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Photo-caged agonists of the nuclear receptors RARγ and TRβ provide unique time-dependent gene expression profiles for light-activated gene patterning

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Abstract—Light-activated gene expression systems hold promise as new tools for studying spatial and temporal gene patterning in multicellular systems. Photo-caged forms of nuclear receptor agonists have recently been shown to mediate photo-dependent transcription in mammalian cells, however, because intracellularly released agonists can rapidly diffuse out of cells, the photo-initiated transcription response is only transient and limited to only a few hours in reported examples. Herein we describe a photo-caged thyroid hormone receptor agonist that provides a robust 36 h transcription response to a single irradiation event. These findings are in contrast to a closely related system, which uses a caged retinoic acid receptor agonist, which provides only a short transcription response. Comparison of the two systems, show that the duration of transcription response is not controlled by the rate of diffusion of free ligand out of the cell, but perhaps by the duration of ligand-induced transcription/stability of the active transcription complex.

1. Introduction

Many methods are currently available to remotely regulate the expression of specific genes and have provided a powerful tool to elucidate the role of specific genes involved differentiation, growth, and homeostasis.[1–8] The actions of many gene products critical for the development and homeostasis are only revealed through their unique spatial and temporal patterns of expression.[9–11] Photo-regulation of signal transduction and/or gene expression provides a powerful new tool for the study of gene function that can provide both spatial and temporal control of gene expression. Several studies have demonstrated that expression of some genes can be regulated by light using photo-caged enzymes, enzyme substrates, or enzyme inhibitors involved in signal transduction pathways.[12–15] Recently, Haselton and later Tsien reported a new strategy to directly control gene expression with light using photo-caged nucleic acids that are transcriptionally (or translationally) silent until uncaged with light.[16,17] The application of such systems to multicellular systems often are not practical due to the difficulties of efficiently delivering of caged biopolymers into cells. A new and promising light-activated transcription system that uses plant phytochrome was recently reported by Quail and co-workers.[18] This system requires addition or co-expression of the plant phytochrome and has thus far only been demonstrated in yeast.

Recently, we described a new approach to regulating gene expression using photo-caged hormones, which target nuclear hormone receptors (NHRs). NHRs function as ligand-dependent transcriptional regulators that directly bind DNA and up-regulate gene transcription in response to binding small molecule hormones.[19,20] Several examples of chimeric receptors in which the DNA binding domain of NHRs have been exchanged for DNA binding domains from other NHRs or heterologous proteins allow one to target the actions of hormones to virtually any transgene of interest. In

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addition, ligand receptor engineering has been used to create selective and functionally orthogonal agonist-NHR pairs that can be used to independently regulate gene transcription without effecting endogenous receptors.[21–26] Together these observations suggest that NHRs represent a novel platform to construct selective photo-inducible transcriptional regulators.

In our initial study a photo-caged form of estradiol was used to mediate light-dependent expression of estrogen responsive genes.[27] Caged hormones (or NHR agonists) readily diffuse across cell membranes and therefore have many advantages over methods, which employ caged biopolymers, which can be difficult to introduce into multicellular systems. Caged hormone receptor agonists have been used to spatially resolved patterns of reporter gene expression,[28] however, when hormones are locally uncaged within multicellular systems, the duration of transcription response is only transient, presumably because the released hormone ultimately diffuses out of the cell. In the case of our previously reported nitroveratryl estradiol, intracellular uncaging provides a relatively weak (40% of max) and short duration reporter gene response that is limited to a few hours. Lawrence and coworkers using a caged ecdysone and cells expressing the ecdysone receptor obtained transient expression profiles that were slightly longer and more intense but were still of relatively short duration ($\geqslant$50% max expression for 2 h).[28] These short duration transcription responses are often too short to provide a practical method to study the effects of gene patterning without having to constantly re-expose the expressed pattern.

Although it is difficult to directly compare the duration of gene response between these two studies, which involve different cell types and different reporter gene constructs, it is intriguing to consider that other hormone-receptor pairs might be able to provide a longer and more robust transcription response to transient photo-patterning. Toward this goal we have studied the properties of caged retinoic acid receptor (RAR) and thyroid hormone receptor (TR) agonists in closely related experimental systems using the same cell line and exploiting the well-known phenomenon that RAR and TR can both regulate reporter gene expression from the identical DR4 hormone response element.[29–32]

2. Results

2.1. Photo-stable agonists for TR and RAR

In previous work we demonstrated that a photo-caged form of estradiol could be used to control transactivation function from the steroid hormone receptor, estrogen receptor (ER) in an exposure dependent manner.[27] In this study we sought to examine the characteristic properties of light-activated gene expression mediated by two members of the nuclear receptor family, which activate gene transcription in a related mechanism that is distinct from that of steroid hormone receptors. Coincidentally, the natural ligands for the nuclear receptors, TR, RAR, RXR, and VDR, are light sensitive. Retinoic acid and calcitriol contain light-sensitive polyenes and triiodothyronine contains aryl iodides sensitive to photoisomerization or degradation. Therefore, we synthesized **3** and **4** as potential photo-caged analogs of the known synthetic agonists of RAR and TR, **1** and **2** (GC-1) (Fig. 1).[33,34]

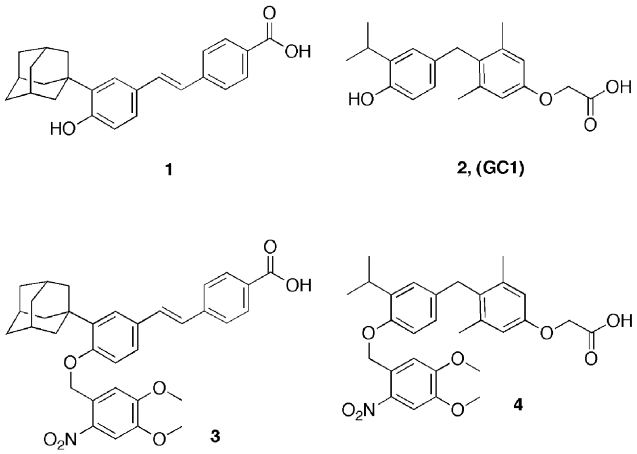


Figure 1. Light-stable agonists and photo-caged analogs.

