Chemistry & Biology, Vol. 9, 1347–1353, December, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00288-0

Spatially Discrete, Light-Driven Protein Expression

Weiying Lin,[1] Chris Albanese,[2,4] Richard G. Pestell,[2,4] and David S. Lawrence[1,3]
[1]Department of Biochemistry
[2]Departments of Medicine and Molecular Biology
The Albert Einstein College of Medicine of Yeshiva University
Bronx, New York, 10461

Summary

Transgene-based inducible expression systems offer the potential to study the influence of any gene *at any point* during an organism's lifetime. However, the expression of individual genes is both temporally and spatially (i.e., cell/tissue)-regulated. The inducible gene expression systems devised to date do not offer fine spatial control over gene expression. We describe herein the creation and study of a light-activatable, ecdysone-inducible gene expression system. We have constructed the first example of a caged ecdysteroid, which is virtually inactive as an inducing agent in a luciferase-based gene expression system. However, upon exposure to brief illumination, the caged ecdysteroid is rapidly converted into active β-ecdysone. Caged β-ecdysone is cell permeable, can be intracellularly photouncaged, and, in combination with spot illumination, can be used to drive spatially discrete protein expression in a multicellular setting.

Introduction

Knockout and knockin animal models are commonly employed to assess the biological role of specific proteins in the context of a multicellular organism. However, expression of individual genes is a temporally and spatially (i.e., tissue) specific phenomenon that can influence both normal and abnormal biological processes. For example, it has not been possible to study the role of certain genes (cyclin D1, Stat5A, prolactin receptor) in mammary gland tumorigenesis because transgenic mice missing these genes lack proper mammary gland development [1–3]. To overcome these limitations, systems have been engineered that inducibly regulate the transgene of interest or excise the targeted gene of choice [4,5]. These inducible constructs allow gene expression patterns to be temporally controlled. The characteristics of an ideal inducible transgenic system include low basal level expression and robust induction of the transgene, the lack of secondary or deleterious effects of the inducing agent, tissue-specific targeting, and the ability to sustain transgene induction. These characteristics are particularly important in the delivery of embryonic lethal, transforming, or otherwise toxic genes. For example, potent oncogenes such as Myc exhibit a wide range of biologic effects, and therefore the ability to control both the temporal expression profile and the activity of the gene is critical.

The early inducible transgenic lines relied on the administration of heavy metals or naturally occurring steroid hormones, such as glucocorticoids, to provoke transgene expression [6]. However, heavy metals are toxic and the glucocorticoids regulate a variety of endogenous genes, thereby complicating interpretation of the biological response to the inducing agent. More recently, a variety of additional inducing agents have been described including tetracycline (*tet* operon system) [7], IPTG (*Lac* operon repressor system) [8], FK1012 (FKBP inducible system) [9], tamoxifen (estrogen receptor system) [10], and ecdysone receptor agonists (ecdysone receptor inducible system) [11,12]. However, many of these inducible systems are plagued by difficulties such as mosaic induction, toxicity, background transgene expression, sluggish clearance, and poor expression of the transactivator.

In 1996, Evans and his colleagues described an ecdysone-inducible gene expression construct [11]. Ecdysone, the insect molting hormone, triggers metamorphosis by binding to and activating the nuclear heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP). The activated complex, in association with an ecdysone-responsive element (EcRE), subsequently drives gene expression. In the mammalian construct, the EcR and retenoid X receptor (RXR; the mammalian homolog of ultraspiracle protein of USP) are constitutively produced. The gene of interest, which is linked to the EcRE, is expressed upon introduction of ecdysone (or structurally related analogs). The advantages of this system include low basal expression, high inducibility (up to four orders of magnitude), and the fact that ecdysteroids are not toxic and do not affect mammalian physiology.

Inducible gene expression systems, as they are currently devised, provide temporal control over when the gene of interest is activated. However, fine spatial control over where gene expression is induced is problematic. A partial solution to the spatial control over gene expression issue is to employ tissue-selective promoters [12]; however, this technology is not applicable to studies of gene expression within specific tissue microenvironments. We described herein a light-activatable form of ecdysone and its application to the spatial regulation of gene expression in a multicellular setting. The advantages of the ecdysone gene activation system as described above, in conjunction with light-driven spatial control over gene expression in a multicellular environment demonstrated herein, could open a new avenue to studies of the effect and influence of protein expression on phenotype as a function of tissue microenvironment.

[3] Correspondence: dlawrenc@aecom.yu.edu
[4] Present address: Lombardi Cancer Center, Georgetown University Medical Center, Research Building, Room E501, 3970 Reservoir Road, NW - Box 571468, Washington, District of Columbia, 20057.

