≪Research Note≫

Characterization and Expression of Turkey Prolactin Regulatory Element Binding in the Anterior Pituitary Gland and Pancreas During Embryogenesis

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The PRL regulatory element-binding (PREB) protein is a transcription factor that was originally cloned from the rat anterior pituitary gland and characterized as a regulator of the PRL promoter. It is also strongly expressed in several extrapituitary tissues; however, its functional role is not well understood to date. In this study, we aimed to clone and characterize the turkey PREB gene and investigate its mRNA expression in the anterior pituitary gland and pancreas during embryogenesis. Based on the conserved sequence of chicken and mammalian PREB cDNAs, a turkey PREB cDNA fragment was obtained, and after sequencing of the fragment, the 5′- and 3′-ends of mRNA were amplified and determined. To identify the PREB gene structure, polymerase chain reaction (PCR) amplification was performed. The turkey PREB gene consists of 9 exons and 8 introns, and it encodes a 411-amino-acid protein. The expression of PREB mRNA in the anterior pituitary gland was measured during embryogenesis. Levels of PREB mRNA significantly increased at embryonic day 22, with maximum levels being detected on day 25 of ontogeny, which correlated with similar changes in levels of PRL mRNA. The highest level of PREB mRNA was detected on day 19 in the pancreas. However, the highest level of insulin mRNA was detected at embryonic day 25. These results indicate that PREB may be involved in the expression of PRL mRNA in the anterior pituitary gland, whereas insulin mRNA may be expressed independently of the expression of PREB mRNA in the pancreas during embryogenesis.

Key words: mRNA, pancreas, pituitary gland, PREB

Introduction

In chickens, the prolactin regulatory element-binding protein (PREB) gene encodes a 1.4-kb transcript, which is translated into a transcription factor and contains 3 WD motifs (Hiyama *et al*., 2015). These WD repeats are involved in the binding of PREB to the prolactin (PRL) promoter to regulate transcription. PREB was originally isolated by Southwestern screening on the basis of its binding capacity to a Pit-1-binding site (1P) in the rat proximal PRL promoter (Fliss *et al*., 1999). The Pit-1 transcription factor plays important roles in the mRNA expression of anterior pituitary hormones,

and Pit-1 mutations have been shown to reduce PRL-, GH-, and TSH-producing cells, leading to dwarfism (Li *et al*., 1990; Radvick *et al*., 1992). Although Pit-1 binds to multiple sites in the rat PRL promoter, high levels of expression of PRL mRNA in the anterior pituitary gland require additional transcription factors (Savage *et al*., 2003). Additive effects of Pit-1 and PREB expression vectors on the proximal region of the rat PRL promoter have been reported (Fliss *et al*., 1999). This additive effect suggests that Pit-1 and PREB independently exert actions on the 1P element. In addition, vasoactive intestinal polypeptide (VIP) is a physiological releasing factor of PRL in birds (Macnamee *et al*., 1986) and both plasma PRL and PRL mRNA levels in the anterior pituitary gland are highly correlated to the hypothalamic content of VIP or levels of VIP receptor mRNA in the anterior pituitary gland (Sharp *et al*., 1989; Chaiseha and El Halawani 1999; Kansaku *et al*., 2001). The effects of VIP are mediated through a VIP response element (VRE) originally identified in turkey (Kang *et al*., 2004) and generally conserved in various avian species (Kansaku *et al*., 2008). The core

sequence of the avian VRE is similar to the PREB binding motif of the mammalian PRL promoter (Hiyama *et al*., 2009). This may suggest that in addition to potential interactions with Pit-1 binding elements, avian PREB may also interact with the VRE to regulate PRL mRNA expression in anterior pituitary glands.

In chickens, changes in levels of PREB mRNA in the anterior pituitary gland have been shown during embryonic development and different reproductive stages (Hiyama *et al*., 2015). Interestingly, increased levels of PREB mRNA have been found to precede those of PRL mRNA. Conversely, levels of PREB mRNA have shown similar changes to levels of PRL mRNA at different reproductive stages. Although PREB mRNA is predominantly expressed in the anterior pituitary gland, it is also strongly expressed in mammals in various tissues such as the heart, skeletal muscle, pancreas, liver, placenta, and salivary gland (Fliss *et al*., 1999; Taylor Clelland *et al*., 2000). The expression of PREB in extra-pituitary tissues may indicate the possible involvement of regulatory function of PREB in the expression of various target genes as a transcription factor.

In mammals, the function of PREB in extra-pituitary tissues is not well understood. However, the effect of PREB on insulin (INS) gene expression has been identified using pancreatic islets (Ohtsuka *et al*., 2006). Several lines of evidence have suggested that PREB has functions in pancreatic cell transcription: 1) PREB is present in the nuclear extract; 2) mRNA expression of the PREB gene is detected in the pancreatic islet cells MIN6 and INS-1; and 3) PREB binds to the A3 elements of the INS promoter. Binding of PREB to the A3 element has been confirmed by super-shift assays using antisera against recombinant PREB. In addition, PREB protein accumulation in pancreatic islets has been induced by glucose (Muraoka *et al*., 2009). These results indicate the contribution of PREB to INS gene transcription and INS secretion in response to glucose stimulation. In birds, the expression of PREB in other tissues has not been examined. Thus, whether PREB contributes to gene expression in different tissues such as the pancreas in birds remains unknown.

