Inducible Gene Expression in Cell Cultures and in Microencapsulated Cells

Yong Yu

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Inducible Gene Expression in Cell Cultures and in Microencapsulated Cells

by

Yong Yu

A Dissertation submitted in partial satisfaction of the requirement for the degree of Doctor of Philosophy in Anatomy

June 2002
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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<tbody>
<tr>
<td>ADS</td>
<td>artificial death switches</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion medium</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>complement deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>DIM</td>
<td>digital imaging microscopy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>Ec</td>
<td>Ecdysone</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FM</td>
<td>fluorescence microscopy</td>
</tr>
<tr>
<td>G418</td>
<td>geneticin</td>
</tr>
<tr>
<td>GAL4-DBD</td>
<td>yeast GAL4-DNA binding domain</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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</table>
GHRH  growth hormone releasing hormone
HEMA-MMA  2-hydroxyethyl methacrylate / methyl methacrylate
HEPES  N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid
hGH  human growth hormone
hPR-HBD  human progesterone receptor hormone binding domain
hPR-LBD  human progesterone receptor ligand binding domain
HSV  herpes simplex virus
IPTG  isopropyl b-D-thiogalacto-pyranoside
IRES  internal ribosomal entry site
LB  Luria-Bertani medium
LSCM  laser scanning confocal microscopy
LTR  long terminal repeats
Luc  luciferase
MOPS  3-(N-morpholino)-propane-sulfonic-acid
murA  muristerone A
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PES  polyether-sulfone
PLL  poly-L-lysine
rAAV  recombinant adeno-associated virus
RLU  relative light units
Ruc  Renilla luciferase
SEAP  secreted human placental alkaline phosphatase
TAE  Tris acetate EDTA buffer
Tet  tetracycline
TGFβ1 transforming growth factor β1
UAS  upstream activation sequence
VEGF vascular endothelial growth factor
VP16 viral transcription trans-activator 16
v/v  volume to volume ratio
w/v  weight to volume ratio
ABSTRACT

Study of Inducible Gene Expression in Cell Cultures and in Microencapsulated Cells Using a Renilla Luciferase-Aequorea gfp Fusion Gene Construct

by

Yong Yu

Doctor of Philosophy, Graduate Program in Anatomy
Loma Linda University, June 2002
Dr. Aladar Szalay, Chairperson

The mifepristone-regulated transcriptional activation system provides a means to exogenously control gene expression in transformed mammalian cells in a temporal and spatial fashion. Such a promoter activation system consists of three components: The first of these is a steroidal inducer drug, mifepristone. This drug binds to the second component, a chimeric transcription factor complex, consisting of the mutant human progesterone receptor fused to the yeast GAL4 DNA-binding domain and the Herpes simplex virus protein VP16 activation domain. The third component is a synthetic promoter, consisting of a series of GAL4 recognition sequences upstream of the adenovirus major late E1B TATA box, linked to a Renilla luciferase – Aequorea green fluorescent protein (GFP) fusion gene (ruc-gfp). The transcription of the promoter-marker gene cassette is activated by the mifepristone-bound chimeric transcription factor complex. The mifepristone-activated gene expression of luciferase and GFP was monitored by a low light video camera and a UV microscope respectively. Using this activation system in transiently transfected cells, we
observed a 10-25 fold mifepristone-dose-dependent activation of both luciferase and GFP expression which lasted up to 38 hours after the initial addition of the inducer drug, but no induction in cells not treated by mifepristone. Further, to facilitate the development of the mifepristone-induced transcriptional activation system in cellular implants for future in vivo studies, we used biocompatible calcium alginate/poly-L-lysine (PLL) beads to encapsulate cells, which may provide immune protection for cells during prolonged cellular transplantation. Using GFP as a marker, we monitored the growth and proliferation of encapsulated cells in culture dishes over a long period of time, up to two and a half months. We also showed that the ruc-gfp fusion gene expression in encapsulated mammalian cells could be regulated in a mifepristone dose-dependent manner, with induction kinetics similar to those in non-encapsulated cells in culture. From these results, we conclude that the mifepristone-regulated ruc-gfp fusion gene expression system may facilitate studies answering fundamental biological and therapeutic questions experimentally. In addition, real-time monitoring of regulated gene expression from alginate-encapsulated cells may provide a useful tool for designing cellular implantation-based protein therapy for clinical applications.
CHAPTER ONE

INTRODUCTION

The development of recombinant DNA technology along with the advances of modern cell biology, molecular biology, and biochemistry have helped the emergence and development of a new method of disease treatment strategy, namely, gene therapy. Although the original meaning of the term “gene therapy” emphasizes the delivery of genetic material to the cells of an organism to replace a defective or missing gene, in recent years, “gene therapy” has been used more broadly as a general term to describe any therapy that utilizes recombinant DNA technology for disease prevention or treatment using gene-based, recombinant protein-based, or transformed cell-based therapeutic systems.

In general, gene therapy strategies can be divided into two major categories: viral-based and non-viral based. In viral-based therapies, the transgenes are delivered to mammalian cells using viral vectors such as adenovirus (Kozarsky and Wilson 1993; Vorburger and Hunt 2002), adeno-associated virus (Smith-Arica and Bartlett 2001), retrovirus (Hu and Pathak 2000), lentivirus (complex retrovirus), Vaccinia virus (Carroll and Moss 1997; Broder and Earl 1999), alphavirus (Schlesinger 2001), simian virus-40 (SV-40) (Strayer 2000), coronavirus (Enjuanes et al. 2001), and herpes virus (Lachmann and Efstatiou 1999; Delecluse and Hammerschmidt 2000; Burton et al. 2001). In non-viral based therapies, naked plasmid DNA (Liu and Huang 2002), liposome- or artificial polymer-encapsulated plasmid DNA (Nicolau and Cudd 1989; Madsen and Mooney 2000; Fenske et al. 2001) and protein (Trif et al. 2001), mRNA (Bettinger and Read 2001), purified recombinant proteins (Russell
and Clarke 1999), and ex vivo-modified cells (Jones and Thomson 2000; Bhatia and Porter 2001) are used as carriers to deliver transgenes to the organism for therapeutic protein expression and disease treatment.

There are several factors that are crucial for the success of a gene therapy. They are: (1) regulated expression or controlled release of the therapeutic protein; (2) efficient and targeted delivery of the therapeutic protein to the appropriate cells without harm to normal tissues; (3) a stable system with minimal host immune rejection, which allows for prolonged treatment. Firstly, the non-physiological constitutive expression of exogenous proteins may provide a high level of concentration, although this may be harmful to normal cellular physiology in the long term. In many cases, the success of the therapy is highly dependent on the dose of the delivered proteins (Low 1993; Schmidt et al. 1995; Naffakh and Danos 1996; Agha-Mohammadi and Lotze 2000a; Jeschke et al. 2001). To exogenously regulate the levels of therapeutic proteins, naturally occurring or synthetic, inducible promoters have been successfully used to express the transgenes (Clarkson 1997; Harvey and Caskey 1998; Agha-Mohammadi and Lotze 2000b). Secondly, in addition to regulating the level of therapeutic protein expression, another factor that will greatly improve the efficiency of therapy is targeted transgene delivery, targeted transgene expression, and targeted delivery of therapeutic proteins (Curiel 1999; Peng 1999; Schaffer and Lauffenburger 2000). A fusion of therapeutic proteins to tissue specific antibodies (Strom et al. 1990; Haber 1992; Xiang 1999) and linking of transgenes to tissue-specific promoters (Lu and Steiner 2000; Lee et al. 2001) are two examples of targeting systems that have been designed to increase the delivery specificity of
foreign molecular or cellular materials after systemic injections. And thirdly, just as in organ transplantation, an inevitable challenge for gene therapy is the immune rejection of non-self foreign molecular and cellular materials. This is particularly problematic for stable long-term therapies. Several approaches have been used to overcome this problem. One of them is to genetically or chemically modify the foreign materials to decrease immune rejection. Expression of nonclassical human leukocyte antigens on xenogeneic cells, for example, will dramatically reduce natural killer cell-mediated cytotoxicity by the host and increase the survival of the implanted cells (Carosella et al. 1999; Lopez-Botet et al. 2000). In addition to genetic or chemical modifications, another approach to reduce host immune rejection is to encapsulate the foreign materials, such as cells, in biocompatible polymers (Han et al. 2000; Uludag et al. 2000; Cascone et al. 2001). The application of these polymers not only shields the foreign cells from the components of the host immune system but also provides a stable environment that allows the free passage of therapeutic proteins, nutrients, and wastes. A successful gene therapy strategy can be developed only when all three of the above issues have been taken into consideration.

In this study, we developed a system which addressed two of the above three issues, and may help in designing efficient and effective gene therapy protocols in the future, namely (a) the exogenous regulation of transgene expression and (b) the microencapsulated-cell systems for immuno-protection. More specifically, we describe for the first time the capability of monitoring exogenously regulated gene expression using a mifepristone-induced promoter activation system in real time. Non-invasive imaging of gene expression is made possible using the light-emitting
fusion protein consisting of *Renilla* luciferase and *Aequorea* green fluorescent protein (Ruc-GFP). This fusion product allows both qualitative and quantitative measurement of transgene activities. Also, we describe for the first time the monitoring of controlled protein expression in alginate-microencapsulated cells in real-time using the Ruc-GFP fusion protein.

In the following sections we provide a brief review of inducible promoter systems for regulated gene expression, of mammalian cell encapsulation in biocompatible polymers, and of Ruc and GFP.

**Review of Inducible Promoter Systems**

*Inducible Promoter Systems for Regulated Gene Expression in Eukaryotic Cells*

Many naturally occurring ligand-dependent transcriptional regulatory systems have been reported and well studied. One of the best examples is hormone-mediated gene expression regulation. Steroid hormones such as estrogen, glucocorticoids, and progesterone, secreted by endocrine cells into the blood circulation travel to and enter cells at a distant location. The hormones then bind to their respective receptors, and the hormone/receptor complex interacts with specific DNA sequences to activate or repress transcription (Almawi et al. 1996). With the development of synthetic hormone analogues, one can mimic and manipulate the regulatory effect of these hormones through exogenous injection of these analogues (Curran and Wagstaff 2001). Other examples of naturally occurring transcriptional regulation systems in eukaryotic cells include the heat-induced expression of heat shock proteins (Tanguay 1988; Lohr et al. 2001; Vivinus et al. 2001), the stress-induced expression of the multidrug resistance gene, *mdr1* (Kohno et al. 1989; Chin et al. 1990; Uchiumi et al. 1989).
1993; Walther et al. 2002), and the glucose-regulated protein gene, *grp* (Gazit et al.
1995).

Although the naturally occurring promoters have been used initially to regulate
gene expression in mammalian cells, the high basal transcriptional levels in the
absence of inducing signals limit their application in gene therapy (Vourc'h et al.
1989; Yarranton 1992). To overcome this problem, synthetic promoters have been
designed that can be activated by chimeric transcription factors derived from
functional domains of prokaryotic, eukaryotic, and viral origins. Two basic
components are required for designing artificial inducible promoter systems: First, an
exogenously added chimeric transcriptional factor able to bind specifically to the
inducible promoter sequence, which cannot be bound and activated by endogenous
transcriptional factors. The chimeric transcription factor should also exhibit a
minimal level of immunogenicity. Second, an exogenously added chemical drug is
needed to bind to the transcriptional factor and reversibly activate the inducible
promoter. This drug should be able to be delivered orally, to readily enter cells, and
to be non-toxic to the host. In an ideal inducible promoter system, transcription
should be at a minimal level in the absence of the drug. However, the addition of the
drug should trigger a robust dose-depend activation of gene expression. Regulated
gene expression from inducible promoters has been successfully demonstrated in
eukaryotic cells and animals using ecdysone (No et al. 1996; Saez et al. 2000),
tetracycline and doxycycline (Shockett et al. 1995; Chen et al. 1998; Saitoh et al.
1998), rapamycin (FK506) (Ho et al. 1996a; Rivera et al. 1996; Liberles et al. 1997),
IPTG (Hu and Davidson 1987), and mifepristone (Wang et al. 1994; Delort and
as the inducer drugs. As noted by Senner et al. (2001), the selection of the most appropriate system for regulated transgene expression depends on the experimental procedures, the application, and the gene to be regulated. Each system has advantages and disadvantages, which should be taken into consideration when selecting inducible promoter systems.

The use of natural steroid hormones and receptors for regulated gene expression from inducible synthetic promoters is complicated by the fact that endogenous hormones may also bind the synthetic promoter and interfere with transcriptional activation. Ecdysone (Ec, also known as β-ecdysterone and 20-hydroxyecdysone) is a *Drosophila* steroid hormone (Fig. 1.1). Christopherson et al. (1992) demonstrated that Ec specifically interacts with Ec receptor (EcR), which cannot be bound by endogenous human hormones. No et al. (1996) made further modifications in both the transcriptional factor constructs and the inducible synthetic promoter in the reporter construct to generate the currently used version of the Ec-regulated promoter system. The chimeric transcriptional factors are provided as two separate proteins on two different plasmid constructs. One construct encodes the retinoid X receptor (RXR), which is the mammalian homolog of *Drosophila* Ultraspiracle protein (USP), linked to either a constitutive CMV promoter or a tissue specific promoter. The second construct contains a fusion gene, encoding a mutant EcR and the *Herpes simplex* virus (HSV) VP16 transcriptional activation domain, also linked to either a CMV promoter or a tissue specific promoter. Simultaneous expression of both proteins in the same cells will not form the active chimeric transcriptional activator complex unless the inducer drug Ec is added (Yao et al. 1993). The synthetic
Figure 1.1. The chemical structures of ecdysone and muristerone A. Ecdysone (Ec; a phytoecdysteroid) is an insect molting hormone. Muristerone A (murA) is an analog of Ec. The lipophilic Ec and murA molecules efficiently penetrate all tissues, including the brain, and have short half-lives in vivo. These properties of Ec and murA allow rapid and potent induction of gene expression, making them desirable as inducer drugs, which are rapidly cleared from the circulation.
Ecdysone

Muristerone A
inducible promoter construct consists of four copies of a 29-base-pair DNA sequence of a mutant ecdysone response element fused to a minimal promoter able to drive the expression of the downstream target gene. In the presence of the inducer Ec, the Ec-bound-EcR-VP16 will form a heterodimer with RXR to produce the active transcriptional factor complex, which will then bind to the synthetic promoter to activate the transcription of the target gene. The Ec-regulated promoter system has both a low basal activity and an up-to-20,000-fold induction of transcriptional activation in cell culture experiments (No et al. 1996). The regulated expression of many proteins, including the expression of chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), luciferases (No et al. 1996; Karns et al. 2001), enhanced GFP (Luers et al. 2000), human nerve growth factor, NGF (Patrick et al. 2001), PTEN tumor suppressor (Stolarov et al. 2001), p53 (Abeyesinghe et al. 2001), and Ras GTP-binding proteins (Plows et al. 2002), were successfully demonstrated in cell cultures and in live animals. The disadvantage of the system is that it requires the over-expression of RXR, which may interfere with normal signal transduction pathways that are responding to retinoid and thyroid hormones (Constantino et al. 2001).

Inducer drugs other than Ec, such as muristerone A (No et al. 1996; Patrick et al. 2001), a synthetic analog of Ec, and other ecdysteroids, such as ponasterone A (Saez et al. 2000), have also been successfully applied to induce transcriptional activation from the same inducible system. It was shown, however, that muristerone A and ponasterone A may interfere with cytokine signaling and ultimately the growth and/or survival of mammalian cells (Constantino et al. 2001). Therefore, less toxic Ec analogs may need to be synthesized.
The tetracycline (tet)-induced regulatable system is another inducible promoter system that has been widely used to regulate transgene expression in mammalian cells. Tetracycline (Fig. 1.2) or its derivative doxycycline, dox (Fig. 1.2), is used as the inducer drug. The tet-regulated systems are divided into two groups: allosteric off-switch (tet-off), and allosteric on-switch (tet-on), which are commercially available as Tet-Off™ and Tet-On™ (Expression Systems & Cell Lines, Clontech, Palo Alto, CA). Both utilize the two regulatory elements derived from the *Escherichia coli* tet-resistance operon: the tet repressor protein (TetR) and the tet operator DNA sequence (*tetO*).

In the two-plasmid tet-off system (Fig. 1.3), the chimeric transcriptional activator plasmid carries a fusion gene between the TetR sequence and the sequence of HSV VP16 transcriptional activation domain. This fusion gene is under the control of CMV promoter. The inducible synthetic promoter in the reporter plasmid construct consists of *tetO* fused to a minimal promoter upstream of the reporter transgene sequence. In the absence of tetracycline, the chimeric transcriptional activator can bind to the *tetO* sequences in the inducible promoter and allow transcription. In the presence of tetracycline, however, the chimeric transcriptional activator-tet complex can no longer bind to the *tetO* sequences and the transcription is therefore turned off (tet-off). A low basal activity and an up to 100,000-fold transcriptional activation have been reported in cell culture experiments when tetracycline is withdrawn from the culture medium (Gossen and Bujard 1992). Transcriptional activation of the tet-off system has also been demonstrated in animals. Regulated expression of signal transduction pathway genes, oncogenes, and reporter genes has been successfully
Figure 1.2. The chemical structures of tetracycline and doxycycline. Tetracycline (tet) and doxycycline (dox) are broad-spectrum antibiotics that inhibit bacterial growth by reversibly binding to the 30S ribosomal subunit and inhibiting protein synthesis. Tet is prepared from the cultures of certain Streptomyces spp. Dox is a synthetic derivative of tet.
Tetracycline

Doxycycline
demonstrated in animal studies using the tet-off system (Saez et al. 1997; Gingrich and Roder 1998; Jaisser 2000). In addition to the two-plasmid system, Schultze et al. (1996) also demonstrated tight control of gene expression in transgenic mice using a single-plasmid tet-off system with the chimeric transcriptional activator expression cassette and the inducible promoter-transgene fusion construct in cis configuration. The disadvantage of the tet-off system is that the continued presence of tetracycline is required to maintain the “off” state, which may not be desirable for long-term applications.

The principle of the tet-on (reverse tet) system (Fig. 1.3) relies on the isolation of TetR mutants from E. coli that require the presence of tetracycline to allow the binding of the TetR to the tetO sequences (Gossen et al. 1995). The tet-on system is a two-plasmid system. The chimeric transcription activator plasmid consists of a fusion between the mutant TetR gene and the sequence encoding the viral VP16 activation domain under the control of the CMV promoter. The second plasmid carries the sequence of the target gene under the control of an inducible promoter, which contains seven copies of tetO fused to the human CMV immediate early minimal promoter. Regulated gene expression from the two-plasmid tet-on system has been demonstrated in transgenic mice (Kistner et al. 1996; Puttini et al. 2001). For example, long-term regulated delivery of erythropoietin from implanted cells has been shown in live mice (Bohl et al. 1997). The limitation of the tet-on system is the residual binding of TetR to tetO in the absence of tetracycline. To address this problem, further technical improvements were conducted to reduce the basal expression level without altering the inducibility of the system. Zhu et al. (2001)
Figure 1.3. Tetracycline (or doxycycline)-induced inhibition or activation of gene expression using “tet-off” and “tet-on” (reverse tet) systems, respectively. In the “tet-off” system, without tet or dox, the tet repressor of the chimeric transcriptional activator can bind to the tetO sequences in the synthetic promoter of the reporter plasmid, and the VP16 transcriptional activation domain of the chimeric transcriptional activator can activate gene expression from the minimal CMV promoter. In the presence of tet or dox, the chimeric transcriptional activator cannot bind to the synthetic promoter and therefore there is no activation of gene expression. In the “tet-on” (reverse tet) system, on the other hand, the mutant chimeric transcriptional activator will bind to the synthetic promoter and activate gene expression only in the presence of tet or dox.
a. tet-off system

b. tet-on (reverse tet-regulated) gene expression system
successfully utilized a tetracycline-controlled transcriptional silencer (tTS), a fusion protein containing the Tet repressor and the KRAB-AB domain of the kid-1 transcriptional repressor, to tighten the control of tet-induced gene expression in transgenic mice.

