Expression of proglucagon and proglucagon-derived peptide hormone receptor genes in the chicken ∗

Mark P. Richards *, John P. McMurtry

Animal Biosciences and Biotechnology Laboratory, United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Building 200, Room 206, BARC-East, Beltsville, MD 20705-2350, USA

Received 9 October 2007; revised 13 December 2007; accepted 16 January 2008

Available online 26 January 2008

Abstract

To better understand how the proglucagon system functions in birds, we utilized a molecular cloning strategy to sequence and characterize the chicken proglucagon gene that encodes glucagon, glucagon-like peptide (GLP)-1 and GLP-2. This gene has seven exons and six introns with evidence for an additional (alternate) first exon and two promoter regions. We identified two distinct classes of proglucagon mRNA transcripts (PGA and PGB) produced by alternative splicing at their 3'-ends. These were co-expressed in all tissues examined with pancreas and proventriculus showing the highest levels of each. Although both mRNA classes contained coding sequence for glucagon and GLP-1, class A mRNA lacked that portion of the coding region (CDS) containing GLP-2; whereas, class B mRNA had a larger CDS that included GLP-2. Both classes of mRNA transcripts exhibited two variants, each with a different 5'-end arising from alternate promoter and alternate first exon usage. Fasting and refeeding had no effect on proglucagon mRNA expression despite significant changes in plasma glucagon levels. To investigate potential differences in proglucagon precursor processing among tissues, mRNA expression for two prohormone convertase (PC) genes was analyzed. PC2 mRNA was predominantly expressed in pancreas and proventriculus, whereas PC1/3 mRNA was more highly expressed in duodenum and brain. We also determined mRNA expression of the specific receptor genes for glucagon, GLP-1 and GLP-2 to help define major sites of hormone action. Glucagon receptor mRNA was most highly expressed in liver and abdominal fat, whereas GLP-1 and GLP-2 receptor genes were highly expressed in the gastrointestinal tract, brain, pancreas and abdominal fat. These results offer new insights into structure and function of the chicken proglucagon gene, processing of the precursor proteins produced from it and potential activity sites for proglucagon-derived peptide hormones mediated by their cognate receptors.

Published by Elsevier Inc.

Keywords: Chicken; Gene expression; Glucagon; Glucagon-like peptide-1; Glucagon-like peptide-2; Proglucagon; Prohormone convertase; Receptor

1. Introduction

Glucagon and two glucagon-like peptides (GLP-1 and GLP-2) comprise the predominant members of a group of peptides that are derived from a common precursor protein via proteolytic processing. In mammals, the proglucagon precursor is encoded by a single gene which expresses a single mRNA (Kieffer and Habener, 1999). Analysis of mammalian proglucagon gene sequence and structure revealed that glucagon, GLP-1 and GLP-2 are each encoded by a separate exon (Heinrich et al., 1984; White and Saunders, 1986). This genomic organization suggests that the three peptide hormones evolved from ancestral glucagon-like sequence as a result of exon duplication (Irwin, 2001). Non-mammalian vertebrates have been reported to exhibit more complex mechanisms for proglucagon gene regulation involving tissue-specific mRNA splicing and differing coding potential of duplicate genes.
that give rise to multiple proglucagon mRNA transcripts and precursor proteins (Irwin, 2001).

Post-translational proteolytic processing of the proglucagon precursor protein is mediated by prohormone convertase (PC) enzymes and this mechanism is responsible for the tissue-specific production of a variety of proglucagon-derived peptides in mammals (Kieffer and Habener, 1999; Sinclair and Drucker, 2005). The expression of specific PC genes (e.g., PC1/3, PC2) and the resulting proteolytic activity of these enzymes in different tissues determine how the proglucagon precursor is processed. For example, the production of glucagon from proglucagon in pancreatic islet α-cells requires the action of PC2, whereas PC1/3 is essential for the nutrient-dependent production of GLP-1 and GLP-2 by enteroendocrine cells in the intestine and selected neurons within the brain (Dey et al., 2005; Dhanvantari et al., 1996; Kieffer and Habener, 1999; Rouille et al., 1995; Sinclair and Drucker, 2005).

Glucagon, a 29 amino acid peptide hormone produced by the α-cells of the pancreatic islets in response to reduced levels of blood glucose, plays an important counter-regulatory role in maintaining glycemic control and energy balance through its effects on glucose, lipid and amino acid metabolism (Jiang and Zhang, 2003). Glucagon-like peptide-1 is an incretin hormone that regulates blood glucose and promotes glucose homeostasis by stimulating pancreatic insulin synthesis and secretion, and islet cell proliferation and neogenesis while inhibiting the secretion of glucagon (Drucker, 2001). In addition, GLP-1 controls nutrient absorption through its inhibitory effects on gastric emptying and food intake regulation (Kieffer and Habener, 1999). Potential functions for GLP-2 have only been reported for mammalian species in which it is thought to play a role in intestinal growth and nutrient absorption by maintaining the integrity of epithelial cells (Burrin et al., 2003; Drucker, 2001). Secreted by enteroendocrine L-cells in response to the presence of intestinal nutrients, GLP-2 has been found to promote crypt cell proliferation and suppress apoptosis in mucosal epithelial cells (Burrin et al., 2003; Estall and Drucker, 2006).

The physiological effects of glucagon, GLP-1 and GLP-2 on regulating metabolism are mediated by a specific set of related G-protein-coupled receptors (GPCRs) belonging to the glucagon–secretin receptor class II family of GPCRs (Mayo et al., 2003). Genes encoding each of these receptors have been identified, cloned and sequenced for a number of species and their expression in different tissues has been characterized (Irwin, 2005; Irwin and Wong, 2005; Kieffer and Habener, 1999; Mayo et al., 2003). Moreover, the unique functions and specific binding characteristics of each of these receptors have been extensively studied in mammals using native hormone ligands as well as specific agonists and antagonists (Drucker, 2001; Kieffer and Habener, 1999; Sinclair and Drucker, 2005). It has been postulated that receptor–ligand specificity evolved separately following duplication of the glucagon-like peptides and their corresponding receptors (Irwin and Wong, 2005; Irwin, 2005). This conclusion is supported by the observation that, in fish, GLP-1 exhibits biological activity similar to glucagon due to its binding by a receptor that possesses signaling characteristics resembling the glucagon receptor rather than the GLP-1 receptor (Irwin and Wong, 2005; Plisetskaya and Mommesen, 1996).