Although the gene structure of PREB is conserved between mammals and chicken (9 exons and 8 introns), the length of the chicken PREB gene is less than 2 kb. In contrast, the length of the mammalian PREB gene is 3−4 kb (NC_000071.6, NW_005393697.1, NC_010445.3, NC_006599.33, NC_005105.4, and NC_000002.12). This difference in length is because of the length of introns. Introns of the mammalian PREB gene are generally longer than 100 bp, whereas those of chickens are less than 100 bp, with the exception of intron 5. By the whole genome shotgun sequence analysis, PREB gene was estimated in two altricial birds (Budgerigar NW_004847627 and Tibetan ground-tit NW_005087685). The length of budgerigar gene is 2.17 kb whereas that of Tibetan ground-tit is 3.6 kb. Budgerigar and Tibetan ground-tit belong to Passriformes and Psittaciformes, respectively. However, these two orders are phylogenetically distant order to Galliformes (Hackett *et al*., 2008). The difference of length of PREB gene may indicate that length of PREB gene varies during evolution or differentiation of species of bird. Since PREB gene of the precocial bird was only characterized in the chicken, it is unknown whether the short length of the PREB gene is a common feature of precocial birds, Galliformes or Phasianidae.

Accordingly, in the present study, we aimed to clone and characterize the turkey *PREB* gene and characterize levels of PREB mRNA in the anterior pituitary gland and pancreas during embryogenesis to examine the possibility of co-regulation and/or relationships in the control of PRL and INS mRNA expression.

Materials and Methods

Tissue Sampling and RNA Isolation

Anterior pituitary glands were collected from adult turkeys obtained from a local turkey breeding company (Couvoir Unik Inc., Mont-Saint-Gregoire, QC, Canada), snap-frozen in liquid nitrogen, and stored at −80℃ until RNA extraction. Handling of turkey eggs and sampling of embryonic anterior pituitary glands were in accordance with the guidelines for animal experimentation of McGill University. Total RNA was extracted using RNA isolation reagent (TRIzol; Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instruction. Genomic DNA was extracted as described previously (Kansaku *et al*., 2005). The amount of total RNA and genomic DNA was estimated using a spectrophotometer (GeneQuant; GE Healthcare, Little Chalfont, UK).

Cloning of Turkey PREB cDNA

Total RNA (1 μg) isolated from the anterior pituitary gland was denatured at 65℃ for 10 min using random hexamer primers and reverse transcribed with 200 units of SuperScript III (Life Technologies Corporation) in a 20-μL mixture. Based on the sequences of chicken PREB cDNA, human PREB cDNA, and rat PREB cDNA (Fliss *et al*., 1999; Taylor Clelland *et al*., 2000; Hiyama *et al*., 2015), primers (S1: TTCCCCAGCCTGGAGAAGGT, A1:CTGACATCGAGG-CAGGAGAC) were designed. The reverse-transcribed product was subjected to 35 cycles of polymerase chain reaction (PCR) amplification using DNA polymerase (AccuPrime Taq DNA polymerase; Life Technologies Corporation) in a total volume of 50 μL. PCR was performed using PTC-100 (MJ Research, Inc., Waltham, MA, USA), and the amplification profile consisted of 2 min of denaturation at 94℃ for the first cycle and 30 s per cycle thereafter, 20 s annealing at 55℃, and 20 s extension at 68℃ for the first 34 cycles, followed by 1 min extension on the final cycle. Amplified PCR products were purified (NucleoSpin Gel and PCR Clean-up; Takara, Shiga, Japan) and directly sequenced on both strands by the dideoxy method (Sanger *et al*., 1977) using the ABI 3710 sequencer (Applied Biosystems, Foster City, CA, USA). Following sequence analysis, the 5′-end of cDNA was amplified by the rapid amplification of cDNA end (RACE) method (Frohman *et al*., 1989). After reverse transcription using the A1 primer, primary RACE-PCR was performed using the primers A2 (TCCCACTTGGTCAGGTA-
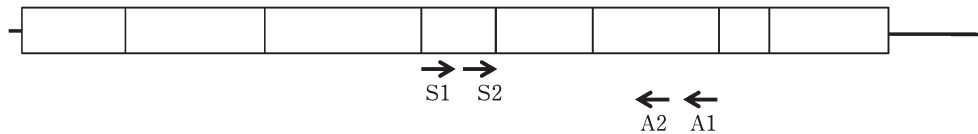
Fig. 1. Primer locations and cloning strategy for turkey PREB cDNA. Open boxes represent putative exon regions.
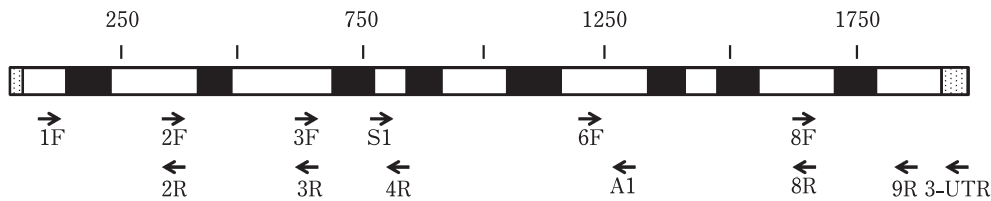

