Developmental Regulation of a Turkey Intestinal Peptide Transporter (PepT1)

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ABSTRACT A cDNA encoding a turkey intestinal peptide transporter, tPepT1, was isolated from a turkey small intestinal cDNA library. The tPepT1 cDNA encodes a 714-amino acid protein with 12 predicted transmembrane domains. The amino acid sequence of tPepT1 is 94.3% identical to chicken PepT1 and approximately 60% identical to PepT1 from rat, sheep, rabbit, and human. Using a 2-electrode voltage-clamp technique in Xenopus oocytes expressing tPepT1, Gly-Sar transport was pH dependent but was independent of Na+ and K+. For the dipeptides Gly-Sar and Met-Met, the evoked inward currents indicated that the transporter was saturable and had high affinity (0.69 ± 0.14 mM and 0.23 ± 0.04 mM, respectively) for these substrates. However, transport of the tetrapeptide, Met-Gly-Met-Met, exhibited apparent substrate inhibition at high substrate concentrations. To study developmental regulation of PepT1 mRNA in turkey embryos, embryos (6 males and 6 females) were sampled daily from 5 d before hatch to the day of hatch (d 0). The abundance of PepT1 mRNA in the small intestine was quantified densitometrically from Northern blots after hybridization with full-length tPepT1 cDNA as probe. A 3.2-fold increase in PepT1 mRNA was observed in intestinal tissue from 5 d before hatch to d 0. This increase in PepT1 mRNA abundance indicates that the PepT1 gene is developmentally regulated and that there may be an important role for PepT1 in the neonatal poult.

(Key words: peptide transporter, PepT1, developmental regulation, turkey, embryo)

INTRODUCTION

A significant fraction of dietary amino nitrogen is absorbed as intact oligopeptides rather than free amino acids (Ganapathy et al., 1994). In poultry, uptake of Leu in vivo is more rapid in the form of the dipeptide glycyl-leucine rather than as a mixture of Gly and Leu (Boorman and Freeman, 1976). The intestinal H+-dependent oligopeptide transporter (PepT1) is a membrane protein that plays an important role in transporting small peptides arising from digestion of dietary proteins in the small intestine (reviewed by Daniel, 2004). PepT1 cDNA from rabbit (Fei et al., 1994), human (Liang et al., 1995), rat (Saito et al., 1995), mouse (Fei et al., 2000), sheep (Pan et al., 2001), and chicken (Chen et al., 2002) have been cloned. Another member of the H+/peptide transporter family, PepT2, has also been cloned (Boll et al., 1996). PepT2 is specifically expressed in the kidney and functions to reabsorb filtered peptides, peptide-derived antibiotics, and peptides produced as a result of the action of luminal peptidases. In mammals, PepT1 shows approximately 45% sequence identity to PepT2. A search of the chicken genome sequence database revealed no chicken PepT2 homolog that was similar in size to mammalian PepT2 (729 to 740 amino acids).

Expression of PepT1 mRNA has been observed in a variety of tissues. PepT1 mRNA is detectable in the small intestine of sheep, cows, pigs, and chickens and the omasal and ruminal epithelium of sheep and dairy cows with little expression in liver and kidney (Chen et al., 1999). Expression patterns of PepT1 in the rabbit small intestine are not the same in different sections of the small intestine or in different areas along the crypt-villus axis (Freeman et al., 1995). Ultrastructural localization of PepT1 in the rat small intestine showed that PepT1 was abundant in the absorptive epithelial cells of the villi in the small intestine (Ogihara et al., 1999). Although PepT1 is exclusively located in the apical brush border of enterocytes in prenatal and mature rats, PepT1 could be detected in the subapical cytoplasm and basolateral membrane of enterocytes immediately after birth (Hussain et al., 2002).

