile duct
Liver
Stomach
Pancreas
Duodenum
Jejunum
Transverse colon
Descending colon
Ascending colon
Ileum
Vermiform appendix
Sigmoid
Rectum
Anus

Figure 2. Projections of Enteric Neurons

Diagrammatic representation of neurons and neuronal projections within the small intestine. The neurotransmitters and/or neuropeptides which have been identified in the guinea pig small intestine are shown. DRG, dorsal root ganglion; LM, longitudinal muscle; MP, myenteric plexus; CM, circular muscle; SM, submucous plexus; M, mucosa. (Adapted from Costa M., Furness, J.B., and Llewellyn-Smith, I.J., "Histochemistry of the Enteric Nervous System," in Johnson, L.R. (ed.), Physiology of the Gastrointestinal Tract, 2nd Edition, Raven Press, New York, 1987, pp. 10-40).


DRG
ACh
ACh
ACh
LM
MP
CM
SM
M
NA
CCK
DYN
ENK
GRP
VIP
+
ACh(SP?)
ChAT
CCK
CGRP
NPY
SOM
DYN
ENK
NPY
VIP
DYN
GRP
VIP
SP
CGRP
NA
NPY
NA
SOM
ChAT
CCK
CGRP
NPY
SOM
VIP
DYN
ChAT
ChAT
SP

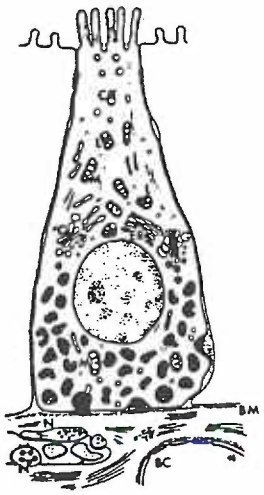
pancreatic bicarbonate secretion which neutralizes stomach acid entering the small intestine at the same time that acidification of the duodenal lumen by acid from the stomach stimulates secretion of cholecystokinin, which in turn causes secretion of bile from the gallbladder to emulsify dietary fat entering the duodenum with the acid. Unlike endocrine tissue in most of the rest of the body, which is organized in glands or islets, the enteroendocrine system is diffuse (4). The endocrine cells are scattered throughout the simple mucosal epithelium of the luminal wall of the GI tract (Figure 3). These cells have been divided into many different subtypes, one of which is the motilin-producing cell or M-cell (5), based on either the electron microscopic appearance of their secretory granules, or the hormone they produce. Many of these cells also contain serotonin and are thus referred to as APUD (amine precursor uptake and decarboxylation) cells (6). They also produce a chromaffin reaction (reduction of bichromate ion) and therefore are sometimes called enterochromaffin cells by analogy with adrenal chromaffin cells. There is, however, debate in the literature as to whether the M-cell is (7), or is not (8) an enterochromaffin cell.

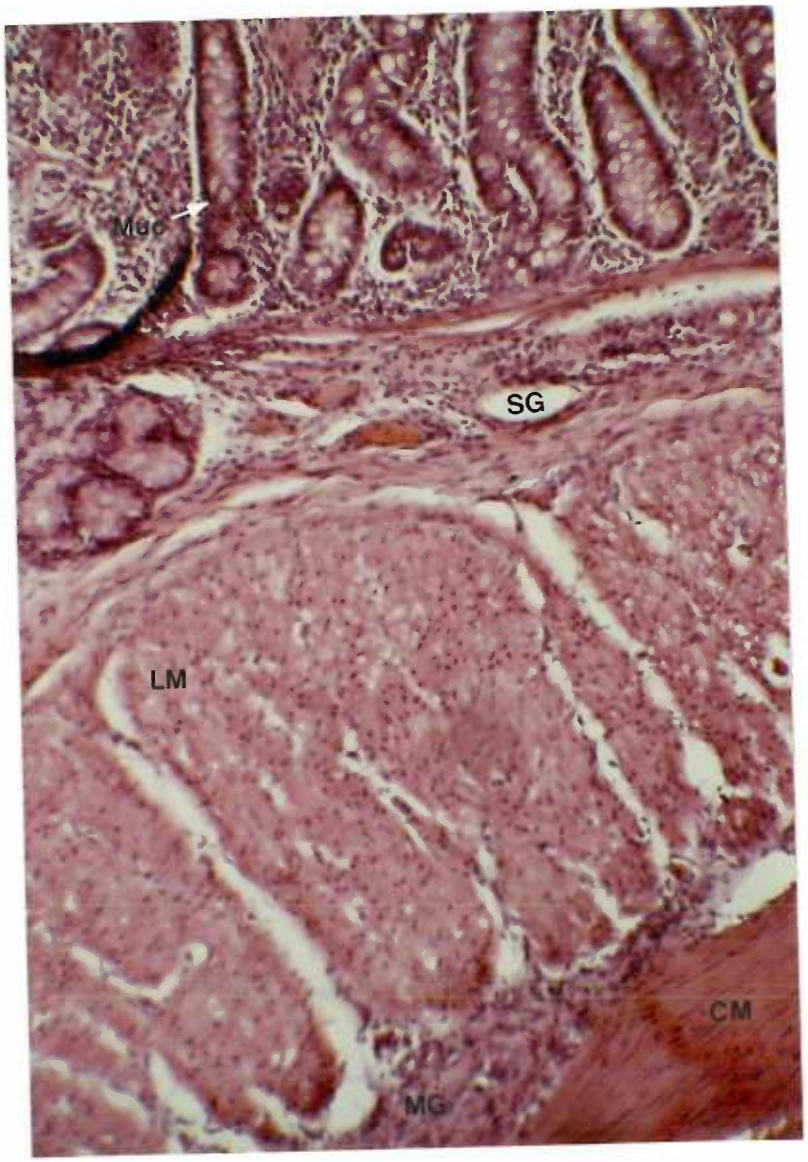
Initial Description of Motilin

One of the earliest gastrointestinal hormones postulated was a factor which would stimulate the motility which is a prominent feature of the mammalian digestive tract. The name motilin was given to this factor in 1905, but it was not until 1972 that "motilin" was purified from porcine duodenum by Brown

Figure 3. Illustration of an Intestinal Endocrine Cell

Top: Schematic representation of a polarized mucosal epithelial cell organized for secretion towards the basilar surface. BM, basement membrane; N, nerve; BC, blood capillary. Adapted from Reference 102. **Bottom:** Photomicrograph of a hematoxylin and eosin stained section of porcine duodenum with the luminal surface at the top of the photograph. CM, circular muscle; MG, myenteric ganglion; LM, longitudinal muscle; SG, submucosal ganglion; Muc, mucosal layer (the arrow points to the approximate location of an intestinal endocrine cell).


BM
N
BC


Muc
SG
LM
CM
MG

and coworkers; the complete amino acid sequence was determined in 1974 (9). The amino acid sequence of porcine motilin is FVPIFTYGELQRMQEK ERNKGQ. This 22 amino acid peptide was purified from secretin and cholecystokinin-pancreozymin in a duodenal protein extract (10) by gel filtration as a fraction which would stimulate gastric contractions. Porcine and human motilin have identical amino acid sequences, but canine motilin differs from these at 5 of 22 sites located in the middle of the molecule (11); the canine sequence is FVPIFTHSELQKIREKERNKGQ (amino acid changes underlined; see also Figure 21). These are the only three species in which the motilin amino acid sequence is known. This is an important point because as described below, the effects of motilin on gastrointestinal functions show great variations in different organs and in different species. Although all of the physiologic and biochemical studies on motilin function have been carried out using synthetic porcine motilin (or synthetic analogues of the porcine sequence) virtually none have been done in the pig and only a subset in the human.

Anatomy and Physiology

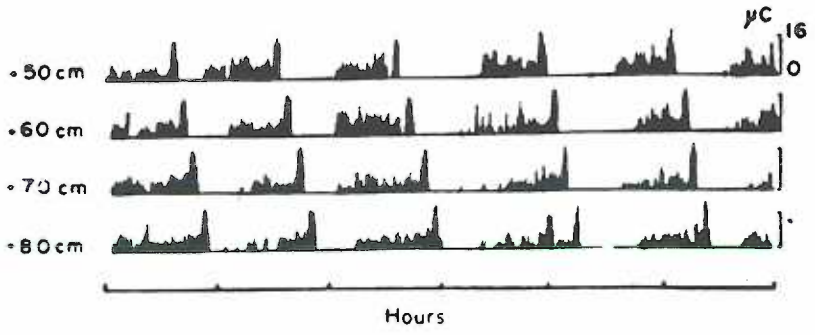
Radioimmunoassay and immunohistochemical studies using antibodies generated against the synthetic porcine sequence soon established that motilin is present in highest levels in the duodenum in all species studied, including primate (12), and that immunoreactive motilin is present in mucosal enteroendocrine cells in the intestine (13). Motilin immunoreactivity has also

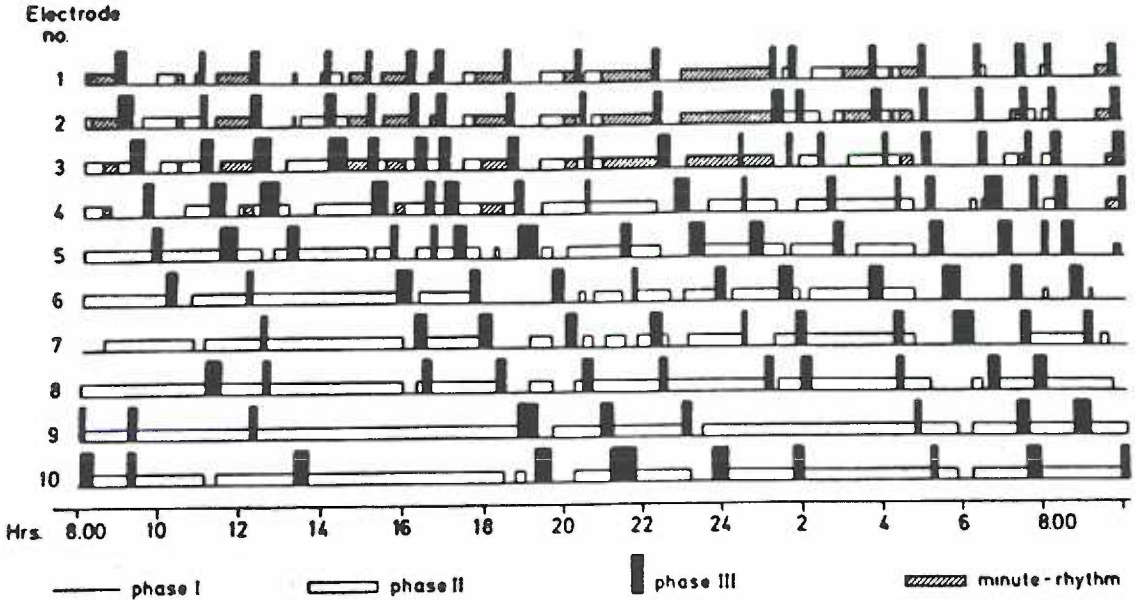
been measured within the muscle layers of the canine duodenum (14). Motilin-containing cells have been detected by immunohistochemistry in the lower small intestine, colon, and bile duct, and significant levels of immunoactivity relative to that in the duodenum have also been measured in primate stomach, gallbladder, and adrenal gland (15). These cells appear very early in human development, a few appearing in the duodenum at least as early as 14-15 weeks gestation (16). The measurement of immunoreactive motilin in peripheral blood and the correlation of peaks of serum motilin levels with a specific gastrointestinal activity suggests that this peptide functions as a hormone. The best functional correlate of motilin secretion appears to be the appearance of the migrating motor complex (MMC). The MMC (Figure 4) is a cyclic pattern of contractile activity which occurs throughout the gastrointestinal tract during postprandial and interdigestive periods. The complex consists of three phases: Phase I is a period of quiescence; Phase II consists of intermittent low intensity, intermediate duration contractions, and Phase III is a brief period of high intensity, short duration contractions. The entire complex travels aborally through the tract. Just prior to each of these muscle contractions a corresponding electrical depolarizarion can be measured; thus the MMC is also referred to as the Migrating Myoelectric Complex. This highly ordered pattern of muscular activity in the gastrointestinal tract is controlled by the enteric nervous system. The MMC is also a stereotypic activity which can be measured in all species studied to date. These include human (17), monkey (18), pig (19), calf (20), horse (21), kangaroo (22), wallaby (22), opossum (23), rat (24), guinea pig (25), and

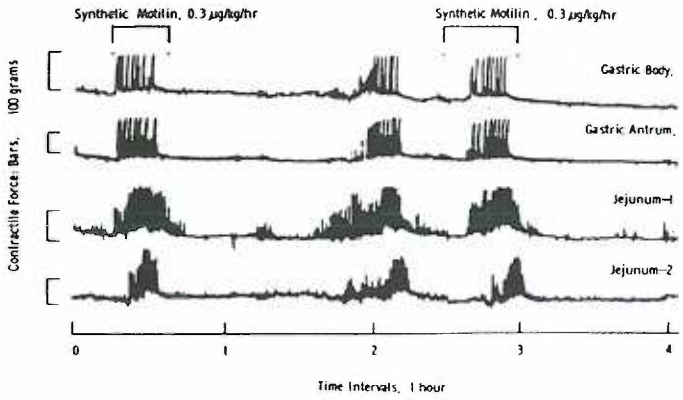
Figure 4. Relation of Motilin Secretion to the Migrating Myoelectric Complex

Top: Temporal distribution of myoelectric spike potentials in the sheep jejunum (From Weisbrodt, N.W., "Intestinal Motility" in Johnson L.R. (ed.), Physiology of the Gastrointestinal Tract, Raven Press, New York, 1987, pp. 631-663). **Middle:** Temporal distribution of MMC spike potentials at 10 different sites throughout the human intestinal tract (duodenum to ileum) illustrating the aboral migration of MMC complexes. (From Fleckenstein, P. and Oigaard, A., Electrical Spike activity in the human small intestine, Am. J. Dig. Dis., 1978, 23:769-775.) **Bottom:** Stimulation of premature interdigestive myoelectric complexes by infusion of motilin. (From Itoh, Z., et al., Motilin-induced mechanical activity in the canine alimentary tract, Scand. J. Gastroenterol., 1976, 39(Suppl. 11):93-110).

The Migrating Myoelectric Complex (MMC)







chicken (26). Interspecie differences are found in the relative durations of each phase and in the effects of feeding on the initiation and propagation of the cycle, but the basic patterns of contractions and myoelectric activity are similar, suggesting that similar neural and hormonal mechanisms are responsible for producing the MMC.

It has been proposed that the primary function of motilin is the initiation of the MMC in the small intestine. The evidence for this is that 1) peaks in plasma motilin levels correspond to the appearance of the MMC in the duodenum in the dog (27), 2) highest motilin levels occur just prior to and during Phase III of the MMC in the opossum (28), 3) intravenous infusion of motilin initates premature activity fronts in man and dog (29), and 4) infusion of motilin antibodies delays the MMC activity fronts in the antrum and duodenum (30). However, neither motilin infusion (31) nor immunoneutralization (32) has an effect on the rate of MMC initiation in the pig; motilin therefore does not appear to be necessary for passage of the MMC through the porcine upper small intestine. Furthermore, in the human there is an imperfect correlation between motilin secretion and the MMC, as some duodenal Phase III contractions occur in the absence of motilin peaks (33, 34). This may be explained by the finding that only motor complexes which originate in the stomach are correlated with motilin peaks in man (35).

That motilin has a causal role in duodenal MMC initiation in any organism has been questioned by Sarna et al; who reported that premature Phase III activity

could be induced by infusion of morphine (36). These morphine-induced contractions are associated with a rise in plasma motilin. However, while morphine could overcome a disruption in the MMC caused by a meal or somatostatin infusion, motilin could not (37). These authors concluded that motilin release in the duodenum is a result of MMC activity, that is, of duodenal contractions, and suggested that rather than initiate the duodenal MMC, motilin may act to potentiate MMC contractions or to coordinate its initiation with other motor and secretory events occuring in the region; for example bile duct and gallbladder contractions, and pancreatic secretion (38). In support of this possibility are the findings that motilin can induce MMC-like activity in the Spincter of Oddi (39) as well as the lower esophageal spincter (40); and stimulate gastric pepsin (41) and pancreatic bicarbonate and protein secretion (42). Alternatively, motilin may function as a positive feedback mechanism to potentiate the MMC in the upper small intestine by further stimulating the gastric antrum contractions which caused its release.

Nevertheless, motilin clearly can stimulate muscular contractions in the duodenum. Application of the synthetic motilin analogue 13-norleucine motilin (13-Nle-M) causes contraction of isolated muscle strips from rabbit and human duodenum at nanomolar concentrations which are not blocked by application of hexamethonium, tetrodotoxin, or atropine (43,44). Motilin's contractile action is therefore not dependent upon neural pathways. This is supported by biochemical studies which indicate that the motilin receptor is present on muscle cells. Bormans et al. measured highly specific binding of

^{125}I-motilin to rabbit antral and duodenal smooth muscle (45). High affinity motilin binding was measured in human antrum and duodenum, but not in canine tissue using either canine or porcine motilin (46). In contrast to the case in rabbit and human, motilin cannot stimulate contractions in duodenal muscle strips from dog (47), rat, or guinea pig (43). However, injection of motilin in the dog *in vivo* leads to typical MMC activity which is sensitive to cholinergic blockade and partially sensitive to ganglionic blockade (48). Poitras et al. measured a contractile effect of synthetic canine motilin in isolated canine duodenal muscle (49), but the dose for half-maximal response was on the order of 10^{-5} M compared with the 10^{-9} M EC_{50} for the receptor in rabbit and human muscle measured previously. Interestingly, erythromycin is a motilin receptor agonist in rabbit and human preparations *in vivo*, while it has no effect on duodenal strips from dog or rat (50). Consideration of all of this data questions the presence of a motilin receptor on canine smooth muscle, and suggests that in the dog, the receptor is present on neuronal cells, presumably excitatory preganglionic interneurons which synapse on nicotinic cholinergic enteric neurons. Although the effects of motilin on muscle cells can be blocked by agents which inhibit the influx of extracellular calcium (43,44), or by removal of extracellular calcium (44), recent data suggests that binding of motilin to its receptor causes a release of intracellular calcium stores (51).

Implications in Gastrointestinal Pathophysiology

An understanding of the mechanisms underlying normal gastrointestinal motility and the role that motilin may have in mediating or regulating this process is of much interest because there are a number of dysmotility syndromes described in humans. Accordingly, abnormalities in motilin synthesis or secretion have been implicated in a number of gastrointestinal disorders; these disorders are generally characterized by abnormal motility. Elevated plasma motilin levels have been measured in patients with the irritable colon syndrome (52) and in infants with infantile colic (53). Elevated motilin levels have also been reported in a series of patients with chronic renal failure; 39 of 39 chronic renal patients undergoing dialysis had elevated plasma motilin levels compared with 22 normal individuals or 12 renal transplant patients who had normal levels, and it was suggested that this abnormality may play a role in the problems with gastrointestinal and/or esophageal motility seen in some end stage renal patients (54). Elevated motilin levels have also been seen in two types of carcinoma. Five patients in a series of 37 pheochromocytomas had elevated plasma motilin (55) and plasma from 17 of 20 patients studied with small cell carcinoma of lung had greatly elevated motilin levels (56). In this latter study three tumors examined contained low levels of immunoreactive motilin, so it was not clear whether the source of motilin was the tumor or was the result of increased secretion from the gastrointestinal tract stimulated by bombesin, which stimulates motilin secretion and is often secreted by these tumors. The hyperactive

Lower than normal levels of plasma motilin have been measured in patients with chronic idiopathic constipation while other gastrointestinal peptides appeared to be at normal levels (57). A study of 20 patients with peptic ulcer disease resulting from idiopathic delay in gastric emptying showed that all but two had no MMC activity fronts originating from the stomach or the duodenum, or both, and had lowered motilin peak frequency and amplitude (58). Motilin levels are also decreased during postoperative ileus, but they return to normal coincident with the end of ileus.

Reduced gastrointestinal motility is often a feature of advanced diabetes mellitus which is thought to be secondary to autonomic neuropathy and which results in a number of gastrointestinal symptoms. However, a study of diabetic patients with autonomic neuropathy revealed basal motilin levels which were 2-3 times higher than normal controls or diabetics without autonomic neuropathy, while the normal fall in plasma motilin levels in response to insulin injection was prolonged fourfold in these patients (59). Thus, abnormalities in motilin secretion may have a role in the gastrointestinal stasis seen in these patients.

Motilin in the Central Nervous System

Soon after localization of motilin cells in the gastrointestinal tract, motilin immunoreactivity was detected in the canine pituitary and central nervous system

Motilin in the Central Nervous System

Soon after localization of motilin cells in the gastrointestinal tract, motilin immunoreactivity was detected in the canine pituitary and central nervous system (15). These studies were extended in the rat CNS where motilin immunoreactivity was detected in a number or brain regions, particularly in the cerebellum (60); however, it was noted that in the rat the predominant form of this immunoreactivity was a large molecular weight specie, with a small amount in a form similar in size, but not identical to, the porcine form (61). Motilin immunoreactivity detected in porcine cerebellar extracts has the same retention time as synthetic porcine motilin on HPLC (62). The cerebellar motilin immunoreactivity was shown to be localized in Purkinje neurons in rodent and human brain by immunohistochemistry (63). Motilin immunoreactivity was also identified in guinea pig and human anterior pituitary somatotrophs (64). However, in addition to size heterogeneity, not all anti-synthetic porcine motilin antibodies were able to detect CNS motilin immunoreactivity, while other antibodies detected it in only some species. This heterogeneity in CNS motilin immunoreactivity (65) has led to the use of the term "motilin-like immunoreactivity" (MLIR) in reference to the immunoreactive material in the CNS.

Motilin synthesis in neural tissue is not a surprising possiblity. Substance P was identified in gastrointestinal cells and the central nervous system in the 1930's (66), and since then virtually every other gastrointestinal hormone described has also been localized in neurons; often in CNS neurons. Many are produced in enteric neurons in addition to mucosal enteroendocrine cells, such as somatostatin,

found in the GI system (70). Often, however, these peptides exist in different processed forms in the two tissues. For example, cholecystokinin (CCK) exists in the GI tract in several large molecular weight forms, including CCK-58 and CCK-39, while the predominant brain form is CCK-8 (71). Overall, it can be said that the occurance of GI hormones in the CNS is the rule, rather than the exception (72). Yet, because of discrepancies in the immunologic data, as well as more recent analysis of motilin gene expression (see below), the existence of motilin in the cerebellum has been questioned (73,74).

Characterization of cDNA Clones Encoding the Motilin Precursor Protein

While progress has been made towards understanding the function of motilin in the gastrointestinal tract at the physiologic level, very little work has been done on the molecular biology of the motilin gene. cDNAs for porcine (74) and human (75) motilin were cloned in 1987. The predicted amino acid sequence derived from nucleic acid sequence analysis of these cDNA clones indicated that motilin is synthesized as a precursor protein with a structure typical of peptide hormone precursors (76). This precursor protein structure consists of a signal sequence, followed immediately by the 22 amino acid motilin sequence, and a 66 amino acid C-terminal sequence (70 amino acids in the pig) separated from motilin by a Lys-Lys dipeptide. The nucleotide sequence and predicted amino acid sequence of human prepromotilin are shown in Figure 5. The two cDNAs have 88% nucleotide sequence identity over the motilin-encoding region as expected, since the two hormones have

Figure 5. Nucleotide Sequence of the Human Motilin cDNA and Predicted Amino Acid Sequence of Prepromotilin

Top: Nucleotide sequence of the cloned human motilin cDNA and the predicted translation product. The 22 amino acid motilin molecule is shaded and the dibasic cleavage site is boxed. **Bottom:** Schematic representation of the structure of the human motilin mRNA, including 5' untranslated sequence, signal sequence, motilin moiety, Lys-Lys cleavage site, C-terminal peptide and 3' untranslated sequence.

