Review article

A New Reporter Gene Technology: Opportunities and Perspectives

Gholam Reza Salehi Jouzani[1,2] and Irina Vasilievna Goldenkova*[2]

[1] *Department of Microorganisms, Biosafety and Gene Bank, Agricultural Biotechnology Research Institute of Iran (ABRII), Mahdasht Road, 21525-1897, Karaj, I.R. Iran* [2]*Vavilov Institute of General Genetics, Russian Academy of Sciences, Gubkina St., 3 GSP-1, 119991 Moscow, Russia*

Abstract
The paper summarizes the current status of the reporter gene technology and their basics. Reporter gene technology is widely used to monitor cellular events associated with gene expression and signal transduction. Based upon the splicing of transcriptional control elements to a variety of reporter genes, it "reports" the effects of a cascade of signaling events on gene expression inside cells. The principal advantage of these assays is their high sensitivity, reliability, convenience and adaptability to large scale measurements. The reporter gene systems β-galactosidase (LacZ), luciferases (LUC), β-glucoronidase (GUS), green fluorescent protein (GFP) and GFP-like proteins, widely used presently, are compared. The potentialities of a new reporter system, based on thermostable lichenase (β-1, 3-1, 4-gluconases) of Clostridium thermocellum are described in detail. Advantages and disadvantages of the used reporter systems, and also the opportunities for development and application of bifunctional reporter systems are considered. Perspectives of reporter genes for studying gene expression regulation in prokaryotic and eukaryotic organisms are elucidated.
Keywords: *Reporter genes; β-galactosidase (LacZ); Luciferase (LUC); β-glucoronidase (GUS); Green fluorescent protein (GFP); GFP-like proteins, Lichenase (LicB).*

Table of contents:

Correspondence to:* **Irina V. Goldenkova, Ph.D.
Tel: +7 95 1320874; Fax: +7 95 1351289
E-mail: irengold@vigg.ru

INTRODUCTION

In recent years genomic sequences of many species (viruses, prokaryotes and eukaryotes) were determined. However, revealing the nucleotide sequence does not always give a clear idea about its functional significance. A study of regulation of genes expression is very important for understanding functional mechanisms operating within the organism. Gene expression can be regulated at different levels, i.e. at chromatin organization, transcription, translation and processing.

In any living cell external and internal signals are realized through activation of different transcription factors and affect gene expression. Receptors located on the cell surface (or inside it) implement perception of such signals. Subsequent signal transduction can be

implemented by different pathways and result in the interaction of transcription factors with specific nucleic acid sequences (response elements) in the promoter regions of the appropriate genes. The application of chimeric sequences, in which such regulator elements control expression of the reporter gene, allows to observe signaling, to reveal the concrete participants of transduction and to evaluate their effect on expression and regulation of genes (the reporter gene methodology). Such an approach facilitates considerably the realization of many studies, as it is much easier to find and observe the product of a reporter gene rather than of protein encoded by the gene under study.

A variety of reporter gene systems are available and used at present. The most widely used systems employ chloramphenicol acetyltransferase (CAT) from *Escherichia coli* (Gorman, 1985), β-galactosidase (LacZ) from *E. coli* (An *et al.,* 1982), bacterial and firefly luciferases (Lux) (Ow *et al.,* 1986 and Olsson *et al.,* 1988), β-glucuronidase (GUS) from *E. coli* (Jefferson *et al.,* 1986, 1987, 1997), and the green fluorescent protein (GFP) from *Aequorea victoria* (jellyfish) (Chalfie *et al.,* 1994).

The choice of a reporter system is determined by a number of important criteria. One of these is the availability of simple and highly sensitive methods for a qualitative and quantitative assay of reporter protein activity in the presence of other cellular components, which obviates the need for multiple purification steps prior to assay. Among other critical factors are the ability to follow dynamic changes in gene expression, the absence of similar activities in the cells of the studied organism, and the sizes of the reporter gene and its product (Joyeux *et al.,* 1997). The choice of a suitable reporter system also depends upon the goals of each particular experiment. For instance, if one needs to monitor gene expression in real time in a single living cell, the GFP reporter system is probably the most suitable. If, however, monitoring in real time is not a priority, and it is necessary only to determine transcriptional activity and the inducibility of a promoter or another regulatory element, other reporter systems characterized by highly sensitive and simple measurement of the reporter protein activity are preferable. For bacterial and yeast cells the LacZ system is the system of choice, while for mammalian cells LacZ, β-lactamase, and Lux systems may be used (Naylor *et al.,* 1999 and Rutter *et al.,* 1995).

The thermophilic bacterium *Clostridium thermocellum* produces a high molecular weight extracellular multienzyme complex that hydrolyzes polysaccharides containing 1,3 and 1,4 mixed-linkage β-glucans (Piruzian *et al*., 1985 and Schwarz *et al*., 1987). Lichenase from *C. thermocellum* is a thermostable enzyme that specially hydrolyzes β-1,4 linkages adjacent to β-1,3 linkages in mixed-linkage β-glucans, but does not attack pure 1,3 or 1,4 linkages. β-1,3;1,4-glucans are not found in a lot of prokaryotic and eukaryotic organisms. These findings suggested that thermoactive β-glucanase can be used as a suitable reporter enzyme in many prokaryotic and eukaryotic organisms (Goldenkova, 2002; Piruzian *et al*., 1998 and 2000).

The potentialities of different reporter genes and types of their application are very broad. The aim of this paper is to provide an overview of this technology, including the types of reporter genes commonly in use and the new reporter gene lichenase, and to consider the main applications and prospects for this technology in the future.

Methodology of reporter systems: The term reporter gene is used to define a gene with readily measurable phenotype that can be distinguished easily over a background of endogenous proteins. All genes have an upstream promoter sequence that can be isolated and placed in front of a heterologous reporter gene. This type of promoter-reporter construct can be introduced into cells and the activity of the reporter gene product monitored in the living cell by different methods. Reporter gene technology involves controlling the activity of such genes by defined cis-regulatory sequences, which are responsive to alterations in gene regulation and expression in host cells. A number of hormones and growth factors have been shown to stimulate target cells by activating second messenger pathways that in turn regulate the phosphorylation of specific nuclear factors to alter gene transcription (Rutter *et al.,* 1995 and 1998; Jefferson *et al.,* 1997).

For example, extracellular signals may be detected by receptors at the cell surface, including G-protein coupled receptors, ion channel linked receptors, receptors containing intrinsic enzymatic activity, or receptors that recruit intracellular tyrosine kinase. These receptors activate intracellular signal transduction pathways and lead to the phosphorylation of a variety of transcription factors by different protein kinases, which then bind specifically to response elements in the promoter regions of hormone-responsive genes. To understand the relationship between the activation /inhibition of different pathways and their effects on gene expression, specific response elements have been fused to reporter genes. Activation of appropriate signal transduction pathways then alters the expression of the reporter gene, providing a simple method for monitoring their effects on gene expression. For example,

figure 1 shows that promoter activity is regulated by signal from receptor on the plasmatic membrane and thus the expression level of the reporter gene depends on power of this signal (Goldenkova, 2002; Suto and Ignar**,** 1997 and Rutter *et al.*, 1995).

It should be noted that reporter genes encode proteins which have some unique features or unique enzymatic activities and owing to those they can be easily identified in mixtures of endo/exocellular proteins.

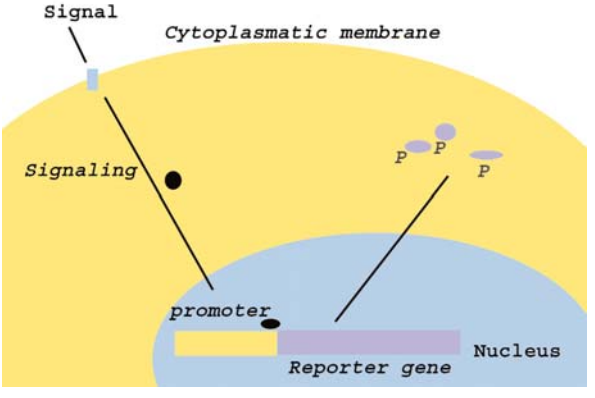

Figure 1. Principles of reporter gene detection in living cells, P: Denotes a reporter protein adopted from Rutter *et al.*, 1998.