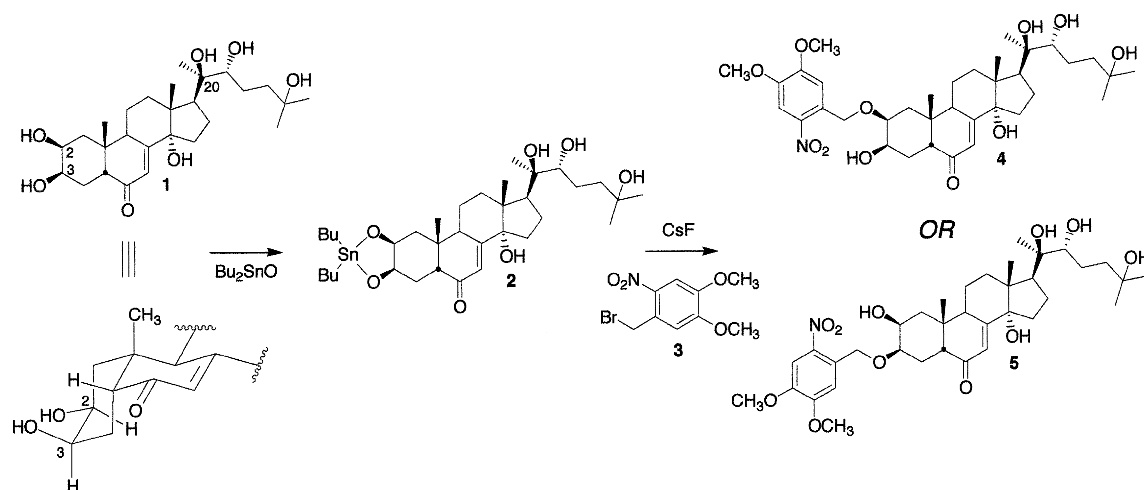

Figure 1. Synthesis of Caged β-Ecdysone 4 from β-Ecdysone 1

Results

Synthesis and Characterization of Caged β-Ecdysone 4

We initially sought to prepare a biologically inactive form of β-ecdysone **1** that, upon photolysis, would furnish the active ecdysteroid. A wide variety of ecdysteroid derivatives have been reported and, in general, the presence of free hydroxyl groups at the C-2, C-3, and C-20 positions are required for biological activity [13] (Figure 1). Consequently, we envisioned that modification of one (or more) of these alcohol moieties, with a photosensitive substituent, should furnish an inactive ecdysteroid analog that ultimately could be "switched on" using high intensity light. An alkylated, as opposed to an acylated, ecdysone would enjoy the advantage of enhanced biological stability. However, to the best of our knowledge, we know of no report describing the alkylation of any of the hydroxyl functionalities on ecdysone or its structurally related congeners. Indeed, our initial attempts to alkylate β-ecdysone with the caging agent **3** using standard methodology failed to furnish a modified ecdysteroid derivative (e.g., acetonitrile/N-methyl morpholine/**3**).

The acetonide of the C-2/C-3 diol has been described [14], and, consequently, we wondered whether the corresponding tin acetal **2** could be formed in situ. Tin-based acetal intermediates have been extensively employed in carbohydrate chemistry to furnish monoalkylated derivatives in high yield and high regioselectivity [15]. Although the dibutylstannylene acetal **2** appeared to form with ease, attempted alkylation with **3** furnished the desired monoalkylated product in only 12% yield. We subsequently discovered that **3** is prone to decomposition in the presence of base ($KHCO_3$, Et_3N). Although alkylation of stannylene acetal alcohol moieties generally requires fairly vigorous conditions, there have been reports that added nucleophiles promote the desired reaction under mild conditions [16]. Indeed, we obtained a single monoalkylated β-ecdysone (high resolution mass spectrometry and a single peak by HPLC) in 90% yield when the alkylation was conducted in the presence of CsF.

Previous studies using a variety of acylating agents suggest that the 2-OH of ecdysteroids is by far the most reactive of the hydroxyl moieties on the ecdysteroid nucleus [13]. In addition, extensive studies with a multitude of *cis*-1,2-diol tin acetal-monosaccharide intermediates revealed that the preferred site of alkylation proceeds (often exclusively) at the equatorial hydroxyl group [15]. The three-dimensional structure of ecdysone has been described, and its stereochemical rendering is shown adjacent to **1** [17]. The methyl substituent at the ring A/ring B junction lies axial relative to the B ring, but equatorial relative to ring A. Consequently, the C-2 and C-3 hydroxyl moieties are arranged equatorially and axially, respectively, on ring A. Based upon these considerations, we predicted that alkylation should occur predominantly, if not exclusively, at the equatorial C-2 alcohol to furnish **4**. However, an examination of the ^1H NMR spectrum, in combination with previously reported proton chemical shifts [18], suggested that alkylation may have transpired at the C-3 hydroxyl position **5**. The latter conclusion arises from the obvious alkylation-induced chemical shift of the C-3 hydrogen (Figure 2A). Given the difference between the predicted **4** and the apparent products **5**, we obtained the complete ^1H and ^{13}C chemical shift assignments for β-ecdysone using a combination of ^1H-^1H double quantum filtered correlation spectroscopy (DQF-COSY), short-range ^1H-^{13}C heteronuclear single quantum correlation spectroscopy heteronuclear multiple quantum correlation spectroscopy (HSQC), and long-range ^1H-^{13}C (HMBC) correlation NMR spectroscopies. The HMBC spectrum of the caged β-ecdysone **4** reveals an obvious coupling between the benzyl methylene protons of the photolabile substituent and the carbon at the C-2 position on the ecdysteroid nucleus (Figure 2B). By contrast, no such coupling is observed between the benzyl methylene protons and the C-3 carbon. Therefore, we conclude that alkylation proceeds at the C-2 hydroxyl to furnish **4**. The most dramatic alkylation-induced change in the 1D spectrum (Figure 2A), namely the chemical shift of the C-3 proton, is best rationalized by invoking a deshielding effect induced by the adjacent C-2 hydroxyl benzyl substituent.

