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1 **Regulation of the Ovine MT₁ Melatonin Receptor Promoter: Interaction between**
2 **Multiple Pituitary Transcription Factors at Different Phases of Development**

3

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23 The sequence of the ovine MT₁ gene described in this study has been submitted to the

24 Genbank database under accession number [AY524665](#)

25 Key words: Pitx-1; Egr-1; pars distalis; gonadotroph; pars tuberalis; E-box

26

27

28 **Abstract**

29

30 Pineal secretion of melatonin provides a neuroendocrine representation of the light-
31 dark cycle, which is used to synchronise daily and annual rhythms of physiology and
32 behaviour. In mammals, melatonin primarily acts through MT₁ melatonin receptors
33 that exhibit a highly restricted tissue distribution. Expression of MT₁ receptors is
34 subject to developmental and circadian control, which likely modulates the
35 physiological actions of melatonin. To investigate the mechanisms controlling MT₁
36 expression we cloned the proximal 1.5kb region of the ovine MT₁ promoter.

37 Sequence analysis revealed putative *cis*-elements for transcription factors
38 involved in pituitary development, namely Pitx-1 and Egr-1, and multiple putative E-
39 boxes, which are involved in both circadian and developmental gene regulation.
40 Nuclear protein from ovine pars tuberalis (PT) cells, a site of high endogenous MT₁
41 expression, stimulated gene expression from a MT₁ expression construct, indicating
42 the presence of a functional promoter. Pitx-1 was strongly expressed in the ovine PT
43 and stimulated MT₁ promoter activity in transfection assays. Co-transfection with
44 Egr-1 induced promoter-specific effects: Pitx-1-stimulated MT₁ activity was inhibited,
45 whereas β LH promoter activity was enhanced.

46 In addition to *Pitx-1* the circadian clock genes *Clock* and *Bmal1* were also
47 expressed in the PT. However, despite multiple putative E-boxes in the MT₁
48 promoter, transfected *Clock* and *Bmal1* were unable to regulate either basal or Pitx-1-
49 stimulated MT₁ promoter activity.

50 The current data, in conjunction with our previous study of the rat MT₁
51 promoter, suggests a general model in which melatonin receptor expression in the
52 mammalian pituitary is determined by the developmentally changing balance between
53 stimulatory and inhibitory transcription factors. Furthermore, our data suggest that
54 circadian variation in MT₁ gene expression does not depend upon the direct action of
55 circadian clock genes on E-box *cis*-elements.

56 1. Introduction

57

58 The nocturnal secretion of melatonin from the pineal gland provides an endocrine
59 representation of both circadian and seasonal time (Simonneaux and Ribelayga,
60 2003). In mammals, melatonin acts via two major receptor subtypes, of which MT₁
61 (previously termed Mel_{1a}) is the more prevalent (Reppert et al., 1996). Throughout
62 life, melatonin receptor populations exhibit considerable plasticity. During early
63 stages of development, melatonin receptors are transiently expressed in multiple
64 neural and endocrine tissues (Davis, 1997). In adult animals, many melatonin
65 receptor populations exhibit 24-hour variation in receptor density (Piketty and
66 Pelletier, 1993; Gauer et al., 1994; Recio et al., 1996; Schuster et al., 2001; Poirel et
67 al., 2002). Currently, the significance of this plasticity and the mechanisms that drive
68 changes in melatonin receptor expression are poorly understood.

69 The best characterised region of foetal melatonin receptor expression is the
70 pituitary pars distalis (PD). In rodents, melatonin binding sites are present in the
71 pituitary after approximately 12-15 days of gestation (Rivkees and Reppert, 1991;
72 Williams et al., 1991; Duncan and Davis, 1993) and decline over the first 2-3 weeks
73 of neonatal life (Vanecek, 1988; Williams et al., 1991; Vanecek and Kosar, 1994). In
74 the ovine PD, melatonin binding is present from 31 days of gestation through to birth,
75 although labelling becomes more diffuse with gestational age (Helliwell and
76 Williams, 1994). Current evidence suggests that these binding sites correspond to
77 MT₁ receptors expressed in gonadotroph cells (Martin and Klein, 1976; Martin et al.,
78 1982; Johnston et al., 2003b; 2006). The development and regulation of this cell type
79 is dependent on many transcription factors, including pituitary homeobox-1 (Pitx-1;
80 Szeto et al., 1999) and the lineage specific factors early growth response factor-1

81 (Egr-1; Lee et al., 1996; Topilko et al., 1998). Pitx-1 is of particular interest as it
82 stimulates promoter activity of many pituitary-specific genes (Tremblay et al., 1998)
83 and is also highly expressed in the pituitary pars tuberalis (PT) (Lanctot et al., 1999),
84 an important site of melatonin action in seasonal mammals (Hazlerigg et al., 2001 ;
85 Johnston, 2004).