Requirements for reporter gene systems: Reporter genes and their products must meet a number of requirements. One of these is simplicity and high sensitivity of quantitative and qualitative methods for determination reporter protein activity against the background of other cell components. Another important property is the ability to conduct dynamic observations of both increase and decrease of gene expression (in this case, important characteristics are half-life and/or duration of protein maturation), the absence of similar activities in the cells under study and also the size of reporter gene and its product (for convenience of cloning and analysis of reporter protein expression). An important property of a reporter protein is its ability to tolerate other proteins fused at its N- and C-termini without a loss of activity (Jefferson *et al.*, 1986, 1987 and George *et al.*, 1997). A number of genes have been used and will be used for analysis of different aspects of gene expression, some of which are discussed below.

Characteristic of reporter systems: advantages and disadvantages: In general, reporter genes have the advantage of low background activity in cells but amplify the signal from the cell surface to produce a highly sensitive and often easily detectable response. The choice of a reporter, however, will depend on the cell line used (endogenous activity), the nature of the experiment (e.g. dynamics of gene expression versus transfection efficiency) and the adaptability of the assay to the appropriate detection method (e.g. single cell imaging versus biological screen).

Chloramphenicol-Acetyltransferase (CAT) from *Escherichia coli:* The gene encoding chloramphenicol-acetyltransferase (CAT) from *E. coli* was discovered owing to the ability of cells to neutralize the action of chloramphenicol, an inhibitor of protein synthesis. CAT catalyzes the reaction of acetyl group transfer from acetyl-coenzyme A (acetyl-CoA) to hydroxyl group of chloramphenicol (at carbon position 3). The transfer of acetyl group to the hydroxyl group at position 1 occurs spontaneously. Thus, repeated acetylating becomes possible at position 3 (Fig. 2).

Acetylated and not acetylated forms of chloram-

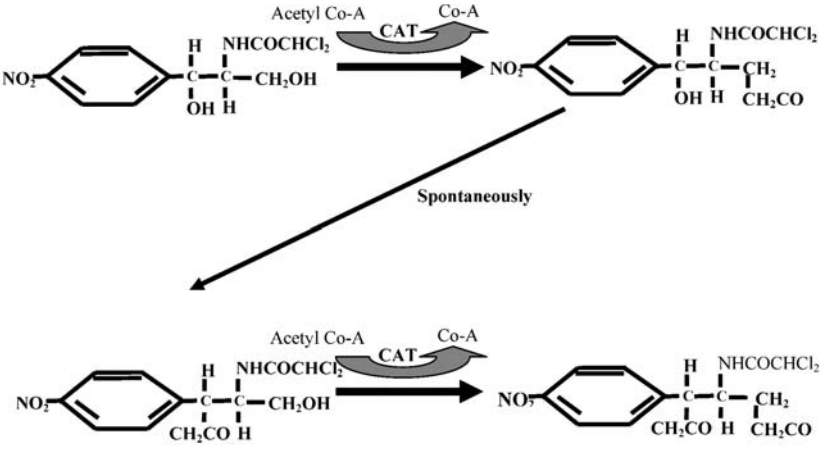

Figure 2. Reactions catalyzed by chloramphenicol acetyltransferase (CAT).

phenicol have different solubility in organic solvents and can be easily separated by thin-layer chromatography. The enzymatic activity may be assayed using radio-labeled [^{14}C]-chloramphenicol, and then the acetylated form is separated and its radioactivity is measured (Gorman, 1985). Also, [^{3}H]-acetyl-Co A, which is cheaper than [^{14}C]-chloramphenicol, can be used. In this case solvents are chosen, in which both forms of chloramphenicol are soluble, but acetyl-CoA is insoluble. To eliminate possible endogenic deacetylating activities, the investigated extracts can be treated at 60°C for 10 minutes which does not have any effect on CAT activity, by contrast to other, less thermostable similar enzymes of the studied organism (Crabb *et al.,* 1987). One of the advantages of this reporter system is the absence of background activity in the majority of model organisms. However, the assay relies on radiochemicals, and although an automated ELISA is now available, the linear range and sensitivity of the assay are not as broad as for other reporters (Bronstein *et al.,* 1994 and Pazzagli *et al.* 1992).

Bacterial (*Vibrio harveyi* and *V. fishery*) and *Photinus pyralis* luciferases (Lux): The name luciferase refers to a family of enzymes that catalyze the oxidation of various aldehyde substrates (e.g. luciferin, coelenterazine) resulting in emission of light. The most commonly used luciferases for reporter gene assays are the bacterial luciferases (from *Vibrio harveyi* and *V. fishery),* the firefly luciferases and more recently the Renilla luciferases (Baldwin *et al.*, 1984 and Endebrecht *et al.*, 1984). The reaction of substrate oxidation by luciferase is shown below. Here R is an aliphatic part containing no less than 7 carbons; FMN is flavin mononucleotide, and $FMNH_2$, reduced FMN.

$$RCHO + O_2 + FMNH_2 \longrightarrow RCOOH + FMN + hv\ (490\ nm)$$

Also the luciferase from the firefly *Photinus pyralis*, which catalyzes ATP-dependent oxidation of various substrates (luciferin, coelenterazine) accompanied by light emission at 490 nm, has been used as a reporter gene (de Wet *et al.*, 1985 and 1987). The intensity of light can be measured and it is directly proportional to quantity of luciferase in the sample. Sensitivity of the method is limited only by background noise of the measuring device. The activity of luciferase can be assayed *in vivo* because the substrates (luciferin esters for the *P. pyralis* luciferase and coelenterazine for the *Renilla* luciferase) can penetrate mammalian cell membranes (Craig *et al.*, 1991).

Bacterial luciferases are generally heat-labile and dimeric proteins, which limits their use as reporters in mammalian cells. A sensitive reporter system capable of monitoring transcriptional activity within living bacterial cells has been developed recently using bacterial luciferase. In this system, the aldehyde substrate required for the assay is expressed constitutively inside the cell. However, the linear range of this assay is still only about 3 orders of magnitude (Manen *et al.*, 1997; Pazzagli *et al.*, 1992). The firefly luciferase, on the other hand, has been one of the most popular reporter genes in mammalian cells because of its high sensitivity and broad linear range (up to 7-8 orders of magnitude). A limitation of the original assay arose from the requirement to lyse the cells prior to addition of the substrate luciferin and detection of the response (Joyeux *et al.*, 1997; Welsh and Key, 1997). However, the use of membrane-permeable and photolysable firefly luciferin esters has obviated the need for cell disruption. Secondly, the development of "glow" reagents (e.g. LucLite, Packard) has increased the duration and stability of the flash response such that it can be detected in a scintillation counter, making it also suitable for high throughput screening (George *et al.*, 1997, 1998; Lorenz *et al.*, 1996 and Suto *et al.*, 1997). Renilla luciferase may be a particularly appropriate reporter for intact (living cells) systems because this luciferase catalyses the oxidation of coelenterazine, which is membrane permeable.

β-Galactosidase (LacZ) from *E. coli:* β-Galactosidase (LacZ) from *E. coli* is a tetramer protein, which catalyzes hydrolysis of different β-galactosides, including lactose. β-Galactosidase is a well-characterized bacterial enzyme and has been one of the most widely used reporter genes in molecular biology as a monitor of transfection efficiency. The schemes of reaction catalyzed by β-galactosidase (a) and β-glucoronidase (b) are shown in figure 3 ("R" is a sugar residue).

The polypeptide chain of the monomer contains 1023 amino acid residues and the molecular mass of the protein product is about 116 kDa. An interesting feature of this enzyme is that its active site is formed by two subunits, thus, one native enzyme carries four active sites. The activity of β-galactosidase is assayed using o-nitrophenyl-β-D-galactopyranoside as a substrate. This enzyme is also suitable for histochemical assays; in this case the substrate used is 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). The enzyme hydrolyzes this substrate to indolyl, with the subsequent oxidation to indoxyl and formation of a derivative of indigo dye having blue color. Ferrocyanide or mixture of ferricyanide and ferro-

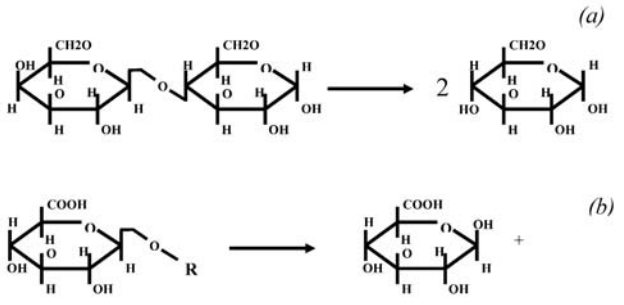

Figure 3. A scheme of reactions catalyzed by β-galactosidase (a) and β-glucoronidase (b), "R" is a sugar residue.

cyanide are used as catalysts of this reaction (Alam and Cook, 1990).

