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COMMENTARY

Reporter Gene Technology: The Future Looks Bright

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ABSTRACT. Reporter gene technology is widely used to monitor the cellular events associated with signal transduction and gene expression. Based upon the splicing of transcriptional control elements to a variety of reporter genes (with easily measurable phenotypes), it "reports" the effects of a cascade of signalling events on gene expression inside cells. The principal advantage of these assays is their high sensitivity, reliability, convenience, and adaptability to large-scale measurements. This review summarises the current status of reporter gene technology including its role in monitoring gene transfer and expression and its development as a biological screen. With the advances in this technology and in detection methods, it is likely that luciferase and green fluorescent protein will become increasingly popular for the non-invasive monitoring of gene expression in living tissues and cells. Such techniques will be important in defining the molecular events associated with gene transcription, which has implications for our understanding of the molecular basis of disease and will influence our approach to gene therapy and drug development. BIOCHEM PHARMACOL **58**;5:749–757, 1999. © 1999 Elsevier Science Inc.

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Knowledge about how cells communicate with each other has increased dramatically in recent years. Transient signals generated by extracellular stimuli (e.g. polypeptide and steroid hormones, cytokines, and neurotransmitters) interacting with receptors either at the cell surface or inside the cell are converted into long-term changes in gene expression by the activation of a variety of transcription factors. It is known that there are several signal transduction pathways [1, 2] that contribute to the regulation of gene transcription in mammalian cells by stimulating the interaction of these transcription factors with genetic sequences called response elements in the promoter regions of responsive genes [3–5]. By attaching response elements to reporter genes, alterations in reporter gene activity mediated by such response elements provide a way of selectively monitoring the activation of particular second messenger cascades and their influence on gene expression and regulation inside the cell (reporter gene technology). The variety of reporter genes available and their application is very broad—from the temporal and spatial control of gene expression to the characterisation of receptors and development of drug screening and gene delivery systems. The aim of this review is, therefore, to provide an overview of this technology including the types of reporter genes commonly in use and their main applications, and to consider the prospects for this technology in the future.

REPORTER GENE TECHNOLOGY

The term reporter gene is used to define a gene with a readily measurable phenotype that can be distinguished easily over a background of endogenous proteins [6]. Generally, such reporters are selected on the basis of the sensitivity, dynamic range, convenience, and reliability of their assay [6–9]. Reporter gene technology involves controlling the activity of such genes by defined *cis*-regulatory sequences (response elements), 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 [3].

For example, extracellular signals may be detected by receptors at the cell surface (Fig. 1), including G-protein coupled receptors, ion channel linked receptors, receptors containing intrinsic enzymatic activity (e.g. receptor tyrosine kinases), or receptors that recruit intracellular tyrosine kinases (e.g. JAK† proteins) [1]. These activate intracellular signal transduction pathways and lead to the phosphorylation of a variety of transcription factors by different protein kinases (e.g. cAMP-dependent protein kinase A, protein kinase C, and receptor tyrosine kinases), which then bind specifically to response elements (e.g. CREs, TREs, and SREs, respectively) in the promoter regions of hormone-responsive genes [1, 5, 10]. In the case of steroid hormones, the receptors themselves act as transcription factors binding directly to hormone response elements to alter gene transcription [11]. There are also

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† *Abbreviations*: JAK, Janus activated kinase; GFP, green fluorescent protein; cAMP, cyclic AMP; CRE, TRE, and SRE, cAMP-, phorbol ester-, and serum-response element; CAT, chloramphenicol acetyltransferase; HTS, high throughput screening; HSV, herpes simplex virus; and GPCRs, G-protein coupled receptors.