86 Putative *cis*-elements for these gonadotroph-associated transcription factors
87 have recently been identified in the rat MT₁ promoter (Johnston *et al.* 2003a). Pitx-1
88 and Egr-1 were subsequently shown to be potent regulators of MT₁ promoter activity
89 (Johnston et al., 2003b). The MT₁ receptors in rat gonadotroph cells appear to be
90 down-regulated by the onset of hypothalamic gonadotrophin-releasing hormone
91 (GnRH) secretion, suggesting that the establishment of a mature hypothalamic input
92 pathway shuts down melatonin sensitivity in these cells in this species (Johnston et
93 al., 2003b). Whether the same pathway of developmental control also applies in
94 larger non-rodent species, in which developmental organisation is far more advanced
95 at the time of birth (precocial), or in other endocrine tissues showing transient
96 melatonin receptor expression, remains to be established.

97 Despite the developmental loss of melatonin receptors in PD gonadotroph
98 cells, MT₁ mRNA remains strongly expressed in the “PT-specific” thyrotrophs of the
99 adult PT (Klosen et al., 2002; Dardente et al., 2003). Daily rhythms of both MT₁
100 expression and melatonin binding sites have been reported in the PT and
101 suprachiasmatic nuclei (SCN) of the hypothalamus (Piketty and Pelletier, 1993; Gauer
102 et al., 1994, Recio et al., 1996; Schuster et al., 2001; Poirel et al., 2002). Furthermore,
103 both of these tissues rhythmically expresses multiple circadian clock genes (Reppert
104 and Weaver, 2001; Lincoln et al., 2002; Hazlerigg et al., 2004; Johnston et al., 2005;
105 von Gall et al, 2005), whose possible role in melatonin receptor expression has not yet

106 been investigated. These clock genes could interact directly with the MT₁ promoter,
107 or modulate the activity of other factor(s), such as Pitx-1 expressed in the PT, to
108 further regulate receptor plasticity.

109 In order to further explore the mechanisms through changes in MT₁ expression
110 occur we have cloned a fragment of the ovine MT₁ promoter. We identified putative
111 *cis*-elements for a range transcription factors, including those involved in circadian
112 gene expression, and in pituitary differentiation. We then assessed the functional
113 relevance of these elements using a combination of *in vitro* transcription and reporter
114 assays, together with analysis of gene expression *in vivo*.

115

116

117 **2. Materials and Methods**

118

119 *2.1. Cloning and sequence analysis*

120

121 An ovine genomic library (Barrett et al., 1994) was screened with a ³²P-labelled
122 oligonucleotide for exon 1 of the ovine MT₁ receptor sequence. Hybridising plaques
123 were plaque purified with several rounds of replating and screening. One clone was
124 analysed by restriction digestion followed by Southern analysis for a fragment likely
125 to contain ~2kb of sequence upstream of the coding region start site. Based on the
126 known restriction digestion pattern for the ovine MT₁ sequence, a NotI/SphI fragment
127 was deemed to fulfil these requirements. This fragment was cloned into the NotI/SphI
128 site of the vector pGEM-T (Promega, UK). The sequence of the cloned fragment was
129 determined in both directions using ABI dye terminator sequencing reagents and

130 analysed on an ABI 377 DNA sequencer. The fragment was 1.68kb in length
131 containing 111bp of coding sequence.

132 Restriction fragments were prepared with lengths as indicated in the
133 manuscript, and subcloned into the luciferase reporter vector pGL3-Basic (Promega
134 UK), according to the manufacturer's protocol. These fragments covered regions -
135 1534 to -26, -716 to -26, -440 to -26 and -90 to -26, relative to the translation start
136 site.

137 Putative transcription factor *cis*-elements were selected using MatInspector
138 software (Quandt et al., 1995; Cartharius et al., 2005).

139

140 2.2. *In vitro* transcription run off assay

141

142 Nuclear extracts from a primary cell culture of ovine PT cells were prepared using the
143 protocol of Andrews and Faller (1991) using up to 10^7 cells. Briefly cells were
144 resuspended in 400 μ l of ice cold buffer A (10mM HEPES-KOH pH 7.9 at 4°C,
145 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, 0.2mM PMSF) and allowed to swell on ice
146 for 10min. Cells were then spun at full speed in a microfuge for 10sec at room temp.
147 The nuclear pellet was then resuspended in 200 μ l of buffer C (20mM HEPES-KOH
148 pH 7.9 at 4°C, 1.5mM MgCl₂, 420mM NaCl, 25% glycerol, 0.2mM EDTA, 0.5mM
149 DTT, 0.2mM PMSF). The nuclear extract was then spun at full speed in a microfuge
150 for 2min. The resultant supernatant was dialysed overnight in buffer D (24mM
151 HEPES pH 7.9 at 25°C, 24% (v/v) glycerol, 120mM KCl, 0.24mM EDTA, 0.6mM
152 DTT, 0.5mM PMSF. Dialysis was carried out in 0.5-3ml cassettes (3500MW,
153 Pierlabo Scientific) according to the manufacturer's protocol.