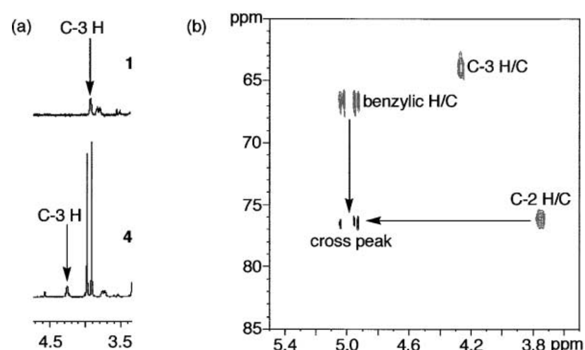

Figure 2. 1D and 2D NMR Spectra of Caged β-Ecdysone 4

(A) The 1D ^{1}H NMR spectrum of β-ecdysone **1** (upper) and caged β-ecdysone **4** (lower). The alkylation induced change in the chemical shift of the C-3 proton is highlighted.

(B) The HMBC 2-D spectrum of caged β-ecdysone **4**. Long-range coupling between the benzylic methylene protons (CH_2) of the dimethoxy-nitrobenzyl substituent and the C-2 carbon is present. By contrast, no such coupling is observed between CH_2 and the C-3 carbon.

Photoconversion of Caged β-Ecdysone 4 to β-Ecdysone 1

A preliminary assessment of the light-induced conversion of the caged analog **4** to β-ecdysone **1** was performed using a 24-well plate system. Wells in the plate were selectively photolyzed for various time intervals using a Hg arc lamp. Aliquots from the wells were subsequently analyzed by HPLC to assess formation of **1** (data not shown). A maximal photoconversion of 60% was achieved after 1 min of photolysis. Longer periods of photolysis did not improve the overall yield of photoconversion. The photochemical quantum yield ($\phi = 0.034$) was determined using ferrioxalate actinometry [19].

Light-Driven Luciferase Expression in Transiently Transfected 293T Cells

We employed a luciferase-based expression system to examine the biological activity of β-ecdysone and its alkylated analog **4**. The 293T cell line was transiently transfected to constitutively express EcR/RXR and inducibly express (upon exposure to β-ecdysone) luciferase. Both ecdysone and its caged counterpart were predissolved in methanol and then added to culture media to furnish a final methanol concentration of 2%. Methanol, at this concentration, does not appear to induce any untoward effects on cell viability. Compound **4** was added to the transfected 293T cells, the cell culture subsequently illuminated, and luciferase activity assessed (Table 1). There is little observable luciferase activity with culture media alone. Exposure of the cells to the bioactive β-ecdysone **1** generates a nearly 90-fold induction of luciferase. By contrast, the caged analog **4** furnishes a slight 6-fold induction of activity over that of culture media alone. However, 1 min photolysis of cells treated with **4** induces a dramatic enhancement of luciferase formation, which is approximately 60% of the expression displayed by the bioactive species **1**. The latter is consistent with our observation that a 1 min photolysis time window converts approximately 60% of the caged derivative **4** into its bioactive counterpart **1**. Finally, illumination in the absence of ligand fails to induce luciferase production. These experiments demonstrate that light can be used to activate the EcR/RXR gene expression system. The time-dependence of β-ecdysone-induced luciferase expression following photolysis is shown in Figure 3. Cells were preincubated with the caged β-ecdysone **4** for 16 hr, illuminated for 1 min, and then lysed at various time points following photolysis. Maximal gene expression is observed at 16 hr. We also assessed whether **4** is cell permeable and undergoes intracellular uncaging upon photolysis. The 293T cell line was preincubated with **4** for 16 hr, the media subsequently removed, and the cells washed with PBS. Ecdysone-free media was then added, the cell culture incubated for various time points, and tested for luciferase activity. The gene expression kinetic profile is nearly identical for the two sets of experiments [(**4** + photolysis) versus (**4** + washing + photolysis)] during the first 5 hr, which is consistent with the notion that caged β-ecdysone **4** is intracellularly liberated (Figure 3). However, we do note that (**4** + washing + photolysis)

Table 1. Luiferase Expression in 293T Cells Transiently Transfected with Plasmids that Code for Constitutively Expressed EcR/RXR and Inducibly Expressed Luciferase in the Presence and Absence of β-Ecdysone **1** and Its Caged Analog **4**

Experimental Conditions	Fold Luciferase Induction
culture media/2% MeOH	-
β-ecdysone **1**/culture media/2% MeOH	88 ± 9
caged β-ecdysone **4**/culture media/2% MeOH	6.5 ± 0.1
caged β-ecdysone **4**/culture media/2% MeOH/1 min hν	50 ± 4
culture media/2% MeOH/1 min hν	0.9 ± 0.1

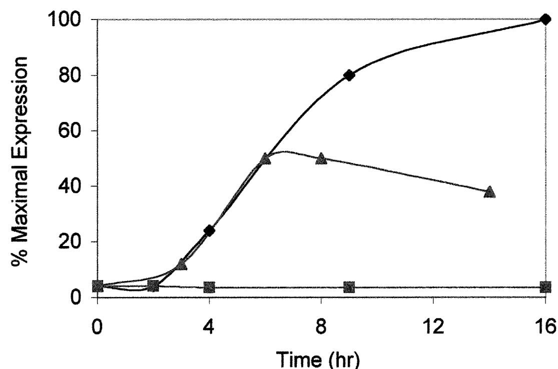

Figure 3. Generation of Luciferase Activity as a Function of Time Following Photolysis

293T cells that had been transiently transfected with plasmids encoding constitutively expressed EcR and RXR and inducibly expressed luciferase were exposed to caged β-ecdysone **4** for 16 hr and then either (◆) illuminated or (▲) first washed to remove extracellular **4** and then illuminated. Control luciferase expression in the absence of ligand is shown as well (■). Percent of maximal expression is normalized relative to the yield obtained for the 1 min photolysis time period.

