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Novel luminescent reporter systems

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NOVEL LUMINESCENT REPORTER SYSTEMS

A Thesis

Presented to

The Faculty of the Department of Biology

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Anupama Yellamraju Kondepudi

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ABSTRACT

NOVEL LUMINESCENT REPORTER SYSTEMS

by Anupama Y. Kondepudi

This thesis examines existing genetic reporter systems and demonstrates the development and applicability of three novel luminescent based reporter systems : Secreted Alkaline Phosphatase (SEAP), Tartrate Resistant Acid Phosphatase (TRAP) and Green Fluorescent Protein (GFP), as gene reporters.

Chemiluminescent detection based SEAP assays appear to be linear, sensitive, convenient, easy to use, fast acting and have kinetic stability. Experiments were successfully conducted to normalize the SEAP reporters with β -Galactosidase using CHO-K1 cells for transfection. It was found that the TRAP vector was linear with similar sensitivities for both the chemiluminescent and colorimetric assays, thereby not showing an inherent advantage for the chemiluminescent technique. Attempts to achieve normalization with the SEAP vector were not successful since neither of the enzymes could be detected in the conditioned medium. The unique properties of GFP make it the first reporter system which can be used to show gene expression *in vitro*, *in vivo* and in real time. GFP gene expression was successfully achieved in several mammalian cell lines. Overall, GFP appears to be stable, sensitive and can fluoresce in the absence of other proteins, substrates and cofactors.

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CHAPTER 1

INTRODUCTION

A genetic reporter system can be described as a method to study regulation in gene expression. Reporter systems are used to understand eukaryotic gene expression using different sequences such as promoters or the sequences of a gene for transcription factors incorporated into a vector or genetically engineered construct. They can be excellent tools for studying the process of transcription activation and the measurement of protein activity.

Other methods of measurement of gene expression can be by the use of RNA analysis (mRNA levels) or protein analysis. These are often not practical to study many different gene constructs and are time consuming in contrast to reporter systems. Furthermore, such techniques require the modification of the analyzed gene to be distinguished from the native gene.

GENETIC REPORTER SYSTEMS

In a genetic reporter system, the approach is to gauge changes in transcription by linking the presumed cis-acting sequence(s) from the gene under study to the coding sequence for an unrelated reporter gene [1]. Once the chimeric reporter construct is introduced into an appropriate cell type or tissue, an indirect estimate of the induction in gene expression directed by the regulatory sequences is obtained by measuring the reporter gene product. The DNA

fragment from the gene of interest is placed upstream of the reporter gene in a vector which lacks endogenous promoter activity, thereby allowing for the testing of complete promoters. For the activator binding sites, the heterologous sequence is placed in a vector proximal to a basal promoter which contains sequences required for recognition by the basic transcription machinery. A reporter vector containing a basal promoter and possibly an upstream enhancer could also have a potential use as a positive control to normalize transfection efficiencies of experimental reporter constructs.

Reporter assays can be broadly classified into two types : *in vitro* (immunological based) and *in vivo* (histochemical based) [2]. *In vitro* reporter assays can be defined as procedures in which the reporter protein is quantified using either cell or tissue lysates containing the reporter or as in the case of secreted protein, the culture medium from transfected cells. Such assays direct quantitation of the reporter protein via enzymatic or immunological methods, thereby providing an indirect estimate of the transcriptional activity of the reporter vector encoding the protein.

When the reporter protein is detected in either live cells or tissues or in cells or tissues which have been fixed for histochemical staining, the gene expression assays are classified as *in vivo*. *In vivo* gene expression is regulated by an inducible promoter or enhancer element to which the reporter gene is linked. Transfection is usually the method by which the reporter gene linked to a promoter element via recombinant plasmid is introduced into cells. The product of the reporter gene will then provide a quantitative measure of the gene

expression level. This type of reporter system is well suited for assays which use precipitating or luminescent signal based substrates. The data obtained from *in vivo* assays are less quantitative than *in vitro* reporter systems but they provide important information on the cell-type specificity of promoters and enhancers and the tissue distribution of specific transcription factors.

Several criteria determine the selection of a proper transcription reporter gene. These include : (1) It should be of high sensitivity, fast acting and low cost, (2) have a wide linear range to facilitate detection of both small and large changes in promoter activity, (3) should be easily assayed (4) should not alter the physiology of the transfected eukaryotic cell, (5) should transcribe for a stable protein except in cases where a lack of stability is a desired feature and (6) should be able to be expressed in various cell systems or types.

Some of the reporter systems which meet the above criteria to varying degrees are Chloramphenicol Acetyltransferase (CAT), Firefly Luciferase, Secreted Alkaline Phosphatase (SEAP), Human Growth Hormone (HGH), β -Galactosidase (β -Gal), Tartrate Resistant Alkaline Phosphatase (TRAP) and Green Fluorescent Protein (GFP). Of these, CAT, SEAP and HGH are *in vitro* reporter systems, GFP is an *in vivo* system while TRAP, Firefly Luciferase and β -Gal can be both *in vitro* and *in vivo* systems. These are described in more detail in the following sections.

Each of the above reporter genes and their corresponding assay systems have advantages as well as weaknesses or limitations which determine the specific

applicability of them to a particular problem. In the case of CAT, Firefly Luciferase and β -Gal assay systems, the mammalian cells have to be lysed for assaying gene activity. This can be cumbersome and eliminates the use of the cells for simultaneous RNA level analysis. For this reason, secreted reporter genes have been designed to ease the assaying capabilities and permissibility of other types of analysis. A few such assay systems include HGH, SEAP, Firefly Luciferase and potentially a novel reporter system such as TRAP. Table 1.1 illustrates a brief comparison of the various reporter systems [3].

Chloramphenicol Acetyltransferase (CAT)

This reporter system measures activity of the *chloramphenicol acetyltransferase* (CAT) prokaryotic gene. It is the most common method for assaying gene expression in terms of CAT activity. The CAT enzyme catalyzes the transfer of acetyl groups from acetyl CoA to chloramphenicol. The CAT gene was first adapted for use in mammalian systems [4] and then modified vectors were developed which contain a polylinker for promoter insertion [5,6]. There are two non-isotopic detection methods for quantifying CAT - ELISA [7] and Fluorescent [8]. The ELISA based technique has the advantage of directly measuring CAT protein levels instead of CAT activity. The fluorescent based assay has the advantage of not only being more sensitive than the radioactive assays but it also has a wider linear range.

Table 1.1 Comparison of Reporter Gene Assays [3]

Reporter Gene	Detection Method	Detection Limit (Molecules)	Advantages	Disadvantages
CAT	ELISA	1.25×10^9	Non Radioactive	Small Range
	Isotopic	5.00×10^7	Widely Used	High Cost Radioactive High Cost Small Range Labor Intensive
β -Gal	Color Fluorescent	3.00×10^8 6×10^5	Widely Used	Poor Sensitivity
	Luminescent	4×10^3	High Sensitivity Wide Range Simple	Auto fluorescent
HGH	Radio-Immunoassay	3×10^8	Secreted into Media	Radioactive High Cost Low Sensitivity
Luciferase	Luminescent	$10^4 - 10^5$	Simple High Sensitivity Wide Range	Protein Stability
SEAP	Color	1.1×10^8	Secreted into Media	Poor Sensitivity
	Luminescent	3.3×10^4	Secreted into Media High Sensitivity Wide Range	
GFP	Fluorescent	Not Available	Live Cells Species Indep. No additional Products Photo bleaching not a problem	Weak Signal Intensity for specific cases

The primary advantage of using the CAT gene as a reporter in mammalian cells is its ease of use and high sensitivity. There is minimal competitive inhibition in most eukaryotic cells since CAT is a prokaryotic enzyme. This results in CAT assays being highly sensitive with high signal to background ratios [9]. It is possible to detect CAT activity driven by a promoter whose expression is too weak to be detected directly by RNA analysis. This characteristic along with the ease of use and sensitivity of the assay for CAT activity has resulted in the CAT reporter gene being widely used. The CAT assay is linear with enzyme concentrations over two to three orders of magnitude, without dilution of the enzyme or adjustment of the incubation time. Linearity is important for both time and activity, in order to get an accurate measure of CAT activity.

CAT has a number of disadvantages including its radioactive nature, high cost, and being labor intensive. The basic protocol utilizes an assay which requires [^{14}C] chloramphenicol and thin layer chromatography. This adds to the cost of the assay. There are alternate protocols using phase extraction [10] which could potentially lead to reduced costs.