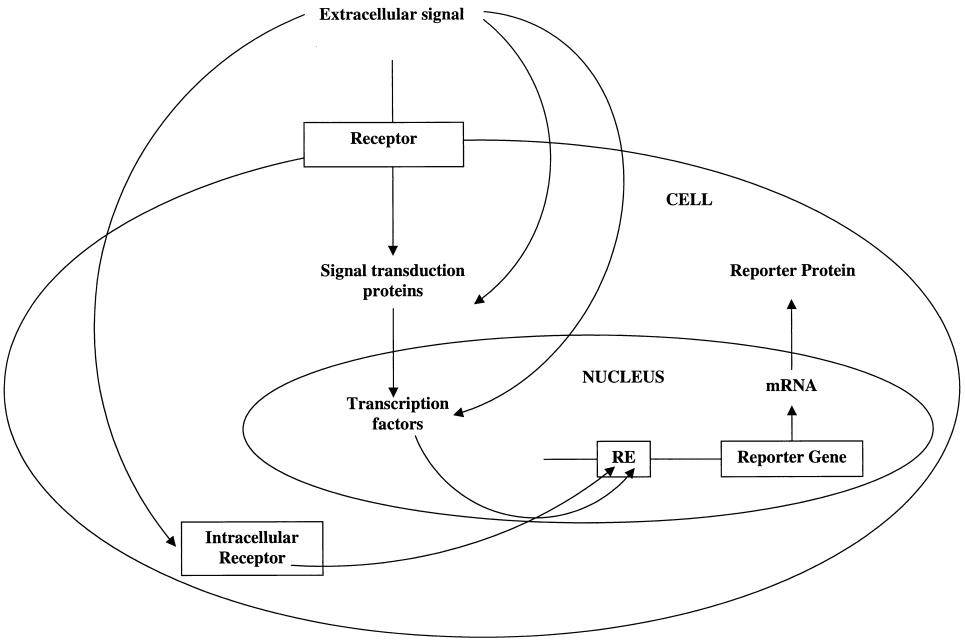

FIG. 1. Schematic representation of a cell-based reporter gene assay. Extracellular signals acting on receptors, signal transduction proteins, or transcription factors activate response elements (RE) that modulate the expression of the reporter gene.

several direct activators or inhibitors of signal transduction proteins [12] and even transcription factors themselves [5] that may be important for the development of disease therapies. To understand the relationship between the activation/inhibition of different pathways and their effects on gene expression, specific response elements have been fused to genes encoding reporter proteins. 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.

REPORTER GENES

Table 1 summarises some of the advantages and disadvantages of the most commonly used reporter genes and further details about specific assays and detection methods have been the subject of reviews elsewhere [7–9, 13, 14]. 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, often easily detectable, response. The choice of 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) [8, 9].

CAT was the first reporter gene used to monitor transcriptional activity in cells [7]. CAT is a bacterial enzyme that can detoxify chloramphenicol, an inhibitor of prokaryotic protein synthesis, by catalysing the trans-

TABLE 1. Comparison of commonly used reporter genes

Reporter gene	Advantages	Disadvantages
Chloramphenicol acetyltransferase (CAT) (bacterial)	No endogenous activity. Automated ELISA available.	Narrow linear range; use of radioisotopes; stable.
β-Galactosidase (bacterial)	Well characterised; stable; simple colorimetric readouts; sensitive bio- or chemiluminescent assays available.	Endogenous activity (mammalian cells).
Luciferase (firefly)	High specific activity; no endogenous activity; broad dynamic range; convenient assays.	Requires substrate (luciferin) and presence of O_2 and ATP.
Luciferase (bacterial)	Good for measuring/analysing prokaryotic gene transcription.	Less sensitive than firefly; not suitable for mammalian cells.
Alkaline phosphatase (human placental)	Secreted protein; inexpensive colorimetric and highly sensitive luminescent assays available.	Endogenous activity in some cells; interference with compounds being screened.
Green fluorescent protein (GFP) (jellyfish)	Autofluorescent (no substrate needed); no endogenous activity; mutants with altered spectral qualities available.	Requires post-translational modification; low sensitivity (no signal amplification).

fer of acetyl groups from acetyl CoA to the 3-hydroxyl position of chloramphenicol. The enzyme is stable [15, 16], and there is no endogenous expression in mammalian cells. However, the assay relies on radiochemicals, and although an automated ELISA is now available, making the assay more manageable [9], the linear range and sensitivity of the assay are not as broad as for other reporters [7, 17].

