

Real-time imaging of gene promoter activity using an adenoviral reporter construct demonstrates transcriptional dynamics in normal anterior pituitary cells

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Abstract

Although analysis of luciferase activity using luminescence imaging has provided new insights into the dynamic regulation of gene expression in living tissues, studies *in vitro* have relied on stably transfected clonal cell lines, limiting the choice of cell type and species, or DNA microinjection, which is arduous and highly selective. We report here the first use of a recombinant adenovirus in which the firefly luciferase reporter gene was regulated by the prolactin gene promoter, to study temporal dynamics of promoter activity. This vector was used to infect the pituitary GH3 cell line, and also primary cultures of Syrian hamster pituitary cells. We show that adenovirally transduced cells retained normal regulation of the promoter-

reporter transgene by appropriate signals. Furthermore, microscopic imaging studies indicated that both clonal and primary pituitary cells were transduced efficiently, giving readily detectable luminescence signals in real-time over long periods. Finally, analysis of single-cell expression patterns indicated that prolactin promoter activity was highly dynamic with pulses in gene expression, revealing that the transcriptional instability seen in clonal cells is a feature of normal pituitary cells. Adenoviral vectors offer a valuable tool for studies of gene regulation where conventional transgenesis and clonal cell lines are not available.

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Introduction

Reporter genes have been widely used in studies of mechanisms of gene regulation in cultured cells *in vitro*, but until recently very little has been known of the temporal patterns of promoter activation in normal tissues studied in real-time. The firefly luciferase reporter gene has become widely used for assays of promoter function, and its activity can be monitored by microscopic luminescence imaging (White *et al.* 1990, 1995, Craig *et al.* 1991, Rutter *et al.* 1998). Using real-time microscopic imaging of a stably transfected rat pituitary cell line, we have recently demonstrated that prolactin (PRL) promoter activity in individual living cells is not stable and exhibits unexpected 40-fold fluctuations from hour to hour (Takasuka *et al.* 1998). Although different individual cells display markedly different mRNA content and peptide secretion rates as determined by *in situ* hybridization and haemolytic plaque assays (Hofland *et al.* 1991), our data indicated that this heterogeneity is subject to rapid

dynamic change (Takasuka *et al.* 1998). The mechanisms for the instability of PRL gene transcription are unknown, but recent imaging and biochemical data indicate that nuclear hormone receptors are recruited to and released from chromatin very rapidly in response to hormonal signals (McNally *et al.* 2000, Shang *et al.* 2000). This leads to the conclusion that transcriptional regulation may be highly dynamic, and the inherent instability of this process may be an important aspect of endocrine regulation.

To date our studies have relied on stably transfected cell lines (Takasuka *et al.* 1998, McFerran *et al.* 2001, Norris *et al.* 2003), but it is vital that this work be extended to normal cells, to allow better understanding of physiological processes, and applied to other species. Pioneering studies using microinjection of individual pituitary cells have revealed important information about pituitary gene regulation in relation to endocrine signals (Castano *et al.* 1996, Villalobos *et al.* 1998), but the microinjection procedure is technically difficult and inevitably entails the selection of a small number of cells for any given experiment. In an

attempt to develop an alternative approach, with wider application for species in which conventional transgenesis is not yet established, we developed a viral vector to deliver reporter genes efficiently into primary cells (Castro *et al.* 1997, Stone *et al.* 2000). We have used a recombinant adenovirus (RAd) containing a transcription unit in which the luciferase reporter gene is regulated by a 4400 bp PRL gene promoter fragment. Here, we describe the first use of an adenoviral vector to investigate dynamic temporal regulation of gene promoter activity in primary cells in culture. We used as a model anterior pituitary cells from the Syrian hamster, a seasonal mammal that exhibits major changes in PRL gene expression under different photoperiodic conditions, and our previous work has demonstrated marked heterogeneity of response of lactotrophic cells to intra-pituitary secretagogues (Stirland *et al.* 2001).

Materials and Methods

Development of PRL-luciferase adenoviral reporter vector

We generated an RAd based on Ad type 5, in which the E1 region and part of the E3 region were deleted, and into which we cloned a luciferase reporter gene linked to a -4429/+14 bp human PRL promoter fragment (RAd-hPRL-luc). Briefly, a 6081 bp PRL-luc cassette was excised from the p5000/PRL-luc⁺ plasmid (Takasuka *et al.* 1998) using an XbaI digest. This fragment was cloned into the XbaI site in the pΔE1sp1a shuttle plasmid (Microbix Biosystems, Toronto, ON, Canada). This shuttle vector was co-transfected with pBHG10 (containing the rest of the adenoviral genome) into the 293 cell line, which expresses the E1 protein and allows propagation of the RAd, as previously described (Shering *et al.* 1997, Southgate *et al.* 2000).