The third type of regulated gene expression system is based on chemical dimerizers and their binding proteins. These dimerizers, such as rapamycin (Sigma-Aldrich, St. Louis, MO; Fig. 1.4), were originally used as immunosuppressants by dimerizing and inactivating cellular proteins that are involved in T cell activation in the immune response pathways (Sigal and Dumont 1992). The cellular protein with which rapamycin interacts is immunophilin FK506-binding protein 12 (FKBP12). Similar to rapamycin, FK506 is another drug with an immunosuppressant function (Inamura et al. 1988; Bierer et al. 1991). Using synthetic homodimers of FK506 (FK1012 or AP1510; Fig. 1.4; Ariad, Cambridge, MA), any two proteins with the FKBP domains can be brought together by the same homodimer (Spencer et al. 1993). Using heterodimers of FK506 and cyclosporin A (FK-CsA) as a bridge, any proteins with FKBP domains can interact with proteins containing cyclophilin domains (Belshaw et al. 1996). Also, a heterodimer between FKBP and the lipid kinase homolog FKBP12-rapamycin-associated protein (FRAP) can also be formed through the mediation of rapamycin. Based on this principle, Rivera et al. (1996) designed an inducible promoter system for regulated gene expression in mammalian cells and transgenic animals (Fig. 1.5). This system consists of three plasmid constructs. The first plasmid carries the expression cassette of a fusion gene between the chimeric ZFHD1 DNA-binding domain (Pomerantz et al. 1995) and three
Figure 1.4. The chemical structures of the dimerizers rapamycin, FK1012, and AP1510. Rapamycin (23,27-Epoxy-3H-pyrido(2,1-c)(1,4)oxaazacyclohentriacontine) is an analog of the immunosuppressant drug FK506. It is prepared from the culture of Streptomyces hygroscopicus. FK1012 and AP1510 are synthetic homodimers of FK506. These small dimerizer drugs are able to enter most tissues in the body, including the brain and the fetal circulatory system, and each has a short half-life *in vivo* (Clackson 1997).
Figure 1.5. Transcriptional activation using the rapamycin, FK1012, and AP1510 dimerizers. In the rapamycin-induced transcriptional activation system, a yeast GAL4 DNA binding domain (DBD) is fused to the FK506 binding protein (FKBP), and an HSV VP16 transcriptional activation domain is fused to the FKBP-rapamycin-associated protein (FRAP). Both chimeric proteins are expressed under a constitutive promoter. Without rapamycin, even though DBD can bind to GAL4 DNA binding sites in the synthetic promoter of the reporter plasmid, no transcription is activated. When rapamycin is added, rapamycin mediates the formation of heterodimers between FKBP and FRAP, bringing in the transcriptional activation domain which activates gene expression. FK1012 and AP1510 are dimerizers that induce the activation of gene expression in a similar manner as rapamycin.
Rapamycin-induced activation of gene expression.

Off

FK1012- or AP1510-induced activation of gene expression.

Off

FKBP-rapamycin-associated protein (FRAP)
GAL4 DNA-binding domain (DBD)
dimerizer drug
HSV VP16 transcriptional activation domain

On

FK506 binding protein (FKBP)
GAL4 DNA-binding sites
Minimal promoter
Transgene cDNA
FKBP12 repeats under the control of the CMV promoter. The second plasmid carries the expression cassette of a fusion gene consisting of the coding sequence of the FRAP rapamycin-binding domain (FBP) and the coding sequence of the NFκB p65 carboxyl-terminal transactivation domain under the control of the CMV promoter. The third plasmid is the reporter plasmid carrying a synthetic promoter consisting of 12 copies of ZFHD1-binding sequences and a CMV minimal promoter to control the expression of the downstream transgene. In the presence of rapamycin, the ZFHD1-FKBP(3) can bind to FBP-p65 to form the transcription activator complex. The binding of the ZFHD1 portion of the complex to the ZFHD1-binding sequences in the synthetic promoter of the reporter plasmid will bring in the transcription activation domain of p65 and activate transcription of the transgene. Using this system, induced gene expression at levels of 1,000 to 10,000 fold has been demonstrated in cell culture experiments (Rivera et al. 1996; Pollock et al 2000). Activated gene expression from this inducible promoter system has also been successfully demonstrated in mice (Magari et al. 1997; Rivera et al. 1999) and rhesus monkeys (Ye et al. 1999). The limitation of this system is that rapamycin regulates gene expression at doses that are immunosuppressive by binding to endogenous FKBP and FRAP, which restricts its use for long-term therapy. However, this problem has been resolved by using synthetic analogs of rapamycin that cannot bind to endogenous FRAP but only to complement mutant FRAPs (Clackson 1997). Similar inducible promoter systems (Pollock and Rivera 1999) have also been designed using FK1012 (Ho et al. 1996b), AP1510 (Amara et al. 1997), and FK-CsA (Belshaw et al. 1996) as inducer drugs.
The fourth type of regulated gene expression system is based on the binding of the bacterial lac repressor to the lac operator sequence originally derived from *E. coli*. This isopropyl β-D-thiogalactoside (IPTG, Fig. 1.6)-inducible system is based on two plasmids. The repressor plasmid carries the lac repressor-encoding the *lacI* gene driven by a eukaryotic constitutive promoter such as the Rous sarcoma virus long terminal repeat. The reporter plasmid carries the transgene sequence linked to a lac operator-containing SV40 early promoter-enhancer. Although the IPTG-induced lac operator-repressor system has mainly been used to induce gene expression in prokaryotic cells, it was also shown to be functional in mammalian cells (Hu and Davidson 1987) and in live animals (Wyborski and Short 1991; Wu et al. 1997).

Regulated expression of mammalian signal transduction proteins and reporter proteins has been reported (Lin et al. 1997). Modifications in the *lacI* gene make the lac operator-repressor system even more suitable for regulated gene expression in mammalian cells and animals (Fieck et al. 1992; Scorable and Stambrook 1997; Cronin et al. 2001). Up to 60-fold induction of transgene expression has been reported in cell culture experiments (Figge et al. 1988). To further increase the induction level, Hu and Davidson (1990) utilized the human metallothionein-IIA gene promoter, instead of the SV40 early promoter-enhancer, in the inducible promoter and integrated the lac operator sequence in the middle of the promoter. An approximately 100-fold induction over the basal expression level was observed. In addition to the human metallothionein-IIA gene promoter, the murine 3-phosphoglycerate kinase 1 gene promoter (Hannan et al. 1993) and the human polypeptide chain elongation factor 1 alpha (EF-1 alpha) promoter (Edamatsu et al.
Figure 1.6. The chemical structure of IPTG. Re-drawn from Johnson and Church (2000).
1997) have also been used for the insertion of the lac operator sequences. They also showed a low basal level of gene expression in the absence of IPTG and robust induction of gene expression when IPTG was added. The limitation of the IPTG-induced lac operator-repressor system is that the induction level is significantly lower than that in ecdysone-, tetracycline- and rapamycin-induced systems, which can reach expression levels four orders of magnitude above their respective basal activities. Furthermore, demethylating agents are required for the transcription of the lacI gene in some strains of transgenic animals (Wyborski et al. 1996). Nevertheless, the fine-tuned dose-dependent induction of gene expression by metabolically stable IPTG makes the lac operator-repressor system adaptable for regulated gene expression studies.

Mifepristone-Induced Transcriptional Activation System

The human progesterone receptor (hPR) binds endogenous progesterone and other hormones. To apply hPR and its hormone ligands to the design of regulated gene expression systems, a 42 amino acid deletion mutation was introduced at the carboxyl terminus of hPR so that it no longer was able to bind to endogenous progesterone but rather only to the synthetic progesterone analog, mifepristone, also known as RU486 or Mifeprex (Fig. 1.7; Vegeto et al. 1992). The original mifepristone-inducible gene expression systems were described by Wang et al. (1994) and Delort and Cappechi (1996). The “GeneSwitch” system described by Wang et al. (1994) is a two-plasmid system, consisting of a chimeric transcriptional activator plasmid and a reporter plasmid (Fig. 1.8). More specifically, the chimeric transcriptional activator plasmid carries the expression cassette of a fusion gene encoding the HSV VP16 transcription
Figure 1.7. The chemical structure of mifepristone (RU486 or Mifeprex) and progesterone. Mifepristone is a synthetic analog of progesterone. Mifepristone can bind to the mutated progesterone receptor ligand binding domain, which is no longer able to bind endogenous progesterone.
Mifepristone (RU486 or Mifeprex)

Progesterone
Figure 1.8. Mifepristone-induced activation of gene expression. The chimeric transcriptional activator consists of a yeast GAL4 DNA-binding domain (DBD) fused to a mutant progesterone receptor ligand binding domain (HPR-LBD) and a VP16 viral transcriptional activation domain. Without mifepristone, the steric hindrance effect of HPR-LBD prevents the DBD from binding to the GAL4 DNA-binding sites in the synthetic promoter, and therefore no activation of transcription occurs. In the presence of mifepristone, on the other hand, the binding of the inducer drug to HPR-LBD triggers a conformational change in HPR-LBD and removes the steric hindrance effect. This allows the subsequent binding of DBD to the GAL4 DNA-binding sites in the synthetic promoter and the activation of transgene expression by the VP16 transcriptional activation domain.
Mifepristone-induced gene expression activation system

**Off**

- Mutant progesterone receptor ligand binding domain (HPR-LBD)
- GAL4 DNA-binding domain (DBD)
- Transgene cDNA
- VP16 viral transcription activation domain

**On**

- GAL4 DNA-binding sites
- Minimal promoter
- Transgene cDNA
activation domain (residues 411-487) fused to the carboxyl terminus of the yeast GAL4 DNA-binding domain (residues 1-94) and also to the amino terminus of the truncated hPR ligand binding domain (hPR-LBD) (residues 640-891). The fusion activator gene is under the control of the constitutive CMV promoter. Residues 1-94 of the GAL4 DNA-binding domain contain the DNA binding sites, dimerization sites, and nuclear localization signals. On the reporter plasmid, the inducible synthetic promoter consists of four copies of the consensus 17-mer GAL4 binding sequences of yeast origin fused to the adenovirus E1B TATA box minimal promoter which control the expression of the target gene. In the absence of mifepristone, the chimeric transcriptional activator is not able to bind to the synthetic promoter to activate target gene expression. When mifepristone is added, it is believed that the binding of the inducer drug to the hPR-LBD induces allosteric change in the hPR-LBD and removes its steric hindrance effect over the GAL4 DNA-binding domain. Therefore, the GAL4 DNA-binding domain can bind to GAL4 binding sequences in the synthetic promoter, and the transcription of the target gene is activated by the incoming VP16 transactivation domain.

The “GeneSwitch” system has been used to express human growth hormone (Draghia-Akli et al. 2002), lacZ (Wang et al. 1994), erythropoietin (Abruzzese et al. 2000), and transforming growth factor β1 (TGFβ1) (Wang et al. 1999). However, since the protein expression analyses in these studies were carried out by Western blots using cell lysate or by staining using fixed samples, it is virtually impossible to follow the induction kinetics in the same group of cells over a period of time. In this study, we used Renilla luciferase and GFP as living markers to non-invasively
visualize the mifepristone-regulated gene expression process. In addition to visualization, the luciferase marker also allows real-time quantification of gene expression. Therefore, using the light-emitting proteins, the kinetics of induced gene expression in the same group of cells can be monitored over a period of time.

A similar two-plasmid-mediated mifepristone-regulated gene expression system (UAS/TAXI system) was constructed by Delort and Capecchi (1996). This UAS/TAXI system is essentially the same as that of the “GeneSwitch” system except for a few minor differences. Both inducible systems are based on two plasmids. The chimeric transcriptional activator plasmid carries a hybrid transcriptional activator construct encoding the fusion gene of the GAL4 DNA-binding domain, a truncated hPR-LBD, and an HSV VP16 activation domain linked to the CMV promoter. The reporter plasmid carries a transcriptional activator-dependent marker gene fusion construct using the GAL4 upstream response elements and a minimal synthetic promoter (UAS) linked to the marker gene. One of the differences between the two systems is that, in the chimeric transcriptional activator plasmid of the UAS/TAXI system, the hPR-LBD has a 35 amino acid deletion in the carboxyl terminus. However in the HPR-LBD of the “GeneSwitch” system there is a 42 amino acid deletion in the carboxyl terminus. Another difference is that in the chimeric transcriptional activator, the VP16 acidic transactivation domain spans residues 415-486 in the UAS/TAXI system, whereas in the “GeneSwitch,” the VP16 transactivation domain spans residues 411-487. In the reporter plasmid constructs, there are five GAL4 upstream response elements along with a minimal promoter sequence from the Drosophila hsp70 gene in the UAS/TAXI system. However, there
are four copies of the GAL4 upstream response elements linked to the adenovirus major late E1B TATA sequence in the “GeneSwitch.” Also, in the UAS/TAXI reporter plasmid, the GAL4 DNA binding domain spans residues 1-147 whereas in the “GeneSwitch” system, it spans residues 1-94. Despite these differences, the UAS/TAXI system has been demonstrated by Delort and Capecchi (1996) to be functionally comparable to the “GeneSwitch” system in regulating gene expression in the presence of mifepristone.

Mifepristone has been used clinically in conjunction with a low dose of prostaglandin for termination of early pregnancy (Brogden et al. 1993). Mifepristone treatment is also beneficial in treating unresectable, hormone-dependent tumors and disorders of the female reproductive system that are resistant to conventional chemotherapy and irradiation (Koide 1998). For example, mifepristone long-term treatment of refractory Cushing’s syndrome caused by an ACTH-secreting pituitary macroadenoma in extremely ill patients has been demonstrated (Chu et al. 2001). In our studies, we use mifepristone, a progesterone analog, as an inducer drug to bind and induce conformational changes in the mutant progesterone receptor, which is part of the chimeric transcriptional activator. This allows the subsequent binding of the chimeric activator to the synthetic promoter in the target gene construct and activates gene expression.

The pharmacological kinetics and safety profile of mifepristone have been carefully evaluated over the years. After ingestion, mifepristone is quickly absorbed and has a half-life of 25 to 30 hours (Heikinheimo 1997). The initial metabolism of mifepristone is catalyzed by cytochrome P450 enzyme CYP3A4 to yield
monodemethylated, didemethylated, and hydroxylated metabolites, which are in similar concentrations to the original drug. The three metabolites and the original drug are active as antagonists against endogenous progesterone. Long-term usage of mifepristone is considered to be safe, as demonstrated in clinical trials (Glasier 1998). No toxic effects were observed in humans even after long-term medical use in doses up to 20 mg/kg (Handerson 1987). It was demonstrated that the serum concentration of mifepristone after oral intake of 200 mg was estimated to be about 5 μM in adult humans (Heikineheim et al. 1989). In our experiments, the optimal concentration of mifepristone used was 250 nM, which corresponds to an oral dose of 10 mg. This is significantly lower than the doses (600 – 800 mg) used for pregnancy termination. Therefore, mifepristone can be applied safely in human subjects for regulating gene expression.

**Mammalian Cell Microencapsulation**

**Overview**

Cell therapy utilizes implanted cells or tissues to secrete the deficient therapeutic proteins to treat human diseases. The challenge facing allogenic or xenogenic cell and tissue transplantation is immune rejection. To suppress such rejection, the recipient host is subjected to daily immunosuppressant drug treatment. However, the immunosuppression therapies are often accompanied by severe adverse effects, such as nephrotoxicity (Olyaei et al. 1999; Olyaei et al. 2001). Although new drugs are being developed to maximize the efficiency of immunosuppression and improve patients’ quality of life (MacDonald 2001), the endogenous immunosuppressant nature of the drug therapy inevitably puts patients at constant risk. To address this
problem, a new approach to protect cells from immune rejection is encapsulation using biocompatible polymers (Aebischer et al. 1991; Chang 1997; Wang 1998; Rihova 2000; Uludag et al. 2000). These polymers physically isolate the cells from immune attack. In addition to providing physical isolation, these polymers also possess other features essential for successful cell therapy. For example, the pore size of the polymers can be adjusted to allow for the passage of nutrients and therapeutic molecules while excluding components of the immune system. The external surface morphology and the mechanical strength of the polymers are also carefully designed both to optimize the interaction between microcapsules and host, and to optimize the durability of the capsules, respectively. There are three major systems of encapsulation, including intravascular macrocapsules, extravascular macrocapsules, and extravascular microcapsules (de Vos et al. 2002). In this study, we focus on extravascular microcapsules, which possess many advantages over the first two types of encapsulation. One of main advantages is the high surface to volume ratio. This allows better diffusion of nutrients, therapeutic proteins, chemical stimulants, etc., into and out of the capsules.

The biocompatible materials for cell encapsulation can be divided into two major groups: membrane materials and matrix materials. The AN69 hollow fiber (polyacrylonitrite-sodium methallylsulfonate; Hospal, Meyzieu, France) is an example of membrane material used to encapsulate cells and tissues. The AN69 copolymer was originally used as hemodialysis membrane (De Paepe and Ringoir 1982). It is highly biocompatible. The hollow fiber used for cell encapsulation is typically 1 cm long and made of 8% AN69 polymer with an internal diameter of 100
µm, a wall thickness of 140 µm, and a molecular weight cut-off of 80 kDa (Prevost et al. 1995). After the loading of cells, the fiber is closed with surgical clips and ready for implantation. So far, the immuno-isolation of hepatocytes (Balladur et al. 1995; Sarkis et al. 2001), of pancreatic islets (Honiger et al. 1994; Prevost et al. 1997), and of transformed fibroblast cells (Serguera et al. 1999) using AN69 hollow fiber encapsulation has been successfully demonstrated in animals and human patients. These encapsulated and implanted cells and tissues are used to restore metabolic function, thus paving the way for the further development of bioartificial organs for clinical applications. Other examples of membrane materials that have been used for cell microencapsulation include PAN/PVC membranes (Shoichet and Rein 1996), polysulphone (PSU) polymers (Lembert et al. 2001; Petersen et al. 2001), and polyurethane (Zondervan et al. 1992; Ward et al. 1993).

In addition to membrane materials that provide an outside capsule, matrix materials are also used in cell encapsulations to provide the cells with an internal environment that mimics the matrix structure of the extracellular space existing in living organisms. Similar to the extracellular matrix, the matrix materials used in cell encapsulation are designed to provide the cells with both physical support as well as functional support for cell proliferation, polarity, secretion, etc. Calcium-crosslinked alginate is one of the most widely used matrix materials for cell microencapsulation (Peirone et al. 1998; Uludag et al. 2000). Chitosan (Zielinski and Aebischer 1994), cellulose (Merten et al. 1991; Dautzenberg et al. 1999), and agarose (Iwata et al. 1992; Hayashi et al. 1996) are other examples of matrix materials used for mammalian cell encapsulation.
Cell and Tissue Encapsulation by Alginate/poly-L-lysine

Alginate is the most common material employed for microencapsulation. It is a polysaccharide composed of negatively charged mannuronic and guluronic acids (Fig. 1.9), which can be gelatinized in polymerization buffer by linking guluronic acid molecules together in the presence of divalent cations, such as Ca\(^{2+}\). Alginate can be used either alone or in combination with poly-L-lysine (PLL). The positively charged PLL interacts with the negatively charged mannuronic and guluronic acids on the surface of gelatinized alginate to form a PLL membrane. The PLL membrane will not only provide stability to the alginate beads but also control pore size by varying the molecular weight and the concentration of PLL used, and also by varying the incubation time and temperature of PLL solutions with alginate beads (King et al. 1987; Vandenbossche et al. 1991; Vandenbossche et al. 1993). Finally, the PLL-covered alginate beads are treated in alginate or other solutions containing negatively charged ions to neutralize any excessive amount of positively charged lysine residues. This external layer of alginate augments the strength and biocompatibility of the alginate beads. In our studies, the alginate beads or alginate-microencapsulated cells were all prepared using an INOTECH encapsulation apparatus (Fig. 1.10).