Fig. 2. Primer locations and strategy for characterization of the turkey PREB gene. Open and closed boxes represent exons and introns, respectively.

Table 1. List of Primers to characterize the turkey PREB gene

Primer name	sequence	position
1F	CTTTCCCGCTCTACACCGT	35–53
2F	ACATCCTGCGGCTTTGCGTA	350–369
3F	AAGAGGTGACGGTGGAGA	548–565
6F	TGCTACCTGACCAAGTGGGA	1230–1249
8F	GAGCTGTTGGCAGGGAATGA	1615–1634
2R	TACGCAAAGCCGCAGGATGT	369–350
3R	TCTCCACCGTCACCTCTT	565–548
4R	AGCGTGATGTCCTCGATCTC	1461–1445
8R	TCATTCCCTGCCAACAGCT	1633–1616
9R	GAAGCCAGGGAAGGCAATCT	1861–1842
3-UTR	TCTGGGTTTAATGGTAGGGC	1401–1382

Positions of foward primers and reverse primers were cited from accession number LC036258. Position of 3-UTR was sited from accession number LC36259.

GCA) and abridged anchor primers from the RACE kit. Similarly, the 3′-region was cloned with the 3-RACE kit (Life Technologies Corporation) using primers S1 and S2 (ACCGCTACCAGGCCTGCCGCT). The cloning strategy and primer locations are indicated in Fig. 1. The nucleotide sequence of PREB cDNA and sequence of deduced amino acids were analyzed using DNASIS Pro (Hitachi Software, Tokyo, Japan), and BLAST search of NCBI.

Characterization of the Turkey PREB Gene

To determine the structure of the turkey *PREB* gene, 11 specific primers based on the turkey PREB cDNA were synthesized. The primer location and sequence are indicated in Fig. 2 and Table 1, respectively. Using two different primer sets (1F-A1 and S1-3UTR), turkey PREB fragments were amplified and sequenced using synthesized primers after purification. PCR was performed using AccuPrime Taq DNA

polymerase. The amplification profile consisted of 2 min denaturation at 94℃ for the first cycle and 20 s per cycle thereafter, 30 s annealing at 58℃, 75 s extension at 68℃ for the first 34 cycles, and 5 min extension on the final cycle.

Measurement of Levels of PREB and Candidate Target Gene mRNA in the Anterior Pituitary Gland and Pancreas

In total, 30 fertilized eggs were obtained from a local company (Couvoir Unik Inc.) and incubated at 37.5℃ in a humidified incubator. Following decapitation, the anterior pituitary glands and pancreas were collected from developing embryos on days 19, 22, 25, and 27 of incubation and on the hatching day. Six of developing embryos or chicks was used for tissue sampling. The anterior pituitary glands and pancreas were snap-frozen in liquid nitrogen and stored at −80 ℃. Two anterior pituitary glands were pooled at each day ($n=3$) and used for RNA extraction. On the other hands, 4 to 6 samples of pancreas were used to determine the levels of PREB and INS mRNA. Total RNA was extracted as described previously. After measurement of RNA amount by O.D., 1 μg of total RNA was reverse-transcribed and PCR amplification of PREB, PRL, INS, and 18S was performed. The position and sequence of primers for PREB, PRL, and INS are listed in Table 2. Commercially available primers (Universal 18S Internal Standard; Ambion, Austin, TX, USA) were used to amplify 18S cDNA. Fragments of PREB cDNA from the anterior pituitary gland and pancreas of embryos were amplified by 37 cycles of PCR. Fragments of PRL, INS, and 18S cDNA were amplified by 27, 27, and 20 cycles of PCR, respectively. Amplification was performed using AccuPrime Taq DNA polymerase. The amplification profile of PRL, INS, and 18S was 2 min denaturation at 94℃ for the first cycle and 20 s per cycle thereafter, 30 s annealing at 59℃, and 30 s extension at 72℃. After the electrophoresis of PCR products, the signal intensity of each PCR product was determined using public domain software (ImageJ Ver.

Table 2. **List of PCR amplified gene and sequence of primers**

Gene		Primer sequence	length (bp)
PREB	Sense	TGCAATGGAACGAGAACCTG	249
	Anti-Sense	ATGTGCCCGAATCACTGAC	
PRL	Sense	GCAAACTCAGCAGATTCATCAC	139
	Anti-Sense	AGCCTTCCAGAGGATGGTATC	
INS	Sense	TCTGGCCCTCCTTGTCTTTTC	240
	Anti-Sense	TCAACAATCCCTCGCTTGAC	

10.2) (Schneider *et al*., 2012).