154 The MT₁ promoter-luciferase constructs were linearised with Sall, facilitating
155 a run off transcript of 2kb. The *in vitro* transcription assay was carried out in a
156 volume of 100ul containing 12mM HEPES (pH 7.9), 12% glycerol, 0.3mM DTT,
157 0.12mM EDTA, 60mM KCl, 12mM MgCl₂, 600μM each rNTP, 10-20μg/ml
158 linearised DNA template. The transcription reaction was incubated at 30°C for 90min
159 at which point RQ1 DNase (Promega UK, 2U per ug DNA) was added to the reaction
160 and incubated for a further 30-60min at 37°C. The synthesised RNA was purified
161 with the RNeasy kit (RNA clean-up protocol, Qiagen UK). The RNA was eluted in
162 30μl DEPC water and concentrated by overnight ethanol precipitation at -20°C
163 following the addition of a one tenth volume of 3M NaAc pH5. The RNA was then
164 resuspended in 5ul of DEPC treated water and prepared for electrophoresis on a
165 denaturing formaldehyde 1% agarose gel. Following electrophoresis, the RNA was
166 transferred to a nylon membrane (Dupont UK). The RNA was UV cross-linked. The
167 membrane was then probed with a ³²P-labelled DNA fragment containing the
168 luciferase coding sequence prepared from the plasmid pGL3 basic (Promega, UK).

169

170 2.3. Transient transfections

171

172 Assays were performed as described previously (Johnston et al., 2003b). Briefly,
173 COS-7 cells at 50-80% confluence were transfected with appropriate plasmid
174 combinations, using FuGene6 (Roche), according to the manufacturer's protocol.
175 Forty-eight hours after transfection, cells were washed, enzymatically detached and
176 pelleted. Cells were then resuspended in DMEM and aliquots were either assayed for
177 β-galactosidase activity or lysed in Steady-Glo reagent (Promega) prior to

178 measurement of luciferase activity. Each treatment was performed in triplicate per
179 experiment.

180

181 2.4. *In situ hybridisation*

182

183 Twenty micron coronal sections were cut through the ovine PT and stored at -80°C.
184 Radioactive *in situ* hybridisation was then performed as described previously
185 (Messenger *et al.* 1999). Hybridised sections were then exposed to autoradiographic
186 film (Kodak X-omat) for 5 days. Resultant images were digitised using an Epson
187 transmittance scanner.

188 The riboprobes used in this study have been previously used for analysis of
189 gene expression (Messenger *et al.*, 1999; Lincoln *et al.*, 2002; Johnston *et al.*, 2003a).

190 The templates for *Clock* and *Bmal1* probe synthesis were cloned by reverse
191 transcriptase PCR of total RNA prepared from ovine PT tissue. The riboprobe
192 template for Pitx-1 was cloned by PCR from a Pitx-1 expression vector supplied by
193 Dr J Drouin. All templates were ligated into pGem-T-easy (Promega, UK), according
194 to the manufacturer's instructions. The cDNA templates corresponded to bases 556-
195 1009 of Genbank accession number AF000998 (*Clock*), 1273-1677 of Genbank
196 accession number AF070917 (*Bmal1*), and 544-1140 of Genbank accession number
197 NM_011097 (*Pitx-1*).

198

199 2.5. *Statistical analysis*

200

201 Transfection data are expressed as mean \pm SEM. Data were analysed with SigmaStat
202 2.0 software (Jandel Corp.), using 1-way ANOVA with Student-Newman-Keuls post-
203 hoc analysis. Statistical significance was defined as $P < 0.05$.

204

205

206 **3. Results**

207

208 *3.1. Sequence analysis of the ovine MT₁ promoter*

209

210 We cloned a 1.68kb region of the ovine MT₁ gene, extending 1.57kb upstream of the
211 putative translation start site. Primary sequence analysis revealed multiple putative
212 *cis*-elements for transcription factors essential to gonadotroph development (Table 1)
213 and also multiple E-box hexamers (CANNTG).

214 The 1.68kb sequence has been deposited in Genbank under accession number
215 AY524665.

216

217 *3.2. Ability of the ovine MT₁ promoter fragment to support transcription*

218

219 We next determined whether the cloned MT₁ sequence was capable of supporting
220 gene expression. An *in vitro* transcription assay was performed using full length (-
221 1534bp) and truncated (-716bp) MT₁-luciferase constructs, in conjunction with
222 nuclear extract from the ovine PT, a key site of melatonin receptor expression *in vivo*
223 (Morgan *et al.* 1994). Luciferase mRNA transcription was strongly supported by both
224 MT₁ promoter constructs, but only weakly supported by the pGL3-basic vector and
225 absent following omission of either DNA or nuclear extract from the assay (Fig. 1).