Cell culture

All animal procedures were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986. Primary pituitary cell cultures were prepared from male Syrian hamsters (9–12 weeks of age; University of Manchester breeding colony), as described previously (Stirland *et al.* 2001). Briefly, the pars distalis (PD) region of the anterior pituitary gland was dissected and fragments were washed and dispersed enzymatically using collagenase (Life Technologies, Inc., Paisley, Strathclyde, UK) and DNase I (Sigma Chemical Co., Poole, Dorset, UK). Cell yield was routinely $0.45 \pm 0.06 \times 10^6$ cells/pituitary gland (mean \pm S.E.M.), and viability exceeded 85%. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with 25 mM Hepes (Sigma), supplemented with 10% fetal calf serum (FCS; Harlan Sera-Lab Ltd, Loughborough, Leics, UK), 4 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin-streptomycin (Life Technologies).

The rat pituitary GH3 cell line was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, Wilts, UK) and cultured in DMEM with 10% FCS as before (Takasuka *et al.* 1998). These cells were used for comparisons with adenovirus-infected GH3 cells and primary cells.

Luciferase assays of adenovirus-infected pituitary cell lysates

Primary pituitary cells were plated at $4\text{--}5 \times 10^4$ cells/well in 24-well tissue culture plates (Costar UK Ltd, High Wycombe, Bucks, UK), and cultured for 3 or 8 days at 37 °C in 5% CO₂, then washed and recounted, before adenoviral infection. Pituitary GH3 cells were plated at $2\text{--}5 \times 10^4$ cells/well for 3 days, washed and recounted, before infection. For initial experiments to optimize adenoviral transduction with RAd-hPRL-luc, cells were infected with a range of different multiplicity of infections (MOIs, i.e. infective virus particles per cell) from 0–300 for 2 or 3 days before cell lysis for determination of luciferase activity. For experiments with effectors, both GH3 cells and PD cells were infected at MOI 30 for 30 h, and the medium was then changed to serum-free (DMEM with 0.25% BSA) medium for a further 17 h prior to the experiment. Cells were then incubated with forskolin (Sigma), an activator of the cAMP signalling pathway, and/or the glucocorticoid, dexamethasone (Sigma), for 8 h before cell lysis. Cell lysates were prepared and luciferase activity measured over a 2 s period in the presence of luciferin and ATP (Sigma), as a measure of PRL promoter activity in RAd-infected cells, using a Berthold-Lumat LB9501 luminometer (Berthold-Lumat, St Albans, Herts, UK), all as previously described (Stirland *et al.* 2001).

Microscopic imaging of individual living pituitary cells

Imaging experiments were performed as previously described (Takasuka *et al.* 1998, McFerran *et al.* 2001, Norris *et al.* 2003). Cells were seeded (5×10^4 and 1×10^5 cells/dish for GH3 and primary PD cells respectively) onto 35 mm coverslip dishes (MatTek Corp., Ashland, MA, USA) which had been pretreated with 0.1 or 0.25 mg/ml poly-L-lysine (Sigma), and cultured for 3 days in DMEM+10% FCS medium at 37 °C, 5% CO₂ before infection with RAd-hPRL-luc at MOI 30. After a further incubation period (30–47 h), cells were transferred to a heated stage of a Zeiss-Axiovert microscope (Carl Zeiss, Welwyn Garden City, Herts, UK) in a humidified 37 °C chamber in 5% CO₂ for the remainder of the experiment. Luciferin (1 mM; Bio-Synth, Inc., Staad, Switzerland) was added at the start of the experiment.

Luminescence imaging employed a Hamamatsu VIM photon-counting CCD camera ($\times 10$, 0.5 numerical aperture objective) (Hamamatsu, Hamamatsu City, Japan) and output was analysed with Argus-50 software (Hamamatsu). Bright-field images were taken using

differential interference contrast to allow localization of the cells. Images were collected using 30 min integration times at 30 min or 1 h intervals. Centre of gravity images were used for quantification, using either total photon counts from the whole field or photon counts derived from defined cellular areas (325 pixels). Hormonal stimulation of cells commenced at least 15 h after addition of luciferin to allow luciferase activity to fall to a steady baseline (Takasuka *et al.* 1998, McFerran *et al.* 2001, Norris *et al.* 2003). Forskolin was used at a final concentration of 5 μ M, and a luciferin concentration of 1 mM was used in all experiments.