Analysis of Results

Proportions of PREB, PRL, and INS normalized to 18S were analyzed by one-way analysis of variance. The significance of differences between means was assessed using the least-significant difference test. Statistical analyses were performed using the commercially available package of the Statistical Analysis System (SAS Institute, 1999). Significance was denoted by $P < 0.05$.

Results

Cloning of Turkey PREB cDNA

Approximately 380 bp of a partial turkey PREB cDNA clone was initially obtained by PCR amplification. Through a combination of partial cDNA, 5′ RACE, and 3′ RACE, a sequence encoding a turkey PREB protein was identified. The methionine codon at nucleotides 11−13 was the likely site for translation initiation because of the similarity of the sequence of the initial 20 amino acid residues to that of chicken PREB. Of the 1422 bp cloned, 10 bp of 5′ UTR, 159 bp of 3′ UTR, and 1233 bp ORF encoding 411 amino acids were characterized. A highly conserved polyadenylation signal (Proudfoot & Brownlee 1976) was detected at 11 nucleotides from the poly (dA) tract. The complete nucleotide sequence and deduced amino acid sequence of the turkey PREB cDNA are shown in Fig. 3. A very high similarity of both cDNA and amino acid sequences to those of chicken were observed. Thus, the tendency of similarity of cDNA and amino acid sequences of chicken to those of mammalian species was also observed in turkey. The similarity of cDNA and amino acid sequences to those of other previously identified PREB proteins is listed in Table 3.

Characterization of the Turkey PREB Gene

Using two different primer sets, 1F-A1 and S1-3UTR, 1284 bp and 1273 bp were amplified, respectively, yielding 1951 bp of the PREB gene sequence. The *PREB* gene consisted of 9 exons and 8 introns, and the structure and exon–intron boundaries are shown in Fig. 4. The general structure of the turkey PREB gene shows high similarity to the chicken PREB gene, including the intron length. Comparison of the intron length and similarities of introns between turkey and chicken were listed in Table 4.

Measurement of Levels of PREB, PRL, and INS mRNA in the Anterior Pituitary Gland and Pancreas

The expression of PREB, PRL, and INS mRNA in the developing anterior pituitary gland was assessed by semi-quantitative RT-PCR (Fig. 5). Levels of 18S mRNA were also determined as an internal control to provide relative values to 18S. The lowest level of PREB mRNA occurred on day 19 of embryogenesis and increased on day 22, reaching a maximum level on day 25. Levels did not change between days 25 and 27 of embryogenesis but significantly decreased on the hatching day in comparison with day 25. Low levels of PRL mRNA were detected on day 19, but subsequently, a significant increase was detected from day 22 to the hatching day. The highest level of PRL mRNA was detected on day 25, and subsequently, a significant decrease was progressively observed until hatch (Fig. 6).

In the pancreas, the highest levels of PREB mRNA were detected on day 19. Subsequently, the levels significantly decreased on day 22 and were maintained until hatch. Conversely, the lowest levels of INS mRNA were detected on day 19 of embryogenesis and the highest levels were observed on day 25 (Fig. 6). A significant decrease was observed on the hatching day.

Discussion

Sequencing of turkey PREB cDNA revealed a high degree of similarity at both the nucleotide and amino acid levels to chicken PREB (96% and 97%, respectively). Comparison of chicken PREB with mammalian PREBs showed that the upper region (amino acids 1−217) is less well conserved than the lower region (amino acids 218−411) (Hiyama *et al*., 2015). Similar to the latter observation, a comparison between turkey and chicken showed the majority of differences in amino acids were confined to the upper region (11 substitutions) in contrast to the lower region (4 substitutions). Similarly, an amino acid substitution was detected in the WD1 motif located at position 146−176, whereas WD2 and WD3 completely matched between chicken and turkey. This suggests that the lower region of the PREB protein is more functionally conserved evolutionarily than the upper region and that the WD repeat units 2 and 3 are more involved than WD1 in affecting gene regulation.

The total length of the PREB gene (from the ATG codon to the stop codon) in both chicken and turkey was less than 2 kb. In addition, the difference in the length between turkey and chicken is only 28 bp, which is due to intron similarities. The sequence similarity of introns was more than 75%, with the exception of intron 3 (Table 4). Thus, the turkey and chicken PREB genes are relatively smaller in size than mammalian PREB genes (3−4 kb). However, this small size

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                                                                                     cgttctcgca

ATGGCGCCGCGTCCGACCGAGCTGTACCGGGCGCCTTTCCCGCTCTACACCGTGCGGCTGCACCCGCGCCGCGGCCTCGCCATCACTGCT
MetAlaProArgProThrGluLeuTyrArgAlaProPheProLeuTyrThrValArgLeuHisProArgArgGlyLeuAlaIleThrAla       30

