Oestrogenic regulation of an *egr-1* transgene in rat anterior pituitary

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Abstract

The C₂H₂ zinc-finger transcription factor Egr-1 has previously been shown to play an essential role within the endocrine system as a molecular determinant of LH β -subunit synthesis in anterior pituitary gonadotrophs. The extent to which Egr-1 may be a dynamic mediator of changes in gonadotroph function during the oestrous cycle is currently unclear. We have recently produced a novel rat transgenic model of *egr-1* gene function in which proximal regions of the rat *egr-1* gene drive expression of a reporter gene. In the present study, we have investigated the expression and physiological regulation of our *egr-1/d4* enhanced green fluorescent protein (EGFP) transgene in the female rat pituitary gland. *In situ* hybridization analysis has revealed anterior pituitary-specific expression that is limited to a sub-population of cells that includes immunohistochemically defined gonadotrophs. Expression of the transgene is up-regulated 5-fold following ovariectomy. The transgene also exhibits regulated expression during the oestrous cycle, mRNA levels being significantly raised on pro-oestrus. Using an explant culture system, we have also demonstrated a direct stimulatory effect of 17 β -oestradiol on anterior pituitary transgene and *egr-1* expression. The acute response of *egr-1* to an oestrogenic stimulus is attenuated by the MEK (MAPK kinase) inhibitor U0126, and is accompanied by increased levels of phospho-p44/42 MAPK protein, suggesting regulation of *egr-1* through a MAPK-linked pathway in the pituitary. These findings provide further evidence of cyclical endocrine regulation of *egr-1* in the rat, demonstrate that proximal sequences of the *egr-1* gene mediate endocrine-regulated expression, and indicate a novel pathway through which pituitary transcriptional responses to oestrogen may be mediated.

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Introduction

Early growth response gene-1 (*egr-1*; also termed *NGFI-A*, *zif268*, *krox-24*) is one member of a transcription factor gene family that includes three related genes (*egr-2*, *3* and *4*; see O'Donovan *et al.* 1999). Extensive analysis of the roles of the DNA-binding protein Egr-1 has revealed important, and unique, roles in fertility (Lee *et al.* 1996, Topilko *et al.* 1998), memory (Jones *et al.* 2001), and the ischaemic stress response (Yan *et al.* 2000). The first of these roles is of major relevance to endocrinology, because fertility is Egr-1-dependent due to transcriptional regulatory activity of Egr-1 on the pituitary hormone gene that codes for the luteinizing hormone (LH) β -subunit (Lee *et al.* 1996, Topilko *et al.* 1998; see also Dorn *et al.* 1999,

Tremblay & Drouin 1999, Wolfe & Call 1999, Sevetson *et al.* 2000, Duan *et al.* 2002). Recent studies have shown that Egr-1 may also affect fertility through regulation of LH receptor gene expression (Topilko *et al.* 1998, Yoshino *et al.* 2002).

The functional association between Egr-1 and the reproductive axis has been explored mainly at the developmental level using null-mutant mice (Lee *et al.* 1996, Topilko *et al.* 1998), but our recent studies have provided evidence of an additional association, at a physiological level. Thus, we have described cyclical changes in anterior pituitary Egr-1 activity that correlate with cyclical changes in the reproductive axis in adult female rats (Slade & Carter 2000). In other studies (Knight *et al.* 2000), we have shown that the nuclear 75 kDa form of Egr-1 protein is primarily co-localized with LH

β -subunit-expressing cells in adult rat anterior pituitary. Taken together, these findings indicate that *Egr-1* might play a role in mediating changes in gonadotroph function during the rat oestrous cycle, possibly contributing to the cyclical changes in LH release (Butcher *et al.* 1974, Fink 1979). This potential role is obscure, however, because gonadotroph function is regulated at multiple levels, and many different molecular factors have now been shown to be associated with this axis (see Brown & McNeilly 1999). Currently there is no direct evidence to link physiological changes in *Egr-1* expression (Slade & Carter 2000) with changes in gonadotroph gene (LH β -subunit, or otherwise) expression because the null-mutant genetic models (Lee *et al.* 1996, Topilko *et al.* 1998) lack *Egr-1* prior to the establishment of reproductive cycles, and adult-onset genetic models have yet to be developed. Consequently, further studies are required to address the physiological role of *Egr-1* in the pituitary.