Quantification and analysis

Data from the 24-well experiments were analysed by one-way ANOVA, followed by post-hoc comparison by Newman–Keuls multiple comparison test. Results are presented as the means \pm S.E.M. Differences of $P < 0.05$ were considered statistically significant. For analysis of temporal patterns of promoter activity, cluster analysis (Veldhuis & Johnson 1986) was used to determine statistically significant pulses of gene expression, and approximate entropy (ApEn) analysis (Hartman *et al.* 1994) was used to test for the degree of randomness in the fluctuations observed, as previously described (McFerran *et al.* 2001). Data were analysed using the SPSS computer program.

Results

Validation of viral transduction

In initial experiments, RAd-PRL-luc vectors were tested in the rat GH3 pituitary cell line, and then in primary cultures of hamster pituitary PD cells. Cells were infected with increasing amounts of vector (MOI) and luciferase expression measured by luminometric assays of cell lysates (Fig. 1). The optimal MOI differed according to cell type: high MOIs appeared to reduce transgene expression in GH3 cells but not primary PD cells. Luciferase activity was normally higher in the transduced GH3 cells than the PD cells, but this may partly reflect the restriction of reporter gene expression by the PRL promoter to just the lactotrophic cells among the mixed cell populations in primary pituitary cultures, whereas in the clonal somatotrophic GH3 cell line, all of the cells are capable of activating the transgene (Southgate *et al.* 2000, Davis *et al.* 2001, Smith-Arica *et al.* 2001). From these experiments, an MOI of 30 was selected for subsequent studies.

Hormonal regulation of the adenovirally transduced promoter-reporter construct

The behaviour of the PRL promoter in the context of the adenoviral vector was tested using a series of known regulators of PRL promoter activity (Berwaer *et al.* 1991),

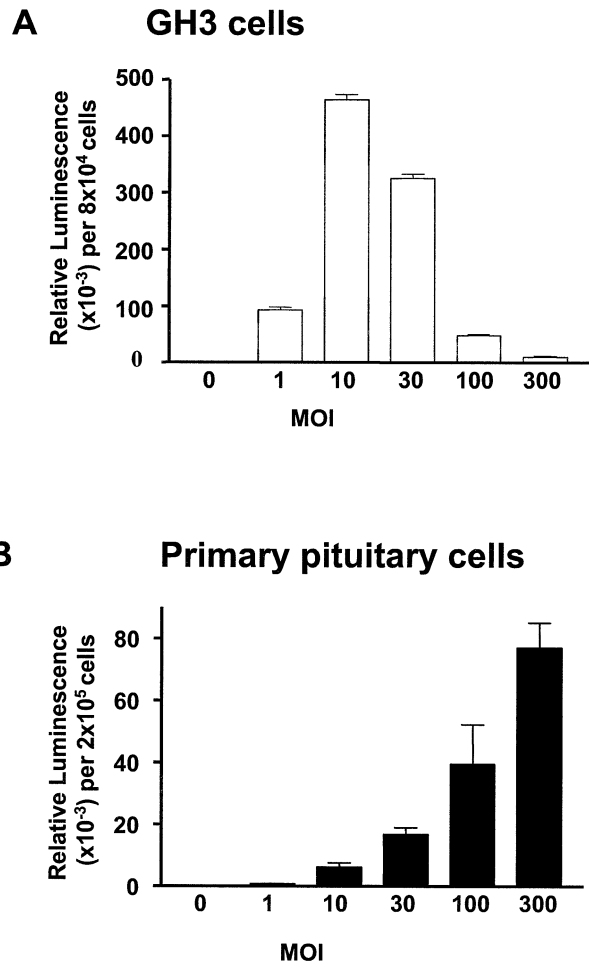


Figure 1 Effect of MOI on luminescence in (A) clonal GH3 pituitary cells and (B) primary hamster pituitary cells in culture. (A) Pituitary GH3 cells were cultured for 3 days, washed and recounted, and infected with RAd-PRL-luc at MOIs of 0–300. After 72 h, infected cells were washed and lysates assayed for determination of luciferase activity. Data are means \pm S.E.M., $n=3$. (B) Primary hamster pituitary PD cells were cultured for 8 days, washed and recounted, and infected with RAd-PRL-luc at MOIs of 0–300, with triplicate wells per MOI. After 53 h, cells were washed and lysates assayed for determination of luciferase activity. Data are means \pm S.E.M., $n=3$.