Compared to mammalian species, there has been relatively little investigation of the proglucagon-derived peptides and their cognate receptors in birds. Previous studies in chickens, which possess a single proglucagon gene, found two different cDNA clones isolated from pancreatic and intestinal cDNA libraries (Hasegawa et al., 1990; Irwin and Wong, 1995). The pancreatic cDNA encoded glucagon and GLP-1, but not GLP-2; whereas, the intestinal cDNA contained coding sequence for all three peptides. These observations suggested that regulation of chicken proglucagon gene expression involves tissue-specific mRNA splicing in addition to the downstream post-translational proteolytic processing that determines which peptides are produced from the precursor and ultimately secreted (Yue and Irwin, 2005). Although glucagon and GLP-1 appear to function similarly in both birds and mammals (Hazelwood, 1984; Honda et al., 2007; Shousha et al., 2007; Tachibana et al., 2004, 2006, 2007), as of yet, there have been no reports of a physiological role for GLP-2 in birds. Moreover, there are only a limited number of reports in chickens on the expression of proglucagon mRNA and GLP-1 immunoreactive staining in brain and ocular tissues (Feldkaemper et al., 2004; Tachibana et al., 2005), glucagon receptor mRNA in the retina (Buck et al., 2004) or indirect evidence for functional GLP-1 receptors using agonists and antagonists of GLP-1 such as N-terminal GLP-1 peptide fragments or exendin 5–39 (Furuse et al., 1998; Tachibana et al., 2001). Despite the fact that the draft chicken genome contains requisite sequence for proglucagon and proglucagon-derived peptide receptor genes (Irwin and Wong, 2005; Yue and Irwin, 2005), there has been no systematic attempt to characterize the expression of these genes in different chicken tissues. Therefore, the objectives of this study were to: (1) further characterize the chicken proglucagon gene structure and sequence; (2) to determine possible mechanisms contributing to the expression of multiple proglucagon mRNA transcripts and precursor protein processing in different tissues; and (3) to study the expression of proglucagon-related peptide receptor genes in different tissues.

2. Materials and methods

2.1. Animals, tissues and nucleic acid isolation

Male broiler chicks (Gallus gallus) were reared from day of hatch to 3 wks of age in heated battery/brooder units. All birds received a standard starter poultry ration and water ad libitum. At 3 wks, tissue samples were collected, snap frozen in liquid nitrogen and stored at −80 °C prior to RNA isolation. In a separate experiment, 30 broiler chickens (3-wk-old males) were divided into five groups of six birds each and subjected to the following treatments: (1) fed ad libitum, (control), (2) fasted for 24 h
(S24), (3) fasted for 48 h (S48), (4) fasted for 24 h (S24-RF24), (5) fasted for 48 h and refed for 24 h (S48-RF24). All birds had free access to water throughout the experimental period. Upon termination of the experiment, samples of pancreas, proventriculus, duodenum and brain were collected and snap frozen in liquid nitrogen prior to RNA isolation. Total RNA was isolated from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA). Blood was collected in the presence of EDTA via cardiac puncture. Following centrifugation (1800g for 30 min at 4 °C), plasma was obtained and stored at −80 °C prior to analysis. Chicken genomic DNA was either purchased (Novagen, Madison, WI) or was prepared from whole blood using the Easy DNA extraction kit (Invitrogen). All protocols involving the use of animals received prior approval from the USDA, ARS, Beltsville Animal Care and Use Committee.

2.2. Nucleotide sequencing

Pancreatic and duodenal total RNA and reverse transcription-polymerase chain reaction (RT-PCR) were used to derive the two proglucagon cDNA sequences (PGA and PGB) including the complete coding region (CDS) as well as the 5′- and 3′-untranslated regions (UTRs). Genomic DNA templates and a primer-directed (primer walking) PCR strategy was used to derive gene sequence. Primer sets based on cDNA sequence reported for chicken proglucagon from pancreas (GenBank Accession No. Y07539) and intestine (GenBank Accession No. S78477) or from previously sequenced PCR products were used to generate a series of overlapping PCR products. PCR products were evaluated by agarose gel (1.5%) electrophoresis and bands of the appropriate size were excised from the gel and purified using a GenElute gel extraction kit (Sigma Chemical Co., St. Louis, MO) or purified directly from the PCR sample by a GenElute PCR clean-up kit (Sigma). Purified PCR products were subjected to bi-directional automated fluorescent DNA sequencing utilizing a Beckman Coulter CEQ 8000XL Genetic Analysis System using the dye terminator cycle sequencing kit (Beckman Coulter, Inc., Fullerton, CA).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription (RT) reactions (20 μl) consisted of: 1.0 μg total RNA, 50 units Superscript II reverse transcriptase (Invitrogen), 40 units of an RNAse inhibitor (Invitrogen), 0.5 mM dNTPs and 100 ng random hexamer primers. Polymerase chain reaction (PCR) was performed in 25 μl reactions containing: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.0 units of Platinum Taq DNA polymerase (hot start), 0.2 mM dNTPs, 2.0 mM Mg2+ (Invitrogen), 10 pmol of each gene specific primer (Table 1) and 1.0 μl of the RT reaction. When relative quantitative RT-PCR was performed, 5 pmol each of an appropriate mixture of primers:competimers specific for 18S rRNA (QuantumRNA Universal 18S Standards kit, Ambion, Inc., Austin, TX) was added along with the gene specific primer pair. Thermal cycling parameters were: 1 cycle 94 °C for 2 min, followed by 30–40 cycles, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min. Under these conditions, reactions were conducted within the linear phase of amplification for each product. All new PCR products were initially verified by direct sequencing to ensure fidelity of the amplification.

