INVITED COMMENTARY

Reporter Genes and Imagene

The rapid progress in molecular genetics over the past three decades has given scientists and researchers the ability to sequence, characterize, and manipulate genes, almost at will. These accomplishments have been possible by the development of various strategies to characterize the spatial organization of DNA sequences and the qualitative and quantitative measurement of gene expression. Methods for the direct measurement of gene expression include Southern, Northern, and Western blot and in situ hybridization techniques. These techniques are now complemented or, in some instances, replaced by the reporter gene technology.

Reporter genes provide an elegant alternative method of genetic analysis that is overall easier, less expensive, more accurate, and more quantitative than the standard hybridization techniques. The general concept of a reporter gene is simple: a defined nucleotide sequence is introduced into a biologic system and yields a readily measurable phenotype (protein) on expression. Reporter genes are introduced into cells using transfection protocols. Common cellular transfection protocols include $^{2+}PO_4$, diethylaminoethyl-dextran, lipid-based and electroporation techniques, retroviruses, and adenovirus vectors carrying the reporter gene in their recombinant genome. Transfected genes are transcriptionally active over 24–72 h. In most instances, the reporter gene's product provides enzyme activities that are not normally found in eukaryotic cells and can be detected by assaying their substrate.

There is now a broad range of reporter genes whose role has expanded from the analysis of the *cis*-acting genetic sequences mediating RNA transcription to the characterization of molecular events that define phenotype expression and, more recently, to the monitoring of the delivery, efficiency, and stability of gene transfer. The most widely used reporter genes encode chloramphenicol acetyltransferase, β-galactosidase, glucuronidase, firefly and bacterial luciferase, alkaline phosphatase, green fluorescent proteins, cytosine deaminase, and herpes simplex virus type 1 thymidine kinase (HSV1-tk). The measurable phenotype can be assayed by radioisotopes, absorbance, fluorescence, bioluminescence, and chemiluminescence. Cellular, real time imaging of these substrates is accomplished by digital imaging microscopy and highly sensitive photon-counting cameras such as the charge-coupled device (*1*).

In vivo, visualization of gene expression using MRI is currently under investigation (*2*,*3*). Because of its unique ability to label and detect metabolic substrates, nuclear medicine provides the right tools to assess repetitively, noninvasively, and quantitatively the expression of transfected genes in tissues and organs. In vivo imaging of gene expression (also called Imagene (*4*)) was reported for the first time in an experimental animal model by Tjuvajev et al. (*5*). Using HSV1-tk as a marker gene, ^{14}C-labeled 5-iodo-2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyluracil (FIAU) as the marker substrate, and autoradiography, Tjuvajev et al. were able to show a preferential accumulation of FIAU in a mouse bearing rat glioma cells transduced with the recombinant STK retrovirus carrying HSV1-tk.

Over the past 5 y, two reporter gene techniques have emerged for in vivo imaging of transgenes. The first approach is based on the initial work of Tjuvajev et al. (*5*) and takes advantage of the promiscuity of the HSV1-tk enzyme for various purine and pyrimidine substrates and the development of radiolabeled analogs of these substrates.

The second approach is based on the expression of a transmembrane receptor as the reporter gene and its cognate receptor ligand as the marker. This method was developed by the UCLA Gene Imaging Consortium using the dopaminergic type 2 receptor and ^{18}F-fluoroethylspiperone as the ligand (*6*).

HSV1-TK SYSTEM

In this issue of *The Journal of Nuclear Medicine*, Iyer et al. (*7*) describe an interesting improvement in the HSV1-tk PET reporter gene imaging technique that they have contributed to establish over the past few years. They also introduce the concept of imaging two reporter genes (HSV1-tk and dopamine type 2 receptor [D_2R]) in the same animal.