to confirm that the transgene expression was behaving in an appropriate manner. A series of agents were tested in both GH3 cells and primary PD cells after adenoviral infection. Forskolin induced a dose-dependent 3- to 4-fold induction of PRL promoter activity, and dexamethasone inhibited this induction, as previously found in transient expression studies (Berwaer *et al.* 1991) (Fig. 2). The PRL secretagogues thyrotrophin-releasing hormone and basic fibroblast growth factor also induced luciferase expression in a dose-dependent manner, as expected (data not shown). We also found reproducible inductions in PRL promoter activity in both adenovirus-infected GH3 cells

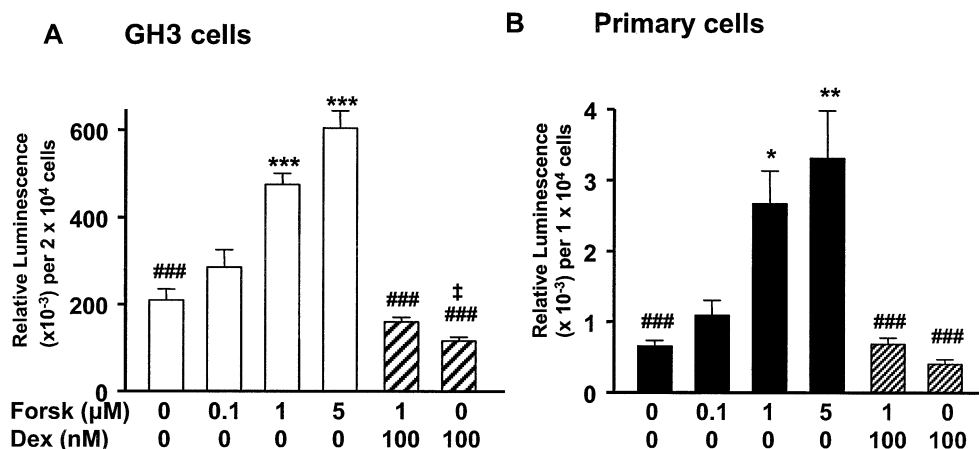


Figure 2 Dose responses of adenovirally encoded PRL promoter-luciferase to forskolin and dexamethasone in adenovirus-infected clonal pituitary GH3 cells and primary hamster pituitary cells in culture. Pituitary GH3 cells (A) or primary hamster pituitary cells (B) were cultured in DMEM with 10% FCS. After 3 days, cells were washed and re-counted and infected with rAd-PRL-luc at MOI 30. After 30 h, the culture medium was replaced with serum-free DMEM with 0.25% BSA. After a further 17 h (i.e. 48 h after infection), cells were incubated with forskolin (forsk, 0–5 µM) and/or dexamethasone (Dex, 100 nM) for 8 h and lysates assayed for determination of luciferase activity. Data are means ± s.e.m., $n=4$ wells per treatment. Forskolin induced PRL promoter activity (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs unstimulated cells), and this was prevented by dexamethasone (#### $P<0.001$ vs 1 µM forskolin, $P<0.05$ vs unstimulated cells).

and primary pituitary cells in the presence of conditioned medium from cultures of hamster pituitary pars tuberalis (PT) cells: for primary cultures of rAd-infected hamster PD cells, PT-conditioned medium stimulated PRL promoter activity 1.9-fold (1938 ± 146 , vs 1018 ± 55 relative luminescence units per 2×10^4 cells, $n=4$, $P<0.001$). This is consistent with our previous observations using a GH3 cell line stably transfected with a hPRL-luc reporter gene construct, and demonstrates that the PT can drive PRL promoter activity in normal lactotrophic cells (Stirland *et al.* 2001).

Luminescence microscopy of adenoviral luciferase expression: GH3 cells

The temporal dynamics of PRL promoter activity were then studied by luminescence microscopy in clonal pituitary GH3 cells after infection with rAd-PRL-luc. Luminescence imaging commenced following the addition of luciferin: forskolin (5 µM) was added to the culture medium after 23 h, and imaging continued up to 42 h. Microscopic images and quantitative luminescence data are shown in Fig. 3, with a bright-field image shown at