2.4. Rapid amplification of cDNA ends (RACE)

Total RNA from pancreas and duodenum (1.0 μg) was used to prepare 3′- and 5′-RACE ready cDNA using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA). PCR was performed using Platinum Taq DNA polymerase with 3.5 mM Mg2+ (Invitrogen) and touchdown PCR. The following gene specific 3′- and 5′-RACE primers initially derived from chicken proglucagon sequence

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oligonucleotide primers for the analysis of chicken proglucagon and related gene transcripts by RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene transcript</td>
<td>Primer sequence</td>
</tr>
<tr>
<td>1. Proglucagon gene</td>
<td></td>
</tr>
</tbody>
</table>
| PGAe1 | Forward: 5′-GGATCCTCAAGGGTTTCCAT-3′  
Reverse: 5′-CAAAGGAAACGTGAAGTACA-3′ | 658 |
| PGAe1 | Forward: 5′-ACCATTGGTGAAACCCAAT-3′  
Reverse: 5′-CAAAGGAAACGTGAAGTACA-3′ | 625 |
| PGBe1 | Forward: 5′-GGATCCTCAAGGGTTTCCAT-3′  
Reverse: 5′-TGTTGGCTGGACACTTCAGA-3′ | 704 |
| PGBe1 | Forward: 5′-GCCATTGTCGAACCCAAAT-3′  
Reverse: 5′-TGATTTCTCCTCCTAAGGTTC-3′ | 671 |
| PGT | Forward: 5′-ACCAAGGCACATTCCCAAGT-3′  
Reverse: 5′-TTCTTGGGACTGCTGACCT-3′ | 496 |
| PGB | Forward: 5′-CACGACACCAATGGGAAAACGTGAAGTACA-3′  
Reverse: 5′-TGATTTCTCCTCCTAAGGTTC-3′ | 451 |
| 2. Proglucagon-derived peptide receptor genes | | |
| Glucagon receptor | Forward: 5′-GTCCCAGCCACACTTCTC-3′  
Reverse: 5′-GCAAGGACCACATGCTCG-3′ | 269 |
| GLP-1 receptor | Forward: 5′-GTGGTGCGAGAAGGATAGT-3′  
Reverse: 5′-GCGGTCCTGCCGTCTGCT-3′ | 441 |
| GLP-2 receptor | Forward: 5′-GCGGTCCTGCCGTCTGCT-3′  
Reverse: 5′-CAGCAATGGAATCAGCACA-3′ | 196 |
| 3. Prohormone convertase (PC) genes | | |
| PC 1/3 | Forward: 5′-ATGCGTGTTTGGGAATC-3′  
Reverse: 5′-CAAGAACGACATTCAC-3′ | 408 |
| PC 2 | Forward: 5′-CTCACCTTCAAAGGAACCA-3′  
Reverse: 5′-CCACCTTGGAGACATTCAC-3′ | 401 |
reported for cDNAs from pancreas (Genbank Accession No. Y07539) or intestine (Genbank Accession No. S78477) were used with total RNA extracted from pancreas and duodenum:

3' RACE (pancreas): 5'-GACCTGTGTAATGAGGCGA-3'  
3' RACE (duodenum): 5'-AACCTCTGATTCGACAA-3'  
5' RACE (pancreas): 5'-TTGATTGGGAATTTGCTATT-3'  
5' RACE (duodenum): 5'-TGTATTCTCCAAAAGGTCTC-3'

Different RACE primers were used for pancreas and duodumen RNA samples because previous reports had suggested that distinct mRNA transcripts were produced by each tissue (Hasegawa et al., 1990; Irwin and Wong, 1995). The use of primers anchored in different regions of the proglucagon CDS ensured that the products to be sequenced from the individual RACE reactions reflected the full complement of mRNA transcripts present in each tissue.

2.5. Cloning

Cloning of selected PCR and RACE products containing both the 5' and 3' UTRs was performed using a TOPO TA Cloning kit with the pCR 2.1 TOPO vector (Invitrogen). The inserted DNA was sequenced using M13 forward and reverse primers.

2.6. Proglucagon gene promoter sequence analysis

Using nucleotide sequence generated for the chicken proglucagon gene (GenBank Accession No. DQ185929), BLAST-N searches of the chicken genome (http://www.ensembl.org/Gallus_gallus/index.html) were performed to establish intron/exon boundaries and genomic structure. The chromosomal location of the proglucagon gene was established as was sequence in the 5' flanking region upstream of the first exon (promoter). Transcription start sites were predicted using a neural network promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html). Flanking (promoter) sequence included 1200 bp of genomic sequence upstream from the inferred transcription (mRNA) initiation site which was set at +1. This region was scanned for TATA boxes and putative transcription factor binding sites with the aid of the MatInspector program (http://www.genomatix.de). Only the core sequence containing the most highly conserved positions for each transcription factor binding site is presented.

2.7. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF)

Analysis of PCR products was accomplished using CE-LIF as described previously (Richards and Poch, 2002). Aliquots (2 μl) of RT-PCR samples were first diluted 1:100 with deionized water. A P/ACE MDQ (Beckman Coulter, Inc.) equipped with an argon ion LIF detector was used. Capillaries were 75 cm I.D. x 32 cm μSil-DNA (Agilent Technologies, Palo Alto, CA). EnhanceCE™ dye (Beckman Coulter, Inc.) was added to the DNA separation buffer (Sigma, St. Louis, MO) to a final concentration of 0.5 μg/ml. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity at 8.1 kV for 4 min. P/ACE MDQ software (Beckman Coulter, Inc.) was used to calculate peak areas for the PCR products separated by CE.

2.8. Relative quantitation of mRNA levels

The level of gene expression in different tissues was determined as the ratio of integrated peak area for each PCR product relative to that of the co-amplified 18S rRNA internal standard (Ambion, Inc.). Values are presented as the mean ± SEM of six individual determinations.

2.9. Plasma glucose and hormone analyses

Plasma glucose was determined using a commercial kit and glucose analyzer (YSI Inc., Yellow Springs, OH). Plasma insulin was determined using a specific radioimmunoassay as previously described (McMurtry et al., 1983). Highly purified chicken glucagon was purchased from Litron Laboratories, Ltd. (Rochester, NY). Chicken GLP-1 (7-36) amide was purchased from BACHEM Bioscience Inc. (King of Prussia, PA). Plasma glucagon and GLP-1 concentrations were determined using kits (No. GL-32K, mammalian glucagon radioimmunoassay; No. EGPLP-35K, mammalian GLP-1 ELISA) obtained from Linco Research, Inc. (St. Charles, MO). As recommended by the vendor for GLP-1 analysis, blood was collected in the presence of a DPP-IV inhibitor (No. DPP4, Linco Research Inc.). For glucagon analysis, an aliquot of plasma was stored in the presence of 1000 KIU of aprotonin (Sigma).