226

227 *3.3. Promoter regulation by gonadotroph-associated transcription factors*

228

229 The homeobox transcription factor Pitx-1, previously shown to stimulate the rat MT₁
230 promoter (Johnston et al., 2003b), was highly expressed in the ovine PT (Fig. 2A), a
231 well-defined site of MT₁ expression. Primary sequence analysis revealed a putative
232 proximal *cis*-element for Pitx-1 within the promoter regions of the ovine MT₁ gene
233 (Fig. 2B), suggesting a conserved role for Pitx-1 in melatonin receptor regulation. In
234 addition, putative *cis*-elements for the gonadotroph-associated factor Egr-1 was
235 observed within the ovine MT₁ sequence (Table 1).

236 The ability of these transcription factors to regulate oMT₁ promoter activity
237 was examined using transient transfection experiments. Basal promoter activity was
238 strongly stimulated by co-transfection of COS-7 cells with full-length (-1534bp)
239 oMT₁ reporter construct and Pitx-1 (Fig. 2C). This stimulation persisted in a
240 truncated (-716bp) promoter construct, but was greatly attenuated in a promoter
241 construct (-90bp) that didn't contain the putative Pitx-1 *cis*-element (Fig. 2C). Low
242 levels of Pitx-1-enhanced promoter activity have been previously reported for
243 negative control vectors and likely represent a non-specific effect on reporter activity
244 (Tremblay and Drouin, 1999; Johnston et al., 2003b).

245 In contrast to the effect of Pitx-1, basal oMT₁ promoter activity was not
246 altered by transfection with Egr-1 (Fig. 3A). Co-transfection of Pitx-1 and Egr-1
247 revealed that Pitx-1-stimulated promoter activity was strongly inhibited by Egr-1 (Fig.
248 3A). This effect of Egr-1 was dose-dependent (Fig. 3A) and qualitatively different
249 from its strong synergistic interactions with Pitx-1 at the LH β promoter (Fig. 3B) and
250 reported previously by others (Dorn et al., 1999; Tremblay and Drouin, 1999).

251 In order to map functional *cis*-elements for Egr-1, we repeated Ptx-1/Egr-1 co-
252 transfection experiments using truncated promoter constructs. Due to the requirement
253 of Pitx-1 stimulation to observe Egr-1 activity, we conducted experiments using -716
254 and -440bp fragments (Fig. 4A). For both constructs, Egr-1 potently inhibited Pitx-1-
255 stimulated promoter activity (Fig. 4B, C). These data indicate that functional binding
256 sites for both Pitx-1 and Egr-1 lie within the proximal region of the ovine MT₁
257 promoter, consistent with the *in silico* primary sequence analysis (Table 1).

258

259 3.4. Promoter regulation by circadian clock gene elements

260

261 Finally we examined the possible role of putative E-box elements observed within the
262 oMT₁ promoter (*data not shown*) in the reported daily rhythms of PT melatonin
263 receptor expression (Piketty and Pelletier, 1993). The mRNA of circadian clock
264 transcription factors Clock and Bmal1 were both expressed in the ovine PT (Fig. 5A,
265 B). However, despite their ability to stimulate arginine vasopressin (AVP) promoter
266 activity (Fig. 5c), Clock and Bmal1 were unable to stimulate either basal (Fig. 5C) or
267 Pitx-1-stimulated (Fig. 5D) oMT₁ promoter activity *in vitro*.

268

269

270 4. Discussion

271

272 Studies of melatonin receptor expression suggest that multiple neuroendocrine tissues
273 are transiently sensitive to melatonin secretion in developing mammals. The function
274 of these receptor populations is poorly understood. We have studied a 1.5kb fragment
275 of the ovine MT₁ promoter in an attempt to understand the molecular and cellular

276 mechanisms driving expression of this receptor. Our data show that key transcription
277 factors involved in pituitary gland development potently modulate MT₁ promoter
278 activity in a promoter-specific manner. These observations support the hypothesis
279 that transient melatonin sensitivity in developing neuroendocrine tissues is closely
280 related to events involved with cell differentiation. In contrast, the circadian clock
281 genes *Clock* and *Bmal1* were not able to regulate MT₁ promoter activity, suggesting
282 that 24-hour changes in melatonin receptor expression are not directly driven by
283 circadian clock genes acting via E-boxes.