β-Galactosidase is a well-characterised bacterial enzyme and has been one of the most widely used reporter genes in molecular biology as a monitor of transfection efficiency. Simple colorimetric assays of poor sensitivity and narrow dynamic range have largely been replaced with more sensitive and adaptable bio- or chemi-luminescent assays. It has the advantage over CAT in that the assays tend to be simpler and do not involve the use of radioisotopes. However, there can be endogenous activity in mammalian cells, which can be reduced at higher pH values [6].

Luciferase refers to a family of enzymes that catalyse the oxidation of various substrates (e.g. luciferin and coelenterazine), resulting in light emission. The most commonly used luciferases for reporter gene assays are the bacterial luciferases, the firefly (*Photinus pyralis*) luciferase, and more recently the Renilla luciferase (from the bioluminescent sea pansy, *Renilla reniformis*) [7, 9]. The bacterial luciferases are generally heat-labile, dimeric proteins, which limits their use as reporter genes in mammalian cells. A sensitive reporter system capable of monitoring transcriptional activity within living bacterial cells has been developed recently using bacterial luciferase, where the aldehyde substrate required for the assay is expressed constitutively inside the cell [18]. However, the linear range of this assay is still only about 3 orders of magnitude [17, 18]. 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) [19, 20]. Deficiencies in the original assay arose from the requirement for cell lysis prior to the addition of the substrate luciferin and the detection of the flash response. In the first instance, the use of membrane-permeable and photolysable firefly luciferin esters [21, 22] has removed the need for cell disruption. Secondly, the development of "glow" reagents (e.g. LucLite, Packard) [9, 23–25] 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 (HTS—see below). Renilla luciferase [26] may be a particularly appropriate reporter for intact (living cell) systems because this luciferase catalyses the oxidation of coelenterazine, which is membrane permeable. Like other luciferases, there is no endogenous activity in mammalian cells.

SEAP (secretable form of alkaline phosphatase) is a mutated form of placental alkaline phosphatase. This reporter gene has the advantage that the protein is secreted from the cell and can be detected by sampling the culture medium. Therefore, the cells remain intact and viable for further experimentation [9, 10]. Inexpensive colorimetric assays have largely been superseded by automated chemiluminescent assays, which are suitable for HTS applications.

Aequorin and GFP are proteins that have been isolated from the bioluminescent jellyfish *Aequorea victoria*. In the presence of Ca^{2+} and the cofactor coelenterazine, aequorin emits blue light with a peak at 469 nm, which can be detected using conventional luminometry [27]. GFP, however, is unique among light-emitting proteins in that it is autofluorescent and, therefore, does not require the presence of any cofactors or substrates for the generation of its green light (510 nm); thus, it provides a convenient means by which intracellular events can be studied in intact living cells. The gene for GFP was cloned in 1991 [28], and several GFP mutants are now available, which exhibit improved fluorescence properties over wild-type GFP [13, 20]. The greatest advantage of GFP is that in the absence of cell lysis, noninvasive monitoring of gene expression in living tissues is possible.

Many of these commonly used reporter genes have been used in combination. For example, SEAP has been used in conjunction with luciferase and β-galactosidase to normalise transfection efficiency, and β-galactosidase and Renilla luciferase have been combined with firefly luciferase for multiple readouts from a single well [7, 9] and dual detection of gene transcription [29, 30]. The reporter gene chosen will largely depend upon the suitability of the assay for a particular investigation (i.e. sensitivity, reliability, detection, and reporter dynamics) [8, 9]. The stability of the reporter gene will determine whether it is appropriate for transcriptional kinetic studies (to monitor changes in gene expression) and HTS or preferable for all-or-none type expression in gene transfer experiments. For example, firefly and Renilla luciferases have relatively short half-lives (3 and 5–6 hr, respectively) compared with CAT (50 hr) in mammalian cells as a result of their sensitivity to proteolysis [17], making these proteins more suitable for kinetic and transfection efficiency experiments, respectively. Likewise, wild-type GFP, which has a much narrower linear range of fluorescence signals and greater stability than luciferase, is less suitable for the quantitative measurement of gene transcription. The lack of enzymatic amplification of GFP tends to make it less sensitive than luciferase and can limit its use to highly expressed genes. Moreover, the accumulation of GFP in cells makes it less suitable for HTS assays.