The mammalian glucagon radioimmunoassay was tested using chicken glucagon as shown in Fig. 1 and Table 2. The dose-response curve of native chicken glucagon was nearly identical to human glucagon (Fig. 1). Furthermore, dose-response curves of chicken and turkey plasma were parallel to both glucagon standards. To determine the recovery of added chicken glucagon in chicken plasma, the following experiment was conducted. Plasma was obtained from 3-wk-old male chickens and pooled. Chicken glucagon was added to each plasma pool (n = 5) in the amounts listed in Table 2. The recovery of added chicken glucagon from chicken plasma averaged 97%. Similar experiments were conducted to validate and determine the suitability of the mammalian GLP-1 ELISA assay for measuring chicken GLP-1. The results are shown in Fig. 2 and Table 3. The dose-response curves of synthetic chicken GLP-1 and chicken plasma were parallel to human GLP-1 (Fig. 2). As with the glucagon validation, a similar recovery experiment was carried out using chicken GLP-1. The recovery of added chicken GLP-1 from chicken plasma averaged 82% (Table 3).

Table 2

<table>
<thead>
<tr>
<th>Added chicken glucagon (pg/ml)</th>
<th>Recovered (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>130 ± 4</td>
</tr>
<tr>
<td>25</td>
<td>152 ± 3</td>
</tr>
<tr>
<td>50</td>
<td>176 ± 7</td>
</tr>
<tr>
<td>100</td>
<td>223 ± 7</td>
</tr>
<tr>
<td>200</td>
<td>316 ± 10</td>
</tr>
</tbody>
</table>

Chicken glucagon dissolved in saline was added to chicken plasma pools and assayed for glucagon content as described in Section 2.9.

Values represent mean ± SEM of five determinations.
strategy involving primer-directed PCR. Direct sequencing of overlapping PCR-generated DNA fragments identified and confirmed a total of 12,876 contiguous nucleotides that included the complete CDS and the UTRs of the corresponding mRNA transcripts produced by this gene, as well as a small amount of 5’- and 3’-flanking sequence (GenBank Accession No. DQ185929). The chicken proglucagon gene has 7 exons (8 including an additional alternate first exon, AE1), 5 of which contain most of the coding sequence and 6 introns that together span 11.93 kbp on chromosome 7 (Fig. 3). This arrangement differs from the human gene which has 6 exons (4 of which contain the majority of the coding region) and 5 introns with a transcriptional unit that spans 9.4 kbp (White and Saunders, 1986). Table 4 summarizes exon and intron sizes for the chicken as compared to the human proglucagon gene as well as general characteristics for the mature mRNA transcripts. In chickens, glucagon, GLP-1 and GLP-2 are each encoded by separate exons (exons 3, 5 and 6, respectively) as they are in other vertebrate proglucagon genes.

There are two important features distinguishing the chicken proglucagon gene from mammalian genes. One concerns the fourth exon consisting of 54 bp which is not present in other vertebrate proglucagon genes with the exception of the Gila monster (Chen and Drucker, 1997; Yue and Irwin, 2005). This extra exon contains the majority of the coding sequence for intervening peptide-1 (IP-1), a peptide segment that divides glucagon from GLP-1 in the proglucagon protein precursor. Chicken IP-1, at 24 amino acids, is four times larger than its human counterpart (Hasegawa et al., 1990; Irwin and Wong, 1995; White and Saunders, 1986). Another difference concerns exon 5 (E5, Fig. 3). This exon contains an internal splice donor site at position 8852 (GenBank Accession No. DQ185929) that results in two different exons (E5A, the entire non-spliced exon containing a TAAA stop codon and flanking 3’-UTR that is a terminal exon or E5B, the alternatively spliced and smaller exon that is joined with E6 and E7) leading to the generation of two classes of mRNA transcripts (PGA and PGB, respectively) each with different 3’-ends derived from either E5A or E7 sequence (Fig. 3).

Fig. 4 depicts a computer-assisted search of 1200 bp upstream (5’) of the presumptive transcription initiation site (start of exon 1 set at +1) of the chicken proglucagon gene indicating the presence of a proximal (immediate) and a distal (alternate) promoter. In addition, evidence was found for an alternate first exon (AE1). No other vertebrate species has been reported to utilize an alternate promoter and alternate first exon, making the chicken unique in this respect. Yue and Irwin (2005) reported a transcription initiation site (+1a*) that results in a 34 bp AE1 for the chicken gene. However, our results suggest a different transcription initiation site (+1a²?) further upstream from the
site predicted by Yue and Irwin (2005) that produces a longer (96 bp) AE1.

Both promoter regions in the chicken proglucagon gene contained a number of identifiable cis-acting control elements that serve as putative binding sites for transcription factors. Putative TATA boxes were predicted at 24 bp upstream from the start of exon 1 (+1) and 27 bp upstream of the start of AE1 (+1a?). Putative transcription factor binding sites were identified in this region for the following transactivators: Cdx-2; Pdx-1; Pax-1,-2, -3 and -6; Pbx-1; CREB-1; C/EBP; AP-1; HNF-3; NeuroD-1; Isl-1; Meis-1 among others. Previous work described the interaction of a variety of transcription factors with the first 300 bp of the rat proglucagon gene promoter region which was sufficient for expression when reporter gene constructs were transfected into pancreatic islet cell lines (Drucker, 2003; Kieffer and Habener, 1999). This region was reported to contain a TATA box and five discrete transcriptional control elements of 20–40 bp (Kieffer and Habener, 1999). Interaction among specific transcription factors is considered to be crucial in regulating proglucagon gene expression not only during tissue development, but also among the different tissues in which the gene is expressed (Liu et al., 2006). The types of transcription factors regulating mammalian proglucagon gene expression include helix–loop–helix proteins, members of the hepatocyte nuclear family and homeodo-

main proteins. A cAMP-binding protein site (CREB-1) has also been reported to control the response of this gene to changing cAMP levels (Kieffer and Habener, 1999). Sites for all of these factors were detected in the chicken proglucagon immediate and alternate promoter regions (Fig. 4) indicating that the chicken gene potentially could be regulated in a similar way. For example, it has recently been reported that Pbx-1 functions as a co-activator for Cdx-2 in regulating expression of the proglucagon gene in pancreatic islet A cells and intestinal L cells (Hill et al., 1999). Fischer et al. (2006) reported the co-expression of Pax6 in four types of glucagon-expressing retinal neurons in the chicken. Yue and Irwin (2005) tested portions of the 5′-flanking sequence of the chicken proglucagon gene for transcription of transiently transfected reporter gene constructs in different rodent pancreatic and intestinal glucagon-expressing cell lines. They found that the immediate promoter region produced significantly more activity than did the distal (alternate) promoter region. However, a construct containing both regions combined was more active in pancreatic islet cells than one containing only the immediate promoter region of the chicken gene (Yue and Irwin, 2005).
3.3. Molecular cloning of four distinct proglucagon mRNA transcripts

Two classes of proglucagon mRNA transcripts, designated PGA and PGB, were cloned (Fig. 3) and the corresponding cDNAs sequenced (GenBank Accession Nos. DQ185929, DQ185930, DQ185931). Class A mRNAs (PGA) that code for glucagon and GLP-1 resemble lower vertebrate transcripts such as those found in fish, while the class B transcripts (PGB) that code for glucagon, GLP-1 and GLP-2 are more like mammalian transcripts (Table 4). Further, each class contained two transcripts that include sequence derived from either exon 1 or AE1. Thus, our results indicate that alternate promoter and first exon usage combined with alternative splicing at the 3'-end produced a total of four proglucagon mRNA transcript variants in the chicken.