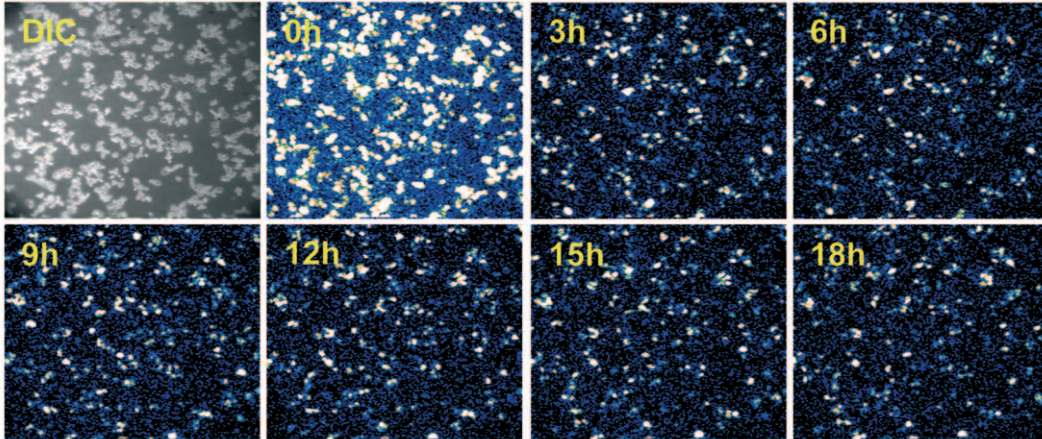
the time of luciferin addition. The addition of luciferin generated a transient increase in luminescence, which then settled to steady baseline levels (Fig. 3A). Forskolin treatment generated a sustained rise in luciferase expression in the total population of cells in the microscope field (Fig. 3B). Quantitative analysis revealed that within the overall population most individual cells showed a transient transcriptional response to forskolin, but the temporal patterns of this response varied widely from cell to cell (Fig. 3C and D). This heterogeneity in response is similar to that noted in earlier work using GH3 cells stably transfected with luciferase reporter genes (Takasuka *et al.* 1998, Norris *et al.* 2003) and thus confirms that the adenoviral transduction approach reveals similar patterns of transcriptional instability and heterogeneity in normal cells.

Luminescence microscopy of adenoviral luciferase expression: primary PD cells

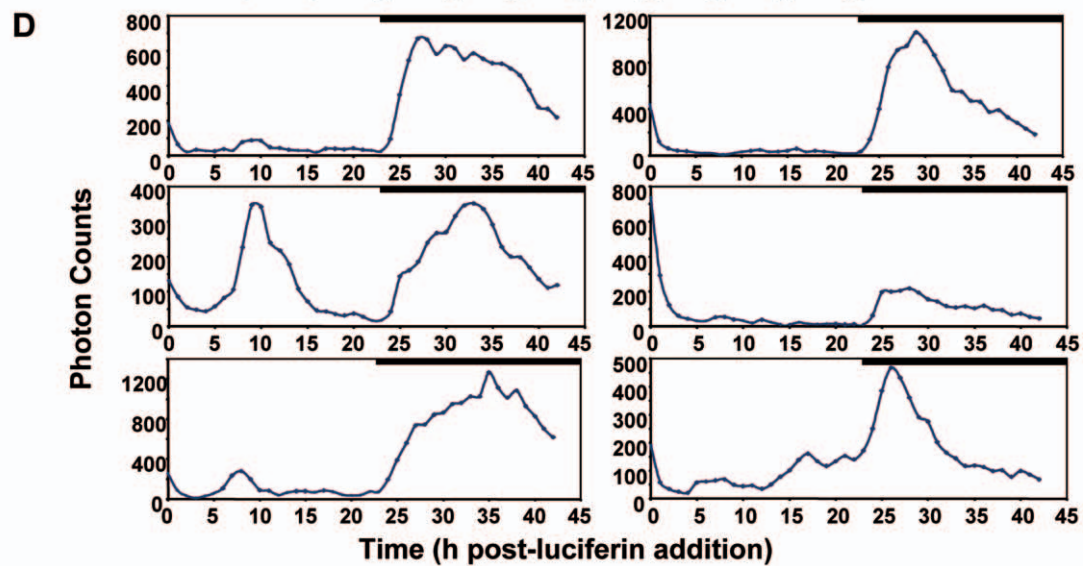
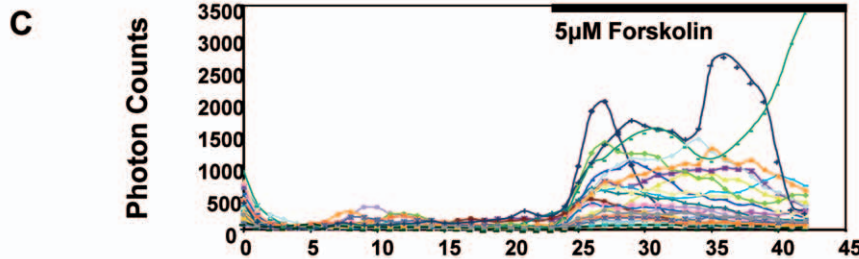
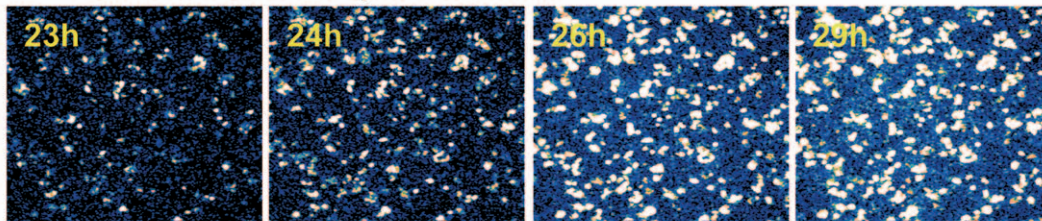
Following validation in the GH3 cell line, normal hamster pituitary PD cells were studied by luminescence microscopy. Cells were imaged for 30 h after luciferin