With reporter proteins and detection systems being constantly improved, luminescent and fluorescent assays are becoming more prevalent because of the ability to visualise reporter gene activity inside cells. Reporters such as GFP and luciferase, which provide a highly sensitive but nondestructive way of monitoring gene transfer and expression, are becoming increasingly popular [reviewed in Ref. 20]. Several forms of firefly luciferase, in which the protease-sensitive regions of the protein have been mutated, yield larger and more stable signals compared with the wild-type protein [31, 32]. Similarly, modifications in GFP

have produced mutant proteins with increased stability [33], altered spectral properties [34], and signal intensity [34–36], which are useful for monitoring the expression and localisation of multiple intracellular proteins [13].

APPLICATIONS

Gene Transfer and Expression

PROMOTER ANALYSIS. Reporter gene technology was first used as a method for analysing the activity of *cis*-acting genetic elements such as enhancers and promoters in the upstream regions of genes. Indeed, there are many recent studies utilising the technology in this way. For example, transcription elements responsible for the basal and tissue-specific expression of receptor genes (e.g. β_1-adrenergic [37, 38], m1 muscarinic [39], luteinising hormone [40], CC chemokine [41], angiotensin II type 1 [42], and interleukin-2 [43] and -5 [44] receptor genes) have been identified using reporter gene technology, as well as in genes that are targets for human disease (e.g. galactocerebrosidase [45] and mammaglobulin [46] genes). Modular promoter sequences responsive to estrogens [47], androgens [48, 49], thyroid hormone [38], Ca^{2+} [50], nitric oxide [42], and various transcription factors [51] that are responsible for regulating gene expression have also been identified and used to selectively alter the level of expression of different genes [48, 51].

GENE DELIVERY. Reporter genes have been used as markers for monitoring gene transfer using a number of different transformation technologies. 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 [20]. For example, fluorescence activated cell sorting (FACS analysis) has been very powerful in detecting gene transfer in plants [52], yeasts [53], and mammalian cells [54] using GFP as a marker. Although much reporter technology is utilized on cells grown in culture, more recently it has been used to monitor the expression and effects of genes in transgenic plants and animals. In transgenic mice, GFP has been developed as a marker for promoter mapping and gene regulation [55], whereas β-galactosidase has been used to monitor the fate of neural grafts [56] and the *in vivo* mutations involved in ageing [57]. Likewise, β-glucuronidase has been used to monitor agrobacterium-mediated transformations in *Brassica carinata* [58] and to identify the factors affecting transient expression in wheat protoplasts [59].

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 [20], cationic liposomes [60], electrical stimulation [18, 61], and peptide-mediated delivery [62]. Much of this work has used reporter genes (such as β-galactosidase, CAT, luciferase, and GFP) to construct HSV-based [63, 64] or retroviral [65] vectors and to evaluate modes of gene delivery [66, 67] for the treatment of diseases such as Parkinson's disease [63], cystic fibrosis [67–70], and cancer [65, 71].

IMAGING OF GENE EXPRESSION. Historically, the spatial organisation of gene expression in plants and animals was measured using colorimetric or fluorimetric assays employing β-glucuronidase and β-galactosidase as markers [8]. However, the development of luciferase and GFP as non-invasive markers of gene expression, combined with ease of detection using sensitive charge-coupled device (CCD) imaging cameras and fluorescence microscopy, has allowed for temporal and spatial information about gene expression even at the single cell level. Such applications already have been reviewed in detail elsewhere [20], and so the focus here will be on more recent advancements in this field.