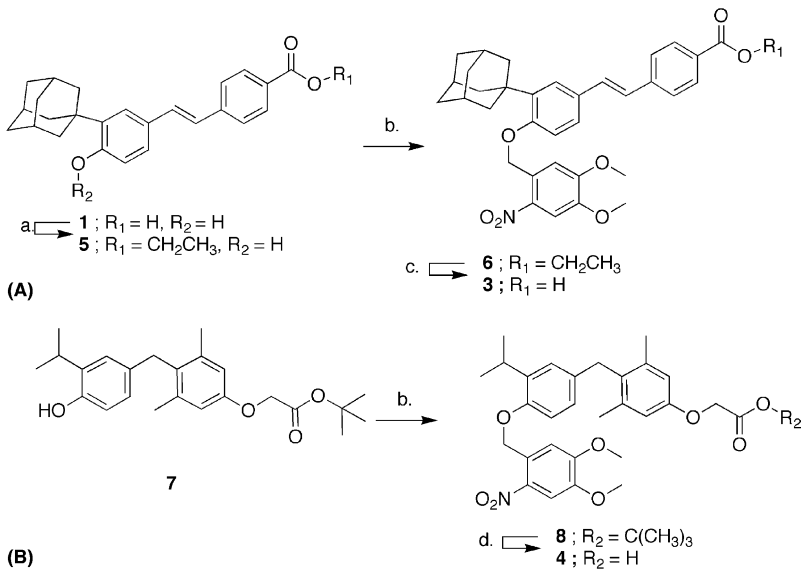
2.2. Photo-caged agonists 3 and 4 efficiently release photo-stable agonists in vitro

The stilbene **1** was first reported by Charpentier et al. as a potent RARγ agonist.[33] Under our assay conditions using transiently transfected HeLa cells, **1** has an EC_{50} of 467 nM with our DR4-luc reporter. This compound is relatively stable to irradiation with a fluorescent laboratory UV lamp showing less than 1% decomposition after 180 s of irradiation. Similarly, the potent halogen-free thyromimetic GC1,[34] **2** (EC_{50} = 48 nM), shows less than 11% decomposition after 180 s of irradiation, demonstrating that **1** and **2** are stable to conditions of photo-deprotection. The caged analogs **3** and **4** were easily synthesized from the previously reported compounds **1** and **7** (Scheme 1),[33,34] and were confirmed to contain less than 0.2% of the parent agonists by HPLC.

The caged compounds **3** and **4** were photo-deprotected under cell free conditions to determine the rate and efficiency with which caged compounds were converted to agonist products upon irradiation. Methanolic solutions of compounds **3** and **4** (100 μM) were placed in polystyrene cell culture plates and exposed in an analogous manner used in our cell culture experiments (see Experimental). HPLC analysis confirms that **3** and **4** efficiently liberate the agonists **1** and **2** with greater than 90% conversion efficiency. However, the rate of deprotection of **4** ($1.1 \times 10^{-2}\,s^{-1}$) is three times slower than that of **3** ($3.1 \times 10^{-2}\,s^{-1}$) such that only 20% of **4** is converted to **2** during a 90 s exposure (Fig. 2).

2.3. Photo-caged compounds 3 and 4 are stable under cellular conditions

The successful application of **3** and **4** to mediate light-activated gene transcription requires that our photo-



Scheme 1. Synthesis of caged agonists **3** and **4**. Reagents and conditions: (a) H_2SO_4, EtOH, reflux (86%), (b) K_2CO_3, nitrovarearyl bromide, DMF (91%), (c) LiOH, THF/H_2O (75%), (d) NaOH, rt (95%).

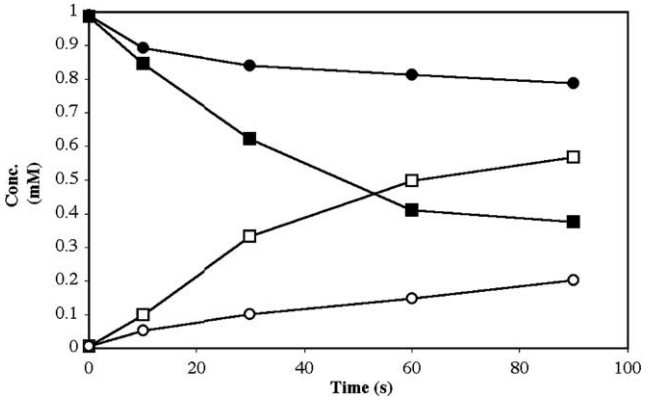


Figure 2. Exposure dependence of photo-decomposition and product formation. Compounds **3** (■) and **4** (●) are converted with greater than 90% efficiency to their parent agonist forms **1** (□) and **2** (○) but at different rates.

caged ligands be stable under cellular conditions, only releasing active ligand upon irradiation. To test the cellular stability of our photo-caged compounds, we simulated intracellular conditions by placing our compounds in HeLa cell lysates for 36 h at 37 °C. Because even trace quantities of the active ligand could activate gene transcription, solutions of the lysate-treated compounds were evaluated for their ability to activate reporter gene expression in cell based transactivation assays with cells expressing their corresponding receptors. Cells grown in the presence of lysate-treated compounds **3** and **4** did not show a significant increase in gene expression compared to cells grown in the presence of untreated compounds at the same concentration. In addition, cells grown in the presence of either lysate-treated or untreated caged compounds show essentially equivalent reporter gene expression after irradiation with light, indicating that the caged compounds were neither degraded nor prematurely uncaged under intracellular conditions (Fig. 3).

2.4. Photo-caged analogs 3 and 4 do not act as agonists or antagonists with receptors below 1 μM

The successful control of gene expression using caged agonists requires that the caged compounds do not act as agonists prior to irradiation. HeLa cells transiently transfected with reporter vector and either pSG5hRARγ or pSG5hTRβ were treated with increasing concentrations of caged agonists **3** and **4**. Only a small increase in reporter gene expression is observed at concentrations at or below 1 μM demonstrating that introduction of the nitroveratryl group onto the phenol hydroxyl of **1** or **2** effectively blocks (or reduces) the potent agonist properties of the parent agonists (Fig. 4). At high concentrations, greater than 2500 nM, of **3** and **4**, a more significant increase in reporter gene activity is observed suggesting that the caged analogs are possibly very weak agonists or perhaps more likely contain trace quantities of active agonists **1** or **2**. High concentrations of **3** (3000 and 4000 nM) were observed to cause visible signs of toxicity. Therefore, caged agonists were used at or below concentrations of 1000 nM.

Compounds **3** and **4** were also evaluated for their ability to act as potential antagonists in competition with the parent agonists **1** and **2**. HeLa cells transiently transfected with either pSG5hRARγ or pSG5hTRβ were grown in the presence of either 800 nM of agonist **1** or 100 nM agonist **2** were treated with increasing concentrations of caged analogs **3** and **4**. No change in reporter gene expression was observed below 1000 nM indicating that the caged compounds do not act as effective antagonists below 1 μM (Fig. 5). In vitro radio-ligand competition binding assays confirmed that compound **3** has an apparent K_d greater than 8 mM.