The genome of herpes simplex virus 1 is characterized by the presence of a promiscuous thymidine kinase gene (HSV1-tk) whose product has relaxed substrate specificity. In contrast to its mammalian counterpart that phosphorylates thymidine preferentially, HSV1-tk can phosphorylate not only thymidine but also purine pentosides and a wide diversity of nucleoside analogs that are not phosphorylated efficiently by cellular kinases such as acylguanosine and uracil derivatives (*8*).

Clinicians have taken advantage of this characteristic and used HSV1-tk as a suicide, killer gene to treat herpes-like viral infections with synthetic purine nucleosides such as acyclovir and ganciclovir. These analogs, on

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phosphorylation by HSV1-tk, cellular guanylate kinase, and other cellular enzymes, interfere with herpes simplex virus DNA polymerase, blocking the replication of the viruses. These triphosphate analogs also enter and block cell DNA synthesis by inhibiting a cellular DNA polymerase, but to a lesser extent. This latter characteristic has been exploited in the treatment of certain cancers by transfecting the cancer cells with a vector carrying the HSV1-tk suicide gene and administering acyclovir or ganciclovir (*9–13*).

A similar approach has been exploited by several groups to image and monitor the adenoviral-mediated delivery and expression of HSV1-tk in mammalian cells and organs (*5,14–16*). ^{18}F positron-labeled acylguanosine derivatives such as 8-fluoroacyclovir (FACV), 8-fluoroganciclovir (FGCV), 9-[(3-fluoro-1-hydroxy-2-propoxyl)methyl]guanine (FHPG), and 9-(4-fluoro-3-hydroxymethylbutyl)guanine (FHBG) are currently being investigated as substrates for positron imaging of HSV1-tk expression (*15,17–19*). Penciclovir, another highly selective antiherpes virus agent with a satisfactory safety profile, is used by Iyer et al. (*7*) to compare its "reporting ability" with that of FACV and FGCV. According to these authors, fluorinated penciclovir (FPCV) has many advantageous characteristics over FACV and FGCV. In vitro, it is converted two times faster to its monophosphate derivative than is FACV by HSV1-tk and it does accumulate at a much higher level than does FGCV in cells expressing HSV1-tk. It is stable in mouse plasma and rapidly cleared from the blood pool compartment. FPCV retention in mouse-bearing tumors that express HSV1-tk is also significantly greater than is FGCV accumulation in such tumors. FPCV retention in HSV1-tk–transfected liver correlates well with the amount of virus injected (i.e., ultimately the amount of HSV1-tk enzyme synthesized).

Improvement of the HSV1-tk reporting technique can be approached by optimizing each component of the system. Gene therapy and reporting of a specific target require exquisite specificity for the vector carrying either the therapeutic gene or the reporter gene (or both). Several potentially useful transfer systems such as retrovirus, pseudotyped retrovirus, liposomal–DNA complex, herpes simplex virus, and adenovirus carriers have been developed. Among these vectors, adenoviral vectors have emerged as the safest and most practical in vivo. The ability to easily insert a foreign therapeutic or reporter gene (or both) within their genome and to express at the surface of their capsid a particular peptide by mutagenesis makes them extremely attractive and suitable for specific, guided gene therapy targeting (phage display library).

The importance of the adenovirus target specificity is not addressed here. Cell-specific gene delivery by viral vectors is limited by the promiscuous tropism of the parent virus. Strategies to alter the adenoviral tropism include, among others, selection from vector display libraries, insertion of short targeting peptides and larger polypeptide-binding domains into the coat proteins, display of an immunoglobulin-binding domain as a genetic fusion to the coat protein and then use of a monoclonal antibody to crosslink the vector with the target cell, and replacement of the coat proteins of the virus with the coat proteins of another virus that already has a desired host range (*20*). There is every reason to believe that the refinements of these viral engineering strategies will ultimately benefit greatly the reporter gene imaging techniques.