GGAGGCGGAGGAGCGGCCAAAACCGGGATCGGCAATGGCGTGCACTTCCTGCAGCTGGAGCGGATCGGGGGGCGGCTGAGCGCGTCGCTG
GlyGlyGlyGlyAlaAlaLysThrGlyIleGlyAsnGlyValHisPheLeuGlnLeuGluArgIleGlyGlyArgLeuSerAlaSerLeu       60

CTGCATTGCCACGACACCGAGACCCGCGCCACCATGAGCATGGCGCTGAGCGAGGACTTCATCGCGGCGGGGCAGGACCACAGCTGCCAC
LeuHisCysHisAspThrGluThrArgAlaThrMetSerMetAlaLeuSerGluAspPheIleAlaAlaGlyGlnAspHisSerCysHis       90

ATCCTGCGGCTTTGCGTACGGCGGACGGCGCGGGAGGCGGCGATGCCGCGGCGGAGAAGGGCCCGCGCAGGCGGAAGGGCGCCGGCCCC
IleLeuArgLeuCysValArgAlaAspGlyAlaGlyGlyGlyAspAlaAlaAlaGluLysGlyProArgArgArgLysGlyAlaGlyPro       120

GCGGAGCGCGGTGAGACGCGGAGCAGCGGCGAAGAGGTGACGGTGGAGAGCCTGCACCGCGTGCGCACCGACTTCAGCCCCGACGCGCTG
AlaGluArgGlyGluThrArgSerSerGlyGluGluValThrValGluSerLeuHisArgValArgThrAspPheSerProAspAlaLeu       150

CAGAAGGCCGTGCGCTTCAGCGCCGACGGCGCGCTGCTCGTCACCGGCGGTGCCGACGGATTCCTGCGGCTCTGGGAGTTCCCCAGCATG
GlnLysAlaValArgPheSerAlaAspGlyAlaLeuLeuValThrGlyGlyAlaAspGlyPheLeuArgLeuTrpGluPheProSerMet       180

AAGAAGGCGCTGGAGTTCAAGGCGCACGATGGGGAGATCGAGGACATCACGCTGGGCCCCGACAATAAGGTGGTGACGGCGGGACGGGAC
LysLysAlaLeuGluPheLysAlaHisAspGlyGluIleGluAspIleThrLeuGlyProAspAsnLysValValThrAlaGlyArgAsp       210

CTGCAGTGCCGCGTGTGGCAGCGGGACCAGCTGGTGACAGGGCTGCAATGGAACGAGAACCTGCCCGGCATCCCCAGCACCGCGTACCGC
LeuGlnCysArgValTrpGlnArgAspGlnLeuValThrGlyLeuGlnTrpAsnGluAsnLeuProGlyIleProSerThrAlaTyrArg       240

TACCAGGCCTGCCGCTTCGGGGCGGTGGAGGACGACCGCGGAGCGCTGCGGCTCTACACGGTGCAGGTGCCCCACAAGCGAGAACGTCGC
TyrGlnAlaCysArgPheGlyAlaValGluAspAspArgGlyAlaLeuArgLeuTyrThrValGlnValProHisLysArgGluArgArg       270

CCGCCGCCCTGCTACCTGACCAAGTGGGACGGGGGCACCTTCCTGCCGCTGCTGACACGGCCCTGCGGCTCTGAAGTCATCTCCTGCCTC
ProProProCysTyrLeuThrLysTrpAspGlyGlyThrPheLeuProLeuLeuThrArgProCysGlySerGluValIleSerCysLeu       300

TCCGTCAGTGATTCGGGCACATTCCTGGGGCTGGGCACCGTCACCGGCTCTGTCGCTGTGCACATCGCCTTCTCGCTTCAGAGGTTGTAC
SerValSerAspSerGlyThrPheLeuGlyLeuGlyThrValThrGlySerValAlaValHisIleAlaPheSerLeuGlnArgLeuTyr       330

TACGTGAAGGAGGCACATGGCATTGTCGTCACCGATGTGGCCTTCGTGCCTGAGAGCGAGCATGGACGGGAGCTGTTGGCAGGGAATGAG
TyrValLysGluAlaHisGlyIleValValThrAspValAlaPheValProGluSerGluHisGlyArgGluLeuLeuAlaGlyAsnGlu       360

GCAGCACTGCTCAGTGTGGCCGTGGACAGCCGCTGCAAGCTTCATCTGCTCCCTGCCCGCCGGTCGCTACCTGTCTGGCTGCTGCTGCTG
AlaAlaLeuLeuSerValAlaValAspSerArgCysLysLeuHisLeuLeuProAlaArgArgSerLeuProValTrpLeuLeuLeuLeu       390