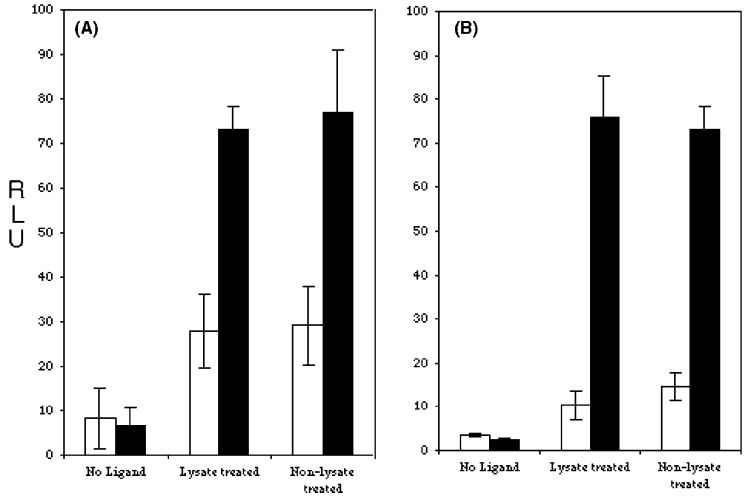


Figure 3. Treatment of caged analogs **3** and **4** with cell lysates does not effect transactivation function in unexposed cells (white bars) or irradiated cells (black). (A) HeLa cells transiently transfected with pSG5hRAR and **3**. (B) Cells transiently transfected with pSG5hTRβ and treated with **4**.

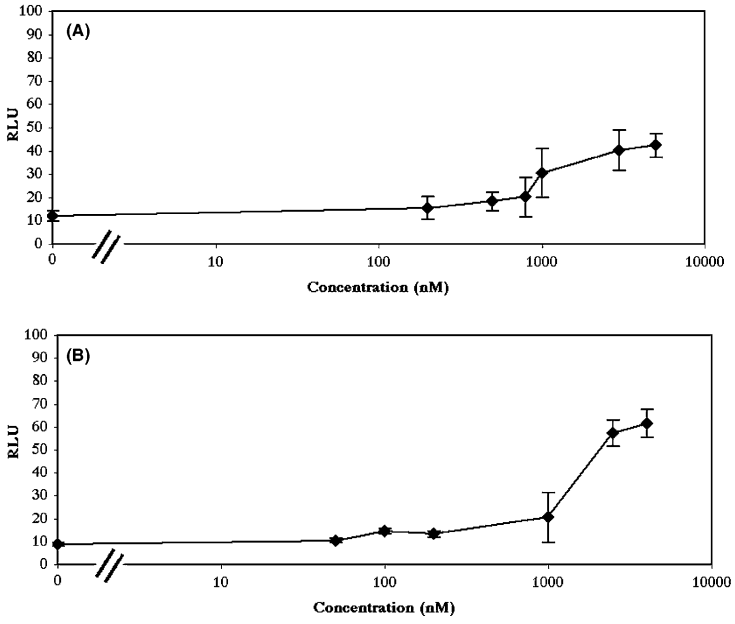


Figure 4. Compounds **3** and **4** show almost no transcriptional activity below 1000 nM in cells expressing (A) RAR or (B) TR.

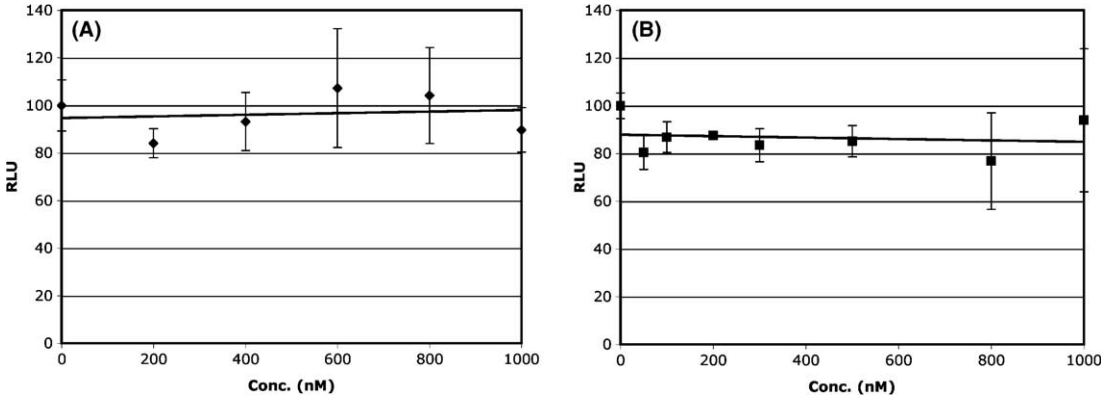


Figure 5. Caged compounds **3** and **4** do not antagonize RAR or TR dependent transactivation. (A) Cells expressing RAR treated with 800 nM **1** and increasing concentrations of **3**. (B) Cells expressing TR treated with 100 nM **2** and increasing concentrations of **4**.

2.5. Caged ligands 3 and 4 can mediate exposure dependent RAR and TR dependent transactivation response

A fluorescent UV lamp (λ_{max} = 365 nm), described previously, was used to irradiate cells directly through the polystyrene lid of a standard cell culture plate, which acts as a filter for potentially damaging short wavelength UV (<345 nm).[27] Cells transiently transfected with reporter and either pSG5hRARγ or pSG5hTRβ grown in the absence of ligand did not display any exposure dependent reporter gene expression. Exposure times greater than 5 min were accompanied by some visible changes in cell morphology or viability. Therefore, substantially shorter exposure times of 90 s or less were used to avoid undesirable effects of prolonged irradiation.

Cells transiently transfected with pSG5hRARγ or pSG5hTRβ were grown in the presence of 800 nM **3** or 500 nM **4**, such that a 90 s exposure would be expected to liberate **1** or **2** at a concentration approximately twice the EC_{50} of these ligands for their respective receptors. Both ligands show an exposure dependent production of the reporter gene in cells expressing their corresponding receptors (Fig. 6). Compound **3** mediates RAR-dependent reporter gene expression, approaching a 4-fold induction of reporter gene expression or 90% of the maximum inducible expression level with a 90 s exposure. Although maximal level of gene expression mediated by **3** is large, the induction of gene expression relative to unexposed cells is relatively modest, 4-fold induction, and is largely limited by the significant background activity observed in the absence of exposure. Cells treated with 500 nM **4** show exposure dependent TR-mediated reporter gene expression corresponding to a 24-fold induction, essentially attaining the maximum possible gene expression with a 90 s exposure.

