Identification and expression of the motilin precursor in the guinea pig

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Abstract Motilin has never been isolated from rodents, the most frequently used laboratory animals, despite several attempts. We have isolated and sequenced the motilin precursor from duodenal mucosa of guinea pig (GenBank accession number AF323752) and studied its expression in several tissues. The percent homology with human motilin is the lowest yet observed due to several unique substitutions in the C-terminal end. As expected, the precursor was present in the gut mucosa with the exception of the gastric corpus. It was also present in medulla oblongata, nucleus of the solitary tract, hypophysis, spinal cord, hypothalamus, and cerebellum but not in the cerebral cortex. For the first time we demonstrated motilin expression in the thyroid. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Motilin; Homology; Precursor; Distribution; Thyroid

1. Introduction

Motilin is a 22 amino acid polypeptide, first purified and sequenced from porcine duodenum [1]. Motilin stimulates gastrointestinal motor activity and, at least in man and in dog, it seems to be involved in the regulation of the fasting motility pattern [2]. In other species its role is less clear and has not been studied in detail. In this respect it is striking that little information is available on the effects of motilin in commonly used small laboratory animals such as rat, mouse and guinea pig.

There are probably two reasons for this. Firstly, porcine motilin has no or only weak effects on gastrointestinal contractility in these species [3]. Secondly, although motilin-like immunoreactivity has been demonstrated in rat and guinea pig brain [4], attempts to isolate the peptide [5] or to identify its precursor [6] failed, suggesting the primary structure of motilin in rodents was different. The two reasons may be related, as only bioactivity may be species-specific. For example, canine but not porcine motilin has an effect on strips from canine small intestine [7].

We now report the first successful identification of a rodent motilin.

2. Materials and methods

2.1. Oligonucleotides

All PCR primers and the hybridization probes were synthesized by Gibco (New York, NY, USA). The oligo-dT_{16} anchor primer and the PCR anchor primer were from the 3′/5′ RACE (rapid amplification of cDNA ends) kit (Boehringer Mannheim, Germany). Primers were designed using the program PRIMER in the GCG program package from BEN (Belgian European Molecular Biology Net node) and were based on the known sequences of the precursor in other species. All primers and probes used are listed in Table 1.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from 1 g mucosa of the duodenum of the guinea pig by the TRIzol Reagent method (Gibco, New York, NY, USA). First strand cDNA was synthesized from 1 μg total RNA using 200 units Superscript II RNase H^- Reverse Transcriptase (Gibco, New York, NY, USA) and the oligo-dT_{16} anchor primer from the RACE kit. The reaction mixture was first incubated at 42°C for 1 h, followed by incubation at 95°C for 10 min.

2.3. PCR

2.3.1. PCR amplification of the middle portion of the motilin cDNA sequence. The first strand cDNA pool of guinea pig intestine served as a template for the PCR using 0.5 units of *Thermus aquaticus* (Taq) DNA polymerase (Pharmacia Biotech, Uppsala, Sweden), the degenerate primer Gmot.for (0.5 μM) and the PCR anchor primer (0.5 μM). Amplification was performed at 94°C (1 min), 53°C (1 min), 72°(1 min) for 38 cycles, followed by an extension step at 72°C for 10 min. Further amplification was obtained in a semi-nested PCR with degenerate primers Gmot.for and Gmot.rev. The PCR was analyzed by electrophoresis on a 1.5% agarose gel and, after Southern blot on a Biodyne® A membrane (Gibco, New York, NY, USA), hybridized with a ^{32}P-labeled rabbit motilin cDNA probe (fragment 69–363 of rabbit motilin cDNA X63860). The rabbit cDNA fragment was labeled using the Rediprime probe kit (Amersham Life Sciences, Buckinghamshire, UK) and [α-^{32}P]dCTP (ICN, Costa Mesa, CA, USA).