284 **There are 2 main subtypes of mammalian melatonin receptor, MT₁ and**
285 **MT₂. Our study has focussed on the MT₁ receptor, which exhibits far greater**
286 **expression within the neuroendocrine system (Reppert et al., 1996).**
287 **Furthermore, although some species of hamster are believed to lack a functional**
288 **MT₂ receptor, their neuroendocrine physiology remains sensitive to effects of**
289 **melatonin, indicating that MT₁ is also the most functionally relevant subtype in**
290 **this system (Weaver et al., 1996).** We have recently shown that the rat MT₁
291 promoter contains multiple putative *cis*-elements for gonadotroph-associated
292 transcription factors (Johnston et al., 2003a), and that some of these factors potently
293 regulate promoter activity (Johnston et al., 2003b). In this paper, we show that
294 putative elements for the transcription factors Pitx-1 and Egr-1 are also located in the
295 ovine MT₁ promoter, suggesting a possible conservation of promoter regulation
296 between species. This high level of similarity also extends to the regulation of MT₁
297 promoter activity. Both the rat and ovine promoters are strongly stimulated by Pitx-1
298 *in vitro* which, combined with the high level of Pitx-1 expression in the PT,
299 strengthens the body of evidence suggesting that Pitx-1 is an important regulator of
300 pituitary melatonin receptor expression *in vivo*.

301 Egr-1 strongly attenuates Pitx-1-stimulated activity of both rat and ovine MT₁
302 promoters. This is in marked contrast to the high level of synergism between Pitx-1
303 and Egr-1 at the gonadotroph-specific LHβ promoter (Tremblay and Drouin, 1999;
304 Johnston et al., 2003b). The expression of Egr-1 in gonadotroph cells is stimulated by
305 the onset of pulsatile GnRH secretion (Dorn et al., 1999; Tremblay and Drouin, 1999),
306 which occurs during the same time window as the developmental decrease in
307 melatonin receptor expression in the rat (Vanecek, 1988; Williams et al., 1991; Ebling
308 and Cronin, 2000). In sheep, the formation of the hypothalamic-pituitary portal
309 circulation and the appearance of GnRH terminals occur by 45 and 60 days of
310 gestation, respectively (Matwijiw et al., 1989; Polkowska, 1995), leading to strong
311 expression of LHβ in the pituitary by day 70 of gestation (Brooks et al., 1992).
312 Although some melatonin binding sites remain in the ovine PD throughout gestation
313 until birth, these sites appear diffuse from 59 days of gestation (Helliwell and
314 Williams, 1994). We therefore predict that this change in the pattern of PD melatonin
315 binding sites is due to the developmental activation of pulsatile GnRH secretion
316 inducing Egr-1 expression in ovine gonadotroph cells. Thus the relationship between
317 GnRH secretion and MT₁ expression in the developing PD may be conserved between
318 species, irrespective of the length of gestation *per se*.

319 Melatonin receptors are strongly expressed in the adult PT of all species thus
320 far examined, with the possible exception of humans (Morgan et al., 1994). Co-
321 localisation studies have revealed MT₁ mRNA in the “PT-specific” thyrotrophs of the
322 rodent PT (Klosen et al., 2002; Dardente et al., 2003). The failure to observe co-
323 localisation between MT₁ and LHβ mRNAs in adult animals is consistent with our
324 hypothesis that GnRH inhibits gonadotroph MT₁ expression, at least in rodent species.
325 Furthermore, the continued high expression of Pitx-1 in the PT of adult animals

326 (Lanctot et al., 1999; Johnston et al., 2003a; this study) suggests that the retention of
327 MT₁ expression in PT-specific thyrotrophs is a result of continued expression of
328 stimulatory factor(s), such as Pitx-1, coupled with an insensitivity to developmental
329 cues, such as GnRH secretion, that turn off expression in other pituitary cell types.

330 Diurnal rhythms of melatonin receptor expression have been reported in the
331 PT of different species (Piketty and Pelletier, 1993; Gauer et al., 1994; Recio et al.,
332 1996; Schuster et al., 2001). To date, evidence suggests that this phenomenon is at
333 least partially driven by melatonin-induced receptor autoregulation (Gauer et al.,
334 1994; Barrett et al., 1996; Guerrero et al., 2000; Schuster et al., 2001). However, the
335 identification of putative E-box elements in the oMT₁ promoter suggested to us that
336 rhythmic melatonin receptor expression might be additionally driven by the direct
337 interaction between the ovine MT₁ promoter and circadian clock genes recently
338 reported in the PT of multiple mammalian species (Lincoln et al., 2002; Hazlerigg et
339 al., 2004; Johnston et al., 2005; von Gall et al., 2005). Such a mechanism is believed
340 to drive the rhythmic expression of some “circadian output” genes, which do not form
341 a core part of the molecular circadian mechanism (Jin et al., 1999; Ripperger et al.,
342 2000).