CTGTGTGCAGCGCTCATCGTGGGTAGCATCGTGCTGCTGCAGATTGCCTTCCCTGGCTTCCTCTAGgcaccctccctgccacactgggggg
LeuCysAlaAlaLeuIleValGlySerIleValLeuLeuGlnIleAlaPheProGlyPheLeu***                               411

gcggatggacctggtcctgggcatgctgtgggagtgggactgccttgggatgcagcaccggcatggcgatgggacacagcgtgccagtgg
ctgggctggagctgctccttggccctaccattaaacccagaatgtgaaaaaaaaaaaaaaaa
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Fig. 3. **Nucleotide and deduced amino acid sequences of turkey PREB cDNA.** Nucleotides are numbered on the right side of the sequence. Untranslated regions are reported in lowercase characters, and the open reading frame is reported in uppercase characters. Asterisks indicate the translation stop codon. Predicted WD repeats are underlined. The poly (dA) signal is indicated by a double line. GenBank/EMBL/DDBJ Bank accession number: LC036259.

of the PREB gene is not a common characteristic of birds because the budgerigar and Tibetan ground-tit genes are 2.17 kb and 3.6 kb, respectively. Since expression of incubation behavior is an unfavorable trait both in chicken and turkey, comparison of changes of PREB mRNA levels and gene structure was conducted to investigate the relationship to PRL mRNA expression. Cloning and characterization of PREB gene in the different species such as guinea fowl (Galliformes Numidae) is necessary to speculate that the short length of the PREB gene may be a feature of Galli-

Table 3.　Sequence similarity of PREB cDNA and amino acids

	Turkey	Chicken	Human	Dog	Bovine	Rat	Mouse	Zebra
Turkey		0.96	0.67	0.67	0.69	0.66	0.67	0.6
Chicken	0.97		0.66	0.66	0.69	0.66	0.67	0.59
Human	0.68	0.68		0.94	0.93	0.9	0.89	0.6
Dog	0.67	0.67	0.92		0.94	0.89	0.88	0.61
Bovine	0.68	0.68	0.91	0.91		0.91	0.91	0.6
Rat	0.66	0.66	0.88	0.87	0.88		0.97	0.59
Mouse	0.66	0.65	0.88	0.86	0.86	0.95		0.59
Zebrafish	0.61	0.61	0.6	0.6	0.6	0.6	0.59	

Values above and under the diagonal line represent similarity of entire sequence of amino acids and cDNA, respectively.

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ATGGCGCCGCGTCCGACCGAGCTGTACCGGGCGCCTTTCCCGCTCTACACCGTGCGGCTGCACCCGCGCCGCGGCCTCG

CCATCACTGCTGGAGGCGGAGGAGCGGCCAAAACCGGGATCGGCAATGGCGTGgtacagtgctgcgcggctccggggggg

tcgggagggttgcggggctccccaggcgcgggggctgagcggcggtgtccccgcagCACTTCCTGCAGCTGGAGCGGATC

GGGGGGCGGCTGAGCGCGTCGCTGCTGCATTGCCACGACACCGAGACCCGCGCCACCATGAGCATGGCGCTGAGCGAGG

ACTTCATCGCGGCGGGGCAGGACCACAGCTGCCACATCCTGCGGCTTTGCGTACGGGCGGACGGCGCGGGAGGCGGCGA

TGgtgagcggggatgcgggcgacgggtgccgggtgccgggtgcggagcgctcaaccggcggctctccgtccccgcagCC

GCGGCGGAGAAGGGCCCGCGCAGGCGGAAGGGCGCCGGCCCCGCGGAGCGCGGTGAGACGCGGAGCAGCGGCGAAGAGG

TGACGGTGGAGAGCCTGCACCGCGTGCGCACCGACTTCAGCCCCGACGCGCTGCAGAAGGCCGTGCGCTTCAGCGCCGA

CGGCGCGCTGCTCGTCACCGGCGGTGCCGACGGATTCCTGCGGCTCTGGGAGgtgggagcggggagggagggcccgggg

ggggtcacctatcggggacccgctGACGGCCGCCGCAGTTCCCCAGCATGAAGAAGGCGCTGGAGTTCAAGGCGCACGA

TGGGGAGATCGAGGACATCACGCTGGGCCCCGACAATAAGgtgggtgaggggcgcgggctgggggcacgaagccccgca

cggtcccgtgagccgcagccgtcgtgtgccccgcagGTGGTGACGGCGGGACGGGACCTGCAGTGCCGCGTGTGGCAGC

GGGACCAGCTGGTGACAGGGCTGCAATGGAACGAGAACCTGCCCGGCATCCCCAGCACCGCGTACCGCTACCAGGCCTG

CCGgtgggtgcggggctggggggtggcccggggggctgtgccgtaggacggtggcggtgaccgcacggggccgccccctgg

cagcgcgatcgcggctcacctctgctgccccgtgccagCTTCGGGGCGGTGGAGGACGACCGCGGAGCGCTGCGGCTCT

ACACGGTGCAGGTGCCCCACAAGCGAGAACGTCGCCCGCCGCCCTGCTACCTGACCAAGTGGGACGGGGGCACCTTCCT

GCCGCTGCTGACACGGCCCTGCGGCTCTGAAGTCATCTCCTGCCTCTCCGTCAGgtgtgtgggggaggtcccacccccccc