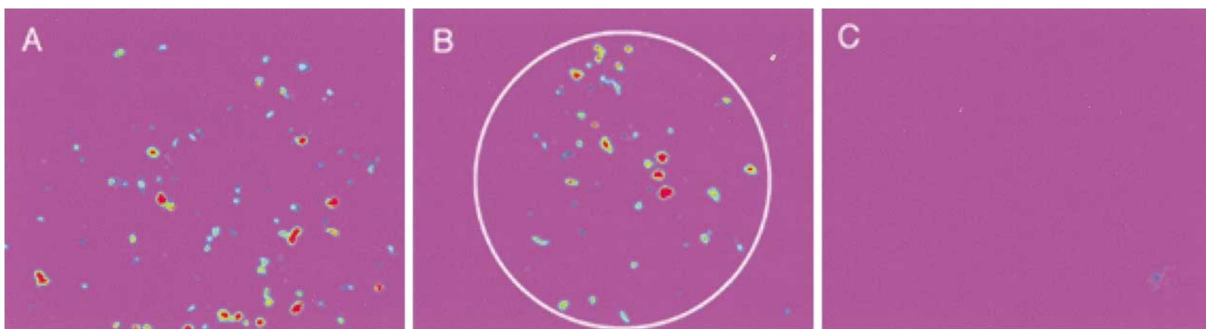

Figure 4. Light-Driven, Spatially Discrete Luciferase Expression

(A) Transfected 293T cells were exposed to β-ecdysone **1** and subsequently probed for luciferase expression.

(B) Transfected 293T cells were exposed to **4**, spot illuminated (~0.25 mm^2) for 10 s, and then probed for luciferase expression.

(C) The experiment as described in (B) outside of the region of illumination. (Olympus IX-70 at 10×, N.A. 0.3).

conditions achieve only 50% of the maximal activity displayed by (4 + photolysis) conditions.

Light-Driven, Spatially Discrete Luciferase Expression in Transiently Transfected 293T Cells

We examined whether the combination of **4** and spot illumination induces gene activation in a spatially discrete fashion. Transiently transfected 293T cells were incubated with **1** for 16 hr, fixed, permeabilized, exposed to a luciferase antibody, and stained with an Alexa-labeled secondary antibody. As expected, luciferase expression (20% transfection efficiency) was observed throughout the general cell population (Figure 4A). By contrast, cells incubated with the caged analog **4** under identical conditions failed to exhibit detectable luciferase expression (data not shown). 293T cells were also exposed to **4**, the media replaced with fresh media to remove extracellular **4**, and spot illuminated (~0.25 mm^2) for 10 s. The cells were then returned to the incubator for 6 hr and subsequently analyzed for luciferase expression. Only those regions exposed to UV light display luciferase production (Figures 4B and 4C).

Discussion

The analysis of protein function in living animals has been and continues to be an exceedingly difficult endeavor. Even when function can be assigned to a specific protein, the phenotypic consequences of activation may not only be cell type specific, but can vary according to both when (e.g., embryo versus adult) and where (e.g., specific tissue microenvironments) activation/expression occurs. As a result, there is increasing interest in the development of methodologies to assess protein function with respect to both temporal and spatial parameters. However, the issue of spatial control has only recently begun to be addressed. One strategy is the use of light to control where gene activation/protein expression transpires. To the best of our knowledge, the first example of light-driven gene expression was reported by Minden and his colleagues in 1997 [20]. These investigators caged GAL4VP16, a transcription factor, via the modification of lysine residues with 6-nitroveratrylcholoroformate. The photoactivatable transcription factor was employed to examine cell fate in *Drosophila* embryos. Haselton and his colleagues prepared 1-(4,5-dimethoxyl-2-nitrophenyl)diazoethane multimodified plasmids encoding either luciferase or green fluorescent protein [21]. The latter were delivered via particle bombardment (rat skin) or liposome transfection (HeLa cells). Recently, Okamoto et al. prepared a coumarin caged mRNA encoding various proteins, including green fluorescent protein, β-galactosidase, and the transcription factor Engrailed2a, which were microinjected into Zebrafish embryos [22]. An obvious advantage of the caged gene-based strategy is that it does not require the use of transgenic animals. Furthermore, it may ultimately be possible to simultaneously introduce and therefore switch on multiple genes. However, limitations include the need to resort to special delivery methods, which hampers application to large multicellular organisms. These characteristics suggest that the caged gene approach should prove ideal for the study of short-term biological phenomena in small cell populations (e.g., embryogenesis).

Koh and his colleagues recently reported the preparation of a caged estradiol, which was used to activate the expression of an estrogen response element-controlled luciferase gene in transiently transfected HEK293 cells [23]. This strategy offers an exciting tool for examining the effect of hormones on endogenous gene expression profiles. Light-activatable, cell-permeable small molecules possess the advantage of ready delivery by simple passive diffusion. In addition, in conjunction with the well-established *Cre/loxP* recombinase strategy [24], a single treatment could potentially elicit permanent changes in gene expression patterns. We describe herein the construction and analysis of a caged cell-permeable ecdysteroid. β-ecdysone and its structural congeners have no known effect on mammalian physiology. Consequently, only ecdysone response element-controlled transgenes should be activated upon photogeneration of the active ecdysteroid. We also demonstrate, for the first time, that a small, caged cell-permeable molecule can be used to drive gene expression in a spatially discrete fashion.