343 In our transfection assays, the clock genes *Clock* and *Bmal1* were unable to
344 modulate the activity of basal or Pitx-1-stimulated promoter activity. This result
345 therefore reinforces the requirement of an appropriate E-box hexamer and/or flanking
346 regions to generate a functional “circadian E-box” *cis*-element, as has been discussed
347 elsewhere (Kyriacou and Rosato, 2000; Munoz et al., 2002). Although our data
348 support the hypothesis that rhythmic melatonin receptor expression is primarily driven
349 by receptor autoregulation (Gauer et al., 1994; Barrett et al., 1996; Guerrero et al.,
350 2000; Schuster et al., 2001), it is possible that daily MT₁ regulation occurs via

351 circadian transcription elements other than E-boxes, such as D-boxes and retinoid
352 orphan receptor elements (ROREs; Ueda et al., 2005). Indeed, we have identified
353 putative D-box elements within the MT₁ promoter sequence and these are the subject
354 of ongoing work.

355 Although the putative E-box elements do not appear to be involved in
356 circadian regulation of MT₁ expression, we cannot rule out functional significance of
357 these promoter regions. Indeed, basic helix-loop-helix transcription factors, which act
358 via E-boxes, are involved in both lineage commitment and tissue-specific
359 transcription in multiple tissues, including the pituitary gland (Crews, 1998; Poulin et
360 al., 2000; Lamolet et al., 2004). It is therefore possible that the developmental
361 regulation of MT₁ expression in the pituitary and/or other tissues that transiently
362 express melatonin receptors is at least partially due to the interaction between E-boxes
363 and basic helix-loop-helix proteins.

364 The data described here and in previous work (Johnston et al., 2003a; 2003b;
365 2006) provide strong evidence for the regulation of melatonin receptor expression by
366 transcription factors essential to pituitary development. The significance of the
367 transient appearance of melatonin receptors in many developing tissues (Vanecek,
368 1988; Rivkees and Reppert, 1991; Williams et al., 1991; Helliwell and Williams,
369 1994) is not understood, but it probably allows foetal tissues to synchronise to one
370 another and to the light dark cycle in response to maternal melatonin secretion (Davis,
371 1997). How this enhances the developmental process is unclear, but the
372 demonstration that circadian clocks have a direct influence on the cell cycle (Dekens
373 et al., 2003; Matsuo et al., 2003) suggests that the role of melatonin may be to
374 optimise the control of cell proliferation in rapidly developing structures. Testing this
375 idea is an important goal for future studies.

376

377

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379

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390

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611 central melatonin binding sites in the rat. *Endocrinology* 128, 2083-2090.

612 Table 1. Putative *cis*-elements for gonadotroph-associated transcription factors in the
 613 oMT₁ promoter.

614

Factor	Position	Sequence	Orientation
Egr-1	-174 to -158	CCGCACGGGGGCGCTGT	+
	-120 to -104	CCCGGGCGGGGCGGCAG	+
	-60 to -44	AGGCGGCGGGGCGGGAG	+
Pitx-1	-338 to -322	AACCTAAGCCCCCTCC	-

615

616 For each element, core similarity = 1.0 and matrix similarity > 0.86. Positions are
 617 expressed relative to ATG.

618 **FIGURE LEGENDS**

619

620 Fig 1. Nuclear protein extracts from ovine pars tuberalis cells drive transcription from
621 ovine MT₁ promoter sequences. Ovine PT nuclear extract stimulates transcription
622 from both -1534 and -716bp MT₁ promoter constructs. Representative
623 autoradiograph of an *in vitro* transcription assay (see Materials and Methods for
624 details). Lane 1: full-length (-1534bp) MT₁ construct; Lane 2: truncated (-716bp)
625 MT₁ construct; Lane 3: no DNA; Lane 4: pGL3-basic plasmid; Lane 5: no cell extract.
626

627 Fig 2. Pitx-1 stimulates ovine MT₁ promoter activity. (A) Autoradiograph showing
628 expression of *Pitx-1* mRNA in the ovine PT. Scale bar =1 mm. (B) Schematic
629 representation of the ovine MT₁ promoter; P, Pitx-1 consensus sequence. Putative
630 Pitx-1 *cis*-elements were identified using MatInspector software (Quandt et al., 1995;
631 Cartharius et al., 2005). (C) Pitx-1 stimulates ovine MT₁ promoter activity in
632 transient transfection assays. Strong promoter activation occurs in full-length (-
633 1534bp) and truncated (-716bp) promoter fragments that contain the putative Pitx-1
634 *cis*-element, but is greatly reduced in a further truncated (-90bp) construct that lacks
635 this site. The dose of Pitx-1 expression vector used was 250ng per well. Values are
636 relative to the same reporter construct without co-transfection of Pitx-1. *P<0.05 vs
637 fold induction of the -90bp promoter construct.