caacccctccatctgctcccagccccgctcactgcccccttccccacagTGATTCGGGCACATTCCTGGGGCTGGGCAC

CGTCACCGGCTCTGTCGCTGTGCACATCGCCTTCTCGCTTCAGgtgagtgcggcacagggagggcacagggtgggcaca

gagggccaccccacctcgtgcgtgtcccccttcagAGGTTGTACTACGTGAAGGAGGCACATGGCATTGTCGTCACCGA

TGTGGCCTTCGTGCCTGAGAGCGAGCATGGACGGGAGCTGTTGGCAGGGAATGAGGCAGCACTGCTCAGTGTGGCCGTG

GACAGCCGCTGCAAGCTTCATCTGCTCCCTGCCCGCCgtatgtgtgtggggctggggggtcgggccaccccccaggacc

ccaccccccactcagcctcctctcccccactccagGGTCGCTACCTGTCTGGCTGCTGCTGCTGCTGTGTGCAGCGCTCA

TCGTGGGTAGCATCGTGCTGCTGCAGATTGCCTTCCCTGGCTTCCTCTAGgcaccctccctgccacactgggggggcgga

tggacctggtcctgggcatgctgtgggagtgggactgccttgggatgcagcaccggcatggcgatgggacacagcgtgc

cagtggctgg
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Fig. 4.　**Nucleotide sequence of turkey PREB gene.** Exon sequences are shown in uppercase characters, and intron sequences are shown in lowercase characters. GenBank/EMBL/DDBJ Bank accession number: LC036258.

Table 4. **Comparison of length of introns and sequence similarities**

	Intron 1	Intron 2	Intron 3	Intron 4	Intron 5	Intron 6	Intron 7	Intron 8
Turkey	81	75	65	75	114	74	71	76
Chicken	85	76	80	75	125	68	76	73
Similarity (%)	78	86	60	81	75	77	79	84

length of introns were listed in bp.
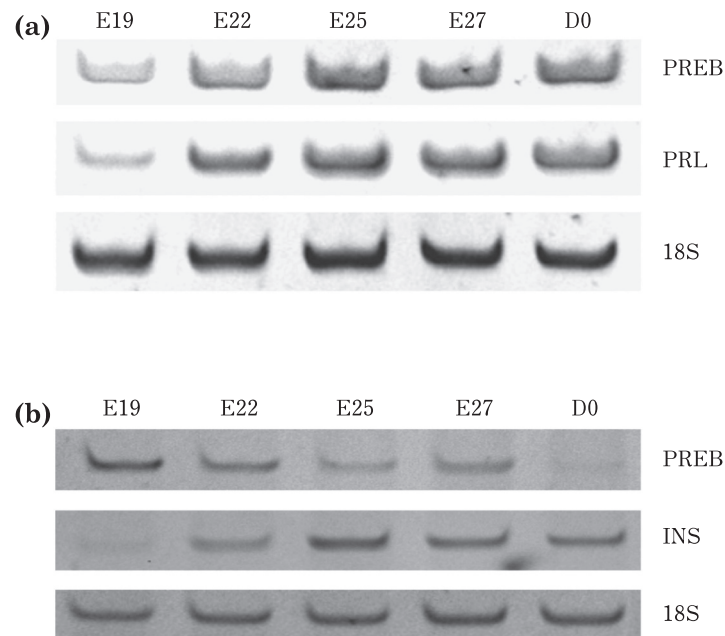
Fig. 5. **Example of RT-PCR analysis of turkey PREB, PRL, INS, and 18S.** PCR products were electrophoresed on 15% polyacrylamide gels. (a) PCR products from embryonic anterior pituitary gland. (b) PCR products from embryonic pancreas.

formes.

Changes in mRNA levels of PREB and PRL in the anterior pituitary gland were identified during embryogenesis. Due to the high GC contents of PREB cDNA, PREB cDNA had not been amplified under the real time PCR condition. Thus, semi-quantitative RT-PCR method which previously applied to measure the chicken PREB mRNA level (Hiyama *et al*., 2015) was used. Levels of PREB mRNA were low during the middle stage of embryogenesis and increased toward the end of embryonic development. The general tendency of changes observed in the turkey anterior pituitary gland was similar to that observed in chicken (Hiyama *et al*., 2015). However, some differences were observed. In the turkey, levels of PREB mRNA reached a maximum on day 25 (3 days before hatch) and subsequently did not change significantly between days 25 and 27, whereas in chicken, the highest levels were detected on day 20 (1 day before hatch: stage 45). In addition, significant increases in levels of mRNA between days 18 (stage 44) and 20 were observed in chicken. Difference of changes of mRNA levels of PREB

and PRL between chicken and turkey was unknown. Although features of developmental stage of chicken embryo have been reported (Hamburger and Hamilton 1951), differences of late stage of developing embryo between chicken and turkey have been shown by the detailed observation (Mun and Kosin 1960). Since the changes of chicken mRNA levels was observed between stage 42 and 45, the difference of a day showing maximum levels between chicken and turkey may not largely affects embryo development.