Gene expression of PepT1 can be regulated markedly by diet and development (Meredith and Boyd, 2000). PepT1 gene expression is induced by selective amino acids.
acids and peptides in vitro (Shiraga et al., 1999). Under various conditions of malnourishment, expression of PepT1 is greatly enhanced (Ihara et al., 2000). Nielsen et al. (2001) have shown that growth factors can influence PepT1 gene expression. In the rat, intestinal expression of PepT1 is induced postpartum (Shen et al., 2001). Miyamoto et al. (1996) showed that PepT1 mRNA was most abundant in 4-d-old rats and levels decreased to adult levels by d 28 of age. Although some studies have investigated the developmental regulation of peptide transport in mammals, little has been done in poultry. In a recent study conducted with broilers in our laboratory, PepT1 mRNA was observed to increase dramatically from embryonic d 18 to the day of hatch (H. Chen, Virginia Tech, personal communication). The objectives of the present study were to clone and characterize by electrophysiology the activity of turkey PepT1 and to determine if the gene is developmentally regulated in the turkey embryonic small intestine.

MATERIALS AND METHODS

Birds and Tissue Sampling

Turkey eggs2 were incubated with rocking at 41°C. Each day from d 23 of incubation (E23) to the day of hatch (d 0), turkey embryos were killed by decapitation, and sex was determined by gonadal inspection. The entire small intestine was removed, washed with ice-cold PBS, and then minced. Approximately 0.2 g of tissue was immediately immersed in 2 mL of ice-cold TriReagent3 for total RNA extraction. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Construction and Screening of a Turkey Intestinal cDNA Library

The turkey intestinal cDNA library was constructed using the ZAP Express cDNA synthesis system4 starting with poly(A)+ RNA extracted from turkey small intestinal tissue. Only cDNA >400 bp were used for library construction. Recombinant phage DNA were packaged with Gigapack III Gold packaging extract4 and introduced into XL1-Blue MRF′ Escherichia coli. The primary phage library was plated out immediately on a series of 150-mm NZY agar plates (50,000 plaques/plate) for screening.

Positive clones were identified by plaque hybridization using Magna nylon transfer membranes5 and [32P]-labeled chicken PepT1 cDNA as probe. Hybridization was carried out for 16 h at 42°C in a solution containing 50% formamide, 5× Denhardt’s solution, 6× SSPE (1× SSPE = 0.15 mM NaCl, 10 mM Na2HPO4, and 1 mM EDTA), 0.5% SDS, and 10 μg/mL yeast tRNA. Posthybridization washing was done with 5× SSPE containing 0.5% SDS at room temperature for 15 min and twice with 1× SSPE containing 0.5% SDS at 42°C for 15 min. Positive clones were subjected to 3 more rounds of screening using the same conditions. The positive plaques identified after screening of the cDNA library were used to generate the excised pBK-CMV phagemid containing the cDNA insert. DNA sequencing was performed on an ABI 377 automated DNA Sequencer using BigDye (version 2.0) Terminator Ready Reaction kit.6

A structural model of tPepT1 was constructed according to the Kyte and Doolittle (1982) hydrophathy analysis using a window of 21 amino acid residues via the Tmpred service program from the European Molecular Biology Network. Putative protein glycosylation and phosphorylation/dephosphorylation sites of tPepT1 were predicted on the basis of consensus amino acid sequences as substrate specificity determinants for protein kinases and phosphatases (Kennelly and Krebs, 1991). The consensus amino acid sequences for cAMP-dependent protein kinase A (PKA) site prediction were R-R/K-X-S/T, R-X2-S/T or R-X-S/T. The consensus amino acid sequences for protein kinase C (PKC) site prediction were (R/K1–3,X2–0) – S/T – (X2–0,R/K1–3) or (R/K1–3,X2–0) – S/T. The consensus amino acid sequence for N-linked glycosylation site prediction was N–X–T/S (X = any amino acid).

Northern Blot Analysis

Total extracted RNA was quantified by absorbance at 260 and 280 nm and stored at −80°C. For Northern blot analysis, 20 μg of total RNA were separated on 1% agarose gels in 2.2 M formaldehyde and stained with ethidium bromide. The size-fractionated RNA was then transferred to a Nylon membrane and cross-linked with ultraviolet light at 0.30 J/cm2. After prehybridization for 2 h in a solution containing 50% formamide, 5× Denhardt’s solution, 6× SSPE, 0.5% SDS, and 10 μg/mL yeast tRNA at 42°C, RNA blots were hybridized for 16 to 18 h under identical conditions with the addition of radiolabeled tPepT1 cDNA as probe.