Simple colorimetric assays of poor and narrow dynamic range have largely been replaced with more sensitive and adaptable bio- or chemiluminescent assays. Application of this reporter allows conducting tissue-specific assays of genes expression. The LacZ reporter system is widely employed for efficient monitoring of transformation of bacterial and mammalian cells. This reporter has the advantage over CAT in that the assays tend to be simpler and do not involve the use of radioisotopes. However, there may be an endogenous β-galactosidase activity in mammalian cells, which can be reduced at higher pH values (Alam and Cook, 1990).

***E. coli* β-glucoronidase (GUS):** The *E. coli* β-glucoronidase (GUS) (EC.3.2.1.31) is a hydrolase that catalyzes the cleavage of a wide variety of β-glucuronides. There are many commercial substrates for assaying glucoronidase activity spectrophotometricaly (P-nitrophenyl glucuronide (PNPG)), flourometricaly (4-methyl umbelliferil glucuronide (MUG)) and histochemically (5-bromo-4-chloro-3-indolyl glucuronide (X-GLUC)) (Jefferson *et al.*, 1987). This enzyme has a monomer molecular weight of about 68.2 kDa (603 amino acid residues), although under certain conditions of SDS-PAGE it migrates a bit slower than would be predicted (around 74 kDa) (Jefferson *et al.*, 1986 and 1987). The behavior of the native enzyme on gel filtration columns indicates that it is probably a tetramer. β-glucoronidase is very stable and will tolerate many detergents, widely varying ionic conditions and general abuse.

Among disadvantages of this reporter system the following may be noted: the GUS activity is inhibited by some heavy metals ions (Cu^{2+} and Zn^{2+}); the protein is characterized by considerable half-life period (GUS is reasonably resistant to thermal inactivation with a half-life at 55°C of about two hours), therefore quick transcription changes of the reporter gene will not result in quick changes of the reporter protein activity (Mascaronhas and Hamilton, 1992; Quaedvlieg *et al.*, 1998). Besides, diffusion of hydrolysis products of X-gluc in plant cells and hydrolysis of this substrate by plant intra-cell peroxidases were showen. All this can results in severe artifacts (Guivarch *et al.*, 1996 and Taylor, 1997). Moreover, GUS assays are destructive to the plant material, which is inconvenient for following the expression of a given gene in time.

Green fluorescent protein (GFP) from Aequoria victori: The green fluorescent protein is a unique *in vivo* reporter capable of emitting green light (509 nm) when illuminated by ultraviolet or blue light (395, 475 nm). GFP, as can be deduced from its cDNA, is a 238 amino acid protein with an apparent molecular weight of about 27-30 kDa on SDS-PAGE; it forms a three-dimensional β-barrel structure (Chalfie *et al.*, 1994). GFP has a unique property that it forms a chromophore of three amino acids within its primary structure and, in contrast to other bioluminescent molecules, functions independently of cofactors (Fig. 4) (Brejc *et al.*, 1997; Cody *et al.*, 1993 and George *et al.*, 1998). Its chromophore is formed by cyclisation and oxidation of the three amino acids Ser65, Tyr66 and Gly67. The wild type GFP has two absorption maxima: a major peak at 395 nm and a minor one at 475 nm. Excitation at either of the two wavelengths results in emission of green light at 508 nm. These fluorescence properties have been changed by genetic engineering leading to several mutants. A wide range of mutant forms of GFP have been developed that have altered spectral properties (e.g. Tyr66 to His, or "blue fluorescent protein", BFP; λ_{ex} = 383 nm, λ_{em} = 447), considerably increased brightness (e.g. Ser65 to Thr; λ_{ex} = 489 nm, λ_{em} = 511 nm) or increased folding and stability (e.g. Tyr145 to Phe) in mammalian cells (Table 1) (Hein *et al.*, 1994). In theory, therefore, using relatively inexpensive equipment it should be possible to use GFP as a reporter for studying gene expression in single living cells without disturbing their integrity.

The greatest advantage of GFP is that in the absence of cell lysis, noninvasive monitoring of gene expression in living tissues is possible, the protein can be visualized directly without the addition of exogenous substrates or cofactors and it is not toxic. The physicochemical properties of GFP suggest that it is not suitable for monitoring rapid changes in gene expression (over the minute to hour range). This is largely because, when it is expressed in cells, maturation of GFP (i.e., the folding and formation of the fluorescent

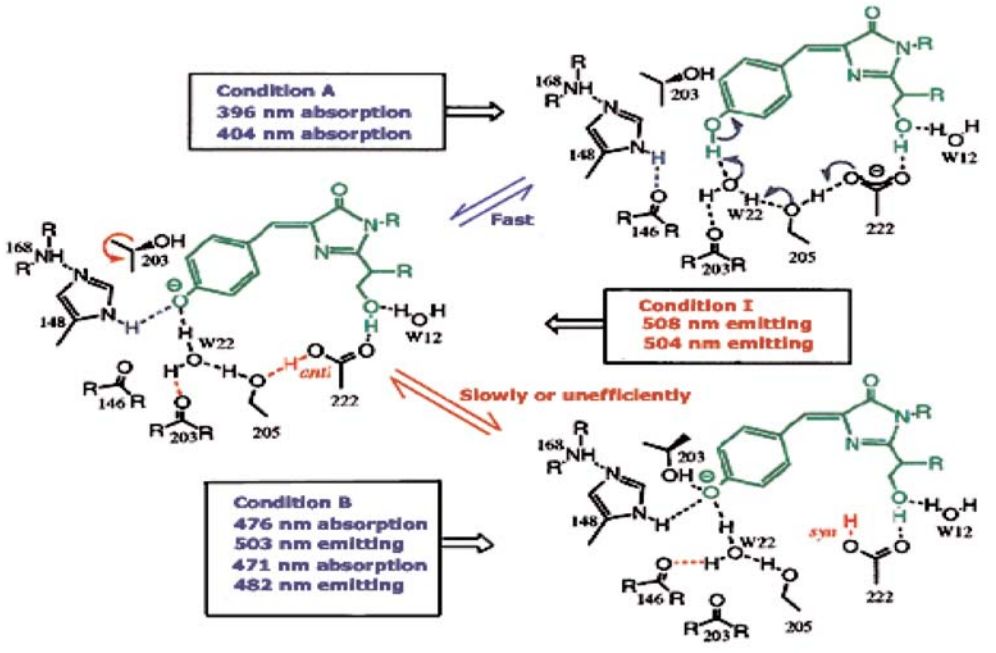

Figure 4. The mechanism of GFP photoisomeric changes. The condition "A" occurs predominantly in the wild type GFP, condition "B" in mutant S65T and condition "I" is a transient state.

Table 1. Properties of the wild type green fluorescent protein (GFP) and it mutants.

Denotation	Amino acid replacement	Light absorption, (nm)	Light emittion, (nm)
GFP	------	395 and 475	510
S65T	Serine 65- threonine	490	511
EBFP	Tyrosine 66- histidine	380	440
EGFP	Phenylalanine 64- lysine, serine 65-threonine	488	507
EYFP	Serine 65- glycine, valine 68- lysine, serine 72- alanine	513	527
ECFP	Tyrosine 66- tryptophan	433; 453	475; 501

Note: Only mutations in the chromophore region which result in changes of absorption and emitting spectra are presented.

protein) requires a finite time, generally 2-4h, although this problem is less acute with mutant GFP variants. Furthermore, the expression of GFP can readily be detected when driven by strong, promoters usually viral, but the utility of GFP for studying the activity of weaker mammalian promoters is considerably limited. Another problem, which has been suggested as a drawback to the use of GFP, is the stability of the protein. A more serious problem is that fluorescence measurements pose problems for quantitation, given day to day variation of the detection apparatus (e.g. the performance of the lamps, detecting camera, etc.); the lack of linearity between protein concentration and fluorescence intensity; and the interference from cellular auto-fluorescence (Sniezko *et al*., 1998 and Stern *et al.*, 1998).