In the case of GFP, the creation of mutant isoforms of the protein with altered spectral properties [34–36] has not only permitted the simultaneous tracking of different proteins within living cells [13, 72] but has 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 [72]. Such techniques have been used to monitor the dimerisation of the pituitary specific transcription factor Pit-1 [73] and the conformational changes associated with Ca^{2+} binding to calmodulin [74, 75] using a calmodulin binding sequence flanked by two GFP variants. pH-sensitive GFP mutants have also been developed that alter their fluorescent properties in acidic environments. By creating appropriate fusion proteins, these pH indicators have been targeted to specific subcellular locations including the cytosol, Golgi apparatus, mitochondria, and secretory vesicles [76–78] to provide the first measurements of organellar pH. Such approaches will be useful for studying the mechanisms controlling pH in such organelles in living cells [72] and will provide sensitive monitors of processes such as synaptic transmission by exploiting the changes in pH associated with vesicular endocytosis and recycling [78]. Similarly, the targeting of Ca^{2+}-sensitive fusion proteins [74, 75] to specific cellular locations could permit *in vivo* imaging of intracellular alterations in Ca^{2+} levels. While GFP has been used successfully for the spatial imaging of gene expression in living cells, luciferase remains the reporter of choice for dynamic measurements of gene transcription (temporal gene expression) because of its high sensitivity and relatively short half-life resulting in its rapid turnover within cells. It has been used to measure circadian rhythms in plants [79] and as a dynamic monitor of gene transcription in insects [80] and mammalian cells [81, 82].

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 localisation, 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.

Biological Screens for Drug Discovery

Advances in molecular biology have led to an increasing number of orphan receptors being discovered and cloned whose ligands and signal transduction mechanisms are unknown and are potential new targets for drug discovery [83]. The reliability, reproducibility, sensitivity, and adaptability of reporter gene technology to HTS has made cell-based assays an increasingly attractive alternative to *in vitro* biochemical assays [14, 19, 84, 85]. The principal advantage of these cell-based assays is that they are robust [9, 23–25, 86] and can provide information about ligand–receptor interactions as well as signalling pathways not achievable with conventional receptor binding and second messenger assays [87]. 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 [19]. Perhaps the greatest advantage of the reporter gene assay is its ability to monitor events both before (signalling event) and after (protein synthesis) gene expression [8].

CHARACTERISATION OF RECEPTORS AND THEIR LIGANDS. Reporter gene technology has been used for the cloning [88] and the functional expression and characterisation of both membrane-bound [89, 90] and intracellular receptors [91, 92] [reviewed in Ref. 19]. Such research has been constructive in identifying both agonist and antagonist ligands capable of altering receptor activity in living cells. A good example would be the superfamily of GPCRs, which are of therapeutic interest and have been studied in detail using this technology. Stable cell lines expressing reporter and receptor DNAs have been instrumental in pharmacologically characterising endogenous [23] and recombinant GPCRs linked to the adenylyl cyclase [25, 93–95] as well as the phospholipase C [96, 97] signalling pathways. With the large number of receptor clones and cell types now available, there is clearly a need for a generic reporter assay in which ligands for potentially any GPCR can be screened. Using the Ca^{2+}-sensitive photoprotein aequorin as a reporter, an assay has been developed to detect agonist activity at a variety of GPCRs (expressed both transiently and stably) in CHO cells by coexpressing the promiscuous G-protein (Gα16) and the apoaequorin reporter gene. The activation of any membrane-bound receptor by an agonist activates the Gα16 protein, which leads to the elevation of intracellular Ca^{2+}. In the presence of aequorin and the membrane-permeable substrate coelenterazine, a flash response is produced [86]. Although the flash-type emission rapid response requires a relatively sophisticated luminometer with a multiple injector in front of the light detector [7], improvements in the stability of the response will make it suitable for large screening programs. Similarly, colorimetric assays (RSATs—receptor selection and amplification technology), which involve the cotransfection of the receptor of interest with a β-galactosidase marker gene, have also been used to measure ligand–receptor interactions (notably in the muscarinic acetylcholine receptors) through agonist-induced cellular proliferation [98].