There are many advantages of using alginate/PLL microcapsules for cellular encapsulation. The small alginate matrix is more reliable than a single large membrane, which is more prone to be torn. The small alginate beads also have a large surface area compared to a single large encapsulation membrane. The pore size of the alginate/PLL beads can be adjusted to allow for free diffusion of nutrients, wastes, and therapeutic proteins, while at the same time excluding immunoglobulins.
Figure 1.9. The chemical structure of alginate. Alginate is a polysaccharide sodium salt (Chaplin 2002).
M: β-(1-4)-linked D-mannuronic acid
G: α-(1-4)-linked L-guluronic acid
Figure 1.10. The microencapsulation apparatus. (1) syringe pump; (2) syringe (containing cells and alginate mixture); (3) nozzle; (4) vibration generator; (5) alginate/cell droplets; (6) encapsulator cylinder; (7) bypass cup; (8) calcium polymerization solution; (9) drainage valve; (10) alginate bead collection flask; (11) stir rotor; (12) bypass cup rotor; (13) electrostatic charge generator; (14) electrode; (15) waste passage. Please refer to Chapter 2 of the Materials and Methods section for a detailed protocol of the operation of the encapsulator apparatus.
Cells microencapsulated in spherical alginate/PLL beads can be implanted into animals by direct injection into the peritoneal cavity or by subcutaneous implantation with minimal invasive surgery. Further modifications of alginate/PLL encapsulation techniques have shown promising results in reducing host immune response and increasing the stability of the alginate/PLL capsules. A high rather than low guluronic acid content of alginate is used to improve the stability of the bead to avoid swelling and breakage after implantation (De Vos et al. 1996). Also, instead of saline and citrate, Ca$^{2+}$-free Krebs-Ringer bicarbonate buffer (KRBB) and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) are used as incubation media to provide prolonged secretion of therapeutic proteins from encapsulated cells and tissues (Fritschy et al. 1991). Furthermore, instead of Ca$^{2+}$, barium (Ba$^{2+}$) (Schrezenmeir et al. 1993; Petruzzo et al. 1997; Duvivier-Kali et al. 2001) and strontium (Sr$^{2+}$) (Thu et al. 1996; Wideroe and Danielsen 2001) have been used as the divalent cations for alginate polymerization. Unlike Ca$^{2+}$, which can only bind to guluronic acids, Ba$^{2+}$ can bind to both guluronic and mannuronic acids, and therefore provides a high level of crosslinking and increased durability. Strontium alginate also showed a considerably higher chemical stability than Ca$^{2+}$-alginate for long-term usage in vivo (Wideroe and Danielsen 2001). And lastly, instead of the traditional spherical beads, non-homogeneous planar alginate beads with a higher concentration of alginate in the periphery than in the center core have been produced (Thu et al. 1996a; Thu et al. 1996b). The non-homogeneous beads have great stability and allow the modulation of bead permeability in the absence of PLL. In summary, these
material and procedural modifications will further facilitate the application of alginate in cellular encapsulation and implantation.

Lim and Sun (1980) were the first to employ alginate-based encapsulated islets to reverse insulin-dependent diabetes in rats. Subsequently, promising results in treating type I diabetes were obtained in large animals (Soon-Shiong et al. 1992; Soon-Shiong et al. 1993; Calafiore et al. 1999; Lanza et al. 1999) and in human patients (Soon-Shiong et al. 1994). In addition to treating diabetes, many other metabolic diseases could be treated using alginate-encapsulated cell therapy. For example, hormone deficiency diseases were treated in animals using encapsulated endocrine cells (Sun et al. 1987). Secretion of functional parathyroid hormones (Gaumann et al. 2001), human growth hormone (hGH) (Chang 1997; Peirone et al. 1998a; Ross et al. 1999; Stockley et al. 2000), and testosterone (Machluf et al. 200) by encapsulated cells has been demonstrated in cultures and in animal models, which could be applied clinically in treating human diseases. In addition to hormones, the delivery of other recombinant gene products, such as lysosomal enzymes (Ross et al. 2000), adenosine deaminase (Hughes et al. 1994), human coagulation factor IX (Liu et al. 1993; Hortelano et al. 1996; Garcia-Martin et al. 2002), inducible nitric oxide synthase (iNOS) (Xu et al. 2002), endothelial cell growth factor (Ko et al. 1995), and endostatin (Read et al. 2001), from alginate-encapsulated cells appears to be a promising strategy for the potential treatment of human diseases.
**Renilla Luciferase (Ruc) and Aequorea GFP as in vivo Markers**

**Ruc**

The sea pansy *Renilla reniformis* is a bioluminescent soft coral living in shallow water off the coast of North America. Upon mechanical stimulation, the sea pansy displays blue-green bioluminescence, which is the result of a photon-emitting reaction catalyzed by *Renilla* luciferase (Ruc). Ruc is one of the members of the luciferase family of proteins isolated from a large variety of organisms (Hastings 1996; Greer and Szalay 2002). The ruc cDNA, cloned and sequenced by Lorenz et al. (1991), is 1196 base pairs in length, and encodes a 314 amino acid glycoprotein of 34 kDa. Ruc catalyzes the oxidation of coelenterazine, the luciferin of *Renilla reniformis* (Fig. 1.11). The coelenterazine substrate consists of an imidazolopyrazine structure \{2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl)-8-benzylimidazo [1,2-a]pyrazin-3-(7H)-one\}, which emits blue light with a peak at 480 nm when oxidized by Ruc (Hori et al. 1973; Matthews et al. 1977). Unlike firefly luciferase (DeLuca and McElroy 1974; de Wet et al., 1987), Ruc does not require cofactors or ATP for activity. It should be noted that the *in vitro* Ruc assay is sensitive to the ionic strength of the buffer (Ward and Cormier 1978), but relatively insensitive to variation in pH and temperature.

Recombinant Ruc was first expressed in *E. coli* (Lorenz et al. 1991), and was determined to have identical characteristics to native Ruc based on Western blot and luminescence emission spectra analyses. Although native Ruc is a glycoprotein, glycosylation is not required for the enzymatic activity of Ruc. Recombinant Ruc expressed in *E. coli* strains, which lack a glycosylating enzyme (Littman et al. 1985),
Figure 1.11. Bioluminescence reaction catalyzed by *Renilla* luciferase. In the presence of molecular oxygen, coelenterazine is oxidized to coelenteramide, with a concomitant release of carbon dioxide and blue light (peak at 480 nm).
is functional in catalyzed luminescent light-emitting reactions. Subsequently, functional Ruc was successfully expressed in *Candida albicans* (Srikantha et al. 1996), in plants (Mayerhofer et al. 1995), in mammalian cells (Lorenz et al. 1996; Liu et al. 1997; Liu et al. 2000; Yu et al. 2001; Timiryasova et al. 2001), embryos (Wang et al. 1996), and adult animals (Yu et al. 2001; Bhaumik and Gambhir 2002).

**Ruc as a Biomarker**

Compared to conventional molecular markers, such as β-galactosidase-based staining or fluorescent dye, there are many advantages associated with Ruc as a biomarker. First, Ruc is a biomarker that can be monitored in real time. The expression of Ruc is indirectly measured by assaying the luciferase enzymatic activity. Since the luciferase assay is based on light emission, no invasive procedures, such as staining, are required for detection. Due to the availability of highly sensitive photon counting instruments, Ruc can be assayed with a higher sensitivity than GFP fluorescence. Also, the latter often suffers from strong interference by intrinsic sample fluorescence. Second, luciferase gene expression can be easily quantified based on photon emissions (Liu et al. 1997; Liu et al. 2000). Quantification of GFP expression, on the other hand, is difficult. Third, no direct toxicity of Ruc has been observed in either cell culture studies (Lorenz et al. 1996) or in animal studies (Bhaumik and Gambhir 2002). However, it has been suggested that overexpression of GFP can cause apoptosis in certain cell cultures (Liu et al. 1999a).

One of the limitations of using Ruc as a biomarker is that the Ruc-catalyzed light-emitting oxidation reaction is dependent on the presence of molecular oxygen. Therefore, when the oxygen supply is limited, such as in poorly vascularized tissues,
luciferase activity is also restricted. Another limitation associated with Ruc is the need of the substrate coelenterazine for light emission. Efficient delivery of coelenterazine could be problematic in animal studies (Bhaumik and Gambhir 2002). In addition, since the coelenterazine substrate is generally prepared in ethanol or methanol, injection of an excessive volume is toxic to the animals.

**Luciferase Imaging Techniques**

Photon emission from Ruc-catalyzed oxidation of coelenterazine can be monitored using any of the following instruments or similar models: (1) a Turner Designs TD 20e luminometer (Turner Designs, Sunnyvale, CA), (2) a SPEX FluoroMax spectrofluorometer (Instruments S.A. Inc., Edison, NJ), (3) a Hamamatsu ARGUS 100 low light imager (Hamamatsu, Japan), or (4) an EG&G Nightowl low light imager (Berthold Technologies, GmbH and Co. KG, Bad Wildbad, Germany). The TD 20e luminometer has sensitivity and versatility specifically suited for use with *Renilla* and firefly luciferases. To detect luminescence light emission using the luminometer, samples suspended in luciferase assay buffer are transferred into scintillation vials. Coelenterazine is either injected into the vials by a Hamilton syringe or by pipetting. It is important to determine the linear range of the luminometer since high intensity light results in signal saturation. Light intensity is proportional to luciferase concentration in the range between $10^{-16}$ M (=10 pg/L) and $10^{-8}$ M (=1 mg/L). A standard curve of relative light units vs. luciferase concentration can be plotted using serial dilutions of purified *Renilla* luciferase (Sigma, St. Louis, MO) in luciferase assay buffer. A supplement of 1 mg/ml BSA is added to the solution to prevent luciferase from attaching to the container surface. The
luminescence intensity (reported in relative light units) can be converted into photons per second by calibrating the luminometer relative to a radioactive $^{63}\text{Ni}$ light standard, which emits light in the 460-480 nm wavelength range (O’Kane and Lee 1990). The Rue assay using a spectrofluorometer is carried out in quartz cuvettes. Immediately after the addition of coelenterazine, samples are scanned across a broad wavelength centered on 480 nm. The results of photon emission can be reported as counts per second. Data can be collected using software provided by the system (DataMax for Windows™). All of the data can be exported as ASCII XY data and processed using the SigmaPlot for Windows version 4.0 software or using Microsoft Excel 2000 for Windows. To detect luminescence light emission using low light imagers (by Argus100 or by Nightowl), samples are prepared in titer plates. After the addition of coelenterazine, the titer plate is put in the dark box of the low light imager. Photon collection is performed for a specified period of time to obtain a low light image. Then, a photographic image is taken with either the low light imager or a Fuji Finepix4900 digital camera. The low light image is superimposed over the photographic image using Adobe Photoshop 5.0 software to record the location of luminescent activity. The luminescence emission can also be quantified by the low light imager and reported as total counts of photon emission for a specified duration of time in a designated area.

*Aequorea GFP*

The cDNA of GFP was cloned from the jellyfish *Aequorea victoria* (Prasher *et al.*, 1992). At pH 7.0, excitation of the wild type GFP by light with a wavelength of 398 nm induces emission of bright green fluorescence with a peak at 508 nm (Ward
and Bokman, 1982). Since the energy needed to generate fluorescence can be acquired through exogenous light absorption, no endogenous or exogenous substrate is needed. Therefore, upon exposure to excitation, its presence and movement can be visualized externally based on fluorescence in real time in live cells and animals, without the need of fixation or staining procedures. A native GFP molecule is relatively stable and can remain functional for up to 24 hours in cells. It is stable upon heating up to 65 °C as well as after treatment with 4% paraformaldehyde (Cubitt et al., 1995). No mammalian homologue of GFP exists, thus there is no endogenous interference with introduced GFP fluorescence. The unique combination of features present in this small protein of approximately 200 amino acid residues makes it an ideal candidate for use as a biomarker.

To enhance fluorescence and extend or decrease the stability of the wild-type protein at higher temperatures, a number of laboratories have modified the native GFP structure (Heim et al., 1994; Delagrave et al., 1995; Ehrig et al., 1995; Heim et al., 1995; Cormack et al., 1996; Crameri et al., 1996; Heim and Tsien, 1996; Yang et al., 1996; Zhang et al., 1996; Kimata et al., 1997; Li et al., 1998; Yang et al., 1998; Stauber et al., 1998; Ito et al., 1999). The coding sequences for mutants have also been designed with codon changes to optimize expression in the mammalian system (Zolotukhin et al., 1996). Mutants of GFP have been generated with 20 to 35 fold higher intensity than wild type GFP (Cormack et al., 1996). Enhanced GFP (EGFP) is a mutant form of GFP that offers an equal or higher sensitivity of expression detection than that of β-galactosidase staining in cell cultures (Zhang et al., 1996).
To simultaneously monitor the fate of more than one protein in the same cell, GFP color variants emitting light at different wavelengths have been constructed and applied successfully in many studies (Siemering et al., 1996; Zernicka-Goetz et al., 1997; Ellenberg et al., 1999; Gibbs and Schmale, 2000; Hadjantonakis and Nagy, 2001). GFP variants with increased stability are not always desirable as reporter proteins. In gene expression studies to monitor target transcription or translation, a reporter protein with quick turnover is essential. To achieve this goal, Li et al. (1998) constructed a destabilized chimeric EGFP protein by fusing the degradation domain of mouse ornithine decarboxylase to the C terminus of EGFP, resulting in a shortening of the EGFP half-life to about 2 hours. Other GFP variants with rapid degradability have also been described (Chiang et al., 2001). These GFPs with shorter half-lives are excellent markers for real-time gene induction studies.

Another advantage of GFP as an in vivo marker protein is that individual cells or subpopulations can be easily monitored, due to its strong fluorescent emission. For example, the contraction of individual cardiomyocytes can be visualized based on GFP fluorescence in live embryos (Fleischmann et al., 1998). Therefore, GFP-transformed cells can be traced spatially and temporally in a nondestructive fashion throughout the entire experiment. Expression of GFP under ubiquitous promoters has been observed in a variety of tissues, without display of cell or tissue preference. Furthermore, GFP labeled cells can be isolated from cell cultures or animals by fluorescence activated cell sorting (FACS) and used for in vitro studies of isolated cell types. Although high levels of GFP expression may be toxic (Hanazono et al., 1997), no abnormalities associated with organismal development have been linked to
modest GFP expression in animals transformed with a gfp expression construct
(Okabe et al., 1997; Pratt et al., 2000; Chan et al., 2001).

Application of Aequorea GFP as a Biomarker

Labeling cells with GFP.

Classical cell labeling studies have utilized techniques including chimeric tissue
transplants (Beresford, 1983; Ambler et al., 2001), intracellular injection of
carbocyanine dyes (Di I, DiA or DiO) and intracellular injection of fluorescent dyes
(Seleck and Stern, 1991; Clarke and Tickle, 1999). Mapping dynamic processes is
limited in transformed cells and in chimeric transplants since visualization based on
histological staining is not possible in the living cells or organisms. Similarly, the
carbocyanine dyes generally require tissue processing for visualization. Fluorescent
dyes, on the other hand, can be monitored in living cells and organisms. However,
diffusion of the injected dye during cell growth limits its usefulness as a long-term
marker. In contrast, cells can be transiently and stably transformed using GFP
expression constructs, and subsequent GFP expression allows the monitoring of cell
growth and proliferation.

There are several ways to label cells with GFP. Individual cells or cell groups can
be injected with purified cRNAs. This method allows the cytoplasmic translation and
the generation of GFP. However, the cRNA is rapidly degraded, leaving only the
stable protein to be passed on to subsequent progeny cells (Peters et al. 1995).

Another method of labeling cells with GFP is to deliver GFP-encoding gene
constructs intracellularly by transient transformation, thus allowing passage of the
transgene to progeny cells. Transgene delivery can be achieved by a variety of
methods including direct DNA injection, electroporation, or viral transduction. Promoters used for this purpose can be ubiquitous, such as the chicken β-actin promoter, or tissue-specific. Examples of promoters used for ubiquitous GFP expression include the *Drosophila* polyubiquitin promoter (Davis *et al.*, 1995), the cytomegalovirus (CMV) promoter (Takeuchi *et al.*, 1999; Damjanovski *et al.*, 2001; Nutt *et al.*, 2001), the *Xenopus* heat shock protein 70 (Xhsp70) promoter (Wheeler *et al.*, 2000), the *Xenopus borealis* cytoskeletal actin (CSK) promoter (Kroll and Amaya, 1996), the ROSA26 (R26) promoter (Kisseberth *et al.*, 1999), and the β-actin promoters from carp (Gibbs and Schmale, 2000), from medaka (Hamada *et al.*, 1998), and from chicken (Ikawa *et al.*, 1995b; Okabe *et al.*, 1997; Shimada *et al.*, 1999; Okada *et al.*, 1999). Examples of promoters used for tissue-specific GFP expression include the muscle creatine kinase (MCK) promoter (Ju *et al.*, 1999), the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter (Zhuo *et al.*, 1997), the murine type II collagen (*col2*) promoter in chondrocytes (Grant *et al.*, 2000), and the L7 promoter in Purkinje cells (Tomomura *et al.*, 2001). An alternative method for targeting specific tissues without using tissue-specific promoters is to implant stably transformed cells of that tissue type or transformed totipotent cells. For instance, Arnhold *et al.* (2000) established a D3-derived embryonic stem cell line transformed with a chicken β-actin promoter-*gfp* construct. After the implantation of these cells into the rat striatum, the migration and differentiation of these GFP-labeled embryonic stem cells were successfully monitored. The authors further described a subpopulation of these GFP-labeled cells that differentiated into neurons positive for
the neuronal marker Thy-1, and also into astrocytes positive for glial fibrillary acidic proteins.

**Analysis of promoter activation using promoter-gfp fusion constructs.**

It has been confirmed repeatedly that the specificity of transgene expression from tissue-specific promoters correlates with the tissue in transformed organisms (Kroll and Amaya, 1996; Fleischmann *et al.*, 1998; Anderson *et al.*, 1999; Fukushige *et al.*, 1999; Offield *et al.*, 2000; Koster and Fraser, 2001). Therefore, linking the gfp cDNA to DNA elements with promoter activity, followed by analysis of the in vivo location of transgene expression, constitutes a valid approach to obtaining tissue-specific function of regulatory sequences. The general strategy is to isolate DNA fragments of the 5' and 3' flanking regions of the gene of interest. After sequential resection of these flanking sequences, each fragment is linked with a gfp cDNA or by fusion between the gene of interest and a gfp cDNA. The resulting constructs are injected into early stage embryos (e.g. one-cell stage, or individual blastomeres) to analyze GFP expression patterns. Using this method, studies have demonstrated that GFP can be used to help identify regulatory regions that provide spatial and temporal control of gene expression (Long *et al.*, 1997; Krempen *et al.*, 1999; Meng *et al.*, 1999; Drivenes *et al.*, 2000; Kusakabe and Suzuki, 2000; Ryffel and Lingott, 2000; Anyanful *et al.*, 2001; Liu and Green, 2001; Martin *et al.*, 2001; Ryu *et al.*, 2001). Furthermore, reporter gene systems have been designed to allow inducible GFP expression from promoters that can be activated by exogenous inducers. For example, Mattingly *et al.* (2001) demonstrated that the environmental toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces the expression of gfp under the control
of the aryl hydrocarbon receptor (AhR) promoter. This finding shows that the promoter-\textit{gfp} fusion system may become a powerful tool to study promoter activation and transcriptional regulation (Arnone \textit{et al}., 1997).