2.6. Intracellular uncaging provides a transient transactivation response

The application of photo-caged hormone receptor agonists to form spatially discrete patterns of gene expression may be limited by the diffusion of ligand out of cells after being intracellularly uncaged. To simulate the conditions expected when compounds are uncaged intracellularly in only a local subpopulation of cells, HeLa cells were grown in six-well plates with media containing either the caged compounds **3** or **4** for 12 h. Just prior to irradiation, the media was removed; the cells were washed twice with PBS buffer and the media replaced with fresh media without caged agonist such that the only caged compound present during irradiation is intracellular. The fluorescent property of the parent agonist **1** allows us to directly visualize the likely cellular localization of structurally related caged analogs **3** and **4**. Cells grown in the presence of **1**, show intense intracellular fluorescence demonstrating that such compounds are likely localized in the cytoplasm and not strongly associated with the membrane or cell surface after media exchange (Fig. 7).

HeLa cells pretreated with 1000 nM **3** and 'washed' prior to irradiation for 90 s, show a unique temporal dependence of gene expression. After exposure, luciferase activity increases over the initial 4 h at a rate similar to cells treated with **3** but not washed prior to exposure. Gene product increases to a maximum at 6 h (54%), and then decreases presumably as the result of the loss of intracellular ligand due ultimately to diffusion (Fig. 8A). The

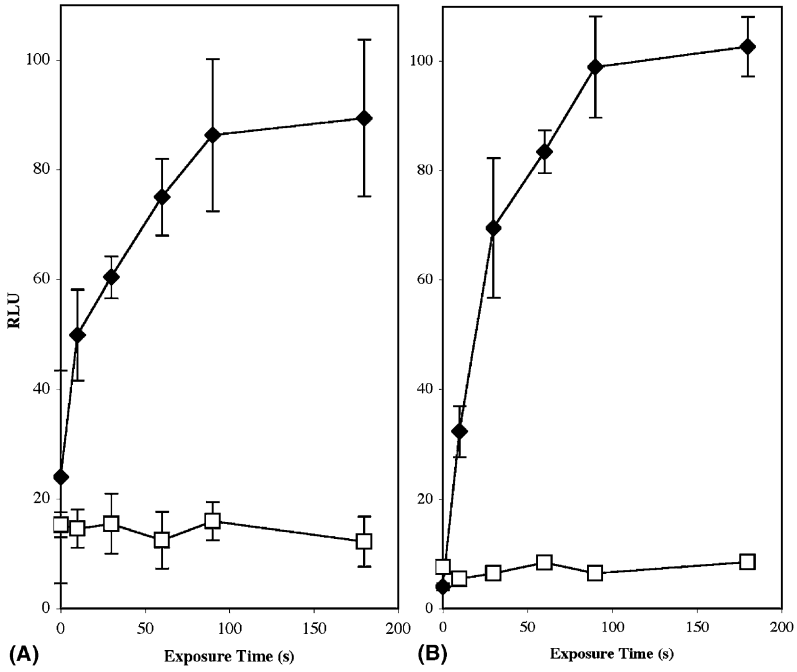


Figure 6. Reporter gene activity determined 24 h after irradiation is exposure and ligand dependent. (A) Cells expressing hRARγ with 800 nM **3** (♦) or with no added ligand (□). (B) Cells expressing hTRβ with 500 nM **4** (♦) or with no added ligand (□).

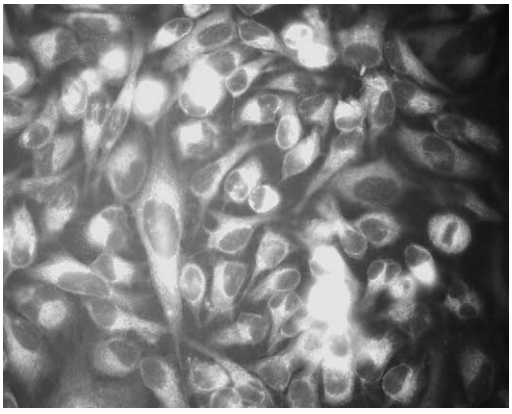


Figure 7. Fluorescence micrograph of HeLa cells incubated with **1** and observed under upright microscope with dipping lens.

duration of transcription response, measured as the length of time gene product is 50% of the maximum level or greater, is limited to approximately 2 h. This is comparable with the duration of transcription response previously reported for ER and EcR mediate gene patterning,[27,28] therefore, similar to ER and EcR, this light-activated RAR system likely does not provide a practical method to pattern expression in multiple tissues without having to constantly rephoto-pattern the system.

2.7. Cells expressing TR provide a long duration response

HeLa cells expressing TR and pretreated 500 nM **4** display a unique expression profile that differs substantially from that observed with RAR and **3** in both intensity and duration of gene response. In this case reporter gene reaches almost 80% the maximum expression level in 12 h and then decreases. Transcription is sustained over 50% of the maximum level for over 36 h, substantially longer than has been attained for any caged hormone studied thus far (e.g., ER, RAR, or EcR) (Fig. 8B).[27,28] These studies show that dramatically different time-dependent transcription profiles can be attained by different nuclear receptors even when using the same cell line and reporter gene. Although 100% of the maximal expression level is not attained, it is important to recognize that genes are generally not expressed at their maximal levels and the similar profiles of time-dependent gene expression are observed in many cellular processes such as cell division or differentiation.[35] Therefore, the light-activated transcription system composed of **3** and TR provides a physiologically significant expression response to a single, exposure event offering substantial practical advantage over existing systems. Furthermore, caged nuclear receptor agonists can provide unique time-dependent transcription profiles that are distinct from those expected from direct photo-activation of caged DNA or caged proteins involved in signal transduction pathways.