2.3.2. Amplification of the 3′ and 5′ cDNA ends. This was done with the 3′/5′ RACE kit (Boehringer Mannheim, Germany), using primers based upon the known sequence of the middle portion. The RACE approach takes advantage of the poly-A tail at the 3′ end of mRNA to prepare cDNA extended with an anchor sequence. A specific primer (Gp1.for) for the known middle portion and a primer corresponding to the anchor sequence were then used to amplify a terminal segment containing the unknown sequence. For the 5′ end, cDNA is extended with a poly-A tail to allow the same strategy to be used. Here cDNA was synthesized from total RNA using the gene-specific primer GpSP1.rev and 30 units of the thermostable DNA polymerase from *Carboxydothermus hydrogenoformans* (Roche, Mannheim, Germany) (65°C for 30 min). The single strand cDNA (ss-cDNA) was purified by the High Pure PCR Product Purification kit (Boehringer Mannheim, Germany). A homopolymeric A-tail was added to the 5′ end of the ss-cDNA by terminal transcriptase (Boehringer Mannheim, Germany). Tailed ss-cDNA was amplified using the gene-specific primer GpSP2.rev and the oligo-dT_{16} anchor primer from the kit, followed by a semi-nested PCR using the gene-specific reverse primer GpSP3.rev and the PCR anchor primer.

Analysis of PCR products was performed as described above except that the probes used for hybridization were GpSP4.rev for the 5′ cDNA end and Gp2.for for the 3′ cDNA end. The oligonucleotide probes were labeled using [γ-^{32}P]ATP (ICN, Costa Mesa, CA, USA) and T_4 polynucleotide kinase (Eurogentec, Belgium) followed by pu-

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Table 1
Primers used to amplify the motilin precursor

Identification	Sequence	Position[a]
Gmot.for	5′-GAAGSCTTYGTNCCNATHTTYAC-3′	83
Gmot.rev	5′-GTACTTTTCCAGCTGYCT-3′	304
GpSP1.rev	5′-CTGCTGCCTTTGACCTCTGC-3′	194
GpSP2.rev	5′-CTGTACGCTCAGGGACTTCCT-3′	175
GpSP3.rev	5′-TAGCCTTTTGTTCTGCTCCCT-3′	154
GpSP4.rev (probe)	5′-GTGTCCTCCGCAGCTCGCTG-3′	128
Gp1.for	5′-ACACAGGAAAGGGAGCAGAAC-3′	125
Gp2.for (probe)	5′-AGGCAGCAGGGCGGCTGGAGCCCC-3′	186
Gp.for	5′-TTCCAATCTTCACTTACAGCGAG-3′	93
Gp.rev	5′-CAATTTCCACTGGAGCAGTC-3	269
PCR anchor primer	5′-GACCACGCGTATCGATGTCGAC-3′	
Oligo-dT$_{16}$ anchor primer	5′-GACCACGCGTATCGATGTCGAC(T)$_{16}$-3′	

N = A+C+T+G; Y = C+T; S = C+G; H = A+T+C.
[a]Numbers indicate the 5′ base of each primer. The numbering refers to the guinea pig precursor.

rification on a NucTrap probe purification column (Stratagene, La Jolla, CA, USA). The obtained 5′ or 3′ RACE products were cloned as described in Section 2.4.

2.4. Cloning and sequencing of cDNA

PCR products that hybridized with the selected probe were ligated into the pCR™ 2.1 plasmid vector and transformed into *E. coli Invα* competent cells (TA cloning kit, Invitrogen, San Diego, CA, USA). After growing on LB plates containing 50 μg/ml ampicillin, the clones were grown separately in LB liquid medium containing ampicillin in 96 well plates and transferred to a Biodyne® A membrane. Screening of positive clones was performed by hybridization with the same ^{32}P-labeled oligonucleotide probe or the ^{32}P-cDNA fragment used for the analysis of the PCR products. The plasmid DNAs from positive clones were extracted and purified by the Mini Qiagen Plasmid kit manual (Qiagen, CA, USA). Sequencing was performed by an AB1377 automatic fluorescent sequencer based on the dideoxy chain termination method (Eurogentec, Belgium).