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     AGACAAGTAGAGAGACTCCTCCAGACCCACTCAGACCACGTGCACGCCCTCCAAG   55

MET Val Ser Arg Lys Ala Val Ala Ala Leu Leu Val Val His Val
ATG GTA TCC CGT AAG GCT GTG GCT GCT CTG CTG GTG GTG CAT GTA 100

Ala Ala Met Leu Ala Ser Gln Thr Glu Ala Phe Val Pro Ile Phe
GCT GCC ATG CTG GCC TCC CAG ACG GAA GCC TTC GTC CCC ATC TTC 145

Thr Tyr Gly Glu Leu Gln Arg Met Gln Glu Lys Glu Arg Asn Lys
ACC TAT GGC GAA CTC CAG AGG ATG CAG GAA AAT GAA CGG AAT AAA 190

Gly Gln Lys Lys Ser Leu Ser Val Trp Gln Arg Ser Gly Glu Glu
GGG CAA AAG AAA TCC CTG AGT GTA TGG CAG AGG TCT GGG GAG GAA 235

Gly Pro Val Asp Pro Ala Glu Pro Ile Arg Glu Glu Glu Asn Glu
GGT CCT GTA GAC CCT GCG GAG CCC ATC AGG GAA GAA GAA AAC GAA 280

Met Ile Lys Leu Thr Ala Pro Leu Glu Ile Gly Met Arg Met Asn
ATG ATC AAG CTG ACT GCT CCT CTG GAA ATT GGA ATG AGG ATG AAC 325

Ser Arg Gln Leu Glu Lys Tyr Pro Ala Thr Leu Glu Gly Leu Leu
TCC AGA CAG CTG GAA AAG TAC CCG GCC ACC CTG GAA GGG CTG CTG 370

Ser Glu Met Leu Pro Gln His Ala Ala Lys
AGT GAG ATG CTT CCC CAG CAT GCA GCC AAG TGATGGCCACGCTGGGGAG 419

AAGGTGGACAGATTTGGGAGGCCCCTCCTGCCCAAGTGAGGCCCTGGGAATTTACAGAG 478

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AAAGCATGAAATATACAG - (A)n 555
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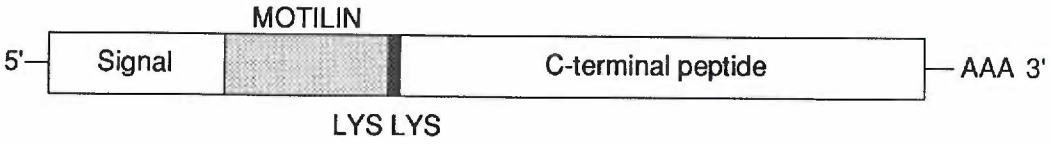


complete amino acid sequence identity. Both contain a Lys-Lys proteolytic cleavage site while the C-terminal extensions have only 54% amino acid and 75% nucleotide identity.

The C-terminal region of the porcine promotilin molecule contains a pentapeptide sequence (74) which is also present within a peptide called PHI-27 cleaved from proglucagon (77). This finding has implications in terms of the evolution of the motilin precursor, discussed below. Bond et al. used the cloned porcine cDNA to probe poly-A^+ RNA from various porcine tissues and only detected motilin mRNA in the duodenum. Interestingly, no hybridizing specie was detected in cerebellar or adrenal RNA (74), suggesting that the motilin-like immunoreactivity detected in the cerebellum may represent a crossreactive peptide sequence not encoded by the motilin gene.

Structural Relation of Motilin to Other Gastrointestinal Hormones

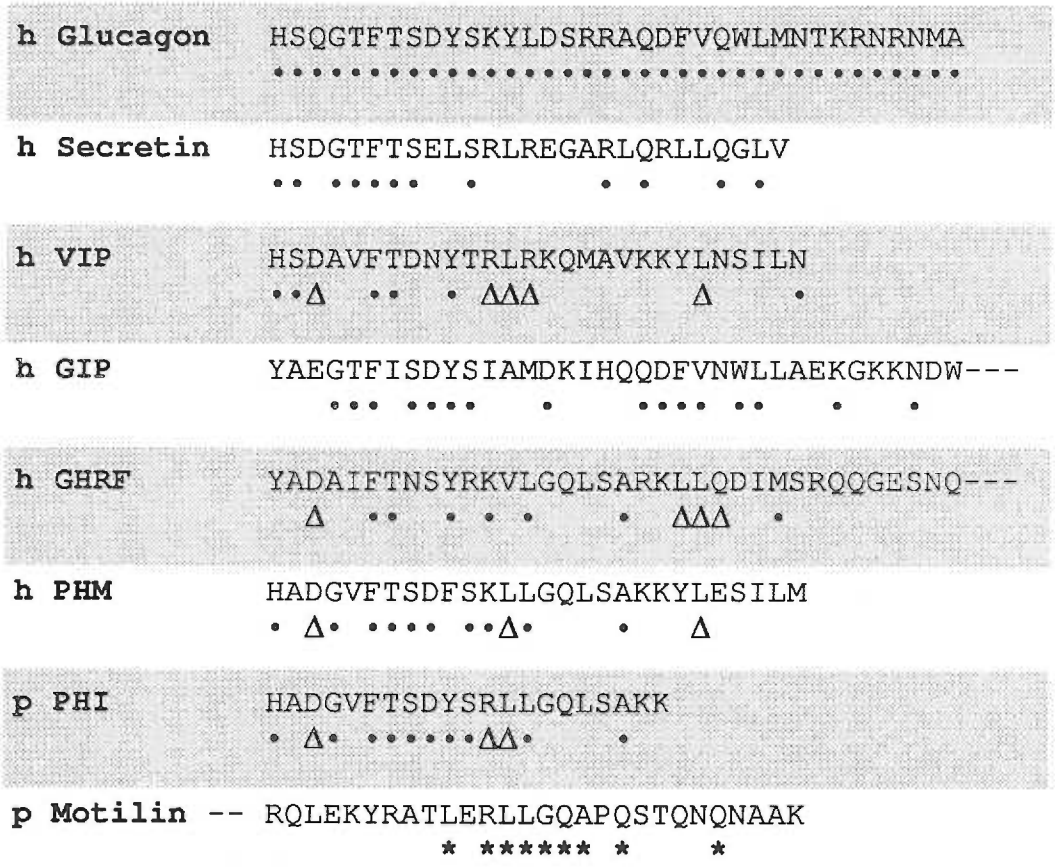
Comparison of the amino acid sequences of gastrointestinal hormones, or their prohormone precursors, indicates that many have significant regions of homology to one another. Furthermore, some of these molecules have homology with other proteins of unrelated function. For example, secretin shares significant amino acid homology with glucagon, growth hormone, pepsin, rennin, trypsin, chymotrypsin and elastin. Adelson has pointed out that all of these related molecules are secreted by embryologically related tissues and that, in fact, virtually all secreted proteins are synthesized by cells

derived from embryonic entoderm, while some may or may not be produced in tissues of different origin (78). Furthermore, all of these "enterosecretory" proteins, as well as the clotting factor proteins secreted from the liver, are activated or processed at similar sites by similar enzymes. These observations suggest that all enterosecretory proteins had a common evolutionary origin in the primordial gastrointestinal tract.

The various gastrointestinal hormones have been grouped into six families based upon amino acid homology with three hormones -- motilin, somatostatin and galanin -- not belonging to any group (72). The largest group is that of the glucagon-secretin family of hormones. Secretin shares identity with glucagon at 12 of its 27 amino acids and the other members of the group have various degrees of homology with these two (Figure 6). Two other members, vasoactive intestinal polypeptide and PHM/PHI-27, are synthesized as part of a common precursor (79) and are encoded on adjacent exons (80). The striking homology between the members of this family suggests that they have arisen by a process of gene duplication and divergence (81). The presence of a five amino acid sequence in the porcine motilin precursor also present in the rodent PHI-27 now places motilin within the glucagon-secretin family of GI hormones.

Figure 6. Amino Acid Homologies of the Glucagon-Secretin Family of Gastrointestinal Hormones

Amino acids which are identical in the corresponding position of the glucagon molecule are undermarked with a dot (•) and amino acids which are identical in the corresponding position of the secretin molecule are undermarked with a triangle (Δ). Amino acids in the porcine motilin molecule which share identity in the corresponding position with any of the other members of the glucagon-secretin family are undermarked with an asterisk (*). h, human; p, porcine; VIP, vasoactive intestinal polypeptide; GIP gastric inhibitory peptide; GHRF, growth hormone releasing factor; PHM, peptide histidine-methionine; PHI, peptide histidine-isoleucine.


h Glucagon HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNMA
h Secretin HSDGTFTSELSRLREGARLQRLLQGLV
h VIP HSDAVFTDNYTRLRKQMAVKKYLNSILN
h GIP YAEGTFISDYSIAMDKIHQQDFVNWLLAEKGKKNDW---
h GHRF YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQ---
h PHM HADGVFTSDFSKLLGQLSAKKYLESILM
p PHI HADGVFTSDYSRLLGQLSAKK
p Motilin -- RQLEKYRATLERLLGQAPQSTQNQNAAK

Overview

In summary, motilin is a 22 amino acid gastrointestinal hormone with only partly delineated function, but which plays an important role in normal motility in the mammalian small bowel and is apparently involved in a number of gastrointestinal disorders in humans. The research described in this thesis was undertaken to isolate and characterize the human motilin gene as a prelude to studying the expression of this gene in the human and nonhuman primate. Important first steps had been taken in the study of motilin gene expression with the cloning of two mammalian cDNAs, and the work described here used information obtained from that earlier work as a starting point. Molecular cloning and determination of the intron-exon organization of the human motilin gene is described in Chapter 1 (Manuscript #1). Another description of the structure of a cloned human motilin gene was also recently published with the same intron-exon structure as the clone described here (82). A cloned fragment of the human gene was next used as a hybridization probe to detect the presence of motilin mRNA in a variety of human and monkey tissues. This work established that the human clone recognizes the monkey motilin mRNA in the duodenum and that motilin mRNA is transcribed in several other gastrointestinal and extra-gastrointestinal tissues. Expression of the motilin gene in the gastrointestinal system is examined in more detail at the tissue and cellular level in Chapter 2 (Manuscript #2). Data indicating that there is a single motilin gene in the human and evidence for the presence of a related gene is presented in Appendix 1. While human and nonhuman

primate motilin are expected to be very similar, earlier immunologic data suggests that the rodent form of the molecule differs significantly from the primate and porcine forms. Evidence in support of this at the nucleic acid level is presented in Appendix 2. Data indicating that the motilin gene may be transcribed in the primate cerebellum is included in Appendix III. Chapter 3 describes work using immunohistochemical techniques to examine the production of motilin peptide in the monkey duodenum in relation to other GI hormones. The results of this work indicate that Substance P is produced in a subset of motilin cells.

RESULTS AND DISCUSSION

Chapter 1: Structure and Expression of the Human Motilin Gene

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Abstract

The human motilin gene was isolated from a human genomic library and its structure determined by restriction mapping and DNA sequence analysis. The gene consists of 5 exons separated by 4 introns spanning approximately 9 kb of genomic DNA. Exon I encodes the 5' untranslated portion of the motilin mRNA. Exons II and III encode the signal peptide and the 22 amino acid motilin peptide; codons encoding the motilin moiety are split by an intron. The C-terminal motilin-associated peptide (MAP) is largely encoded by Exons III and IV with the last two amino acids of the motilin precursor and the 3' untranslated region encoded by Exon V. Thus, the motilin gene has an unusual structure in which a small bioactive peptide is encoded on two distinct exons. Examination of the expression of the human and non-human primate motilin gene by Northern hybridization analysis indicates that it is expressed in a number of gastrointestinal and extra-gastrointestinal tissues.

Introduction

Motilin is a 22 amino acid peptide which has been purified from porcine, canine and human small intestine (Brown et al.; Reeve et al., 1985). The peptide is present in highest concentration in the duodenum, where it is present in enteroendocrine cells of the duodenal mucosa (Pearse et al., 1974), and in lesser amounts throughout the small intestine. Motilin-like immunoreactivity (MLIR) has also been detected in colon and gallbladder (Yanihara et al., 1978), adrenal, pituitary and CNS tissues (O'Donohue et al., 1981). In the gastrointestinal system, motilin is at least in part responsible for the initiation of interdigestive myoelectric activity and resulting contractile activity in the stomach and small intestine. Plasma motilin levels undergo regular cyclic fluctuations during fasting; peak levels correlate with the onset of the migrating myoelectric complex (MMC) in the gastric antrum in the human (Bormans et al., 1987). Intravenous infusion of synthetic motilin initiates premature MMC spikes (Vantrappen et al., 1979) and infusion of anti-motilin antiserum results in disappearance of intestinal MMC activity in the canine small intestine (Lee et al., 1983). Motilin also induces contractions of the Spincter of Oddi and gallbladder, as well as contraction of the lower esophageal spincter at least one hundred times as intense as those induced by atropine (Itoh, 1978). The mechanism and site of action of this hormone are not known; however, recent electrophysiologic studies suggest that motilin acts by activation of excitatory opioid pathways in the small intestine of the dog (Fox and Daniel, 1987) while binding studies indicate that motilin may act directly upon smooth muscle cells of the stomach and small intestine in rabbit and human (Strunz et al., 1975). This

action on smooth muscle apparently results in a release of intracellular calcium stores (Matthijs et al., 1989). Motilin release is stimulated by gastrin releasing peptide (GRP) and inhibited by pancreatic polypeptide, somatostatin and secretin (Walsh, 1987).

Abnormal serum motilin levels have been implicated in a number of pathophysiologic states including chronic idiopathic constipation (Sjölund et al., 1986) and idiopathic delay in gastric emptying (Labo et al., 1986). Elevated motilin levels have been reported in patients with chronic renal failure, small cell carcinoma of the lung and adrenal medullary pheochromacytoma, and may play a role in the disordered gastrointestinal motility seen in these patients.

Molecular characterization of the porcine motilin cDNA indicates that the peptide is synthesized as part of a prohormone precursor consisting of a signal peptide preceding the 22 amino acid motilin moiety and a 66 amino acid C-terminal peptide separated from the C-terminus of motilin by a Lys-Lys dipeptide (Bond et al., 1988). The C-terminal motilin-associated peptide (MAP) is predicted to be released after proteolytic cleavage at the dibasic site. This precursor structure is conserved in the human (Seino et al., 1987). We report here the structure of the human motilin gene and its expression in human and non-human primate tissues.

Materials and Methods

Screening of human genomic library

300,000 clones from a human genomic library (Maniatis et al., 1978) were plated and screened to isolate clones containing human motilin DNA. A 37 base oligonucleotide complementary to a portion of the human motilin cDNA sequence (Seino et al., 1987) was synthesized on an Applied Biosystems Model 380A DNA Synthesizer. This and all other oligonucleotides used in this work were purified by acrylamide gel electrophoresis and 5'-end labelled with ^{32}P-ATP (ICN) using polynucleotide kinase (Boehringer-Mannheim). Phage plaques were screened in duplicate by *in situ* hybridization on nylon membranes (Genescreen, NEN/DuPont) as previously described (Adelman et al., 1985).

Southern blot analysis of motilin genomic DNA clones

Positive phage plaques were purified by subsequent rounds of low density screening and phage DNA was purified from liquid culture by polyethylene glycol/NaCl precipitation followed by phenol extraction and ethanol precipitation (Yamamoto et al., 1970). Purified DNA was cut with a variety of restriction enzymes, electrophoresed on a 1.2% agarose gel and transferred to a nylon membrane (Nytran; Schleicher and Schuell) by capillary blotting. The resulting Southern blot was probed with the motilin-specific radiolabelled oligonucleotide in 50% formamide at 37^{o}C to determine the location of restriction sites relative to the region of the gene

encoding the motilin sequence. Similar Southern blots were subsequently probed with 32P-labelled oligonucleotides complimentary to different regions of the human motilin cDNA to determine the overall restriction map of the human motilin gene.

DNA sequence analysis

Exon-containing restriction fragments of the human motilin gene were subcloned into pGEM-3Z plasmid vector (Promega), M13mp18, or M13mp19 RF DNA and sequenced by the dideoxy chain termination method (Sanger et al., 1977).

Northern blot hybridization analysis

Surgical or autopsy samples of human and rhesus monkey tissue were snap frozen on liquid nitrogen immediately after removal and total RNA isolated by guanidine extraction and lithium chloride precipitation (Cathala et al., 1983). In most cases poly A(+) mRNA was isolated via oligo-dT cellulose (Type II, Collaborative Research, Inc.) affinity chromatography (Aviv and Leder, 1972). RNA samples were denatured in 50% formamide (Bethesda Research Laboratories), 2M formaldehyde in 20mM HEPES, 1mM EDTA, pH 7.8 and electrophoresed in 1.5% agarose containing 2M formaldehyde in 20 mM HEPES, 1mM EDTA, pH 7.8. After removal of the formaldehyde by diffusion, the gel was blotted onto a Nytran filter and probed with a ^{32}P-UTP labelled cRNA probe in 50% formamide. The probe, consisting of a 1.0 kb fragment of the human motilin gene encoding the first

39 amino acids of the prohormone was transcribed *in vitro* from a pGEM-3Z Riboprobe vector (Promega) using SP6 RNA Polymerase (Bethesda Research Laboratories). After overnight hybridization under stringent conditions (50% formamide, 65^{o}C), the blot was washed in 0.1X SSC, 0.1% SDS and 1mM EDTA at 70^{o}C and exposed to X-Ray film (Kodak X-Omat AR). The Northern blot was also probed with a cRNA probe for rat cyclophilin under identical conditions as a control for RNA integrity (Danielson, et al.).

Results

Isolation of human motilin genomic DNA clones

Screening of a human genomic library with a motilin-specific oligonucleotide identified two hybridization positive clones, hMG28-1 and hMG28-2. Restriction analysis indicated that hMG28-2 contained approximately 14.0 kb of human genomic DNA with three internal EcoRI sites. Southern hybridization analysis with oligonucleotides 2,3,4, and 5 (see Fig. 1B) indicated that the coding regions for motilin and the N-terminus of MAP were contained in a 6.6 kb EcoRI fragment while the C-terminus of MAP and the 3' untranslated portion of the human mRNA were encoded within EcoRI fragments of 1.2 kb and 2.7 kb, respectively. Oligonucleotide 1 (Fig.1B) complementary to the 5' untranslated portion of the motilin mRNA hybridized with a 7.6 kb EcoRI fragment of hMG28-1. These hybridization positive EcoRI fragments were subcloned into plasmid vectors for further characterization. The restriction map of the human motilin gene determined by Southern hybridization analysis using the various radiolabeled oligonucleotides is shown in Figure 1A.

Structure and nucleotide sequence of the human motilin gene

The strategy used to sequence the human motilin gene is shown in Figure 7A. Comparison of the genomic DNA sequence with the human cDNA sequence allowed identification of exon-intron junctions. In each case these junctions conform to the consensus GT/AG donor/acceptor

sequences used in RNA splicing (Breathnach and Chambon, 1981). The nucleotide sequence obtained indicates that the human motilin gene consists of five exons separated by four introns. The nucleotide sequence of the five exons and immediately flanking intron sequence, as well as 350 bp of 5' flanking sequence, is shown in Figure 7B. The DNA flanking the 5' end of the motilin gene contains the consensus promoter sequence, TATAA, 32 bp upstream from the putative transcription initiation site (cap site), which is indicated at nucleotide +1 in Figure 7B. The sequence upstream from the TATA box contains a CCAAT sequence at position -221 and GC-rich sequences homologous to known SP-1 binding sites at nucleotide -40 (GGGAGG) and -224 (ACGCCT). In addition, there is a core consensus cAMP-responsive element (CRE) CGTCA sequence at nucleotide -22.

The overall organization of the gene is shown schematically in Figure 8. The 5' untranslated region of the human motilin mRNA is encoded almost entirely on a single exon (Exon I), as is the signal peptide (Exon II). Motilin, however, is split by intron B into two exons (Exons II and III) between the codons for amino acids 14 and 15 of the motilin peptide. Exon III also encodes the 31 N-terminal amino acids of MAP. Intron C splits MAP nearly in half with all but the last three amino acids encoded by Exon IV. The last two MAP codons and DNA encoding the 3' untranslated portion of the human motilin mRNA reside on Exon V. A consensus polyadenylation signal (Proudfoot and Browness, 1976), AATAAA is located within this 3' untranslated region (See Figure 7B).

Expression of the motilin gene in human and rhesus monkey

Northern blot hybridization analysis of poly A+ RNA isolated from human and rhesus monkey duodenum revealed a strongly hybridizing band of approximately 700 bases (Fig. 9). A weakly hybridizing band of approximately 1.6 kb was also apparent in human duodenum. Bands of the same molecular weight as the duodenal motilin mRNA were detected in poly A(+) RNA extracted from monkey colon, liver, adrenal and kidney, and in total RNA isolated from monkey testis. No hybridization to monkey lung or cerebellum poly A(+) mRNA, total RNA from monkey pituitary, or to human cerebellum poly A(+) was detected. A band corresponding to rat cyclophilin mRNA was observed in all tissues examined, indicating that intact RNA was present in each lane. Cyclophilin, or prolyl cis-trans isomerase (Takahashi et al., 1989), mRNA is present in all of the tissues examined, although not necessarily in equivalent amounts. This corresponds to similar observations made in studies of cyclophilin mRNA distribution in rat tissues; for example, cyclophilin mRNA is present in lower levels in rat lung than in other tissues (Danielson et al., 1988).

Discussion

Cloned DNA containing the human motilin gene has been isolated and characterized. The exonic structure of the gene has been determined by DNA sequence analysis. This gene contains five exons separated by four introns containing consensus donor and acceptor splice sequences. The structure of this gene is unusual in that the sequence encoding the 22 amino acid hormone motilin, which is the purified and apparently biologically active form, is split near its center by an intron. This is a surprising finding since polyprotein precursor structure is generally reflected at the genomic level. Functional domains cleaved from the prohormone precursor at structurally distinct sites are usually encoded on a single exon. (Douglass et al., 1984). The few exceptions to this pattern involve intron splits near one of the ends of a functional domain. For example, all but the last three amino acids of the 26 amino acid gastrin-releasing peptide (GRP) are encoded on a single exon (Spindel et al., 1987).

The genomic structure of motilin may have a significant functional correlate. Since the 22 amino acid peptide (in the human and presumably other species) is encoded on two exons, motilin may in fact comprise two functional domains. Furthermore, the structure of the human motilin gene presented here may represent the basis for the interspecies heterogeneity in motilin structure which has been observed or inferred by others (Shin et al., 1980). Thus, it is interesting to note that although there is complete amino acid identity between human and porcine motilin, 5 of 22 amino acids are different in canine motilin. All five of these changes occur at codons expected to be encoded by Exon II. In addition, there is immunologic

evidence for molecular heterogeneity between rodent and human/porcine motilin. (Smith et al., 1980). For example, antisera specific to the C-terminus of porcine motilin can measure comparable levels of MLIR in the rat duodenum, but N-terminal specific antisera cannot detect any MLIR in rat duodenum (O'Donohue et al., 1981). This apparent difference in primary structure between rat and porcine motilin would also correspond to differences in nucleotide sequence in Exon II.

The nucleotide sequence of the five exons of the human motilin gene is almost identical to that reported for the human cDNA (Seino et al., 1987). However, three differences in amino acid codons were found (Fig. 1B): nucleotide 92, G instead of C; nucleotide 95, G instead of C; and nucleotide 254, G instead of C. All of these codon differences occur in the third base position and do not result in any amino acid changes. They likely represent silent polymorphisms in the human genome.

The region immediately upstream from Exon I contains a consensus TATA box likely used in transcription initiation at position -32 relative to the putative cap site. While we have not precisely defined the cap site, the presence of a purine-rich segment approximately 30 bp downstream from a TATA box, corresponding to the reported 5' end of the human motilin cDNA, allows us to predict the transcription start site region. A TATA box 25-30 bases upstream from a stretch of purines is also present beginning at nucleotide -312 relative to the proposed cap site. However, the sequence (TATATT) fits the consensus less well, and transcription initiation from this site would yield a mRNA at least 300 bases longer than that which is observed by Northern analysis. This region is therefore not likely a major promoter of the human motilin gene.