3. Discussion

3.1. The magnitude and duration of transactivation response may be dependent upon the duration of active transcription

The caged agonists **3** and **4** provide exposure dependent RAR and TR mediated reporter gene expression cultured cells. This exposure dependence, suggests that light-activated gene expression using hormone receptors

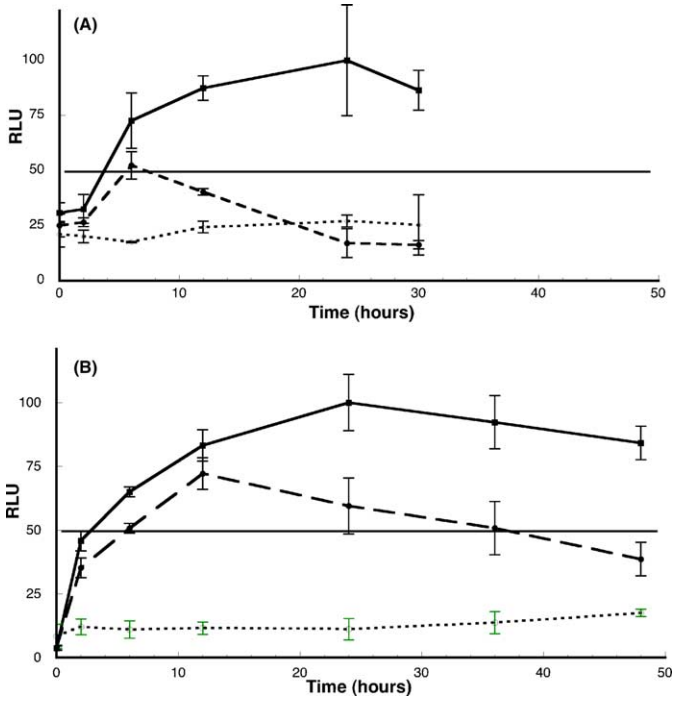
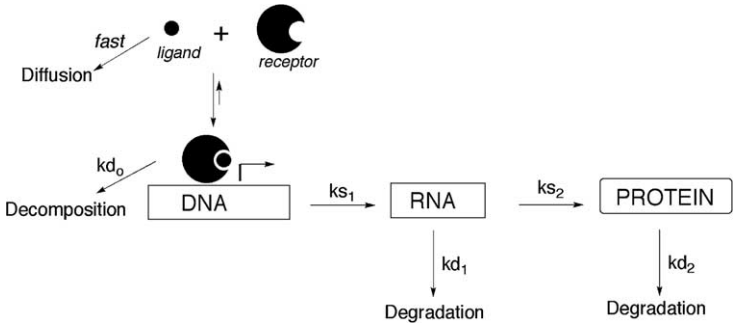


Figure 8. Time dependence of luciferase formation after 90 s exposure. (A) Cells expressing RARγ pretreated with 800 nM **3**. (B) Cells expressing TRβ pretreated with **4**; (■) ligand uncaged in media and cells; (●) ligand uncaged in cells only after prewashing; (○) not irradiated.

may be used to create both spatially resolved binary patterns and potentially gradients of inducible gene products in multicellular systems.

For these studies, the identical reporter gene construct and cell line was used to measure both RAR and TR mediated gene expression. These systems provides a unique opportunity to explore the factors that may lead to different time-dependent transcription profiles that are independent of cell type or the nature of the reporter gene. Presumably, gene product formation triggered by intracellularly released hormone receptor agonist stops as the result of the loss of intracellular ligand. However the duration of reporter gene response obtained with GC1 and TR is too long to be attributed simply to the rate of free ligand diffusion out of the cell, which would be expected to be very fast, on the order of $2 \times 10^5\,s^{-1}$ for diffusion across a lipid bilayer.[36] Furthermore, agonists **1** and **2**, though not identical, are structurally very similar, and would be expected to diffuse across membranes at comparable rates, yet they exhibit markedly different time dependent transcription profiles.

In an attempt to better understand the factors leading to the different time-dependent transcription responses from RAR and TR, a mathematical model for transcription/translation developed by Hargrove and Schmidt was fit to our two systems.[37] The kinetic parameters governing the RAR and TR systems are significantly underdetermined. However, relative and qualitative



Scheme 2. The formation of protein product is represented as an intermediate between transcription/translation and protein degradation. The duration of transactivation response is limited by the decomposition of the receptor associated transcription complex (k_{d0}).

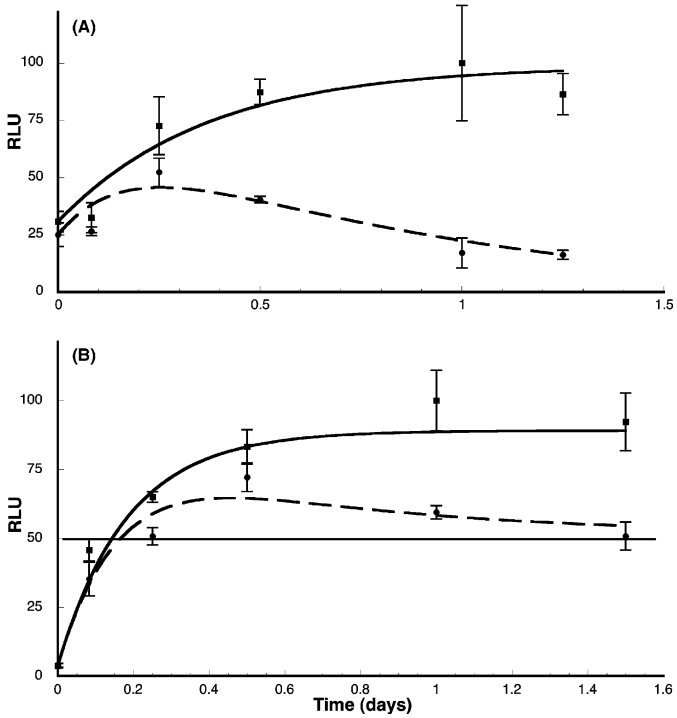


Figure 9. Transcription/translation models fit to observed time-dependent transcription profiles using the equation of Hargrove and Schmidt (■, solid lines): (A) RAR with 8, $k_{s1} = 0.34\,pmol/g/d$, $k_{s2} = 10{,}000\,pmol/d/pmol\ RNA$, $k_{d1} = 2.77\,d^{-1}$, $k_{d2} = 5.54\,d^{-1}$, $P_0 = 114\,pmol/g$, $R_0 = 0.08\,pmol/g$, and $R^2 = 0.93$. (B) TR with 5, $k_{s1} = 0.51\,pmol/g/d$, $k_{s2} = 10{,}000\,pmol/d/pmol\ RNA$, $k_{d1} = 2.77\,d^{-1}$, $k_{d2} = 5.54\,d^{-1}$, $P_0 = 13.9\,pmol/g$, $R_0 = 0.18\,pmol/g$, and $R^2 = 0.99$; Intracellular uncaging fit as first order exponential decay of the transcription complex (●, dashed line): (A) RAR with 8, $k_{d0} = 0.18\,d^{-1}$ (half-life = $3.14\,d^{-1}$), $a = -0.66\,pmol/g$, $k_{s1} = 0.34\,pmol/g/d$, $k_{s2} = 10{,}000\,pmol/d/pmol$ RNA, $k_{d1} = 2.77\,d^{-1}$, $k_{d2} = 5.54\,d^{-1}$, $P_0 = 92.7\,pmol/g$, $R_0 = 0.08\,pmol/g$, and $R^2 = 0.71$. (B) TR with 5, $k_{d0} = 0.025\,d^{-1}$ (half-life = $0.27\,d^{-1}$), $a = -0.36\,pmol/g$, $k_{s1} = 0.51\,pmol/g/d$, $k_{s2} = 10{,}000\,pmol/d/pmol$ RNA, $k_{d1} = 2.77\,d^{-1}$, $k_{d2} = 5.54\,d^{-1}$, $P_0 = 13.9\,pmol/g$, $R_0 = 0.18\,pmol/g$, and $R^2 = 0.99$.

insight into the functions controlling the duration of reporter gene expression in response to a transiently introduced stimulus can be approximated, using literature values of mRNA and protein half-lives because the mRNA and protein products are identical for both systems.