Furthermore, CAT curtails the use of cells for other purposes such as RNA analysis since cells have to be harvested, lysed, and extracted prior to analysis. It also precludes the analysis of gene expression using one culture over a period of time. It is said [4] that stable transfectants are hard to isolate with the CAT reporter gene probably due to its half life of 50 hours in mammalian cells. This can be a hindrance when inducible enhancers are being studied since the CAT

gene activity may surpass or mask the effects of the inducible enhancer molecule. Hence, reporter genes with shorter half-lives such as Firefly Luciferase are currently being utilized.

Firefly Luciferase

The first non-isotopic genetic reporter system with widespread applicability to mammalian cells was developed by cloning the *luc* gene from the firefly *Photinus pyralis* [11]. The luciferase gene derived from the firefly *P.pyralis* catalyzes a bioluminescent reaction. The reaction includes luciferin as a substrate and Mg^{2+} as cofactor. ATP and molecular O_2 are needed for enzyme-substrate degradation which results in light emission.

Mammalian cells are transfected with the luciferase gene and the cell lysates harvested. The gene activity is measured using a luminometer. The light signals decay rapidly but the signal gives a direct correlation to the luciferase gene activity. A liquid scintillation counter can also estimate the light signal. The original luciferase assay is typically 10 to 1000 times more sensitive than the corresponding radioactive CAT assay. This is somewhat intricate to perform and lacks easy reproducibility primarily due to the quick "flash" kinetics of the photon emission. This problem has been addressed by developing a modified assay by the inclusion of coenzyme A in lysates to make the light signal more stable [12].

The above methods are *in vitro* in nature and recent studies have demonstrated *in vivo* applications as well. It has been shown that Firefly Luciferase activity can be detected in live cells using soluble luciferase substrates capable of crossing the plasma membrane [12]. Once across the membrane and inside the cell, the substrates are converted into firefly luciferin which in turn serves as the substrate for the Firefly Luciferase encoded by the gene. However, in its present form, the *in vivo* assay lacks both sensitivity and the ability to perform real time analysis.

However, in contrast to the above application, a secreted luciferase reporter enzyme has been described [13] which originates from a small marine crustacean *Vargula hilgendorffi*. With the luciferase cDNA, a vector was constructed to monitor gene expression in mammalian cells.

β -Galactosidase (β -Gal)

β -Gal is encoded by the *Escherichia coli lacZ* or β -Galactosidase gene. The β -Gal enzyme catalyzes the hydrolysis of various β -galactosidases. There are both *in vivo* and *in vitro* based β -Gal assay formats using specific substrates. A colorimetric assay was developed using ONPG (o-nitrophenyl β -D-galactopyranoside) as the substrate and a fluorometric assay using MUG (4-methylumbelliferyl- β -D galactoside) as a substrate [2]. The more sensitive method includes a 1,2-dioxetane based substrate [12]. This improved method has led to a 50,000 fold more sensitive assay compared with the colorimetric method.

Sensitivity can also be improved by using assay conditions which minimize the endogenous enzyme activity resulting from eukaryotic β -Galactosidase [2].

In vivo levels of the β -Gal reporter protein can be determined in both prokaryotic and eukaryotic cells and tissue sections using the precipitating substrate X-Galactosidase [1]. The reaction with X-Gal results in a rich blue color which is easily distinguishable from the background in most applications. Detection of β -Gal in live cultured cells is achieved using fluorescein di- β -galactopyranoside (FDG) [2]. Another application of *in vivo* β -Gal studies is the so called "enhancer trap" studies in which positional effects of reporter integration into the chromosomes of transgenic organisms is used to locate enhancer sequences [1].

In addition to its use as a genetic reporter system, β -Gal expression can be used as an internal control to normalize the variability of other reporter assays. In such a scenario, the β -Gal must be expressed under the control of a constitutive promoter. Such an internal control should be such that it does not hamper the activity of the reporter gene of interest. Examples where such normalization could be used is for CAT, Firefly Luciferase and SEAP assays [1]. Subsequently, the normalization of β -Gal transfections was demonstrated using Firefly Luciferase [14].

Human Growth Hormone (HGH)

Somatotropic cells of the anterior pituitary gland secrete Human Growth Hormone or HGH and reporter vectors have been designed to include this gene . When mammalian cells are transfected with the HGH encoding genetic reporter vector, the hormone or protein can be measured in the conditioned medium expressed transiently or in stable cell lines. The drawbacks of the HGH assays include the use of radio-isotopes in the assay system, high cost and poor sensitivity [2]. However, the HGH system may be used as an internal control in the form of a normalizing reporter gene for other secreted reporter genes such as SEAP.

Secreted Alkaline Phosphatase (SEAP)

There are several alkaline phosphatases present in mammals including a phosphatase isozyme - placental alkaline phosphatase which occurs primarily in the placenta of higher primates. The SEAP (secreted alkaline phosphatase) encodes for a modified form of placental alkaline phosphatase. Hepatic and intestinal cells have been found to have significant quantities of endogenous SEAP [1]. In contrast, the SEAP gene transfected cells easily secrete the SEAP gene product which is a truncated form of placental alkaline phosphatase lacking a membrane anchoring sequence [15].

The SEAP gene transcribed protein is a 489 amino acid peptide, lacking the carboxyl 24 amino acid of the normal protein to improve the efficacy of secretion

[16]. The levels of SEAP activity detected in culture medium were shown to be directly proportional to the intracellular concentrations of SEAP mRNA and protein [17]. SEAP is heat stable and resistant to L-homoarginine, a phosphatase inhibitor. Hence, endogenous phosphatase can be inactivated by including L-homoarginine in assay buffers of SEAP conditioned medium and the SEAP activity alone can be measured when incubated at 65 °C.

SEAP can be directly measured from cell conditioned media. The expression of SEAP from cells transfected with the SEAP gene can be detected as early as 24 hours post transfection [18]. To account for the variability in transfection between replicate samples a second plasmid or vector like acid phosphatase or TRAP could perhaps be used in co-transfection experiments.

The original SEAP assay [17] was colorimetric in nature, therefore fast, simple to perform and inexpensive. However, it lacks sensitivity and has a narrow dynamic range. The sensitivity can be improved to a level comparable to that of the conventional bioluminescent Firefly Luciferase assay using a two step bioluminescent technique [16]. Further improvements in sensitivity are obtained using a chemiluminescent method [19]. In addition to improving sensitivity for the SEAP assay, the use of chemiluminescence results in an increase in the linear range.

Tartrate Resistant Acid Phosphatase (TRAP)

TRAP is a lysosomal enzyme. It is an iron containing acid phosphatase secreted by porcine uterine epithelium. Takahashi et al. [20] suggested that TRAP is an essential element for the differentiation of mouse marrow cells - primarily osteoclasts and osteoblasts. TRAP was obtained from human placenta [21]. They also inferred that the amino acid structure of TRAP consists of 304 amino acids with an unglycosylated molecular weight of 34,193 kDa.

It was shown that TRAP from the spleen is transcribed from the same gene as the uteroferrin secreted from the uterus. It was further shown that the two proteins' primary structure is identical and that humans have the same TRAP gene as porcine TRAP [22]. It was also demonstrated that uteroferrin is secreted rather than being retained intracellularly primarily due to its overexpression in the uterine endometrium [23]. A secreted uteroferrin vector was transfected into CHO cells, and conditioned medium was used to measure the enzyme activity using a colorimetric assay.

The use of TRAP as an expression gene was suggested [24] and a simple method described for determining transfection efficiency when transfected with CMV driven TRAP DNA. Transfection efficiency can be determined using cell staining with a TRAP positive stain. TRAP was shown to be involved in bone resorption [25]. It acts as a medium of communication between accessory cells and osteoclasts. Since endogenous TRAP levels will be negligent in most cell types, it

could potentially be used as a more efficient reporter gene to clearly distinguish transfected cells from untransfected cells.

Green Fluorescent Protein (GFP)

Several bioluminescent jellyfish produce light as the result of energy transfer to green fluorescent proteins or GFPs. One such GFP originates from the jellyfish *Aequorea victoria* which fluoresces *in vivo* after receiving energy from Ca^{2+} activated photoprotein aequorin [26]. The green luminescence is due to the direct energy transfer from one protein to another requiring no additional gene products or substrates. There is a great interest in this technique since it is possible to introduce fluorescent labeling to intact cells, tissues, organisms, and sliced specimens in a genetic manner.