2.5. Distribution of the motilin precursor in guinea pig

Total RNA was extracted by the TRIzol reagent procedure (Gibco, New York, NY, USA) from several tissues. Preparation of cDNA and semi-nested amplification was performed as described in Section 2.3.1, except that the primers Gp.for and Gp.rev were used (Table 1). PCR products were analyzed on a 1.5% agarose gel. Southern blot hybridization was performed using a ^{32}P-guinea pig motilin oligonucleotide probe (GpSP1.rev).

3. Results and discussion

3.1. Isolation and sequencing of cDNA encoding the guinea pig motilin precursor

Semi-nested PCR of cDNA prepared from total RNA isolated from the duodenal mucosa of the guinea pig with the degenerate primers (Gmot.for, Gmot.rev) and the PCR anchor primer resulted in four cDNA products (~180 bp, ~220 bp, ~310 bp, ~430 bp). Southern blot hybridization analysis revealed that only the ~220 bp product hybridized with the ^{32}P-rabbit cDNA probe. This band also had the expected size (222 bp). PCR products were subcloned and positive clones were sequenced. The length of the cloned cDNA fragment was 222 bp and its sequence was used to design primers for the amplification of the 3′ and 5′ cDNA ends. Cloning and sequencing of the amplified 3′ and 5′ cDNA ends revealed sequences of 367 and 154 bp.

Many problems were encountered in obtaining the 5′ cDNA end. Positive PCR products were only obtained if the reverse transcription reaction was performed with the thermostable DNA polymerase from *C. hydrogenoformans* which can be used at a high temperature (65°C). This may indicate that a secondary structure in a GC-rich region of the

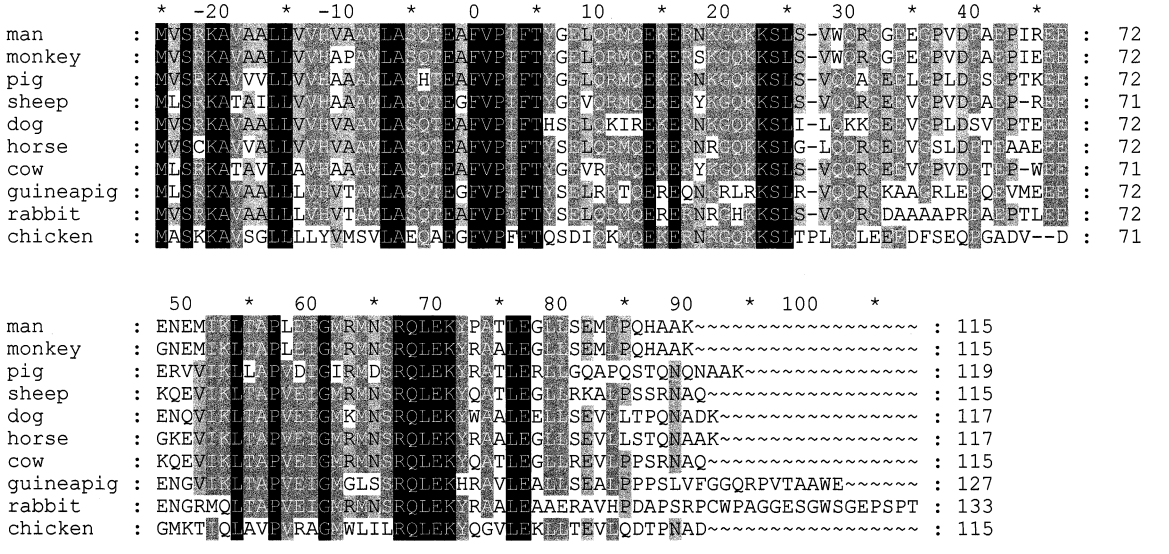

Fig. 1. Alignment of the amino acid sequences of the motilin precursor from different species. The most conserved region between species is indicated with a dark background. Amino acids are numbered relative to the motilin peptide.