It has been previously demonstrated, using a two compartment mathematical model, that the amount of active protein $P(t)$, is represented as an intermediate between mRNA and protein products (Scheme 2). This model shows that the maximal amount of gene product formed, the 'fold induction', and the half-life for formation of protein are critically dependent on the mRNA and protein stabilities, in addition to the rates of transcription and translation.

Since the same DR4-luciferase reporter was used in the TR and RAR studies, k_{s2}, k_{d1}, k_{d2} should be identical. Thus the rate of transcription, k_{s1}, could be responsible for the differences in the temporal responses for TR and RAR. The rates of transcription were determined by fitting the data for the intra and extracellular deprotection experiments of RAR and TR to the mathematical model of Hargrove and Schmidt, using literature values for the half-life of luciferase mRNA and protein, and a generic rate of protein synthesis.[37–39] Our experimental data fits reasonably well to this model. The results obtained from the regression analysis shows that the rate of transcription of RAR is approximately 66% of TR based on the maximum absolute luciferase activity of RAR is approximately 60% that of TR, and $P_{max} = \frac{k_{s1}k_{s2}}{k_{d1}k_{d2}}$ (Fig. 9, solid lines).

In this study, we have selectively released the agonists **1** and **2** intracellularly by removing the extracellular caged compound **3** and **4** prior to irradiation. Under identical conditions, it was determined that the duration and magnitude of the reporter gene response was much greater with TR + 2 then RAR + 1. In an attempt to gain further insights into the molecular events responsible for controlling these differences in reporter gene response, the intracellular only deprotection was fit to a modified form of the Hargrove and Schmidt equation, where active transcription was to a first approximation modeled as a first order exponential decay, k_{d0}, while keeping the kinetic parameters derived above constant. The results of the regression analysis of the intracellular only deprotection of TR + 2 and RAR + 1, show that the rate of decay of active transcription by RAR is approximately seven times faster than that of TR, which is $3 \times 10^{-7}\,s^{-1}$. Using this model, it can further be shown that the different rates of transcription, k_{s1} observed for TR and RAR is insufficient to account for the very large difference in the duration of transcription response (data not shown).

There are several potential molecular events that may account for the differences in the apparent rate at which transcription decreases for the RAR and TR based systems including disassembly/decomposition of the transcription complex, the off-rate of the ligand from the receptor and diffusion of the ligand out of the cell.[40–49] As noted above, the diffusion of ligand out of the cell is clearly not controlling the rate of decay of active transcription because the rate of diffusion of free ligand out of the cell is expected to be approximately 10-orders of magnitude faster than the modeled rate of decomposition. This suggests that that the rate of loss of active transcription complex, perhaps via proteolysis or chaperone mediated disassembly of the transcription complex and/or ligand off-rate likely control the rate of transcription loss and ultimately the duration of reporter gene response to intracellularly released hormone.

4. Conclusion

New methods to create and control spatial and temporal patterns of expressed gene products may serve as a powerful tool to elucidate the function of the many genes, which elicit their function only through their unique spatial and temporal patterns of expression. Light-activated gene expression using caged agonists of NHRs provides several unique advantages over current methods to photo-pattern gene expression in multicellular systems, however, the application of this method to some systems is potentially limited by the relatively short duration of expression response reported thus far. It is now demonstrated under similar experimental conditions that the duration of transcription response is receptor dependent. Under identical conditions RAR shows a relatively short duration response whereas thyroid hormone receptor agonist that provides a robust 36 h reporter gene response to a single irradiation event. This is far longer (16 times) than any other system NHR-dependent system thus far. Comparison of the RAR and TR dependent systems show that the duration of transcription response is not controlled by the rate of diffusion of free ligand out of the cell, but rather by the rate of transcription loss by the inactivation of the active transcription complex.

5. Experimental

5.1. General

All compounds were purchased from Aldrich chemical company unless otherwise noted. NMR spectra were recorded on a Bruker DRX-400 spectrometer. Chromatography was performed using ICN SiliTech (60 Å) Flash Silica. HeLa cells were obtained from ATCC (American Type Culture Collection) maintained at the University of Delaware Biology Core Facility. Luciferase reporter gene activity of transiently transfected HeLa cells was determined using the Dual-Luciferase Reporter Assay System (Promega #E1960) following the manufacturer's protocol. DR4-Luc$^+$ is derived from pGL3 (Promega), an enhanced luciferase promotor by inserting three nestled copies of the DR4 hormone response element (TGACCTAAAATGACCTAAAATGACCTAAAATGACCT) upstream of a minimal thymidine kinase promotor (tk) corresponding to bases −113 to +31.

5.2. Transient transfixion assays (general)

Twenty-four hours before transfection, HeLa cells were seeded at a density of 40,000 cells per well in six-well culture plates (Costar# 3516) and grown in Dulbecco's Modified Eagle Medium, DMEM (without phenol red) supplemented with 10% fetal bovine serum (FBS; Cellegro, lot# SBF30-1126) and gentamycin. Three hours prior to transfection, the media was changed to DMEM containing 10% charcoal-resin stripped FBS. FBS was depleted of endogenous hormones according to the procedure of Samuels et al.[50] Transfections were performed by calcium phosphate method using pSG5hRARγ or pSG5hTRβ as receptor DNA, DR4-Luc$^+$ (reporter), and pRLCMV (control). Twenty hours after the transfection, the media was removed and replaced with media containing an appropriate concentration of ligand. All manipulations involving compounds **2** and **4** were performed in the dark with the use of a photographic darkroom safelight. The cells were allowed to incubate in media containing caged ligand for 12 h prior to exposure (see Exposure Protocol). After exposure the cells were allowed to incubate for 24 h before harvesting by passive lysis. Cell extracts were immediately assayed using the Dual Luciferase Assay (Promega) using a Dynex luminometer. Bioluminescence activity is reported in relative light units (RLU) determined as the ratio of the firefly luminescence divided by the luminescence of the renilla luciferase control.