Pure GFP has similar spectral properties as that of the protein expressed *in vivo*, absorbing blue light and emitting bright green which is detectable using a fluorescence microscope, UV light box or by fluorescence activated cell sorting (FACS). The spectral properties (absorption and emission wavelengths) of GFP are similar to that of fluorescein - an absorption or excitation wavelength of 395 nm and an emission wavelength of 590 nm. Hence normal FITC filter sets can be used to visualize GFP incorporated cells under a microscope.

In the early 1960's, it was demonstrated that the green light is emitted from *Aequoria victoria* (jellyfish) umbrella margin [28]. With the advent of recombinant DNA technology, recombinant GFP (rGFP) was made [28]. GFP recombinant

type may exist in either an oxidized or reduced form [26]. Furthermore, GFP can be used to investigate the process of embryo genesis. The implications for bCD mRNA localization from the spatial distribution of exu protein in *Drosophila* oogenesis have been studied [29].

As with Firefly Luciferase, variant GFPs with shifted emission wavelengths may lead to the study of multiple transcriptional events in the same cell population. In addition, attempts are being made to increase the fluorescence or brightness of the protein via mutagenesis [30]. There has been work performed on the development of shifted mutants to GFP, one of which fluoresces blue. The GFP gene was mutagenized using random mutagenesis and screened for variants with altered spectra. Optimized combinatorial mutagenesis and digital imaging spectroscopy has been used to create and examine a library of red shifted GFP mutants [31]. The spectrally shifted mutants were identified by their green luminescence when excited at 490 nm wavelength but not at 410 nm.

GFP helps to visually trace gene expression without the use of additional substrates or inducers. Protein localization is hence possible within a living cell or organism. GFP does not require techniques which kill the cells being studied and therefore gene expression and the cascade of events after gene expression can be studied in real time. It is this ability to monitor transcriptional changes in real time which is a critical aspect of GFP based reporter systems [32]. It is also been suggested that GFP can be used to develop fluorescent zebrafish eyes, flies, yeast and even people if engraftment of GFP tagged stem cells in bone marrow becomes possible [33].

RESEARCH OBJECTIVE : LUMINESCENT REPORTER SYSTEMS

Of the above described reporter systems, the potential for novel reporter systems using chemiluminescent or bioluminescent methods is very high. It has been shown that the use of chemiluminescent substrates enables the ultra sensitive detection of enzymes compared to more traditional spectroscopic methods [12]. Bioluminescent systems such as GFP appear to have great promise as an *in vivo* reporter system [28].

Chemiluminescent substrates emit visible light upon enzyme induced decomposition. Typically, chemiluminescent substrates can be used to detect alkaline phosphatase or alkaline phosphatase conjugates. In the presence of secreted enzymes such as SEAP, TRAP and β -Gal, the substrate becomes unstable, decomposes and emits light. The intensity of the light signal is proportional to the enzyme concentration and measured using a luminometer. With the use of chemiluminescent substrates in assays, low levels of gene expression can be more readily measured thereby enhancing reporter gene assay sensitivity. Such systems typically have greater sensitivity than traditional methods which are radioactive or colorimetric in nature. The characteristic features of non-radioactivity, higher sensitivity, wide dynamic range and ease of handling makes the use of chemiluminescent substrates a useful tool for gene detection.

In the case of bioluminescent based systems such as GFP, the primary advantage is that it is possible to introduce fluorescent labeling to intact cells, tissues, organisms, and sliced specimens in a genetic manner. No additional substrates or gene products are necessary, resulting in a simplistic and fairly robust method of detection. This enables gene expression to be studied in real time.

The primary objective of this research work is to review existing and study different luminescent based reporter systems. Such techniques will enhance and simplify the study of different promoter elements as well as enhancers which influence eukaryotic gene expression. This research shall explore the development and applicability of three such gene reporter systems : SEAP, TRAP and GFP.

CHAPTER 2

MATERIALS AND METHODS

In performing studies on genetic reporter systems, there are several standardized experimental protocols used. This chapter describes details on the preparation of cell cultures, transfections, use of chemiluminescent detection techniques, and the various experimental protocols or assays. SEAP and TRAP use chemiluminescent substrates and hence use a luminometer to detect luminescence, and for GFP a fluorescent microscope is used to detect fluorescence. While the transfection methods are common for the different reporter systems, the specific reporter genes have different experimental assays and these are also presented in detail.

CELL CULTURE

The cell lines of interest are : CHO-K1, NIH3T3, COS and HeLa. The following section will describe the details of cell culture for these above cell lines. All the medium reagents were obtained from Gibco BRL, Gaithersburg, MD. Conditions for growth include an incubation temperature of 37 °C in a humidified atmosphere of 5 % CO₂.

CHO-K1

CHO-K1 cells are derived from Chinese Hamster (*Cricetulus griseus*) Ovary with a morphology similar to epithelial cells. Culture medium included 90% Ham's F-12 , 10% Fetal bovine serum and 1% antibiotic-antimycotic from GIBCO-BRL.

CV-1

CV-1 cells are kidney cells from African green monkey, *Cercopithecus aethiops*. Monolayers of fibroblast-like CV-1 cells facilitate the transfection of SV40 based GFP vectors. The cell culture medium contains 90% Minimum essential medium (MEM) with Earle's BSS or balanced salt solution , 10% Fetal bovine serum and 1% antibiotic-antimycotic from GIBCO-BRL.

COS-1

COS-1 cells are established from CV-1 Simian cells transformed by an origin-defective mutant of SV40 coding for wild-type t antigen. This makes them suitable hosts for propagation of SV40 based GFP vectors. The cells were cultured in 90% DMEM Dulbecco's modified Eagle's medium , 10% Fetal bovine serum , 1% antibiotic-antimycotic from Gibco BRL.

NIH / 3T3

NIH Swiss mouse embryo cultures are the source of the contact-inhibited NIH/3T3 cell line. The cells were cultured in (DMEM) Dulbecco's modified Eagle's medium, with 4.5 g/L glucose, 10% new born calf serum, 1% antibiotic-antimycotic from GIBCO-BRL.

HeLa

HeLa cells are epithelial like in morphology established from a human cervical epitheloid carcinoma. The cells were passaged in 90% Minimum essential medium (MEM) with Earle's BSS or balanced salt solution and non-essential amino acids , 10% Fetal bovine serum and 1% antibiotic-antimycotic from GIBCO-BRL.

TRANSFECTION PROCEDURES

Important aspects of transfection include the efficient transfer of recombinant plasmid into cells and the expression of this recombinant plasmid. There are two ways of transfection for studying gene expression : stable and transient. In stable transfections, a clonal cell line or stable transfection can be generated containing the sequence or factor of interest integrated into the chromatin. Stable transfections are time consuming since a dominant selection marker is used to isolate stable transfectants. Typically, the parental cells with the gene of interest and selection marker gene are grown in selection medium before individual colonies are isolated and expanded into cell lines. Transient transfection is a method by which gene expression regulated by promoter or enhancer elements can be quantified in 24 hours to 72 hours. Also, there is no need for a selection marker or establishment of stable clonal cell lines to study gene expression.

2×10^5 cells were plated on a 60mm tissue culture dish with a 22mm X 40mm glass coverslip. The cells were grown for 18-24 hrs in an appropriate growth medium supplemented with serum. To reach an optimal confluency of 40-60%,

the cells were incubated at 37°C in a 5% CO₂ incubator. This was the standard seeding protocol used for all cell types to maintain an average of 10-15% transfection efficiency from experiment to experiment.

Transfection solutions for each cell line include 4 µg of the plasmid DNA diluted into 200µl serum-free medium and 20 µg Lipofectin™ reagent (Gibco BRL) diluted into 200µl serum-free medium. The diluted plasmid-Lipofectin™ solutions were combined, gently mixed and incubated for 20 minutes at room temperature.

Prior to the application of Lipofectin™-plasmid complex on to the cells, the cells were washed twice with serum-free medium to remove any residual serum from the respective cell culture medium. The cells were incubated with the transfection solution for 8 hrs at 37°C in a CO₂ incubator during which the Lipofectin™-plasmid complex fuses with the cells for efficient uptake resulting in enhanced gene expression. Subsequently, the transfection solution is replaced with the appropriate growth medium specific for a given cell line. The cells were allowed to recover for a period of 36 hrs at an incubation temperature of 37°C in a CO₂ incubator.