GFP-like proteins: Recently, six GFP-like proteins from non-bioluminescent species of corals, *Anthosoa*, have been cloned. Two of them (zFP538 and drFP583) have light emitting peaks in the yellow and red regians, which distinguishes them from *A. victoria* GFP, whose emission peak lies in the green regain. These proteins are convenient reporters for simultaneous tracking the expression of two regulator elements. Their important characteristic is also a very high sta-

bility in the cells, much higher than that of EGFP (Enhanced GFP). Another protein (dsFP593), with the emission peak at 593 nm was cloned from the coral *Discosoma*. Using the "error-prone PCR" method of mutagenesis, mutants drFP583 and dsFP593 were obtained, which encode proteins with unique spectral properties; and ds/drFP616 with emission peak in the far red area (615 nm) and E5 (on the basis drFP583), which changes fluorescent spectrum from green (at the initiation of expression) through yellow and orange to red (at the termination of expression). Thus, E5 can act as a fluorescent clock, allowing to obtain information about spatial and temporary activity of the studied regulator element. Green fluorescence will indicate a recent activation of the regulator element, yellow and orange fluorescence will mean that expression from the regulator element occurs continuously, whereas cells and tissues having red fluorescence will suggest cessation of the expression after a period of activity (Terskikh *et al.*, 2000).

Lichenase from *Clostridium thermocellum*: A novel reporter system usable in bacteria, yeasts, plants and mammals, based on thermostability of lichenase *C. thermocellum*, was developed in the laboratory of functional genomics, Vavilov Institute of General Genetics, Russian Academy of Sciences, headed by professor Piruzian (Piruzian *et al.*, 1998, 2000 and 2002).

The capabilities of this reporter system will be described in more detail due to the fact that it has been proposed recently and has not been used widely since then. The thermophilic bacterium *C. thermocellum* produces a high molecular weight extracellular multienzyme complex which hydrolyzes polysaccharides containing β-1,3 and β-1,4 mixed-linkage β-glucans (Piruzian *et al.*, 1985 and Schwarz *et al.*, 1987). Lichenase from *C. thermocellum* is a thermostable enzyme that specially hydrolyzes β-1,4 linkages adjacent to β-1,3-linkages in mixed-linkage β-glucans, but does not attack pure β-1,3 or β-1,4 linkages (Fig. 5). β-1,3;1,4-glucans are not found in many prokaryotic and eukaryotic organisms, suggesting that the thermoactive β-glucanase can be used as a suitable reporter enzyme in many prokaryotic and eukaryotic organisms (Goldenkova, 2002; Piruzian *et al.*, 1998 and 2000). The small size of the gene and its product facilitates DNA manipulations and protein fusions in the course of reporter gene engineering.

The detection of the thermostable reporter enzyme is simple and sensitive; in particular, the *in situ* zymogram technique and plate test are very sensitive, quick, inexpensive and convenient for primary screening of

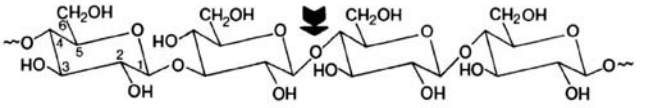

Figure 5. A scheme of reactions catalyzed by lichenase.

transgenic organisms. Furthermore, molecular masses of fused proteins in protein fusion studies can be simply determined by staining the gels with Congo red. There are some sensitive methods for quantitative and qualitative assaying of lichenase activity. Lichenase activity is measured in a plate test according to protocol by Teather and Wood (1982). Bacterial or yeast colonies are seeded onto plates containing an appropriate medium. The colonies that appear are covered with agarose containing 0.1% lichenan. The plates are then incubated for 1-5h at 65°C, stained for 15 min with 0.5% Congo red solution and washed with 1 M NaCl until transparent spots of the hydrolyzed substrate become apparent.

Lichenase activity in the lysates is determined as described previously at 65°C using lichenan as substrate (Piruzian *et al.*, 1998). Reducing sugars released from the substrate are assayed with the dinitrosalicyl reagent as per the method of Wood and Bhat (1988). The reaction mixture contains 200 μl of 0.5% lichenan and 100 μl of the protein sample. It is incubated for 10-20 min, then 1.2 ml of the dinitrosalicyl reagent is added, and the mixture is heated at 100°C for 15 min. The concentration of the colored product is determined with a spectrophotometer. The enzyme is characterized by the zymogram method described earlier (Piruzian *et al.*, 1998). The bands of active enzyme appear as clear spots since the dye binds only to unhydrolyzed lichenan.

The quantitative assay can measure about 25 ng of lichenase in 10 minutes. Because of high stability of lichenase (5h at 65°C), the incubation period can be extended, thus increasing the sensitivity.

When promoter activity is measured by the quantitative and qualitative methods, lichenase shows the sensitivity and measurement ranges equivalent to those of β-galactosidase. Like other widely used reporters, such as luciferase, CAT, β-glucuronidase and β-lactamase, lichenase also requires a substrate. The thermostability of the enzyme allows to get rid of endogenic activities, as the majority of enzymes lose activity at the optimum temperature of this enzyme (about 65°C). All these properties of the bacterial lichenase have allowed offering a new reporter system based on lichenase or other prokaryotic and eukaryotic model organisms (Piruzian *et al.*, 2002).

A study of expression of the modified bacterial lichenase gene (designated *licBM2*) in *E. coli*, yeast, mammalian and plant cells has shown that the enzyme has many properties indispensable for a reporter system (Salehi Jouzani *et al*., 2005). The thermal stability of lichenase allows an easy assay of expression of *licBM2* using both qualitative and quantitative methods against a background of thermo-labile enzymes of these organisms. In all cells lichenase is produced in the active form (Fig. 6 and 7, zymogram and plate test) (Goldenkova *et al*., 2002).

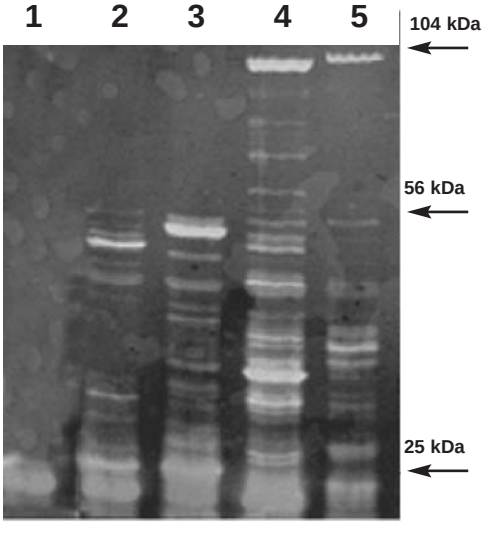

Figure 6. Zymograms of protein extracts obtained from bacterial, yeast and mammalian cells in the presence of 0.1% lichenin. Lane1: protein extract of *E. coli* cells carrying a plasmid with gene *licBM2 from* lanes 2 and 3: protein extracts of mammalian cells (line PC12) carrying plasmid with the hybrid genes from *licBM2-gfp* (lane 2) and *gfp-licBM2* (lane 3). Lanes 4 and 5: protein extracts from yeast cells carrying a plasmid with the hybrid genes *licBM2-gus* (lane 4) and *gus-licBM2* (lane 5). The arrows showing the appropriate molecular masses mark bands of lichenase activity and the bifunctional proteins LicBM2-Gfp, Gfp-LicBM2, LicBM2-Gus and Gus-LicBM2.

Results of the plate test demonstrate that lichenase gene can be offered as a potential selective marker for selection of bacterial and yeast transformants, and of transgenic plants. Because products of the licBM2 substrate (lichenan), unlike the non-hydrolyzed substrate, are not colored with Congo red, lichenan can be used as a convenient substrate for selection of bacterial and yeast colonies, expressing licBM2 gene under control of constitutive and inducible promoters. Such selection of clones is very convenient for fast selection of bacterial and yeast transformants.

One of the key situations of gene expression regulation occurs at turning transcription on and off. Our studies show that lichenase is suitable for such experiments on bacterial and yeast cells as well as plants (Piruzian *et al.*, 1998 and 2002). In these experiments, the LacZ promoter (bacteria), the GAL1 promoter, which is strictly induced by galactose and repressed by glucose (yeast cells) and the promoter of RuBiCS small subunit, which is induced by light (plant), were employed. When inducible promoters were used for expression of the lichenase gene, in all studied cells fast changes of enzyme activity in response to the appropriate inductor or repressor were observed. These results show that lichenase can be used in dynamic studies.