A CCAAT, sequence important for basal transcription in some genes, is present at nucleotide -321; this is further upstream than is usually the case for functional CAAT boxes, although this site has an adjacent GC box often present in proximity to CAAT boxes in other genes. Another potential SP-1 site (GGGAGG) resides adjacent to the TATA box at nucleotide -40 and several other GC-rich regions closely resembling SP-1 binding sites are present upstream of the TATA box. The presence of a core consensus CRE (CGTCA) at nucleotide -22 is noteworthy. However, compared with other known CREs this would be unusually close to the promoter region for a functional CRE. A fragment of the cloned human motilin gene containing Exon II readily detects a human duodenal mRNA of approximately 700 bases by Northern blot hybridization analysis. This size is in reasonable agreement with the 556 bp human cDNA described by Seino et al. which did not contain a poly-A tail and did not define the 5' end of the cDNA. Also, porcine motilin mRNA appears to be approximately 700 bases in length as determined by similar analysis (Bond et al., 1988). In addition, a minor 1.6 kb band is present in human duodenal mRNA. This band has not been seen in any other tissue examined. It may represent a weakly crossreacting species of mRNA, a partially spliced, or an alternately spliced form of the motilin message; neither possibility can be excluded at this time. Duodenal mRNA isolated from rhesus monkey contains motilin mRNA identical in size and apparent abundance to that of human duodenum. Because motilin expression in the duodenum appears to be very similar in human and nonhuman primate, monkey tissue was used to examine the tissue-specific expression of the motilin gene. As expected, motilin mRNA levels are highest in monkey duodenum, but motilin mRNA is also detectable in the

consistent with immunologic localization of motilin in mammalian colon in lower levels than those seen in small intestine. A significant amount of motilin mRNA is also present in liver; however, we cannot say which hepatic cell type expresses motilin mRNA. Since motilin has been localized by immunohistochemistry in bile duct tissue, it may be that the motilin mRNA is expressed in bile canaliculi which ramify throughout the liver parenchyma, as opposed to hepatocytes.

The motilin gene is also expressed in a number of non-gastrointestinal tissues, such as the adrenal gland. This expression is presumably in the adrenal medulla and is consistent with detection of MLIR in adrenal tissue (Yanihara, 1978) and with the report of elevated circulating motilin levels in a subset of patients with tumors of the adrenal medulla (Vinik et al, 1986). Motilin mRNA was also observed in the kidney. Significant motilin levels in the kidney have not been reported, although there is a report of elevated circulating motilin levels in a group of patients with renal disease (McCleod and Track, 1979). Motilin mRNA is also readily detectable in monkey testis total RNA. Subsequent Northern blots using poly A(+) testis RNA indicates that levels in the testis are comparable to those seen in liver tissue (data not shown). While motilin has not been previously localized in lung, a series of patients with oat cell tumors of the lung have been described with extremely high circulating motilin levels which could not explained by elevated GRP (Noseda et al., 1987). However, motilin mRNA is not detected in normal primate lung in the present study.

Motilin mRNA was not detected in monkey pituitary total RNA or in poly A(+) RNA isolated from human or monkey cerebellum. This result is inconsistent with the identification of MLIR in pituitary and central nervous

inconsistent with the identification of MLIR in pituitary and central nervous system (CNS) tissue, notably the cerebellum (Nilaver et al., 1982). However, motilin mRNA was also not detected in porcine cerebellum using a cloned porcine motilin cDNA probe (Bond et al., 1988). One explanation for the discrepancy is that the polyclonal antisera used to detect MLIR in the CNS recognize a crossreacting specie of peptide. In fact, the existence of motilin peptide in CNS tissue has been questioned by a number of authors because of variable crossreactivity to motilin antisera and differences in chromatographic mobility of MLIR between gut and brain extracts. Nevertheless, MLIR in the central nervous system has been independently detected in rodent, porcine, ovine and primate species by several groups using independently derived antisera. Another possibility is that the level of motilin mRNA expression in these tissues is lower than the sensitivity of our hybridization analysis. When the levels of MLIR of the same chromatographic mobility as the 22 amino acid peptide detected in duodenum were compared directly by RIA in the same specie (dog), pituitary and cerebellar levels were 10% and 3%, respectively, of that detected in duodenum (Poitras et al., 1987). It is therefore possible that motilin mRNA is synthesized at a very low level in brain and pituitary tissue.

In summary, we have described the cloning and characterization of the human motilin gene. The gene has an unusual structure which may explain the structural and species heterogeneity observed in this peptide. The motilin gene is expressed in a wide variety of tissues, but does not appear to be expressed at any significant level in neural tissue. Further studies will be directed at characterization of upstream regulatory regions of the gene and examination of its expression at the cellular level

Acknowledgements

We thank Mr. David Stein and Ms. Valerie Stallbaumer for oligonucleotide synthesis and Ms. Chris Bond and Dr. Gajanan Nilaver for useful discussions throughout this project. We are grateful for the excellent assistance of Ms. Vicki Robertson and Ms. June Shiigi for artwork and appreciate the help of Ms Wendy Manley in preparation of the manuscript. This work was supported by NIDA Grant DA-04154 (to J.D.) and NIH Grant HD24562-01 (to J.PA.). D.D. is supported by the Medical Research Foundation of Oregon.

Figure 7: Restriction Maps of Overlapping Human Motilin Genomic Clones, Sequencing Strategy, and Nucleotide Sequence of Human Motilin Gene Exons and Adjacent Intron Sequences

A: Restriction map of human motilin gene and sequencing strategy. Overlapping clones are shown with positions of pertinent restriction sites. Solid boxes depict exons and lower arrows indicate extent and direction of sequence data obtained. B: Nucleotide sequence of human motilin gene exons, immediately adjacent intron sequences, and flanking DNA. The putative transcription start site is indicated with an arrow and designated position +1; the short purine-rich region containing the proposed cap site is overlined. Numbers refer to nucleotide positions upstream from the cap site (negative) and to nucleotide positions in the human motilin cDNA. The TATAA sequence is within a shaded box and the AATAAA polyadenylation signal is underlined. The base corresponding to the end of the human motilin cDNA is indicated with an arrow at position 556. The encoded amino acid sequence of the motilin precursor is shown with the motilin moiety boxed. Sequences to those used for oligonucleotide probes are overlined; the oligonucleotides used were:

1) 5' GGGCGTGCACGTGGTCTGAGTGGGT 3'
2) 5' CCTCTGGAGTTCGCCATAGGTGAAGATGGGGACGAAG 3'
3) 5' GGGCTCGGCAGGGTCTACAGGACCTTCCTCCCC 3'
4) 5' GCATCTCACTCAGCAGCCCTTCCAGGGTGGCCGGG 3'
5) 5' CCCAGGGCCTCACTTGGGCAGGAGGGGCCTCCC 3'

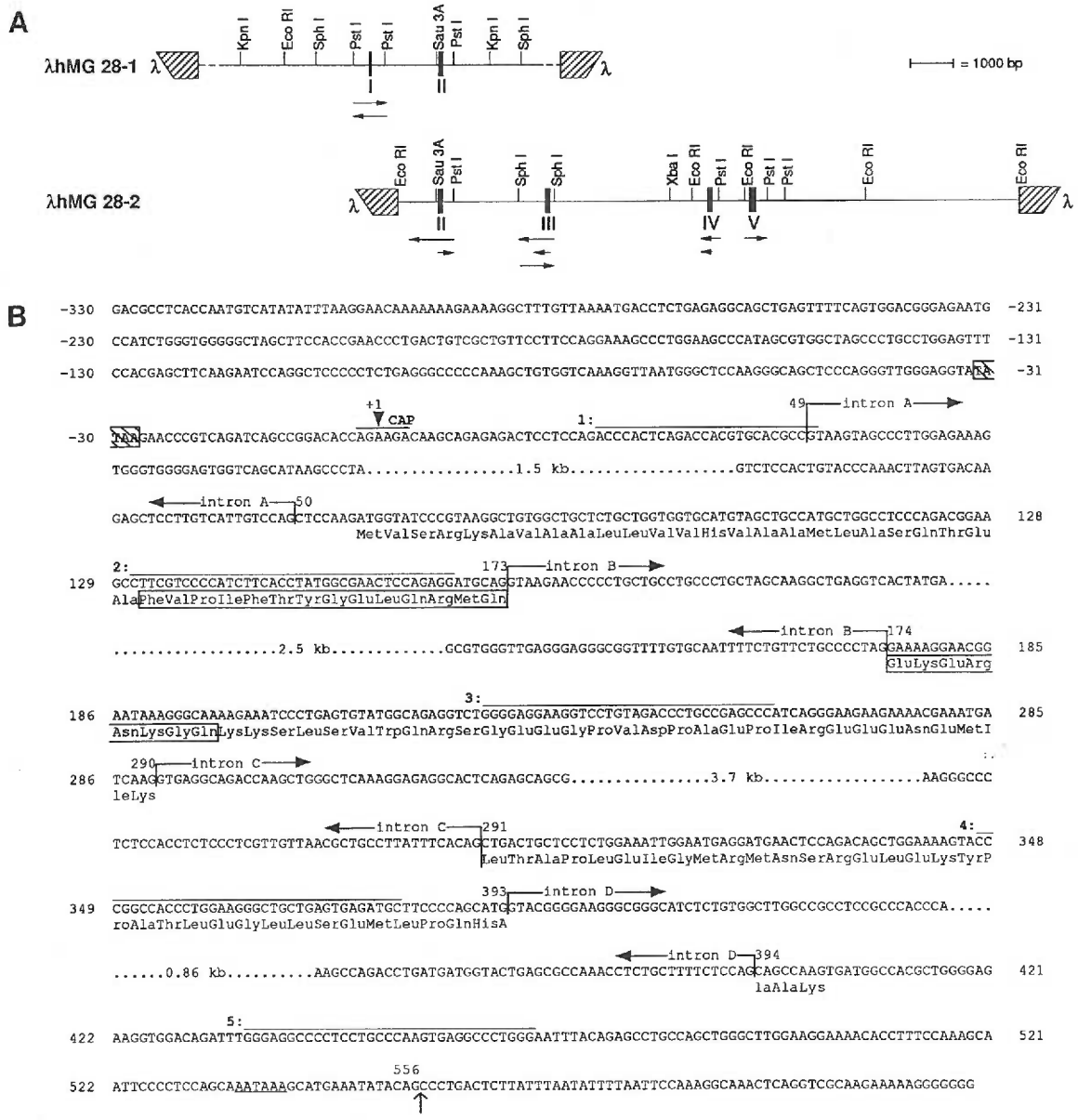

A
λhMG 28-1
λhMG 28-2
Kpn I
Eco RI
Sph I
Pst I
Sau 3A
Xba I
= 1000 bp
B
CAP
intron A
intron B
intron C
intron D
MetValSerArgLysAlaValAlaAlaLeuLeuValValHisValAlaAlaMetLeuAlaSerGlnThrGlu
AlaPheValProIlePheThrTyrGlyGluLeuGlnArgMetGln
GluLysGluArg
AsnLysGlyGlnLysLysSerLeuSerValTrpGlnArgSerGlyGluGluGlyProValAspProAlaGluProIleArgGluGluGluAsnGluMetI
leLys
LeuThrAlaProLeuGluIleGlyMetArgMetAsnSerArgGluLeuGluLysTyrP
roAlaThrLeuGluGlyLeuLeuSerGluMetLeuProGlnHisA
laAlaLys

Figure 8: Intron-Exon Structure of the Human Motilin Gene

Human motilin gene and its relation to prepromotilin structure. Exons are represented by solid boxes and numbered with roman numerals; capital letters refer to introns. Regions of the precursor split by introns are depicted, including the amino acids adjacent to these junctions. MAP is motilin-associated peptide and 5' UT and 3' UT refer to the untranslated portions of the human motilin mRNA.

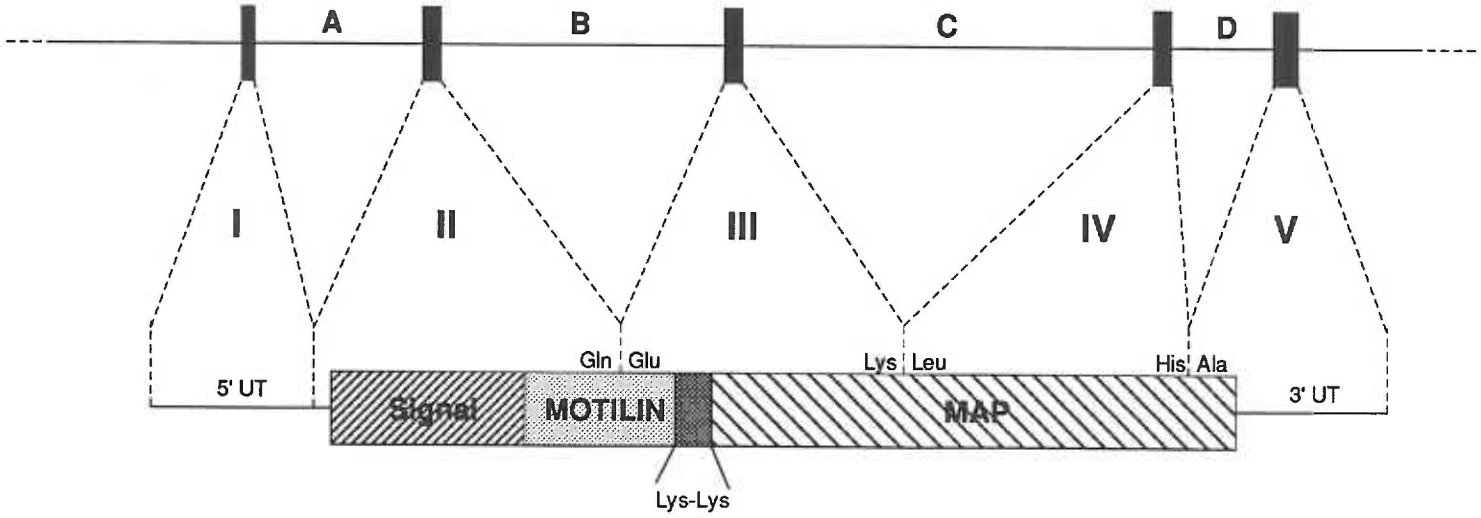
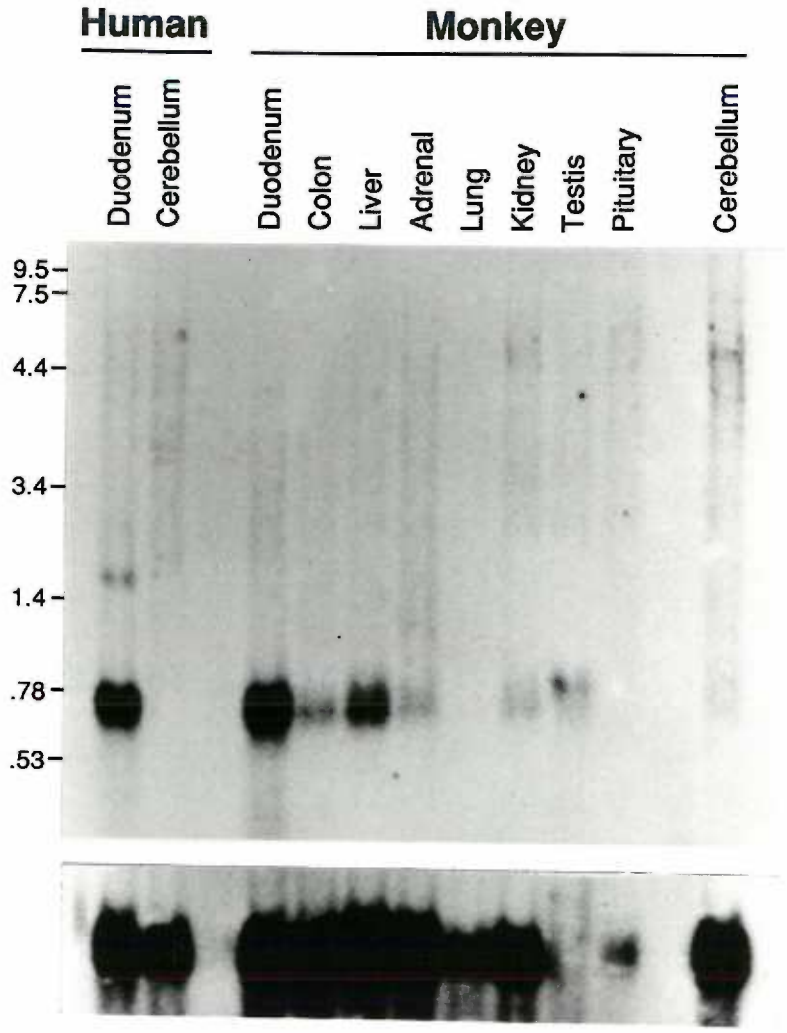

A
B
C
D
I
II
III
IV
V
5' UT
Gln Glu
Lys Leu
His Ala
3' UT
Signal
MOTILIN
MAP
Lys-Lys

Figure 9: Northern Blot Hybridization Analysis of Human and Rhesus Monkey Poly-A+ RNA Isolated from Various Tissues

Northern blot analysis of motilin gene transcription in human and non-human primate tissues. The blot was probed under stringent conditions with a cRNA probe derived from the human motilin gene. RNA molecular weight standards were run on the same gel and stained separately with acridine orange. Their size in kilobases and mobilities are shown to the left of the blot. The lower panel shows the same Northern blot described above which was washed free of radiolabel and reprobed with a rat cyclophilin cRNA probe. The bands obtained are of the appropriate molecular weight for cyclophilin RNA.


Human
Monkey
Duodenum
Cerebellum
Duodenum
Colon
Liver
Adrenal
Lung
Kidney
Testis
Pituitary
Cerebellum
9.5
7.5
4.4
3.4
1.4
.78
.53

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Chapter 2: Expression of Motilin Messenger RNA in the Primate Gastrointestinal System

RUNNING TITLE: Motilin Gene Expression in Gut

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This work was supported by NIDA Grant DA-04154 (to J.O.D.) and NIH Grant HD24562-01 (to J.P.A.). D.D. is supported by the Medical Research Foundation of Oregon.

Abbreviations used in this paper: bp, base pairs; BSA, bovine serum albumin; cDNA; complementary DNA; cRNA, complementary RNA, EDTA, ethylenediamine tetraacetic acid; HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; kb, kilobases; MMC, migrating myoelectric complex; mRNA, messenger RNA; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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Abstract

The tissue distribution and cellular localization of motilin gene expression in the primate gastrointestinal system was studied using a fragment of the cloned human motilin gene as a probe. Northern blot hybridization and *in situ* hybridization analysis were used to detect the presence of motilin mRNA, and immunohistochemistry was used to detect motilin immunoreactivity. Motilin mRNA levels were highest in the duodenum, with the transcript detected only in mucosal cells containing motilin peptide. Lower levels of motilin transcript were detected throughout the gastrointestinal tract, including the esophagus and stomach, and in accessory tissues such as the sublingual gland and bile duct. A related mRNA specie of the same apparent size as the motilin transcript was detected in liver and gallbladder. The results of this study show that the motilin gene is transcribed in the same mucosal cells in the primate lower gastrointestinal tract that express motilin peptide. However, motilin mRNA is also synthesized in the upper gastrointestinal tract, suggesting that the motilinergic effects described in these tissues may be mediated by small populations of motilin-secreting cells acting locally.

KEY WORDS: gene; Northern analysis; in situ hybridization; immunohistochemistry

Introduction

Normal gastrointestinal function relies upon precise coordination of motility and secretion. An increasing body of evidence indicates that enteric neurons, which synthesize a diverse array of peptides considered to act as neurotransmitters or neuromodulators, provide the direct stimulus and control of these events (1). However, little is known about the mechanisms whereby contraction and secretion are coupled. Motilin is a 22 amino acid peptide initially purified from porcine and canine duodenum which is thought to play a major role in the initiation of the interdigestive migrating myoelectric complex (MMC). Plasma motilin levels show cyclic fluctuations during interdigestive periods and peak levels have been correlated with the appearance of the MMC in the duodenum in a number of species (2,3,4). Intravenous infusion of motilin can initiate premature activity fronts (4), and infusion of motilin antibodies delays the MMC activity fronts in the antrum and duodenum (5). The appearance of MMC activity, however, is not always accompanied by motilin peaks (6), and normal duodenal MMC activity can be induced by agents other than motilin such as morphine (7). Furthermore, the peak motilin levels measured in humans correlate with the appearance of the MMC in the stomach, rather than in duodenum (8). Also, while motilin injection does not initiate Phase III MMC activity during the fed state in the dog, morphine injection overcomes the disruption in MMC cycling induced by feeding, resulting in the reappearance of motilin cycling (9). It has therefore been argued that motilin is released in response to muscular contractions in the duodenum (9). This is further supported by the finding

that motilin cycling can be blocked by atropine or tetrodotoxin *ex vivo* (10). Sarna et al. have proposed that motilin may function to coordinate secretory and motor events in the upper small intestine with the appearance of the MMC in this region (9).

Motilin can stimulate rabbit and human smooth muscle contraction *in vivo* even after complete denervation (11), and motilin binding activity has been measured in isolated stomach smooth muscle (8). Thus, motilin apparently can act directly to stimulate smooth muscle contraction. Synthetic porcine motilin causes contraction of the Sphincter of Oddi (12) and contraction of the lower esophageal spincter 100 times as strong as that induced by atropine (13). Although motilin immunoreactivity has been measured within small bowel muscle, implying its presence in enteric neurons (14), most investigators have localized motilin immunoreactivity only in the mucosal layer, where it is believed to be in enterochromaffin cells (15). Highest levels of motilin immunoreactivity and the largest population of motilin immunoreactive cells have consistently been found in the duodenum, while lower levels of immunoreactivity have been measured throughout the rest of the lower intestinal tract. However, low levels have also been measured in the upper gastrointestinal tract in some species, especially in stomach (16). Motilin immunoreactive cells have also been detected in bile duct (17), and motilin-like immunoreactivity has been measured in the primate adrenal (16), human adrenal tumors (18), pituitary (19) and central nervous system (20). There has not, however, always been a correlation between the presence of motilin-like immunoreactivity and motilin mRNA (21). Thus, while the peptide apparently functions in extraduodenal tissues, it is not clear based on immunologic data whether the peptide is actually

synthesized outside of the duodenum. Localization of motilin mRNA provides an alternate and complementary approach to determining the sites of motilin biosynthesis.

We previously cloned the human motilin gene and used a fragment of the gene to detect motilin mRNA in a range of human and monkey tissues (22). The cloned human gene recognized a specie of poly-A^+ RNA of approximately 700 bases in both human and monkey duodenum; the size predicted for the human motilin transcript based upon the published cDNA sequence (23). Thus, the human motilin gene provides a specific probe with which to examine motilin gene expression in the human and nonhuman primate gastrointestinal system. The current study was therefore undertaken to examine the tissue distribution of motilin mRNA in the primate gastrointestinal system in greater detail, and to determine its site of synthesis at the cellular level.

Materials and Methods

Preparation of Radiolabelled Probes

Genomic DNA fragments encoding portions of the human motilin precursor were subcloned into the plasmid pGEM-3Z (Promega Biotech Co.), which contains promoter sequences for SP6 and T7 RNA Polymerases on either side of a multiple cloning site. Radiolabelled cRNA transcripts complementary to motilin mRNA were transcribed *in vitro*. The standard reaction contained 1 ug of linearized template DNA and was carried out in a

solution containing 40 mM Tris-HCl pH 7.0, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine and 0.5 mM each of rGTP, rATP, and rCTP, 7.5 uM rUTP, 100μCi ^{32}P-rUTP or ^{35}S-rUTP (6.25 μM), 10 mM dithiothreitol, 100 μg/ml BSA, 1 unit of placental RNAse inhibitor (RNAsin, Promega Biotech C.), or InhibitACE (5' to 3' Inc.), and 1 unit of RNA polymerase in a total volume of 20 μl at 40^{o}C for 45 minutes. All solutions were made in diethylpyrocarbonate treated water to inhibit contaminating RNAse activity. After incubation the reaction volume was increase to 150 μl and extracted with phenol:chloroform, adjusted to 2M ammonium acetate, and precipitated with three volumes of ethanol. The labelled probe was resuspended in 200 μl 0.1% SDS, 50% formamide and the incorporation of radiolabel determined by liquid scintillation counting.

Northern Blot Hydridization Analysis

Surgical or autopsy samples of human and rhesus monkey tissue were snap frozen in liquid nitrogen immediately after removal and total RNA isolated by guanidine extraction and lithium chloride precipitation (24). Poly A^{+} mRNA was isolated by oligo-dT cellulose (Type II, Collaborative Research, Inc.) affinity chromatography (26). RNA samples were denatured in 50% formamide (Bethesda Research Laboratories), 2M formaldehyde in 20mM HEPES, 1mM EDTA, pH 7.8 and electrophoresed in 1.5% agarose containing 2M formaldehyde in 20 mM HEPES, 1mM EDTA, pH 7.8. After removal of the formaldehyde by diffusion, the gel was blotted onto a Nytran filter (Schleicher and Scheuell) and probed with a ^{32}P-UTP labelled cRNA probe in 50% formamide, 5% SDS in 0.4M sodium phosphate, pH 7.2. The

probe was transcribed from plasmid pGM-2 containing Exon II of the human motilin gene encoding the first 39 amino acids of the prohormone (22). After overnight hybridization under stringent conditions (50% formamide, 65^{o}C), the blot was washed in 0.1X SSC, 0.1% SDS and 1mM EDTA at 68-70^{o}C and exposed to X-Ray film (Kodak X-Omat AR) with an intensifying screen at -70^{o}C. The Northern blot was also probed with a cRNA probe for rat cyclophilin under identical conditions as a control for RNA integrity (26).

