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Hormonal regulation of platypus Beta-lactoglobulin and monotreme lactation protein genes

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ABSTRACT

Endocrine regulation of milk protein gene expression in marsupials and eutherians is well studied. However, the evolution of this complex regulation that began with monotremes is unknown. Monotremes represent the oldest lineage of extant mammals and the endocrine regulation of lactation in these mammals has not been investigated. Here we characterised the proximal promoter and hormonal regulation of two platypus milk protein genes, Beta-lactoglobulin (*BLG*), a whey protein and monotreme lactation protein (*MLP*), a monotreme specific milk protein, using *in vitro* reporter assays and a bovine mammary epithelial cell line (BME-UV1). Insulin and dexamethasone alone provided partial induction of *MLP*, while the combination of insulin, dexamethasone and prolactin was required for maximal induction. Partial induction of *BLG* was achieved by insulin, dexamethasone and prolactin alone, with maximal induction using all three hormones. Platypus *MLP* and *BLG* core promoter regions comprised transcription factor binding sites (e.g. STAT5, NF-1 and C/EBPα) that were conserved in marsupial and eutherian lineages that regulate caseins and whey protein gene expression. Our analysis suggests that insulin, dexamethasone and/or prolactin alone can regulate the platypus *MLP* and *BLG* gene expression, unlike those of therian lineage. The induction of platypus milk protein genes by lactogenic hormones suggests they originated before the divergence of marsupial and eutherians.

1. Introduction

Monotremes are the only living representatives of the egg laying subclass of prototherian mammals, which diverged from the theria (placental mammals) about 166–220 million years ago (Mya) (Lefèvre et al., 2010b). Theria subsequently split into marsupials and eutherians approximately 140 Mya (Lefèvre et al., 2010b). The monotreme lineage comprises three extant species, the platypus (*Ornithorhynchus anatinus*) and two genera of echidnas, short-beaked echidna (*Tachyglossus aculeatus*) and the long-beaked echidna (*Zaglossus* spp.) (Musser, 2005). The monotreme reproductive strategy is unique among mammals exhibiting a fascinating combination of reptilian and mammalian characters, as they lay eggs, yet have fur and females produce milk for their hatchlings (Graves, 1996). Platypus gestation is 15–21 days (Hawkins and Battaglia, 2009; Holland and Jackson, 2002), after which two to three eggs are laid in an earthen nesting burrow and eggs hatch after 10–12 days of incubation (Griffiths, 1978; Hawkins and Battaglia, 2009). The short-beaked echidna has a gestation of 21 days and a single egg is laid in a pouch and the young hatches after 10–10.5 days (Griffiths et al., 1969; Morrow et al., 2009). Following hatching monotreme mothers produce nutrient rich milk which sustains the young for a period of 114–145 days in the platypus (Grant and Griffiths, 1992; Hawkins and Battaglia, 2009; Holland and Jackson, 2002) and 160–210 days in the echidna (Morrow et al., 2009; Morrow and Nicol, 2012).

The monotreme mammary glands differ from therians by lacking nipples (Griffiths et al., 1969, 1973). The milk produced by monotreme mammary glands emerges from a cluster of ducts (areolae or milk patch) onto the female's abdomen and the young lick the milk directly from the abdominal surface (Griffiths, 1978). Monotreme young are born at an underdeveloped stage and milk must provide all the nutrients to support the large extent of growth that occurs outside the uterine environment of the mother (Blackburn, 1993). Moreover, given their strategic position of being the lowest mammals in the mammalian tree of evolution, monotreme milk is regarded as an ancestral form of milk. The hormonal

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regulation of mammary gland development has not been studied. Observations of seasonal variation in plasma progesterone and 17β-oestradiol during platypus pregnancy suggested that endocrine control of pregnancy in monotreme mammals was conserved and must have evolved in parallel to mammary gland development throughout marsupial and eutherian lineages (Hughes and Carrick, 1978; Nicol et al., 2005).

We previously reported the monotreme milk cells transcriptome and identified caseins (alpha-casein, beta-casein and kappa-casein) and whey proteins (β-lactoglobulin, lactoferrin, lysozyme, alpha-lactalbumin and other novel proteins) (Lefèvre et al., 2010a). Recently, we have characterised a highly expressed monotreme lactation protein (MLP) in monotreme milk with antibacterial properties and which is expressed throughout lactation period (Enjapoori et al., 2014).

The hormonal regulation of milk protein gene expression varies among mammals, but generally requires prolactin (PRL), adrenal glucocorticoids (usually cortisol) and insulin (Neville et al., 2002; Qian and Zhao, 2014). PRL is an essential endocrine regulator that primarily functions through binding to cell membrane prolactin hormone receptor (PRLR) and imposing a signature of milk protein gene expression (Watson and Burdon, 1996). The requirement of insulin for milk protein gene expression is supported by several observations in marsupial and eutherian species (Menzies et al., 2009, 2010; Neville et al., 2013). In mouse mammary gland explant culture, it was shown that insulin was absolutely required for casein gene expression (Bolander et al., 1981). Glucocorticoids alone have little to no ability to induce expression of milk protein genes (Puissant and Houdebine, 1991; Welte et al., 1994). However, it is well established that prolactin and glucocorticoid cooperate in regulation of milk protein gene transcription (Chomczynski et al., 1986; Doppler et al., 1989; Mukhopadhyay et al., 2001).