Posthybridization washing conditions included twice in 5× SSPE with 0.5% SDS at room temperature for 15 min, twice in 1× SSPE with 0.5% SDS at 42°C for 15 min, and twice in 0.1× SSPE with 1% SDS at 65°C for 15 min. Dried filters were exposed to K-type imaging screens, which were then scanned using a FX laser scanner.7 To correct for differences in RNA loading, membranes were stripped of the tPepT1 probe and rehybridized to a [32P]-labeled 18S rRNA probe. The densities of hybridizing bands were quantified using volume tools of Quantity One Quantification Software.8

In Vitro Transcription of cRNA

cRNA was synthesized using the RNA transcription kit mMESSAGE mMACHINE9 according to the manufac-
turer’s protocol. For sense cRNA synthesis, plasmid containing the cDNA insert was linearized using XbaI and transcribed in vitro by T3 RNA polymerase in the presence of an RNA cap analog. For antisense cRNA synthesis, plasmid containing the cDNA insert was linearized using EcoRI and transcribed in vitro by T7 RNA polymerase in the presence of an RNA cap analog. The resultant cRNA was purified with phenol/chloroform, precipitated with ethanol, resuspended in nuclease-free water and stored frozen at −80°C. Concentration was determined by ultraviolet spectrophotometry, and the cRNA was verified by denaturing 1% agarose-formaldehyde gel electrophoresis and visualization using ethidium bromide staining.

**Electrophysiology**

Healthy *Xenopus* oocytes at stage V were dissected from ovarian tissue and manually defolliculated. Membrane potential was measured on approximately 10% of these defolliculated oocytes bathed in standard buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 6.0). Only batches of defolliculated oocytes with a resting membrane potential (Vₘ) more negative than −30 mV were used for injection the next day. With a microinjection system, cRNA was injected into the vegetal pole of each oocyte near the polar interface. Antisense cRNA or water was used as a control. The injected oocytes were incubated in culture solution at 17°C for 1 to 7 d. The 2-electrode voltage-clamp technique was used to characterize the induced peptide transport activity in oocytes injected with sense or antisense cRNA. All responses were monitored by a 2-electrode voltage-clamp amplifier⁹ and analyzed with a MacLab,¹⁰ which is an analog-digital converter and software system that uses an Apple MacIntosh computer for data acquisition. *Xenopus* oocytes were injected with varying amounts of tPepT1 cRNA (4, 8, 16, 32, and 64 ng). The current in the oocytes in response to 1 mM Gly-Sar (pH 6.0) perfusion was examined from d 1 to d 7 after injection. For functional characterization, 64 ng of sense cRNA or antisense cRNA were injected into oocytes and electrophysiologic measurements were carried out 3 to 6 d after injection. Only oocytes with a resting Vₘ more negative than −30 mV were used for recordings. An oocyte was perfused continuously with buffer with or without peptide at a rate of 1.2 mL/min using a gravity feed perfusion system.¹¹ All peptide substrate solutions were prepared by dissolving the peptides in standard buffer. All experiments were performed at room temperature (~21°C).

**Calculations and Statistics**

The kinetic parameters, including the Michaelis-Menten constant (Kₛ), the maximal velocity (Iₘ₉₉), and all other calculations (linear as well as nonlinear regression analysis) were performed using PRISM.¹² Data from Northern blots and data for ion and pH dependency of tPepT1 in cRNA-injected *Xenopus* oocytes were subjected to analysis of variance procedures for 2-way factorial in completely randomized designs. Other data were evaluated using 1-way ANOVA by using the general linear models procedure of SAS software,¹³ and orthogonal contrasts were used to detect linear and quadratic (curvilinear) effects.

**RESULTS**

**Sequence and Predicted Structure of the Turkey Intestinal PepT1 cDNA**

The cloned turkey intestinal PepT1 (tPepT1) cDNA was 2,921 bp with an open reading frame of 2,142 bp, a 72-bp 5’ UTR, and a 707-bp 3’ UTR. The translation start codon was consistent with the Kozak consensus sequence, GCCGCC/(A/G)CATGG for eukaryotic mRNA (Kozak, 1987). At the 3’ end, the cDNA included a polyadenylation signal (AATAAA) 19 nt preceding the polyA tail. The mRNA isolated from turkey small intestine hybridized to the tPepT1 cDNA probe at 2.9 kb, thus the isolated tPepT1 cDNA was likely full length. The encoded tPepT1 protein was predicted to have 714 amino acids with a molecular mass of 79.4 kDa and a pI of 5.9. The amino acid sequence of tPepT1 was 94.3% identical to chicken PepT1 and 63.2, 64.9, 64.6, 63.4, and 62.8% identical to mouse, rat, sheep, rabbit, and human PepT1, respectively (Figure 1).