Interestingly, different changes of PRL mRNA levels during developmental stages were previously reported using different strain or breed of the chicken. Decrease of PRL mRNA levels between embryonic day 20 and hatching day was observed in the Gifujidori (Ishida *et al*., 1991), whereas no changes of PRL mRNA levels was observed in the White Leghorn (Hiyama *et al*., 2015). Similarly, increase of PRL mRNA levels between 27 of embryonic day and day of hatch was reported in the turkey (Bédécarrats *et al*., 1999). However, decrease of PRL mRNA levels was detected in this study. Thus, difference of changes of PRL mRNA levels
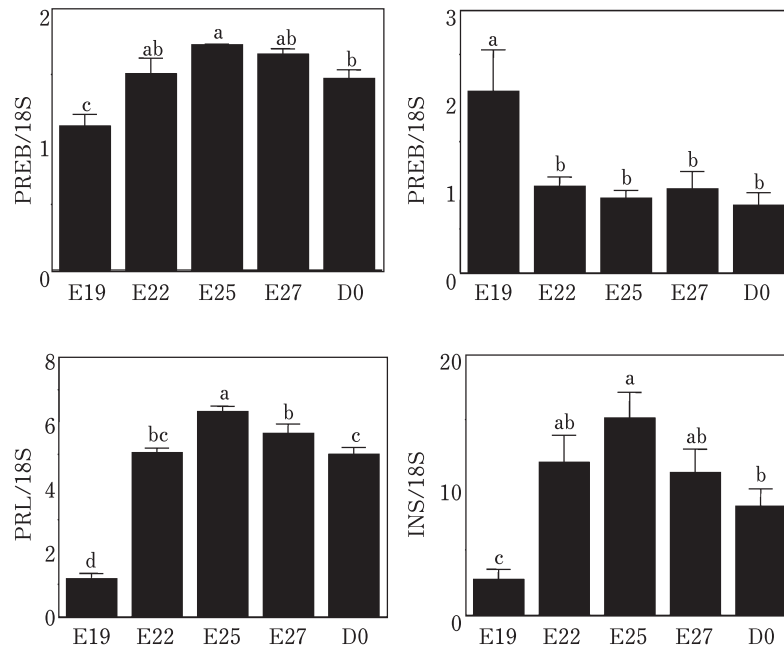
Fig. 6. **Changes in PREB, PRL, and INS mRNA expression during embryogenesis.** Means with different letters are significantly different from each other ($P<0.05$). Values represent mean$\pm$SEM.

may reflect difference of breed or line and under regulation of PREB. Because the regulatory region of the PREB gene has not been cloned and identified, the factors that affect PREB mRNA expression in the anterior pituitary gland remain unknown. To characterize the regulatory mechanism, cloning and functional characterization of the PREB promoter region is necessary.

Levels of PRL mRNA increased in concert with changes in levels of PREB, and the highest levels of both PRL and PREB were observed on day 25 (Fig. 5). The increase in PRL is likely to involve increases in Pit-1 mRNA, as previously demonstrated at a comparable time during embryogenesis in chicken (Hiyama *et al*., 2015). Pit-1 binds to the PRL promoter (Ohkubo *et al*., 2000, Kang *et al*., 2004), and additive effects of both Pit-1 and PREB increase PRL transcription (Fliss *et al*., 1999). Thus, it is possible that increases in the Pit-1 and PREB transcription factors would result in the increased levels of PRL observed in the present study.

In mammals, PREB is highly expressed in extrapituitary tissues such as the pancreas, where it acts in INS and glucokinase regulation (Ohtsuka *et al*., 2006; Muraoka *et al*., 2009). However, no studies have been performed in avian species. In the present study, the highest levels of pancreatic PREB mRNA were detected on day 19 of embryogenesis; however, these significantly decreased on day 22 and were subsequently maintained at lower levels until hatch (Fig. 6). Conversely, low levels of INS mRNA were detected on day 19, which subsequently increased between days 19 and 22 and reached a maximum on day 25. This change in INS tran-

scription is in agreement with other studies of chicken embryogenesis (Bagnell *et al*. 1989; Serrano *et al*., 1989; Alarcón *et al*., 1998); however, the associated changes in PREB have not been previously documented. In rats, PREB forms part of the transcriptional complex to partly regulate INS and GK gene transcription in response to glucose (Ohtsuka *et al*., 2006; Muraoka *et al*., 2009). It is open to speculation that PREB would play a similar role in turkey during embryogenesis and the transition to *ex ovo* life. Further studies are required to determine whether PREB functions as a *cis* or *trans* transcription factor in modulating pancreatic gene function in birds.

In conclusion, changes in expression levels of PREB mRNA are tissue dependent. The expression of PREB mRNA in the anterior pituitary gland may be related to the expression of PRL mRNA. However, the relationship between the expression of pancreatic INS mRNA and PREB mRNA requires further investigation.

Acknowledgments

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