It has proved possible to use lichenase in experiments on secretion and transport of proteins, at least in plant cells, using a sequence encoding the leader peptide of extensin (from carrot). This peptide is capable of providing effective secretion of proteins into the intercellular spaces of plants. The results on testing lichenase activity in the intercellular space open up the opportunity of studying secretion and compartmentalization of proteins in plant cells. Background activities as well as changes in growth of transformed cells were not observed in any of the systems studied.

Bifunctional reporter systems: It should be noted

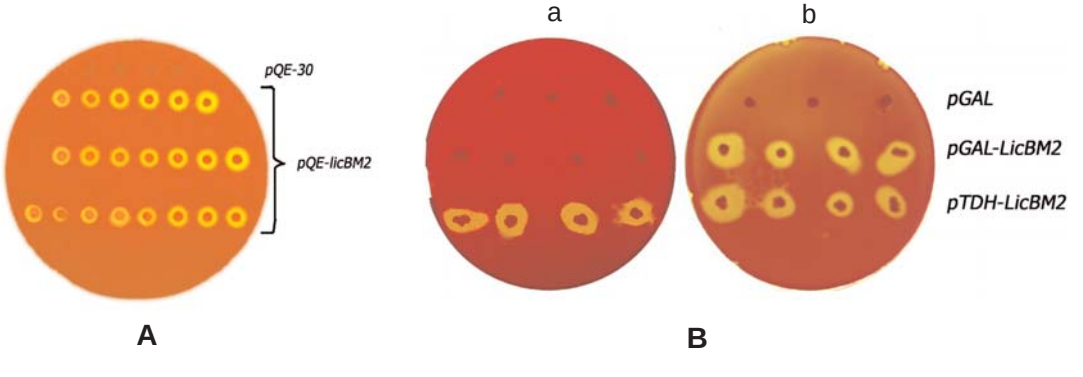

Figure 7. A plate test for assay of lichenase activity in bacterial cells (A) and yeast cells (B) grown on a medium containing glucose (a) and galactose (b).

Table 2. Properties of GFP, LicBM2 and GUS reporter systems.

Denotation	Amino acid replacement	Light absorption, (nm)	Light emittion, (nm)
GFP	------	395 and 475	510
S65T	Serine 65- threonine	490	511
EBFP	Tyrosine 66- histidine	380	440
EGFP	Phenylalanine 64- lysine, serine 65-threonine	488	507
EYFP	Serine 65- glycine, valine 68- lysine, serine 72- alanine	513	527
ECFP	Tyrosine 66- tryptophan	433; 453	475; 501

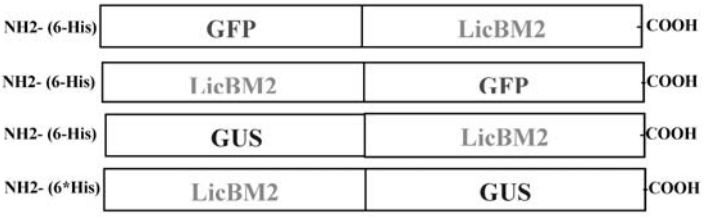

Figure 8. Bifunctional reporter proteins. GFP denotes green fluorescent protein, GUS, β-glucuronidase, LicBM2, Lichenase; N and C are the N-terminal part and the C-terminal part of the bifunctional protein.

that none of the reporter systems used is universally applicable; each has its own advantages and disadvantages, which may limit its use in some cells of model organisms and in certain types of studies. Therefore, it is desirable to have a number of reporter systems available for the same cells. The studies on the modification of the widely used reporter systems, elaboration of the methods used for measuring reporter activity and screening of new reporters are continuing. These discussed limitations of reporter systems can be solved with application of the strategy described below.

In the table 2 the advantages and disadvantages of some reporter systems, GFP, GUS and the offered by us licBM2 are summarized so that to find out what system can be more appropriated for a particular study. The data suggest that by uniting two reporter systems, limitations of one can be compensated with advantages of the other. For this purpose we investigated a possibility to use fused sequences in order to bring together advantages of two reporter systems.

Bifunctional reporter proteins were constructed on the basis of lichenase (LicBM2) and green fluorescent protein (GFP), and also of lichenase and *E. coli* β-glucuronidase (GUS) (Fig. 8). Our results with these systems showed that as bifunctional hybrid proteins, lichenase, GFP and GUS save their main properties: lichenase remains active at 65°C, GFP shows fluorescent property, and GUS hydrolyzes appropriate substrates (Fig. 9).

These experiments also have demonstrated that lichenase tolerates quite long sequences in the N- and C- termini and can be utilized for translational fusion with sequences of studied proteins. Thus, in bifunctional proteins lichenase remains active and the bands of its activity correspond to molecular masses of hybrid proteins (104 kDa for GUS-LicBM2 and LicBM2-GUS; 56 kDa for GFP-LicBM2 and LicBM2-GFP) (Fig 9). This property allows applying the zymogram method for lichenase instead of Western blotting. The data that the main properties of lichenase (activity) and GFP (fluorescence) are preserved in bifunctional hybrid proteins suggest proposing them as more appropriate bifunctional reporters for assaying regulation of genes expression in cells of prokaryotic and eukaryotic organisms.

Application of new reporter gene technology (lichenase and other reporters): The methodology of reporter systems allows to control the temporary and spatial expression of genes during such complex processes as the cell cycle, the effects of hormones, growth factors, nutrient materials and environmental factors on the cell; to study mechanisms of sorting, transport and endocellular localization of proteins; to

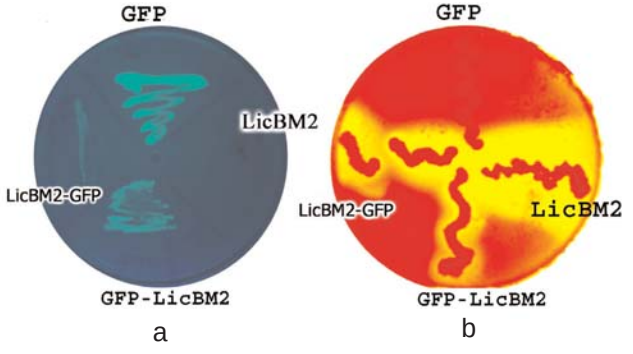

Figure 9. A plate test for the assay of GFP (a) and LicB (b) activity within GFP-LicBM2 and LicBM2-GFP hybrid proteins.

conduct analysis of cell lines for medicinal sensitivity or resistance; to assay gene delivery, and for other purposes. The main areas of reporter system applications are examplified below.

Studying activity and induciblity of promoters and regulator elements (transcriptional reporters): The reporter gene technology was first used as a method to study the activity of cis-acting genetic elements such as enhancers and promoters in the upstream regions of genes. Those reporter systems that have highly sensitive and simple quantitative assays of reporter protein activity and, at the same time, allow conducting dynamic monitoring of turning on and off transcription are used. These genes should have short product half-life and/or maturation time, so that the quick changes of reporter gene transcription can be followed. Various cis- or trans acting elements, such as enhancers and repressors can participate in regulation of transcription. Their participation during regulation of gene expression can also be studied using reporter genes, placing these sequences before the gene fused with promoter, after it or even inside the sequence encoding reporter protein.

Reporter systems help to study the circadian rhythms of plants (Michelet and Chua, 1997) as well transcription regulations of many insect (Brandes *et al.*, 1996) and mammalian genes (Rutter *et al.*, 1995 and 1998; Watson *et al.*, 1998 and White *et al.*, 1995). Reporter systems allow to pinpoint functions of regulator sequences in regulation of gene expression in response to action of different factors (hormones, interleukins, transcription factors, etc.) and also to identify genes whose products are responsible for some human diseases (galactocerebrosidase, mammaglobin). Modular promoter sequences responsive to estrogens, androgens, thyroid hormone, Ca^{2+}, nitric oxide and various transcription factors that are responsible for regulating gene expression have also been identified and used to selectively alter the level of expression of different genes (Bamberger *et al.* 1997 and Luzi *et al.*, 1997).