Hydropathicity analysis indicated that tPepT1 was structurally similar to other cloned PepT1. Turkey PepT1 was predicted to have 12 transmembrane domains with the amino and carboxyl termini on the cytoplasmic side of the membrane and a large extracellular loop of 203 amino acids between transmembrane domains 9 and 10 (Figure 2). The alignment of turkey PepT1 with chicken PepT1 showed 94.8% identity in the transmembrane domains and 88.2% identity in the large extracellular loop. The large extracellular loop contained 7 potential N-linked glycosylation sites at positions Asn 414 (NVT), Asn 423 (NVT), Asn 453 (NRS), Asn 485 (NFT), Asn 508 (NIT), Asn 526 (NYT), and Asn 546 (NCT). An additional putative N-linked glycosylation site was observed at position Asn 56 (NLS) in the extracellular loop between transmembrane domains 1 and 2. The tPepT1 protein also contained one potential site for PKC-dependent phosphorylation in the intracellular loop between transmembrane domains 6 and 7 (Ser 272, S-E-K) and one potential site for PKA-dependent phosphorylation in the intracellular loop between transmembrane domains 8 and 9 (Thr 365, R-K-I-T).

**Developmental Regulation of PepT1 mRNA in Turkey Embryonic Intestinal Tissue**

Northern blot analysis indicated that there was a quadratic increase (P < 0.001) in PepT1 mRNA abundance

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¹°Model TEV-200, Dagan, Minneapolis, MN.
¹⁰AD Instruments, Milford, MA.
¹¹Model BPS4, Ala Scientific Instruments, Westburg, NY.
¹²GraphPad, San Diego, CA.
¹³Version 8.0, SAS Institute Inc., Cary, NC.
FIGURE 1. Alignment of the predicted amino acid sequence of turkey PepT1 with chicken, human, mouse, rat, rabbit, and sheep PepT1 using the Clustal W (1.81) multiple sequence alignment program. Dots (.) indicate gaps introduced to maximize alignment. Amino acids identical to the chicken sequence are indicated by dashes (–).

with age in turkey embryonic small intestinal tissue from E23 to d 0 (Figures 3 and 4). PepT1 mRNA was barely detectable in turkey embryonic small intestine at E23 but increased 3.2-fold from E23 to d 0. No differences were observed in intestinal tPepT1 mRNA abundance between male and female birds.
**Functional Characteristics of tPepT1**

Peptide transport activity in tPepT1 cRNA injected *Xenopus* oocytes was assayed using the 2-electrode voltage-clamp technique by measuring inward currents when the oocytes were perfused with 1 mM Gly-Sar in standard buffer at pH 6.0. In preliminary experiments, the optimal amount of cRNA and optimal time for tPepT1 expression were determined to be 64 ng of injected cRNA assayed on d 3 to 6 after injection (data not shown). As observed with other PepT1, peptide transport by tPepT1 was pH dependent and Na⁺ and K⁺ independent (Figure 5). The optimal pH for Gly-Sar transport was pH 6.5. In all experiments, control oocytes injected with antisense cRNA or water did not display any current when perfused with the substrates (data not shown).

Transport kinetics of tPepT1 were investigated by perfusing the cRNA-injected oocytes with 0.05 to 10 mM Gly-Sar in standard buffer at pH 6.5. Gly-Sar induced currents were saturable and fit Michaelis-Menten kinetics ($r^2 = 0.997$), resulting in a substrate affinity ($K_t$) of 0.69 ± 0.14 mM and a maximal transport rate ($I_{\text{max}}$) of 188.6 ± 8.8 nA (Figure 6A). The dipeptide Met-Met induced currents that were also saturable over a Met-Met concentration range of 0.05 to 10 mM. The $K_t$ and $I_{\text{max}}$ for Met-Met uptake were determined to be 0.23 ± 0.04 mM and 194.4 ± 7.2 nA. Different from the dipeptides, tPepT1 transport of the tetrapeptide, Met-Gly-Met-Met, showed increased substrate uptake at low substrate concentrations from 0.05 to 0.5 mM followed by inhibition at higher concentrations from 1 to 10 mM. The maximum current was 33.8 nA, which was observed at 0.5 mM (Figure 6B).