CHEMILUMINESCENT ASSAYS - SEAP, TRAP and β-Gal

Chemiluminescent detection techniques are used for SEAP, TRAP and β-Gal assays. This section briefly describes the chemiluminescent substrates used followed by the actual assay procedures for SEAP, TRAP and β-Gal.

Chemiluminescent Substrates

Chemiluminescent substrates such as CSPD™ and AMPGD™ (obtained from TROPIX Inc., Bedford, MA) emit visible light upon enzyme induced decomposition. The intensity of the light signal is proportional to the enzyme concentration and measured using a luminometer. CSPD™ substrate was used to quantitate SEAP and TRAP enzymes. In the case of β -Gal, since the assay has to be performed at higher pH levels, the chemical substrate used was AMPGD™ which is more stable at higher pH levels.

SEAP Gene Expression Assay Procedure

Transfection procedures were performed as described above. Within 24 hours after transfection with the pSEAP Control Vector, SEAP activity can be detected. Details on the specific SEAP vectors are provided in the following chapters on SEAP. At pre-determined time points, samples of the conditioned media are transferred to microcentrifuge tubes for storage (frozen or on ice) until they are ready for assaying. In order to assay for SEAP activity, the samples are diluted with buffer, heated for 30 minutes at 65 °C and incubated in buffer containing L-homoarginine for 5 minutes. The CSPD substrate is then added and the resulting mixture incubated for 10 to 60 minutes. The signal can be detected by either a luminometer or by brief exposure to X-ray film. This procedure is illustrated in Figure 2.1 [34].

Figure 2.1 Schematic of SEAP Gene Expression Assay Procedure
(Reproduced with permission from ClonTechniques [34])

β-Gal Gene Expression Assay Procedure

The cis-regulatory elements of interest need to be first cloned into the appropriate β-Gal vectors and the experimental constructs transfected into either adherent or suspended host mammalian cells via any common transfection method. Transfection procedures were performed as described above. Twenty four to seventy two hours after the transfection, cell lysates are prepared and the lysates incubated with AMPGD™ containing reaction buffer for approximately 60 minutes. The chemiluminescent signal is measured 5 to 15 seconds later with a tube or plate luminometer. Alternatively the assay can be performed using a 96 well microtiter plate and the resulting signal monitored via exposure of x-ray film. Overall, the entire protocol is fairly simple, easy and quick to perform. This procedure is illustrated in Figure 2.2. [35]

TRAP Assay Procedure

As stated earlier, the use of a chemiluminescent method of detection has greater sensitivity than a traditional colorimetric method. However, it is important to be able to compare the two methods in order to determine the differences in sensitivity and linear range.. The colorimetric assay was first performed and subsequently modified to develop the chemiluminescent version. All experiments for methods development were conducted using 10^{-7} g of pure uteroferrin or TRAP.

Figure 2.2 Schematic of β -Gal Gene Expression Assay Procedure
(Reproduced with permission from ClonTechniques [35])

TRAP Colorimetric Assay

The enzyme substrate reaction was determined in a 96 well flex vinyl microtiter plate. Forty μl of 100 mM substrate was added to 10 μl of pure enzyme along with 50 mM Tris pH 7.0 HCl and 1 mM β -mercaptoethanol. This ensures the inhibition of endogeneous acid phosphatases. TRAP being tartrate resistant, will not be inhibited when the reaction mixture is pre-incubated at 37 °C for 20 minutes. Ten (10) μl of 1.0 M sodium acetate (pH 5.2) is added to the above reaction to lower the reaction pH. This is followed by the addition of *p* nitro phenyl phosphate (40 mM), a phosphatase substrate dissolved in 50 mM sodium acetate. Following an incubation period of 20 min at 37 °C, 100 μl of 50 mM NaOH was added. The NaOH inhibits the enzyme substrate reaction with an increase in pH. Subsequently, the microtiter plate reader was used to determine absorbance at 405 nm.

Chemiluminescent Assay of TRAP

In a 500 μl microfuge tube, 20 μl of pure enzyme was put into untransfected medium. Twenty ml of 1M Tris-HCl, pH 7.0, 1 mM β -mercaptoethanol and 0.1 M Na tartrate were added and incubated for 20 minutes at 37° C to inactivate endogenous tartrate sensitive phosphatases. This is followed by the addition of 10 μl of 1M Na acetate at a pH of 4.0 in order to adjust the final pH to 4.0. 100 μl of 25 mM CSPD diluted in 1.0 M Tris-HCl, pH7.0 was added to the samples and incubated once again for 20 minutes at 37° C. One hundred (100) μl of 0.5 N NaOH with 10% Emerald EnhancerTM (stock 10 mg/ml) was used to raise the

Figure 2.3 Schematic of TRAP Gene Expression Assay Procedure

reaction pH to more than 9.0. The light emission was measured using a Turner model TD-20e luminometer. Background values obtained with conditioned medium from untransfected cell culture medium or added enzyme were subtracted from all values. Figure 2.3 illustrates the TRAP assay procedure in the form of a simple flowchart.

Chemiluminescent Detection

The light emitted from the above assays was detected and measured using a luminometer. A Turner model 20E luminometer was used for these purposes.

FLUORESCENT MICROSCOPY

Fluorescent microscopy is used to study cells, tissues or micro-organisms using microscope equipped with a source of ultraviolet rays. Typically these cells exhibit fluorescence which results from the excitation of Green Fluorescent Protein (GFP) present in the cells transfected with the GFP plasmid.

A Zeiss axioscope 50 was used for viewing GFP fluorescence from GFP-C1 transfected mammalian cells. Most observations were made using 40 X oil immersion objectives. Photographs were taken using KODAK Ektachrome 400 ASA film. The filter sets used were standard fluorescein isothiocyanate (FITC) filters from Chroma Technology Corporation . The maximum excitation and emission wavelengths were 485 nm (filter range 460-510 nm) and 540 nm (filter range 515 - 565 nm). The use of a long-pass emitter filter (520-750 nm) helps in

distinguishing the autofluorescence as a yellow hue as compared to the fluorescent green expressed by GFP-C1 transfected cells. There is no observable photo bleaching of the GFP signal over an extended period of time.

CHAPTER 3

SECRETED ALKALINE PHOSPHATASE (SEAP)

INTRODUCTION

SEAP is a secreted form of human placental alkaline phosphatase [16]. Changes in the intracellular concentrations of SEAP mRNA and protein have a directly proportional relationship to the levels of SEAP activity detected in the cell culture medium. There are several positive aspects to using the secretion properties of SEAP in the form of a transcription reporter system. These advantages include : (1) Only requires the researcher to assay the culture medium for the reporter protein, without having to prepare cell lysates. (2) Study of the kinetics of gene expression using the same cultures by repeatedly sampling the medium, since the transfected cell cultures remain intact, thereby eliminating the need for multiple cultures and the elimination of sample variability. (3) The ability to use the cells for RNA and protein studies after the samples have been taken for SEAP assays. (4) Possibility of automating sample collection using cultures grown in standard 96 well microtiter plates. (5) Non-radioactive in nature since there are no radioisotopes required. SEAP may not be suitable if experimental designs affect secretory capacity of target cells or when cells expressing low levels of SEAP are to be used.

SEAP AS A CHEMILUMINESCENT GENETIC REPORTER SYSTEM

In order to effectively use SEAP as a reporter system, one of the first steps is to construct a recombinant plasmid or vector such that the SEAP gene is placed under the control of the *cis*-acting regulatory region of interest. There are four SEAP based vectors designed to function as an integrated set of plasmids together with other components which form the basis for a complete reporter system (Table 3.1) [33].