Use of target protein-GFP fusions to study protein function during embryonic development.

Mutational analysis and protein over-expression have been frequently used to study how individual proteins affect cell function. However, one of the main problems of using conventional staining or dye methods for these studies is that experimental observations are made based on information obtained from cells and organisms at different time points. Therefore, interpretation of such results is difficult since observations were made on different cells or organisms. Moreover, incorrect conclusions may be drawn unless a sufficient number of specimens have been examined. To overcome these difficulties, new techniques need to be developed so that cellular events can be studied in real-time. This can be accomplished by fusing a \textit{gfp} cDNA to the gene of interest. Using GFP as a live marker, information can be obtained on cellular, subcellular, and tissue localization of the protein of interest (Marshall \textit{et al}.
1995; Htun \textit{et al}.
1996; Cormack 1998; Phillips 2001), on onset and duration of protein expression at a certain stage of cell proliferation and differentiation (Godwin \textit{et al}.
1998; Friedman \textit{et al}.
2000; Mateus and Avery 2000), on agonist induced protein translocation and aggregation (Fejes-Toth \textit{et al}.
1998; Zhu \textit{et al}.
1998; Liu \textit{et al}.
1999b) and on many other signal transduction events (Kallal and Benovic 2000; Zaccolo and Pozzan 2000; Chiesa \textit{et al}.
2001; Kabuta \textit{et al}.
2002; Sato \textit{et al}.
2002).
Fluorescence-based GFP detection in living cells and organisms may present some experimental difficulties. A few words of caution: Most of the gfp fusion constructs lack introns and 5' and/or 3' untranslated flanking regions of the target gene, which may contain the potential regulatory sequences (Inoue and Thomas, 2000). Therefore, the GFP reporter protein expression patterns may not represent those of endogenous proteins. In addition, the synthesis and maturation of GFP requires at least two hours. Due to this time lag, it is difficult to determine the precise time of transcriptional activation in short time intervals during dynamic cell growth (Davis et al., 1995; Timmons et al., 1997; Hazelrigg et al., 1998; Fay et al., 1999; Tanaka et al., 2001). Furthermore, many GFP variants, such as EGFP, are very stable in vivo. GFP fluorescence, which may be the result of the accumulation of current and previous episodes of GFP expression, may not accurately reflect the transcriptional activation of an endogenous target gene at a given point in time (Arnone et al., 1997). Currently, due to the possibility of "transgenic mosaicism" (Palmiter et al., 1984; Chan et al., 1999), one cannot rule out that tissue-specific GFP expression is the result of uneven distribution and expression of a transgene in different cells of the organism. Cell type specificity of an unknown promoter can be verified only when a statistically significant number of transiently transformed organisms have been analyzed.

**GFP Imaging Techniques**

GFP expression in organisms and single living cells can be visualized by a conventional wide-field fluorescence microscope with GFP filter sets (e.g. the Nikon Endow GFP Longpass Emission (FGP(R)-LP) filter combinations). However, this
method of imaging can be complemented with laser scanning confocal microscopy (LSCM) due to LSCM’s out-of-focus correction and optical sectioning capabilities for three-dimentional image reconstruction. More recently, two-photon laser scanning microscopy (TPLSM) has been developed (Denk et al., 1990) to increase tissue penetration ability and decrease photobleaching, photodamage, and autofluorescence that cannot be resolved using LSCM. Periasamy et al. (1999) have compared three imaging techniques (TPLSM, LSCM, and conventional widefield digital deconvolution microscopy (DDM)) for studying cells in deep tissues. TPLSM shows the most promising deep tissue imaging (>30µm) capability that allows high-resolution time-lapse microscopy. The advantages and disadvantages of some of the GFP imaging techniques are summarized in Table 1.

**Renilla Luciferase – Aequorea GFP Fusion Protein**

The *Renilla* luciferase – *Aequorea* gfp fusion gene (ruc-gfp) was constructed by Wang et al. (2001). There is a 15-base-pair linker bridging the *Renilla* luciferase cDNA with the cDNA of GFP in frame. The fusion gene was linked to either a CMV promoter or a chicken β-actin promoter for expression in mammalian cells. Western blot analysis confirmed the expression of the fusion protein. A transformed Chinese hamster ovary cell line stably expressing the Ruc-GFP fusion protein was also established. Fluorescence *in situ* hybridization (FISH) analysis located the ruc-gfp fusion gene on a single chromosomal locus. The recombinant fusion protein was demonstrated to be functional. Luminescence resonance energy transfer (LRET) was established. Fluorescence *in situ* hybridization (FISH) analysis located the ruc-gfp
Table 1. GFP imaging techniques

<table>
<thead>
<tr>
<th>TECHNIQUES</th>
<th>DESCRIPTION</th>
<th>ADVANTAGES</th>
<th>DISADVANTAGES</th>
<th>REFERENCES</th>
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<tr>
<td>Digital imaging microscopy (DIM)</td>
<td>- wide-field fluorescence microscopy - using sequentially focused images and computational algorithms to reconstruct high-resolution 3-D images</td>
<td>- weaker illumination requirement than laser scanning confocal microscopy, low photobleaching and photodamage - fast image acquisition - high spatial resolution 3-D images</td>
<td>Rizzuto et al. 1998; Howell et al. 2000; Yang et al. 2001</td>
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<td>Single-photon laser scanning confocal fluorescence microscopy (LSCM)</td>
<td>- high-intensity short wavelength excitation of fluorescence at a given focal plane</td>
<td>- optical sectioning ability through thick tissues for 3-D image reconstruction - can be configured to perform dual-color imaging with GFP variants - Although cellular photodamage is likely, frequent exposure of organisms to low laser power confocal imaging generally does not interfere with organismal development.</td>
<td>- high photobleaching and phototoxicity to cells on the focal plane as well as to the cells above and below the focal plane</td>
<td>Zernicka-Goetz et al. 1997; Cooper et al. 1999; Dailey et al. 1999; Ellenberg et al. 1999; Kulesa and Fraser 1999; Robb and Wylie 1999; Webb 1999; Kiehart et al. 2000; Paddock 2000; Gautier et al. 2001; Reynaud et al. 2001</td>
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<tr>
<td>Two-photon or multiple-photon laser scanning fluorescence microscopy</td>
<td>- use of a pulsed long excitation wavelength, such as an infrared (IR) laser beam, to excite fluorescence at a focal plane - simultaneous excitation of the fluorescence molecule by two photons</td>
<td>- high sensitivity with high signal/noise ratio due to low background fluorescence - low photobleaching or phototoxicity - almost no excitation of cells outside of focal plane - great depth of tissue penetration - time-lapse kinetic studies - 3-D and 4-D (repetitive 3-D) image reconstruction</td>
<td>- expensive system</td>
<td>Potter et al. 1996; Periasamy et al. 1999; Parnass et al. 2000; Huang et al. 2001; Lansford et al. 2001</td>
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fusion gene on a single chromosomal locus. The recombinant fusion protein was demonstrated to be functional. Luminescence resonance energy transfer (LRET) was observed in the fusion protein (Wang et al. 2001). In the LRET studies, spectroscopic analysis of cells expressing the Ruc-GFP fusion protein in the presence of coelenterazine substrate resulted in two emission peaks at 471 nm and 503 nm, which correspond to the emission maxima of Ruc and GFP respectively. The GFP peak was the result of intramolecular LRET from Ruc to GFP in the absence of any exogenous excitation light. In addition to ruc-gfp, a second fusion gene, namely gfp-ruc, has also been constructed and expressed in mammalian cells (Wang et al., unpublished data). In this second fusion, the cDNA of GFP was fused to the 5’ end of the Renilla luciferase cDNA with a linker of 21 base pairs in between. Interestingly, GFP-Ruc exhibited better LRET than Ruc-GFP, by showing a stronger emission peak at 503 nm in the presence of coelenterazine. It was reasoned that since the carboxyl terminus of GFP is more physically and functionally flexible than the amino terminus (Ormo et al 1996; Youvan and Michel 1996; Li et al. 1997; Wachter et al. 1997), the fusion of Ruc to the carboxyl terminus of GFP allows a greater probability of intramolecular proximity between the Ruc and GFP domains, and hence a more efficient LRET.

Effect of the GFP Tag on the Structure and Function of the Protein Fusion Partner

Most proteins when fused to GFP exhibit no interference with protein folding and function during cell growth and organism development (Okada et al., 1999). Mouse embryos and embryos of C. elegans, expressing GFP fusion proteins, developed into normal adults. It has also been shown that target protein-GFP fusion products can
rescue the fusion partner null phenotype (Wang and Hazelrigg, 1994; Endow and Komma, 1996; Fukushige et al., 1999; Jiang and Sternberg, 1999; Chen et al., 2000; Friedman et al., 2000; Du and Chalfie, 2001; Glenn and Searles, 2001). For example, Ezrin::GFPc (GFP fused to the carboxy terminus of full-length Ezrin) has been shown to behave like the endogenous protein in mouse embryos, accumulating at the apical pole of the blastomeres during compaction and persisting at this localization during the later stages in outer layer cells (Dard et al., 2001). In another example, similar to endogenous decapentaplegic (Dpp), the Dpp::GFPc fusion protein can form an active gradient in the wing imaginal disc and restore normal wing patterning and development in mutant dpp Drosophila (Teleman and Cohen, 2000). GFP fusion proteins can be generated by attaching GFP as a tag to either the N-terminus or the C-terminus of a given target protein, by inserting the gfp cDNA within the target protein encoding sequence (Siegel and Isacoff, 1997; Biondi et al., 1998; Yamao et al., 1999, Wang et al. 2001), or by inserting target protein encoding sequences into the GFP encoding DNA fragment (Baird et al., 1999). Analysis of the literature revealed that most of the fusion proteins have the target proteins connected to the N terminus of GFP without using linker sequences. It has been suggested that a linker peptide is helpful when the C terminus of GFP is fused to the target protein (Cubitt et al., 1995). In the examples of Ezrin::GFPc and Dpp::GFPc fusion proteins, the presence of GFP at the carboxyl-terminal end of the target proteins did not interfere with target protein function and localization (Teleman and Cohen, 2000; Dard et al., 2001).

Although the GFP fusion proteins usually exhibit normal GFP and target protein functions, the 27 kDa GFP tag, a relatively large moiety, may interfere with the native
structure and function of the target protein. It has been shown that cells expressing
GFP fusion proteins behave somewhat differently than those cells expressing the
target protein alone (Mao et al., 2000). For example, gap junction formation is
 crucial in normal neuronal differentiation, trophoblast differentiation, and in
coordinating cardiac development. In the gap junction experiments, although
connexin 43-GFP fusion proteins were shown to form functional channels, a lower
level of electrical coupling was observed in cells expressing connexin 43-GFP than in
cells expressing connexin 43 alone (Mao et al. 2000). The C terminus of connexin 43
is thought to act as a gating particle (Bruzzone et al. 1996), which may explain why
tagging GFP to this portion of the protein may interfere with connexin function (Mao
et al. 2000). In addition, the connexin 43-GFP transformed cells were more easily
pulled off the coverslip by the microelectrodes than connexin 43 cells indicating that
they may be less adherent to neighboring cells (Mao et al. 2000). In another study,
the human slow poke (hSlo) K+ channel protein was tagged with GFP at the N-
terminus of its α-subunit and was expressed transiently in human embryonic kidney
293 cells (Meyer and Fromherz, 1999). Whole cell patch-clamp data indicated the
fusion protein formed functional voltage-gated channels. However, the additional
presence of the GFP tag lowered the threshold of Ca2+ mediated K+ channel
activation, thus indicating a lowering of the voltage threshold of gating. In a third
example, the expression of the fusion form of the γ-aminobutyric acid (GABA) ρ1
receptor with GFP took longer than ρ1 receptor alone in Xenopus oocytes (Martinez-
Torres and Miledi, 2001). Also, cells with ρ1 receptor::GFP fusion proteins exhibited
a smaller GABA current than those with wild type ρ1 receptor. The authors reasoned
that this difference was due to the decreased assembly of functional p1 receptors on the cell surface when fused with GFP. When gfp was inserted into the middle of the gene encoding the cAMP-dependent protein kinase regulatory subunit, not surprisingly, a moderate disturbance of fusion protein folding was observed as indicated by changes of the cAMP binding capacity (Biondi et al., 1998). It was also found that cells over-expressing GFP are more prone to undergo apoptosis (Liu et al., 1999a). An over-expression of GFP was found to interfere with normal embryonic development (Sussman 2001). Therefore, it is important to be cognizant of these negative effects of GFP when interpreting data obtained by using target protein-GFP fusions.
CHAPTER TWO
MATERIALS AND METHODS

Plasmid Vectors

"GeneSwitch" Plasmid Vectors

The construction of reporter plasmid p17x4-TATA-ruc-gfp has been described previously (Yu et al. 2001). The chimeric transcriptional activator plasmid (PAP CMV-GL914VPc’SV) was a gift from Dr. Y. Wang (Department of Cell Biology, Baylor College of Medicine, Houston, TX). We used these two plasmids to express the ruc-gfp fusion gene under the induction of mifepristone.

Sequencing vector, pBluescript KS (+)II (Strategene, La Jolla, CA)

This plasmid vector is a double-stranded DNA of 2.9 kb. It is a derivative of the pUC plasmid (Vieira and Messing 1982; Norrander et al. 1983; Yanisch-Perron et al. 1985), which has a prokaryotic F1 replication origin. We used this vector to clone various cDNAs into the multiple cloning sites for DNA sequencing.

pLEIN (Clontech, Palo Alto, CA)

This plasmid vector is a retroviral shuttle vector carrying an egfp expression cassette under the control of the constitutive 5’ LTR promoter. It is derived from the pLXIN retrovirus shuttle vector, a double stranded DNA of 6.1 kb, which contains multiple cloning sites downstream of the 5’ LTR promoter for expression of the gene-of-interest. Following the multiple cloning sites is an internal ribosomal entry site (IRES) and a neomycin resistance gene. The 5’ LTR promoter controls the expression of both the gene-of-interest and the neomycin resistance gene. In addition to the neomycin resistance gene for selection of stable transformants in eukaryotic
cells, this vector also carries an ampicillin resistance gene for selection in bacteria. We used this plasmid to produce retrovirus, which carries the ruc-gfp fusion gene expression cassette.

**pcDNA 3.1 (Invitrogen, Carlsbad, CA)**

This plasmid vector is a double-stranded DNA of 5.4 kb. The pcDNA 3.1 is designed for high-level, constitutive expression of a gene-of-interest under the control of the cytomegalovirus (CMV) enhancer-promoter in mammalian cells. It has large multiple cloning sites downstream of the CMV enhancer-promoter, followed by a bovine growth hormone (BGH) polyadenylation signal and a transcription termination sequence. The pcDNA 3.1 has a SV40 origin for episomal replication in cell lines that express the large T antigen, such as COS1 and COS7 cells. It also has a pUC origin for replication in bacteria. The pcDNA 3.1 carries the neomycin resistance gene for selection of stable transformants in mammalian cells and the ampicillin resistance gene for selection in bacteria. We used this plasmid to constitutively express the Ruc-GFP fusion protein in mammalian cells.

**pCX (Ikawa et al. 1995)**

This plasmid vector is a double-stranded DNA of 3.8 kb. It carries a CMV immediate early enhancer and a chicken β-actin promoter for high-level constitutive expression of a gene-of-interest in mammalian cells. A single EcoRI cloning site is located downstream of the chicken β-actin promoter for cloning a gene-of-interest. Downstream of the EcoRI site is the polyadenylation signal and transcriptional termination sequences from the rabbit β-globin gene. The pCX plasmid vector has replication origins for both bacteria and mammalian cells (SV40 ori). The plasmid
carries the ampicillin resistance gene for selection of stable transformants in bacteria. There is no drug-resistance marker in this plasmid for selecting stable transformants in mammalian cells.

**cDNA Sequences**

*The Renilla Luciferase cDNA (Genbank Access Number M63501)*

The *rue* cDNA, cloned and sequenced by Lorenz et al. (1991), is 1196 base pairs in length. It encodes a 314 amino acid glycoprotein of 34 kDa.

*The “humanized” Aequorea GFP cDNA (Genbank access number U50963) (Zolotukhin et al. 1996)*

“Humanized” GFP is synthesized with optimized codon usage for expression in mammalian cells. It encodes a polypeptide of 238 amino acids with a calculated molecular weight of 26.9 kDa.

*The Renilla Luciferase-Aequorea GFP Fusion Gene (Wang et al. 2001)*

The coding sequence of *rue* is fused in frame to the 5' terminus of the *gfp* coding sequence. There is a short 15 bp linker between the two genes.

**Bacterial Strains**

*E. coli, DH5α [F’/endA1 hsdR17 (rK- mK+) supE44 thi-1 recA1 gyrA (NalR) relA1 Δ(lacZΔM15)lacY1 argF]U169 deoR (φ 80dlacΔ(lacZ)M15)]*

This strain was used as a host for all plasmid construction and amplification.

DH5α was cultured at 37 °C in LB medium with continuous shaking at 200 rpm or on LB agar plates in an incubator.
Mammalian Cell Lines

The COS-7 Cell Line (ATCC: CRL-1651)

COS-7 is a cell line derived from African green monkey kidney tissue. It was transformed by the SV40 virus (Gluzman 1981). COS cells form a fast growing monolayer, which can be cultured in DMEM medium (Cellgro®, Mediatech, Herndon, VA) supplemented with 2 mM glutamine, 10% FBS, and penicillin (100 IU/ml) / streptomycin (100 µg/m) antibiotics, or in supplemented RPMI-1640 medium (Cellgro®, Mediatech, Herndon, VA). COS cells can be easily transformed with plasmid DNAs using many commercially available methods, including calcium phosphate precipitation (Promega, Madison, WI), FuGENE-6 (Roche Diagnostics, Indianapolis, IN), Lipofectamine (Life Technologies, Gaithersburg, MD), and SuperFect (Qiagen, Valencia, CA).

The HT1080 Cell Line (ATCC: CRL-7951)

HT1080 is a human fibrosarcoma cell line derived from human bone. HT1080 cells form a fast growing monolayer, which can be propagated in supplemented DMEM medium or in MCDB302 medium (Sigma-Aldrich, St. Louis, MO). Plasmid transformation of HT1080 cells has been successfully performed using calcium phosphate precipitation, or the FuGENE-6 and Lipofectamine kits.