Immunohistochemistry

Fresh autopsy tissue from Rhesus monkey was immersion-fixed in buffered formalin (overnight at 4^{o}C) followed by cryoprotection in 30% sucrose in 0.05M phosphate buffered saline overnight at 4^{o}C. Cryostat sections were immunostained with a rabbit antiserum to synthetic porcine motilin (N1-4) (20) at a final dilution of 1:1000. Sections were labeled by the avidin-biotin glucose oxidase technique using biotinylated Protein A as the bridging reactant (27). Reaction products were visualized with nitroblue tetrazolium (28). For dual immunohistochemical and *in situ* hybridization labeling experiments, sections were labeled with aviden-horseradish peroxidase and the reaction was visualized with diaminobenzidine, which resulted in a brown colored reaction product. Negative controls consisted of identical sections incubated in normal (nonimmune) rabbit serum in place of the immune serum and treated in parallel with the specifically labeled sections in subsequent steps.

in situ Hybridization

Cryostat sections (prepared as above) were mounted on silanized slides (29), delipidated, air dried, and rehydrated in 2X SSC (1X SSC is 0.15M NaCl, 0.015M sodium citrate). After removal of excess liquid the sections were incubated in 50 µl prehybridization solution (2X SSC, 50% formamide, 100µg/ml each yeast tRNA and sonicated salmon sperm DNA, 0.1% each SDS, BSA, ficoll, and polyvinyl pyrrolidone) 60 min at room temperature. This solution was removed and replaced with 50 µl prehybridization solution containing 2-4 x 10^5 cpm ^{35}S-labeled cRNA probe and 20 mM dithiothreitol. The sections were coverslipped, sealed with airplane glue, and hybridized overnight at 60^{o}C. All solutions used to this point were treated with 0.1% diethylpyrocarbonate to inhibit RNAse activity and the slides handled with sterile technique. Coverslips were next floated off in 2X SSC and the slides washed in 4L 0.2X SSC, 0.1% SDS at 50^{o}C. After air drying the slides were dipped in NTB-3 emulsion (Kodak, Inc.) diluted 1:1 in H_2O and exposed in a light-tight, dessicated box for 2-4 wk. The emulsion was developed in D-170 developer (Kodak, Inc.) according to manufacturers instructions.

Specificity of hybridization was demonstrated by use of the following controls: 1) sections were pretreated with 50 ug/ml bovine pancreatic RNAse (Sigma Co.) for 30 min at 37^{o}C, followed by extensive rinsing with 2X SSC prior to *in situ* hybridization, and 2) adjacent sections to those hybridized with the antisense cRNA probe were hybridized with an equivalent amount of ^{35}S-labelled sense strand cRNA probe.

Results

Northern Hybridization Analysis of Motilin Transcript Distribution

Northern blot analysis was used to determine the tissue distribution of motilin transcripts. After hybridization with a ^{32}P-labelled cRNA probe corresponding to a Exon II fragment of the human motilin gene encoding the first 39 amino acids of the prohormone molecule (23), a strongly hybridizing specie of poly-A$^+$ RNA of approximately 700 bases was apparent in poly-A$^+$ RNA isolated from Rhesus monkey duodenum (Figure 10A). A lower level of the same molecular weight specie was present in poly-A$^+$ RNA isolated from proximal jejunum, and still lower levels in more distal intestinal tissues. Weakly hybridizing bands of the same size were also detected in tissues oral to the duodenum, including stomach and esophagus. In addition, poly-A$^+$ RNA of the same apparent molecular weight, in levels comparable to that seen in the proximal jejunum, were detected in RNA from liver and gallbladder, and to a lesser extent in bile duct. Each lane contained RNA which hybridized to a ^{32}P-labelled cyclophilin probe indicating that all lanes contained approximately similar amounts of intact RNA (Figure 10C). The presence of higher molecular weight species of hybridizing mRNA suggested that the hybridization/wash conditions were such that the probe could also recognize partially complementary nucleotide sequences present in other species of mRNA. When the Northern blot was washed at slightly higher stringency (70^{o}C/0.1XSSC vs. 68^{o}C/0.1XSSC), the higher molecular weight hybridization signals largely disappeared. Surprisingly, the 700 base hybridization signal detected in liver and biliary tissues also

disappeared, while the bands in upper small intestinal tissues were mainly unaffected (Figure 10B). Faint hybridization signals were apparent in the RNA samples from esophagus, stomach, distal small intestinal tissues and colon upon long exposure. It therefore appears that the highest motilin mRNA levels are present in duodenum and ileum, with lower, but detectable levels present throughout the gastrointestinal tract. Furthermore, the 700 base hybridization signal detected in liver and biliary tract samples may not represent authentic motilin transcripts. These results have been confirmed by polymerase chain reaction analysis using oligonucleotides complementary to the human motilin mRNA to prime single stranded cDNA generated by reverse transcription of mRNA isolated from various primate gastrointestinal tissues. The identity of the amplified products was confirmed by Southern hybridization analysis using a labeled oligonucleotide complementary to a region of the motilin mRNA in between the priming oligonucleotides. The results of these experiments indicate that while authentic motilin transcripts are produced in monkey esophagus, stomach, duodenum, jejunum, ileum and colon, motilin mRNA is not synthesized in liver or biliary tissues (data not shown).

Localization of motilin peptide by Immunohistochemistry

Motilin immunoreactivity was detected in monkey duodenal mucosal cells using an antibody raised against synthetic porcine motilin (Figure 11A,B). These cells were most commonly seen in duodenal sections, but were also occasionally detected in sections of ileum and colon (Figure 11C). Motilin-specific immunoreactivity was only seen in cells in the mucosal layer

Motilin-specific immunoreactivity was only seen in cells in the mucosal layer thoughout the intestinal tract. Repeated attempts to detect motilin-specific immunoreactivity in monkey liver, gallbladder, or bile duct using either an avidin-glucose oxidase conjugate (which gave low-background staining) or a more sensitive aviden-horseradish peroxidase conjugate were consistently negative.

Cellular Localization of Motilin Transcripts by *in situ* hybridization

In situ hybridization was used to determine the sites of motilin mRNA synthesis at the cellular level in the duodenum, where the level of motilin gene transcription is highest. This technique was also combined with immunohistchemistry to correlate the site of mRNA synthesis with that of motilin peptide synthesis. When ^{35}S-labelled antisense cRNA probes were hybridized to monkey duodenal sections *in situ*, clusters of silver grains were observed in mucosal regions. Only background grain densities were observed over Brunner's submucosal glands, muscle layers, or enteric ganglia (Figure 12A). When adjacent sections were pretreated with RNAse, or a sense strand (mRNA) labelled probe was used in a hybridization experiment, only background grains were observed with no clustering of grains, indicating that the grain densities observed with the antisense probe were specific for the motilin mRNA transcript. The mucosal grain densities were localized over discreet mucosal cells whose abundance was comparable to those detected by immunohistochemistry (Figure 12B). The sequential application of motilin antiserum visualized by horseradish peroxidase and the motilin cRNA radiolabeled probe revealed an

sequential application of motilin antiserum visualized by horseradish peroxidase and the motilin cRNA radiolabeled probe revealed an immunohistochemically stained mucosal cell with a dense overlying cluster of silver grains (Figure 13), indicating that motilin peptide and mRNA are present in the same cell. Immunohistochemically stained sections probed with the labeled RNA (sense) probe revealed immunoreactive mucosal cells, but only low levels of background silver grains (data not shown).

Discussion

The experiments described in the present study demonstrate that the motilin gene is expressed in a tissue-specific manner in the primate gastrointestinal system. Northern blot hybridization analysis of poly-A^+ RNA purified from tissues throughout the monkey gastrointestinal system indicates that the highest levels of motilin mRNA are present in the duodenum, with significant levels also present in the proximal jejunum. These findings are consistent with measurements of motilin peptide by radioimmunoassay or immuno-histochemistry by a number of different groups in several species which indicate that motilin is synthesized predominantly in the duodenum. For example, Bryant et al. measured highest levels of motilin in the primate duodenum with lesser and steadily decreasing amounts moving aborally (30).

In situ hybridization with an antisense motilin probe demonstrates that the mRNA encoding the motilin precursor is expressed only in mucosal epithelial cells in the duodenum. The specificity of this hybridization was

confirmed by the absense of any specific hybridization using a probe corresponding to the sense, or motilin mRNA, strand; or after pretreatment with RNAse. The mucosal cells which contain motilin mRNA have the same appearance and location as those which contain motilin immunoreactivity, and in a dual immunohistochemistry/*in situ* hybridization experiment, a single mucosal cell was observed to contain both motilin immunoreactivity and motilin mRNA. Therefore, this cell type is responsible for both production and secretion of motilin in the primate duodenum. The absence of any specific hybridization or immunostaining in any other layer of the monkey intestinal tract indicates that the motilin gene is expressed only in a subset of mucosal epithelial cells. This finding is in agreement with those previous immunohistochemical studies which have detected motilin immuoreactivity only in the mucosa (31).

The synthesis and secretion of motilin within the duodenum is consistent with its putative role in stimulation or coordination of the MMC in the proximal small intestine. However, motilin also has actions in other tissues, for example contraction of sphincter muscle in the esophagus and bile duct (14). Motilin binding has also been measured in human and rabbit stomach (32,8). Although it is possible that all of these actions are mediated via release of motilin in the sphlancnic circulation (i.e. via an endocrine mode), the possibility of a direct action of locally synthesized motilin in these regions cannot be excluded. Consistent with this possibility is our detection of small amounts of motilin mRNA in the monkey upper gastrointestinal tract. Northern blot analysis revealed detectable hybridization signals in samples of esophageal and stomach poly-A^+ RNA. The identity of these transcripts was confirmed by a more detailed analysis using the polymerase chain

reaction to amplify motilin mRNA sequences from the total RNA pool in these tissues. The combined results of Northern analysis and PCR amplification of gastrointestinal RNA indicates that in addition to the relatively high levels of motilin mRNA produced in the duodenum, low levels of motilin mRNA are produced throughout the monkey gastrointestinal tract extending from the esophagus through the colon. The low levels of motilin transcript detected in these tissues may be the result of a very low level of gene expression in many cells, or they may represent a few cells (localized or diffuse) which individually express high levels of motilin mRNA. Only low levels of motilin immunoreactivity have been measured in primate esophagus, while levels comparable to those measured in the duodenum were detected in antral tissue (16). There have been no reports of motilin-positive cells detected by immunohistochemistry in the upper gastrointestinal tract. However, no systematic histochemical examinations of these tissues for motilin cells have been performed in the primate. The other possibility for the absence of previous reports of motilin immunoreactivity in these tissues is that the motilin peptide may be present in a different processed form not recognized by the anti-synthetic motilin antibodies used in these studies. Evidence for such a possibility has recently been presented in the case of canine motilin in which there apparently is an initial proteolytic cleavage within the C-terminal peptide of promotilin, releasing a 6 kd fragment from the promotilin precursor before cleavage of motilin itself (33).

The presence of an mRNA transcript of the same apparent size as the duodenal motilin transcript in liver and gallbladder which hybridized with the human motilin probe in a Northern hybridization experiment was somewhat surprising. Although motilin immunoreactivity has been measured in

gallbladder and bile duct (16,17), motilin is not generally considered to be a hepatic peptide. However, the observation that the hybridization signal could be removed from the liver and gallbladder mRNA samples at slightly more stringent wash conditions (while remaining hybridized with the duodenal form) indicates that this RNA does not represent the duodenal motilin transcript. This was confirmed by analysis of PCR products from these tissues. While motilin-specific products were amplified from other tissues which also contained motilin mRNA as determined by Northern analysis, no such products could be amplified from liver or gallbladder mRNA. Thus, the motilin gene does not appear to be expressed in these tissues.

Nevertheless, the specie of mRNA which hybridized to the motilin probe in liver and gallbladder mRNA must at least be closely related to motilin at the nucleic acid sequence level since the motilin probe did remain hybridized following the initial stringent hybridization/wash conditions. None of the other known gastrointestinal peptides, nor any published nucleic acid sequence in the EMBL and GENBANK databases reveal any significant degree of homology to the human motilin mRNA. Interestingly, Yanaihara measured extremely high levels of motilin by radioimmunoassay in iver (34), although hepatic motilin has not been reported by other means or in other species. The presence of a transcript in monkey liver that is closely related to motilin raises the possibility that a closely related peptide or protein is produced in the liver which could crossreact with anti-motilin antibodies. Such a molecule could result from an alternately spliced from of a motilin primary transcript or from a transcript of a different gene.

In summary, we have examined the expression of the motilin gene in the primate gastrointestinal system and demonstrated that motilin mRNA is transcribed in highest levels in the upper small intestine within mucosal cells which contain motilin immunoreactivity, as well as in lower levels throughout the gastrointestinal tract. These findings suggest that motilin peptide is synthesized throughout the upper gastrointestinal tract at local sites in addition to the duodenum, perhaps to act near these sites of synthesis. A transcript which is related to the product of the motilin gene, but is as yet unidentified, is expressed in monkey liver and biliary tissue.

Figure 10: Northern Blot Analysis of Gastrointestinal Poly-A^+ RNA Using an Exon II-Specific cRNA Probe

A: Northern blot analysis of motilin gene transcription monkey gastrointestinal tissues. Each lane contained 5 μg poly-A^+ RNA. RNA molecular weight standards were run on the same gel, blotted, and stained separately with methylene blue. Their size in kilobases and mobilities are shown to the left of the blot. The blot was probed under stringent conditions (60°C/50% formamide) with a cRNA probe derived from the human motilin gene, washed in 0.1X SSC at 68°C, and exposed 4 days to X-ray film. **B:** The Northern blot described above rewashed in 0.1X SSC at 70°C. and exposed for an equivalent time to X-ray film. **C:** The same Northern blot described above was washed free of radiolabel and reprobed with a rat cyclophilin cRNA probe. The bands obtained are of the appropriate molecular weight for cyclophilin RNA.

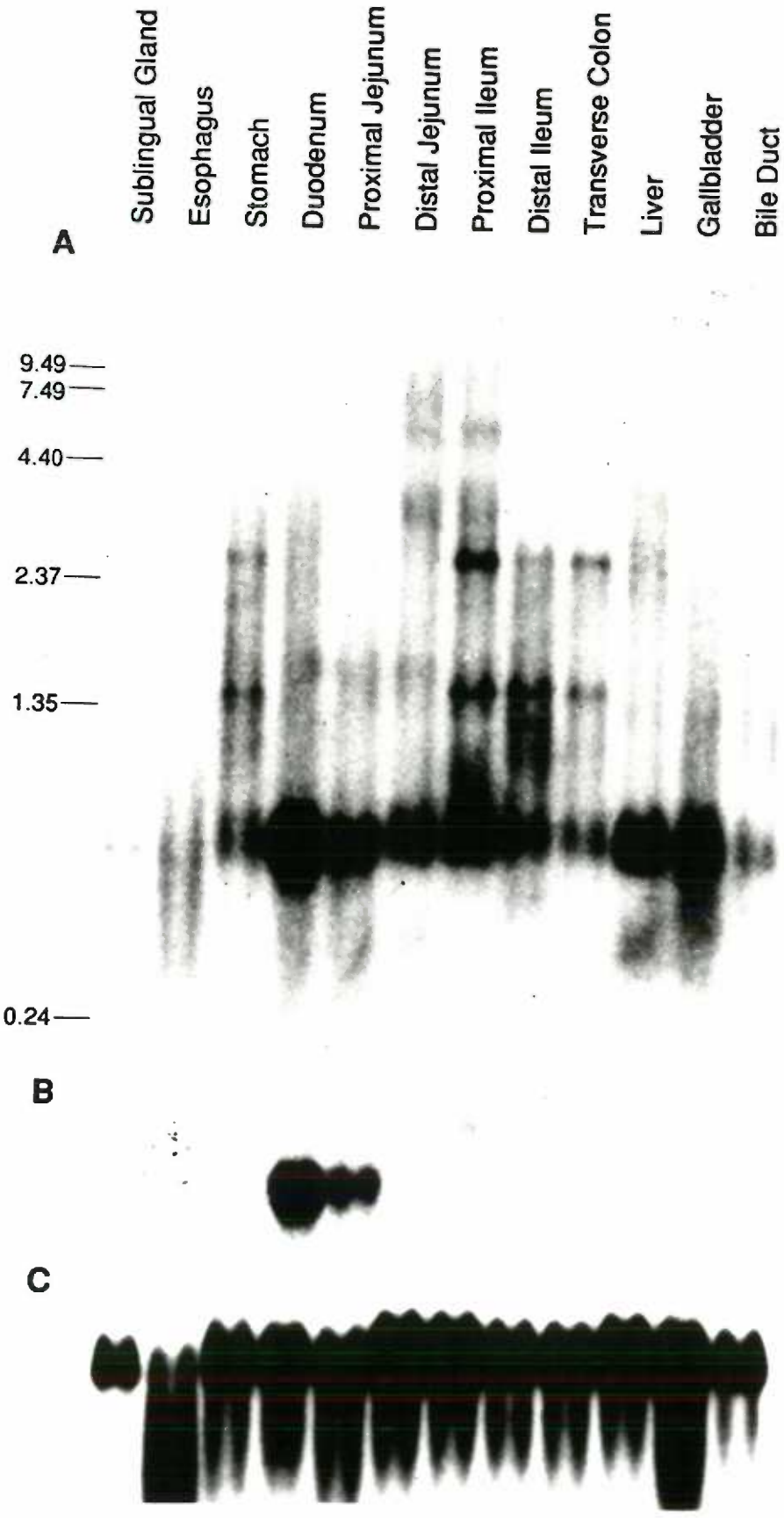

Sublingual Gland
Esophagus
Stomach
Duodenum
Proximal Jejunum
Distal Jejunum
Proximal Ileum
Distal Ileum
Transverse Colon
Liver
Gallbladder
Bile Duct
A
9.49
7.49
4.40
2.37
1.35
0.24
B
C

Figure 11: Immunohistochemical localization of Motilin-containing cells in Monkey Duodenum

A: Low power photomicrograph of a monkey duodenal section containing a relatively large number of motilin immunoreactive cells. 12 μm sections were incubated with motilin antiserum as described and visualized with nitroblue tetrazolium. Calibration bar = 100μm. **B:** High power view of a motilin immunoreactive mucosal cell in monkey duodenum. Calibration bar = 10μm. **C:** High power view of a motilin immunoreactive mucosal cell in monkey descending colon. Calibration bar = 10μm.

C
B
A

Figure 12: Localization of Motilin mRNA in Monkey Duodenum by *in situ* Hybridization

A: Dark field photomicrograph of a section of monkey duodenum incubated with a ^{35}S-labeled antisense motilin cRNA probe. Silver grains are localized only within the mucosal layer over mucosal cells. Calibration bar = 100μm. **B:** High power bright field photomicrograph of a monkey duodenal mucosal crypt incubated as in A and counterstained with hematoxylin and eosin. Silver grains are specifically localized over a single mucosal cell. Calibration bar = 10 μm.

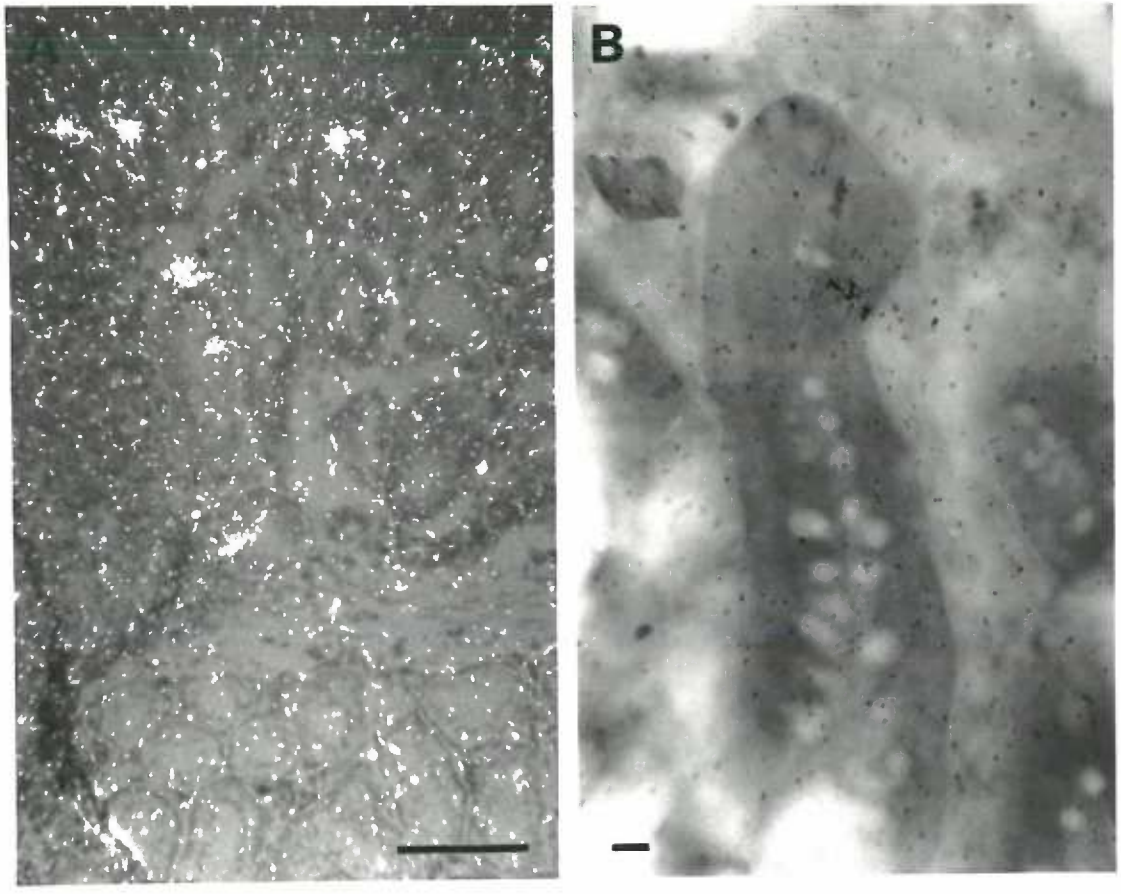
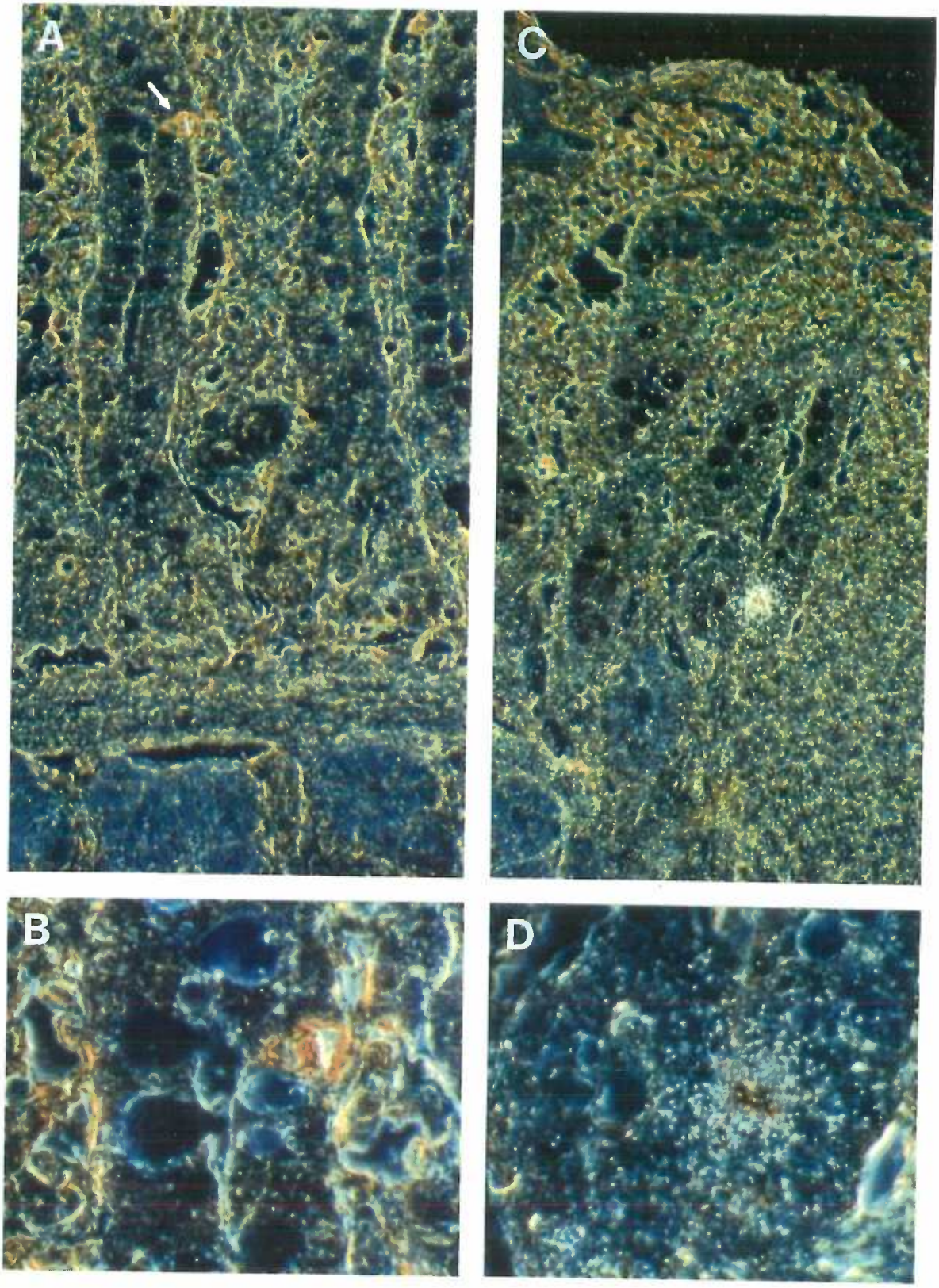

B

Figure 13: Colocalization of Motilin Peptide by Immunohistochemistry and Motilin mRNA by *in situ* Hybridization

A: Darkfield photomicrograph of a section of monkey duodenum sequentially labelled by immunohistochemistry using motilin antiserum visualized with horseradish peroxidase and *in situ* hybridization using a sense strand ^{35}S-labeled motilin riboprobe The arrow points to a labeled motilin immunoreactive cell in the mucosal layer of in intestinal crypt; only a few scattered background silver grains are apparent. **B:** High power view of the immunoreactive cell seen in A. **C:** Darkfield photomicrograph of a section of monkey duodenum sequentially labelled by ithe peroxidase technique using motilin antiserum visualized with DAB and *in situ* hybridization using an antisense ^{35}S-labeled motilin riboprobe. A single mucosal cell is densely labeled with silver grains. **D:** High power view of the *in situ* labeled mucosal cell seen in C showing the underlying brown staining indicating the presence of motilin immunoreactivity.