We have recently begun an investigation of *egr-1* promoter activity with the aim of developing new *in vivo* genetic models. Our transgenic model has demonstrated that 1.6 kb of the rat *egr-1* promoter, together with the *egr-1* intron, can direct cell-specific, and regulatable expression in the brain of rats (Slade *et al.* 2002). In the present study, we have used this model to investigate transgene expression in the anterior pituitary. The use of a promoter-reporter transgenic approach is justified because analysis of transgene expression will inform us of the extent to which the transgene can recapitulate expression of the endogenous *egr-1* gene. The consequent insights into the (*in vivo*) regulatory capacity of the promoter region used in the transgene may, furthermore, have practical applications in the design of secondary transgenic models in which the function of *Egr-1* in the pituitary may be addressed through transgenic targeting. Additionally in this study, we have also investigated the physiological factors that determine both transgene and endogenous *egr-1* expression in the anterior pituitary.

Materials and methods

Animal procedures

All animal procedures were conducted according to UK Home Office regulations, and local ethical

review. Sprague-Dawley rats were maintained in approved laboratory conditions on a 14 h light:10 h darkness cycle. Transgenic rats of the 57C line (Slade *et al.* 2002) were identified by Southern analysis of BglII-cut genomic DNA using an enhanced green fluorescent protein (EGFP) sequence-specific probe (1.4 kb HindIII fragment of pd4 EGFP; Slade *et al.* 2002). In one experiment, transgenic rats were either ovariectomized using standard surgical procedures and injected with vehicle (s.c. sesame oil, daily on days 8–10 after surgery), ovariectomized and injected with 17 β -oestradiol (E_2) (Sigma) (20 μ g/100 g body weight s.c.) or sham-ovariectomized. Following surgery, rats were maintained for 10 days prior to killing. All other experimental rats were killed on particular days of the oestrous cycle as determined by vaginal cytology (dioestrus day 2 for organ culture experiments; pro-oestrus for *in situ* hybridization (ISH) analysis). Animals were killed at 1200 h by decapitation. Anterior pituitary glands were rapidly dissected and rinsed in ice-cold saline before freezing on dry ice prior to subsequent RNA extraction. Alternatively whole pituitary glands were snap-frozen in isopentane cooled with dry ice pellets prior to ISH analysis. Unless otherwise stated, all determinations of gene and protein expression were confirmed with duplicate experimental animals or organ cultures.

Transgene expression analysis

Total RNA extraction and Northern analysis of transgene and *egr-1* transcript expression was performed as described (Slade *et al.* 2002). Blots were sequentially probed with 32 P-labelled cDNA probes specific for EGFP (NotI/SalI fragment of pEGFP-N1), *egr-1* (EcoRI/DraI fragment of the rat NGFI-A cDNA (Milbrandt 1987)) and 18S ribosomal RNA (DecaTemplate, Ambion, Austin, TX, USA), washed, and exposed to either a Phosphor screen (Kodak-K) (EGFP and *egr-1*) or X-ray film (18S). Densitometric analysis of mRNA levels between groups was performed using Imagequant software (Amersham Pharmacia Biotech).

ISH histochemical analysis of transgene transcript expression was performed as described (Slade *et al.* 2002) using antisense and sense 35 S-labelled RNA probes transcribed using the Riboprobe system (Promega). As reported previously (Slade

et al. 2002), ISH was used for transgene expression analysis because, despite robust transcription of the transgene, GFP fluorescence deriving from the transgene reporter is not readily detectable. The transgene-specific ISH probe was derived from a plasmid containing 426 bp of pd4 EGFP (bases 971–1396; Clontech, Palo Alto, CA, USA) in pGEM-11Z (Promega). Probe hybridization (1×10^7 c.p.m./ml) was performed overnight at 56 °C, prior to washing and exposure to a storage phosphor screen (Kodak-K) for 3 days, and subsequent coating in photographic emulsion (LM-1; Amersham Pharmacia Biotech). After 4 weeks, coated slides were developed, counterstained (Mayer's haematoxylin), and viewed with a Leica DM-RD microscope. Images were captured using a Spotcamera (1·30) and Spot Advanced Image software (Spot software 2·2; Diagnostic Instruments, Sterling Heights, MI, USA), imported into Adobe Photoshop (4·0).