5.3. Cell exposure procedure

Cells growing in six-well culture plates were placed below a UV lamp (Spectroline model XX-15A; long wave UV 365 nm). The samples were irradiated for 10–300 s through a 4 mm thick glass plate and the polystyrene culture plate lid, which acted as a filter for short wave length UV. The intensity was determined to be 400 mW.

5.4. Binding assay

The ligand binding domain of RARγ, residues 178–423, was overexpressed in *E. coli* as a N-terminal his_6 tag in pER15b following a modification of the procedure of Moras and co-workers.[51] BL21(DE3) cells grown at 37 °C to an OD_{600} of 0.6–0.7, were induced with 1 mM IPTG and incubated at 25 °C for 3.5 h. Collected cell pellets were resuspended in lysis buffer (Tris–HCl (pH 8.0), 5 mM imidazole, 500 mM NaCl, 0.5 mM PMSF, 2 μg/mL Leupeptin) french pressed twice, sonicated 3×10 s (setting 3, Branson sonicator with microprobe), and incubated 20 min at 0 °C. Centrifugation (10,000 rpm, SS-34 rotor) produced crude soluble cell lysates, which were stored in 10% glycerol at −20 °C. Ligand binding assays were carried out following the procedure of Wecksler and Norman.[52] Briefly, RARγ (LBD) was diluted to a concentration of approximately 2 nM (active protein) in binding buffer (10 mM Tris (pH 8.0), containing EDTA (15 mM), DTT (2 mM), Leupeptin (2 μg/mL), and 10% glycerol) and incubated with [20-methyl-^{3}H] tRA with various concentrations of unlabeled competitor, in the absence or presence of 100-fold excess cold tRA for 16 h at 4 °C. The protein was then absorbed onto hydroxyapatite (0.5 mL) for 30 min on ice, washed four times with 0.8 mL of wash buffer (10 mM Tris (pH 7.5) − 0.5% tritron × 100). The hydroxyapatite pellet was then extracted two times with 0.8 mL of 2:1 methanol/chloroform and dissolved in 15 mL of scintillation fluid. The data was fit to the following equation by non-linear regression analysis using GraphPad InPlot (Graphpad software).

5.5. Intracellular deprotection by prewashing

In order to determine if ligand was being deprotected inside cells, HeLa cells were transiently transfected with respective receptor, reporter, and control. The cells were allowed to incubate for 12 h in the presence of active agonists or their photo-caged analogs. The cells were then rapidly washed twice with PBS buffer, which was replaced with ligand-free media. Immediately after the media was changed, the cells were irradiated with 30 and 180 s to liberate any photo-caged agonists. The cells were allowed to incubate for 0–48 h before harvesting by passive lysis.

5.6. Evaluation of compound stability to cell lysates

In order to determine if the ligands were being deprotected in the cells in the absence of light exposure, compounds were preincubated in cell lysate and assayed for activity. Solutions of **3** and **4** (0.2 μM), in media containing cell lysates from 1.5×10^7 HeLa cells, was allowed to incubate for 36 h at 37 °C. These lysated-treated ligand solutions were diluted to appropriate concentrations in media and added to transfected cells. These cells were compared with transfected cells grown in the presence of **3** and **4** that were not pretreated with cell lysates.

5.7. Synthesis

5.7.1. Ethyl 4-[(*E*)-2-[3-(1-adamantyl)-4-hydroxyphenyl]ethenyl]-benzoate (5). To a solution of **1** (0.45 g, 1.2 mmol) in 5 mL of ethanol was slowly added 0.064 mL of concentrated sulfuric acid (98%). The solution was refluxed for 12 h, neutralized with sodium bicarbonate, and extracted 2×10 mL ether. The combined organic extracts were dried over $MgSO_4$, and evaporated in vacuo to afford 0.416 g (85.6%) of a yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.38 (t, 3H), 1.78 (s, 6H), 2.12 (S, 9H), 4.39 (q, 2H), 6.80 (d, J = 8 Hz, 1H), 6.93 (d, J = 16 Hz, 1H), 7.16 (d, J = 16 Hz, 1H), 7.22 (dd, J = 8, 2 Hz, 1H), 7.34 (d, J = 2 Hz, 1H), 7.51 (d, J = 8 Hz, 2H), 8.01 (d, J = 8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.50, 164.95, 159.15, 142.34, 138.75, 131.51, 129.94, 128.65, 125.91, 125.41, 125.37, 124.99, 111.87, 55.10, 40.53, 37.86, 29.07, 14.37. MS (FAB) calcd for $C_{27}H_{30}O_3$ (MH^+) 402.2165, found: 402.1147.

5.7.2. Ethyl 4-[(*E*)-2-[3-(1-Adamantyl)-4-[(nitroveratryl)oxy]phenyl]ethenyl]-benzoate (6). To a suspension of **5** (0.100 g, 0.25 mmol) and K_2CO_3 (0.069 g, 0.50 mmol) in 1 mL of dry DMF was added dropwise a solution of

(0.069 g, 0.25 mmol) nitroveratryl bromide in 1 mL of dry DMF. The resulting solution was allowed to stir for 12 h and extracted with 2×10 mL ether. The combined organic extracts were dried over $MgSO_4$, evaporated in vacuo, and purified by flash chromatography hexanes/ethyl acetate (50:50) to afford 0.125 g (83.1%) of a yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.40 (t, 3H), 1.78 (s, 6H), 2.11 (s, 9H), 3.87 (s, 3H), 3.99 (s, 3H), 4.39 (q, 2H), 5.63 (s, 2H), 6.81 (d, $J = 8.5$ Hz, 1H), 6.99 (d, $J = 16.3$ Hz, 1H), 7.18 (d, $J = 16.3$ Hz, 1H), 7.26 (s, 1H), 7.28 (dd, $J = 8$, 2 Hz, 1H), 7.46 (d, $J = 2$ Hz, 1H), 7.54 (d, $J = 8.4$ Hz, 2H), 7.80 (s, 1H), 8.01 (d, $J = 8.3$ Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.10, 166.50, 164.95, 162.3, 159.15, 147.7, 142.34, 141.1, 138.75, 131.51, 129.94, 128.65, 125.91, 125.41, 125.37, 124.99, 111.87, 109.40, 107.9, 81.9, 55.10, 40.53, 37.86, 29.07, 14.37. MS (FAB) calcd for $C_{36}H_{40}O_8N$ (MH^+) 614.2729, found: 614.0856.