A
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B
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Chapter 3: Relation of Motilin Synthesis in the Primate Duodenum to other Gut Neuropeptides

Summary

The occurance of a number of putative neurotransmitter peptides in the monkey duodenum has been examined by immunocytochemistry. Vasoactive intestinal polypeptide, somatostatin and substance P immunoreactivity are present in neuronal fibers and in mucosal epithelial cells, implying their synthesis in both cell types, while motilin immunoreactivity is present only in mucosal cells. The mucosal cells expressing motilin, vasoactive intestinal polypeptide, and somatostatin exhibit intense immunoreactivity, and each cell type has a distinct morphology. In contrast, substance P-containing mucosal cells exhibit weak immunoreactivity and do not appear to be of a single structural class. Data is presented indicating that substance P is also present in a subset of motilin-producing cells in the monkey duodenum.

Introduction

A major strategy that multicellular organisms from invertebrates to mammals have exploited to expand their functional repertoire has been to use the same molecules for more than one function. Examples of this are so numerous

that it now must be considered a general phenomenon. This is particularly evident in the family of "brain-gut peptides" which are produced in gastrointestinal endocrine tissues, where they function as hormones, and in central neural tissue, were they function as neurotransmitters or neuromodulators. Functional regulation, and therefore diversity, can be further increased when these two systems (endocrine and nervous) interact with one another. This is the situation in the gastrointestinal system where enteric neurons, which send projections throughout the layers of the gut wall, including the mucosa (see Figure 2), produce some of the same peptides secreted by mucosal endocrine cells. Obviously such a situation also greatly increases the complexity and difficulty of physiologic studies of the gastrointestinal system.

Three peptides in particular - vasoactive intestinal polypeptide (VIP), somatostatin and substance P (SP)- have been localized in both mucosal endocrine cells and enteric neurons (83, 84, 85). Each of these peptides has diverse, and only partially delineated actions. VIP is a strong stimulator of secretion from both intestinal mucosal epithelium and duodenal Brunner's glands (86). Its effect is a direct one as evidenced by the presence of VIP receptors on the basolateral membrane of enterocytes (87). VIP also increases intestinal blood flow (88), and relaxes smooth muscle (89).

Although originally named for its ability to inhibit growth hormone secretion from the pituitary, somatostatin can also inhibit the release of most known gastrointestinal hormones (90). In addition, somatostatin inhibits amino acid

absorption in the human small intestine (91) and has broad effects on gastric and small intestinal motility. In particular, somatostatin inhibits normal cyclic interdigestive motor activity, and ectopic contactile activity induced by motilin (92).

Substance P is considered to be an excitatory neurotransmitter throughout the GI system and contracts gastrointestinal smooth muscle in many species (93). In addition, it increases GI blood flow, and inhibits gastric acid secretion (94) and intestinal ion absorption (95). In some cases it appears that these peptides function as neurotransmitters which are released from neurons, as evidenced by the actions of VIP and substance P on smooth muscle. In other cases it is not clear whether the source of the peptide is a neuron, or an endocrine cell which releases it's contents into the bloodstream. Finally, the mucosal cells can also have a local (paracrine) action, as in the case of gastric somatostatin cells, which have processes that reach to nearby parietal cells, apparently to exert the profound somatostatin-induced inhibition of gastric acid secretion. (96).

In constrast to the three peptides described above, motilin is only produced in mucosal cells (See Chapter 2). Since motilin has a direct contractile effect on smooth muscle in the outer gut wall, it must act by an endocrine mechanism following its secretion from mucosal cells into the bloodstream. However, since some of motilin's actions can be inhibited by neuronal blockade, it is also possible that motilin secretion has a direct neuromodulatory effect on

neuronal fibers present in the submucosal layer immediately adjacent to the mucosal basement membrane (see Figure 3).

An additional level of complexity is added to these systems by the ability of regulatory cells to secrete more than one peptide. The first evidence of colocalization of peptides in enteric neurons was that of somatostatin and cholecystokinin in guinea pig submucous neurons (97), and many more examples have followed. In the mucosa, substance P is produced in enterochromaffin cells which secrete serotonin (85). The observation that motilin is also produced in enterochromaffin cells (7) suggests that at least two different peptides are synthesized in these cells. Kishimoto et al. have described motilin and SP in human duodenum (98).

The studies described in this chapter were undertaken to determine if motilin in the primate duodenum is colocalized with any of the other peptides known to be synthesized in enteroendocrine cells. Single antibody fluorescence immunohistochemistry on thick sections was used to visualize the structure of enteroendocrine cells and mucosal nerve fibers. Based upon the results of these studies, double antibody avidin-biotin immunohistochemistry was used on thin sections to look for colocalization of motilin and substance P immunoreactivity.

Materials and Methods

Tissue Embedding and Sectioning

Fresh autopsy tissue was immersion fixed overnight in buffered formalin at 4°C, followed by cryoprotection in 20% sucrose in 50mM Tris-Cl, pH 7.6 in normal saline (Tris-NS). Tissue for thin sections was cut on a Leitz cryostat at 10-12 μm, mounted on gelatin coated slides, and stored at -70°C. Tissue for thick sections was embedded in a mixture of 6% gelatin and 50% egg yolk, post-fixed in buffered formalin overnight at 4°C, and cut on a vibratome at 75-100 μm.

Immunohistochemistry

The antisera and dilutions used were as follows: Motilin: N1-4 at 1:800, VIP: N1-8,1:1000; somatostatin: RbA ST (EL),1:1000; substance P: RbA SP (GN_1), 1:1000; and neuron-specific enolase (Polysciences, Inc.), 1:1000. For fluorescence immunohistochemistry, thick sections were equilibrated in Tris-NS containing 0.02% bovine serum albumin (BSA, Sigma, Inc.; Fraction V) and 0.1% Triton X-100, and incubated in primary antibody in equilibration buffer overnight at 4°C. The sections were washed in Tris-NS and incubated in fluorescein-conjugated goat anti-rabbit antiserum (Cappel, Organon Teknika, Inc.) diluted 1:500 in Tris-NS, BSA for 30 min. at room temperature. Following extensive rinsing, sections were coverslipped and examined with a Leitz Orthomat™ microscope under fluorescence optics. Controls included

adjacent sections treated in parallel but minus primary antibody, or substitution of normal (nonimmune) rabbit serum for the primary antiserum.

For the double label experiments, specimens were immunostained first with the rabbit anti-motilin antibody as described above except without Triton. Sections were labeled by the avidin-biotin glucose oxidase (GO), technique (99) using biotinylated Protein A as the bridging reactant (100). Briefly, this involves addition of biotinylated Protein A after the primary antibody, which binds to the Fc portion of the bound rabbit anti-motilin IgG. Avidin and a biotinylated enzyme (eg biotinylated GO) are then added together; the avidin binds to the biotinylated Protein A and the biotinylated enzyme binds to the remaining three unoccupied biotin sites on the avidin, providing a specific site for a enzymatic histochemical reaction. Reaction products were visualized with nitroblue tetrazolium. After re-equilibration of the stained sections in Tris-NS, they were incubated in the substance P antiserum overnight at 4^{o}C. The sections were labelled as above with the biotinylated Protein A, avidin-biotin technique, except that a biotin-horseradish peroxidase conjugate (ABC-HRP) was used, and the reaction product visualized with diaminobenzidine.

Results

Fluorescence Immunocytochemistry

Fluorescence immunocytochemistry of thick sections provided superior visualization of the duodenal structures of interest in this study. Whereas only

short segments of the thin beaded mucosal nerve fibers could occasionally be seen in thin (10-20 µm) cryostat sections, complete fiber tracts could be traced throughout all layers of the gut wall in thick sections (Figure 14). Furthermore, the background staining, which would be overwhelming using immunohistochemical staining techniques, was much reduced using fluorescence. In addition, the three dimensional structure of the fluorescence-labeled endocrine cells could be appreciated by focusing up and down through various planes of the section. Thus, while motilin cells appeared to be variously shaped using immunohistochemistry; appearing either triangular shaped, columnar, or possessing a thin axon-like process depending upon the plane of section or the enzyme substrate used, all of the motilin cells detected by fluorescence immunocytochemistry in thick sections were noted to have a single, distinct morphology. These cells possess a long thin apical process which extends at least half the length of the cell and sometimes over two cell lengths extending from the lumen to the larger cytoplasmic region. Towards the basilar end, the cells either narrow briefly or remain the same diameter until they widen into a structure at the basement membrane which resembles a neuronal bouton. The most intense immunofluorescence was invariably localized in this basilar region of the cell (Figure 15). No other specific fluorescence was observed anywhere apart from that in scattered mucosal cells; specifically, no motilin immunofluorescent neurons or fibers were seen.

The preservation of immunoreactive neuron cell bodies and distant fiber tracts in the tissue preparations used was demonstrated by the use of an antiserum

Figure 14: Visualization of the Enteric Nervous System in the Monkey Duodenum by Indirect Fluorescence from an Anti-Neuron Specific Enolase Antibody

Low power photomicrograph of a vibratome section of monkey duodenum immunostrained for neuron-specific enolase. Specific fluorescence is visible in nerve cell bodies in the myenteric and submucous plexus and in neuronal fibers throughout the submucosal layers of the intestinal wall.

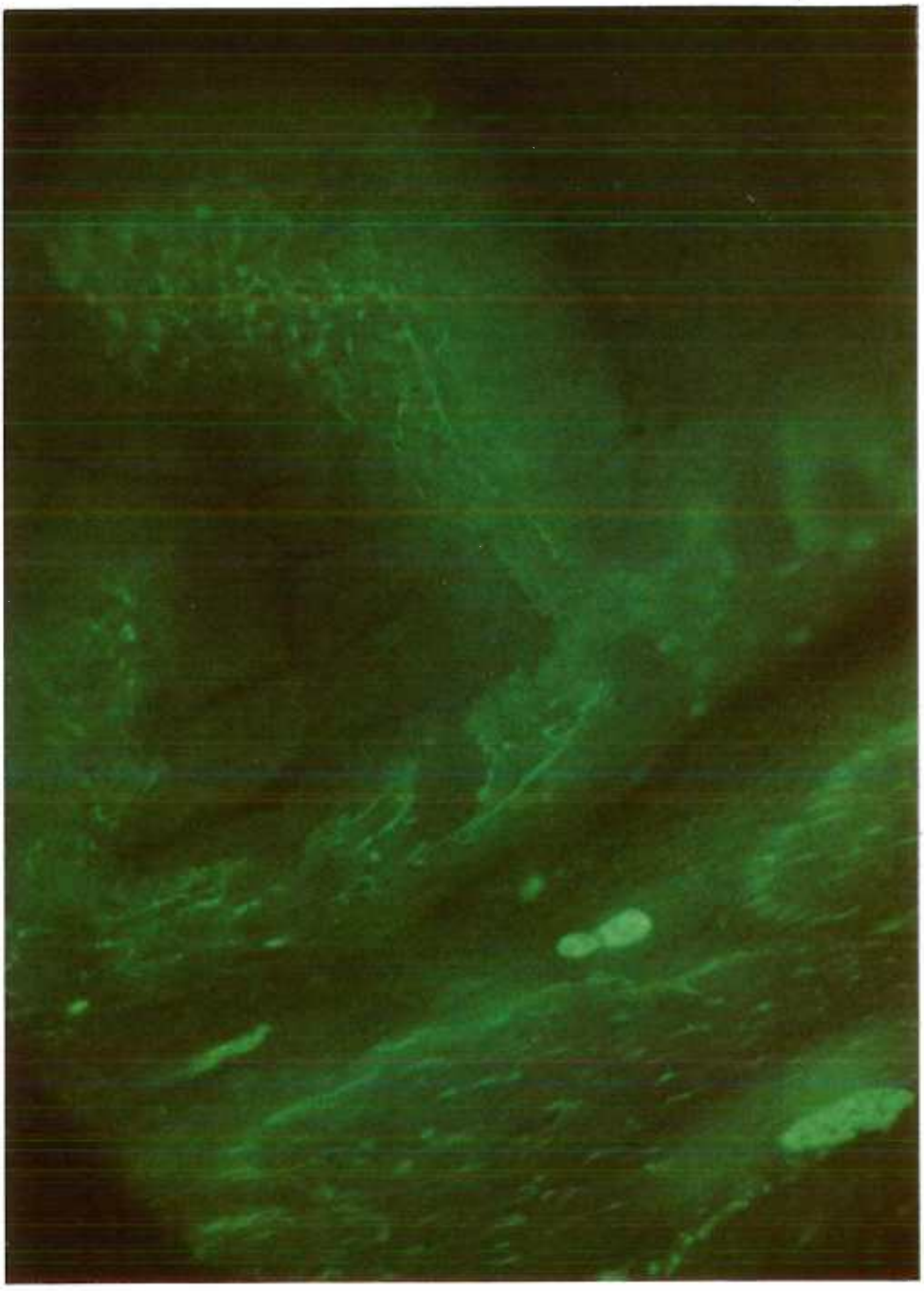
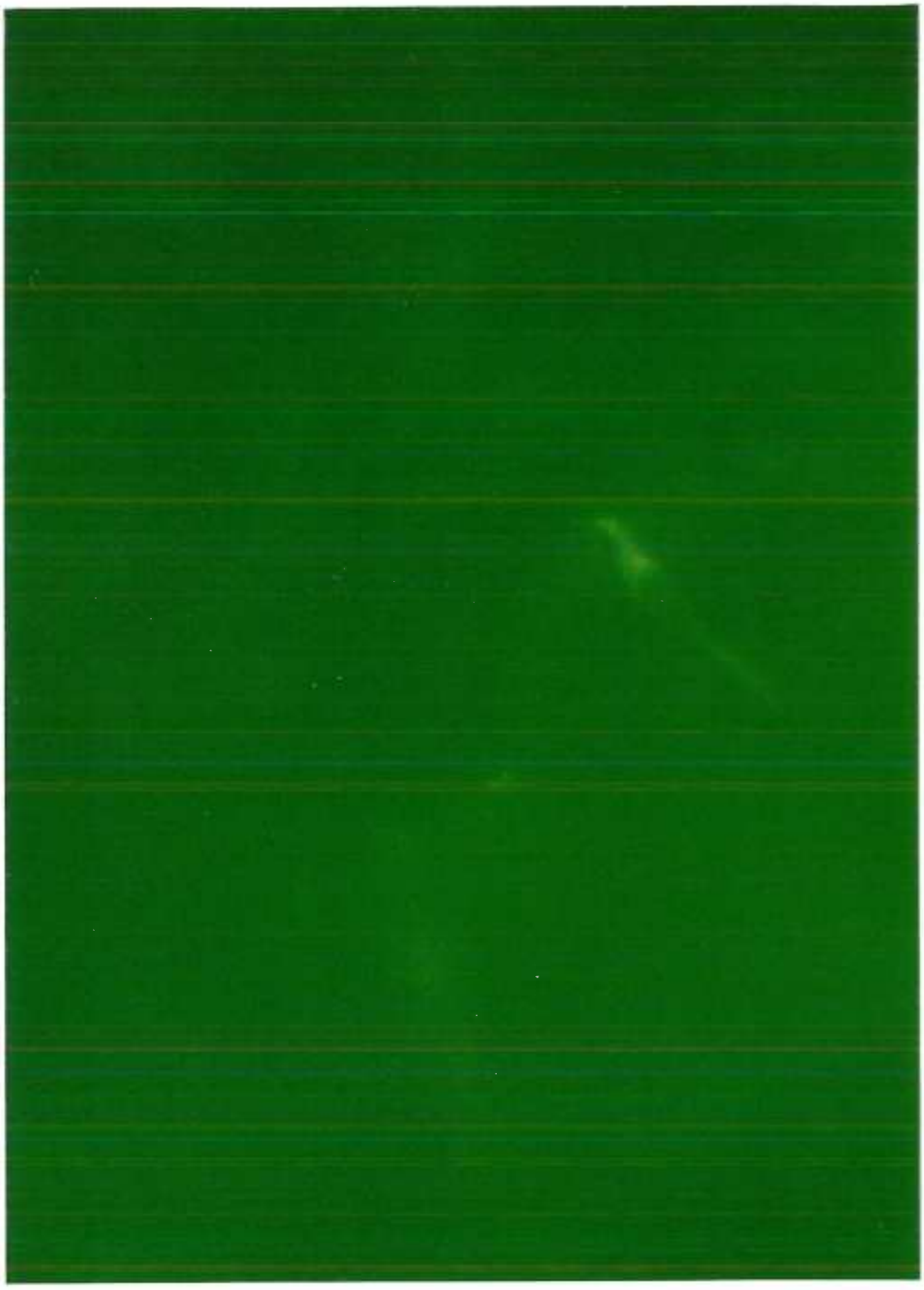


Figure 15: Visualization of a Motilin-Producing Enteroendocrine Cell in Monkey Duodenum by Fluorescence Immunohistochemistry

Photomicrograph of a single motilin immunoreactive cell in a vibratome section of monkey duodenum visualized by indirect fluorescein fluorescence. This cell illustrates the typical appearance of a long, thin luminal process on a motilin cell.



to neuron-specific enolase. This antiserum recognizes an isozyme which is present specifically in neurons (101) and immunostaining of the thick duodenal sections with this serum revealed an extremely dense network of neuronal fibers thoughout the intestinal wall as well as nerve cell bodies in the myenteric and submucus plexuses (Figure 14). On the other hand, VIP, somatostatin and SP antisera selectively labeled a subset of these elements. However, in addition to neurons, each of these antisera labelled mucosal cells. As observed for motilin immunoreactive mucosal cells, cells containing somatostatin and VIP each had a distinctive appearance. VIP-containing cells were small, brightly fluorescent cells with a central nucleus (Figure 16). They had no processes and appeared to be of the closed-type enteroendocrine cell (102), ie. they were not in contact with the lumen. Somatostatin immunoreactive cells were brightly fluorescent and also possessed a thin luminal process similar to that seen on motilin cells. However, compared with the motilin cell, the somatostatin cells widened at the basilar end instead of centrally and the nucleus itself was also more basilar (Figure 17). Occasional somatostatin immunoreactive cells were noted to have additional processes, similar to those described for gastric somatostatin cells (103). Substance P immunofluorescence was also seen in mucosal cells, but it was significantly less intense than that noted in nerve fibers (Figure 18), suggesting that SP is present in lower levels in the mucosal endocrine cells, compared to neurons. The cells which were immunoreactive appeared to be generally triangular in appearance. No mucosal or neuronal fluorescence was detected in adjacent sections incubated without antiserum or with normal rabbit serum, followed by application of fluorescein-conjugated goat anti-rabbit serum.

Figure 16: Visualization of a VIP-containing Enteroendocrine Cells In Monkey Duodenum Visualized by Fluorescence Immunohistochemisty

Indirect VIP immunofluorescence from a mucosal enteroendocrine cell in a vibratome section of monkey duodenum.

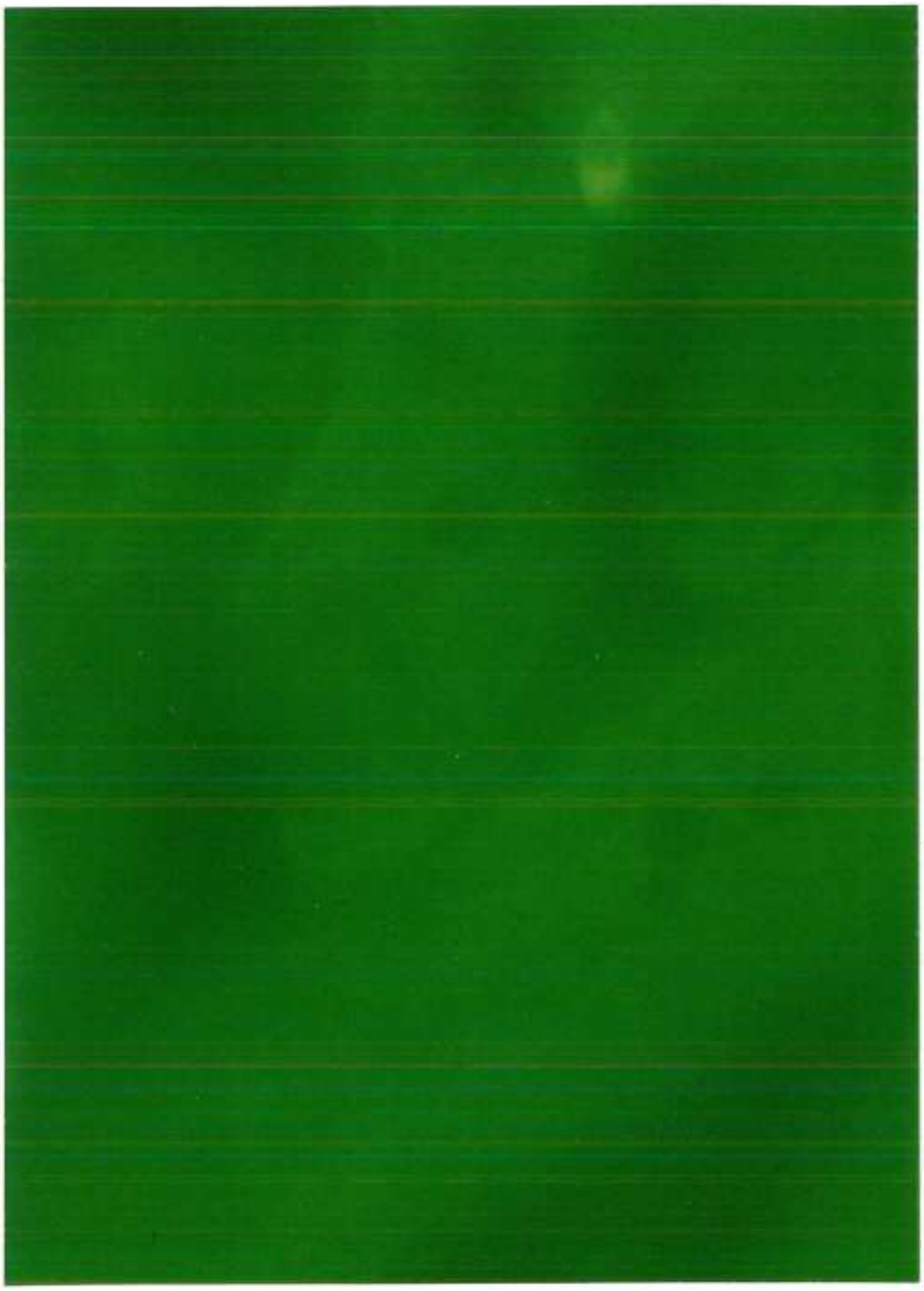


Figure 17: Visualization of a Somatostatin-containing Enteroendocrine Cell in Monkey Duodenum Visualized by Fluorescence Immunohistochemisty

High power photomicrograph of a typical somatostatin immunoreactive mucosal cell in a vibratome section of monkey duodenum.