Dual ISH/immunocytochemistry was performed using a previously described protocol (Allen *et al.* 1997, Morgan *et al.* 2000) adapted for gonadotrophs. All solutions used in the initial immunocytochemical step were prepared in diethylpyrocarbonate-treated water, and also contained 0·1 U/μl RNasin ribonuclease inhibitor (Promega). Sections (8 μm) of 57C rat pituitary were post-fixed in 4% paraformaldehyde in PBS for 5 min, and permeabilized in methanol (−20 °C, 2 min). LH β-subunit immunoreactivity was detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol, using 3,3'-diaminobenzidine as chromogen. The primary antisera to LH β-subunit (NIDDK-anti-rbetaLH-IC-2; AFP22238790 GPOLHB) was diluted 1:1000. Following development of the chromogen, slides were washed, dried and stored at −70 °C prior to ISH analysis (see above). Following development of the photographic emulsion, sections were counterstained with Mayer's haematoxylin.

Explant culture of rat anterior pituitary

Explant culture of rat anterior pituitary fragments was performed as described previously (Carter *et al.* 1993) with some modifications. The media used was phenol red-free DMEM (Life Technologies) supplemented with penicillin (100 U/ml) and

streptomycin (100 μg/ml). Cultures were maintained at 37 °C for 24 h prior to experimental stimuli. Drugs were diluted into the medium to give appropriate final concentrations of 10 nM E₂ (Sigma) and 10 μM MEK (MAPK kinase) inhibitor U0126 (New England Biolabs, Beverly, MA, USA). Control cultures received similar amounts of drug vehicle (ethanol or DMSO).

Immunoblot analysis

Western analysis of p44/42, phospho-p44/42 (ERK1/2 (extracellular signal-regulated kinases)), and phospho-p38 MAPK was performed on whole cell anterior pituitary extracts using a protocol described by the manufacturer of the primary antisera (New England Biolabs). Protein concentrations were determined by a dye-binding method (Bradford 1976), and mass estimates were obtained with reference to Broad Range Protein Markers (New England Biolabs). Primary and secondary (anti-rabbit IgG, horseradish peroxidase-linked) antisera used were used at 1:1000 and 1:2000 dilutions respectively. Protein bands were detected by chemiluminescence (Luminol reagent; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The relative level of protein bands were compared using densitometric analysis (Imagequant, see above).

Results

ISH histochemical analysis demonstrated that the antisense GFP (transgene-specific) probe hybridized to a sub-population (approximately 10–15%) of anterior pituitary cells (Fig. 1A). The cells exhibiting hybridization were often associated into small groups (Fig. 1A). The aggregations of silver grains that define transgene mRNA expression were restricted to the anterior lobe of the pituitary gland (see Fig. 1C). In control experiments, using a sense GFP probe, there was no evidence of hybridization above background either in the anterior pituitary (Fig. 1B) or in other lobes of the gland (not shown). Immunocytochemical analysis of transgenic rat pituitary glands using a previously characterized (Knight *et al.* 2000) LH β-subunit antiserum revealed a sub-population of β-subunit-expressing cells in the anterior pituitary gland (Fig. 1D). Dual ISH/immunocytochemistry (Allen *et al.* 1997, Morgan *et al.* 2000) using the LH β-subunit

Figure 1 Anterior pituitary-specific expression of *egr-1/d4* EGFP transcripts in 57C transgenic rats. ISH histochemical analysis of transgene transcript expression in the pituitary of a female transgenic rat killed on the day of pro-oestrus. Fresh-frozen glands were sectioned (12 μm) and probed with a ^{35}S -labelled RNA probe specific for transgene (GFP) mRNA. Emulsion autoradiographs were exposed for 3 weeks. (A) Representative image of anterior lobe cells probed with antisense GFP probe. Note the presence of a group of cells associated with aggregated silver grains, and other cells associated with a background distribution of silver grains. (B) Representative image of anterior lobe cells probed with sense GFP probe. (C) Representative image of the neural (NL)–intermediate (IL) lobe boundary (dashed line) probed with the antisense probe. (D) Representative image of anterior lobe cells probed with both an LH β -subunit antisera and the antisense GFP probe. Arrow indicates the association of an aggregation of silver grains with cells exhibiting the orange-brown staining that is representative of LH β -subunit immunoreactivity. In this grey-scale image, the aggregated silver grains associated with the LH β -subunit staining are apparent as blacker grains compared with grains not associated with LH β -subunit staining. Scale bar=15 μm.