Figure 3 Imaging of PRL promoter-directed luminescence in the adenovirally transduced GH3 cell line, before and after stimulation with forskolin. Pituitary GH3 cells cultured in DMEM with 10% FCS for 3 days, washed and re-counted, and infected with rAd-PRL-luc at MOI 30. After 30 h, the medium was replaced with serum-free DMEM with 0.25% BSA. After a further 17 h (i.e. 47.0 h after infection), luciferin (1 mM) was added ($t=0$ h on panels A, C and D). Forskolin (5 µM) was added 23 h after the start of imaging, indicated by horizontal bars in panels C and D. Images were collected ($\times 10$ objective) over 30 min integration times at 1 h intervals (A and B). Quantitative data from these images were analysed for 27 single-cell areas in the overall field of cells (C) and for six representative single-cell areas (D). DIC (differential interference contrast) denotes bright-field image.

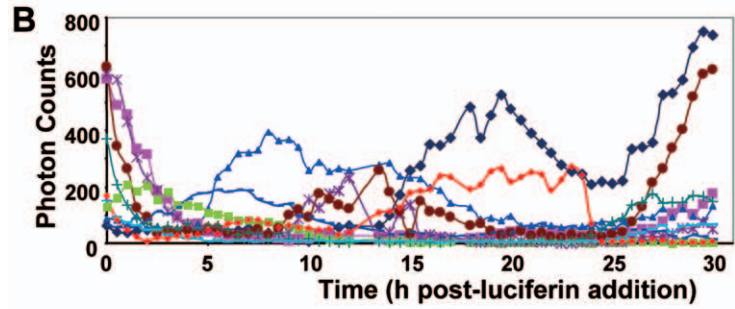
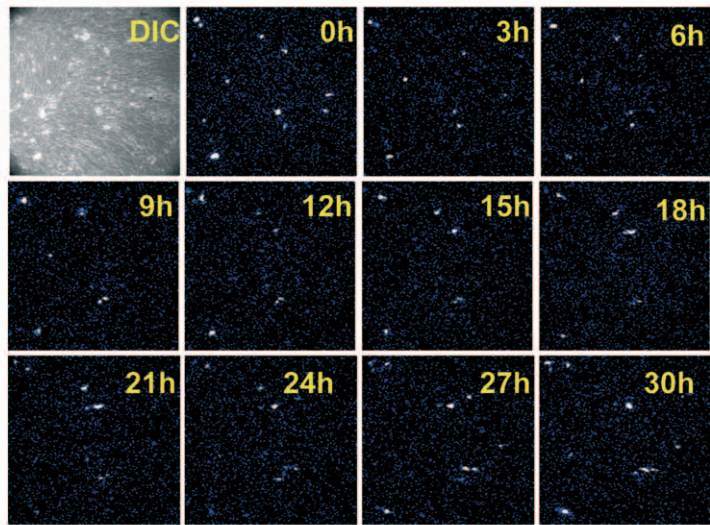
A Unstimulated GH3 cells



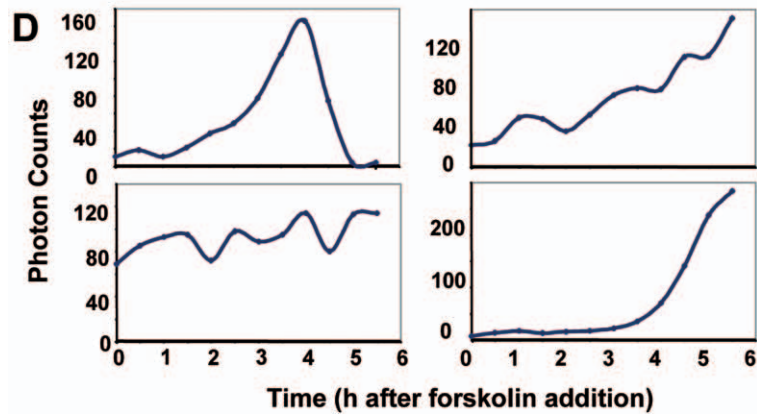
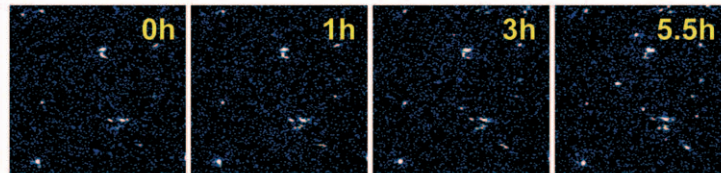
B Stimulation with 5 μ M Forskolin