In contrast, 5'-RACE reactions for both types of RNA yielded a single product which suggested that the same first exon or first exons of similar size (which would not be resolved by the 1.5% agarose gel) were present in the mRNA transcripts. Yue and Irwin (2005) also reported that the two mRNAs were expressed in chickens, but concluded that they were expressed separately; one in pancreas (PGA) and the other in the intestine (PGB). They also observed that exon 1 was the predominant or sole site of transcription initiation in the chicken proglucagon gene. Based on such observations it was concluded that proglucagon gene regulation in chickens involves tissue-specific alternative splicing which produces two distinct mRNA transcripts with different open reading frames (Irwin and Wong, 1995; Irwin, 2001; Yue and Irwin, 2005).

Table 4
Comparison of chicken and human proglucagon gene characteristics

<table>
<thead>
<tr>
<th>Gene feature</th>
<th>Chicken</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Exon size (bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alternate exon 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34/96</td>
<td>—</td>
</tr>
<tr>
<td>Exon 1</td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td>Exon 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>101</td>
<td>101</td>
</tr>
<tr>
<td>Exon 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>162</td>
<td>162</td>
</tr>
<tr>
<td>Exon 4&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>54</td>
<td>—</td>
</tr>
<tr>
<td>Exon 5 (a/b)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1179/138</td>
<td>138</td>
</tr>
<tr>
<td>Exon 6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>144</td>
<td>144</td>
</tr>
<tr>
<td>Exon 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>489</td>
<td>509</td>
</tr>
<tr>
<td>2. Intron size (bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 1</td>
<td>4262</td>
<td>2967</td>
</tr>
<tr>
<td>Intron 2</td>
<td>428</td>
<td>1572</td>
</tr>
<tr>
<td>Intron 3</td>
<td>822</td>
<td>1676</td>
</tr>
<tr>
<td>Intron 4</td>
<td>1575</td>
<td>—</td>
</tr>
<tr>
<td>Intron 5</td>
<td>2148</td>
<td>1368</td>
</tr>
<tr>
<td>Intron 6</td>
<td>754</td>
<td>654</td>
</tr>
<tr>
<td>3. mRNA size (bp)&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA (cDNA)</td>
<td>1591</td>
<td>1592</td>
</tr>
<tr>
<td>Coding exon</td>
<td>456</td>
<td>456</td>
</tr>
<tr>
<td>5'-UTR&lt;sup&gt;e&lt;/sup&gt;</td>
<td>98</td>
<td>37/99</td>
</tr>
<tr>
<td>3'-UTR</td>
<td>1037</td>
<td>1037</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two values are given for alternate exon 1 size due to uncertainty about the exact location of the alternate transcription initiation site in the chicken gene. The human gene does not contain an alternate first exon.

<sup>b</sup> These exons contain coding sequence.

<sup>c</sup> The chicken gene has an additional exon (exon 4) that codes for a larger intervening peptide-1 (IP-1) as compared to human IP-1. The human gene does not possess this exon.

<sup>d</sup> Alternative splicing occurs within exon 5 of the chicken gene giving rise to two classes of mRNA (PGA and PGB) that contain different sizes for exon 5 and different 3'-ends. Exon 5 is the terminal exon for class A transcripts. This does not occur in the human gene.

<sup>e</sup> Four different proglucagon mRNA transcripts are expressed in chickens due to use of an alternate promoter and first exon (AE1) and alternative splicing at the 5'- and 3'-ends of the initial gene transcripts, respectively. The human gene expresses a single mRNA.

<sup>f</sup> Due to uncertainty about the exact size of the alternate first exon (AE1), two values (37 and 99 bp) are listed for 5'-UTR in those chicken mRNA transcripts containing AE1.

1. Sequence used for these calculations was obtained as follows: chicken (GenBank Accession Nos. DQ185929, DQ185930, DQ185931) and human (GenBank Accession Nos. BC005278, NM_002054).
compared to the start site (+1a? set at /C0 754) and exon size (96 bp) for AE1 that we propose (Fig. 4). Moreover, they were not able to obtain experimental evidence for expression of AE1-containing transcripts using either 5\textsuperscript{0}-RACE or primer-directed RT-PCR. Nor were they able to demonstrate any significant \textit{in vitro} activity of the alternate promoter region located upstream of AE1. However, the existence of AE1 and the usage of the alternate promoter in the chicken proglucagon gene are strongly indicated by our results as this is the only mechanism through which AE1-containing transcripts could be produced. Our findings are further supported by an earlier report by Irwin and Wong (1995) of a clone isolated from an intestinal cDNA library that contained sequence virtually identical to the longer AE1 (GenBank Accession No. S78477). Moreover, if the AE1 were 34 bp in size, then the results of 5\textsuperscript{0}-RACE conducted in our study should have yielded two products (one from transcripts containing exon 1 sequence and a smaller size product for those containing sequence from AE1) instead of the single product observed (Fig. 5). Thus, regulation of the proglucagon locus is more complex in chickens than in mammals which express a single mRNA transcript in all tissues and rely solely on cell-specific post-translational processing to generate diversity in proglucagon-derived peptides produced (Kieffer and Habener, 1999).