5′ cDNA end uncouples the reverse transcriptase. Subsequent sequence analysis indeed confirmed that the signal peptide has a high GC content (68%).

The cDNA sequences were confirmed by 15 independent sequences obtained from nine different PCR products from cDNA prepared from six guinea pigs. All gave identical results. The complete nucleotide sequence of the cDNA can be consulted in the GenBank database, accession number AF323752.

Analysis of the cDNA sequence of 491 bp revealed an open reading frame of 381 bp initiated at the 5′ end by AUG. The stop codon TGA is located 378 bp downstream of the start codon and is followed by a 3′ untranslated region of 94 bp. The polyadenylation signal sequence AATAAA is located 15 bp before the poly-A tail. The amino acid sequence deduced from the cDNA sequence indicates that the guinea pig motilin precursor consists of 127 amino acids. As in the other motilin precursors, the sequence starts with a 25 amino acid signal peptide followed by the 22 residues of the bioactive peptide and an 80 amino acid 'motilin-associated peptide' (MAP) (Fig. 1).

3.2. Homology with the motilin precursors from other species

cDNA encoding the motilin precursor has previously been isolated and sequenced from man [8,9], pig [10], rabbit [11], sheep [12], monkey [13], dog, chicken, horse, and cow [6]. In every species the precursor consists of a 25 amino acid signal peptide, 22 amino acid motilin itself and a MAP of variable length. It is clear from Fig. 1 that the guinea pig precursor has a similar structure.

To evaluate the similarity quantitatively, the percent homology with the other precursors was calculated for the three main regions of the precursor using the Wisconsin Genetics Computer Group (GCG) package made available through the Belgian European Molecular Biology Net Node (BEN). The results are summarized in Table 2. While the homology is rather high, as expected, it is of interest to note that for the motilin peptide itself the values, ranging between 50 and 73% for the amino acids and 67 and 79% for the nucleotides, are low when compared with those obtained between the other precursors. Indeed the lowest amino acid homology yet obtained was 64%, and the average homology was 80%.

Table 2
Homology of the guinea pig motilin precursor

	Signal peptide		Motilin		MAP	
	aa	nt	aa	nt	aa	nt
Man	84	85	68	74	57	74
Monkey	80	85	64	74	59	76
Pig	68	84	68	77	51	68
Sheep	80	87	59	74	57	74
Dog	84	90	59	76	57	74
Horse	76	88	68	77	59	73
Cow	76	85	64	77	59	75
Rabbit	92	96	73	79	55	68
Chicken	56	65	50	67	41	54

The reason for this are the large number of substitutions, seven compared to human motilin, with four unique for the guinea pig (Thr^{13}, Gln^{18}, Arg^{21}, and Leu^{22}) and one only shared with the cow (Arg^{11}) and rabbit (Arg^{16}). Structure–activity studies in the rabbit have shown that bioactivity is mostly determined by the N-terminal end, especially residues 1, 4, and 7 [14,15], and it is assumed that the C-terminal region (amino acids 10–22) forms an α-helix which stabilizes the interaction of the N-terminal residues at the active site [16]. It remains to be determined how these changes affect bioactivity.

The MAP starts with a putative endoproteinase dibasic cleavage site, Arg^{23}-Lys^{24}. In all other precursors this is Lys^{23}-Lys^{24} but this change will not affect the endoprotease activity.

The function of the MAP region is unknown. It contains a PEST region rich in proline (P), glutamate (E), serine (S), and threonine (T), which may be subject to rapid intracellular degradation and which extends from residues 54 to 68 in the guinea pig. It also contains a highly conserved region at