A Unstimulated primary pituitary cells



C Stimulation with 5 μ M forskolin



addition, and then for a further 5 h after forskolin stimulation (Fig. 4A). Marked fluctuations in luciferase activity were noted from hour to hour in unstimulated PD cells, indicating that resting normal pituitary cells display highly dynamic patterns of transcription, as previously seen with stably transfected clonal hPRL-luc/GH3 cells. Oscillating patterns were detectable in over 60% of cells (representative experiment shown in Fig. 4B). Cluster analysis confirmed that pulses in gene expression were statistically significant, with cells showing between 1 and 4 pulses (mean 1.5) over 30 h. ApEn analysis of the time-series data confirmed that these fluctuations were non-random (ApEn scores 0.71 ± 0.33 (mean \pm s.d.), compared with 1.22 ± 0.23 for the same data after random shuffling, $P < 0.001$, indicating significant regularity) (Hartman *et al.* 1994). After forskolin treatment individual cells displayed heterogeneous patterns of response, some cases showing transient and others sustained responses to identical stimulation (Fig. 4C and D).

Discussion

Microscopic studies of luciferase reporter gene activation offer valuable insights into dynamic temporal regulation of gene expression, but so far have relied on either clonal cell lines or microinjection of primary cultures of cells (Castano *et al.* 1996, Takasuka *et al.* 1998, Villalobos *et al.* 1998, McFerran *et al.* 2001, Norris *et al.* 2003). Our studies demonstrate for the first time that real-time microscopic imaging of promoter activity can also be readily achieved in normal pituitary cells using an RAd expressing the firefly luciferase gene under the control of an appropriate gene promoter. Our results indicate that adenovirus vectors can be used at low copy number, achieving sufficient transgene expression for detectable luciferase activity to be imaged microscopically over long periods. We also show that the appropriate hormonal regulation of the adenoviral transgene is maintained in transduced cells. Our study thus demonstrates that adenoviral vectors offer a highly efficient route towards studies of real-time gene regulation in normal cells.

Adenovirus vectors are characterized by highly efficient gene transfer into target cells, and can transduce a wide range of cell types, including post-mitotic cells (Stone *et al.* 2000). In the case of the pituitary, the efficacy of adenoviral gene transfer has been demonstrated in a number of studies. All cell types in the mature pituitary gland are effectively transduced by adenoviruses expressing the

β -galactosidase reporter gene (Castro *et al.* 1997), while pituitary hormone gene promoters are able to restrict transgene expression to specific target cell types, in both clonal cell lines and cultured normal pituitary cells (Lee *et al.* 1999, Southgate *et al.* 2000, Davis *et al.* 2001, Smith-Arica *et al.* 2001). Adenoviral vectors using the β -galactosidase reporter gene have been used effectively to report the spatial patterns of transgene expression in different tissues including the pituitary (Lee *et al.* 1999, Southgate *et al.* 2000, Windeatt *et al.* 2000, Davis *et al.* 2001, Smith-Arica *et al.* 2001). The luciferase reporter gene, on the other hand, is preferable for quantitative temporal studies of dynamic gene expression, due to its sensitivity and short half-life (White *et al.* 1995, Takasuka *et al.* 1998, Villalobos *et al.* 1998, Norris *et al.* 2003).

The present data, using the luciferase transgene as a highly sensitive reporter of promoter activity, show that the optimal MOI for infection differed between clonal GH3 cells and primary pituitary cells. The fall in activity using high MOIs in clonal GH3 cells might reflect direct adenovirus toxicity, although previous work suggested this is unlikely (Windeatt *et al.* 2000); alternatively, it might be due to promoter squelching with higher copy numbers per cell, resulting from competition for limiting amounts of cellular transcription factors and co-activators.