antiserum in combination with the antisense GFP probe revealed co-localization of LH β -subunit immunoreactivity with transgene mRNA (Fig. 1D). The distribution and abundance of silver grain aggregations in the dual analysis sections was similar to that observed for single ISH analysis (Fig. 1A), indicating that the specificity of GFP probe hybridization was not compromised in the dual analysis procedure. Co-localization was observed in the majority of LH β -subunit-expressing cells; however, a sub-population (approximately 10%) of

Figure 2 Regulation of *egr-1/d4* EGFP transgene, and *egr-1* transcript expression in 57C rat anterior pituitary gland following ovariectomy, and oestrogen replacement. (A) Representative Northern blot analysis of transgene, and endogenous *egr-1* mRNA in sham-operated (Sh), ovariectomized (Ovx) and ovariectomized, E_2 -treated (E2) rats. Immobilized total cellular RNA extracted from individual glands (12 μg/lane) was probed sequentially with ^{32}P -labelled cDNA probes specific for GFP, *egr-1* and 18S ribosomal RNA. Blots were exposed to a Phosphor screen (Kodak-K). Exposure times were 3 days (GFP and *egr-1*) and 10 min (18S). (B) Histogram showing densitometric analysis of multiple GFP Northern blots, as described in (A). Levels of transgene mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level for sham-operated animals (means $\pm$ S.E.M.). * $P < 0.05$ compared with other groups (ANOVA followed by Duncan's multiple range test). $n=4$ (Sh and Ovx) and 3 (E2) pituitary glands in each group.

β -subunit-positive/GFP mRNA-negative cells was also observed. Conversely, a similar sub-population of GFP mRNA-positive/ β -subunit-negative cells was also found, indicating, firstly, that the aggregations of silver grains are not non-specifically associated with immunopositive cells, and secondly, that the transgene is also expressed in a small population of cells other than gonadotrophs.

The capacity of *egr-1* genomic sequences within the transgene to mediate transcriptional up-regulation in response to physiological (endocrine) stimuli was investigated by observing transgene mRNA levels in ovariectomized rats (Fig. 2), and in rats sampled during the four days of the oestrous cycle (Fig. 3). Following ovariectomy, we found a

Figure 3 Regulation of *egr-1/d4* EGFP transgene transcript expression in 57C rat anterior pituitary glands during the oestrous cycle. (A) Representative Northern blot analysis of transgene mRNA compared with endogenous *egr-1* mRNA. Immobilized total cellular RNA extracted from paired glands (12 µg/lane) was probed sequentially with ³²P-labelled cDNA probes specific for GFP, *egr-1* and 18S ribosomal RNA. Blots were exposed to either a Phosphor screen (Kodak-K; GFP and *egr-1*) or X-ray film (18S). Exposure times were 2 days (GFP, *egr-1*) and 1 h (18S). Samples loaded were: D1, dioestrus day 1; D2, dioestrus day 2; P, pro-oestrus; E, oestrus. (B) Histogram showing densitometric analysis of multiple GFP Northern blots, as described in (A). Levels of transgene mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level for D1 (means ± S.E.M.). * $P < 0.05$ compared with D1 (ANOVA followed by Duncan's multiple range test). $n = 3$ samples of paired pituitary glands in each group.

marked and significant ($P < 0.05$) elevation of transgene mRNA levels in the anterior pituitary (Fig. 2); this response was reversed by treatment with E_2 (Fig. 2). These responses of the transgene were shown to recapitulate regulation of the endogenous *egr-1* mRNA (Fig. 2; Slade & Carter 2000). In intact rats also, we observed regulated expression of transgene mRNA; a significant ($P < 0.05$) up-regulation of transgene mRNA levels was observed on pro-oestrus (Fig. 3), mirroring an endogenous *egr-1* mRNA response (Fig. 3). Transgene mRNA levels were generally observed to be reduced on the subsequent day of oestrus, but the reduction was not as great as that observed for endogenous *egr-1* transcripts, transgene mRNA

levels remaining significantly elevated relative to dioestrous levels (Fig. 3).