Table 3.1 : SEAP based Genetic Reporter System

• pSEAP	Basic Vector
• pSEAP	Enhancer Vector
• pSEAP	Promoter Vector
• pSEAP	Control Vector
• Buffers	Assay and Dilution
• CSPD TM	Chemiluminescent substrate
• Enhancer	Chemiluminescent
• Positive Control	Placental Alkaline Phosphatase

All the SEAP vectors differ only by the insertion of the promoter and/or the enhancer sequences. There is a great deal of flexibility in the analysis of regulatory sequences from the gene of interest since all the pSEAP reporter vectors contain a similar repertoire of cloning sites. A brief description of the four vectors follows :

- **pSEAP Basic** does not have the eukaryotic promoter and enhancer sequences inserted into the vector. It has a multiple cloning site (MCS) upstream of the SEAP gene which allows for the insertion of DNA fragments containing promoters into the vector. The vector backbone provides an f1 origin for single stranded production, and carries a pUC19 origin of replication and an ampicillin resistant gene for propagation and selection in *E.coli*. Upstream of the MCS is a polyadenylation signal which reduces the background level of the transcription. The SEAP coding sequences are followed by a second polyadenylation signal and an intron from SV40. This will ensure effective processing of the SEAP transcript. (Figure 3.1)
- **pSEAP Control** (Figure 3.2) is identical to the pSEAP Basic vector with the insertion of the SV40 early promoter upstream of the SEAP gene and the SV40 enhancer downstream of the SEAP gene. The purpose of this vector is to provide a reference (hence control) to compare the activities of promoters and enhancers using SEAP as a reporter system.
- **pSEAP Enhancer** and **pSEAP Promoter** are the other SEAP vectors which are used in the reporter system. They are identical to the pSEAP Basic except for the insertion of the SV40 enhancer and SV40 promoter sequences respectively in the exact locations shown in the pSEAP control. The transcriptional activity of weak promoters inserted into the MCS of

Figure 3.1 Schematic of pSEAP Basic Vector
 (Reproduced with permission from ClonTechniques [34])

Figure 3.2 Schematic of pSEAP Control Vector
 (Reproduced with permission from ClonTechniques [34])

the pSEAP Enhancer can be potentially increased with the use of an SV40 enhancer. The SV40 promoter can be used to analyze enhancer sequences inserted either downstream or upstream of the SEAP gene in the pSEAP Promoter.

EXPERIMENTAL DETAILS

Once the vectors are constructed, their sizes and presence of the Hind III and Sca I sites were confirmed using restriction enzyme digestion. The recombinant and control plasmids were then transfected into the host cells and the gene expression assays carried out as described in Chapter 2.

SEAP SENSITIVITY AND TIME COURSE

The colorimetric methods for SEAP are based on hydrolysis of *p* nitrophenyl phosphate. However, with the use of chemiluminescent methods for SEAP as described above, the sensitivity increases by almost two orders of magnitude (80 to 100 times). This is illustrated in Figure 3.3 which shows the sensitivity of SEAP detection using a luminometer. Conditioned medium from NIH/3T3 cell cultures was used as described in Chapter 2. Known amounts of purified human placental alkaline phosphatase were added to conditioned medium from the above cell cultures. The light signals were measured using a Turner model TD-20e luminometer with a 15 second integration time. Another interesting observation from Figure 3.3 is that SEAP behaves in a linear fashion over four

Figure 3.3 Sensitivity and Linear Range of SEAP
(Reproduced with permission from ClonTechniques [34])

orders of magnitude demonstrating that small and large changes in promoter activity can be measured. CHO-K1 cell cultures were maintained as described in Chapter 2. A liposome based transfection procedure was used to transfect duplicate cultures with 4.0 mg of the pSEAP Control, Promoter, Basic and Enhancer vectors. Samples of the conditioned media were assayed at periods ranging from 24 to 72 hours post transfection. The chemiluminescent signals were measured with the same luminometer described above and data adjusted to account for the background values obtained from untransfected conditioned media.

The results (Figure 3.4) indicate that there is an approximately linear increase in SEAP activity between 24 and 72 hours for the pSEAP Control and Promoter vectors. There was negligible activity for the pSEAP Basic and Enhancer vectors and these are not shown in the figure. The absence of both eukaryotic promoter and enhancer sequences in the pSEAP Basic vector or the presence of only an SV40 enhancer leads to a lack of transcription or background level transcription of the SEAP gene respectively. The pSEAP Control vector had an activity level which was an order of magnitude (10 times) higher than that of the pSEAP Promoter vector. This illustrates the strong inductive power of the SV40 enhancer resident in the pSEAP Control. The low level basal activity from the pSEAP Promoter vector allows for a clear observation of the further increases in SEAP expression resulting from the cloning enhancer sequences into this vector upstream of the SV40 Promoter.

Figure 3.4 Time Course of SEAP Expression
(Reproduced with permission from ClonTechniques [34])

Similar results were observed when the pSEAP Control Vector was transfected using COS-7 cells. This indicates that it may be possible to use the SEAP gene as a genetic reporter system for other mammalian cell lines such as HeLa, CV-1 and NIH-3T3. Lung, testes and cervical cells may have low levels of SEAP activity exhibiting significant background values.

NORMALIZATION OF SEAP USING β -Gal

Frequently, normalization of reporter systems by another system is conducted to help define transfection efficiency and variation in transfection between replicate samples. This is done by co-transfecting a second reporter plasmid with that of the first. The purpose of normalization is to reduce the variability between transfected replicate samples. Such a normalization procedure has been shown to reduce the standard deviation errors of experimental values by 3 orders of magnitude [1]. The secretory nature of SEAP enzyme allows the use of cells for multiple purposes like RNA analysis. However, β -Gal requires cell lysis since it is intra-cellular. This limits the use of β -Gal in SEAP normalizations.

To illustrate the use of β -Gal in such a role, experiments were conducted to normalize the SEAP reporters with β -Gal. (Figure 3.5). 1×10^5 CHO-K1 cells were used for transfection. One (1) μg of β -Gal Control vector was co-transfected with 1 μg of pSEAP Basic, Control and Promoter vectors using lipofectin as the transfection reagent. Transfections, SEAP assays and β -Gal assays were performed as outlined in Chapters 2, 3 and 4 respectively. Figure 3.5 shows that there was 6.94% efficiency of secretion for the pSEAP Basic, 99.11 % efficiency

for the pSEAP Control and 18.29 % efficiency for the pSEAP Promoter. The control vector had an activity level 10 times the basic vector and 5 times the promoter vector.

CONCLUSIONS

Overall, SEAP appears to be an excellent reporter system which is both convenient, sensitive and acts rapidly as a transcription reporter. SEAP is inexpensive, non-isotopic and cell lysis is not required since SEAP is a secreted protein. SEAP assays using chemiluminescent detection methods are convenient, rapid, linear over 4 orders of magnitude and kinetically stable. However, SEAP may not be suitable for cells which express low levels of placental type alkaline phosphatase and may not be suitable if the experimental design impacts on the secretory capacity of the target cells. If used in a colorimetric manner, the assay can have poor sensitivity but this drawback can be overcome by using a chemiluminescent technique.

The relatively easy two step chemiluminescent SEAP assay allows the endogenous alkaline phosphatase from cells to be inhibited by the use of L-homoarginine and preheating the media at 65 °C. The sensitivity of the chemiluminescent based SEAP assay is determined by constructing a linear range. The assay will allow detection from 10^{-9} to 10^{-13} g of pure enzyme with the detection of activity as early as 24 hours post transfection. Following transfection, the SEAP reporter gene product can be measured to obtain an indirect estimate of the gene expression induced by the regulatory sequence.

Figure 3.5 Normalization of SEAP with β -Gal

CHAPTER 4

TARTRATE RESISTANT ACID PHOSPHATASE (TRAP)

INTRODUCTION

TRAP is a lysosomal enzyme secreted by porcine uterine epithelium during gestation [21]. TRAP is synonymous to Uteroferrin (Uf) which aids in the transportation of iron across the endometrium of the mother to the fetal liver. TRAP cDNA of 1.1 kbp was found to be identical to the nucleotide sequence of a uteroferrin cDNA of 1.42 kbp [22]. The primary difference between TRAP and uteroferrin is that while TRAP is only intracellular, uteroferrin is both intra and extra cellular [21]. Uteroferrin is a secreted form of TRAP. Both are encoded by the same mRNA. In this study, all references to TRAP are porcine based.

Acid phosphatase protein assays were designed using a colorimetric based method of detection [23]. Ling and Roberts used either *p*-nitrophenyl phosphate (pNPP) as a substrate or cell extract protein concentrations were measured with bovine serum albumin as a standard by the Bradford protein assay. A uteroferrin based plasmid vector was constructed with an SV40 early promoter and the expression of uteroferrin verified in transfection experiments showing that the cell conditioned medium had TRAP activity almost exclusively. Cell extracts had no traceable uteroferrin activity.