Maximal biological activity of ecdysteroids requires the presence of free hydroxyl groups at the C-2, C-3, and C-20 positions (see **1**) [13]. We prepared the chemically

modified, *biologically inactive* ecdysteroid **4** via alkylation of one of these alcohol moieties. Subsequent light-based cleavage of the modifying agent from the alkylated ecdysone regenerates the native (and therefore biologically active form) of ecdysone. Although a wide variety of structurally diverse caging agents have been described [25], we chose the 4,5-dimethoxy-2-nitrobenzyl moiety because of its reasonably good photophysical properties (λ_{max} and ϕ).

The ecdysone-inducible gene expression system was employed to assess the ability of β-ecdysone **1**, and its caged counterpart, **4**, to induce luciferase expression in the absence and presence of light. We note that naturally occurring β-ecdysone homologs, such as muristerone and ponasterone A, are 1,000 times more active than β-ecdysone and induce transcriptional activity of up to 20,000-fold in cell-based systems [11,12]. However, since these homologs are expensive, we chose to develop our initial chemistry on the more readily available congener **1**. β-Ecdysone induces a nearly 100-fold enhancement in luciferase activity versus background in 293T cells that were transiently transfected to constitutively express EcR/RXR and inducibly express luciferase (Table 1). By contrast, caged β-ecdysone **4** is virtually inactive. However, incubation of 293T cells with **4** and subsequent illumination for 1 min induces luciferase formation at a level that is nearly 60% of its native bioactive counterpart **1**. The 60% restoration of luciferase activity is consistent with our observation that a 1 min photolysis time window converts approximately 60% of the caged compound **4** into the uncaged β-ecdysone as assessed by HPLC. Various control experiments confirmed that the caged derivative, in conjunction with light, is sufficient to activate the EcR/RXR expression system (see Results).

Spatially discrete gene activation requires that the caged inducing agent is both cell permeable and undergoes ready photo-uncaging within the intracellular environment. We addressed this key point by first examining the time-dependence of β-ecdysone-induced luciferase expression as a function of post-irradiation time. As is apparent from Figure 3, photolysis induces the expected induction of luciferase activity in a time-dependent fashion. An analogous experiment was performed to assess the cell permeability of the caged ecdysteroid. In this case, following incubation with **4**, the cell media was removed and the cell culture subsequently washed with PBS to eliminate all extracellular **4**. Ecdysone-free media was then added and the cells were immediately illuminated. Under these conditions, the ecdysteroid should only be present in the intracellular compartment. Both sets of conditions (extracellular + intracellular caged β-ecdysone versus intracellular caged β-ecdysone only) furnish essentially identical kinetic profiles of luciferase induction over the first 6 hr following illumination. Thereafter, however, luciferase activity levels off and begins to decrease in the cell culture where caged β-ecdysone was removed from the extracellular milieu. The latter result may be due to the egress of intracellular β-ecdysone in the absence of an extracellular β-ecdysone concentration gradient. These experiments suggest that **4** is membrane permeable and intracellularly uncaged following photolysis.

We note that it is possible for intracellularly photouncaged β-ecdysone to migrate out of illuminated cells and into the extracellular environment. Under these circumstances, gene activation could transpire in cells distant from the region of illumination upon exposure to active ecdysone. However, given the small intracellular volume of a typical cell (~1 pL), it is likely that any uncaged β-ecdysone that escapes from its intracellular locale will be too dilute to effect changes in gene expression profiles at remote sites. We explicitly addressed this possibility by examining spatially discrete gene activation using spot illumination of cultured 293T cells.

Luciferase expression in fixed and permeabilized cells was visually identified via the coupled use of a luciferase antibody and a secondary Alexa-labeled antibody. Cells incubated with bioactive β-ecdysone **1** furnish global luciferase expression throughout the cell culture (Figure 4A). Luciferase expression is not observed in the absence of β-ecdysone (data not shown) nor in the presence of caged β-ecdysone without light (see below). By contrast, spatially discrete luciferase expression is observed when 293T cells were incubated with caged β-ecdysone **4**, subsequently washed to remove extracellular (but not intracellular) **4**, and spot illuminated (~0.25 mm^2) (Figure 4B). Regions outside of the zone of illumination fail display luciferase expression (Figure 4C). These results not only confirm that caged **4** and light are required to activate gene expression, but also indicate that a well-defined cellular zone of light-induced protein expression is feasible using this strategy.

Caged small molecules (e.g., ATP, NO, etc.) have proven to be extraordinarily useful as reagents to help define temporal relationships in biochemical-mediated processes [25]. More recently, caged peptides and proteins have been described, and their use as tools to delineate the role of individual proteins in cell-based phenomena is underway [26,27]. Although concerns have been raised about the effect of light and/or the caging agent by-product on cell viability, we, as well as others, have found that light-induced activation of caged compounds (from caged fluorophores to caged enzymes) has no obvious effect on cell viability. Indeed, cells behave as expected when the bioactive species is generated (e.g., see the effect of caged cAMP-dependent protein kinase on cellular phenotype [28]). Some of these concerns arise as a consequence of the relatively long illumination times required for photoactivation in vitro, which are typically on the order of minutes. However, cell-based experiments performed under the microscope require only a few seconds to generate the uncaged species (due to a high photon flux through a narrow spatial window). In addition, although the nitrobenzyl byproduct of the uncaging process is an electrophile, extensive work with a wide variety of caged species has failed to uncover any apparent deleterious effects on cell performance or viability (possibly owing to the high intracellular concentration of glutathione, which may chemically add to and therefore neutralize the by-product) [29,30].