In order to investigate the possibility that oestrogen can directly affect transgene (and *egr-1*) expression in the rat anterior pituitary, a previously characterized (Carter *et al.* 1993) explant culture model was used. In an initial experiment, the effects of 0, 1, 10 and 100 nM E_2 on *egr-1* mRNA levels in wild-type anterior pituitary gland explants were compared. This experiment showed, firstly, that only the 10 and 100 nM concentrations were associated with raised levels of *egr-1* mRNA, and secondly that 100 nM did not exert a greater effect than 10 nM (Fig. 4A). Consequently, a 10 nM concentration of E_2 was used in further cultures of 57C transgenic pituitary glands. In these studies, 10 nM E_2 was shown to effect a significant elevation of both *egr-1* (Fig. 4B) and transgene (Fig. 4C) mRNA. However, the time course of these effects differed in that the elevation of *egr-1* mRNA was transient, falling significantly at 60 min relative to the 30 min time point (Fig. 4B), whereas the levels of transgene mRNA remained significantly elevated at 60 min (Fig. 4C). The rapid, and transient effects of E_2 on *egr-1* expression are suggestive of signal transduction via the MAPK pathway (see Discussion).

Consequently, an additional series of *in vitro* experiments were conducted using a specific inhibitor of this pathway, and it was shown that the MEK inhibitor U0126 effected a significant inhibition of the stimulatory effect of E_2 on anterior pituitary *egr-1* mRNA levels (Fig. 5A and B). In a further study, the effect of oestrogens on anterior pituitary protein kinases was examined, and it was shown that E_2 treatment was associated with significantly raised levels of the phospho-p44/42 (ERK1/2) MAPK (Fig. 5C and D), but did not change levels of phospho-p38 MAPK (Fig. 5C).

Discussion

Previous analysis of our *egr-1* transgenic rat model (Slade *et al.* 2002) showed that the *egr-1/d4* EGFP transgene confers both tissue-specific constitutive expression and inducible expression in the brain. These findings paralleled the results obtained with a recently generated transgenic mouse model (Tsai *et al.* 2000) in which 5'-flanking sequences of the murine *egr-1* gene were shown to direct both

Figure 4 Regulation of *egr-1* and *egr-1/d4* EGFP transgene expression by oestrogen in explant cultured 57C rat anterior pituitary gland. (A) Representative Northern blot analysis of *egr-1* mRNA in extracts of glands treated with E_2 (0, 1, 10 and 100 nM) for 30 min. Immobilized total cellular RNA extracted from paired glands (6 μ g/lane) was probed sequentially with 32 P-labelled cDNA probes specific for *egr-1* and 18S ribosomal RNA. Blots were exposed to either a Phosphor screen (Kodak-K; *egr-1*) or X-ray film (18S). Exposure times were 3 days (*egr-1*) and 1 h (18S). (B and C) Representative Northern blot analyses of (B) *egr-1* mRNA and (C) transgene mRNA in extracts of glands treated with E_2 (10 nM) for 0, 15, 30 or 60 min. Immobilized total cellular RNA extracted from paired glands (6 μ g/lane) was probed sequentially with 32 P-labelled cDNA probes specific for GFP, *egr-1* and 18S ribosomal RNA. Blots were exposed as in (A). Below each Northern blot are corresponding histograms that show densitometric analysis of multiple blots ($n=3$ individual explant cultures/group). Levels of mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level at time 0 (means $\pm$ S.E.M.). * $P<0.05$ compared with time 0 (ANOVA followed by Duncan's multiple range test).

constitutive expression in brain, heart and liver, and hepatectomy-associated gene induction in liver. In the present study, we have now shown that sequences within the rat *egr-1/d4* EGFP transgene confer region-specific, and physiologically regulated expression in the rat pituitary gland. Each of these aspects of transgene expression mirror expression of the endogenous *egr-1* gene (see Slade & Carter 2000, Tsai *et al.* 2000, Slade *et al.* 2002). Accordingly, the body of data that is emerging from

these transgenic studies argues that proximal sequences within the *egr-1* gene are sufficient to recapitulate, at least broadly, expression of the endogenous *egr-1* gene in rodents. Taken together with extensive *in vitro* evidence of the regulatory capacity of the *egr-1* proximal 5'-flanking sequence (Changelian *et al.* 1989, Sakamoto *et al.* 1991, DeFranco *et al.* 1993, Cohen *et al.* 1996, Yan *et al.* 1999, Bernal-Mizrachi *et al.* 2000), it can be further argued that this region alone is sufficient