In general, the major advantage of cell-based reporter gene assays is their adaptability for HTS strategies [9, 86, 87, 98]. Coupled with the availability of cell lines expressing proteins of interest, these reporters can be used to identify and measure the activation of specific signal transduction pathways in highly sensitive, nonradioactive, easy-to-perform functional assays. The development of new reporter proteins, substrates, and detection methods that provide sensitive readouts from intact cells will facilitate the production of reporter cell lines tailored to HTS requirements. This includes their compliance with microtitre format (96-well [9, 23–25, 86, 89]) and 384-well format [99] and automation (loading and measuring) using a robot-operated HTS system connected to a database system [14]. This will provide rapid, reliable, cell-based functional assays for the accurate testing of millions of compounds of potential therapeutic interest to the pharmaceutical industry.

SIGNALLING PATHWAYS. Given the importance of gene transcription in cell regulation, signal transduction proteins and transcription factors also have become important targets for therapeutic intervention [5, 12, 87], and the exploitation of reporter gene-based functional assay 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 [5, 12, 19, 87]. Already, specific inhibitors of G-proteins, kinases, and transcription factors have been identified that have produced potential therapies for a variety of diseases including cancer, inflammatory and cardiovascular diseases, as well as viral diseases [5, 12]. 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 [100]. However, the ubiquitous nature of signal transduction proteins means that such drug treatments may require a combination of approaches to be effective.

There is also academic interest in deciphering how information is transferred from the cell surface to the nucleus, particularly the control of intracellular signalling mechanisms and the crosstalk between various pathways [3]. There are now commercially available signal transduction pathway kits

(PathDetect, Stratagene) based upon reporter gene technology, which can map the involvement of a protein in different signal transduction pathways including the JNK (c-jun N-terminal kinase), MAPK (mitogen-activated protein kinase), and protein kinase A (cAMP-dependent kinase) pathways [101–103]. These systems could also be used to study the *in vivo* effects of a new gene or drug candidate on a particular signal transduction pathway.

Inevitably, as the genome project progresses, the focus will shift from discovering genes to monitoring their *in vivo* function. Systems that are sensitive to transcriptional changes within cells and are amenable to HTS will be pivotal in this role. These newly discovered genes may encode novel receptors, protein phosphatases or kinases, and other regulatory proteins that are involved in intracellular signalling cascades. However, such reporter gene technology will have to be well-characterised with respect to signal generation and crosstalk, since there have been a number of reports highlighting the potential limitations of such reporter gene systems in a variety of cell lines [104–108].

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 [109, 110]. 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 [111], ions (e.g. arsonites and antimonites) [112], metals, and agrochemicals [113]. Yeast-based reporter gene systems using either β-galactosidase [114] or luciferase [115–117] have been developed to determine the potency and levels of synthetic estrogens and xenoestrogens (e.g. nonylphenol, bisphenol A) in the environment that may act as endocrine disruptors to humans and wildlife. Sensitive luciferase-based reporter gene assays have been used to determine the toxic potency of mixtures of dioxins in the blood plasma of ducks [118] and for the large-scale measurement of metabolites of vitamin D [119] in human blood without the need for prior purification.

FUTURE PROSPECTS

The challenge facing cell and molecular biologists is to decipher how cellular events occur and are regulated at the single cell and organismal level. This will require the development of simple, sensitive, noninvasive methods for the visualisation of cellular events, and 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 [13, 19, 20]. 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 (e.g. HTS) measurements. They are compatible with all biological systems including bacteria, yeast, insects, plants, and animals. They also produce comparable results to traditional assays [24, 98] and additional information about signalling events as well as gene and protein expression inside living cells [19, 87]. With the current advancements in this technology, it is likely that the largest impact will be made in the drug discovery process, approaches to gene delivery 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 [120], combined with improvements in detection methods, will make reporter gene technology one of the most versatile techniques for understanding of intracellular signalling events and the molecular basis of disease. Such technology in turn should provide both novel targets as well as high throughput screening platforms for the discovery of novel therapeutics.

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