5.7.3. [(*E*)-2-[3-(1-Adamantyl)-4-[(nitroveratryl)oxy]phenyl]ethenyl]-benzoic acid (3). A solution of **6** (0.100 g, 0.25 mmol) and 0.069 g (0.12 mmol) of LiOH in 2 mL THF/H_2O (4:1) was allowed to react for 6 h at ambient temperature. The reaction mixture was extracted 3×10 mL ether. The combined organic extracts were dried over $MgSO_4$, evaporated in vacuo, and purified by flash chromatography hexanes/ethyl acetate (40:60) to afford 0.075 g (75.1%) of a yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.78 (s, 6H), 2.11 (s, 9H), 3.87 (s, 3H), 3.99 (s, 3H), 5.63 (s, 2H), 6.81 (d, $J = 8.5$ Hz, 2H), 6.99 (d, $J = 16.3$ Hz, 1H), 7.18 (d, $J = 16.3$ Hz, 1H), 7.26 (s, 1H), 7.28 (dd, $J = 8$, 2 Hz, 1H), 7.46 (d, $J = 2$ Hz, 1H), 7.54 (d, $J = 8.4$ Hz, 2H), 7.80 (s, 1H), 8.01 (d, $J = 8.3$ Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.10, 166.50, 164.95, 162.3, 159.15, 147.7, 142.34, 141.1, 138.75, 131.51, 129.94, 128.65, 125.91, 125.41, 125.37, 124.99, 111.87, 109.40, 107.9, 81.9, 40.53, 37.86, 29.07. MS (FAB) calcd for $C_{34}H_{35}O_8N$ (MH^+) 585.2453.

5.7.4. [3,5-Dimethyl-4-(3′isopropyl-4′-nitroveratryl-benzyl)phenoxyl]*tert*-butylacetate (8). To a solution of 0.0461 g (0.12 mmol) of **7**, and 0.0248 g (0.18 mmol) of K_2CO_3 in 10 mL of DMF was added 0.0331 g (0.12 mmol) of nitroveratryl bromide. The resulting solution was allowed to stir at room temperature for 24 h. The reaction mixture was extracted 3×10 mL methylene chloride and dried over $MgSO_4$ and concentrated in vacuo. Purified by flash chromatography hexane/ethyl acetate (80:20) to yield 0.628 g (91.1%) as a white crystalline.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (s, 9H), 1.55 (d, $J = 8.10$ Hz, 6H), 2.21 (s, 6H), 3.40 (qt, $J = 8.35$ Hz, 1H), 3.92 (s, 2H), 3.98 (d, 6H), 4.50 (s, 2H), 5.44 (s, 2H), 6.62 (s, 1H), 6.69 (d, $J = 8.24$ Hz, 1H), 6.80 (d, $J = 8.35$ Hz, 1H), 6.98 (s, 1H), 7.48 (s, 1H), 7.78 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.10, 168.52, 156.04, 152.70, 141.16, 138.61, 137.60, 133.37, 129.48, 128.64, 126.10, 125.60, 114.20, 112.5, 109.4, 107.9, 82, 55, 34.01, 29.29, 28.28, 27.18, 23.01, 20.73.

5.7.5. [3,5-Dimethyl-4-(3′isopropyl-4′-nitroveratryl-benzyl)phenoxyl]acetic acid (4). To a solution of 0.0628 g (2.07 mmol) of **8** in 5 mL of methanol was added 1 mL of 1 N NaOH. The reaction mixture was allowed to stir for 1 h at ambient temperature and then acidified with 1 N HCl (10 mL) and extracted 2×10 mL ether. The combined organic extracts were dried over $MgSO_4$ and concentrated in vacuo. The resulting residues was purified by flash chromatography hexane/ethyl acetate (90:10) to yield **4** (95.2%) as a white crystalline.

^{1}H NMR (400 MHz, $CDCl_3$): δ 1.55 ($J = 8.10$ Hz, 6H), 2.21 (s, 6H), 3.40 (qt, $J = 8.21$ Hz, 1H), 3.92 (s, 2H), 3.98 (d, 6H), 4.50 (s, 2H), 5.44 (s, 2H), 6.62 (s, 1H), 6.69 (d, $J = 8.24$ Hz, 1H), 6.80 (d, $J = 8.35$ Hz, 1H), 6.98 (s, 1H), 7.48 (s, 1H), 7.78 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.10, 168.52, 156.04, 152.70, 141.16, 138.61, 137.60, 133.37, 129.48, 128.64, 126.10, 125.60, 114.20, 112.5, 109.4, 107.9, 34.01, 29.29, 28.28, 27.18, 20.73.

6. Non-linear regression analysis

Time-dependent reporter gene expression was fit to the Hargrove and Schmidt model for transcription/translation. Protein expression based on the model shown in Scheme 2, is given by

$$P(t) = \frac{k_{s1}k_{s2}}{k_{d1}k_{d2}} - \left(\frac{k_{s1}k_{s2}}{k_{d1}k_{d2}} - P_0\right)e^{-k_{d2}t} - k_{s2}\left(\frac{k_{s1}}{k_{d1}} - R_0\right)\left(\frac{e^{-k_{d2}t} - e^{-k_{d1}t}}{k_{d1} - k_{d2}}\right)$$

Identical values for k_{s2}, k_{d1}, k_{d2} were used in multiple iterations to attain a self-consistent set of starting mRNA values for both TR and RAR data using non-linear regression analysis (Kaleidagraph, Synergy Software). Values for k_{s1} were derived for RAR and TR (0.32 and 0.51 pmol/g/d, respectively).

The decomposition of the transcription complex that results from intracellular—only deprotection was to a first approximation modeled as a first order exponential decay using a modified form of the Hargrove and Schmidt equation.

$$P(t) = \frac{k_{s1}(e^{-k_{d0}t} + a)k_{s2}}{k_{d1}k_{d2}} - \left(\frac{k_{s1}(e^{-k_{d0}t} + a)k_{s2}}{k_{d1}k_{d2}} - P_0\right)e^{-k_{d2}t} - k_{s2}\left(\frac{k_{s1}(e^{-k_{d0}t} + a)}{k_{d1}} - R_0\right)\left(\frac{e^{-k_{ds}t} - e^{-k_{d1}t}}{k_{d1} - k_{d2}}\right)$$

Non-linear regression analysis using the parameters derived above, were used to determine the apparent first order rates of decay for RAR and TR (0.18 d^{-1} and 0.025 d^{-1}, respectively).

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