Figure 5 The stimulation of *egr-1* expression by oestrogen involves the MAPK pathway. (A) Regulation of *egr-1* expression by oestrogen in explant cultured rat anterior pituitary gland. Representative Northern blot analyses of *egr-1* mRNA in control glands (C), or glands treated with E₂ (10 nM) for either 30 or 60 min (E30 and E60), or treated with drug vehicle (V) or U0126 (10 μ M, U01) for 2 h prior to treatment with E₂. Immobilized total cellular RNA extracted from individual explant cultures (6 μ g/lane) was probed sequentially with ³²P-labelled cDNA probes specific for *egr-1* and 18S ribosomal RNA. Blots were exposed to either a Phosphor screen (Kodak-K; GFP and *egr-1*) or X-ray film (18S). Exposure times were 3 days (*egr-1*) and 1 h (18S). (B) Histogram showing densitometric analysis of multiple blots as in (A) ($n=3$ individual explant cultures/group). Levels of mRNA were corrected against the equivalent level of 18S RNA, and expressed as fold difference over the level in control cultures (means $\pm$ S.E.M.). * $P<0.05$: U0126 vs vehicle (ANOVA followed by Duncan's multiple range test). (C) Regulation of phospho-p44/42 MAPK by oestrogen in explant cultured rat anterior pituitary gland. Representative Western blot analyses of whole cell protein extracts (15 μ g/lane) derived from either control (C) glands, or glands treated with E₂ (10 nM) for either 5, 15 or 30 min. p-p44/42, phospho-p44/42 MAPK; p44/42, p44/42 MAPK; p-p38, phospho-p38 MAPK. (D) Histogram showing densitometric analysis of multiple blots as in (C) ($n=3$ individual explant cultures/group). Levels of phospho-p44/42 MAPK were corrected against the equivalent level of p44/42 MAPK, and expressed as fold difference over the level in control cultures (means $\pm$ S.E.M.). * $P<0.05$ compared with controls (ANOVA followed by Duncan's multiple range test).

to confer an appropriate pattern of expression. However, the presence of consensus, albeit functionally unproven, *cis*-acting element sequences within the *egr-1* intron (incorporated in the rat, but not the mouse transgene) that include a core serum response element (SRE) at base 1444, and an AP-1-like element at base 1682 of the rat *egr-1*

gene (Acc. No. J04154; Changelian *et al.* 1989) suggests that a more extensive analysis of the contribution of the *egr-1* intron sequences should be conducted. In this context, our transgenic studies of the melatonin-regulating *arylalkylamine N-acetyltransferase* gene (Burke *et al.* 1999) have demonstrated an important role of intronic

sequences for appropriate expression of this gene in the pineal gland.

The demonstrated restriction of Egr-1/d4 EGFP transgene expression to cells of the anterior lobe of the pituitary indicates that the transgene contains sufficient regulatory information to discriminate the embryologically distinct cells of the neural and intermediate pituitary lobes. Within the anterior lobe, transgene transcripts are further restricted to a sub-population of cells indicating that the transgene is also able to mediate cell-type-specific transcriptional control. Thus, using a previously validated (Morgan *et al.* 2000) dual ISH/immunohistochemical technique we have demonstrated co-localization of the transgene transcript with LH β -subunit immunoreactivity. This finding reflects our demonstration of Egr-1 protein in rat gonadotrophs (Knight *et al.* 2000), and indicates a remarkable fidelity of cell-type-specific expression that is conferred by *egr-1* sequences in the transgene. It should be noted that we have also observed cell-type-specific expression of the *egr-1*/d4 EGFP transgene in brain (Slade *et al.* 2002). The full extent of the cellular specificity of transgene expression in the anterior pituitary will, however, require considerable further analysis in which, firstly, the small population of transgene-positive/LH β -subunit-negative cell type(s) are characterized, and secondly the co-localization of transgene and *egr-1* transcripts is quantified. It is apparent from our results, however, that the former population of cells is limited in number and would not appear to constitute a major anterior pituitary cell type. A similar distribution of expression is observed for Egr-1 protein (see Knight *et al.* 2000).