Figure 18: Immunohistochemical Visualization of Substance P-Containing Neuronal Fibers and Mucosal Cells in Monkey Duodenum

Photomicrograph of a vibratome section of monkey duodenum cut in cross section through the mucosal villi exibiting indirect Substance P immunofluorescence. Specific fluorescence in seen in neuronal fibers surrounding the villi and in small triangular shaped mucosal cells (arrow).

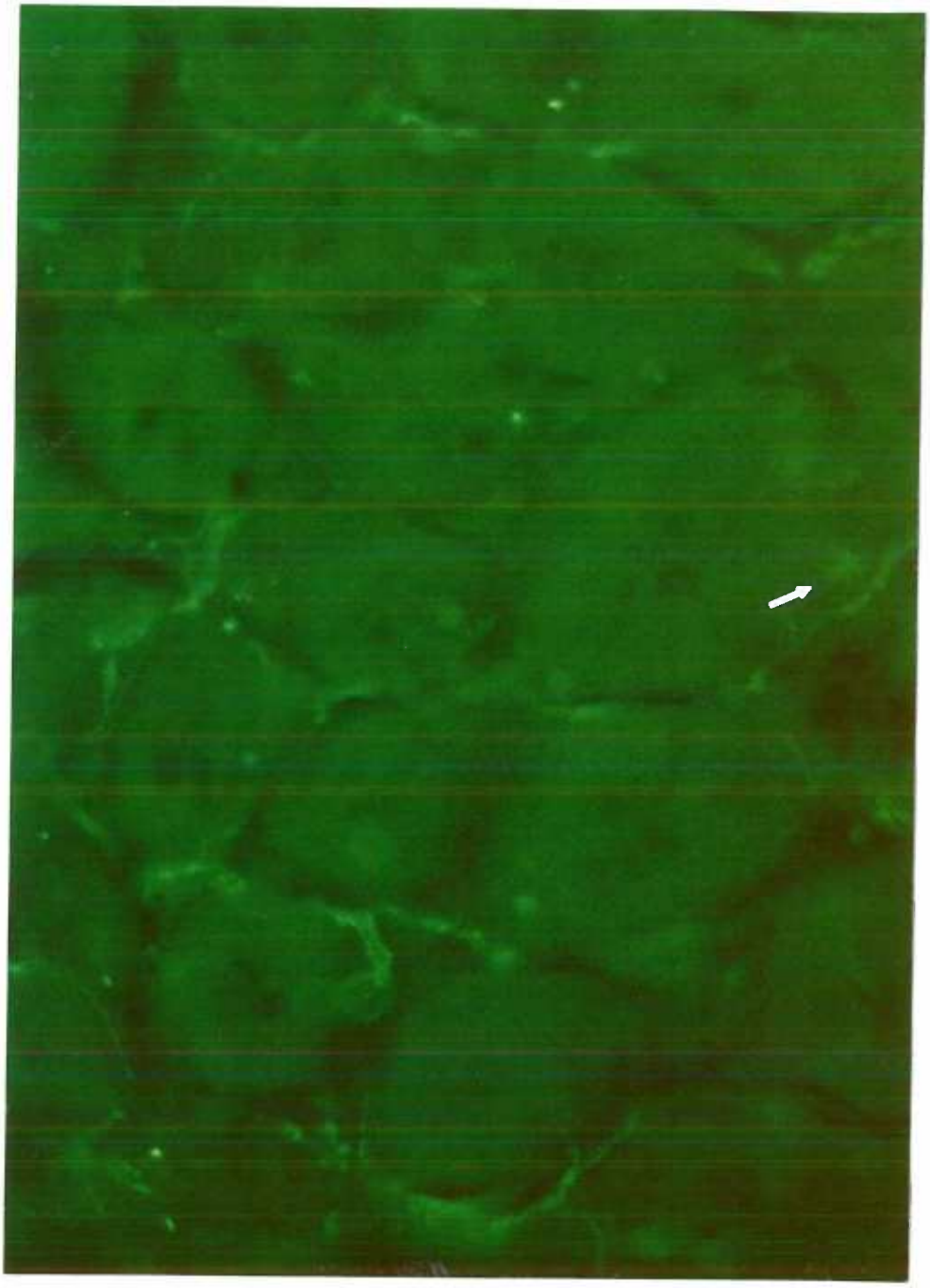


Figure 19: Colocalization of Motilin and Substance P in a Subset of Enteroendocrine Cells in Monkey Duodenum

A: Photomicrograph of a motilin immunoreactive mucosal cell in a cryostat section of monkey duodenum visualized with nitroblue tetrazolium. **B:** Two adjacent Substance P immunoreactive cells in the same section as in A visualized with diaminobenzidine. **C:** Colocalization of motilin (Blue) and Substance P (brown) immunoreactivity in a cryostat section of monkey duodenum

A

B

C

Double-Label Immunohistochemistry

Thin cryostat sections labeled first with motilin antibodies and visualized with nitroblue tetrazolium, followed by substance P antibodies visualized with diaminobenzidine, exibited three kinds of immunoreactive cells. Most obvious were those stained dark blue indicative of the presence of an anti-motilin/glucose oxidase conjugate (Figure 19A). However, although some of these cells contained only blue reaction product, others which were lightly stained blue also contained a brown color, indicating the presence of the anti-substance P/HRP conjugate (Figure 19B). In addition, a few mucosal cells were only stained brown (Figure 19C). After washing these sections for 15 min. in 50% ethanol, which dissolves the blue tetrazolium salt, but not the brown diaminobenzidine polymer, some of the previously dark blue cells were seen to contain underlying brown color, while others contained only residual blue.

Discussion

The results of the immunofluorescence studies described are consistent with previous work in other species indicating that VIP, somatostatin and substance P may exist in mucosal enteroendocrine cells in addition to neurons in the gastrointestinal tract. In addition, the inability to detect motilin fluorescence in nerve cell bodies or fibers is consistent with results using conventional immunohistochemistry (Chapter 2) and is in agreement with most other studies of motilin cellular localization in the small intestine. The

distinct morphology of three cell types - the motilin cell, the VIP cell, and the somatostatin cell - was quite striking and argued against any colocalization of these peptides. However, SP reactive cells appeared to belong to a less distinct morphologic class. This may be in part because these cells exibited less intense fluorescence than the others and were therefore more difficult to visualize. This observation suggests that substance P is produced in these enteroendocrine cells in low levels because 1) the immunofluorescence intensity of the SP cells in the mucosa was significantly less intense that that of nearby SP fibers, which exibited the most intense immunofluorescence of any peptides studied, and 2) VIP and somatostatin immunoreactive mucosal cells exibited levels of immunofluorescence comparable to that observed in the corresponding nerve fibers.

The inability to clearly distinguish substance P cells from motilin cells, coupled with published reports that both substances are present in serotonin-containing enterochromaffin cells, suggested the possibility that substance P could be present in motilin-containing cells. Double-label immunohistochemistry was therefore performed using motilin and SP antisera. The detection of cells containing reaction products indicative of both motilin and substance P, as well as cells containing one or the other product alone, indicates that a subpopulation of motilin-containing enteroendocrine cells also contain substance P. This double-label experiment, however, might be considered ambiguous because both primary antibodies were raised in rabbits, and both detection methods involved reagents (Protein A, avidin and biotin) which potentially could crossreact with those used for the other

antibody. Thus, it is possible that the reagents used to visualize the anti-SP antibodies added in a second immunologic step were in fact reacting with residual unoccupied rabbit IgG molecules or Protein A-bound avidin remaining from the initial application of motilin antibodies. That this was in fact not occuring in these experiments is indicated by the following: First, all of the reactants used in the immunohistochemical reactions are in large excess and are not depleted in these reactions. Second, the HRP reaction used to visualize substance P is very efficient and more sensitive that the glucose oxidase (GO) reaction used to visualize motilin (105). Thus, if motilin reactivity remained after (partial) reaction of the GO substrate, it should be present in all cells equally. Since cells staining only blue were present, the brown DAB reaction product present in some cells was not due to residual motilin reactivity. Finally, the occurance of mucosal cells which only stain brown also argues against this possibility.

The occurance of a population of cells containing motilin and substance P is consistent with the previous identification of substance P in mucosal cells (106, 107). However, Sternini et al. recently studied the distribution of SP mRNA in the rat small intestine by *in situ* hybridization and could detect specific hybridization only over enteric neurons (108). Their inability to detect substance P mRNA in mucosal cells may be due to species differences between rat and monkey (studied here) or human, rabbit and tupaia studied previously. Alternatively, SP mRNA levels in endocrine cells may have been too low to be detected by their *in situ* hybridization technique. This possibility is consistent with the current observation of low levels of SP immunoreactivity

in endocrine cells compared with enteric neurons. The colocalization of motilin and substance P is also consistent with previous results localizing motilin (7) and substance P (85) in enterochromaffin cells. A previous study found evidence of motilin and Substance P colocalization in duodenal mucosal cells (98 However, a different study concluded that these peptides are not produced in the same cells in human duodenum and rabbit bile duct (104). The results obtained here indicate that motilin and substance P are colocalized in a subset of enteroendocrine cells. However, it is not clear whether the discrepancies in earlier reports were due to differences in sensitivity of the methods employed or to species differences. The functional significance of the co-secretion of motilin and substance P implied by the current study is unclear; substance P has several actions which could augment or complement that of motilin, such as smooth muscle contraction or increase of intestinal blood flow. The possibility that substance P is also produced by endocrine cells containing VIP or somatostatin also exists, but was not addressed in these studies.

Conclusions

The occurance of VIP, somatostatin, substance P and motilin in enteroendocrine cells has been investigated by immunocytochemical techniques in the monkey duodenum. The results indicate that while VIP, somatostatin, and motilin are produced by distinct cell types, substance P is also present in a subset of motilin cells.

SUMMARY AND CONCLUSIONS

This thesis describes the isolation and characterization of the human motilin gene. Southern analysis of human genomic DNA using a fragment of the cloned human motilin gene indicates that there is a single motilin gene in the human genome. DNA sequence analysis of this gene demonstrates that prepromotilin is encoded by five exons. The most surprising result from this work is the finding that the 22 amino acid motilin molecule is encoded on two separate exons; a very unusual situation for small peptide hormones. Northern hybridization analysis of intestinal RNA isolated from other species using a fragment of the human gene as a probe indicates that there are significant nucleotide sequence differences between rodent motilin mRNA and the relatively well conserved human, monkey and porcine motilin mRNAs. This is consistent with previous immunologic data which suggested that the rodent form of motilin differs from porcine and human motilin at the amino acid level; probably at the amino terminus of the molecule. These inferred amino acid changes and the known differences in the amino terminal portion of canine motilin suggest that motilin may consist of two structural domains, with one being more conserved throughout evolution than the other. The finding that motilin is encoded on two exonic domains provides support for this idea.

Isolation of the human motilin gene provided a tool with which to study its expression. Rhesus monkey tissue was used to examine motilin gene expression as a model for the expression of this gene in human and nonhuman primate; the validity of this approach is supported by the finding

that the human motilin probe recognizes a mRNA in monkey duodenum of the same size as the human motilin transcript. Northern hybridization analysis of a range of human and monkey tissues indicates that motilin gene expression is highly tissue specific. Motilin mRNA is by far most abundant in the duodenum in both human and monkey; however, low levels were detected in a few other tissues including adrenal, esophagus, stomach, lower small intestine and colon. The demonstraton of motilin mRNA in the upper gastrointestinal tract suggests that some of the physiologic effects of motilin which have been measured in these tissues, such as the gastric antrum, may be due to locally secreted motilin rather than from duodenal motilin. While it initially appeared that motilin mRNA was also relatively abundant in the liver and gallbladder, reexamination of the hybridization signal under very stringent conditions indicated that the liver transcript is not the same as the duodenal motilin transcript. Thus, it appears that a related transcript, perhaps from a different gene, is expressed in the primate liver and biliary system.

In situ hybridization was performed using a motilin gene fragment to localize the cellular site of mRNA synthesis, and immunohistochemistry was used to visualize the location of motilin peptide in the monkey duodenum. The results of these studies show that motilin gene expression within the duodenum is restricted to a specific type of mucosal endocrine cell. Immunohistochemical studies also demonstrated that Substance P is present in a subset of mucosal cells which synthesize motilin.

In contrast to previous immunologic detection of motilin-like immunoreactivity in cerebellum, no motilin mRNA could be detected by Northern analysis of human or monkey cerebellar RNA. This result is in agreement with a previous result for porcine cerebellum. However, in polymerase chain reaction experiments, a cDNA could be amplified from monkey cerebellum using motilin specific oligonucleotides complementary to Exons II, III, IV and V of the human motilin gene, whereas no specific amplification occured using an Exon I oligonucleotide as a primer. Thus, it appears that an alternately spliced form of a motilin transcript may be expressed in cerebellar tissue.

In summary, the work presented in this thesis represents the first step towards an understanding of of the molecular biology of motilin in humans. The cloned gene has provided a tool with which to examine the normal expression of the motilin gene; and with this tool these initial observations can be extended to study the normal regulation of motilin gene expression, as well as its abnormal expression in disease states such as gastrointestinal carcinomas. The results obtained also point towards additional experiments; most notably isolation of the motilin-related transcripts in cerebellum and liver using a human motilin gene fragment as a probe. Characterization of these molecules will likely identify new bioctive peptides and may well reveal the source of the elusive "motilin-like immunoreactivity."

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Appendix I: Southern analysis of the human motilin gene

Summary

Southern blot hybridization analysis of human genomic DNA was performed to determine the number of copies of the motilin gene present within the human genome. Human genomic DNA was isolated and cut at discreet restriction sites into fragments which could be separated by gel electroporesis. The separated fragments were transferred to and immobilized on a nylon membrane in a Southern transfer and probed with a labeled fragment of the cloned human motilin gene. The results obtained are consistent with the occurance of a single gene encoding the motilin precursor in the human genome. However, evidence for the presence of another closely related gene was also obtained.

Materials and Methods

Isolation of genomic DNA

Peripheral blood was obtained by antecubital venipuncture, collected into a sterile tube containing 1 ml 0.5M EDTA, and immediately placed on ice. The blood was centrifuged for 15 min at 3000 rpm at 4^{o}C. The upper buffy coat was removed along with a small amount of contaminating red cells and mixed gently with an equal volume of 0.64M sucrose, 2% (w/v) Triton X-100, 10 mM

$MgCl_2$ in 20 mM Tris-Cl pH 7.6 (2X Sucrose-Triton) and homogenized with six strokes in a dounce homogenizer. The volume was increased to 50 ml with 1X Sucrose-Triton and placed on ice for 10 min. Nuclei from the lysed leukocytes were pelleted by centrifugation at 1000xg for 20 min at 4°C. Nuclei were then gently resuspended in 6 ml normal saline containing 2.5 mM EDTA and SDS at a final concentration of 1% (w/v). After 5 min on ice, Proteinase K (Boeringer-Mannhein) was added at a concentration of 150 µg/ml and the solution incubated at 37°C for 4 hr. The solution was then extracted gently and exhaustively with equal volumes of a 1:1 mixture of buffered phenol pH 8.0 containing 0.1% 8-hydroxyquinoline and chloroform until a clear interface was present. The aqueous phase was extracted with an equal volume of chloroform and then dialyzed overnight against 2 liters of 10 mM NaCl, 1 mM EDTA in 10 mM Tris-Cl pH 7.5 at 4°C. The solution was made 2M in ammonium acetate and the DNA precipitated with three volumes ice cold ethanol. After rinsing with 3 times 1 ml 70% ethanol and drying gently under vacuum, the DNA was resuspended in 3 ml 10 mM Tris-Cl pH. 8.0, 0.1 mM EDTA and its concentration determined by measuring absorbance at 260 nm.

DNA fragment isolation and purification

200 µg of plasmid pEXII, containing a 160 bp Sac I-Nhe I fragment of the human motilin gene corresponding to Exon II, was cut with Sac I and Acc I in a standard restriction reaction in Sac I buffer for 2 hr at 37°C. The reaction was then run directly on a 1.5% agarose gel in 40 mM Tris-acetate, 0.002 M EDTA, stained with 0.5 µg/ml ethidium bromide, and visualized over an

ultraviolet lamp. The approximately 160 bp band was cut out of the gel and weighed. 2.75 volumes (v/w) of 1M sodium iodide were added to the fragment and placed at 55°C for 5 minutes to melt the agarose. The released DNA was purified on finely ground silica using Glassmilk™ reagents (Bio 101, Inc.) according to manufacturers instructions. Purified DNA was eluted in 20 µl H_2O and its concentration determined by measuring absorbance at 260 nm.

Labeling of DNA Probe

Double stranded DNA fragments were labelled by the random-priming method of Feinberg and Fogelstein (109). Briefly, hexanucleotides of random sequence were annealed with the denatured DNA template followed by incorporation of labelled and unlabelled nucleotides by the Klenow fragment of DNA Polymerase I. The standard reaction contained 5-15 ng of template DNA, 25 mM each of dATP, dGTP, and dTTP, approximately 10 uM ^{32}P-dCTP (6000 Ci/mmole), 5 ug of random hexamers, and 10 Units of Klenow enzyme in 25 mM Tris-Cl pH 8.0, 25 mM $MgCl_2$, 25 mM 2-mercaptoethanol. The DNA template was denatured by incubation at 100°C for 10 minutes followed by rapid cooling on ice for 3 minutes. A mixture containing all the other reaction components minus enzyme were added on ice and the reaction initiated by addition of 1 µl enzyme (10 Units). The reaction mixture was incubated at 37°C for 60 minutes. Labelled probe was separated from unincorporated label over a G-50 Sephadex (2 ml pasteur pipet) column equilibrated in 10 mM Tris-Cl pH 8.0, 1 mM EDTA (TE) and 10 µg yeast tRNA. Labelled DNA in the peak fractions (3, 200 µl fractions immediately after void

volume) in TE was filtered through a 0.2 μm disposable filter and added directly to the prehybridization solution.

DNA Restriction and Southern Blotting

20 μg of purified genomic DNA was cut with various restriction enzymes in a reaction containing a fivefold excess of enzyme in the buffer recommended by the manufacturer for that enzyme in a total volume of 200 μl for 2 hrs at 37°C; after 1 hr an additional 10 Units of enzyme were added to the reactions. DNA was purified from the reactions by phenol/chloroform extraction as described above and precipitated from 0.3M sodium acetate, pH 5.2 with three volumes ethanol. The rinsed and dried DNA was resuspended in 40 μl H_2O, mixed with 1/20 volume loading dye containing 20% (w/v) ficoll and 0.1% bromphenol blue, and electrophoresed through a 1% agarose gel in TEA until the dye front was 2 cm from the bottom of the gel. As a control for the sensitivity of hybridization, lanes containing 1ng, 0.1 ng, and 0.01 ng of pExII plasmid in 20 μg of salmon sperm DNA were loaded, along with molecular size standards. After electrophoresis the gel was stained in 0.5 μg/ml ethidium bromide and photographed.

The gel was subsequently washed in approximately 2 x 20 volumes of the following solutions: 1.5M NaCl, 0.5M NaOH; 3M NaCl. 0.5M Tris-Cl, pH 7.5; 3M NaCl, 0.3 M sodium citrate. The denatured DNA was then transferred to a nylon membrane (Nytran, Schleicher and Scheuell) by capillary blotting (110) overnight at room temperature. The transferred DNA was immobilized on the

membrane by irradiation with ultraviolet light (Stratolinker, Stratagene Inc.) for 12 sec. followed by baking for 1 hr. in a vacuum oven at 80°C.

Southern Blot Hybridization

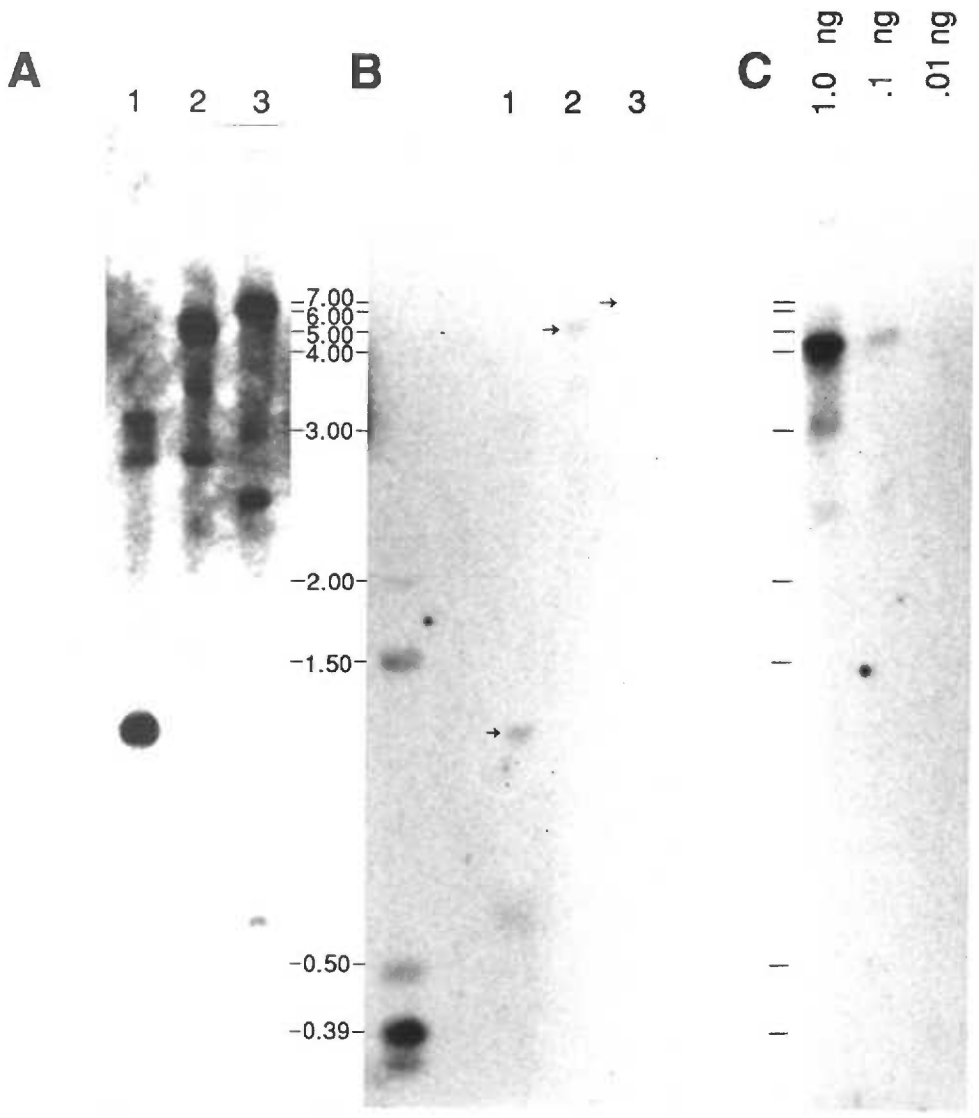
The Southern blot was prehybridized in 50% formamide, 1M NaCl, 2% SDS for 2 hr. at 42°C. Labelled DNA fragment was added directly the the hybridization bag and hybridization allowed to proceed overnight (12 hrs) at 42°C. The blot was washed under moderately stringent conditions in 1 liter 0.2X SSC (1X SSC is 0.15M NaCl, 0.015M sodium citrate) at 42°C and exposed to X-Ray film. Subsequently, the blot was washed at increasing stringency to a final stringency of 0.1X SSC at 60°C.