However, as with most colorimetric based assays, this form of reporter system was not very sensitive. Hence, the development of a chemiluminescent based reporter system using TRAP along with CSPD as a substrate was conceptualized and appropriate experimental methods developed. Furthermore, using CSPD as the substrate would allow for the use of TRAP to normalize for SEAP activity, since CSPD is also used with SEAP. In comparison to β -Gal, TRAP is secreted acid phosphatase. Cell lysis is not required and the collection of conditioned media from SEAP + TRAP transfected cells can be used for analysis.

CONSTRUCTION OF TRAP VECTOR

One of the first steps in using TRAP as a reporter system is to construct a plasmid using site directed mutagenesis. Based on work from the literature [23], a 4.7 kbp pCMV-Uf plasmid was developed. TRAP secretion is pivotal to the use of TRAP in normalizing SEAP transfections. The use of this modified plasmid in mammalian cell lines did not result in any TRAP secretion into the cell culture medium or into the cell extract. Hence, the CMV-TRAP sequence was checked against the original sequence obtained from [23] and the cDNA sequence did not have a one to one correspondence with the modified vector.

There was an absence of a cytosine C which would destroy the PM1I site resulting in the shift of ATG out of the TRAP reading frame. The expected PM1I sequence was : AGCTTATGGACACGTGGACGGTACTGCT whereas the sequence from the modified plasmid had a C missing where indicated in bold : AGCTTATGGACAGTGGACGGTACTGCT. This C needed to be inserted into

the sequence. In order to enable the insertion of the C as well as incorporate a PMLI site into the TRAP gene, site directed mutagenesis was performed using a transformer kit obtained from Clontech Laboratories, Palo Alto CA.

Such a kit efficiently introduces specific base changes such as the addition of a cytosine and the corresponding guanine into the double stranded pCMV Uf / TRAP plasmid. In this process, two oligonucleotide primers are simultaneously annealed to one strand of a denatured double stranded pCMV/TRAP plasmid. As a result of the site directed mutagenesis, a TRAP plasmid was finally constructed as shown in Figure 4.1.

EXPERIMENTAL DETAILS

Once the vectors were constructed, their sizes and the presence of the Hind III and Sca I sites were confirmed using restriction enzyme digestion. The recombinant and control plasmids were then transfected into the host cells and the gene expression assays carried out as described in Chapter 2.

TRAP SENSITIVITY AND LINEAR RANGE

Figure 4.2 shows the absorbance of the uteroferrin as a result of the colorimetric assay over a range. Figure 4.3 shows a linear range of the chemiluminescent assay using pure enzyme to show sensitivity. Similar to the colorimetric assay, of the chemiluminescent assay, range also varies from 5 ng to 200 ng with the

Figure 4.1 Schematic of pCMV-TRAP Vector

Figure 4.2 Linear Range of TRAP Colorimetric Assay

Figure 4.3 Linear Range of TRAP Chemiluminescent Assay

mid-point being 100 ng. From Figures 4.2 and 4.3, it can be noted that the sensitivity of the TRAP vector in the chemiluminescent assay has the same order of magnitude as that of the colorimetric assay. This is attributed to the use of CSPD as the chemiluminescent substrate with TRAP. CSPD has an optimal working pH of greater than 7.0 but TRAP is an acid phosphatase which works better at acid pH conditions. As newer chemiluminescent substrates are developed which work well at low pH conditions, the sensitivity of chemiluminescent based TRAP vectors will improve.

pH PROFILE FOR TRAP AND CROSS TALK WITH SEAP

A pH profile of TRAP-CSPD (enzyme-substrate) reaction was evaluated in order to optimize the enzyme substrate reactions (Figure 4.4). A pH of 4.0 seems to be the most optimal. Estimation of the cross-talk or interference of the TRAP reporter system with the SEAP assay protocol is shown in Figure 4.5. There appears to be no cross-talk between TRAP and SEAP and therefore, TRAP can be selectively assayed. This indicates that TRAP can be used in conjunction with SEAP when pure enzymes are mixed in mock transfected conditioned medium.

DETECTION OF TRAP IN MAMMALIAN CELLS

In order to demonstrate the expression of the TRAP gene in mammalian cells, the TRAP vector was transfected into CHO-K1 cells using procedures described

Figure 4.4 pH Profile for TRAP-CSPD Reporter System

Figure 4.5 Estimation of Cross Talk between SEAP and TRAP

Figure 4.6 Expression of TRAP in CHO-K1 Mammalian Cells

in Chapter 2. Figure 4.6 shows that there is a linear increase in TRAP activity from 24 hours to 72 hours post transfection.

NORMALIZATION AND TIME COURSE EXPERIMENTS

Similar to the normalization of SEAP using β -Gal, it was decided to attempt similar experiments with TRAP. However, the results were not conclusive. When the pSEAP control vector was co-transfected with the pCMV/TRAP vector, there is an inhibition of the SEAP and TRAP activity as illustrated by Table 4.1. This indicates an absence of SEAP or TRAP in the medium of the co-transfected cells. If these two vectors are transfected independent of each other as shown in the time course experiments, individual expressions of SEAP and TRAP (Figures 3.3 and 4.6 respectively) can be measured.

One reason for such behavior can probably be attributed to the competitive inhibition of SEAP and TRAP in the secretory pathways or alternatively either of them are being blocked in the vesicles of the cell membrane thereby inhibiting secretion into the cell culture medium. It is further presumed that the vector construction strategy is favorable since gene expression was measured when SEAP and TRAP were transfected independently. RNA analysis and Northern blotting experiments would define if mRNA synthesis is occurring when SEAP and TRAP are co-transfected.

Table 4.1 : Co-Transfection of SEAP and TRAP
(Units : RLU)

Time (hrs)	SEAP			TRAP		
	Untransfected	Transfected	Co-Transfected	Untransfected	Transfected	Co-Transfected
24	9.3	128.2	1.2	89.34	239.34	355.51
48	23.28	486.9	-1.47	151.38	708.27	984.46
72	0.653	512.6	2	2575	3602	1880

CONCLUSIONS

TRAP has been shown to be an expression vector in the literature [23]. This was verified using both a colorimetric assay as well as a chemiluminescent assay. The secretion of TRAP into the conditioned medium instead of being retained intracellularly enhances the usage of TRAP as a reporter gene. It was found that the sensitivity of the TRAP vector in the chemiluminescent assay has the same order of magnitude as that of the colorimetric assay. The range varies from 5 to 200 ng with the mid point being approximately 100 ng.

Although the TRAP - CSPD enzyme substrate reactions were optimized by evaluating a pH profile, the system is not efficient. This could be attributed to the instability of CSPD at low pH values. It is expected that improved chemiluminescent substrates which work in an optimal fashion at lower pH conditions will increase the sensitivity of TRAP considerably.

The construction of the TRAP vector reinforced its use as an expression vector. As a result of the transient transfection assays, it is apparent that TRAP activity can be detected as early as 24 hours post transfection. However, the use of TRAP to normalize SEAP transfections was not successful. In co-transfection experiments of TRAP and SEAP using CHO-K1 cells, there was no enzyme activity observed. The utility of TRAP as a complete reporter system has value but is limited by its inability to normalize SEAP vectors. Further work is needed in this regard.

CHAPTER 5

GREEN FLUORESCENT PROTEIN (GFP)

INTRODUCTION

The green fluorescent protein (GFP) is rapidly gaining popularity as a reporter method to monitor gene expression *in vivo* and in real time. Unlike other luminescent based reporter systems, GFP will exhibit fluorescence in the absence of other substrates, proteins or cofactors. Its fluorescence is both stable, species independent and can be monitored in a non-invasive manner in living cells. GFP based systems use already existing technologies and imaging methods such as fluorescent microscopes, filters, fluorescent cell sorting and automated flow cytometers. In traditional fluorescent studies, labeling is done by purifying the proteins and conjugating to fluorescein and rhodamine. However, in living cells, it is difficult to get conjugates across the cell membrane. In contrast, GFP proteins are small, bright, accurate and easy to manipulate even inside living cells and tissues.

GFP is a naturally occurring protein emitted from the jellyfish, *Aequorea victoria* and emits green light with a wavelength of 509 nm when excited by ultra-violet light with a wavelength of 395 nm. Fluorescent light is emitted when energy is transferred from the Ca^{2+} activated photoprotein aequorin to the green fluorescent protein (GFP) [36].

Pure GFP has similar spectral properties to the protein expressed *in vivo* where it absorbs blue or ultra violet light and emits green light which is detected using a fluorescent microscope or UV light box. The cloning of the GFP gene and its subsequent expression in heterologous systems demonstrated that GFP could be used as a reporter system [36]. GFP expressed in either prokaryotic or eukaryotic cells yields a bright green fluorescent light.