Molecular Cloning Techniques Used in Plasmid Construction

In modern molecular and cellular biology research, transgenes are routinely introduced into prokaryotic and eukaryotic hosts using plasmid vectors as carriers. There are four steps involved in recombinant plasmid preparation: transgene and linearized plasmid DNA preparation, DNA ligation, bacterial transformation, and
positive clone identification. To prepare the transgene-containing fragment and the linearized plasmid, both the transgene DNA and the plasmid DNA are digested with restriction enzymes, and purified by agarose gel electrophoresis, if necessary. There are three methods by which the transgene and plasmid DNA can be prepared. In the first method, they can be digested with restriction enzyme(s) that yield identical overhangs (sticky ends) at the 5' and 3' ends of both. Since these identical overhangs are complementary with each other, the annealed fragments can be ligated together with DNA ligase. In general, we used a transgene-containing fragment to plasmid ratio of 5 to 1 to increase the probability of transgene insertion into the plasmid. A problem associated with this method of ligation is the high self-ligation of the plasmid alone. A dephosphorylation step may be included by treating the linearized plasmid with alkaline phosphatase. The incidence of plasmid self-ligation can be greatly reduced without the phosphate groups at the termini. Another problem associated with this method of ligation is that, since all the overhangs are identical, the fragment could be inserted into the plasmid in either orientation. If the transgene fragment is a cDNA sequence, it must have the same orientation as the promoter on the plasmid DNA. The orientation of the transgene insert can be confirmed using restriction enzyme digestions and agarose gel electrophoresis. The second method for preparing the transgene fragments and linearized plasmid DNA is to digest them with two different restriction enzymes so that they have compatible ends with each other but without the ability for self-ligation. Since there is only one orientation in which the transgene can be inserted, this method of ligation is very efficient. The third method for preparing transgene fragments and linearized plasmid DNA is to blunt-
end the DNA after restriction enzyme digestion. This is especially necessary when
the protruding ends of the transgene fragment are not compatible to the ends of the
linearized plasmid. Blunt-ending of the transgene fragment can be performed by
either filling in the ends using a DNA polymerase or by trimming the ends using a
single strand DNA exonuclease. Blunt-end ligation of DNA is a difficult cloning
strategy due to the very high levels of plasmid self-ligation. It is much less efficient
than the ligation method using compatible sticky ends. Dephosphorylation of the
linearized plasmid vector by phosphatase may help to decrease the incidence of self-
ligation and to improve the transgene insertion efficiency. Furthermore, in
experiments when the protruding ends of the insert are not compatible with the ends
of the plasmid vector, an alternative cloning strategy may be applied. In this strategy,
restriction sites that are used to linearize the plasmid vector are inserted into the PCR
primers, which are then used to amplify the transgene insert. Following restriction
digestion by the same enzyme(s), both the PCR fragment containing the transgene
and the plasmid vector have compatible ends generally resulting in greatly improved
ligation efficiency. After the ligation step, the DNA is transformed into competent
bacteria (usually E. coli DH5α) either by the heat shock method or by
electroporation. Positive clones are identified using restriction digestion of isolated
plasmids and examination of fragment lengths separated by agarose gel
electrophoresis.

**Bacterial Transformation with Plasmid DNA**

The transformation of circular plasmid DNA into bacteria (E. coli) was performed
using either the heat shock method or electroporation. We used the following
protocol, which was modified from Inoue et al. (1990), to prepare competent \textit{E. coli} for plasmid transformation by heat shock. A single colony of \textit{E. coli} DH5\textalpha{} was used to inoculate 500 ml of SOB medium in a 2 L flask. Cells were grown at room temperature with vigorous shaking (250 rpm) to an OD\textsubscript{600} of 0.6. The cells were incubated on ice for 10 min before being pelleted by centrifugation at 3,000 rpm for 10 min at 4 °C. We resuspended the pellet in 160 ml ice-cold TB buffer, and placed it on ice for 10 min. We centrifuged the cells again at 3000 rpm for 10 min at 4 °C. The cell pellet was then gently resuspended in 40 ml of ice-cold TB. During gentle swirling, 2.8 ml of 0.2 μm-filtered DMSO were added to the cells. After incubation on ice for another 10 min, the cells were aliquoted into Eppendorf tubes (200 μl each) and immediately frozen in liquid nitrogen. Frozen competent \textit{E. coli} bacteria were stored in a -80 °C freezer. To transform plasmid DNA into competent \textit{E. coli} by heat shock, frozen competent cells were thawed on ice for 10 min. Plasmid DNA (<20 μl in volume) was added to the cells, and the mixture was kept on ice for another 30 min. At the end of that time, the plasmid DNA/\textit{E. coli} mixture was heat shocked in a 42 °C water bath for 30 sec. After the addition of 0.8 ml of SOC, the cells were transferred to a 37 °C incubator and shaken vigorously for 1 h. At the end of incubation, the desired amount of transformed cells was spread on agar plates with antibiotic selection and incubated in the 37 °C incubator overnight. On the second day, colonies were counted to determine transformation efficiency.

To prepare competent \textit{E. coli} for plasmid transformation using electroporation, 1 L of SOB was inoculated with a single colony of bacteria, and the bacterial cells were cultured at 37 °C with moderate shaking (150 rpm) to an OD\textsubscript{600} of 0.3. The bacterial
cells were kept on ice for 30 min, and then pelleted by centrifugation at 3000 rpm for 10 min at 4 °C. Bacterial cells were washed sequentially with the following: an equal volume of ice-cold 1 mM HEPES (pH 7.0), an equal volume of ice-cold water, one-half volume of ice-cold water, and finally 30 ml of ice-cold 20% glycerol washing solution. After each washing step, the bacteria were pelleted by centrifugation at 3000 rpm for 10 min at 4 °C. At the end of washing procedures, the pellet was gently suspended in 2 ml ice-cold 10% glycerol stock solution. The bacterial suspension was then aliquoted (50 µl each) into Eppendorf tubes and frozen in liquid nitrogen, and then stored in a −80 °C freezer. To transform plasmid DNA into E. coli by electroporation, competent cells were thawed on ice for 10 min. Plasmid DNA (<5 µl) was added to the cells. The mixture was transferred into a 0.1 cm electroporation cuvette (Bio-Rad, Hercules, CA) and electroporated at 1.8 kV, 25 µF, and 200 Ω using a Gene Pulser apparatus and Pulse Controller (Bio-Rad, Hercules, CA). Immediately after electroporation, 0.9 ml of SOC was added to the cells, and the cells were incubated in a 37 °C incubator with shaking for 1 h. At the end of incubation, a desired amount of bacterial cells was spread on agar plates with antibiotic selection. After overnight incubation at 37 °C, colonies were counted to determine transformation efficiency.

**Purification of the DNA from an Agarose Gel**

We used the GENECLEAN DNA purification kit (Bio101, Vista, CA) to purify the DNA from a TAE agarose gel following a modified protocol. A slice of agarose gel (<500 µl in volume) containing the plasmid DNA was excised under UV illumination and transferred to an Eppendoff tube. Three volumes of 6 M NaI
solution was added to the gel slice, and placed in a tube in a 65 °C dry bath for 5 min to melt the gel. The tube was then transferred to ice for 10 min. Then, 20 μl of glassmilk were added, and mixed by gentle vortex. The tube was then kept on ice for 10 min to allow binding between the glassmilk and the DNA. The glassmilk/DNA complex was then pelleted by centrifugation at 12,000 rpm for 5 sec. The supernatant was removed and the pellet washed with 1 ml of New Wash solution. The wash and pelleting steps were repeated twice. The DNA was eluted in 20 μl of water and incubated at 65 °C for 2 min. After elution, the glassmilk was pelleted by centrifuging at 12,000 rpm for 5 sec. The supernatant containing the DNA was carefully transferred to a new tube.

**Large-Scale Plasmid DNA Purification**

We used the QIAfilter Maxi Prep kit (Qiagen, Valencia, CA) to purify large amounts of plasmid DNA (300 μg to 1 mg) from transformed *E. coli* using the following protocol. Bacteria were inoculated in 250 ml of LB medium in the presence of antibiotic selection and cultured overnight at 37 °C with vigorous shaking (250 rpm). The bacteria were harvested by centrifugation at 6000 rpm for 15 min. The bacteria were resuspended in 10 ml of Buffer P1 (containing RNase A). Then, 10 ml of Buffer P2 were added and mixed well by swirling the centrifuge bottle clockwise and counterclockwise. Ten ml of ice-cold Buffer P3 were added and mixed well by swirling the bottle. The lysate mixture was then transferred to the barrel of the QIAfilter cartridge and incubated at room temperature for 10 min. Ten ml of Buffer QBT were used to equilibrate a QIAGEN-tip 500 column. The plunger was inserted into the QIAfilter cartridge to filter the cell lysate into the equilibrated
QIAGEN-tip 500 column. After allowing the lysate to flow through the column, the column was washed with 30 ml of Buffer QC. This step was repeated once. The purified plasmid DNA was eluted into a new 50 ml centrifuge tube using 15 ml Buffer QF. Isopropanol (10.5 ml) at room temperature was added. This was mixed well and immediately centrifuged at 11,500 rpm for 30 min at 4 °C. The plasmid DNA pellet was washed with 5 ml of 70% ethanol. After centrifugation, the pellet was air-dried and redissolved in 200 μl sterile water. The DNA concentration was determined using a spectrophotometer at 260 nm.

**Mammalian Cell Transformation with Plasmid DNA (Calcium Phosphate Precipitation Method)**

Transformation of the double-stranded plasmid DNA into mammalian cells was performed using the calcium phosphate transformation method (Profection Mammalian Transfection Systems, by Promega, Madison, WI). The calcium phosphate transformation method can be used both for transient transformation as well as for long-term stable transformations of adherent cell lines. This technique involves the coprecipitation of plasmid DNA with calcium phosphate crystals, formed by mixing CaCl₂ and HEPES phosphate buffer. Graham and van der Eb (1973) were the first to show that the entrance of DNA into mammalian cells is greatly enhanced when coprecipitated with calcium phosphate crystals. Since then, plasmid DNA transformation using the calcium phosphate precipitation method has been successfully demonstrated in a large variety of cell lines. Therefore, it is routinely used as the method of choice for both transient and stable transformations. The following is a brief description of the protocol of the calcium phosphate precipitation method we used for mammalian cell transformation. The cells were
seeded in 10 cm culture dishes, and grown to 50-80% confluency. Three hours before the transformation experiment, the old medium was replaced with fresh growth medium. Frozen HBS buffer (containing HEPES) was thawed and brought to room temperature. Two 15 ml tubes were prepared. To the first tube, 62 µl of 2 M CaCl₂ and plasmid DNA were added. The volume was adjusted to 500 µl using sterile water. To the second tube, 500 µl of 2 × HBS were added. Working in a tissue culture hood, the DNA CaCl₂ mixture from the first tube was added dropwise to the HBS in the second tube, which was continuously vortexed. The mixture was incubated at room temperature for 30 min. After briefly vortexing the mixture again it was added dropwise to the cells. The cell culture plates were incubated at 37°C for 12 to 17 hours after transformation, at which time fresh medium was added. The transformed cell cultures were analyzed 48 to 72 hours after transformation for protein expression.

**Luciferase Assays**

The activity of recombinant Rue was assayed using the following protocol. Mammalian cells were suspended in 500 µl of luciferase assay buffer (0.5 M NaCl, 0.1 M potassium phosphate, 1.0 mM EDTA, pH 7.5) (Matthews et al. 1977). Coelenterazine substrate (Molecular Probes, Eugene, OR, and Nanolight Technology, Pinetop, AZ) was added to the samples to a final concentration of 0.1 to 10 µM, and the samples were then analyzed using a luminometer, a spectrophotometer, or a low light imager. Since coelenterazine can freely diffuse into mammalian cells, cell lysis is not required to assay intracellular Rue activity. However, if desired, a luciferase
lysis buffer (125 mM Tris, pH 7.6, 0.5% Triton X-100) may be used to lyse cells prior to luciferase assays.

**Fluorescence Microscopy**

The principle of fluorescence microscopy is based on the fact that fluorescent molecules (or fluorophores) absorb light of a given wavelength and subsequently emit light at a longer wavelength. The fluorescence microscope is a modification of the conventional optical microscope. There are two types of fluorescence microscopes: epifluorescence microscopes and transmission fluorescence microscopes. Three components are needed to construct a fluorescence microscope: 1) a source of excitation light, usually a mercury arc lamp, that emits strong short wavelength light; 2) an excitation barrier filter, which excludes undesirable wavelengths of light and allows passage of only the one that excites the fluorophores; 3) an emission barrier filter, which allows the passage of only the long wavelength light emitted from the fluorophores. The fluorescence image can be viewed through the eyepiece or through a CCD camera. The quality of the images is dependent on the choice of filter set.

In our laboratory, we used an upright Carl Zeiss Axioplan microscope (Thornwood, NY) equipped with a Model 1200 power supply for visualizing fluorescence of cells on slides. Cells were first grown on sterile coverslips, which were precoated with a layer of polylysine, in tissue culture dishes. Before transfer to glass slides, the cells can be fixed with either 4% formaldehyde or with 70% ethanol. However, fixation is not necessary. Then, the coverslip was picked up by a pair of sterile forceps and laid down on a glass slide with the cell-coated surface facing the slide. The coverslip was then sealed with Fluoromount-G* Slide Mounting Medium (Fisher Scientific, Tustin,
CA), but this step is not necessary. We used both an inverted Carl Zeiss Axiovert TV100 microscope equipped with a Model 1600 power supply and a 35 mm camera and a Leica MZ8 stereo fluorescence microscope equipped with a SONY DKC-5000 3 CCD digital camera for visualizing fluorescence in tissue culture dishes.

Mammalian Cell Microencapsulation by Alginate/PLL

The detailed methods of cell microencapsulation have been described previously (Martinsen et al. 1992; Read et al. 1999; Serp et al. 2000). In our laboratory, the following procedures were used in a typical cell encapsulation experiment. The cell microencapsulation device (Fig. 1.10) used in our laboratory is the Encapsulator (Inotech AG, Switzerland). The autoclaved encapsulation cylinder was assembled under a laminar flow hood. The encapsulation cylinder was filled with 225 ml of sterile polymerization solution. A cell culture with approximately $5 \times 10^6$ cells was centrifuged and the pellet was suspended in 7.5 ml of sterile MOPS washing buffer. The cell suspension is added to 15 ml of 1.5% sodium alginate solution, which has been prefiltered through a filter with pore size of 50 μm, and mixed gently so as not to form bubbles. A 60 ml syringe was filled with the cell-alginate suspension and attached to the syringe pump. Control of the flow of alginate to the Encapsulator nozzle was achieved by adjusting the speed of the syringe pump, which was initially set at 14.3 units. The turbo button was turned on to initiate the flow of cell-alginate. After initiation of flow, the syringe pump speed was reduced to about 10.9 units. The vibration frequency used to create individual beads was set initially at 700 Hz for the 300 μm-sized nozzle. The electrostatic charge system was turned on and the initial voltage was set to 1 kV. The electrostatic charge aids in dispersing the beads. The
pump speed and/or the vibration frequency were adjusted in combination to achieve a stable bead chain with beads of the desired size flowing down into the bypass cup. Increasing the frequency and decreasing the speed of the syringe pump results in formation of smaller beads and *vice versa*. After the formation of the desired stable bead chain, the bypass cup was removed allowing the bead chain to fall into the polymerization solution, which was constantly agitated by a stir bar. The electrically controlled bead disperse point was adjusted to about 5 cm below the electrode. Shortly before the syringe pump was empty, the bypass cup was moved back into the bead stream. The syringe pump control, vibration control, and electrostatic charge control were all turned off at this point. The beads were allowed to polymerize for 5 min under constant stirring. The drain valve was opened to release the alginate beads encapsulating cells into a collection flask. The beads were allowed to settle to the bottom of the flask for 5 min. Excess polymerization solution was carefully aspirated, leaving just sufficient liquid to cover the cell-alginate beads. Fifty ml of 0.05% PLL, which had been prefILTERED through a 0.2 μm filter, was added to the cell-alginate beads, and stirred for 10 min. The aspiration procedure was repeated again. The cell-alginate/PLL beads were washed with 100 ml of MOPS washing buffer for 1 min and again with 150 ml of MOPS washing buffer for 5 min. The washing solution was removed and 50 ml of 0.03% of sodium alginate solution was added to the beads, and stirred for 5 min. The beads were sequentially washed again with 100 and 150 ml of MOPS washing buffer for 1 and 5 min, respectively. After removing the liquid, cell culture medium was added to the beads. While being agitated by swirling, the
medium containing the beads was aliquoted into cell culture dishes. The alginate/PLL-encapsulated cells were incubated at 37 °C in the presence of 5% CO₂.
CHAPTER THREE

STUDY OF INDUCIBLE GENE EXPRESSION IN VIVO USING A RENILLA LUCIFERASE – AEOUROEA GFP (RUC-GFP) FUSION GENE CONSTRUCT

Abstract

In this study, we used a steroid-induced promoter activation system as a molecular switch to study exogenous activation of transgene expression. This promoter activation system consists of three components, (1) a steroidal inducer drug, mifepristone (RU486), which binds the (2) chimeric transcription factor complex, consisting of the mutant human progesterone receptor fused to the yeast GAL4 DNA-binding domain and the herpes simplex virus protein VP16 activation domain, and (3) a synthetic promoter, consisting of a series of GAL4 recognition sequences upstream of the adenovirus major late E1B TATA box, linked to a Renilla luciferase – Aequorea green fluorescent protein (GFP) fusion gene (ruc-gfp). The transcription of the promoter-marker gene cassette is activated by the drug (mifepristone)-bound chimeric transcription factor complex. Monitoring of induced gene expression was carried out by a low light video camera and a UV microscope to detect luciferase and GFP activation, respectively. Using this activation system, we showed that robust and significant levels (close to 30-fold) of ruc-gfp marker gene expression were achieved in cell cultures. The activation of gene expression is mifepristone dependent and is strictly regulated by the chimeric transcriptional activator protein. The establishment of this rapid and noninvasive method of monitoring inducible gene expression in cell cultures will aid investigators in developing imaging procedures to continuously follow gene function in developing organisms and the location of therapeutic proteins during gene therapy procedures. The method should also be of
general use in gene therapy investigations for simultaneous monitoring of the expression levels of light-emitting proteins and therapeutic proteins originating from the activation of identical promoters.

Key Words

Inducible gene expression • Renilla luciferase • Aequorea GFP • Cellular implantation

Introduction

Numerous eukaryotic genes have been successfully overexpressed in cell cultures and in animal models. This technology allows investigators to study gene functions, and address some fundamental questions that could potentially promote the establishment of defined gene therapy procedures. However, constitutive expression of proteins that may be toxic or may have negative effects on cell growth and organism development is not desirable, and the accumulation of such proteins could have detrimental effects. Furthermore, experiments to study the function of individual proteins during specific stages of development of an organism in a spatially and temporally controlled manner cannot be performed in transgenic animals that carry a constitutive promoter driven gene construct. Several laboratories have reported the design of inducible eukaryotic gene expression systems, which include the tetracycline-regulated systems (Gossen and Bujard, 1992), an ecdysone-inducible system (Christopherson et al. 1992; No et al. 1996), an antiprogestin-regulated promoter activation system (Wang et al. 1994; Delort and Capecchi 1996), and a dimerization-based regulatable system (Belshaw et al. 1996; MacCorkle et al. 1998). Among those listed, the two-plasmid-based “GeneSwitch” promoter activation
system (Wang et al. 1994) has consistently given the highest levels of target gene expression upon the addition of a progesterone antagonist (mifepristone) as the inducer drug. In this system, one of the two plasmids carries the chimeric transcription factor cassette, which is expressed constitutively under the CMV promoter (chimeric activator plasmid), and the second plasmid harbors the inducible promoter-target gene fusion cassette (reporter plasmid). The binding of mifepristone to the chimeric transcription factor in the cells allows the binding of the transcriptional factor to the GAL4 promoter, activates the inducible promoter and initiates transcription of the target gene. So far, using this promoter activation system, transgene expressions of secreted human placental alkaline phosphatase (SEAP) (Abruzzese et al. 1999), human growth hormone (hGH) (Burcin et al. 1999), transforming growth factor β1 (TGFβ1) (Wang et al. 1999), vascular endothelial growth factor (VEGF) and erythropoietin (Abruzzese et al. 2000), inhibin A (Pierson et al. 2000), and growth hormone releasing hormone (GHRH) (Draghia-Akli et al. 2002) have been successfully demonstrated. This regulatable system has been shown to be stable both in cell cultures and in transgenic animals. Furthermore, the induction drug mifepristone does not have toxic effects on humans when it is administered over a long period of time at dosages up to 20 mg/kg (~18.6 μM) (Handerson 1997).