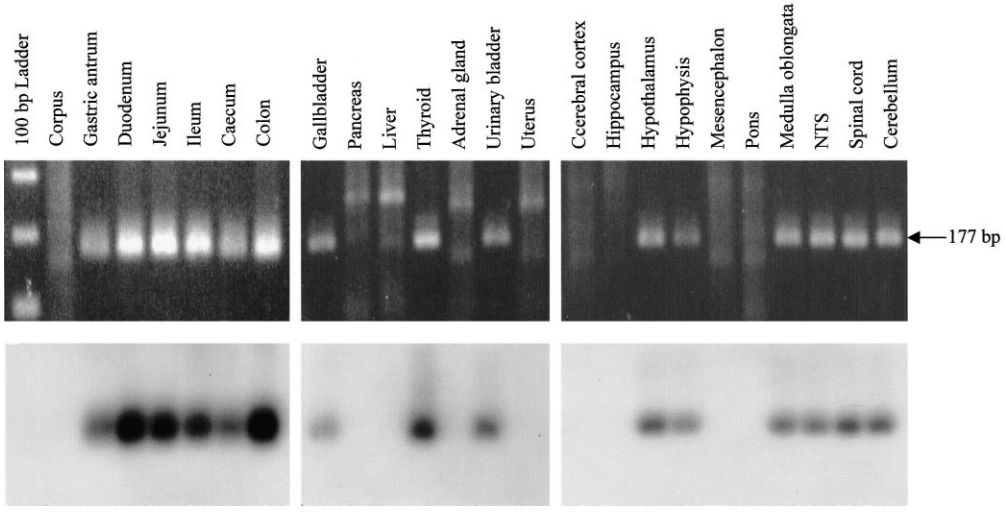

Fig. 2. Demonstration of the presence of motilin mRNA by RT-PCR in different tissues. Top: Agarose gel electrophoresis of amplified cDNA of guinea pig corpus, gastric antrum, duodenum, jejunum, ileum, caecum, colon, gall bladder, pancreas, liver, thyroid, adrenal gland, urinary bladder, uterus, cerebral cortex, hippocampus, hypothalamus, hypophysis, mesencephalon, pons, medulla oblongata, nucleus of the solitary tract, spinal cord, and cerebellum. Motilin products correspond to the 177 bp band. Bottom: Hybridization of the agarose gel with the ^{32}P-motilin oligonucleotide probe (GpSP1.rev). In all preparations only the 177 bp band hybridized.

residues 67–73, although in the guinea pig residue 73 is histidine instead of tyrosine.

An earlier analysis revealed that the homology of the signal peptide and motilin was higher than in the MAP and concluded that the MAP region probably evolved at a different rate [6]. However, as can be seen from Table 2, the situation in the guinea pig is different because the homology is highest in the signal peptide and comparable but lower in motilin and MAP. The significance of this finding, which suggests that motilin and MAP had the same evolutionary rate in this species, remains to be determined.

3.3. Motilin mRNA expression in guinea pig

Expression of mRNA was found in several tissues as illustrated in Fig. 2. For all tissues tested PCRs were performed with mRNA extracted from three animals, on three separate occasions. In every experiment expression was found in the same tissues. For the expression in the colon, thyroid, gall bladder, hypothalamus, nucleus of the solitary tract, medulla oblongata, and spinal cord, where the presence of motilin is controversial or had not been reported until now, the result was confirmed in a fourth animal. All cDNA samples were tested in two independent PCRs.

As can be seen in Fig. 2, motilin mRNA was detected in the duodenum. It should be noted that in pig, dog, man, cat, and rabbit the duodenum is the richest source of motilin, and motilin content decreases distally along the gastrointestinal tract. Although our analysis is not quantitative, the bands decrease in intensity for the more distal parts but surprisingly, a very strong band was obtained in the colon. On the other hand, the gastric corpus did not contain motilin mRNA, as is the case in other species. We also found motilin expression in gall bladder and urinary bladder mucosa, where it has never yet been reported.

The presence of motilin in the central nervous system has been debated for a long time. However, recently the issue has been resolved by the demonstration of motilin mRNA in the rabbit [17] and in the human brain [17]. We also detected motilin expression in the medulla oblongata, nucleus of the solitary tract, hypophysis, spinal cord, hypothalamus, and cerebellum. In rabbit, the motilin receptor has also been described in the hypothalamus and cerebellum [18], and this suggests that motilin may play a central role. Recent studies indeed indicate that the central administration of motilin activates neural pathways in the brain [19]. Finally, it may be noted that we detected motilin mRNA in the thyroid. Motilin expression has never been studied in the thyroid, but we included it in our exploration because the motilin receptor which was recently cloned [20] also originated from the thyroid. It cannot be excluded that motilin may have some role in regulating thyroid function.