This chapter will describe the development of GFP based fluorescent plasmids which can be used for gene expression in different types of mammalian cells. Sensitivity, stability and potential concerns of the fluorescence are also discussed.

GFP AS A BIOLUMINESCENT GENETIC REPORTER SYSTEM

One of the first steps in effectively utilizing GFP as a reporter gene is to develop a line of fluorescent reporter vectors. Figures 5.1 through 5.4 describe a comprehensive set of GFP vectors.

pGFP (Figure 5.1) is primarily intended to be a source of GFP cDNA which can be excised via restriction digestion in the two multiple cloning sites which are on either side of the GFP coding sequence. It is also possible to use PCR to amplify the GFP coding sequences.

pGFP-1 (Figure 5.2) is a reporter vector which monitors the activity of promoters cloned into the multiple cloning sites upstream of the promoterless

Figure 5.1 pGFP cDNA Promoterless Vector
(Reproduced with permission from ClonTechniques [36])

Figure 5.2 pGFP-1 Promoter Vector
 (Reproduced with permission from ClonTechniques [36])

GFP gene. In order to increase translation efficiency in eukaryotic cells, the sequences surrounding the GFP start codon have been converted to a Kozak consensus translation initiation site. The pGFP-1 vector also contains a neomycin/kanamycin resistant cassette which enables the selection of transformed bacterial and eukaryotic cells.

The GFP vector can also be used in the form of a fusion protein as illustrated in Figures 5.3 and 5.4. pGFP-N1 is one of the vectors which can be used to fuse heterologous proteins to the N-terminus of GFP. The same multiple cloning site is present in different reading frames and can allow researchers to create in-frame fusion to a convenient restriction site in the gene of interest. Similarly, pGFP-C1 can be used to create in-frame fusions to the C-terminus of GFP. All the fusion vectors contain the immediate early promoter of cytomegalovirus (CMV) to express fusions in mammalian cells. They also contain the Kozak consensus sequence and the neomycin/kanamycin resistant cassette for the same reasons as expressed for the pGFP-1 vector.

DETECTION OF GFP IN MAMMALIAN CELLS

Once the vectors have been constructed, their ability to be expressed in mammalian cell lines needs to be demonstrated. In order to best illustrate the successful expression the GFP gene in various cell lines, color photographs were taken using a fluorescent microscope equipped with a high resolution camera. Details of this equipment are described in Chapter 2.

Figure 5.3 pGFP-N1 Terminal (amino) Protein Fusion Vector
 (Reproduced with permission from ClonTechniques [36])

Figure 5.4 pGFP-C1 Terminal (carboxy) Protein Fusion Vector
 (Reproduced with permission from ClonTechniques [36])

Similar to the other reporter vectors such as SEAP and β -Gal, restriction digestion was conducted to confirm the plasmid sequences as described above. This was performed similarly to the procedure described in Chapter 3 for SEAP. The GFP vectors were transfected in a variety of cell types such as CHO-K1, HeLa, COS-1 and NIH/3T3. In all cases, the cell culture methods were as described in Chapter 2. Standard transfection procedures as described in Chapter 2 were utilized. In all cases, the transfections were transient in nature.

Figure 5.5 shows the GFP fluorescence in living CHO-K1 cells transformed with a mammalian GFP expression vector. There is a clear distinction between the cells which express GFP and those which do not. In a separate experiment using CHO-K1 cells, fixed cells were counterstained using propidium iodide (Figure 5.6). This clearly shows that : (a) GFP fluorescence persists in formaldehyde fixed cells and (b) GFP is well suited for double labeling experiments with other fluorescent markers. Figures 5.7 through 5.9 demonstrate the GFP fluorescence for CHO-K1 cells using a GFP-1 vector, GFP-N1 and GFP-C1 vectors respectively. In the case of the GFP-1 vector, since the vector lacks both a promoter and enhancer, there is no fluorescence due to lack of transcription. Fluorescence is clearly observed in Figures 5.8 and 5.9.

STABILITY AND SENSITIVITY ISSUES WITH GFP

GFP has been found to be very stable in the presence of photo bleaching and is more stable than even fluorescein under the higher intensity illumination of a

Figure 5.5 Detection of GFP in CHO-K1 cells
(Reproduced with permission from ClonTechniques [36])

Figure 5.6 Transient Expression of GFP with Counterstaining
(Reproduced with permission from ClonTechniques [36])

Figure 5.7 Expression of pGFP-1 in CHO-K1 cells

Figure 5.8 Expression of pGFP-N1 in CHO-K1 cells

Figure 5.9 Expression of pGFP-C1 in CHO-K1 cells

fluorescent microscope. GFP needs to be in an oxidized state to fluoresce and hence, strong reducing agents do have an impact by converting the fluorescent GFP into a non-fluorescent state. However this state is reversible under the presence of atmospheric oxygen. GFP is very resistant to heat, alkaline pH, detergents and some common proteases. However, fluorescence is lost if GFP is denatured by high temperature or extremes of pH. In the case of *in vivo*, GFP appears to be stable when expressed in several organisms. However, no measurement of the half-life of GFP has been reported [37].

In fluorescent microscopy, GFP fusion proteins have been found to exhibit greater sensitivity and resolution than staining with fluorescently labeled antibody [30]. In terms of behaving as a quantitative reporter system, since the signal from GFP does not have enzymatic amplification, it will probably have a lower sensitivity than enzyme based reporter systems such as β -Gal, SEAP and Firefly Luciferase. The suitability of GFP as a quantitative reporter system has yet to be fully established [37].

POTENTIAL CONCERNS WITH GFP FLUORESCENCE

There have been cases of placing GFP expression vectors into systems and not detecting fluorescence. Some of the reasons and difficulties with lack of fluorescence include : (1) GFP fluorescence is very sensitive to some nail polishes used to seal coverslips. (2) Some samples may have significant background from autofluorescence. (3) Intense excitation of the GFP over extended period of times

leading to toxic free radical generation in the cell. (4) Fusion of a protein to GFP may cause a red shift in the absorption spectrum [36].

CONCLUSIONS AND FUTURE DIRECTION

The unique properties of GFP make it the first reporter system which can be used to follow gene expression *in vitro*, *in vivo* and in real time. GFP based fluorescent plasmids were developed followed by expression in multiple mammalian cell lines. Fluorescence was observed in CHO-K1, HeLa, COS-1 and NIH/3T3 cell types and also when CHO-K1 cells were counterstained with propidium iodide.

GFP was found to be stable, sensitive and can fluoresce in the absence of other proteins, substrates and cofactors. The expression of the GFP gene has been demonstrated successfully showing its applicability across several mammalian cell lines. Fusions of GFP to heterologous proteins promise to be an excellent tool for studying protein localization and trafficking. Some near term research includes the development of stable transfections and the use of fluorescence activated cell sorting analysis (FACS) as related to GFP.

The use of GFP as a genetic marker has problems associated with it in certain experimental systems. For example, the toxic effects of GFP have not been established. It has not yet been shown to date how long term expression of GFP transfected cells impacts cell viability. Further, some researchers have noted that by altering the GFP chromophore by mutagenesis, there is an altered spectrum

of GFP. This will require the use of modified filter sets to detect new mutant GFPs.

Stable Transfections, Fluorescence Activated Cell Sorting (FACS) Analysis and Sensitivity

Stable transfections are necessary in order to analyze the functionality of GFP or to evaluate the function of fusion proteins constructed by using the GFP vectors. Since it has been shown that the fluorescence spectra of GFP has not been altered by the use of GFP vectors, stably integrated cell lines with GFP have to be derived. A dominant selectable marker such as G418 or Genetecin helps to permit isolation of the stable transfectants. Initial studies for determining the optimal drug concentration have been performed. A range of 0.8 mg/ml to 1.0 mg/ml, G418 appears to be adequate for selection in HeLa, CHO-K1 and NIH/3T3 cell types.

FACS can be used as a tool for the selection of GFP transformed cells. GFP expressed *in vivo* (for example, from living cells transfected with a GFP vector) is detectable by FACS analysis. In addition to detecting the GFP, the FACS analysis enables the isolation of positive GFP clones in a more time effective manner compared to conventional methods. Following the isolation, the DNA can be stably integrated and maintained in the chromatin of cells by using selectable drug resistant markers.