Using the two-plasmid-based GeneSwitch promoter activation system, we studied the induction and expression of a *Renilla* luciferase-GFP (*ruc-gfp*) fusion gene construct. *Renilla* luciferase is an enzyme from a soft coral, *Renilla reniformis* (Lorenz et al. 1991). It catalyzes a chemical reaction using coelenterazine and
oxygen, which emits light at a peak wavelength of approximately 485nm. Due to the simplicity of this process, which allows indirect monitoring of luciferase activity by observing light emission, luciferase has been used as a marker gene in gene expression studies (Yu et al. 2001; Bhaumik and Gambhir 2002; Greer and Szalay 2002). Such light-emitting events can be quantified by imaging techniques and correlated directly with luciferase levels in cells. GFP is a chromophore-containing protein whose cDNA has been isolated and sequenced from the jellyfish *Aequorea victoria*. Unlike luciferase, GFP does not require any substrates or cofactors to be functional. GFP fluorescence can be monitored within living cells using an excitation source provided by UV light. Quantification of GFP signals may be difficult due to poor sensitivity of currently available assays. Nevertheless, since an external substrate is not required for GFP fluorescence in living tissues, use of GFP for studying gene expression in living organisms would be potentially advantageous.

The Ruc-GFP fusion protein has been shown to retain both luciferase and GFP functions in cell cultures (Wang et al. 1997; Liu et al. 2000). We employed the Ruc-GFP fusion protein as marker to monitor gene activation in real time, in living cells and animals and to locate GFP coexpression in individual cells.

Here we describe the construction of a reporter plasmid, which carries a fusion gene, *ruc-gfp* encoding a chimeric protein with luciferase and GFP activity. This plasmid was cotransformed with the chimeric activator plasmid which carries the chimeric transcriptional activator expression cassette into COS cells. The fusion gene expression was studied by exogenous addition of mifepristone. We showed that significant levels of *ruc-gfp* marker gene expression were achieved in cell cultures.
The establishment of this rapid and noninvasive method of monitoring inducible gene expression will aid investigators in developing imaging procedures in future studies to continuously follow gene function in developing organisms and trace the location of therapeutic proteins during gene therapy procedures.

**Materials and Methods**

*Construction of Plasmid Vectors*

Plasmid DNA p17×4-TATA-CAT (from Dr. Y. Wang, Department of Cell Biology, Baylor College of Medicine, Houston, TX) was digested with XhoI and EcoRI. The ends of the larger fragment were blunted with T4 DNA polymerase, and gel-purified using a Geneclean kit (Qbiogene, Inc., Carlsbad, CA). In a parallel reaction, pCEP-4-ruc-gfp (Wang et al. 1997) was double digested with SalI and KpnI and blunted with T4 DNA polymerase. The smaller fragment containing the ruc-gfp sequence was similarly gel-purified. The purified fragments were then ligated together to create the resulting plasmid named p17×4-TATA-ruc-gfp. Using restriction digestions, the correct orientation of the fragments was confirmed.

Plasmid pCX-ruc-gfp was constructed based on pCX-EGFP (Okabe et al. 1997). Plasmid pCX-EGFP was digested with EcoRI. The EcoRI fragment containing the EGFP cDNA was excised, and replaced with the Pmel fragment (containing the ruc-gfp cDNA sequence) from pcDNA-ruc-gfp.

Plasmid pUAS-ruc-gfp was generated by inserting a Pmel fragment of ruc-gfp from pcDNA-ruc-gfp (Wang et al. 1997) into the T4 DNA polymerase-blunted EcoRI fragment of pUAS (Delort and Capecchi 1996).
**Cell Cultures and Transient Transfection Assays**

Six-well microtiter plates were seeded with $5 \times 10^5$ COS cells (monkey kidney cells transformed by SV40 virus). Cells were grown overnight in DMEM supplemented with 10% fetal bovine serum at 37 °C with 5% CO$_2$. On the second day, the culture medium was changed two hours before plasmid DNA addition. The cells were then transformed with designated amounts of DNA, using the calcium phosphate precipitation method (Profection Mammalian Transfection Systems, Promega, Madison, WI). Twenty-four hours after DNA transformation, mifepristone (Steraloids, Inc., Newport, RI) dissolved in absolute ethanol was added to the cultures to initiate induction of gene expression from the inducible promoter. Following mifepristone induction, aliquots of the cell cultures were tested for luciferase activity (using coelenterazine as substrate) and for GFP fluorescence signal (using a Zeiss fluorescence microscope).

**Luciferase Assays**

The culture medium in each well was removed by aspiration and replaced with 1 ml of luciferase assay buffer (0.5 M NaCl; 1 mM EDTA; and 0.1 M potassium phosphate, pH7.4) to which was added 2 μl of coelenterazine from a 0.5 μg/μl methanol stock solution. The cells were then imaged using a Hamamatsu Argus100 Low Light Video Camera (Hamamatsu Instruments, Hamamatsu, Japan) or a Nightowl Low Light Imager (EG&G-Perkin Elmer, Boston, MA). The light emission from luciferase assays was also monitored using a Turner TD 20e luminometer (Turner Designs, Sunnyvale, CA).
Results

*Induced Expression of Ruc-GFP Fusion Protein in Transiently Transfected Cells*

To study the inducible system, we constructed a *ruc-gfp* reporter plasmid based on the reporter vector of the promoter activation system by inserting the *ruc-gfp* fusion gene construct downstream to the inducible promoter (Fig. 3.1). The mifepristone-dependent Ruc-GFP fusion protein expression was tested by detecting luciferase activity in cell cultures. COS cells were either cotransformed with both reporter and transcription activator plasmids or transformed with reporter plasmid construct alone. The inducer mifepristone drug was delivered 24 hours after DNA transformation to initiate transcriptional activation of marker gene expression. The promoter activation by the hybrid transcription factor bound mifepristone complex was allowed to proceed over a 24- to 36-hour period of time, and then the cells were tested for luciferase or GFP expression. The culture medium was removed by aspiration and the cells were washed with serum-free medium. To avoid cell loss or cell damage, the luciferase substrate coelenterazine was added directly in luciferase assay buffer to the plates. Luciferase activity was quickly monitored in attached cells with a Hamamatsu Low Light Video Camera, a Nightowl Low Light Imager, or quantified by the use of a Turner luminometer. Results from such experiments showed that the induction levels of luciferase expression obtained were ~28-fold higher than that of the uninduced controls (Fig. 3.2). These induction levels did not reach the ~ 50-fold levels obtained with the chloramphenicol acetyltransferase reporter gene by Wang et al. (1994).
Fig. 3.1. Description of the plasmid components of the mifepristone activatable Ruc-GFP expressing promoter system. The chimeric transcriptional activator plasmid construct (PAP CMV-GL914VPc'SV) encodes a hybrid protein consisting of a mutated human progesterone binding protein fused to the carboxyl terminus of a yeast GAL4-DNA binding domain (GAL4-DBD) and to the amino terminus of a viral transcription trans-activator (VP16). This expression cassette is under the control of a CMV promoter. The synthetic promoter in the reporter plasmid (p17×4-TATA-ruc-gfp) consists of four GAL4 upstream response elements and a TATA box, controlling the expression of the ruc-gfp reporter gene. Ruc-GFP expression from the reporter plasmid can be induced by the chimeric activator protein in the presence of the inducer drug mifepristone.
Chimeric activator plasmid (PAP CMV-GL914VPc'SV):

![Chimeric activator plasmid diagram]

Reporter plasmid (p17×4-TATA-ruc-gfp):

![Reporter plasmid diagram]
Fig. 3.2. Comparison of luciferase expression levels in cells transformed with both plasmids of the inducible promoter system or with the reporter plasmid alone. Approximately $5 \times 10^6$ COS cells were seeded onto each of the three 10-cm plates the night before transformation. The cells in two plates were each co-transformed with 9 µg of p17×4-TATA-ruc-gfp and 9 µg of PAP CMV-GL914VPC’sV plasmid DNAs. The cells in the third plate were transformed with 9 µg of p17×4-TATA-ruc-gfp DNA alone as the control. The cells were cultured at 37 °C for 24 hours followed by the addition of mifepristone, to a final concentration of 250 nM, to one of the two plates of cells that have been transformed with both plasmids, or to the cells that have been transformed with the reporter plasmid alone. Thirty-six hours after mifepristone addition, the cells from all three plates were tested for luciferase expression in the presence of coelenterazine (1 µg / 10 ml plate) as substrate. Light emission from cells were reported as counts/sec.
transformed with reporter plasmid alone

0 nM

250 nM

[mifepristone]
The levels of luciferase gene expression obtained from the inducible vector system were also compared with data obtained from experiments using a strong constitutive promoter, such as the chicken β actin promoter with CMV enhancer, linked to the ruc-gfp marker gene cassette carried on the pCX-ruc-gfp plasmid DNA in COS cells. In spite of an approximately 28-fold induction, the luciferase signal from the inducible system was approximately 4-fold less intense than that emitted from the β actin promoter driven ruc-gfp fusion gene construct (data not shown). This finding was not surprising, since the chicken β actin promoter was found to be the strongest promoter in COS cells and showed the highest levels of constitutive Ruc-GFP expression in earlier experiments (data not shown).

Mifepristone-induced GFP expression of ruc-gfp fusion cassette from transiently transformed COS cells was also monitored using a fluorescent UV microscope (Fig. 3.3). Approximately 18% of the cells exhibited green fluorescence upon addition of mifepristone to the growth medium at a final concentration of 250 nM. On the other hand, virtually no GFP signal was observed in control cells in the absence of the mifepristone inducer drug. The percentage (~18%) of GFP positive cells transiently transformed with the inducible vector system was markedly lower than the percentage (~30%) of GFP-positive cells transiently transformed with an equimolar amount of marker gene plasmids using pCX-ruc-gfp with the chicken β-actin promoter. Since we used transient DNA transformation procedures, we only followed the induction levels to a maximum of 38 hours after introduction of mifepristone. Therefore, the percentage of GFP-positive cells may be further increased by extending the induction
Fig. 3.3. Comparison of GFP expression in COS cells transformed with both plasmids in the presence or absence of mifepristone induction. Approximately 5 \times 10^6 COS cells were seeded on two 10-cm culture dishes. On the second day, the cells were transiently cotransfected with 40 \mu g of p17\times4-TATA-ruc-gfp and 2 \mu g of PAP CMV-GL914VPC'SV. Twenty-four hours following transfection, mifepristone was added to a final concentration of 250 nM to one of the two plates for transcriptional induction (A). No mifepristone was added to the control plate (B). An additional 24 hours later, the cells were visualized under a Zeiss fluorescence microscope to observe GFP expression.
time, or by repetitive addition of mifepristone, to allow enhanced transcriptional activation.

*The Activation of the Inducible Promoter Driven ruc-gfp Fusion Gene Expression is Strictly Dependent on the Presence of the Chimeric Transcription Factor-Encoding Plasmid DNA*

Since the chimeric transcriptional activator is under the control of a constitutive CMV promoter, it is important to verify the expression levels of Ruc-GFP driven by the inducible promoter without the addition of mifepristone to obtain the basal levels of the inducible promoter. To determine the basal expression levels, COS cells were seeded 24 hours before DNA transformation. Different ratios of the transcriptional activator and Ruc-GFP reporter plasmids were cointroduced into the cells (Fig. 3.4). The concentration of mifepristone (final concentration of 250 nM) was kept constant. After analyzing the transformation results, it became evident that by limiting the amount of transcriptional activator encoding plasmid DNA, the basal expression from the inducible promoter could be kept at a relatively low level. A reporter plasmid to activator plasmid DNA ratio ranging from 20:1 to 100:1, yielded the most optimal induction levels while maintaining a basal level of leakiness at a minimum. The addition of the chimeric activator and reporter plasmid DNA in a 1:1 ratio resulted in higher basal levels and therefore diminished the induced activation of transcription. These results were repeatable in several experiments (Fig. 3.5). Not surprisingly, when the reporter and activator plasmids were used in a ratio of 500:1, the basal level of gene expression was kept at a minimal level, but overall induction levels were also smaller. Interestingly, the presence of an excessive amount of chimeric activator DNA to reporter DNA in a ratio of 20:1 did not result in increased reporter gene
Fig. 3.4. The activation of the inducible promoter driven \( ruc-gfp \) fusion gene expression is dependent on the presence of the chimeric transcriptional activator-encoding plasmid DNA. \( 5 \times 10^5 \) COS cells were aliquot equally and seeded into twelve 3-cm plates 16 hours before DNA transformation. Fixed amounts (2.5 \( \mu \)g) of reporter plasmid (p17\texttimes4-TATA-\( ruc-gfp \)) and various amounts of chimeric activator plasmid (PAP CMV-GL914VPc’SV), as indicated by the numerical ratios, were co-transformed into the cells. For each ratio, cells from one of the two plates were treated with mifepristone (250 nM in final concentration) for 36 hours, while the other plate served as the control.
30000
□ - mifepristone
H + mifepristone
25000
20000
15000
10000
5000
500/140/1 100/1 1/1 20/1
Reporter / inducer plasmid ratio

counts / sec

□ - mifepristone
■ + mifepristone

0 5000 10000 15000 20000 25000 30000

1/20 1/1 20/1 40/1 100/1 500/1

Reporter / inducer plasmid ratio
Fig. 3.5. Effect of mifepristone concentration on promoter activation determined by luciferase assays. (A) Approximately $5 \times 10^6$ COS cells were seeded onto each of the nine 10-cm culture dishes. After 24 hours of cell growth, the cells in each plate were cotransformed with 9 μg of p17×4-TATA-ruc-gfp and 9 μg of PAP CMV-GL914VPC'SV plasmid DNAs. Following further culture for 24 hours, increasing amounts of mifepristone (125, 250, 500, 1000, 4000, 16000, 32000, and 64000 μM in final concentration) were added to the cells to trigger transcriptional activation. Thirty-six hours after mifepristone addition, cells were trypsinized and transferred to 12-well titer plates. One ml of luciferase assay buffer containing 1 μg of coelenterazine was added to each well. Luciferase activity was recorded indirectly as relative light units or counts per second based on light emitting chemiluminescence reactions. (B) A similar mifepristone dose response experiment as shown in (A) except different ratios of chimeric transcriptional activator (2 μg) and reporter (40 μg) plasmid DNAs (i.e. ratio of 1:20) were used.
A. 

![Graph A](image1)

B. 

![Graph B](image2)
expression. This may be the result of saturating the supply of the inducible promoter-reporter gene construct with chimeric transcriptional activator proteins. Further, the expression of Ruc-GFP marker, in cells transformed with reporter plasmid DNA alone, was at same level with or without the addition of mifepristone. This finding suggests that non-specific binding of other transcription factors to the inducible promoter region occurred without the inducer drug (data not shown). Therefore, the activation of reporter gene transcription by the chimeric activator protein is strictly mifepristone-dependent.

**Determination of Mifepristone Concentration Required for Maximal Inducible Promoter Activation**

We set out to determine the optimal concentration of mifepristone that will allow the highest level of activated gene expression. Cells transiently transformed with chimeric activator plasmid DNA and reporter DNA were treated with increasing concentrations of mifepristone (Fig. 3.5). By comparing data from several experiments, we consistently observed the highest levels of reporter gene expression in the presence of 250 nM mifepristone. Based on these findings, we continued to use a 250 nM concentration of mifepristone in all subsequent experiments.

**Kinetics of the Inducible System**

We further examined the time course of mifepristone required for activation of the inducible promoter driven Ruc-GFP expression in cell cultures, by measuring luciferase based photon emissions after the addition of the substrate coelenterazine (Fig. 3.6). Activation of Ruc-GFP expression was evident just three hours after exposure of cell cultures, transformed with both activator and reporter plasmids, to mifepristone. Following the treatment with mifepristone, the activated gene
Fig. 3.6. Determination of mifepristone promoter activation in real time. COS cells from two 10-cm plates (0.6 × 10^7 each plate) were transformed with 60 μg of p17×4-TATA-ruc-gfp and 3 μg of PAP CMV-GL914VPC’SV plasmid DNAs using the calcium phosphate precipitation method. Twenty-four hours after transformation, the cells were aliquot onto twenty-six 3-cm plates. Mifepristone was added by gently mixing into the media of half the plates (13) to start induction. The other half numbers of plates were used as no-induction controls for each time point taken. At each time point, two plates (one with and one without mifepristone induction) were withdrawn from the incubator, and the luciferase activity was determined (panel A). Panel B shows the photon collection image from the light emitting cells in two 3-cm plates measured at the 38-hour induction time point using the Nightowl Low Light Imager. One half μg of coelenterazine was used for luciferase assay in each 3-cm plate.
A.  

![Graph showing counts/sec over hours of mifepristone induction](graph.png)

B.  

![Image showing cell culture at 38 hours](image.png)

@ 38 hours
expression occurred over a linear range during the 17-hour exposure time. Seventeen hours after mifepristone addition, the luciferase activity began to plateau, although the light signal did continue to increase when measurements were made up to 38 hours. In contrast, in the absence of mifepristone, cells containing both plasmid DNAs exhibited only basal levels of luciferase expression throughout the experiment. Therefore, the induction of transcriptional activation in the presence of the inducer mifepristone worked in a time-dependent fashion.

**Discussion**

This paper describes the use of a promoter activation system, which is a three-component gene expression system that is exogenously regulatable. This system is functional both in mammalian cell cultures and in live animals (data not shown). The essential feature of these experiments is the use of a ruc-gfp fusion gene construct linked to the inducible promoter that enables us to monitor the kinetics of the exogenously added, inducer drug-regulated, gene expression process through noninvasive procedures, i.e. by monitoring luciferase gene expression using a low-light video camera or GFP gene expression in individual cells based on fluorescence. Besides allowing noninvasive visualization, the second advantage of the Ruc-GFP marker protein is that the dual labeling allows gene expression to be quantitatively compared using luciferase, based on photon emission, and allows gene expression in individual cells to be localized based on GFP fluorescence. A third advantage of the Ruc-GFP based promoter activation system is that one can quantitatively analyze expression of other target-genes-of-interest indirectly based on Ruc-GFP markers. In order to express the target protein and the Ruc-GFP fusion protein in the same cell in
close to equimolar amounts, the expression of both genes must be activated by the same promoter. This can be achieved by using one inducible promoter linked to the target gene followed by an internal ribosomal entry site (IRES) linked to the \textit{ruc-gfp} fusion gene. In this way, activation of target gene expression can be indirectly monitored based on downstream Ruc-GFP marker protein expression. In addition to IRES, a bi-directional promoter, the tetracycline-regulated bi-directional promoter in the pBI cloning vector (Clontech, Palo Alto, CA), may be used to simultaneously and quantitatively express the target gene and the \textit{ruc-gfp} fusion gene in the same cell.

An alternative solution to using IRES and a bi-directional promoter is to construct a second fusion gene by fusing the target gene in frame to either luciferase or GFP cDNA or to both as long as such a fusion event does not interfere with the original function of any of the fusion partners. Since there is a one-to-one ratio between the two genes, expression from the target gene can be at least semiquantitatively estimated based on luciferase light emission or by determining the GFP fluorescence quantitatively. The obvious disadvantage of using the fusion strategy is that for each gene-of-interest, a new fusion gene needs to be constructed, which is time consuming. However, once such a fusion is completed and characterized, the gene constructs could have great potential in functional studies.

The feasibility of using IRES, enhanced GFP, and fluorescence activated cell sorting (FASC) for systematically characterizing exogenous gene functions in mammalian cells has been demonstrated very recently by Garton et al. (2002).