Fig. 4. Nucleotide sequence of the 5\textsuperscript{0}-flanking region of the chicken proglucagon gene (GenBank Accession No. DQ185929) depicting the locations of exon 1 and the alternate first exon, as well as the immediate and distal (alternate) promoter regions. The transcription initiation site for exon 1 was set at +1 and is highlighted by a box. Two reported transcription initiation sites for alternate exon 1 were set at +1a* (Yue and Irwin, 2005) and +1a? (this study) and highlighted with boxes. Three TATA box motifs were identified 24–27 bp upstream from their respective transcription initiation sites. In addition, putative binding sites for a series of transcription factors in the forward (>) or reverse (<) orientation were identified. Only the most highly conserved region or core sequence of each transcription factor binding site is shown and these are highlighted by italicized bold text and underlining. The sequences for exon 1 and alternate exon 1 are highlighted by uppercase and bold lettering. The first 12 bp of intron 1 are also shown.
3.4. Expression of the proglucagon gene in different tissues

Fig. 6 demonstrates that PGA and PGB mRNAs containing exon 1 sequence are highly co-expressed in pancreas and surprisingly in proventriculus. Transcripts containing AE1 sequence were detected in all tissues analyzed and tended to be more highly expressed in tissues other than pancreas and proventriculus with high levels observed in heart, lung and abdominal fat. Since previous immunocytochemical studies have detected the presence of glucagon and GLP-1 in endocrine cells of the proventriculus from newly hatched chicks (Martinez et al., 1991, 2000), it is not unexpected that proglucagon mRNA would be detected in this tissue. What was unexpected is the high level of mRNA expression in proventriculus relative to other tissues analyzed. The significance of this observation remains to be determined as does the autocrine/paracrine or endocrine role, if any, for the proventriculus in influencing the local production and circulating levels of proglucagon-derived peptides in birds. Along these lines, it has recently been shown that the proventriculus also is an important site of production for the anorexic hormone leptin (Neglia et al., 2008). This lends additional support to potential autocrine/paracrine or endocrine roles for proglucagon-derived peptides produced by the proventriculus.

In order to quantify proglucagon mRNA expression, we utilized primer sets (Table 1) that amplified both exon 1 and AE1 containing transcripts within each class (i.e., total PGA and PGB). Using this approach, we found that a variety of tissues expressed PGA and PGB mRNAs, although at different levels (Fig. 7). There was some indication of tissue-specific mRNA transcript processing with the PGA mRNAs being preferentially expressed in the pancreas and proventriculus. In pancreas, proventriculus and abdominal fat, PGA was predominantly expressed, whereas in duodenum, liver, breast muscle and kidney, PGB was the predominant transcript. The remaining tissues were analyzed: pancreas (2), proventriculus (3), duodenum (4), liver (5), brain (6), heart (7), lung (8), breast muscle (9), abdominal fat (10), kidney (11) and spleen (12).

Fig. 7. Expression of class A and class B proglucagon mRNAs in different tissues from 3-wk-old broiler chickens. Reverse transcription PCR and capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection were used to quantify the level of expression relative to an 18S rRNA co-amplified internal standard. Expression ratio (class/18S) values represent the mean ± SEM of six determinations. Different letters above each bar denote statistically significant (P < 0.05) differences for mean comparisons.
sues surveyed showed about equal levels of PGA and PGB mRNA expression relative to 18S rRNA (Fig. 7).

3.5. Effects of fasting and refeeding on proglucagon expression

Plasma levels of glucagon were significantly ($P < 0.05$) increased during 24 or 48 h of feed deprivation in 3-wk-old broiler chickens, opposite to the changes observed in glucose and insulin and consistent with its counter-regulatory role (Fig. 8). Refeeding birds for 24 h resulted in significant declines in plasma glucagon levels. Plasma GLP-1 levels showed trends similar to glucagon, but no significant changes were found. Expression of total proglucagon mRNA (i.e., classes A and B combined) was determined in pancreas, proventriculus, duodenum and brain in response to feed deprivation and subsequent refeeding. There were no significant effects of altered energy status on proglucagon gene expression in any of these tissues (Fig. 9) suggesting that post-transcriptional effects or changes in glucagon secretion from pancreatic islet α-cells were driving the observed changes in plasma glucagon levels as has been observed in mammals (Kieffer and Habener, 1999).

Previous reports in rodents indicated that intestinal (jejunum and ileum) proglucagon mRNA levels decreased with a 72 h fast and increased upon refeeding (Hoyt et al., 1996); whereas, pancreatic expression of proglucagon mRNA increased in response to a 96 h fast (Chen et al., 1989). Vrang et al. (2008) reported that fasting had no effect on proglucagon expression in the brainstem of rats despite a strong up-regulation in response to genetic obesity. In contrast, Tachibana et al. (2005) reported that proglucagon mRNA expression declined in the brainstem of chickens following a 24 h fast and suggested that this correlated with an increased stimulus to feed due to reduced endogenous GLP-1 production in an area of the brain involved in regulating feeding behavior. Moreover, they also found that proglucagon mRNA was expressed in other regions of the brain, but at lower levels (Tachibana et al., 2005). These reports may explain our finding that fasting and refeeding produced no significant changes in whole brain proglucagon mRNA levels as the specific effects, if
3.6 Expression of prohormone convertase (PC) genes and proglucagon processing

Fig. 10 depicts the expression of PC1/3 and PC2 mRNAs in four different tissues. It is clear that both pancreas and proventriculus preferentially express PC2 which is required for glucagon production, while the expression of PC1/3, which is required for GLP-1 and GLP-2 production, is significantly higher in duodenum and brain as compared to pancreas and proventriculus. The predominant expression of PC2 mRNA in pancreas and proventriculus found in this study is consistent with the preferential production of glucagon by these two organs in chickens. Rawdon and Larsson (2000), using immunohistochemical staining for PC1/3 and PC2 in embryonic chicken and quail pancreatic tissue, reported that PC1/3 immunoreactivity was largely confined to insulin-producing B islet cells; whereas PC2 was localized to both A (glucagon-producing) and B cells with B cells staining more intensely. Their observations support the preferential production of glucagon from the proglucagon precursor in the avian pancreatic islet \(\alpha\)-cells.

The higher levels of PC1/3 mRNA expression observed in duodenum and brain suggest an enhanced production of GLP-1 and GLP-2 by these two tissues. Thus, the pattern of PC mRNA expression among tissues is an important factor reflecting the nature of proglucagon-derived peptides that have either glucose-raising or glucose-lowering effects (Wideman et al., 2007). The higher level of PC2 mRNA expressed in brain may reflect the expanded role of this particular convertase enzyme in the processing of other (i.e., neuroendocrine) prohormone precursors (Ling et al., 2004; Vandenborne et al., 2005; Zhou et al., 1999).