The sensitivity of GFP can be quantified using a fluorometer. This will allow measuring gene expression directly by analyzing cell lysates. However, a linear range of enzyme sensitivity using pure GFP has to be established prior to cell lysate analysis. Isolation of positive stable GFP clones, the use of FACS analysis with GFP and sensitivity analysis of GFP is presently underway as part of a separate study.

Future Direction

Future directions to improve the use of GFP as a genetic reporter system include efforts to increase the intensity of fluorescence, leading to higher detection sensitivity, reduction in the variability of the signal between replicate samples. Variant GFPs with shifted emission wavelengths may allow for the study of multiple transcriptional events in the same cell population.

Some of the potential advantages of GFP as a reporter system include the easier analysis of gene expression, gene targeting/therapy, monitor the effects of drugs, hormones, toxins on the expression of proteins in living cells and the possible tracking of multiple proteins in a living organism. It also offers the possibility to behave as a screening tool by detecting gene induction before changes in the phenotype are apparent .

CHAPTER 6

SUMMARY

The primary objective of this research was to review existing genetic reporter systems and demonstrate the development and applicability of three systems : SEAP, TRAP and GFP, as gene reporters.

Various genetic reporter systems were reviewed and novel techniques using chemiluminescent and bioluminescent methods were reviewed. Brief descriptions on how genetic reporter systems work were presented along with the different types of reporter systems : *in vitro* and *in vivo*, what criteria define a good reporter system and brief comparisons of existing systems.

A literature review was made of the common reporter systems such as Chloramphenicol Acetyltransferase (CAT), Firefly Luciferase, β -Galactosidase (β -Gal), Human Growth Hormone (HGH), and the three systems of specific interest to this study : Secreted Alkaline Phosphatase (SEAP), Tartrate Resistant Acid Phosphatase (TRAP) and the Green Fluorescent Protein (GFP). It was found that the use of chemiluminescent substrates enables the ultra sensitive detection of enzymes compared to more traditional spectroscopic methods. Hence, greater levels of sensitivity forms the primary advantage in the use of luminescence in detection over more traditional methods which are radioactive or colorimetric in nature. This will enhance and simplify the study of different promoter elements as well as enhancers which influence eukaryotic gene expression.

Chemiluminescence based assays will therefore enhance reporter gene assay sensitivity in addition to being simple to use.

SEAP

Since SEAP is a secreted protein, there is no need in SEAP assays, for cell lysis. Activity can be directly measured from the conditioned media. Four SEAP vectors were constructed by co-workers and used in gene expression assays based on chemiluminescent techniques.

A study on the sensitivity of SEAP as a reporter system was performed and time course experiments conducted to quantify SEAP activity over time. The cell lines studied were CHO-K1 and COS-7. It was found that there is an approximately linear increase in SEAP activity between 24 and 72 hours in the case of the control and promoter vectors. The control vector had an activity level 10 times higher than the promoter vector illustrating the strong inductive power of the enhancer resident in the control vector.

It was further found that chemiluminescent detection based SEAP assays appear to be convenient, easy to use, fast acting and have kinetic stability. They are linear in nature over a wide range and have sensitivities over 2 orders of magnitude higher than traditional colorimetric methods using SEAP.

Frequently, normalization of reporter systems by another system is conducted to help define transfection efficiency. This is done by co-transfecting a second reporter plasmid with that of the first. The purpose of normalization is to reduce the variability between transfected replicate samples. Experiments were conducted to normalize the SEAP reporters with β -Gal using CHO-K1 cells for transfection. There was 6.94% efficiency of secretion for the pSEAP Basic, 99.11 % efficiency for the pSEAP Control and 18.29 % efficiency for the pSEAP Promoter. The lacZ gene which encodes β -Gal is used as an internal control to normalize other reporter assays such as SEAP, which helps in eliminating variance in transfection efficiency.

TRAP

A TRAP vector was first constructed based on the literature. However, due to lack of TRAP secretion the plasmids had to be modified by site directed mutagenesis. A colorimetric based assay was performed verifying work done by previous investigators demonstrating that TRAP can be used as a gene reporter system. Following this, a chemiluminescent based assay was designed and developed for the TRAP vector. It was found that the sensitivity of the TRAP vector in the chemiluminescent assay has the same order of magnitude as that of the colorimetric assay. Both assays had linear behavior over a similar range. At present, TRAP does not appear to have an advantage in using the chemiluminescent technique over other traditional methods. However, it is expected that improved chemiluminescent substrates which work in an optimal

fashion at lower pH conditions will increase the sensitivity of TRAP considerably.

Following the assay development, the detection of mammalian cells was performed using the TRAP vector showing that there was a linear increase in TRAP activity from 24 hours to 72 hours post transfection. Attempts to achieve normalization with the SEAP vector were not successful as it was found that neither of the enzymes could be detected in the conditioned medium. This is possibly due to their inhibiting one another in the secretory pathway. This places a serious limitation on the use of the chemiluminescent based TRAP vector as a complete reporter system.

GFP

The unique properties of GFP make it the first reporter system which can be used to show gene expression *in vitro*, *in vivo* and in real time. A line of GFP based fluorescent reporter vectors were developed by co-workers. This was followed by the expression of the GFP gene in several mammalian cell lines including CHO-K1, HeLa, COS-1 and NIH/3T3. Fluorescence was observed in all the four cell lines, showing the system to be fairly robust and independent of cell type. Counter staining with propidium iodide in CHO-K1 cells showed that the GFP system fluorescence persists in formaldehyde fixed cells and that GFP is well suited for double labeling with other fluorescent markers.

Sensitivity, stability and other related concerns regarding GFP were also discussed. Proper detection tools such as appropriate filter sets and fluorescent microscopy are necessary for the use of GFP as a reporter system. Fusions of GFP to heterologous proteins promise to be an excellent tool for studying protein localization and trafficking. Overall, the protein appears to be stable, sensitive and can fluoresce in the absence of other proteins, substrates and cofactors. The applicability of GFP to be a quantitative reporter system is yet to be demonstrated and is under current study. Stable transfections and FACS analysis are also under current study. If these issues can be resolved successfully, it will be one of the most powerful and effective tools to study the expression of gene activity in cells.

IMPACT OF CHEMILUMINESCENCE ON SENSITIVITY

The use of chemiluminescent techniques has lead to an improvement in the sensitivity of specific enzymatic assays. Sensitivity of a reporter system in terms of molecules detected is also a measure of its functionality. The sensitivities of the various reporter systems are best illustrated with the use of a chart (Figure 6.1). This clearly establishes that the use of chemiluminescent methods for SEAP and β -Gal appear to be the most sensitive compared to other techniques.

It is seen that the detection limit in number of molecules for β -Gal and SEAP using chemiluminescent methods is one order of magnitude lower than the

Figure 6.1 Sensitivity of Various Reporter Systems
 (Reproduced with permission from ClonTechniques [35])

bioluminescent luciferase assay, two orders of magnitude lower than a fluorometric based β -Gal assay, and between four and five orders of magnitude lower than CAT, growth hormone and colorimetric β -Gal assays. In order to establish a frame of reference, it should be noted that 10,000 molecules of β -Gal is equivalent to 10^{-13} g. GFP is not illustrated since under the present study, GFP was only being used as a qualitative means of detection and not for quantification purposes.

FUTURE WORK

Improving the sensitivity of genetic reporter systems will always be a direction in which future research work should proceed. In general, in order to enhance the detection of reporter systems, vector designs can be further improved by adding conserved sequences. 5'-non coding sequences compiled from vertebrate mRNAs or "Kozak" consensus sequences can be used [38]

There are several other potential research studies which could be performed such as the development of chemiluminescent substrates which work more optimally in more acidic (lower pH) conditions for TRAP detection. TRAP cannot currently be used to normalize the SEAP vector due to the inhibitory secretory nature of either of the enzymes. It is important to establish the reasons for such inhibitory behavior. Pulse chase experiments could possibly be designed to track enzyme outflow. Additionally, there is a need to evaluate the localization of SEAP and TRAP enzymes in co-transfection experiments. This

will better establish if the two enzymes are competing for transcription machinery.

The quantification of the GFP vector system will enhance its utility as a detection tool. Several researchers have already developed mutants of wild type GFP that can be used to track different proteins. However the use of GFP as an efficient genetic reporter system should be improved by increasing the intensity of fluorescence. Detection sensitivity may be improved by the development of filter sets accommodating for all the variety of GFP mutants available.

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