Similarly, with the aid of IRES, a bi-directional promoter, and light-emitting proteins, a close to uniform population of transformed cells with low basal but high induction
levels of gene expression can be isolated based on luciferase light emission and based on the GFP signal with the use of FACS. Without the help of light-emitting proteins, isolation of cell clones with high levels of induced target gene expression is difficult. To maintain both the reporter and the chimeric transcriptional activator plasmid constructs in the mammalian cell genome, different antibiotic selection genes need to be included in the two plasmid DNAs. After cotransformation, cells are initially grown in medium containing both antibiotics for two weeks. Cells that exhibited basal levels of light emission indistinguishable from untransformed cells are collected and propagated for three days without the addition of antibiotics. Then mifepristone is added to the medium. After continuous culture for another 36 hours, the cell population is analyzed with FACS for identification of cells with the highest level of activated Ruc-GFP expression. These cells are collected and grown in the absence of antibiotics. Cells isolated by the detectable marker based enrichment procedures are expected to be genetically stable for months, which will be valuable in cell implantation and *in vivo* therapeutic uses.

Currently, the monitoring of GFP signal in live animals is limited to structures located close to the surface. This is due to the limited penetration of the excitation wavelengths that are necessary to induce green fluorescence and due to the strong scattering of fluorescence emission by the tissues. Successful visualization and localization of GFP signal originating from transformed tissues in nude mice at a depth of only 2.2 mm has been reported (Yang et al. 2000). Novel techniques, such as two-photon laser scanning microscopy, may need to be applied to increase the sensitivity and resolution of fluorescence monitoring in cells and in tissues.
A similar two-plasmid-DNA-mediated, steroid-regulated, gene expression system (UAS/TAXI) was constructed by Delort and Capecchi (1996). The principle of the UAS/TAXI system is essentially the same as that of the promoter activation system as described originally by O’Malley and colleagues as the “GeneSwitch” (Wang et al. 1994), with the exception of a few differences. Both systems are based on two plasmids: the chimeric transcriptional activator plasmid which carries the fusion gene of the GAL4 DNA binding domain, the truncated human progesterone receptor hormone binding domain (hPR-HBD), and the herpes simplex virus protein VP16 activation domain which encodes one fusion protein; the reporter plasmid carries a transcriptional activator-dependent promoter-marker gene fusion construct which contains repeats of GAL4 upstream response elements and a minimal synthetic promoter (UAS) linked to the target gene. The differences between the two systems are, in the activator plasmid of the UAS/TAXI system, the hPR-HBD has a deletion of the 35 (rather than 42 as in “GeneSwitch”) carboxyl-terminal amino acids. Further, in the chimeric transcription factor, the VP16 acidic transactivation domain contains residues 415-486 (rather than 411-487 as in “GeneSwitch”). In the reporter plasmid constructs, the differences between the two systems are that there are five (rather than four as in “GeneSwitch”) repeats of GAL4 upstream response elements linked to a minimal promoter sequence from the Drosophila hsp70 gene (rather than the adenovirus major late E1B TATA sequence as in “GeneSwitch”) in the UAS/TAXI system. Also, in the UAS/TAXI reporter plasmid, the GAL4 DNA binding domain spans residues 1-147, in contrast to 1-94 as in the promoter of the “GeneSwitch” reporter plasmid. We have inserted the ruc-gfp marker gene into the
UAS/TAXI system to create the reporter plasmid pUAS-ruc-gfp, and compared its activation in the presence of the chimeric activator plasmid pTAXI and inducer drug mifepristone in transient transformations (Fig. 3.7). Although the inducible gene expression from the UAS/TAXI system was dependent on mifepristone, the activation results we obtained were lower than the activation data with the “GeneSwitch” system. The highest induction levels of Ruc-GFP expression obtained using the UAS/TAXI system in cell culture experiments were merely five fold. In addition, there was a lack of consistency in the levels of gene expression using the UAS/TAXI system from one experiment to the next. We found no apparent optimal concentration of mifepristone that gave the highest level of induction. In animal experiments, we found that UAS/TAXI responded less well to the intraperitoneally delivered mifepristone than when both plasmid DNAs were injected into the limb muscles of nude mice (Fig. 3.7A-c). Based on these comparisons, we concluded that the promoter activation system developed by O’Malley and colleagues (Wang et al. 1994) proved to be more applicable to our gene expression studies.

There may be numerous applications of the mifepristone inducible gene expression system. In gene therapy, constitutive expression of proteins such as hormones and cytokines may not be desirable at all stages of therapeutic applications. An inducible system will therefore provide the investigators and clinicians with the ability to control both the temporal and spatial expression of the therapeutic proteins. Besides controlling the expression of therapeutic proteins, the induction system can also be used to regulate the expression of engineered chimeric proteins such as those encoded by suicide genes, e.g. the artificial death switches (ADS) (MacCorkle et al.
Similar to the activation of other suicide genes such as the Herpes Simplex Virus thymidine kinase gene, activation of ADS leads to cell death. Such suicide genes can be included in gene therapy vectors and delivered to the organism. When the therapy is satisfactory or its continuation is undesired to the host, the treatment can be terminated by activating the expression of ADS proteins in the implanted transformed cells to initiate the pre-programmed suicide process. In addition to its clinical applications, the inducible system can also be enormously valuable in transgenic animal studies. Many functional protein studies in transgenic animals, such as investigating the role of developmentally regulated transcription factors, have proven to be difficult due to perinatal lethality caused by uncontrolled non-physiological over-expression of the gene-of-interest. However, a regulated gene expression system can circumvent such a problem by only activating the expression of a gene after the embryo or fetus reaches a certain developmental stage. Functional studies on transforming growth factor β1 using the mifepristone regulated gene expression system (Wang et al. 1999) have shown the usefulness of such strategies.

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CHAPTER FOUR

MIFEPRISTONE-REGULATED EXPRESSION OF RENILLA LUCIFERASE-AEQUOREA GFP FUSION PROTEIN IN ALGINATE-ENCAPSULATED MAMMALIAN CELLS

Abstract

The mifepristone-regulated transcriptional activation system provides a means to exogenously control gene expression in transformed mammalian cells temporally and spatially. To apply this system successfully in cellular implants into animals for prolonged studies requires the encapsulation of the cells with a biocompatible polymeric device. We used biocompatible calcium alginate/poly-L-lysine (PLL) beads to provide immune protection for cells during cellular implantation. Using Aequorea green fluorescent protein (GFP) as a marker, we monitored the growth and proliferation of encapsulated cells over a long period of time, up to two and a half months. Furthermore, to study regulated gene expression in encapsulated cells, a Renilla luciferase-GFP (ruc-gfp) fusion gene was linked with a synthetic promoter that is inducible in the presence of exogenously added mifepristone. We showed that the ruc-gfp fusion gene expression in encapsulated mammalian cells is regulated in a mifepristone dose-dependent manner. The highest induction by mifepristone occurs at a final concentration of 250 nM. This induction reached a maximal level of approximately 8-9 fold and lasted up to 37 hours after the initial addition of mifepristone. From these results, we conclude that regulated protein expression from alginate-encapsulated cells may provide a useful tool for designing cellular implant-based protein therapy in the future.
Key Words

Microencapsulation · inducible gene expression system · *Renilla* luciferase · *Aequorea* GFP

Introduction

Many metabolic diseases are caused by a deficiency in the production of a key protein. This can be temporarily corrected by injection of the missing protein. However, such recombinant protein therapy can be costly for long-term treatment of the disease. To address this problem, various somatic gene therapy strategies have been developed to allow a sustained delivery of the therapeutic protein (Vervoordeldonk and Tak 2001). These strategies include viral-based transgene delivery, such as recombinant adenovirus (Schneider et al. 2002) and adeno-associated virus (rAAV) (Flotte et al. 1993; Murphy et al. 1997; Snyder 1999; Kawada et al. 2002; Melo et al. 2002), and non-viral-based gene delivery, such as cell transplantation (Grompe 1999; Musgrave et al. 2002). The main disadvantages of virus-based long-term therapy are the associated risks. In fact, it has been reported that long-term delivery of rAAV can be tumorigenic (Donsante et al. 2001). Cell-based therapy, on the other hand, has many advantages over viral-based therapy. These advantages include a much higher carrying capacity of exogenous genetic materials (such as with an artificial chromosome), a lower risk of transgene integration into the host genome, a more stable therapeutic protein production, etc. A major obstacle associated with cell-based therapy however, is the host immune rejection of cellular implants. Genetic modifications of the donor cells have been introduced to reduce host immune rejection (Cozzi et al. 2000; Dorling et al. 2000).
In addition to genetic modifications, a more convenient and cost-effective method is microencapsulation of cellular implants with biocompatible polymers (Uludag et al. 2000). The semipermeable polymer will protect cells from the host immune system, but allow the passage of nutrients, oxygen, metabolic waste, and small therapeutic proteins. Due to the biocompatible nature of the polymers, the recipient is not required to be on immunosuppression medication. In addition to prevention of immune rejection, microcapsules also provide a stable environment, and help to contain the cellular implants in defined locations. The risk of uncontrolled spreading of exogenous cells in the host therefore is greatly reduced. Upon completion of therapy, the cell-containing microcapsules can be conveniently removed.

Biocompatible polymers used for cellular encapsulation can be divided into two major groups: (1) carbohydrate polymers, including alginate (Lim and Sun 1980), agarose (Scheirer et al. 1983), cellulose (Armeanu et al. 2001), glycosaminoglycan (Orgill et al. 1998), and chitosan (Zielinski and Aebischer 1994); and (2) non-carbohydrate polymers, including AN69 (Kessler et al. 1991), 2-hydroxyethyl methacrylate / methyl methacrylate (HEMA-MMA) (Dawson et al. 1987), polyether-sulfone (PES) (Regulier et al. 1998), and poly-acrylonitrile-co-vinyl chloride (Aebischer et al. 1996). Among these, alginate was the first and one of the most widely used polymers for mammalian cell encapsulation. Alginate polymers are composed of guluronic acids and mannuronic acids polymerized by Ca$^{2+}$. After the formation of the alginate core, the polyanionic alginate bead is first coated with a polycationic PLL membrane, and then with an outer layer of alginate. The diameter of the spherical alginate-PLL microcapsules can be set to range from 200 to 2,000
μm. The permeability of the alginate-PLL capsules can be modulated by varying the concentrations of alginate and PLL. Transplantation of alginate-encapsulated cells has helped to treat a variety of metabolic diseases, such as hyperbilirubinemia (Bruni and Chang 1989), type I diabetes (Soon-Shiong 1999), and lysosomal storage deficiency (Ross et al. 2000), either in animal models or in human patients.

In addition to microencapsulation, another possible safety feature of a cell therapy system is that the transgene expression in the cellular implants can be regulated by an inducible promoter, as has been shown by others (Zoltick and Wilson 2001). This allows regulated expression of therapeutic proteins in a time and dose-controlled manner. Unregulated delivery of therapeutic proteins, on the other hand, may backfire and result in adverse metabolic consequences (Al-Hendy et al. 1996). Inducible promoter systems, including the Tet-on system (Saitoh et al. 1998; Hagihara et al. 1999) and the mifepristone system (Serguera et al. 1999) have been introduced in encapsulated cells to control therapeutic gene expression. The mifepristone-regulated promoter system (Wang et al. 1994) has three components: (a) a plasmid carrying the expression cassette encoding a chimeric transcriptional activator under the control of a constitutive promoter; (b) a plasmid carrying a synthetic promoter derived from the yeast GAL4-binding sequence linked to a therapeutic gene or a reporter gene; and (c) the inducer drug, mifepristone. The presence of the inducer drug allows binding of the chimeric transcription factor-mifepristone complex to the inducible promoter to activate transcription. Using this system, controlled gene-transfer studies have been successfully conducted in cell cultures and in live animals (Burcin et al. 1999; Osterwalder et al. 2001; Terada et al.
2001; Draghia-Akli et al. 2002). Although regulated gene expression from encapsulated cells has been reported, no experiments have been conducted to study mifepristone-induced gene expression in alginate-encapsulated cells. In this paper, we utilized Renilla luciferase light emission and GFP fluorescence to investigate real-time gene expression in encapsulated cells. The kinetics of mifepristone dose-dependent induction of the ruc-gfp fusion gene expression in encapsulated cells were also examined in real time.

**Materials and Methods**

**Plasmid Vector**

The construction of the reporter plasmid p17×4-TATA-ruc-gfp was described previously (Yu et al. 2001). The chimeric transcriptional activator plasmid (PAP CMV-GL914VPc’SV) was a gift from Dr. Y. Wang (Department of Cell Biology, Baylor College of Medicine, Houston, TX). A retroviral shuttle vector, pLEIN, carrying an EGFP expression cassette under the control of a constitutive 5’ LTR promoter, was obtained from Clontech (Palo Alto, CA).

**Tumor Cell Lines**

COS cells (monkey kidney cells transformed by SV40 virus; ATCC, Rockville, MD) were cultured in DMEM medium (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 10% (v/v) FBS, 2 mM glutamine, and 1× penicillin/streptomycin. HT1080 human fibrosarcoma cells (ATCC, Manassas, VA) were cultured in F12 minimal essential medium (Cellgro, Mediatech, Inc., Herndon, VA) supplemented with 10% FBS and 1× penicillin/streptomycin.
Establishment of a Stably Transformed HT1080 Cell Line by Retroviral Transduction

PT67 packaging cells (Clontech, Palo Alto, CA) were cultured in DMEM medium supplemented with 10% (v/v) FBS. At 70% confluence, PT67 cells were transformed with the pLEIN vector using the calcium phosphate precipitation method (Profection Mammalian Transfection Systems, Promega, Madison, WI). Twelve hours after the initiation of transformation, fresh medium was added. Retroviral supernatant fluid collected from PT67 cells 48 hours post transformation was filtered through a 0.45 μm filter and added to target HT1080 cells. In addition, polybrene was added to a final concentration of 4 μg/ml. The medium was replaced after 24 hours, and the cells were selected with G418 at 400 μg/ml, increased stepwise over time to 1200 μg/ml in order to select stably transformed cells.

Alginate Encapsulation of Mammalian Cells

Alginate beads were prepared using an Inotech Encapsulator (Dottikon, Switzerland) with a vibrating nozzle. A low viscosity alginate sodium salt (Sigma-Aldrich, St. Louis, MO) was used to produce beads. A detailed description of the Encapsulator and protocols for alginate microencapsulation can be found elsewhere (Serp et al. 2000). In brief, to produce <50 ml of encapsulated cells, 13 ml of 1% alginate solution (1% sodium alginate, 10 mM MOPS, 0.85% NaCl) were mixed with 9.5 ml of cells suspended in MOPS washing buffer (10 mM MOPS, 0.85% NaCl). Droplets of cells were dispersed into 225 ml (10 times the volume of alginate/cell mixture) of Ca²⁺ polymerization solution (10 mM MOPS, 100 mM CaCl₂). After
polymerization for 10 min, the beads were transferred into 50 ml of 0.05% PLL (prepared with MOPS washing buffer) and stirred for 10 min. Then the beads were washed with 100 ml of MOPS washing buffer for 1 min, and again with 150 ml of MOPS washing buffer for another 5 min. The excess washing buffer was aspirated. Then 50 ml of 0.03% alginate solution (0.03% sodium alginate, 10 mM MOPS, 0.85% NaCl) were added to the beads and incubated for 5 min with stirring. The two-step washes with MOPS were repeated again and the excess washing buffer was aspirated. Fresh supplemented DMEM culture medium was added to the beads, which were then aliquoted into 6-well titer plates, and kept in the tissue culture incubator at 37 °C, 100% humidity, and 5% CO2.

**Determination of Mifepristone-Dose Dependency and Kinetics of Inducible Ruc-GFP Expression in Encapsulated COS Cells**

COS cells (5 × 10^6) were seeded in three 10 cm culture dishes and grown to ~70% confluence in supplemented DMEM medium. The cells were then transiently transformed with both plasmids of the inducible promoter system using the calcium phosphate precipitation method. To transform cells in one 10 cm dish, 40 µg of reporter plasmid p17×4-TATA-ruc-gfp and 2 µg of the chimeric transcriptional activator plasmid PAP CMV-GL914VPc’SV were used. Fifteen hours after transformation, the cells were harvested and encapsulated in alginate-PLL beads. Twelve hours after encapsulation, mifepristone was added to the aliquot of the encapsulated cells to a final concentration of 0, 125, 250, 500, 1000, 4000, 16000, 32000, or 64000 nM to initiate induction of gene expression. Twenty-four hours after the addition of mifepristone, the encapsulated cells were assayed for luciferase.
activity and monitored for GFP fluorescence to determine mifepristone dose-dependence at the above molar concentrations.

To determine the kinetics of the inducible promoter system in encapsulated cells, a fixed final concentration of 250 nM of mifepristone was used to induce transcriptional activation. For each experimental culture, one culture without mifepristone was used as a negative control. The cell transformation and encapsulation steps were carried out as described above, except that the luciferase activity and GFP fluorescence in encapsulated cells were examined at 0, 6, 9, 12, 15, 18, 21, 24, 27, 28, 31, and 34 hours after the addition of mifepristone.

**Luciferase Assays**

The culture medium in each well was removed by aspiration and replaced with 1 ml of luciferase assay buffer (0.5 M NaCl; 1 mM EDTA; and 0.1 M potassium phosphate, pH7.4) to which was added 1 µg of coelenterazine substrate from a 0.5 µg/µl methanol stock solution. The luciferase light emission from alginate-encapsulated cells was determined using an Argus100 Low Light Video Camera (Hamamatsu Instruments, Hamamatsu, Japan) and recorded with Image-Pro Plus software (MediaCybernetics, Silver Spring, MD).

**Fluorescence Imaging**

Determination of GFP expression in encapsulated cells was performed using either a Leica MZ8 stereo fluorescence microscope or a Zeiss fluorescence microscope, both equipped with a mercury lamp power supply and a GFP filter (excitation at 470 nm). For the Leica microscope, images were captured using a SONY DKC-5000 3 CCD digital camera and processed with the Adobe Photoshop
5.0 software (Adobe Systems, San Jose, CA). For the Zeiss microscope, images were captured with a conventional camera using 800° ISO color film.

Results

Encapsulation of Mammalian Cells in Alginate Beads

We first evaluated alginate-based mammalian cell encapsulation using HT1080 fibrosarcoma cells stably transformed with a retrovirus-encoded, enhanced gfp construct. The number of cells encapsulated in the alginate beads can be manipulated to be in the range of <50 to >500 cells per bead (data not shown). Typically, we encapsulated 50-100 cells per bead. Initially, the cells were distributed throughout the alginate beads immediately after encapsulation (Fig. 4.1A). Over time, small clusters of green fluorescent cells developed within the beads. During the next two weeks, some clusters of cells predominantly outgrew other groups of cells (Fig. 1B), and formed large green fluorescent clusters. In alginate beads with a diameter of >500 μm, the green fluorescent cells tended to cluster in spindle-shaped islands (Fig. 4.1B-C). In alginate beads with a diameter <200 μm, often the entire bead was filled with a spheroid of green fluorescent cells (Fig. 4.2).

The alginate beads prepared in our experiments consisted of a calcium alginate core, a PLL capsule, and an outer membrane of alginate. Without the PLL capsule, the alginate beads were observed to break apart, starting at two and a half weeks after encapsulation. Green fluorescent cells were observed to grow on the bottom of the culture dish after falling out of the broken beads (data not shown). With the PLL capsule, the alginate beads were much more stable. In most cases, very few cells were released from the beads.
Fig. 4.1. Alginate-PLL microencapsulation of HT1080 human fibrosarcoma cells stably transformed with a GFP-encoding retroviral construct. Individual green fluorescent cells were seen dispersed in the beads (A) immediately after encapsulation when examined under a Leica fluorescence stereomicroscope equipped with a mercury lamp and a GFP filter set. (B) Nine days after encapsulation, clusters of green fluorescent cells emerged and outgrew the other originally encapsulated cells as seen under the Leica microscope. (C) Twelve days post-encapsulation, multiple large clusters of cells exhibiting green fluorescence were observed in the beads when examined under a Zeiss fluorescence microscope. The sizes of these cell clusters eventually reached a steady state. The encapsulated cells exhibited visible green fluorescence for more than two and a half months (data not shown). The images were taken under blue excitation light for green fluorescence and also in the presence of a low level of background illumination to mark the location of the beads. (Bars = 2 mm).
Fig. 4.2. Encapsulated cell viability analysis based on the intensity of GFP fluorescence. HT1080 human fibrosarcoma cells were stably transformed with a GFP encoding retroviral construct. Fifteen days after the beginning of encapsulation, cells losing viability were shown to have dramatically decreased fluorescence intensity (solid arrow), whereas strong fluorescence emission was observed in viable cells when GFP was still actively expressed (dashed arrow; Bar = 2 mm).
**In vivo Viability of Alginate-Encapsulated Mammalian Cells**

We investigated the viability of mammalian cells in alginate-encapsulated beads based on GFP fluorescence. Two weeks after encapsulation, we observed that some large clusters of encapsulated cells were no longer fluorescent (as indicated by the solid arrow in Fig. 4.2), whereas other cells remained strongly fluorescent (as indicated by the dashed arrow in Fig. 4.2). Since the HT1080 fibrosarcoma cells were stably transformed and were selected from a single clone, all of the cells should have the same copy numbers of the gfp construct on their chromosome(s). Therefore, it is reasonable to correlate weaker green fluorescence with lower cellular viability.