Search for new promoters and regulator elements: For these purposes the following strategy is applied: at first a construct is created which contains the reporter gene sequence without the promoter. Such construct is then transferred into cells of the target organism, and the presence of reporter protein in the transformed cells shows that insertion of the reporter gene sequence has occurred in such a manner that a constitutive promoter in the host genome controls the expression. An analysis of mRNA size of the reporter gene allows to evaluate the distance from transcription start point to the beginning of the reporter gene, and then to clone this area. Using the same strategy it is also possible to search for inducible promoters.

Imaging of gene expression (studying the localization of gene products): Reporter systems can be applied to determine localization of the studied gene product. Such studies are done using chimeric sequences which consist of the reporter gene fused in frame with the sequence encoding the product under study, or with a leader peptide or signal a sequence. The signal sequences ensure transport of the protein products to the appropriate cell compartments or to the environments (Rossi *et al.*, 1997).

Historically, the spatial organization of gene expression in plants and animals was measured using colorimetric or fluorimetric assays employing β-glucuronidase and β-galactosidase as markers. However, the development of luciferase and GFP as noninvasive markers of gene expression, combined with ease of detection using sensitive charge coupled device imaging cameras and fluorescence microscopy, has allowed for temporal and spatial information about gene expression even at the single cell level.

In the case of GFP, the creation of mutant isoforms of the protein with altered spectral properties has not only permitted the simultaneous tracking of different proteins within living cells but also allowed the interactions between or within proteins to be measured. Fluorescence resonance energy transfer (FRET) has been used to monitor both intermolecular and intramolecular interactions between proteins in living cells using fast imaging flow cytometry and confocal microscopy. Such techniques have been used to monitor the dimerisation of the pituitary specific transcription factor Pit-1 and the conformational changes asso-

ciated with Ca^{2+} binding to calmodulin using a calmodulin binding sequence flanked by two GFP variants (Naylor *et al.*, 1999).

The key advantage of reporter gene technology for monitoring gene expression and transfer, therefore, is the ability to gain both temporal and spatial information about a particular gene product, even at the single cell level. Imaging of proteins tagged with reporters in live cells will not only help to decipher the subcellular localization, trafficking, and interactions between specific proteins but also will allow for the monitoring of multiple proteins at one time, including changes in their local environment in response to extra- or intracellular events (Goegan *et al.*, 1998; Naylor *et al.*, 1999; Stern *et al.*, 1998 and Wiznerowicz *et al.*, 1997).

Gene delivery: Reporter genes have been used as markers for monitoring of gene transfer and localizations of transgenes (gene therapy) using a number of different transformation technologies, i.e. allow to determine "successful" genes delivery to specific cells. Bicistronic vectors expressing both the reporter and a gene of interest have been used to monitor gene transfer and as a screening strategy to identify successfully transformed cells. For example, fluorescence activated cell sorting has been very powerful in detecting gene transfer in plants, yeasts, and mammalian cells using GFP as a marker. Reporter gene expression had been applied to the field of gene therapy, where it has been used to monitor the delivery, location, and pattern of transgene expression. Genes encoding therapeutic proteins for the treatment of disease have been introduced into mammalian cells using a variety of techniques including viral and retroviral vectors, cationic liposomes, electrical stimulation, and peptide mediated delivery. It allows not only to study the mechanism of action of biologically active substances, but also to develop new medicinal preparations for treatment of many diseases (Eastman *et al.*, 1998; Ferrari *et al.*, 1997; Fink *et al.*, 1997 and Rancourt *et al.*, 1998).

Biological screens for drug discovery: Interactions between reporter genes and ligands and study of signaling pathways: Advances in molecular biology have led to an increasing number of orphan receptors being discovered and cloned whose ligands and signals transduction mechanisms are unknown and are potential new targets for drug discovery. The reliability, reproducibility, sensitivity and adaptability of reporter gene technology to high throughput screening (HTS) have made cell-based assays an increasingly attractive alternative to *in vivo* biochemical assays. The principal advantage of these cell based assays is that they are robust, and can provide information about ligand-receptor interactions as well as signaling pathways not achievable with conventional receptor binding and second messenger assays (Dhundale and Goddard, 1996). They also represent model biological systems that mimic physiological conditions and provide important information about bioavailability and cytotoxicity of compounds. Moreover, the ability to maintain these cells in culture for several weeks allows for long-term observations of any adaptive changes associated with drug resistance and side effects. Perhaps the greatest advantage of the reporter gene assay is its ability to monitor events both before (signaling event) and after (protein synthesis) gene expression. Reporter gene technology has been used for the cloning and the functional expression and characterization of both membrane-bound and intracellular receptors (Naylor *et al.*, 1999 and Strathdee *et al.*, 2000). Such research has been constructive in identifying both agonist and antagonist ligands capable of altering receptor activity in living cells. The strategy of such research encompasses the following steps. Transformed cell lines are obtained in which the reporter gene expression is controlled by, for example, cAMP regulator elements (cycling adenosine monophosphate, cAMP, is one of the first participants in the transduction of many signals). This cell line is then further transfected with plasmids carrying genes which encode the receptor. When there is an interaction between a ligand (the studied substance, a medicinal preparation, toxin, etc.) and such a receptor, the reporter gene expression produces a signal that such interaction has taken place. On the basis of knowledge about cAMP-mediated signal transduction pathways, it is possible to determine the mechanism of action of ligands (Benzakour *et al.*, 1995 and Xu *et al.*, 1997b). Besides, such cell lines allow tracking down changes in cells over long enough periods of time.

Given the importance of gene transcription in cell regulation, signal transduction proteins and transcription factors also have become important targets for therapeutic intervention, and the exploitation of reporter gene-based functional assays systems will play an important role in the discovery not only of new drugs but also of novel targets. It will lead to a better understanding of the molecular basis of disease and provide new therapies based on the pharmacological modification of signal transduction pathways. Already, specific inhibitors of G-proteins, kinase, and transcription factors have been identified that have produced potential therapies for a variety of diseases including cancer, inflammatory and cardiovascular disease, as well as viral diseases (Naylor *et al.*, 1999). A patent

application for a large-scale drug screening strategy for antiviral and antitumour agents based on the inhibition of gene-specific transcription factors has been filed recently. However, the ubiquitous nature of signal transduction proteins means that such drug treatments may require a combination of approaches to be effective. There are now commercially available signal transduction pathway kits based upon reporter gene technology, which can map the involvement of a protein in different signal transduction pathways including the c-jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK), and protein kinase A (Benzakour *et al.* 1995; Xu *et al.*, 1997a; Xu and Zheng, 1997).

Problems of horizontal transfer of genes: The study of probable mechanism of horizontal transgenesis from eukaryotic cells into prokaryotic and *vice versa* using reporter gene methodology is also considerably simplified, as reporter systems possessing fast and simple assays allow to analyze large number of cells that are potential recipients of heterologous sequences.

Toxicology: The availability of stably transfected reporter cell lines has also provided biological screens for measuring cytotoxicity, where the release of the reporter is used as a measure of the poration and hence viability of the cell line. Both β-galactosidase and luciferase reporters have been employed for this purpose using sensitive colorimetric and luminometric detection assays. Similarly, there are biosensors based on reporter gene technology aimed at detecting environmental pollutants in soil including naphthalenes, ions, metals and agrochemicals. Yeast-based reporter gene systems using either β-galactosidase or luciferase have been developed to determine the potency and levels of synthetic estrogens and xenoestrogens in the environment that may act as endocrine disruptors to human and wildlife (Naylor *et al.*, 1999).

Conclusions and future prospects: The challenge facing cell and molecular biologists is to decipher how cellular events occur and are regulated at the single cell and organism level. This will require the development of simple, sensitive and noninvasive methods for the visualization of cellular events. The versatility of the reporter gene technology in this regard has been highlighted in the numerous, although not exhaustive, set of applications covered in this and other reviews (Joyeux *et al.*, 1997; Misteli and Spester, 1997). The advantages of these assays are their high sensitivity and selectivity, simpler manipulation procedures (e.g. reduced purification or cell lysis) and their adaptability to large scale measurement (e.g. HTS). They are compatible with all biological systems, including bacteria, yeast, insects, plants and animals. They also produce comparable results to traditional assays and provied additional information about signaling events as well as gene and protein expression inside living cells (Brauner-Osborne and Brand, 1996; Craig *et al.*, 1991). With the current advancement in this technology, it is likely that the largest impact will be made in the drug discovery and therapy, and the monitoring of intracellular events surrounding gene expression. Subsequent development of more sensitive and selective luminescent reporters, some of which will undoubtedly be discovered in deep sea microorganisms, combined with improvements in detection methods, will make reporter gene technology one of the most versatile techniques for understanding intracellular signaling events and the molecular basis of disease (Cubbit *et al*., 1995; Strathdee *et al.*, 2000; Wildt *et al.*, 1999 and Komakhin *et al.*, 2005). Such technology in turn should provide both novel targets as well as high throughput screening platforms for the discovery of novel therapeutics (Dhundale *et al*., 1996).