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References

[1] Brown, J.C., Cook, M.A. and Dryburgh, J.R. (1973) Can. J. Biochem. 51, 533–537.
[2] Vantrappen, G. and Peeters, T.L. (1989) in: Handbook of Physiology, Motilin, Section 6: The Gastrointestinal System (Makhlouf, G.M., Ed.), pp. 545–558, American Physiological Society, Bethesda, MD.
[3] Tani, S. and Muto, N. (1985) J. Pharmacobiodyn. 8, 793–799.
[4] Beinfeld, M.C. and Korchak, D.M. (1985) J. Neurosci. 5, 2502–2509.
[5] Vogel, L.B. and Brown, J.C. (1990) Can. J. Physiol. Pharmacol. 68, 1124–1130.
[6] Huang, Z., Depoortere, I., De Clercq, P. and Peeters, T. (1999) Gene 240, 217–226.
[7] Poitras, P., Lahaie, R.G., St-Pierre, S. and Trudel, L. (1987) Gastroenterology 92, 658–662.
[8] Seino, Y., Tanaka, K., Takeda, J., Takahashi, H., Mitani, T., Kurono, M., Kayano, T., Koh, G., Fukumoto, H. and Yano, H. (1987) FEBS Lett. 223, 74–76.
[9] Dea, D., Boileau, G., Poitras, P. and Lahaie, R.G. (1989) Gastroenterology 96, 695–703.
[10] Bond, C.T., Nilaver, G., Godfrey, B., Zimmerman, E.A. and Adelman, J.P. (1988) Mol. Endocrinol. 2, 175–180.
[11] Banfield, D.K., MacGillivray, R.T.A., Brown, J.C. and McIntosh, C.H.S. (1992) Biochim. Biophys. Acta 1131, 341–344.
[12] De Clercq, P., Depoortere, I. and Peeters, T.L. (1997) Gene 202, 187–191.
[13] Huang, Z., De Clercq, P., Depoortere, I. and Peeters, T. (1998) FEBS Lett. 435, 149–152.
[14] Macielag, M.J., Peeters, T.L., Konteatis, Z.D., Florance, J.R., Depoortere, I., Lessor, R.A., Bare, L.A., Cheng, Y.S. and Galdes, A. (1992) Peptides 13, 565–569.
[15] Peeters, T.L., Macielag, M.J., Depoortere, I., Konteatis, Z.D., Florance, J.R., Lessor, R.A. and Galdes, A. (1992) Peptides 13, 1103–1107.
[16] Miller, P., Gagnon, D., Dickner, M., Aubin, P., St-Pierre, S. and Poitras, P. (1995) Peptides 16, 11–18.
[17] Depoortere, I., De Clercq, P., Svoboda, M., Bare, L. and Peeters, T.L. (1997) Peptides 18, 1497–1503.
[18] Depoortere, I., Van Assche, G. and Peeters, T.L. (1997) Brain Res. 777, 103–109.
[19] Tang, M., Zhang, H., Han, X., Peeters, T.L., Depoortere, I., Xu, L. and Jiang, Z. (1999) Neurogastroenterol. Motil. 11, 294.
[20] Feighner, S.D., Tan, C.P., McKee, K.K., Palyha, O.C., Hreniuk, D.L., Pong, S.S., Austin, C.P., Figueroa, D., MacNeil, D., Cascieri, M.A., Nargund, R., Bakshi, R., Abramovitz, M., Stocco, R., Kargman, S., O'Neill, G., Van Der Ploeg, L.H., Evans, J., Patchett, A.A., Smith, R.G. and Howard, A.D. (1999) Science 284, 2184–2188.