We found that, even after two to three months of culturing, the majority of the encapsulated cells were fluorescent and therefore putatively viable (data not shown).

**Mifepristone-Induced Ruc-GFP Reporter Expression in Encapsulated Cells**

We evaluated the inducible gene expression system in encapsulated cells. COS cells were transformed with both the reporter (ruc-gfp) and the chimeric transcriptional activator plasmid constructs. The transformed cells were then encapsulated in alginate-PLL beads. To obtain information about the mifepristone-dose dependency of the inducible system, we first treated the encapsulated cells with stepwise increasing concentrations of mifepristone. Luciferase-based photon emission in the presence of the substrate coelenterazine was recorded to quantitatively analyze the levels of activated gene expression. We found in our dose dependency studies that a final concentration of 250 nM mifepristone resulted in the highest level of induction (Fig. 4.3). In general, an approximately 7-8-fold induction was seen when mifepristone was given in a final concentration between 250 nM and
Fig. 4.3. Mifepristone dose-dependent activation of luciferase activity in encapsulated cells. COS cells were transformed with both the reporter and the chimeric transcriptional activator plasmids of the inducible promoter system. Fifteen hours after transformation, the cells were encapsulated in alginate-PLL beads. Various amounts of mifepristone were added to the beads twelve hours after encapsulation to initiate the induction of \textit{ruc-gfp} gene expression. Twenty-four hours later, the encapsulated cells were analyzed for luciferase activity in the presence of the substrate coelenterazine. A final concentration of 250 nM of mifepristone resulted in the highest level of induced luciferase activity at approximately 8 fold.
1000 nM. Excessive amounts of the inducer drug tended to inhibit rather than promote gene activation. These data were very similar to what we had previously reported in the inducible system in cultured mammalian cells (Yu et al. 2001).

In addition to dose-dependency, we also analyzed the kinetics of the inducible gene expression system in encapsulated cells. Since we noted that a final concentration of 250 nM of the inducer drug would result in the highest level of induction, we therefore used that concentration of drug to determine the kinetics of induced gene expression. Induced luciferase activity was shown to increase in a linear fashion during the first twenty-four hours after the addition of mifepristone, but the rate slowed somewhat thereafter (Fig. 4.4A). Thirty-seven hours after the initial induction, an approximately 8-9-fold induction of gene expression was observed (Fig. 4.4A-B). In the absence of the inducer drug, there was a <1-fold increase in luciferase activity, which could be caused by non-specific binding of endogenous transcription factors to the synthetic promoter. At the 37 h induction time point, we qualitatively assayed GFP fluorescence and found weak induced GFP signal in small clusters of cells, but no GFP fluorescence in the negative control cells without mifepristone addition (data not shown).

Discussion

In the present study, we have demonstrated that the long-term viability of alginate-PLL encapsulated mammalian cells can be easily monitored in a non-invasive manner based on GFP fluorescence. We also showed that using a light-emitting fusion gene (ruc-gfp) construct, mifepristone-activated gene expression in
Fig. 4.4. Kinetic analysis of the inducible promoter system in encapsulated cells. COS cells were transformed with both the reporter and the chimeric transcriptional activator plasmids of the inducible promoter system, as was done in the mifepristone dose-dependent experiment. Fifteen hours after transformation, the cells were encapsulated in alginate-PLL beads. A final concentration of 250 nM of mifepristone was used to initiate transcriptional activation twelve hours after the beginning of encapsulation. The control experiments were carried out identically except there was no addition of the inducer drug mifepristone. The kinetics of the induced gene expression in encapsulated cells were investigated based on assaying luciferase activity at different time points (Panel A). Quantitative analysis of luciferase/coelenterazine photon emission was performed using a Hamamatsu Argus100 Low Light Imager. Panel B shows a pseudo-colored image of total photon emission collected over 1 min using experimental and control samples at the 37-hour time point. The left side shows the strong luminescence activity in the presence of mifepristone induction, and the dashed circle on the right indicates the margin of the well of the titer plate where the parallel control experiment was carried out in the absence of mifepristone.
microencapsulated cells could be quantitatively and qualitatively examined in real-time.

To develop a successful gene therapy protocol, one needs to satisfy the following minimal requirements: a long-term delivery of the therapeutic protein; a regulatable therapeutic protein level at desired times; and a minimization of immune rejection of the gene therapy delivery system. In our present study, we focused on developing a cell therapy system that could potentially address some of the above requirements, namely; (1) use of an artificially inducible promoter system that can be activated by the presence of an exogenously added drug, and (2) the encapsulation in biocompatible alginate polymers of the mammalian cells carrying the therapeutic genes.

In our experiments, we found that the long-term viability of encapsulated cells in alginate beads could be determined by imaging GFP fluorescence in real time. This non-destructive method is preferable to other methods of determining cell viability such as conventional propidium iodide staining, trypan blue exclusion, or calcein fluorescence labeling (Live/Dead TM Viability/Cytotoxicity Assay, Molecular Probes, Eugene, OR). In particular, an advantage of the GFP-based method over calcein fluorescence labeling is that the later method requires the exogenous delivery of calcein probes for fluorescence, whereas the detection of GFP activity is based on the fluorescence emission of an endogenous protein. It has been reported that cells encapsulated in the central core of the beads tend to be less labeled using calcein fluorescence (Zhou et al. 1998), which may result in false conclusions. It is important to point out that GFP is rather stable with a half-life of approximately 26 hours, this
may limit its use as a marker for short-term viability analysis, but long-term cell survival can be monitored based on GFP expression. It has been demonstrated that GFP fluorescent light extinction can be used as an indicator of cell apoptosis and necrosis (Steff et al. 2001; Strebel et al. 2001). GFP-based cell viability assays have been developed to facilitate cryobiological applications (Elliott et al. 2000). Using GFP as a viability biomarker of encapsulated cells as shown in our experiments clearly demonstrates its powerful application potential. A word of caution, the GFP-based cell viability assay can only be used to evaluate cells that have been stably transformed by a gfp construct and which are from the same clone. In fact, even in stably transformed cells, part of or the entire integrated exogenous construct can be recombined out of the chromosome to form extrachromosomal arrays. Loss of extrachromosomal arrays may also cause the loss of GFP fluorescence. In our experiments, we applied constant G418 selection pressure to ensure the stability of the construct. In transient transformations, on the other hand, a lower level of fluorescence signal could be caused by variation in copy numbers of the transformed gfp construct, which therefore may lead to false conclusions.

We found that the mifepristone-induced transcriptional activation in encapsulated cells is less robust than free cells in tissue cultures. The highest level of induction based on monitoring luciferase activity is approximately 8-9 fold, which is considerably lower than the ~30 fold induction seen in free cell cultures (Yu et al. 2001). This could be explained by the limited access of mifepristone to the encapsulated cells. Since we used transiently transformed cells in our experiments, we did not test the induced luciferase activity beyond 37 hours after mifepristone
addition. In the future, a cell line stably transformed with both the reporter and the chimeric activator plasmids of the inducible system needs to be established. Using such a cell line, a higher level of induced transgene expression may be achieved when longer exposure times and multiple doses of mifepristone are given.

Although the alginate-based cell encapsulation is considered to be biocompatible, certain components of the alginate beads, such as mannanuronic acid polymers, may provoke a pro-inflammatory response by host monocytes (Flo et al. 2000; King et al. 2001). The recruitment and growth of pericapsular macrophages significantly limits the survival of encapsulated cells in clinical transplantation (De Vos et al. 1993). To address this problem, 15-deoxyspergualin, a macrophage inhibitor, has been used to prolong the survival of encapsulated grafts against macrophages (Hsu et al. 1999). The generation of antibodies against capsules with a high content of mannanuronic acid has also been reported (Kulseng et al. 1999). The guluronic acid-rich alginate, on the other hand, was shown to be much less capable of triggering an antibody response (Kulseng et al. 1999), and therefore is more stable following implantation.

In the future, to mimic the design of polymer-cell-encapsulation-based artificial organs, we plan to analyze the secretion of Ruc-GFP fusion protein from encapsulated cells. A DNA sequence encoding a signal peptide will be inserted in frame at the 5' end of ruc-gfp fusion gene. Real-time protein secretion analysis from mammalian cells based on luciferase has been documented (Liu et al. 2000). Similar experiments will be performed using encapsulated cells. Furthermore, the mifepristone-induced gene expression from encapsulated cells will be ultimately evaluated in animal models. For example, the alginate-encapsulated cells will be
implanted in the subarachnoid space to test mifepristone-induced endorphin secretion into cerebral spinal fluid for potential pain-control therapy, as has been demonstrated in other similar systems (Saitoh et al. 1995; Ishii et al. 2000). Encapsulated cells could also be implanted into the intraperitoneal space to assay the release of therapeutic proteins into the blood for treatment of metabolic diseases, such as obesity which has been treated in animal models by delivering regulated levels of leptin protein (Meinders et al. 1996; Muzzin et al. 1996). These experiments, if successful will help to advance the clinical application of encapsulated cell therapy.
CHAPTER FIVE
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

In this study, we described for the first time the monitoring of regulated gene expression from a mifepristone-induced promoter activation system in real time. Non-invasive imaging of gene expression was possible using the light-emitting Ruc-GFP fusion protein. This allowed both qualitative and quantitative measurement of gene activities. We described the construction of a reporter plasmid that carries a mifepristone-regulated synthetic promoter linked to a ruc-gfp fusion gene encoding a fusion protein with both Renilla luciferase and GFP activities. This plasmid was cotransformed with the chimeric transcriptional activator plasmid into COS cells. The induction of ruc-gfp fusion gene expression was monitored following the exogenous addition of mifepristone. We showed that there was nearly a 30-fold induced increase of ruc-gfp marker gene expression in cell cultures. We showed that the mifepristone-activation of gene expression is dose-dependent and is strictly regulated by the chimeric transcriptional activator protein.

The significance of our findings is that the establishment of this rapid and noninvasive method of monitoring inducible gene expression in cell cultures will aid investigators in developing imaging procedures to continuously visualize gene function in developing organisms in real time. Also our system will facilitate the in vivo localization of therapeutic proteins in animal models and perhaps eventually in human subjects throughout the course of a gene therapeutic modality.
We also, for the first time, demonstrated and quantified mifepristone-controlled protein expression in alginate-microencapsulated cells utilizing the Ruc-GFP fusion protein. In addition, we demonstrated that the long-term viability of alginate-PLL encapsulated mammalian cells can be easily monitored in a non-invasive manner based on GFP fluorescence.

**Future Directions**

The combined application potential of the mifepristone-induced gene expression activation system and the non-invasive monitoring of induced gene expression is enormous. In gene therapy, the mifepristone-regulated system may provide the investigator and clinician with a means to control the expression of the therapeutic proteins, such as hormones and cytokines, in levels similar to physiological conditions. By directly linking the expression of the light-emitting Ruc-GFP fusion protein with the expression of target therapeutic proteins, the *in vivo* levels of the inducibly expressed therapeutic proteins can be modulated in real-time. Besides controlling the expression of therapeutic proteins, the induction system can also be used to control the expression of engineered chimeric proteins such as those from suicide genes, for example, the artificial death switches (MacCorkle et al. 1998). These inducible suicide gene systems may provide another level of safety in future clinical applications of gene therapy. The termination of therapeutic protein expression can be indirectly monitored in real time based on Ruc-GFP light extinction. In addition to its clinical applications, the inducible system can also be enormously valuable in basic transgenic animal research. Since mifepristone-dependent gene expression can be induced in either transgenic embryos or adult...
animals \textit{in vivo}, functional studies of target proteins can be conducted easily with the help of the light-emitting Ruc-GFP fusion protein upon reaching specific stages of development or aging. Finally, it would be of great importance to design an efficient inducible protein secretion system in microencapsulated cells. The induced target protein secretion could be monitored and modulated in real-time with the aid of light-emitting proteins. A better understanding of inducible expression and the secretion of therapeutic proteins by encapsulated cells will facilitate future clinical applications. Ultimately, such microencapsulation-based recombinant cell therapy could even be developed to mimic biocompatible artificial organs.
BIBLIOGRAPHY


Overexpression of matrix metalloproteinases leads to lethality in transgenic *Xenopus laevis*: implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev Dyn 221:37-47


A nuclear GFP that marks nuclei in living *Drosophila* embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. Dev Biol 170:726-729

Microencapsulation of CHO cells in a hydroxyethyl methacrylate-methyl methacrylate copolymer. Biomaterials 8:360-366


The genetic approach to the Epstein-Barr virus: from basic virology to gene therapy. Mol Pathol 53:270-279

A molecular switch to control expression of genes *in vivo*. Hum Gene Ther 7:809-820

Kinetics of the firefly luciferase catalyzed reactions. Biochemistry 13:921-925


Evaluation of hemofiltration with different AN 69 membrane devices using a discontinuous flow-single needle system. Int J Artif Organs 5:87-91

Factors influencing the adequacy of microencapsulation of rat pancreatic islets. Transplantation 62:888-893


Falk MM, Lauf U. (2001) High resolution, fluorescence deconvolution microscopy and tagging with the autofluorescent tracers CFP, GFP, and YFP to study the structural composition of gap junctions in living cells. Microsc Res Tech 52:251-262


140


148


MacDonald A. (2001) Improving tolerability of immunosuppressive regimens. Transplantation 72:S105-S112


Selleck MA, Stern CD. (1991) Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. Development 112:615-626


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APPENDIX

Lists of Buffers and Solutions

Buffer for Agarose Gel Electrophoresis

1 × Tris acetate buffer (TAE): 40 mM Tris-acetate, 1 mM EDTA

6 × Gel-loading buffer: 0.025% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose in water

Bacteria Culture Medium

Luria-Bertani (LB) medium: 1% Bacto tryptone, 0.5% yeast extract, and 1% NaCl; autoclave to sterilize

LB agar plate: LB medium with 1.5% granulated agar

Brain heart infusion (BHI) medium: 3.7% BHI.

Note: All media were sterilized by autoclave at 10 lb/in² and 220 °F for 20 minutes.

Reagents for Preparation of Competent E. coli (Heat Shock Method)

SOB medium: 2% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, and 10 mM MgSO₄; autoclave to sterilize

SOC medium: SOB medium with 20 mM glucose

TB washing buffer: 10 mM Pipes, 55 mM MnCl₂, 15 mM CaCl₂, and 250 mM KCl.

Note: pH is adjusted to 6.7 before the addition of MnCl₂.

Reagents for Preparation of Competent E. coli (Electroporation Method)

SOB medium

SOC medium

HEPES buffer: 1mM, pH 7.0

Glycerol washing solution: 20%
Glycerol storage solution: 10%

**Plasmid Purification Solutions (Alkaline Lysis Method)**

Solution I: 50 mM glucose, 25 mM Tris-Cl, pH 8.0, and 10 mM EDTA
Solution II: 0.2 mM NaOH and 1% SDS (prepared fresh before use)
Solution III: 3 M potassium acetate and 5 M acetic acid
RNase A: 10 mg/ml in 1 mM Tris-Cl buffer, pH 7.5
Ammonium acetate solution: 10 M stock solution
Tris-saturated phenol: phenol covered with a layer of Tris-Cl buffer, pH 8.0
Chloroform and isoamyl alcohol mixture (24:1): mix chloroform and isoamyl alcohol in 24:1 (v/v) ratio and saturate with a layer of Tris-Cl buffer, pH 8.0

**Plasmid Purification Solutions (QIAfilter Maxi Prep Method)**

Buffer P1 (resuspension buffer): 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 μg/ml
RNase A, stored at 4 °C
Buffer P2 (lysis buffer): 200 mM NaOH, 1% SDS, stored at room temperature
Buffer P3 (neutralization buffer): 3 M potassium acetate, pH 5.5, stored at 4 °C
Buffer QBT (equilibration buffer): 750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100
Buffer QC(washing buffer): 1 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol
Buffer QF (elution buffer): 1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% isopropanol

**Plasmid Purification Solutions (GENECLEAN Method):**

Nal: 6 M, pH 6-7.4
Glassmilk
New Wash solution: a solution containing NaCl, Tris, EDTA, and ethanol
**TE Buffer**

10 mM Tris-Cl, pH 8.0, 1 mM EDTA

**Supplemented Dulbecco's Minimum Essential Medium (DMEM)**

DMEM, 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 
μg/ml streptomycin

**Supplemented RPMI1640 Medium**

RPMI1640 medium, 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin

**Phosphate Buffered Saline (pH 7.4)**

58 mM Na₂HPO₄, 17 mM NaH₂PO₄, 68 mM NaCl. Filter through a 0.2 μm filter to sterilize. Store at 4 °C.

**Buffers for Calcium Phosphate Precipitation Method (for DNA Transfer into Mammalian Cells)**

Calcium chloride: 2 M

HBS (HEPES-buffered saline) (2 ×): 50 mM HEPES, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.1

DNAse-free water

Trypsin-EDTA solution (1 ×): 0.05% (w/v) trypsin, 0.53 mM EDTA, prepared in 1 × PBS buffer

**Mammalian Cell Storage Medium**

90% fetal bovine serum, 10% Dimethyl sulfoxide (DMSO) (0.2 μm filtered)

**Bacterial Cell Storage Medium**

Glycerol: 25%

**Trypan Blue Cell Viability Assay Solution**
Trypan blue: 2% (0.2 μm filtered) in phosphate buffered saline

**Luciferase Assay Buffer**

0.5 M NaCl, 0.1 M potassium phosphate, 1 mM EDTA, pH 7.5

**Luciferase Lysis Buffer**

125 mM Tris, pH 7.6, 0.5% Triton X-100

**Antibiotic Stock Solutions**

Ampicillin stock solution: 50 mg/ml in water, sterilized with 0.2 μm filter

Kanamycin stock solution: 50 mg/ml in water, sterilized with 0.2 μm filter

Chloramphenicol stock solution: 34 mg/ml in ethanol

Tetracycline stock solution: 7.5 mg/ml in ethanol

Penicillin / Streptomycin solution: 100 units/ml penicillin, 100 μg/ml streptomycin

Neomycin (G418) solution: 50 mg/ml in water

**Alginate Microencapsulation Solutions**

MOPS washing buffer: 10 nM MOPS, 0.85% NaCl

1.5% sodium alginate solution: 1.5% Na-alginate, 10 mM MOPS, 0.85% NaCl, pH 7.3-7.4

Polymerization solution: 10 mM MOPS, 100 mM CaCl₂

PLL-solution: 0.05% PLL (Molecular weight 15,000-30,000), 10 mM MOPS, 100 mM CaCl₂

0.03% sodium alginate solution: 0.03% Na-alginate, 10 mM MOPS, 0.85% NaCl, pH 7.3-7.4