To understand how the chicken proglucagon precursors might be processed requires amino acid sequence data such as that presented in Fig. 11. Proteolytic cleavages occur at specific pairs of basic residues involving arginine (R) and lysine (K) (Zhou et al., 1999). With the exception of a single basic residue (R) at position 199 (located after GLP-2), it is apparent that there is complete conservation of these cleavage sites in chicken as compared to mammalian precursors (Fig. 11). This suggests that similar or the same mechanisms may be involved in post-translational proteolytic processing of the chicken proglucagon precursor proteins with PC1/3 acting on the C-terminal end to produce GLP-1 and GLP-2 involved in processing the N-terminal end of the precursor to yield glucagon (Dhanvantari et al., 1996; Rouille et al., 1995). In addition, there are other proteolytic cleavage sites conserved within the chicken proglucagon precursor (Fig. 11). These include: (1) a pair of arginine residues at positions 71–72 located within glucagon that are used to cleave a C-terminal fragment, miniglucagon, by a miniglucagon-generating endopeptidase (Dalle et al., 2002); (2) the single arginine residue at position 117 that is required to produce truncated forms of GLP-1 (7–37 and 7–36 amide) by a GLP-1 convertase (Dey et al., 2005; Dhanvantari et al., 1996); and (3) two sites recognized by DPP-IV which produces inactivated forms of GLP-1 and GLP-2 in circulation (Drucker, 2001). Thus, the chicken precursor proteins...
and the proglucagon-derived peptide hormones are presumably subject to the same or a similar post-translational processing regimen as their mammalian counterparts. There are differences that characterize chicken compared to mammalian proglucagon. The chicken produces two different proglucagon precursor proteins from the two classes of mRNA expressed, one that contains glucagon and GLP-1 and another that contains glucagon, GLP-1 and GLP-2, both of which would be subjected to post-translational proteolytic processing. Thus, the level of each precursor protein and the specific actions of the two prohormone convertase enzymes ultimately influence the type and amount of proglucagon-derived peptides produced by a given tissue. Another significant difference involves the production of oxyntomodulin which is a peptide segment derived from the proglucagon precursor that is composed of glucagon and IP-1. Because the chicken precursors contain an elongated IP-1, oxyntomodulin would be larger (55 amino acids) in the chicken compared to the 37 amino acid peptide produced by mammals (Drucker, 2005; Kieffer and Habener, 1999). How this might affect its biological function is not known since there have been no studies to date investigating chicken oxyntomodulin. However, it has been reported that human glucagon and oxyntomodulin are both biologically active in the chicken eye affecting ocular growth (Vessey et al., 2005). Recently, Dey et al. (2005) proposed a three dimensional model of mammalian proglucagon protein structure that positioned the IP-1 segment as an external loop that contains the dibasic amino acid site (K70-R71) for initial cleavage of the precursor by PC2, generating glicentin and the major proglucagon fragment (Fig. 11). The fact that chicken proglucagon contains a larger IP-1 than observed in mammals could influence this proposed processing mechanism mediated by PC2.

Fig. 12 depicts the effects of fasting and refeeding on PC1/3 and PC2 mRNA expression in pancreas, proventricle, duodenum, and brain. Expression of PC1/3 mRNA in pancreas showed a significant \( P < 0.05 \) trend, declining during fasting and increasing with subsequent refeeding. These changes may impact plasma insulin levels during fasting and refeeding since this enzyme has been reported
to play a role in the processing of proinsulin (Zhu et al., 2002). However, expression of PC2 (the enzyme involved in glucagon production) in pancreas was unchanged and only minor changes in PC2 mRNA expression were observed in the other tissues studied with no discernable trends. This data suggests that post-translational processing of the proglucagon precursor proteins in chickens is unaffected by changes in energy status. Thus, it is more likely that the changes observed in plasma glucagon during fasting and refeeding (Fig. 8) result from changes in the regulation of glucagon secretion.

3.7. Expression of proglucagon-derived peptide receptor genes

Expression of genes encoding receptors that recognize glucagon, GLP-1 and GLP-2 was investigated in broiler chickens and tissue-specific expression patterns were found, possibly indicating unique sites of hormone action. The same tissues that expressed proglucagon mRNAs also expressed genes encoding receptors for glucagon, GLP-1 and GLP-2 (Table 5). Liver and abdominal fat exhibited the highest levels of glucagon receptor mRNA expression suggesting that these tissues would exhibit the greatest sensitivity to glucagon. The high mRNA expression level for the glucagon receptor in liver is consistent with the role of glucagon in promoting glucose production by this organ. Mayo et al. (2003) reported that, in mammals, the glucagon receptor was most highly expressed in liver and kidney with a relatively low level of expression in fat tissue. Our results clearly show that the glucagon receptor gene is highly expressed in abdominal fat in broilers (Table 5) and this may indicate a species difference with respect to glucagon action on adipose tissue. Using a radioligand assay approach, Oscar (1995, 1996) reported the presence of glucagon receptors on the surface of chicken adipocytes.
Table 5
Expression of proglucagon-derived peptide hormone receptor mRNAs in different tissues from 3-wk-old male broiler chickens

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Glucagon receptor</th>
<th>GLP-1 receptor</th>
<th>GLP-2 receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreas</td>
<td>0.47 ± 0.05c</td>
<td>1.28 ± 0.09cd</td>
<td>2.60 ± 0.13</td>
</tr>
<tr>
<td>Proventriculus</td>
<td>1.87 ± 0.18bc</td>
<td>1.07 ± 0.05as</td>
<td>3.19 ± 0.24b</td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.41 ± 0.17a</td>
<td>1.40 ± 0.06bc</td>
<td>4.51 ± 0.11a</td>
</tr>
<tr>
<td>Liver</td>
<td>5.52 ± 0.16b</td>
<td>0.59 ± 0.04ac</td>
<td>0.10 ± 0.03b</td>
</tr>
<tr>
<td>Brain</td>
<td>2.13 ± 0.20bc</td>
<td>1.26 ± 0.11bc</td>
<td>3.46 ± 0.24b</td>
</tr>
<tr>
<td>Heart</td>
<td>1.76 ± 0.10cd</td>
<td>0.27 ± 0.10bc</td>
<td>0.43 ± 0.12bc</td>
</tr>
<tr>
<td>Lung</td>
<td>0.70 ± 0.12bc</td>
<td>1.64 ± 0.12bc</td>
<td>0.97 ± 0.14bc</td>
</tr>
<tr>
<td>Breast muscle</td>
<td>2.44 ± 0.06b</td>
<td>0.15 ± 0.04ab</td>
<td>1.07 ± 0.19ab</td>
</tr>
<tr>
<td>Abdominal fat</td>
<td>5.55 ± 0.08a</td>
<td>2.03 ± 0.14ab</td>
<td>1.36 ± 0.23ab</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.83 ± 0.16cd</td>
<td>1.32 ± 0.08cd</td>
<td>0.14 ± 0.02cd</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.89 ± 0.20bc</td>
<td>0.97 ± 0.16bc</td>
<td>1.70 ± 0.03bc</td>
</tr>
</tbody>
</table>

1 Reverse transcription PCR and capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection were used to quantify the levels of proglucagon-derived peptide hormone receptor mRNAs relative to an 18S rRNA internal standard. Expression values represent the mean ± SEM of six determinations. Different letters denote statistically significant (P < 0.05) differences for mean tissue comparisons within each receptor.