Results

The results of this Southern hybridization analysis are shown in Figure 20. After washing at 0.2X SSC/42°C, strong hybridization signals were present at approximately 1.1 kilobases (kb) in the lane containing Pst I-cut DNA, 5.0 kb in the Bam HI lane and 7.0 kb in the Eco RI lane (Figure 20A) In addition, less intense hybridization signals are observed of approximately 3.5 kb in the Pst I lane, 4.0 kb in the Bam HI lane and 2.0 kb/0.5 kb in the Eco RI lane, as well as a signal just under 3.0 kb in both the Pst I and Bam HI lanes. After more stringent washing of the blot at 60°C/0.1X SSC, only the three most intense bands remained (Figure 20B). However, after prolonged exposure of the blot, the bands comigrating at approximately 3.0 kb in the Pst I and Bam

Figure 20: Southern Blot Analysis of Human Genomic DNA

A: Genomic Southern blot probed with a 32P-labeled human motilin genomic probe and washed at moderate stringency (0.2X SSC, 42°C) and exposed for 3 days. Lane 1, Pst I; Lane 2, Bam H1; Lane 3, Eco R1; each lane was loaded with 20 µg of human genomic DNA. **B:** The Southern blot shown in A rewashed at high stringency (0.1X SSC, 60oC) and exposed 3 days. The unlabeled lane shows the positions of DNA molecular weight markers which hybridized with the probe. The positions of the faint, but clearly detectable bands which remained after high stringency wash are indicated by arrows. **C:** Additional lanes of the same Southern blot shown in A and B which were loaded with 20 µg of salmon sperm DNA contianing known amounts of a 4.2 kb plasmid containing a 1.2 kb motilin gene inset. This section the blot was probed with the motilin genomic probe, washed at high stringency and exposed for 3 days to X-ray film.


A
1 2 3
B
1 2 3
7.00
6.00
5.00
4.00
3.00
2.00
1.50
0.50
0.39
C
1.0 ng
.1 ng
.01 ng

HI lanes were still visible. A strong hybridization signal remained after the last wash in the control lane containing 1.0 ng of motilin plasmid DNA, while a much weaker signal, of an intensity comparable to that seen in the experimental lanes, was apparent in the control lane containing 0.1 ng of plasmid DNA. No hybridization signal was detected in the control lane containing 0.01ng of plasmid DNA (Figure 20C).

Discussion

The sizes of the most intense signals after hybridization with the labelled exon II probe correspond to the sizes of the restriction fragments predicted from the restriction map of the cloned human motilin gene (111). Thus, these hybridization signals represent fragments of the human motilin gene. The presence of additional hybridization signals raised the possibility of an additional gene containing motilin-related sequence elements, with a unique pattern of restriction sites. However, these less intense bands disappeared after washing under more stingent conditions. Therefore, they do not share the same level of homology with the genomic probe as the homologous sequence in the motilin gene, and thus cannot be considered as evidence of a second motilin gene. The two bands of the same molecular weight (3 kb) present in low amounts in lanes 1 and 2 (Figure 20A) are most likely due to the presence of a small amount of contaminating plasmid DNA containing recombinant motilin sequences. The reasons for this conclusion are: 1) the same sized band is present in adjacent lanes cut with different restriction enzymes, 2) their apparent molecular weight corresponds to that of the

lineraized pEXII plasmid and 3) similar bands were seen sporadically in other, similar experiments.

A discreet restriction fragment containing exon II of the motilin gene is produced after cutting with the three enzymes shown here, or with two other restriction enzymes (data not shown); each of these single fragments are the size predicted from the restriction map of the cloned gene. This observation strongly suggests the presence a single motilin gene in the human genome. Yet, it is possible that duplicate motilin genes are present which exibit no restriction fragment length polymorphism for the enzymes used. Independent determination of gene copy number may be obtained by comparing the intensity of hybridization of a probe for a gene of known copy number with that of the gene in question, or of the intensity of hybridization of the same probe to a known amount of target DNA. The latter approach was used in this experiment. The 160 bp exon II of the motilin gene represents 8×10^{-8}th of the human genome (Table 1). Therefore, if the motilin gene is present in a single copy, then the 20 µg of genomic DNA present in each lane of the Southern blot contains 1.6 pg of exon II DNA. This can be compared with the known amount of exon II DNA present in the pExII plasmid DNA present in the control lanes, assuming that all of the restriction fragments are transferred with equal efficiency. These amounts of control DNA are approximately 50 pg, 5 pg, and 0.5 pg (Table 1). The presence of a hybridization signal in the 5 pg lane (approximately equivalent to 3 copies), and the observation that this 5 ng signal is slightly more intense than the signals in the genomic DNA lanes, indicates that the technique is sensitive enough to detect a gene present in a

single copy and provides additional evidence for the occurance of a single motilin gene.

Although they do not represent another perfectly homologous motilin gene, the other weakly hybriziding bands observed after the low stringency wash likely represent genomic DNA fragments which have homology with exon II of the motilin gene. The presence of discreet bands argues in favor of a single related gene. The possibility of a motilin-related gene is especially interesting in light of the data presented in Chapter 2 indicating the occurance of a mRNA specie in primate liver and gallbladder which is highly related to the motilin gene transcript.

Conclusions

Genomic Southern hybridization analysis was used to determine the copy number of the human motilin gene. The results indicate that the motilin gene is present in a single copy. However, evidence of a related gene was also obtained.

TABLE 1

Calculations used in Genomic Southern Hybridization Analysis

Exon II of the Human Motilin Gene:

$$\frac{\text{160 bp (Exon II)}}{2 \times 10^9 \text{ bp in human genome}} = 8 \times 10^{-8} \text{ of genome}$$

20 μg genomic DNA(8×10^{-8}) = 1.6×10^{-12} μg Exon II = 1.6 pg Exon II

Exon II sequences in pExII plasmid:

$$\frac{\text{160 bp Exon II insert}}{\text{3000 bp pEXII plasmid}} \approx 0.05$$

pEX II Plasmid DNA loaded on gel	Exon II sequences represented in plasmid DNA loaded on gel
1 ng = 1000 pg	0.05(1000 pg) = 50 pg
0.1 ng = 100 pg	0.05(100 pg) = 5 pg
0.01 ng = 10 pg	0.05(10 pg) = 0.5 pg

Appendix II: Structural homology between primate prepromotilin mRNA and the corresponding mRNA in lower mammals

Summary

RNA isolated from human, monkey, pig, rat and guinea pig duodenum was analyzed by Northern blot hybridization with human and porcine motilin nucleotide probes to determine how well these probes recognize the motilin transcript from other species. The results indicate that the rodent motilin mRNA nucleotide sequence is significantly different from the porcine and primate forms.

Introduction

A number of reports in the literature using radioimmunoassay or immunohistochemistry suggest that there is not a high degree of amino acid sequence conservation between rat and pig motilin. For example, a number of antisera generated against synthetic porcine motilin show very low cross-reactivity with rat duodenal extracts (112). In fact, based upon the amino acid sequences which are known, motilin appears to be less well conserved than many small peptide hormones. For example, the 28 amino acid vasoactive intestinal polypeptide (VIP) is identical in sequence in the human, pig, cow, dog, and mouse; with the chicken form differing at three positions, two of which are very conservative changes. While human and porcine motilin are

identical, canine motilin differs at 5 of 22 positions. These changes occur in the middle of the molecule and are not necessarily conservative (Figure 21). Nevertheless, some anti-porcine motilin antisera recognize motilin-containing cells in the canine duodenum (113). Rat motilin appears to differ from these forms at the N-terminus. Antibodies directed against the C-terminal, and the middle to C-terminal regions recognize a molecule in rat duodenal extracts which co-elutes with porcine motilin on a gel filtration column. Conversely, an antiserum directed against the N-terminus of the porcine molecule detects no motilin-like immunoreactivity in rat duodenum(61).

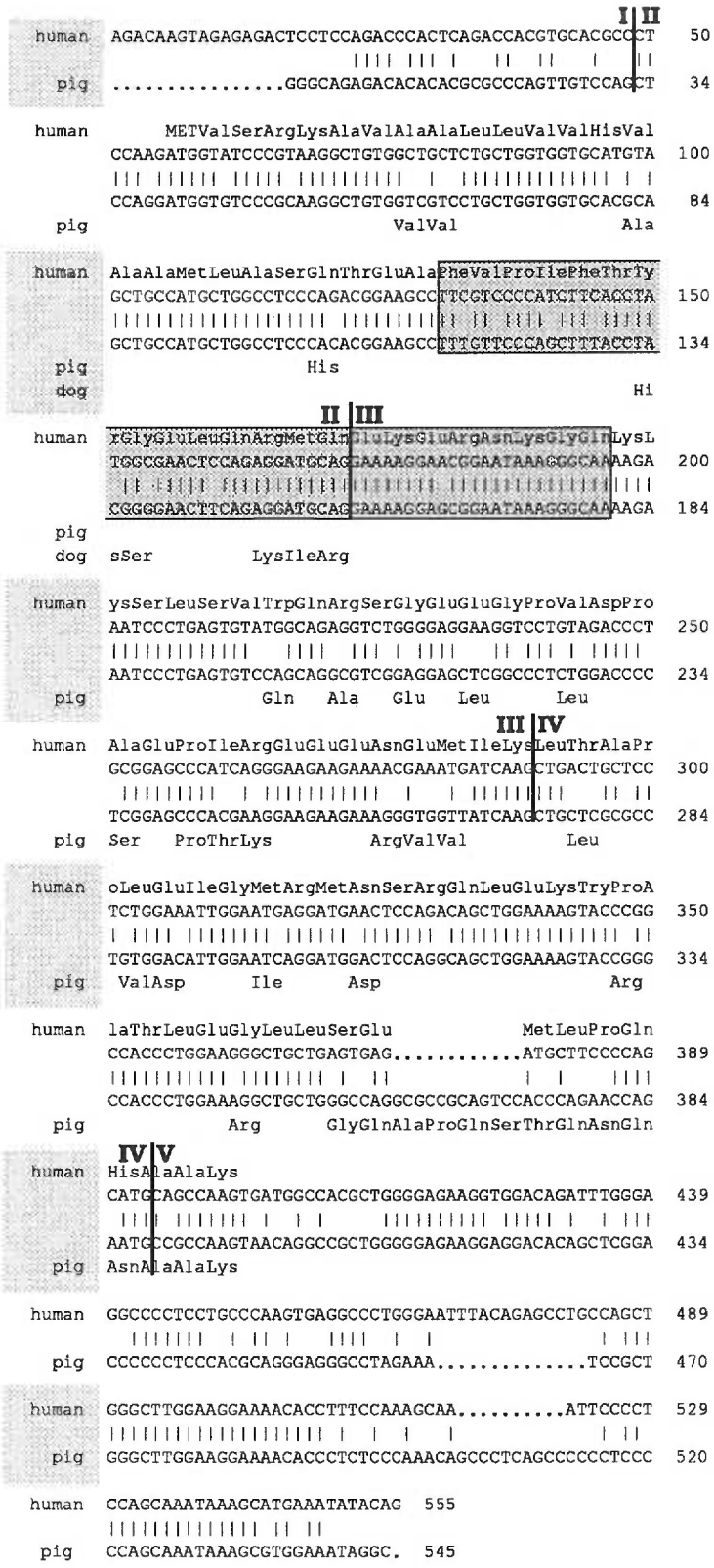
At the nucleotide level, the cDNAs for human and porcine prepromotilin are identical at 58 of 66 nucleotides encoding motilin (88%) and share 78% homology overall (Figure 21). A probe complementary to the motilin-encoding region of the human mRNA should therefore hybridize with the porcine molecule. The Northern blot hybridization experiments described here were carried out to test this prediction as well as the ability of both human and porcine motilin probes to recognize the rodent homologue.

Materials and Methods

RNA isolations and Northern hybridization analysis were carried out as described in Chapter 2.

Figure 21: Amino Acid and Nucleotide Homologies of Human, Porcine and Canine Motilin

Nucleotide sequences of human and porcine motilin cDNA were aligned for maximum homology and positions of nucleotide identity noted by a verticle bar. The predicted translation product of the human motilin mRNA is shown above the nucleotide sequence. Amino acids in porcine prepromotilin which differ from the human sequence are shown below the porcine nucleotide sequence. The five amino acids in canine motilin which differ from the human and porcine form are also noted below the porcine amino acid sequence. The 22 amino acid motilin moiety is shaded and the position of intron-exon junctions in the human motilin gene are marked.


I II
II III
III IV
IV V

Results

Figure 22 shows the Northern analysis of total duodenal RNA isolated from human, rhesus monkey, pig, rat and guinea pig probed with a riboprobe complementary to Exon II of the human motilin gene. After hybridization and subsequent washing at high stringency (0.1X SSC, 70^{o}C) the most intense hybridization signal is present in the human RNA sample with signals also observed in lanes containing monkey RNA. A weak, but detectable hybridization signal is also seen in porcine RNA. No hybridization signal was detectable in either rodent RNA samples, even after prolonged exposure of the blot. To determine if porcine motilin cDNA might recognize the rodent form, a radiolabelled porcine motilin cRNA probe was used as a hybridization probe to analyze poly-A^{+} RNA isolated from rat and guinea pig duodenum. The results of these experiments are shown in Figure 23. After low stringency hybridization (37^{o}C, 50% formamide), the Northern blot was washed at successively higher stringencies with equivalent autoradiographic exposure of the blot after each wash. At the lowest wash stingency of 50^{o}C, 0.5X SSC (Figure 23A), faint hybridization signals of approximately 900 bases in the rat (R_M A^{+}) and 800 bases in the guinea pig (GP_D A^{+} and GP_M A^{+}) were seen in the poly-A^{+} lanes, in addition to an intense signal of approximately 700 bases in porcine poly-A^{+} RNA (P_D). None of these hybridization signals were present in the corresponding poly-A^{-} sample lanes. As the wash stringencies were increased, the faint signals in the rat and guinea pig lanes became less intense. The hybridization signal in the rat sample lanes appeared to disappear first, but both rodent hybridization signals were almost completely eliminated following wash conditions of 0.1X SSC at 68^{o}C (Figure 23B), and

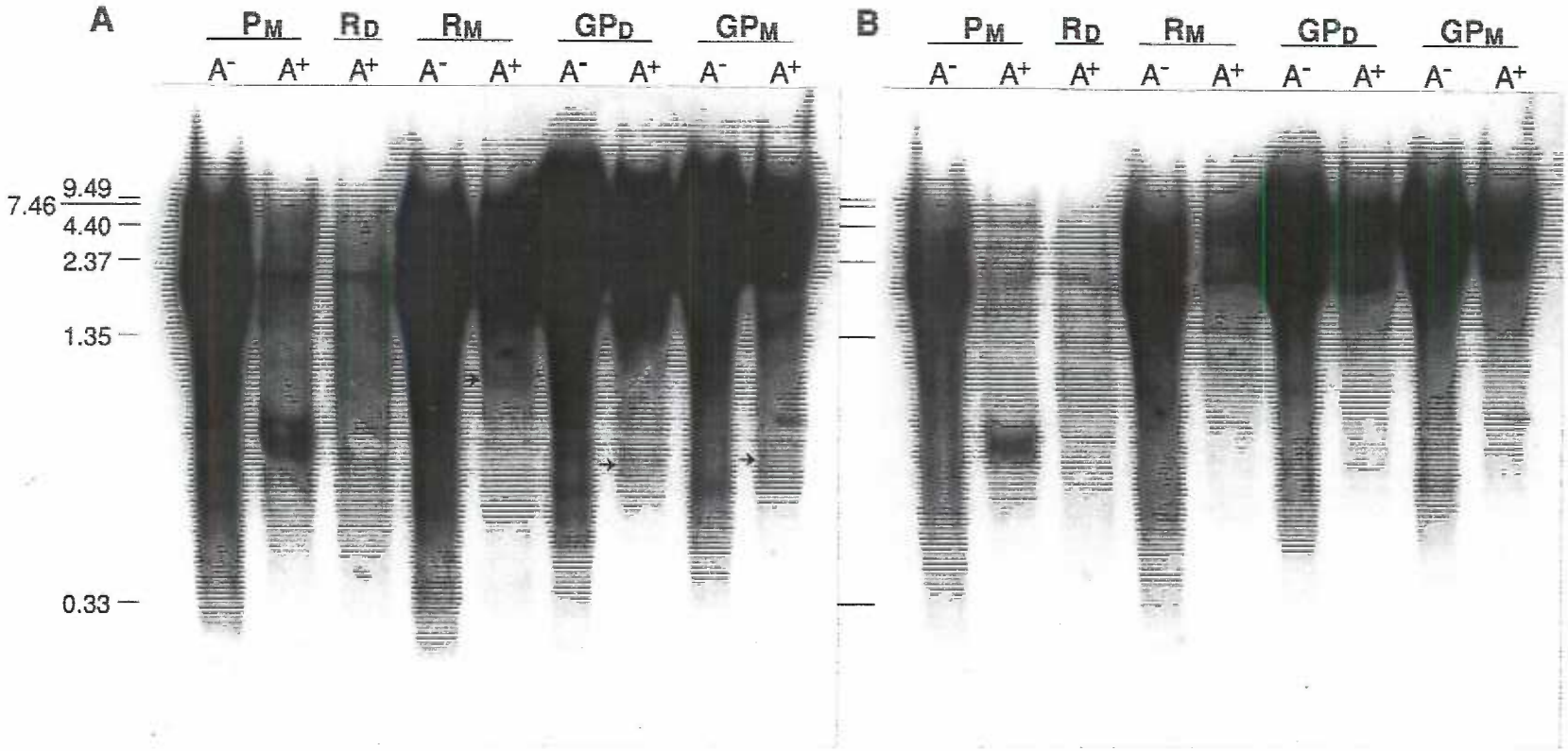
Figure 22: Cross-Specie Northern Hybridization Analysis Using a Human Motilin Gene Probe

20 μg of total duodenal RNA isolated from the indicated species was electrophoresed on a denaturing acrylamide gel, blotted onto a nylon membrane and probed with a ^{32}P-labeled human motilin genomic cRNA probe. The blot was washed at high stringency and exposed to X-ray film for 6 days. RNA molecular weight markers run concomitantly (not shown) indicate that the hybridization signals are approximately 700 bases in length.


Monkey
Human
Monkey
Pig
Guinea Pig
Rat

Figure 23: Northern Hybridization Analysis of Rodent Motilin Transcripts Using a Porcine Motilin cDNA Probe

A: Low stringency hybridization (37°C/50% formamide) and wash (0.5X SSC/50°C) of a Northern blot of porcine and rodent duodenal RNA probed with a ^{32}P-labeled porcine cRNA probe and exposed to X-ray film for 3 days. P_M, porcine mucosa; R_D, rat duodenum; R_M, rat mucosa; GP_D, guinea pig duodenum; GP_M, guinea pig mucosa. Each lane was loaded with 20 µg (poly-A^-) or 5 µg (poly-A^+) RNA. The positions of faintly hybridizing bands present in the rodent poly-A^+ lanes are indicated with arrows. The positions of RNA molecular weight markers in kilobases are indicated to the left of the figure. **B:** The same Northern blot shown in A rewashed at high stringency (0.1X SSC/68°C) and exposed 3 days.


A
PM
RD
RM
GPD
GPM
A- A+ A+ A- A+ A- A+ A- A+
7.46
9.49
4.40
2.37
1.35
0.33
B
PM
RD
RM
GPD
GPM
A- A+ A+ A- A+ A- A+ A- A+

were not detectable after 0.1X SSC at 70°C. The intense porcine hybridization signal remained after the most stringent wash, as expected.

Discussion

As noted previously, the human motilin probe recognizes the nonhuman primate form of the motilin transcript. However, the hybridization signal obtained is always less intense than that observed under identical conditions in an equivalent amount of human RNA Therefore, it appears that there is significant divergence at the nucleotide sequence level in prepromotilin mRNA between these closely related species. The human probe also recognizes porcine motilin mRNA, as predicted from the known sequence homology. It should also be noted that the very weak hybridization signals seen in Figure 22 are the result of analysis of total RNA rather than poly-A+ RNA. 2-4 days of exposure to X-Ray film are required to visualize the motilin transcript in poly-A+ RNA (compared with the cyclophilin transcript, for example, which can be seen in hours) indicating that the motilin transcript is a rare mRNA even in the duodenum, where it is most abundant. This is born out by *in situ* hybridization analysis of monkey duodenum where only a very small percentage of mucosal cells exibit a hybridization signal. Since the poly-A+ fraction represents no more than 3-5% of the total pool of cellular RNA (114), the 20 μg of total RNA in each lane in Figure 22 represents a maximum of one fifth the amount of poly-A^+ RNA normally analyzed (see for example Figure 9). The fact that a hybridization signal is observed at all in a sample of total

RNA suggests that those cells which contain motilin mRNA express it at a high level.

The observations that neither the human, nor the porcine motilin nucleotide probe recognize a motilin transcript in either rat or guinea pig under high stringency hybridization/wash conditions provides strong evidence that the rodent form of motilin mRNA is significantly different from the porcine and human transcript, and lends support to the earlier inferences from immunologic data regarding the primary amino acid sequence of rat motilin. Nevertheless, the results of the low-stringency cross-specie hybridization experiment using a porcine motilin cDNA probe indicate that a motilin transcript is present in both the rat and guinea pig duodenum. The presence of a discreet hybridizing specie of poly-A^+ RNA of approximately the same size as the motilin transcript in pig, monkey and human, suggests that the hybridization signal observed at low stringency hybridization/wash indeed represents the motilin transcript in these species.

The evidence of poor nucleic acid sequence conservation in rodent motilin presented here and the unique exon structure of the human motilin gene described in Chapter 1, coupled with the known amino acid changes in canine motilin and the amino acid changes in rodent motilin inferred from immunologic data, leads to some interesting hypotheses about the evolution of mammalian motilin and about what may represent the biologically active portion of the molecule. Teleologic reasoning argues that those amino acids important for biologic activity will be relatively more conserved throughout evolution. Thus, most small bioactive peptides, as well as functional domains

in larger proteins, are usually highly conserved. It is therefore somewhat surprising how poorly motilin structure is apparently conserved. However, as noted previously, biogically active neuroendocrine and endocrine peptides are generally encoded by single exons, while motilin is encoded by two. The primary structural divergence observed in motilin from different species might therefore be explained by divergence in one exon with a single functionally important domain present on the other exon remaining more highly conserved. Considering this possibility, it is interesting to note that the five amino acid changes in canine motilin all occur in that portion of the molecule encoded by Exon II. Furthermore, the apparent N-terminal differences in rodent motilin would also be the result of changes in Exon II. Thus, this analysis leads to the specific prediction that rat motilin will contain the eight amino acid sequence present at the C-terminus of canine, porcine and human motilin. Furthermore, it is predicted that this sequence is important for the biologic activity of the molecule. Isolation of a rat motilin cDNA will verify or refute this prediction.

Conclusions

The cross-species Northern hybridization analyses described here suggest that motilin mRNA is also synthesized in rat and guinea pig duodenum, but that the rodent motilin mRNA, and therefore the primary amino acid sequence, differs significantly from the human and porcine forms that have been identified to date.

Appendix III: Expression of the motilin gene in primate neural tissue

Summary

The polymerase chain reaction was used to determine if motilin mRNA transcripts could be amplified from poly-A$^+$RNA isolated from Rhesus monkey cerebellum. Oligonucleotides complementary to the human motilin cDNA were used as primers for the polymerase chain reaction. To confirm that the amplified product represented a motilin transcript, reaction products were examined by Southern hybridization analysis using a labelled oligonucleotide probe complementary to a region of the human motilin cDNA in between the two priming oligonucleotides. For each reaction, monkey duodenal poly-A$^+$ RNA was used as a positive control. The results indicate that the duodenal form of the motilin transcript is not present in the monkey cerebellum, in agreement with Northern hybridization analysis of cerebellar RNA. However, an altered form of a motilin gene transcript, or a transcript from a highly related gene, is amplified from monkey cerebellar RNA.