The subsequent development of reporter gene technology will be associated with creating new and modifying already used reporters, as well as modifying methods of activity determination of reporter gene products (Tauriainen *et al.*, 1999). This is due to the fact that none of the used reporter systems is universaly applicable (all of them alongside with advantages have some shortcomings that limit their application for a number of model organisms, cells and in some studies) and on other hand, it is important for researchers to have some reporter systems which can be used in the same cells (Guivarch *et al.*, 1996; Jacobson *et al.* 1994 and Rutter *et al.*, 1998).

Recently, we have demonstrated the application of the *licBM2* gene as a reporter system for prokaryotic and eukaryotic cells by expressing it either as a transcriptional fusion with selected promoters or as a translational fusion with the heterologous genes. The assays available for LicB activity are sensitive, accurate and simple, and can be used for the analysis of various gene fusion systems or for screening of transformants (Goldenkova, 2002 and Komakhin *et al.*, 2005).

Analysis of the expression of *licB* gene in *E. coli*, yeast and mammalian cells has shown that bacterial lichenase exhibits many of the features necessary for a reporter protein. In particular, its thermostability allows one to easily detect and assess the expression of the bacterial *licB* gene by both qualitative and quantitative methods against the background of thermolabile

enzymes in tested cells (Piruzian *et al.*, 1998, 2000)

Taking into account the apparent advantages of this reporter system and other reporter genes in comparison with other methods of studying regulation of gene expression, it may be concluded that they will be used widely in different areas of molecular and cell biology.

References

Alam J, Cook JL (1990). Reporter genes: Application to the study of mammalian gene transcription. *Analytical Biochem*. 188: 245-254.

An G, Hidaka K, Siminovitch L (1982). Expression of Bacterial β-Galactosidase in Animal Cells. *Mol Cell Biol*. 1982, 2: 1628-1632.

Baldwin TO, Berends T, Bunch TA, Holzman TF, Rausch SK, Shamansky L, Treat Ml, Ziegler MM (1984). Cloning of the luciferase structural genes from *Vibrio harveyi* and expression of bioluminescence in *Escherichia coli*. *Biochem.* 23: 3663-3666.

Bamberger CM, Else T, Bamberger AM, Beil FU, Schulte HM (1997). Regulation of the human interleukin –2 gene by the "a" and "b" isoforms of the glucocorticoid receptor. *Mol Cell Endocrinol.* 136: 23-28.

Benzakour O, Kanthou C, Dennehy U, Al Haq A, Berg LP, Kakkar VV, Cooper DN (1995). *Bichem J*, 309: 385.

Brandes C, Plautz Jd, Stanewsky R, Jamison CF, Straume M, Wood KV, Kay SA, Hall JC (1996). Novel features of Drosophila period transcription revealed by real-time luciferase reporting. *Neuron*, 16: 687-692.

Brauner-Obsorne H, Brand MR (1996). Pharmacology of mus-carinic acetylcholine receptor subtypes (m1-m5): High throughput assays in mammalian cells. *Eur J Pharmacol.* 295: 93-102.

Brejc KA, Sixma TK, Kitts PA, Kain SR, Tsein RY, Ormo M, Remington SJ (1997). Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* Green Fluorescent Protein. *Proc Natl Acad Sci USA*. 94: 2306-2311.

Bronstein I, Fortin J, Stanley PE, Stewart GSA, Kricka LJ (1994). Chemiluminescent and bioluminescent reporter gene assays. *Anal Biochem*. 219:169-181.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994). Green Fluorescent Protein as a marker for gene expression. *EMBO J.* 4: 2145-2151.

Cody CW, Prasher DC, Westler WM, Prendergast FG, Ward WW (1993). Chemical structure of the hexapeptide chromophore of the *Aequorea* Green Fluorescent Protein. *Biochem*. 32: 1212-1218.

Crabb DW, Dixon JE (1987). A method for increasing the sensitivity of chloramphenicol acetyltransferase assays in extracts of transfected cultured cells. *Anal Biochem*. 163 : 88-92.

Craig FF, Simmonds AC, Watmore D, McCapra F, White MRH (1991). Membrane permeable luciferin esters for assays of firefly luciferase in living cells. *Biochem J*. 276: 637-641.

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995). Underestanding, improving and using Green Fluorescent Proteins. *Trends Bichem Sci.* 20: 448-455.

Dhundale A, Goddard C (1996). Reporter assays in the high throughput-screening laboratory: A rapid and robust first look? *J Biomol Screen.* 2: 115-121.

Eastman SJ, Tousignant JD, Lukason MJ, Chu Q, Cheng SH, Scheule RK (1998). Aerosolization of cationic lipid: pDNA complexes – *in vitro* optimization of nebulizer parameters for human clinical studies. *Hum Gene Ther*. 9: 43-52.

Endebrecht J, Silverman M (1984). Identification of genes and gene products necessary for bacterial bioluminescence. *Proc Natl Acad Sci USA*. 81: 4154-4158.

Ferrari S, Moro E, Pettenazzo A, Behr JP, Zachello F, Scarpa M (1997) Exgene 500 is an efficient vector for gene delivery to lung epithelial cells *in vitro* and *in vivo*. *Gene Ther*. 4: 1100-1106.

Fink DJ, Poliani PL, Oligino T, Krisky DM, Goins WF, Glorioso JC (1997). Development of an HSV-based vector for the treatment of Parkinsons Disease. *Exp Neurol*. 144: 103-112.

Geogan P, Vincent R., Kumarathasan P, Brook J (1998). Sequential *in vitro* effects of airborne particles in lung macrophages and reporter CAT-gene cell lines. *Toxicol In vitro*. 12: 25-37.

George SE, Bungay PJ, Naylor LH (1997). Evaluation of a CRE-Directed luciferase reporter gene assay as an alternative to measuring cAMP accumulation. *J Biomol Screen*. 2: 235-240.

George SE, Bungay PJ, Naylor LH (1998). Functional analysis of the D2L dopamine receptor expressed in a cAMP-responsive luciferase reporter cell line. *Bichem pharmacol*. 56:25-30.

George SE, Bungay PJ, Naylor LH (1997)/ Functional coupling of endogenous serotonin (5-HT1B) and calcitonin (C1a) receptors in CHO cells to a cyclic AMP-responsive luciferase reporter gene. *J Neurochem.* 69: 1278-1285.

Goldenkova IV (2002). Reporter systems: Possibilities of studying various aspects of gene expression regulation. *Successes Modern Biol.* (in Russian) 122: 6: 514-526.

Gorman CM (1985). High efficiency gene transfer. *DNA Cloning a Practical Approach* (Glover DM, Ed.) pp: 143-190.

Guivarch A, Caissard JC, Azmi A, Elmayan T, Chriqui D, Tepfer M (1996). *In situ* detection of expression of the gus reporter gene in transgenic plants: Ten years of blue genes. *Transgenic Res.* 5: 281-288.

Heim R, Prasher DC, Tsien RY (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc Natl Acad Sci USA*. 91: 12501-12504.

Jacobson RH, Zhang XL, DuBose RF, Matthews BW (1994). Three dimensional structure of beta-galactosidase from *E. coli. Nature*, 369: 761-766.

Jefferson RA (1987). Assaying chimeric genes in plants: The gus gene fusion system. *Plant Mol Biol Rep*. 5: 387-

405.

Jefferson RA, Burgess SM, Hirsh D (1986). Beta-glucoronidase from *E. coli* as a gene fusion marker. *Proc Nat Acad Sci USA.* 83: 8451-8457.

Jefferson RA, Kavanagh TA, Beven MW (1987). Gus fusions: Beta-glucoronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. 6: 3901-3907.

Joyeux A, Balaguer P, Germain P, Boussiox AM, Pons M, Nicholas JC (1997). Engineered cell lines as a tool for monitoring biological activity of hormone analoges. *Anal Biochem*. 249: 119-130.