Significance

The first example of a caged small molecule that targets a nonmammalian receptor has been prepared

using a stannylene acetal-based strategy. The structure of the caged ecdysteroid was unambiguously characterized by 2D NMR analysis. The caged ecdysteroid is nearly inactive as a gene expression-inducing agent. However, upon exposure to brief illumination, the caged steroid is rapidly converted into active β-ecdysone. In addition, the caged ecdysteroid is cell permeable and undergoes intracellular photouncaging. The light-initiated ecdysteroid-based gene activation strategy not only furnishes temporal control over protein expression in living cells but, in combination with spot illumination, also drives spatially discrete protein expression in a multicellular setting. The ecdysteroid gene activation system is endowed with a number of favorable attributes, including low basal activity in the absence of and high inducibility in the presence of the ligand. Furthermore, ecdysteroids do not appear to have any effect on mammalian physiology. These characteristics, in conjunction with light-driven spatial control over where expression occurs, could potentially provide a means to assess the effect and influence of protein expression on phenotype in a spatially well-defined fashion. For example, tumorigenic potential is dependent, in part, on the ability of the tumor to overcome the unique growth suppressive influence associated with the distinctive microenvironment that harbors it [31]. In addition, tumor metastasis is known to be dependent upon the nature of the host microenvironment as well [32]. Consequently, the ability to alter gene expression, in a microenvironment-specific fashion, should prove valuable in assessing the influence of protein function on the progression of both normal and disease-related phenomena. Finally, we note that the strategy developed here could prove applicable to the array of other previously described inducible expression systems as well.

Experimental Procedures

General

β-Ecdysone was purchased from A. G. Scientific. All other reagents and solvents were purchased from Aldrich. Silica gel 60 (40 μm, Baker) was employed for column chromatography. 1D NMR spectra were recorded on a Bruker DRX-300 and 2D spectra on a Bruker DRX-600. Chemical shifts are reported downfield from tetramethylsilane.

Preparation of Caged β-Ecdysone (4)

A suspension of β-ecdysone (20 mg, 41.6 μmol) and dibutyltin oxide (13.5 mg, 54.2 μmol) in anhydrous methanol (5 mL) was heated to reflux for 3 hr under argon. After the solvent was removed under reduced pressure, the residue was subsequently azeotroped with anhydrous benzene (3 × 2 mL). The resulting stannylene acetal was further dried in vacuo for 2 hr before addition of 3 Å molecular sieves (100 mg), CsF (25.2 mg, 166.4 μmol), 1-bromomethyl-4,5-dimethoxy-2-nitrobenzene **3** (20.6 mg, 74.9 μmol), and anhydrous DMF (1 mL). After the reaction mixture was stirred at room temperature overnight, the solvent was evaporated under reduced pressure. The resulting residue was purified by silica gel column chromatography (methylene chloride/methanol: 6/1) to afford **4** as an off-white solid (25.3 mg, 90%). The purity was determined to be >99% by analytical HPLC (retention time 16.2 min on a Vydac C4 column 250 mm × 3.0 mm, monitored at 242 nm; a 15 min linear gradient from 95% A [water] to 50% B [acetonitrile], followed by 50% B for 5 min with the flow rate of 1 mL/min). ^{1}HNMR (300 MHz, CD_3OD): δ 0.89 (s, 3H), 0.99 (s, 3H), 1.19 (s, 9H), 1.20–1.98 (m, 17H), 2.39 (m, 2H), 3.11 (m, 1H), 3.72 (m, 1H), 3.90 (s, 3H), 3.97 (s, 3H), 4.25 (br s, 1H), 4.99 (q, 2H), 5.81 (d, 1H), 7.42 (s, 1H), 7.71 (s, 1H). HRMS (ESI+) calculated for $C_{36}H_{54}NO_{11}$: 676.3697 Found: 676.3674. A series of 2D correlation (COSY) NMR experiments were subsequently performed to assess the site of alkylation on the β-ecdysone framework, including double quantum filtered COSY (DQF-COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple quantum correlation (HMQC) spectroscopies.

Photolysis of Caged β-Ecdysone (4)

A 500 μl 100 μM solution of **4** in 2% methanol/98% water was placed in a 24-well culture plate. Photolysis was performed using an Oriel 200 W Hg arc lamp (model 6283) with a 348 nm filter (Oriel, lot number 51260; 50% internal transmittance at 348 nm and cutoff at 325 nm) to remove short wavelength light and an IR filter to remove heat. Aliquots of 10 μl were removed at different time points of photolysis and were analyzed by analytical HPLC (monitored at 242 nm) employing a Vydac C4 (250 mm × 3.0 mm) column and using the following solvent system: a linear gradient from 95% A (water) 5% B (acetonitrile) to 50% A (water) 50% (acetonitrile) over 15 min followed by 50% A (water) 50% B (acetonitrile) for 5 min with flow rate at 1 mL/min.