Adenoviral vectors offer a valuable alternative to microinjection, which has until now been the only approach for studying promoter activity in normal cells, other than generating transgenic animals. In particular, the high efficiency of adenoviral transduction allows analysis of the transcriptional patterns of almost all individual cells that contribute to the behaviour of a given population. Although microinjection of reporter gene constructs has been valuable in studies of normal cells from animals in different physiological states (Frawley *et al.* 1994, Castano *et al.* 1996, Villalobos *et al.* 1998), it is laborious and necessarily highly selective for a small number of cells that can be successfully injected amongst the overall population, and usually generates very high transgene copy numbers for a short period. Our studies now show that effective transduction of luciferase activity can be achieved using relatively low copy number, which will be critical for detailed studies of transcriptional regulation where squelching phenomena may seriously compromise interpretation of quantitative effects.

Our data now extend previous information on the temporal regulation of PRL gene promoter activity in individual living cells. Using stably transfected pituitary GH3 cells, we have reported that activity of PRL and

Figure 4 Imaging of PRL promoter-directed luminescence in adenovirally transduced primary hamster pituitary cells, before and after stimulation with forskolin. Primary hamster pituitary cells were cultured in DMEM with 10% FCS for 3 days, washed and recounted, and infected with RAd-PRL-luc at MOI 30. After 30 h, luciferin (1 mM) was added ($t=0$ h on panels A and B). Cells were imaged using 30 min integration times every 30 min. Forskolin (5 μ M) was added 35.5 h after start of imaging ($t=0$ h on panels C and D). Quantitative data from these images are shown for the overall field of unstimulated cells (B), and for four representative single-cell areas following forskolin stimulation (D). DIC denotes bright-field image.

growth hormone promoters is not stable within any given individual cell, but varies dramatically from hour to hour, with phasic oscillations in activity (Takasuka *et al.* 1998, Norris *et al.* 2003). Similar findings have been reported in microinjected primary pituitary cells from lactating rats (Villalobos *et al.* 1998, Shorte *et al.* 2001). We have found that periodic oscillations in promoter activity can be seen in cell lines exposed to both pulsed and sustained serum stimulation (McFerran *et al.* 2001), and a recent report using microinjection studies has analysed the periodic nature of 'pulses' in promoter activity in microinjected normal rat pituitary cells (Shorte *et al.* 2001). The present data, while not addressing the mechanisms of pulsatile gene expression, confirm that this phenomenon may be ubiquitous, as it is observed in normal cells using the adenovirus reporter system. Inherent transcriptional instability may therefore be a common feature of normal cellular physiology. For example, in the pituitary gland of photoperiodic species such as the hamster, we have found that patterns of transcriptional heterogeneity in PRL gene expression are altered in response to secreted factors from the PT that modulate seasonal neuroendocrine rhythms (Stirland *et al.* 2001). The present approach offers a route to understanding processes of transcriptional recruitment in tissues exhibiting marked circadian and seasonal rhythmicity in gene expression. Adenovirus-mediated gene expression offers an alternative form of transgenesis in animal species, which could offer valuable physiological models not yet available from conventional transgenic approaches. Furthermore, adenoviruses can also achieve highly efficient gene transfer in intact tissue *in vivo* after stereotaxic injection, both in the rat (Lee *et al.* 1999, Southgate *et al.* 2000) and in large animals such as sheep (Davis *et al.* 2001).

Recent studies have demonstrated that very large numbers of genes exhibit circadian transcriptional periodicity (Panda *et al.* 2002). Non-circadian (ultradian) fluctuations in gene transcription (Shorte *et al.* 2001) may be critical in the endocrine regulation of hormone synthesis. Studies of different systems have indicated that gene transcription within an individual cell can be regulated in either a graded or a stochastic (binary) manner (Hume 2000, Becskei *et al.* 2001, Biggar & Crabtree 2001). Stochastic regulation may be particularly important for endocrine tissues that require rapid secretory and hormone-synthetic responses to environmental cues, in addition to their long-term adaptive or trophic responses. It is important now to extend this understanding to normal mammalian cells and tissue, using technologies such as adenoviral gene transfer. Since imaging of luminescence or fluorescence of transgenic or injected reporter proteins is applicable not only to transcriptional regulation but also to intracellular signalling cascades (Zaccolo *et al.* 2000, Nagai *et al.* 2001), this now raises the possibility of studying intracellular endocrine physiology in populations of normal cells. Ultimately, such studies could extend to *in vivo* systems, in

which viral gene transfer could be followed by luminescence imaging in the intact animal (Yamaguchi *et al.* 2001, Wu *et al.* 2002), providing insight into the temporal regulation of cellular processes during physiological responses at the cellular level.

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