Introduction

Data published from a number of laboratories using immunologic techniques to detect motilin peptide indicate that, like most gastrointestinal peptide hormones, motilin is synthesized in the central nervous system. Motilin-like immunoreactivity (MLIR) has been detected by radioimmunoassay or

immunohistochemistry in rodent, canine, porcine, ovine, and primate neural tissue. However, there is much variability in the distribution and level of the measured immunoreactivity depending upon which antisera is used; all of the antisera used in these studies were polyclonal and were raised against synthetic porcine motilin. In the rodent, MLIR was detected throughout the brain, but with a unique distribution. Highest levels were measured in cerebral cortex and cerebellum using antisera specific for the C-terminus (Antibody (Ab) 4) or middle to C-terminal regions of porcine motilin (Ab 13, 601, 753, 756, and GP) (61), in the anterior pituitary, pineal and retina (antibodies 4,13,9 and GP) (115), and in cerebellum using antibody K123 (73).

However, a number of findings indicate that the source of this rodent motilin-like immunoreactivity differs from porcine motilin. Notably, different antisera measure different amounts of immunoreactive material. For example, only antiserum specific for the N-terminus of porcine motilin (Ab 9) detected significant MLIR in rat anterior pituitary (115). In addition, the peak of motilin immunoreactivity isolated from rat brain exibited a different retention time on a reverse phase HPLC column than that of synthetic porcine motilin, although its chromatogram was the same as that observed for motilin immunoreactivity detected in rat duodenal extracts (61). Lange et al. could not detect a peak of motilin immunoreactivity from rat cerebellar extract co-migrating with either porcine or rat duodenal immunoreactivity on HPLC using an antibody which detected motilin-like immunoreactivity in cerebellar Purkinje neurons (73); and Fratta et al. could detect no rat brain (or intestinal) motilin immunoreactivity by

radioimmunoassay or immunohistochemistry with an anti-porcine motilin serum (Ab M-2) (62).

Variability in brain motilin immunoreactivity has also been seen in other species. Low levels of MLIR were detected in canine brain and moderate levels in pituitary using N-terminal (Ab 7922) and C-terminal (Ab 7921) specific antisera. However, although the gel filtration profile of the brain MLIR was similar to that obtained with canine intestinal motilin immunoreactivity, there was a relatively larger amount of a high molecular weight specie in canine brain extracts (116). On the other hand, a peak of porcine cerebellar MLIR with the same HPLC elution time as synthetic porcine motilin was detected using an antiserum (Ab M-2) which immunohistochemically stained porcine Purkinje neurons (62). In the human, an antibody which detects MLIR in rodent cerebellum (Ab 4), also detected human Purkinje neuron MLIR; this immunoreactivity could be absorbed by synthetic porcine motilin (63). Thus, the immunologic data indicates that there is species-specific and tissue-specific variation in motilin-like immunoreactivity in neural tissue. This variation may be explained by corresponding variations in motilin peptide structure, or by the presence of unrelated, crossreacting peptides. Such a result is not without precident; for example, what was thought to be a high molecular weight form of ß-endorphin detected by Western blot in the cerebellum turned out to be IgG, which shares a pentapeptide sequence with ß-endorphin (117).

The presence of intestinal motilin peptide in the brain has been further questioned by recent data obtained using the cloned porcine motilin cDNA.

Northern blot analysis of porcine cerebellar poly-A^+ RNA failed to detect any transcripts homologous to the prepromotilin transcript detected in porcine duodenum (74). Similarly, no transcript homologous to a segment of the human motilin mRNA (Exon II) was detected in intact human or monkey cerebellar poly-A^+ RNA (see Chapter 1). These data indicate that prepromotilin is not expressed in the cerebellum and support the idea that the MLIR detected in the brain is the result of crossreactivity with a very similar, yet distinct, peptide sequence which is the product of a different gene. Alternatively, prepromotilin mRNA may be present in neural tissue in a different processed form which only encodes part of the 22 amino acid motilin moiety, and which is only partly homologous to the full-length intestinal cDNA or genomic fragment used previously as probes. An example of such differential processing of a gastrointestinal hormone mRNA in neural and endocrine tissue is seen in the case of human gastrin-releasing peptide (118).

To eliminate the additional possibility that a non-polyadenylated motilin transcript is expressed in the cerebellum, Northern analysis of duodenal and cerebellar total, poly-A^+, and poly-A^- RNA was performed. Finally, the possibility that a prepromotilin mRNA is expressed at levels below the sensitivity of Northern blot hybridization analysis must also be considered. To address this latter possibility, as well as to examine the possibility of alternate processing of the primate prepromotilin transcript, the extremely sensitive and specific polymerase chain reaction (PCR) was used to search for authentic motilin gene transcripts in monkey neural tissue.

Materials and Methods

Northern blot hybridization analysis

Isolation of poly-A^+ RNA, preparation of radiolabelled cRNA probes and the procedure used for Northern analysis are decribed in Chapter 2.

Preparation of radiolabeled oiigonucleotides

Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA Synthesizer, purified by acrylamide gel electrophoresis and 5'-end labelled with ^{32}P-ATP using T4 Polynucleotide Kinase (Boehringer-Mannheim) in a reaction containing 500 mM Tris-Cl pH 8.0, 100 mM $MgCl_2$, and 200 mM dithiothreitol, 5-10 pmoles of the oligonucleotide, a twofold molar excess of radiolabel and 1 unit of kinase in a total volume of 50 µl. The labelling reaction was allowed to proceed at least 30 minutes at 37°C. The reaction was then applied directly to a 0.25 ml column of DE-52 anion exchange resin (Whatman, Inc.) preequilibrated in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA and 150 mM NaCl. The column was washed with 15 ml of the equilibration buffer and the labelled oligonucleotide eluted with 1 ml 1M NaCl in 10 mM Tris-Cl, 1 mM EDTA.

Amplification of mRNA by the Polymerase Chain Reaction

Single stranded DNA (sscDNA) complementary to messenger RNA (mRNA) from various GI tissues was synthesized using MMLV Reverse Transcriptase

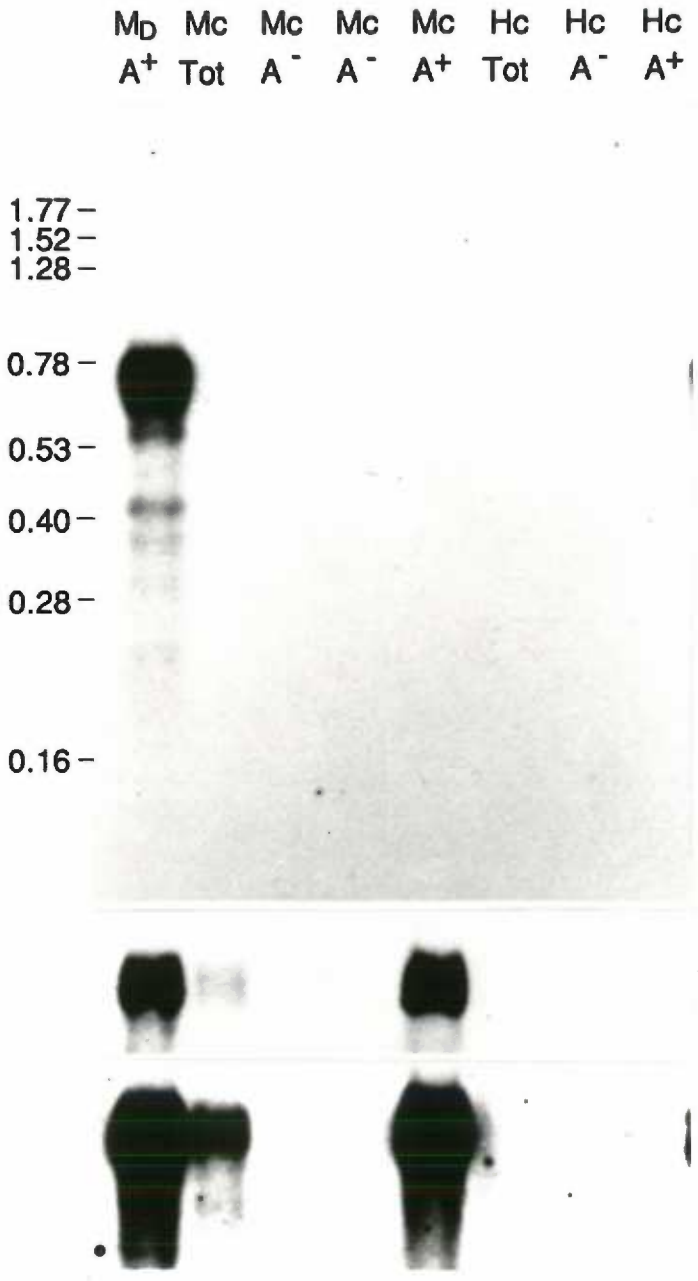
(BRL Inc.) and oligo-dT-primed poly-A^+ RNA essentially according to the method of Gubler and Hoffman (119). The RNA strand was removed by hydrolysis in 1M NaOH for 20 minutes at 65°C. The reaction was neutralized with an equal volume of 1M Tris pH 7.5, adjusted to 2M ammonium acetate and precipitated with three volumes absolute ethanol at -20°C. The pelleted sscDNA was resuspended in 50 µl H_2O and a 5 µl aliquot used as template in a polymerase chain reaction (PCR). PCR reactions were done using oligonucleotides complementary to the human motilin cDNA (described in Figure 25) and were carried out in a standard buffer (120) on a Techne Thermal Cycler. Reaction conditions were: denature 94°C, 60 sec; anneal 64°C, 60 sec; elongate 72°C, 60 sec for 50 cycles followed by a final cycle at 72°C, 4 min. Products were separated by agarose gel electrophoresis in a 3% gel (2% SeaKem NuSieve, FMC, Inc./1% MB Grade Agarose, Bethesda Research Laboratories), visualized with ethidium bromide (50 µg/ml) fluorescence and photographed. The PCR products were then transfered to a Nytran membrane by Southern transfer (110) and probed with a ^{32}P-labelled oligonucleotide internal to the amplified products.

Results

Previous Northern analysis of poly-A^+ RNA isolated from porcine, monkey and human tissue indicated that no motilin gene transcript is expressed in the cerebellum. However, to eliminate the possibility that a non-polyadenylated motilin transcript is expressed in cerebellar tissue, these experiments were repeated using total RNA samples. The results of Northern hybridization

Figure 24: Northern Hybridization Analysis of Primate Cerebellar RNA using a Human Motilin Gene Probe

Upper Panel: Northern blot of monkey duodenal (M_D), monkey cerebellar (M_C) and human cerebellar (H_C) RNA probed with a 32P-labeled human motilin cRNA probe and exposed to film for 5 days. Poly A^+ (A^+) lanes were loaded with 5 µg RNA, total RNA (Tot) and poly-A^- RNA (A^-) lanes were loaded with 20 µg RNA. Positions and sizes (kb) of RNA molecular weight standards are indicated at the left of the figure. **Middle Panel:** The blot shown above washed to remove the motilin hybridization signals, reprobed with a rat cyclophilin probe and exposed to film overnight. The position of these hybridization signals was appropriate for the size of the cyclophilin mRNA. **Bottom Panel:** Re-exposure of the cyclophilin blot for 3 days.


MD A+
Mc Tot
Mc A-
Mc A-
Mc A+
Hc Tot
Hc A-
Hc A+
1.77
1.52
1.28
0.78
0.53
0.40
0.28
0.16

analysis of monkey and human cerebellar RNA are shown in Figure 24. No motilin-specific hybridization is detectable in poly-A^+, poly-A^-, or total RNA fractions of monkey or human cerebellar RNA, while an intense motilin hybridization signal is detected in monkey duodenum poly-A^+ RNA. The integrity of the various RNA samples as well as the effectiveness of the oligo-dT affinity purification of poly-A^+ RNA is indicated by the presence of intense cyclophilin hybridization signals in monkey duodenum and cerebellum poly-A^+ samples, a much less intense signal in monkey cerebellar total RNA, and no detectable hybridization in the poly-A^- fractions. Only a weak cyclophilin hybridization signal is present in the human cerebellum poly-A^+ sample, indicating that this RNA sample was largely degraded. Thus, no transcript homologous to the human motilin exon II probe is detectable in monkey cerebellar RNA.

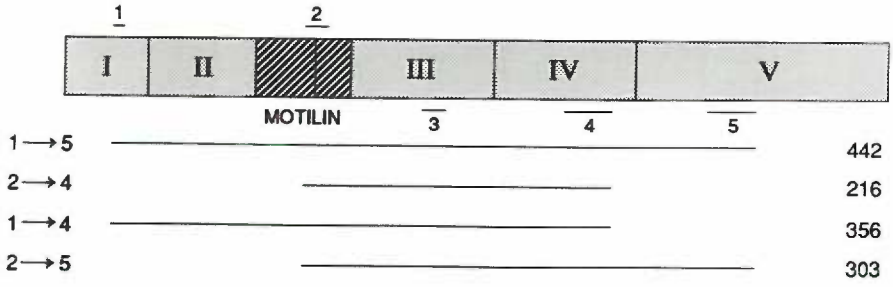
Although the duodenal motilin transcript is not expressed in cerebellum, it is a formal possibility that a motilin gene transcript is expressed which retains some of the motilin coding region, but which does not retain enough nucleotide sequence homology overall to hybridize with the probes used for Northern analysis. To investigate the possibility of alternate motilin transcripts in neural tissue, the polymerase chain reaction was used to try to amplify various regions of the prepromotilin mRNA from the monkey cerebellum. The results of detailed PCR analysis of cDNA synthesized from mRNA from various monkey and human tissues, including cerebellum, are shown in Figure 25. Oligonucleotides complementary to Exon I and Exon V of the human motilin gene were used to amplify the authentic duodenal form of the motilin transcript, and as expected, a product of the predicted size, and which

Figure 25: Amplification of Motilin Transcripts From Monkey Neural Tissue Using the Polymerase Chain Reaction

Polymerase chain reaction amplification of cDNA reverse transcribed from poly-A+ RNA isolated from various human and monkey tissues. The positions of the oligonucleotides (numbered 1-5) used as primers relative to the prepromotilin mRNA (shaded figure) are indicated in the top figure; (+) sense primers are indicated above and (-) sense primers below prepromotilin. Exons are numbered with Roman numerals and their positions indicated. The location of the motilin sequence within propromotilin is indicated by crosshatches. The predicted lengths of the various amplification products are shown below and their lengths in base pairs indicated to the right. All of the Southern blots shown below were probed with 32P-labeled oligonucleotide 3. **A:** Ethidium-stained gel of PCR products resulting from priming with oligonucleotides 1 and 5 (Right) and the corresponding Southern blot (Left). D, monkey duodenum; T, monkey testis; A, monkey adrenal; P, monkey pituitary; C, monkey cerebellum; H, human duodenum. Ethidium-stained products of approximately 450 bp are evident in lanes D and H, and these products hybridize with labeled oligonucleotide 3. **B:** PCR amplification using oligonucleotides 2 and 4. **C:** PCR amplification using oligonucleotides 1 and 4. **D:** (Right); Ethidium-stained gel of PCR products resulting from amplification using oligonucleotides 2 and 5. (Middle); Ethidium-stained gel of human duodenum cDNA amplified with oligonucleotides 1 and 4 (H1) and monkey cerebellum and human duodenum cDNA amplified with oligonucleotides 2 and 5 (C and H, respectively). (Left); Southern blot of middle gel.

The sequences of the oligonucleotides used in these experiments are as follows:

1: 5'- CCCACTCAGACCACGTGCACGCC
2: 5'- GGATGCAGGAAAAGGAACGG
3: 5'- GGGCTCGGCAGGGTCTACAGGACCTTCCTCCCC
4: 5'- GCATCTCACTCAGCAGCCCTTCCAGGGTGGCCGGG
5: 5'- CCCAGGGCCTCACTTGGGCAGGAGGGGCCTCCC


1
2
I
II
III
IV
V
MOTILIN
3
4
5
1→5
442
2→4
216
1→4
356
2→5
303

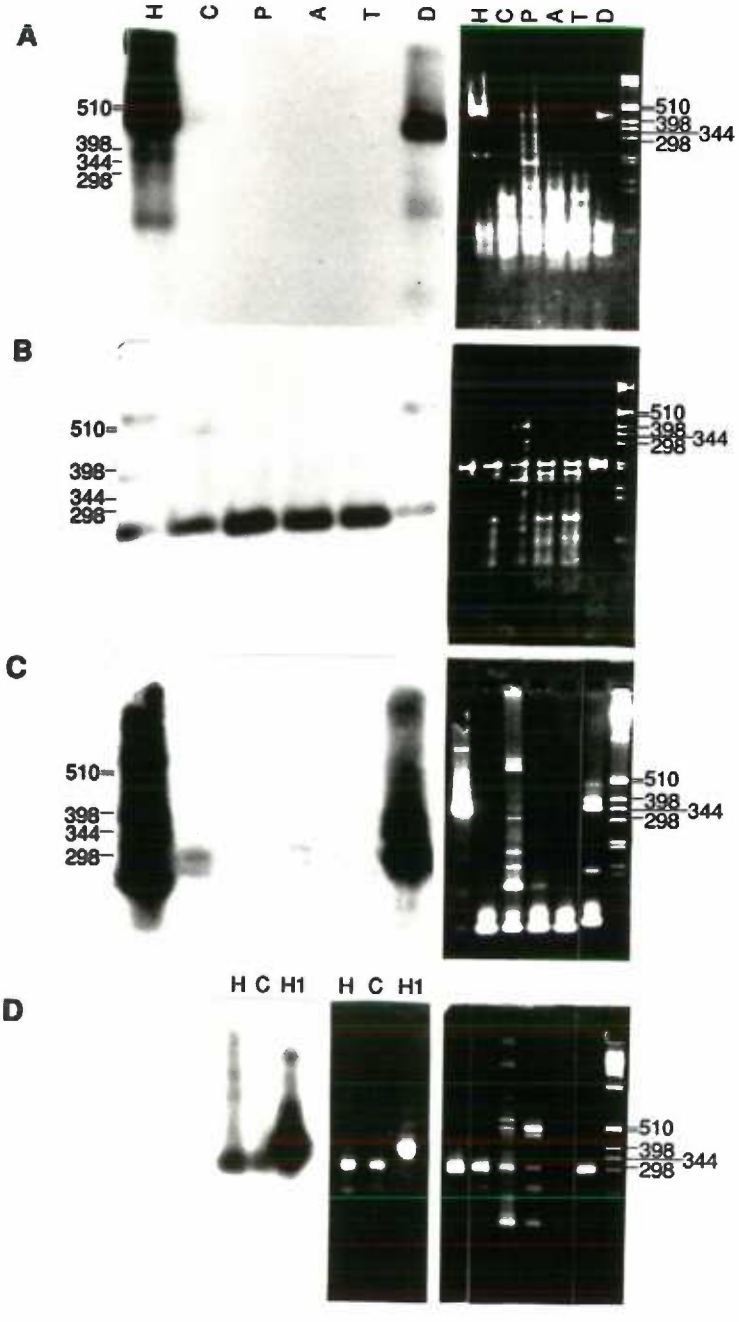

A
H C P A T D
510
398
344
298
B
C
D
H C H1

Figure 26: Summary of Polymerase Chain Reaction Amplification of Motilin Transcripts from Monkey Neural Tissue

Amplification of a product of the predicted length which hybridized with an internal motilin oligonucleotide using the specified oligonucleotide as a primer is indicated by a "+". Failure of an oligonucleotide primer to generate a specific motilin-related product is indicated by a "-".

		Amplification	
Oligonucleotide	Exon	Duodenum	Cerebellum
1	I	+	-
2	II/III	+	+
4	IV	+	+
5	V	+	+
		Hybridization	
3	III	+	+

Discussion

The Northern analysis of cerebellar RNA described here agrees with a previous experiment described in Chapter 1 and with the previously published expression of porcine motilin cDNA (74), and indicates that there is no motilin transcript present the human, nonhuman primate, or porcine cerebellum. This conclusion is confirmed by the results of the PCR experiment using oligonucleotides complementary to Exon I and Exon V of the human motilin gene which indicates that there is not even a very low level of the duodenal form of motilin mRNA in monkey cerebellum. Nevertheless, PCR amplification of cerebellar, adrenal, and pituitary cDNA using Exon II/III, IV, and V oligonucleotides as primers results in major products of the same size as that amplified from the duodenum and which also contain sequences complementary to the Exon III-specific oligonucleotide as determined by Southern hybridization analysis. Thus, it appears that a motilin related transcript is present in these tissues. These results are consistent with the expression of a motilin gene transcript which is alternately spliced at the 5' end. This possibility is very interesting in light of the immunologic evidence described above which suggests that the source of MLIR in the cerebellum does not share homology with the N-terminus of motilin. Since the N-terminal amino acids are encoded by Exon II, such an alternately spliced form would be predicted to contain only part of Exon II. Another possibility consistent with these results is that the amplified transcript is the product of a distinct, but related gene. Data presented in Appendix I indicates that one or more motilin-related genes exist in the human genome. It is, however, difficult to reconcile the absence of a hybridization signal in the Northern blot experiment

with the presence of a cerebellar transcript detected with the polymerase chain reaction which is apparently highly homologous with the motilin cDNA. Since the Northern experiments utilized an Exon II-specific probe, the cerebellar transcript either differs in sequence from the 5' end of Exon II of the motilin gene, or it is too rare to be detected by Northern analysis. These outstanding questions as to the identity of the motilin-related product amplified from monkey cerebellum can only be answered by nucleic acid sequence analysis of the cerebellar transcript.

Conclusions

The existence of a motilin gene transcript in the monkey cerebellum was investigated by Northern hybridization analysis and the polymerase chain reaction. No motilin transcript could be detected on a Northern blot of cerebellar RNA, confirming previous results. Similarly, no specific product corrresponding to the authentic duodenal form of the motilin mRNA could be amplified from cerebellum in a polymerase chain reaction. However, motilin-specific products were amplified from cerebellar cDNA using Exon II/III, IV, and V oligonucleotide primers, suggesting that an alternately spliced motilin gene transcript, or a transcript from a highly related gene, is present in the monkey cerebellum.

SUMMARY OF APPENDICES

Cross-specie Northern analysis described in Appendix II using either a human motilin genomic fragment or the porcine motilin cDNA as probes to identify the motilin transcript in rat, guinea pig and pig indicates that there is significant nucleic acid sequence divergence in mammalian motilin. While the human and porcine probes both recognize the human, monkey and porcine motilin mRNA, they do not detect a rodent transcript in the duodenum under high stringency hybridization and wash conditions. Under low stringency conditions a single transcript is detected in rat and guinea pig duodenal RNA which is assumed to be the rodent motilin homologue. This divergence at the nucleic acid level is consistent with previous immunologic data which suggests that there are significant difference between rat motilin and human/porcine motilin at the amino acid level.

The data presented in Appendix I describing the results of human genomic Southern analysis of the human motilin gene indicate that a single motilin gene is present in the human genome. Thus, the tissue-specific differences in motilin mRNA levels described in Chapters 1 and 2 are due to differences in transcription levels of a single gene rather than activaton of additional genes in certain tissues. However, the results also indicate the presence of an additional sequence or sequences in the human genome which share a significant degree of sequence homology with the motilin gene. This finding is relevant to the results described in Chapter 2 which show that a specie of mRNA is present in monkey liver which is highly homologous to the motilin mRNA expressed in the duodenum. It is plausible that this liver transcript is

the product of one of these motilin-related genes detected by Southern analysis. Furthermore, data presented in Appendix III is consistent with the expression of a gene in the primate cerebellum which contains a region of high homology with the primate motilin gene.

Appendix III describes the results of polymerase chain reaction amplification of mRNAs expressed in primate duodenum and cerebellum. In these experiments, oligonucleotide primers complementary to the human motilin cDNA were used as primers to amplify motilin transcripts. In the duodenal reactions, products of the size predicted from the human motilin cDNA sequence were amplified using combinations of oligonucleotides complementary to regions encoded by exons I,II/III,IV, and V. The specificity of these products was confirmed by showing that they were also contained an internal motilin-complementary sequence. Specific products of the predicted size were also amplified from the cerebellum using exon II/III, IV, and V oligonucleotides, but not with an exon I-specific primer. While these experiments do not identify the cerebellar transcript, they indicate that a transcript which is highly related to the duodenal motilin transcript is present, at least in small amounts, in the monkey cerebellum. This transcript could be the product of a very closely related gene, as noted above for the liver transcript, or it may represent a transcript from the motilin gene which has been alternatey spliced to produce a transcript which differs at the 5' end compared with the duodenal motilin transcript. The identity of this cerebellar transcript must, however, be determined by nucleic acid sequence analysis.