Plasmid Description

The luciferase reporter $(E/GRE)_6$TK81LUC consists of multimeric E/GRE binding sites from $(E/GRE)_4$ΔMTVLuc linked to the minimal TK promoter in the pA_3LUC reporter plasmid. The modified MMTV promoter from MMTVp206 that incorporates the 5′UTR of v-Ha-ras was inserted into a vector containing a modified ecdysone receptor VgEcR (a gift from R. Evans) to form MMTV-VgEcR.

Cell Culture, DNA Transfection, and Luciferase Assays

Cell culture, DNA transfection, and luciferase assays were performed as previously described [33]. Briefly, 293-derived BOSC cells (293T) were seeded into individual wells of a 24-well plate and maintained in Dulbecco's modified Eagles medium with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were transiently transfected with pVgEcR/RXRα and $(E/GRE)_6$TKLUC via the standard calcium phosphate method. The media was changed after 14 hr and the cells treated with 10 μl of 5 mM β-ecdysone **1** in methanol (to furnish a 100 μM final concentration of ecdysteroid). Cells were either left unilluminated or illuminated for 1 min using an Oriel 200 W Hg arc lamp (model 6283) with a 348 nm filter (Oriel, lot number 51260, 50% internal transmittance at 348 nm and cutoff at <325 nm) to remove short wavelength light and two IR filters to remove heat. Cells were subsequently lysed as previously described and the luciferase assay performed at room temperature using an Autolumat LB 953 (EG & G, Berthold). Luciferase content was measured by calculating the light emitted during 10 s of the reaction. The values are expressed in arbitrary light units.

Time-Dependent Luciferase Assays

293T cells were split in a 24-well culture plate (Becton Dickinson Labware, lot 353047) and were transfected by the phosphate calcium method. The cells in 500 μl culture medium were incubated with either 100 μM caged β-ecdysone, 100 μM β-ecdysone, or in the absence of ecdysteroid ligand. After the cells were incubated with caged β-ecdysone for 16 hr, the medium was removed and washed with PBS once. A fresh medium free of ligand was then immediately added just prior to UV light exposure for 1 min. In the "Caged + hv" experiment, the caged β-ecdysone ligand remained in the medium and these cells were likewise illuminated for 1 min. Cells that were not exposed to ecdysteroid ligand were treated in an otherwise analogous fashion. The cells were then returned to the incubator for various time intervals. Incubation was terminated by treating cells with an extraction buffer (1% Triton X-100 and 1 mM DTT in GME). The luciferase assay was preformed as described above.

Immunodetection of Intracellularly Expressed Luciferase

293T cells were transferred to the individual wells of a 24-well culture plate (Becton Dickinson Labware, lot 353047) and transfected as described above. The cells were then incubated with either 100 μM β-ecdysone or 100 μM caged β-ecdysone for 16 hr. The medium

was removed and the cells washed once with PBS before addition of an ecdysteroid free medium. The surface of the prescored wells was spot illuminated (~0.25 mm^2) for 10 s on the light microscope using a 100 W Hg-Arc lamp through an Olympus UAPO 20X/0.40 objective. The cells were then incubated for 6 hr, fixed with 4% formaldehyde in PBS for 60 min, washed with PBS 3 × 5 min, permeabilized with 0.1% Triton X-100 for 10 min, washed with PBS 3 × 5 min, and blocked with 1% normal donkey serum (Jackson ImmunoResearch, catalog number 017-000-121) in PBS for 45 min. The cells were subsequently exposed to 300 μl of anti-luciferase pAb (polyclonal goat antibody, Promega, lot 149040) at a concentration of 40 μg/mL in 1% donkey serum in PBS. After incubation for 2 hr at room temperature in a humidified chamber, the cells were washed with PBS 3 × 15 min and exposed to 300 μl of an Alexa Fluor 568-labeled rabbit anti-goat IgG (20 μg/mL in 1% donkey serum/PBS for 1 hr, Molecular Probes, catalog number A-11079). Unbound antibody was removed by washing with PBS 3 × 15 min and the cells treated with 300 μl of mounting medium (N-propyl gallate at 6 mg/mL in 1:1 glycerol:PBS). Images were taken using an Olympus IX-70 microscope equipped with a CCD camera.

Acknowledgments

This work was supported by the National Institutes of Health (D.S.L. and R.G.P.). We are pleased to acknowledge the Analytical Imaging Facility, the Comprehensive Cancer Center, the Laboratory for Macromolecular Analysis and Proteomics, and the Structural NMR Resource of the Albert Einstein College of Medicine. We thank Dr. Sean Cahill, Mr. Michael Cammer, Dr. Mousumi Ghosh, and Dr. Renjian Zheng for technical assistance and/or advice, and Professors Anne Bresnick, John Condeelis, and Edward Turos for helpful discussions.