Komakhin RA, Abdeeva IA, Salehi Jozani GR, Goldenkova IV, Zhuchenko AA (2005). Thermostable lichenase as a translation reporter. *Genetika* (In Russian), 41: 30-40.

Lorenz WW, Cormier MJ, OKane DJ, Hua D, Escher AA, Szalay AA (1996). Expression of the *Renilla reniformis* luciferase gene in mammalian cells. *J Biolumin Chemilumin.* 11: 31-37.

Luzi P, Victoria T, Rafi MA, Wenger DA (1997). Analysis of the 59-flanking region of the human galactocerebrosidase (GALC) gene. *Biochem Mol Med*. 62: 159-164.

Manen D, Pougeon M, Geysen HM (1997). A sensitive reporter gene system using bacterial luciferase based on a series of plasmid cloning vectors compatible with derivatives of pBR322. *Gene,* 186: 197-2000.

Mascaronhas JP, Hamilton DA (1992). Artifacts in the localization of gus activity in anthers of petunia transformed with a CAMV 35s-gus construct. *Plant J*. 2: 405-408.

Michelet B, Chua N (1997). Improvement of Arabidopsis mutant screens based luciferase imaging in planta. *Plant Mol Biol Rep.* 14: 321-329.

Misteli T, Spestor DL (1997). Application of GFP in cell biology and biotechnology. *Nature Biotech*. 15:961-964.

Naylor LH (1999). Reporter gene technology: The future looks bright. *BiochemPharmacol.* 58: 749-757.

Olsson O, Koncz C, Szalay AA (1988). The use of the *luxA* gene of the bacterial luciferase operon as a reporter gene. *Mol Gen Genet.* 215: 1-9.

Ow, DW, Wood KV, DeLvca M, Helinski DR, Howell SH (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science*, 234: 856-9.

Pazzagli M, Devine JH, Peterson DO, Baldwin TO (1992). Use of bacterial and firefly luciferases as reporter genes in DEAE- Dextran-Mediated transfection of mammalian cells. *Anal Bichem*. 204: 315-323.

Piruzian ES, Goldenkova IV, Musiychuk KA, Abdeev RM, Volkova LV, Kobets NS (2000). *Plant Physiol. (In Russian)* 37: 382-390.

Piruzian ES, Goldenkova IV, Musiychuk KA, Kobets NS, Arman IP, Bobrysheva IV, Chekhuta IA, Glazkova D (2002). A reporter system for prokaryotic and eukaryotic cells based on thermostable *Clostridium thermocellum* lichenase. *Mol Genet Genomics*. 266: 778-786.

Piruzian ES, Monzavi-Karbassi B, Darbinian NS, Goldenkova IV, Kobets NS, Mochulsky AV (1998). The use of a thermoactive -glucanase gene from *Clostridium thermocellum* as a reporter gene in plants. *Mol Gen Genet.* 257:561-567.

Piruzian ES, Mogutov MA, Velikodvorskaia GA, and Akimenko VK (1985). Cloning and expression of the structural gene of cellulolytic complex endoglucanase of Clostridium thermocellum phi 7 in *Escherichia coli* cells. *Dokl Akad Nauk SSSR.* 281:963-5 (In Russian).

Quaedclieg NEM, Schlaman HRM, Admiraal PC, Wijting SE, Stougaard J, Spaink HP (1998). Fusions between GFP and GUS as sensitive and vital bifunctional reporters in plants. *Plant Mol Biol*. 38: 861-873.

Rancourt C, Robertson MW, Wang MH, Goldman CK, Kelly JF, Alvarez RD, Siegal GP, Curiel DT (1998). Endothelial cell vehicles for delivery of cytotoxic genes as a gene therapy approach for carcinoma of the ovary. *Clin Cancer Res*. 4: 265-270.

Rossi F, Charlton CA, Blau HM (1997). Monitoring protein-protein interactions in intact eukaryotic cells by beta-galactosidase complementation. *Proc Natl Acad Sci USA.* 94: 8405-8410.

Rutter GA, Kennedy HJ, Wood CD, White MRH, Tavare JM (1998). Real-time imaging of gene expression in single cells. *Chem Biol*. 5: 285-290.

Rutter GA, White MRH, Tavare JM (1995). Involvement of MAP kinase in insulin signaling revealed by non-invasive imaging of luciferase gene expression in single living cells. *Curr Biol*. 5: 890- 899.

Salehi Jozani GR, Komakhin RA, Piruzian ES (2005). Comparison analysis of expression of wild type and synthetic *cry3a* genes of *Bacillus thuringiensis* in pro- and eukaryotic cells. *Genetika,* (In Russian) 41: 171-178.

Sniezko I, Dobson-Stone C, Klein A (1998). The treA gene of Bacillus subtilis is a suitable reporter gene for the archaeon *Methanococcus voltae. FEMS Microbiol Let*. 164: 237-242.

Stern M., Caplen NJ, Browning JE, Griesenbach U, Sorgi F, Huang L, Gruenert DC, Marriot C, Crystal RG, Gedes DM, Alton EWFW (1998). The effect of mucolytic agents on gene transfer across a CF sputum barrier *in vitro. Gene Ther*. 5: 91-98.

Strathdee CA, McLeod MR, Underhill TM (2000). Dominant positive and negative selection using luciferase, Green Fluorescent Protein and Beta-galactosidase reporter gene fusions. *Biotechniques*, 28: 210-214.

Suto CM, Ignar DM (1997) Selection of an optimal reporter gene for cell-based high throughput screening assays. *J Biomol Screen*. 2: 7-9.

Tauriainen S, Virta M, Change W, Karp M (1999). Measurement of firefly luciferase reporter gene activity from cells and lysates using *E. coli* arsenate and mercury sensors. *Anal Biochem*. 272: 191-198.

Taylor CB (1997). Promoter fusion analysis: An insufficient measure of gene expression. *Plant Cell*, 9: 273-275.

Teather RW, Wood PJ (1982). Use of Congo Red-polysaccharide interactions in enumeration and characterization

of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol*. 43:777-780.

Terkikh A, Fradkov A, Ermakova G, Zaraisky A, Tan P, Kajava AV, Zhao X, Lukyanov S, Matz M, Kims S, Weissman I, Siebert P (2000). Fluorescent timer: Protein that changes color with time. *Science*, 290: 1585-1588.

Watson MA, Darrow C, Zimonjic DB, Popescu NC, Fleminig TP (1998). Structure and transcriptional regulation of the human mammaglobin gene, a breast cancer associated member of the uteroglobin gene family localized to chromosome 11q13. *Oncogene*, 16: 817-824.

Welsh S, Kay SA (1997). Reporter gene expression for monitoring gene transfer. *Curr Opin Biotech*. 8: 617-622.

de Wet JR, Wood KV, Helinski DR, DeLuca M (1985). Cloning of Firefly Luciferase cDNA and the Expression of Active Luciferase in *Escherichia coli*. *Proc Natl Acad Sci USA*. 82: 7870-7873.

de Wet JR, Wood KV, DeLuca M, Helinski DR, Subramani S (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol*. 7:725-737.

White MRH, Masuko M, Amet L, Elliot G, Braddock M, Kingsman AJ, Kingsman SM (1995). Real time analysis of the transcriptional regulation of HIV and CMV promoters in single mammalian cells. *J Cell Sci*. 108: 441-445.

Wildt S, Deuschle U (1999). cobA, a red fluorescent transcriptional reporter for *E. coli*, yeast and mammalian cells. *Nat Biotech*. 17: 1175-1178.

Wiznerowicz M, Fong AZC, Mackiewicz A, Hawley RG (1997). Double-copy bicistronic retroviral vector platform for gene therapy and tissue engineering: Application to melanoma vaccine development. *Gene Ther.* 4: 1061-1068.

Wood TM, Bhat KM (1988). Methods for measuring cellulase activities. *Methods Enzymol*. 160:87-112.

Xu L, Sanchez T, Zheng CF (1997a). *In vivo* signal transduction pathway reporting systems. *Strategies*, 1:1-3.

Xu L, Sanchez T, Zheng CF (1997b). Signal transduction reporting systems using cis-acting enhancer elements. *Strategies*, 10: 79-80.

Xu L, Zheng CF (1997). New fusion trans-activation plasmids for studying signal transduction pathways. *Strategies*, 10: 81-83.