**DISCUSSION**

Turkey PepT1 is biochemically and structurally similar to other cloned mammalian and avian PepT1. Comparison of the amino acid sequences of turkey and chicken PepT1 revealed a high degree of similarity. Chicken and turkey PepT1 have 714 predicted amino acid residues, with 94.8% sequence identity in the membrane-spanning regions and 88.2% sequence identity in the large extracellular loop between transmembrane domains 9 and 10. A lower amino acid similarity in the large extracellular loop between other species has also been reported (Miyamoto et al., 1996; Chen et al., 2002). The functional significance of this variability in the large extracellular loop has not been determined. One possibility is that this loop plays no functional role and thus the region is free to diverge among species. The putative substrate-binding site in
mammalian PepT1 has been mapped to the region spanning transmembrane domains 7, 8, and 9 and the intervening loops (Fei et al., 1998). Histidyl residues His-57 and His-121 have been shown to be essential for substrate recognition and transport activity of PepT1 (Terada et al., 1996; Fei et al., 1997). Although tPepT1 and cPepT1 show only about 60% identity to mammalian PepT1, these histidyl residues are well conserved among species. The presence of PKC and PKA phosphorylation sites, which play an important role in regulation of the maximum transport rate of PepT1 (Brandsch et al., 1994), are also conserved within cPepT1 and tPepT1 proteins. One key difference between tPepT1 and other PepT1 is the calculated isoelectric point of the protein. Turkey PepT1 has an isoelectric point of 5.9, whereas the isoelectric points of chicken (7.5), sheep (6.6), rabbit (7.5), rat (7.4), mouse (8.1), and human (8.6) are higher. The significance of this difference in isoelectric point remains to be determined.

The Xenopus oocyte is a well-defined experimental model used for in vitro expression and analysis of proteins. The functional characteristics of PepT2 determined in Xenopus oocytes were found to be very similar to, if not identical with, the peptide transport activity found in the apical membrane vesicles of rabbit kidney tubular cells (Boll et al., 1996). Chen et al. (2002) further showed that the transport kinetics determined in the oocyte expression system were comparable with that determined in a mammalian cell line, Chinese hamster ovary cells. Expression of tPepT1 in Xenopus oocytes revealed that Gly-Sar transport was pH dependent with an optimal pH of 6.5, which is comparable to the optimal pH of 6.0 to 6.5 for cPepT1. Substitution of Na+ and K+ did not have any effect on peptide transport activity of tPepT1 at any pH tested, which demonstrated that tPepT1 operates independently from these ions but is coupled to H+. These results are consistent with that of previous studies, which indicated that peptide transport activity of PepT1 was driven by an inwardly directed H+ gradient and was independent of Na+, K+, Cl− or Ca2+ (Pan et al., 2001; Chen et al., 2002).