Received: September 19, 2002
Revised: October 25, 2002
Accepted: October 25, 2002

References

1. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. (1987). Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell *49*, 465–475.
2. Ormandy, C.J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., et al. (1997). Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. Genes Dev. *11*, 167–178.
3. Liu, X., Robinson, G.W., Wagner, K.U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. *11*, 179–186.
4. Ryding, A.D., Sharp, M.G., and Mullins, J.J. (2001). Review: conditional transgenic technologies. J. Endocrinol *171*, 1–14.
5. Albanese, C., Sakamaki, T., Wang, J., and Pestell, R.G. (2002). Recent advances in inducible transgenic mice. Semin. Cell Dev. Biol. *13*, 129–141.
6. Gingrich, J.R., and Roder, J. (1998). Inducible gene expression in the nervous system of transgenic mice. Annu. Rev. Neurosci. *21*, 377–405.
7. Shockett, P.E., and Schatz, D.G. (1996). Diverse strategies for tetracycline-regulated inducible gene expression. Proc. Natl. Acad. Sci. USA *93*, 5173–5176.
8. Cronin, C.A., Gluba, W., and Scrable, H. (2001). The *lac* operator-repressor system is functional in the mouse. Genes Dev. *15*, 1506–1517.
9. Belshaw, P.J., Spencer, D.M., Crabtree, G.R., and Schreiber, S.L. (1996). Controlling programmed cell death with a cyclophilin-cyclosporin-based chemical inducer of dimerization. Chem. Biol. *3*, 731–738.
10. Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. Proc. Natl. Acad. Sci. USA *93*, 10887–10890.
11. No, D., Yao, T.P., and Evans, R.M. (1996). Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA *93*, 3346–3351.
12. Albanese, C., Reutens, A.T., Bouzahzah, B., Fu, M., D'Amico, M., Link, T., Nicholson, R., Depinho, R.A., and Pestell, R.G. (2000). Sustained mammary gland-directed, ponasterone A-inducible expression in transgenic mice. FASEB J. *14*, 877–884.
13. Dinan, L., Hormann, R.E., and Fujimoto, T. (1999). An extensive ecdysteroid CoMFA. J. Comput. Aided Mol. Des. *13*, 185–207.
14. Suksamrarn, A., and Pattanaprateep, P. (1995). Selective acetylation of 20-hydroxecdysone. Partial synthesis of some minor ecdysteroids and analogues. Tetrahedron *38*, 10633–10650.
15. Grindley, T.B. (1998). Application of tin-containing intermediates to carbohydrate chemistry. Adv. Carbohydr. Chem. Biochem. *53*, 17–142.
16. Nagashima, N., and Ohno, M. (1991). Selective monoalkylation of acyclic diols by means of dibutyltin oxide and fluoride salts. Chem. Pharm. Bull. *39*, 1972–1982.
17. Huber, R., and Hoppe, N. (1965). Die kristall- und molekulstrukturanalyse des insekternverpuppungshormons ecdyson mit der automatisierten faltmolekulmethode. Chem. Ber. *98*, 2403–2424.
18. Galbraith, M.N., and Horn, D.H.S. (1969). Insect molting hormones: crustecdyson (20-hydroxyecdysone) from *Podocarpus elata*. Aust. J. Chem *22*, 1045–1057.
19. Hatchard, C.G., and Parker, C.A. (1956). A new sensitive chemical actinometer. II. Potassium ferrioxalate as a standard chemical actinometer. Proc. R. Soc. London *A220*, 518–536.
20. Cambridge, S.B., Davis, R.L., and Minden, J.S. (1997). *Drosophila* mitotic domain boundaries as cell fate boundaries. Science *277*, 825–828.
21. Monroe, W.T., McQuain, M.M., Chang, M.S., Alexander, J.S., and Haselton, F.R. (1999). Targeting expression with light using caged DNA. J. Biol. Chem. *274*, 20895–20900.
22. Ando, H., Furuta, T., Tsien, R.Y., and Okamoto, H. (2001). Photo-mediated gene activation using caged RNA/DNA in zebrafish embryos. Nat. Genet. *28*, 317–325.
23. Cruz, F.F., Koh, J.T., and Link, K.H. (2000). Light-Activated Gene Expression. J. Am. Chem. Soc. *122*, 8777–8778.
24. Ryding, A.D., Sharp, M.G., and Mullins, J.J. (2001). Review: conditional transgenic technologies. J. Endocrinol *171*, 1–14.
25. Adams, S.R., and Tsien, R.Y. (1993). Controlling cell chemistry with caged compounds. Annu. Rev. Physiol. *55*, 755–784.
26. Curley, K., and Lawrence, D.S. (1999). Light-activated proteins. Curr Opin. Cell. Biol *3*, 84–85.
27. Marriott, G., and Walker, J.W. (1999). Caged peptides and proteins: new probes to study polypeptide function in complex biological systems. Trends Plant Sci. *4*, 330–334.
28. Curley, K., and Lawrence, D.S. (1998). Photoactivation of a Signal Transduction Pathway in Living Cells. J. Am. Chem. Soc. *120*, 8573–8574.
29. Kaplan, J.H. (1978). Rapid photolytic release of adenosine 5′-triphosphate from a protected analog: utilization by the sodium: potassium pump of human red blood cell ghosts. Biochemistry *17*, 1929–1935.
30. Walker, J.W., Reid, G.P., and Trentham, D.R. (1988). Synthesis and properties of caged nucleotides. Methods Enzymol. *172*, 288–301.
31. Roskelley, C.D., and Bissell, M.J. (2002). The dominance of the microenvironment in breast and ovarian cancer. Semin. Cancer Biol. *12*, 97–104.
32. Liotta, L.A., and Kohn, E.C. (2001). The microenvironment of the tumour–host interface. Nature *411*, 375–379.
33. Wantanable, G., Howe, A., Lee, R.J., Albanese, C., Su, I.-W., Karnezis, A.N., Zon, L., Kyriakis, J., Rundell, K., and Pestell, R.G. (1996). Induction of cyclin D1 by simian virus 40 small tumor antigen. Proc. Natl. Acad. Sci. USA *93*, 12861–12866.