Our finding that transgene mRNA levels are up-regulated following ovariectomy, in an oestrogen-dependent manner, is most likely explained by increased hypothalamic drive to the pituitary following the removal of the negative feedback effects of oestrogen. In this respect, LH-releasing hormone (LHRH)-stimulated *egr-1* expression has been well documented (Tremblay & Drouin 1999, Wolfe & Call 1999, Duan *et al.* 2002). However, our finding that the *egr-1*/d4 EGFP transgene is up-regulated prior to the increase in LHRH pulse amplitude that occurs during the night of pro-oestrus (Sarkar *et al.* 1976) is extremely interesting because our previous studies (Slade & Carter 2000) have implicated transcriptional regulation of the *egr-1* gene by oestrogen, and

recently this mode of *egr-1* regulation has been directly demonstrated, albeit in other cell types (de Jager *et al.* 2001). In the present study, using an explant culture model, we have now demonstrated a direct effect of E₂ on transgene (and *egr-1*) expression at the level of the anterior pituitary gland. Therefore, *cis*-acting sequences within the proximal 5'-flanking, and first intronic region of the rat *egr-1* gene appear to mediate an *in vivo* oestrogenic stimulus. This conclusion may be considered perplexing in view of the absence of consensus oestrogen response elements within this sequence (see Slade & Carter 2000, de Jager *et al.* 2001). However, the recent studies of de Jager *et al.* (2001) have provided evidence that up-regulation of *egr-1* by E₂ is mediated by SREs within the proximal *egr-1* promoter via a mechanism involving the p44/42 MAPK pathway. E₂ signalling via the MAPK pathway is, in fact, consistent with an accumulating body of evidence derived from the study of both pituitary cells (Watters *et al.* 2000) and many other cell types and systems (see Kelly & Levin 2001). By blocking E₂-stimulated *egr-1* expression with a specific inhibitor of the MAPK pathway, and demonstrating an increased abundance of phospho-p44/42 MAPK in E₂-stimulated pituitary glands, we have now provided further evidence of E₂ acting via this pathway. Further studies are now required both to define the cell populations in which MAPK is activated and to establish the functional consequences of the E₂→MAPK→*egr-1* pathway in the pituitary gland.

The *in vitro* evidence described above has indicated one mechanism through which E₂ may influence anterior pituitary gene expression, and consequently effect changes in pituitary cell function during the oestrous cycle. However, the transient up-regulation of pituitary *egr-1* mRNA demonstrated here *in vitro* would not appear to be reflective of a sustained, physiological, *egr-1* response to rising levels of E₂ over the dioestrous–pro-oestrous period, and therefore other factors may modulate the *egr-1* response *in vivo*. It is possible, for example, that *egr-1* mRNA half-life may be differentially regulated *in vivo* (see Cao *et al.* 1992). In this context, the differences observed between the dynamics of the *egr-1* and transgene mRNA responses are interesting, and most likely reflect the absence of destabilizing 3' UTR sequences (Wilson & Treisman 1988) in the transgene transcript. The transient *egr-1* response

may be a phenomenon of the artificially acute E₂ stimulation used in the explant culture experiments. Consequently, although the *in vitro* paradigm has been useful in demonstrating a direct effect of E₂ on *egr-1*/transgene expression at the pituitary level, it may have little further application in probing *in vivo* mechanisms of *egr-1* gene regulation during the oestrous cycle.

In conclusion, we have used a transgenic approach to demonstrate that proximal regions of the *egr-1* gene can direct cell-specific and physiologically regulated expression in the anterior pituitary gland. Expression of the transgene, both anatomical and regulated, recapitulates expression of the endogenous *egr-1* gene, a finding that reflects observations in an *egr-1* transgenic mouse model (Tsai *et al.* 2000). Therefore, although we have used only a single transgenic line (57C) for these studies, it is considered unlikely that the observed expression patterns in the pituitary gland are artefactual. Accordingly, our transgene construct could be useful in future studies that are designed to direct expression of functional cDNAs to a subset of pituitary cells. Our novel evidence of a direct effect of oestrogen on transgene (and *egr-1*) expression at the level of the pituitary, together with previous evidence of *egr-1* gene regulation by LHRH, suggests that the molecular response to these two physiological factors may be integrated, possibly through a co-operative action, at the *egr-1* promoter.

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