To date the hormonal regulation of monotreme milk protein gene expression has not been studied with the exception of the observation that injection of oxytocin induces milk ejection similar to that in marsupial and eutherian mammary glands (Griffiths, 1978). However, it has been assumed that monotreme milk protein genes are hormonally regulated in a similar way to marsupial and eutherian milk protein genes. The conservation of casein genes across mammalian lineages supports the hypothesis for similar hormonal regulation between mammalian lineages (Lefèvre et al., 2009), however, the exact mechanism of hormonal regulation of milk protein gene expression during lactation is still not understood (Griffiths, 1978). Hormonal regulation of monotreme milk protein gene expression may reveal similarities and differences among marsupial and therian lineages with respect to the presence of evolutionarily conserved lactation.

The objective of the current study was to characterise the proximal promoter region of the platypus milk protein genes *MLP* and *BLG*, and investigate the hormonal regulation of *MLP* and *BLG* in a first effort to better understand the endocrine regulation of lactation in monotremes. Our results provide the experimental evidence that a combination of insulin, dexamethasone and prolactin is required for maximal induction of both platypus *BLG* and *MLP* genes as has been observed in many other mammals. However a striking difference was the observation that insulin, dexamethasone and/or prolactin alone had the capacity for partial induction of the expression of these genes.

2. Material and methods

2.1. Sample collection and genomic DNA extraction

Platypus (*O. anatinus*) genomic DNA was a gift from Associate Professor Peter Temple-Smith (Monash University, Australia) and Dr. Andrew Weeks (Melbourne University, Australia). Short-beaked echidna (*T. aculeatus*) genomic DNA was extracted from liver tissue. The tissue was homogenised in DNA extraction buffer (100 mM NaCl, 20 mM Tris–HCl (pH 8.0), 20 mM EDTA and 10% SDS) and treated with RNase A (3 μg) for 2 h at 37 °C. The solution was further incubated with Proteinase K (200 μg/ml) overnight at 37 °C, after which DNA was isolated using a standard phenol/chloroform method. DNA was precipitated with absolute ethanol containing 10% sodium acetate (3 M, pH 5.2). The DNA pellet was washed with 70% ethanol and resuspended in 100 μl TE buffer.

2.2. Cloning of the platypus MLP and BLG promoter and echidna BLG promoter

The 5′ flanking promoter region of platypus *MLP* (2100 bp) and *BLG* (1253 bp) was amplified from platypus genomic DNA using primers based on the location of the transcription start site. Platypus specific primers were used to amplify both platypus *MLP*, *BLG* and echidna *BLG* promoter regions from genomic DNA. PCR was performed on 30 ng of genomic DNA using primers (Table 1) in a 25 μl total volume with Elongase enzyme mix (Invitrogen). Platypus *MLP* promoter PCR was conducted under the following conditions: 3 min denaturation at 94 °C followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min and the reaction was completed with a final extension for 5 min at 72 °C. PCR conditions for platypus and echidna *BLG* promoter were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 90 s, 72 °C final extension for 5 min and 4 °C to terminate the reaction. PCR products were separated on 0.8% agarose gel and purified using Gel extraction kit (Qiagen). Purified PCR products were cloned into TA cloning vector pGEM-T easy (Promega) and sequenced in both directions. The nucleotide sequences reported have been submitted to the GenBank with accession numbers, platypus MLP promoter KM491790, platypus BLG promoter KM491791 and echidna BLG promoter KM491792.

Table 1
Primers used for PCR amplification of the platypus and short-beaked echidna MLP and BLG promoters.

Gene	Primer	Primer sequence 5′–3′	Length	Restriction enzyme site
Primers for TA cloning				
Platypus MLP	F1	TTTGATGCCTGTCTCCTTCC	2100 bp	
	R1	GCCATGGTGAAGAGCAAGTC		
Platypus BLG	F1	ATGGCTGAATTGTCCAAAGC	1253 bp	
	R1	GCCAATGCTCAGCAGAAGA		
Primers for Luciferase constructs				
Platypus MLP	F2	ACAGTggtaccGCTCTGCACACAGTAAGC	2000 bp	KpnI-HF
	R2	CCATctcgagGGTGAAGAGCAAGTCTGGC		XhoI
Platypus BLG	F2	AAGggtaccATGGCTGAATTGTCCAAAGC	1226 bp	KpnI-HF
	R2	CTTCATaagcttTGTGCAAGCAGGGATCTC		HindIII

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