638

639 Fig 3. Egr-1 inhibits Pitx-1-stimulated activity of the ovine MT₁ promoter. (A) Egr-1
640 inhibits Pitx-1-stimulated MT₁ promoter activity in a dose-dependent manner. (B)
641 Pitx-1 synergises with Egr-1 to stimulate the LHβ promoter. All experiments used the
642 full-length (-1534bp) MT₁ promoter construct and a dose of 250ng of each expression

643 vector per well, unless otherwise stated. *P<0.05 vs basal promoter activity; #P<0.05
644 vs Pitx-1-stimulated promoter activity.

645

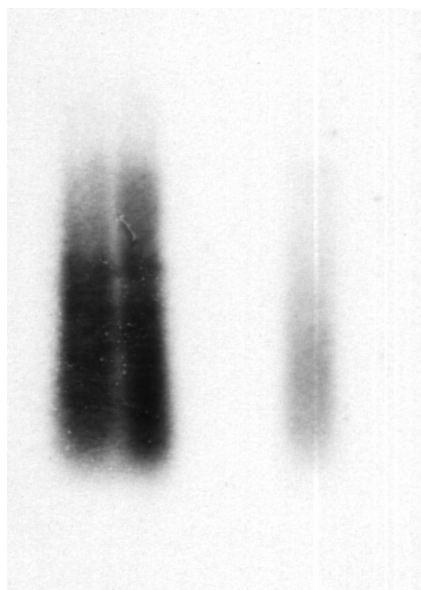
646 Fig 4. Egr-1 inhibits Pitx-1-stimulated activity of truncated ovine MT₁ promoter
647 constructs. (A) Schematic representation of truncated MT₁ promoter constructs. E,
648 putative Egr-1 consensus sequence; P, Pitx-1 consensus sequence. (B-C) Egr-1
649 attenuates Pitx-1-stimulated activity of (B) -716bp and (C) -440bp MT₁ constructs.
650 Experiments used 250ng of each expression vector per well. *P<0.05 vs basal
651 promoter activity; #P<0.05 vs Pitx-1-stimulated promoter activity.

652

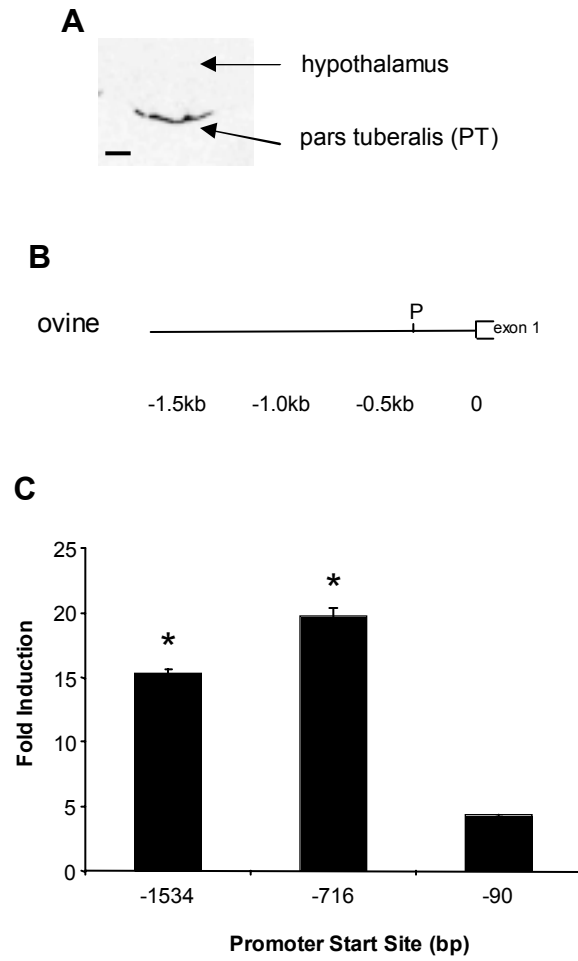
653 Fig 5. The circadian clock genes *Clock* and *Bmal1* stimulate AVP, but not ovine
654 MT₁ promoter activity. Autoradiographs show expression of (A) *Clock* and (B)
655 *Bmal1* mRNA in the ovine PT. Scale bar =1mm. (C) *Clock* and *Bmal1* stimulate
656 basal AVP, but not MT₁ promoter activity. (D) *Clock* and *Bmal1* are unable to
657 stimulate Pitx-1-stimulated MT₁ promoter activity. (C-D) Experiments used the full-
658 length (-1534bp) MT₁ promoter construct and 250ng of expression vector per well.
659 *P<0.05 vs basal promoter activity.