Other groups have studied mRNA expression for glucagon receptor in the chicken eye where it has been reported to mediate glucagon’s effects on ocular growth (Buck et al., 2004; Vessey et al., 2005). Montaron et al. (1994) used radioautography to detect and localize glucagon receptors in duck brain. Honda et al. (2007) recently reported that glucagon, when administered centrally, suppressed food intake and induced hyperglycemia in chicks. Their data suggest that functional glucagon receptors are expressed in the brain (specifically in the neurons that regulate food intake) and our finding of significant expression of glucagon receptor mRNA in whole brain tissue (Table 5) is consistent with that conclusion.

Our study is the first to report GLP-1 and GLP-2 receptor mRNA expression in different chicken tissues. The GLP-1 receptor was most highly expressed in abdominal fat with the lowest levels of expression in heart and breast muscle. Although the existence of GLP-1 receptors has been previously inferred in brain tissue through the use of a receptor antagonist (exendin 5–39) and the actions of centrally administered GLP-1 hormone (Furuse et al., 1998; Tachibana et al., 2001), our study is the first to directly determine GLP-1 receptor gene expression in brain. The fact that proglucagon mRNA expression was co-localized with GLP-1 immunoreactivity in perikarya of brainstem neurons (Tachibana et al., 2005) coupled with inferred expression of GLP-1 receptors suggests an autocrine/paracrine action of GLP-1 and its cognate receptor in the brain. Low levels of GLP-1 receptor mRNA expression in liver, skeletal muscle and heart from broilers are similar to findings reported in mammals (Mayo et al., 2003). Significant expression of GLP-1 receptor in pancreas is consistent with an incretin role for GLP-1 in chickens as reported for mammals whereby GLP-1 stimulates insulin secretion from the pancreas (Drucker and Nauck, 2006).

However, this remains to be demonstrated experimentally in birds.

GLP-2 receptor expression was highest in pancreas, proventriculus, duodenum and brain indicative of GLP-2 effects associated with these tissues (Table 5). Elevated expression of GLP-2 receptor mRNA in broiler gastrointestinal tissues is consistent with the proposed role for GLP-2 in intestinal growth and function (Burrin et al., 2003; Estall and Drucker, 2006). However, a physiological function(s) for GLP-2 in birds has yet to be identified. Interestingly, Shousha et al. (2007) found no effect of GLP-2, administered i.c.v. or i.p., on food intake, body temperature or locomotor activity in Japanese quail. However, it must be noted that this group utilized rat GLP-2 in their experiments which shares only 52% amino acid homology with chicken GLP-2 (Fig. 11). This may not have been sufficient for GLP-2 receptor binding/activation in the avian system. The relatively high level of expression of GLP-2 receptor mRNA in brain is interesting and could indicate a role for GLP-2 in brain-gut signaling reflecting nutrient sensing or in the control of food intake. It could also indicate an autocrine/paracrine system for GLP-2 in avian brain similar to what was suggested for GLP-1. In general, very little is currently known about the regulatory mechanisms governing expression of glucagon, GLP-1 and GLP-2 receptor genes in mammals and nothing as of yet in birds. This is clearly an area that merits further investigation.

### 4. Conclusions

In this study, we cloned, sequenced and characterized the genomic structure of the chicken proglucagon gene. We found that two classes of mRNA composed of four variants are expressed from the proglucagon gene via alternative 3′-end splicing and alternate promoter and first exon usage. Both mRNA classes are co-expressed in different tissues with the highest levels detected in pancreas and proventriculus. The two classes of mRNA exhibit different coding potential with respect to the glucagon-like peptides and when translated would produce two different proglucagon precursor proteins. Expression of PC1/3 and PC2 genes was determined in key tissues that play important roles in the post-translational processing of proglucagon precursor proteins. This work is the first to demonstrate that proglucagon-derived peptide hormone receptors for glucagon, GLP-1 and GLP-2 are widely expressed, with some tissues demonstrating high levels of mRNA for particular receptors indicating potentially important sites of hormone action. The information gained from this study offers new insights into the nature and function of the proglucagon system in chickens.

### Acknowledgments

The authors acknowledge the expert technical assistance of Donna Brocht in blood collection and plasma hormone
analyses and Stephen Poch in DNA sequencing and gene expression analyses.

References

Buck, C., Schaeffel, F., Simon, P., Feldkaemper, M., 2004. Effects of positive and negative lens treatment on retinal and choroidal glucagon and glucagon receptor mRNA levels in the chicken. Invest. Ophthalmo-


Dey, A., Lipkind, G.M., Rouille, Y., Norrbom, C., Stein, J., Zhang, C., Carroll, R., Steiner, D.F., 2005. Significance of prohormone conver-
tase 2, PC2, mediated initial cleavage at the proglucagon interdomain site, Lys70-Arg71, to generate glucagon. Endocrinology 146, 713–727.

Dhanvantari, S., Seidah, N.G., Brubaker, P.L., 1996. Role of prohormone con-


Heinrich, G., Gros, P., Habener, J.F., 1984. Glucagon gene sequence. Four of six exons encode separate functional domains of the rat pre-


Martinez, A., Lopez, J., Barrechea, M.A., Sesma, P., 1991. Immuno-


Montaron, A., Moyal, E., Barre, H., 1994. Radioautographic demon-

stration and localization of glucagon receptors in duck brain. Brain Res. 663, 121–130.

Neglia, S., Arcamone, N., Gargiulo, G., de Girolamo, P., 2008. Immuno-


Richards, M.P., Poch, S.M., 2002. Quantitative analysis of gene expres-
sion by reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection. Mol. Bio-
technol. 21, 19–37.