Early efforts to characterize PepT1 were focused on uptake of peptidomimetic drugs. Recent studies in our lab evaluated the transport characteristics of ovine and chicken PepT1 using peptides containing essential amino acids (mainly Met and Lys; Pan et al., 2001; Chen et al., 2002). The studies on cPepT1 indicated that a wide range of di- or tripeptides, regardless of their molecular weight, electrical charge, or hydrophobicity, could be transported, demonstrating the importance of peptide transport in chickens. In the present study, transport of the hydrolysis-resistant dipeptide, Gly-Sar, in oocytes expressing tPepT1 followed Michaelis-Menten-type kinetics with a $K_t$ of 0.69 ± 0.14 mM and $I_{\text{max}}$ of 189 ± 8.8 nA, which was comparable to that of cPepT1 ($K_t$ 0.47 ± 0.03 nA; $I_{\text{max}}$ 206 ± 3.1 mM). The kinetic study also showed that tPepT1 transport of the dipeptide Met-Met had lower affinity ($K_t$ = 0.23 ± 0.04 vs. 0.08 ± 0.01 mM) and greater transport velocity ($I_{\text{max}}$ = 194 ± 7.2 vs. 113 ± 3.2 nA) when compared with cPepT1. According to the NRC (1994), the methionine requirement of turkeys is 0.25 to 0.55%, which is generally greater than that of chickens (0.19 to 0.3%). It is not clear how PepT1 transport kinetics for Met-containing peptides may be related to the amino acid requirements of an animal.
Dipeptides, as well as tripeptides, are excellent substrates for the intestinal peptide transport system (Pan et al., 2001; Chen et al., 2002). Transport of tetrapeptides occurs to only a small extent, if at all (Ferraris, 1994). In the present study, transport of the tetrapeptide Met-Gly-Met-Met was observed in oocytes expressing tPepT1. However, the transport activity of Met-Gly-Met-Met did not fit with Michaelis-Menten type kinetics. It showed increased transport at low substrate concentrations from 0.01 to 0.5 mM with a maximum transport velocity of 33.8 nA at 0.5 mM, followed by substrate inhibition at higher concentrations from 1 to 10 mM. The substrate phenomenon of inhibition has also been reported for the transfer of nitrate across the membrane of Xenopus oocytes expressing a high affinity fungal nitrate carrier (Zhou et al., 2000). Whether the substrate inhibition reaction is related to a conformational change of tPepT1 at high extracellular tetrapeptide concentrations, which affect the transport affinity and velocity, or because of a second binding site on tPepT1 remains to be studied. We had not previously observed this phenomenon during characterization of sheep (Pan et al., 2001) or chicken (Chen et al., 2002) PepT1.

The expressions of nutrient transport genes are developmentally regulated. Intestinal glucose and amino acid transporters are thought to be present prenatally in humans, guinea pigs, sheep, rabbits, and rats (Pacha, 2000). In rabbits, active uptake of glucose and galactose is increased 3-fold during the final 7 d of gestation with the highest transport rate occurring right after birth but decreases gradually thereafter (Ferraris, 2001). In rats, expression levels of PepT1 increase dramatically at birth, are maximal 3 to 5 d after birth in the duodenum, jejunum, and ileum, and then declined rapidly (Shen et al., 2001). Although considerable progress has been made in understanding the mechanisms for developmental regulation of nutrient transport in mammals, little has been done in poultry. A recent study with broilers in our lab showed that cPepT1 mRNA abundance increased dramatically by the time of hatch when compared with 3 d before hatch (H. Chen, personal communication). In the present study, PepT1 mRNA in turkey small intestinal tissue was detectable 5 d before hatch and increased 3.2-fold from E23 to d 0. A recent study of expression and cellular distribution of PepT1 during development in rat small intestinal epithelium indicated that distribution of PepT1 is exclusively in the apical brush border of enterocytes from prenatal and mature animals. However, immediately after birth immunolocalization of PepT1 extends to the subapical cytoplasm and to the basolateral membrane of enterocytes (Hussain et al., 2002). In another study conducted with broiler chicks, the specific activities of digestive enzymes, such as maltase, sucrase, and aminopeptidase N, were reported to be maximal at hatch (Iji et al., 2001). At birth or hatch, the digestive tract of an animal faces a serious challenge because of the dramatic change of the luminal environment. The early developmental regulation of PepT1 gene expression and digestive enzymes may have a significant physiologic role for adaptation of an animal to the new environment.

In summary, we have cloned tPepT1, which has high homology with chicken PepT1 and encodes for a 714-amino acid protein with 12 transmembrane domains and a pI of 5.9. Functional characterization of tPepT1 in Xenopus oocytes demonstrated that the transport process was pH dependent but Na+ and K+ independent. The transport of the dipeptides, Gly-Sar and Met-Met, into oocytes was saturable with a $K_t$ of 0.69 and 0.23 mM, respectively. Transport of the tetrapeptide Met-Gly-Met-Met by tPepT1 did not fit Michaelis-Menten kinetics but showed a substrate inhibition pattern at high (>0.5 mM) substrate concentration. PepT1 mRNA was expressed in turkey embryos 4 to 5 d before hatch and increased 3.2-fold at day of hatch. These studies reveal the potential importance of intestinal peptide absorption for improved growth and performance of neonatal turkey poults.

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