660

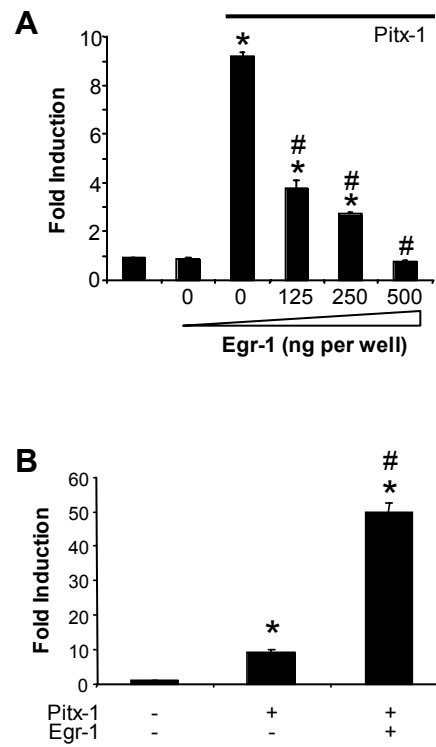
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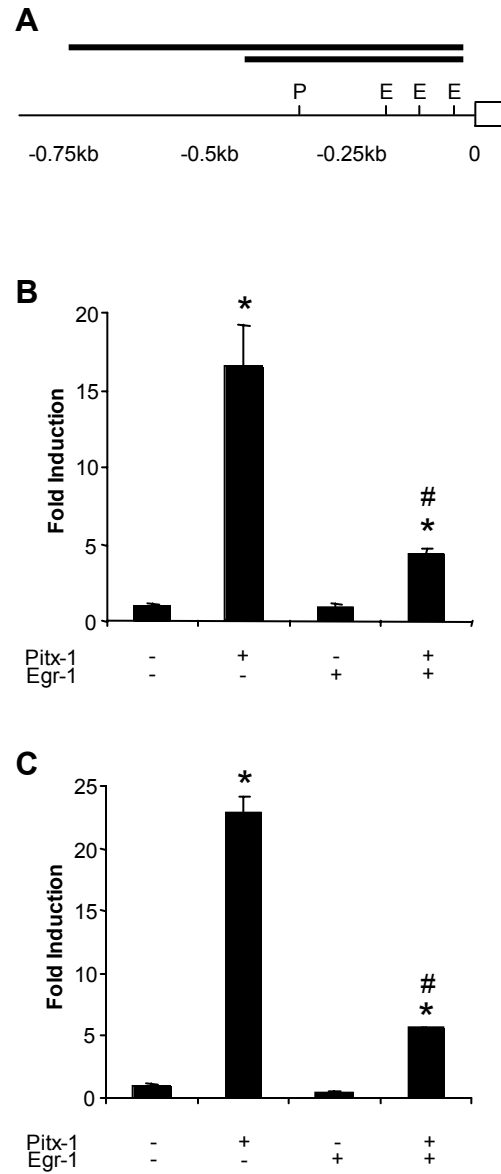
Johnston et al Fig 1



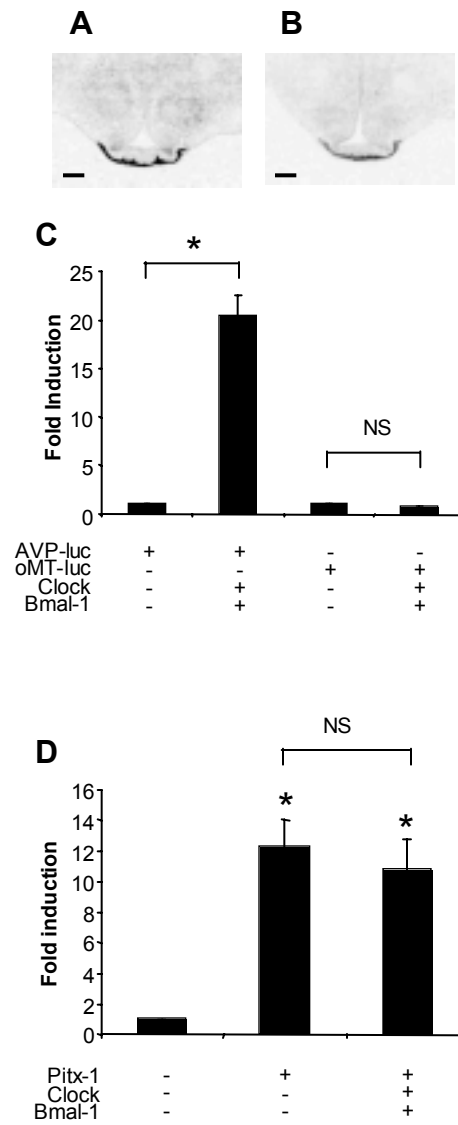
Johnston et al Fig 2



Johnston et al Fig 3



Johnston et al Fig 4



Johnston et al Fig 5