On adenoviral transfection, transgene expression is usually transient and requires repeated infections. Optimization of transgene expression is therefore critical. Transcription of the transgene sequence(s) depends on intracellular and DNA sequence-related factors. When constructing a transcriptionally targeted vector, a significant number of promoters, enhancers, silencers, and locus control sequences are available to choose from. Tissue-specific promoters and regulatory sequences are ideal for obvious reasons. A detailed discussion of transcriptional targeting is outside the scope of this commentary. Transcriptional targeting of viral vectors is not trivial and requires a great deal of construction fine-tuning in the context of a recombinant genome. Although far from ideal because it is not specific, the cytomegalovirus (CMV) promoter element used to drive the expression of HSV1-tk in this work is well characterized and gives a good level of expression of the downstream genes in many cell lines, tumors, and organs (*21,22*).

HSV1-tk salvages thymidine in the metabolism of the virus. The amino acid sequence and three-dimensional structure of the enzyme are well characterized and have been used as a framework to develop mutant enzymes. Various site-directed mutagenesis experiments conducted on HSV1-TK have allowed assignment of specific amino acid residues to specific functional properties (*23*). From this, a range of mutations has been hypothesized to confer the enzyme a higher affinity for various substrate and inhibitors that could be used in imaging. In a separate work, Gambhir et al. (*24*) have described the use of an HSV1-tk (HSV1-sr39tk) that seems to have improved V_{max}/K_m for penciclovir and increases the accumulation in C6 rat glioma cells expressing HSV1-sr39tk by a factor of 20 compared with that of wild-type HVS1-tk.

Over the past 20 y, acyclovir has become the reference drug to develop HSV-tk enzyme inhibitor and treat herpes simplex virus infections. Numerous compounds with higher affinity for the enzyme have been discovered and synthesized. Only a few have reached clinical use. For imaging purposes, two main classes of substrates have been investigated: the acylguanosine and the uracil derivatives. Acylguanosine derivatives such as acyclovir, ganciclovir, 9-[(1-hydroxy-2-propoxyl)methyl]guanine, and 9-(3-hydroxymethylbutyl)guanine are typically labeled with ^{18}F for positron imaging studies. Uracil nucleoside derivatives such as FIAU and fluoromethyl-β-arabinofuranosyluracil can be labeled with ^{11}C, ^{124}I, ^{131}I, and ^{123}I for PET or SPECT studies (*5,16,18,25,26*).

Most studies using the HSV1-tk reporter gene technique have come short of addressing a critical factor for the enzyme substrate accumulation. It is now well established that the HSV1-tk substrates are mediated by multiple membrane transporters, essentially peptide transporters (*27*). Therefore, it is reasonable to believe that the net uptake of HSV1-tk substrate inside the target cells not only reflects the enzyme activity but also the transport across the cell membrane. The design and labeling of the ideal HSV1-tk reporter substrate will have to take this important parameter into account. Similar to the therapeutic use of these HSV-tk substrates, the in situ delivery of the imaging agents might be critical for the successful assessment of transgene imaging. Interventional nuclear medicine with local delivery of diagnostic or therapeutic (or both) labeled agents is an interesting avenue that will definitely help the Imagene approach. Ultimately, the selection of the ideal reporter substrate for HSV1-tk imaging will depend on all of these factors and the absence of any significant systemic side effects.

RECEPTOR–LIGAND REPORTING SYSTEM

The D_2R–3-(2′-fluoroethyl)spiperone (FESP) reporter system described by Iyer et al. (*7*) belongs to the membranous receptor–ligand category of gene reporters. In this approach, a membranous receptor gene is expressed in the target cell and represents the reporter gene. The labeled cognate receptor ligand is then used to image the expression of the transduced gene. MacLaren et al. (*6*) have shown the usefulness of the D_2R–FESP reporting technique. Iyer et al. have shown the ability of the reporter gene technique to image two reporters in the same animal using the HSV1-tk and the D_2R–FESP systems concomitantly. As stated by the authors, it is not unreasonable to believe that some day this technique might be useful to assess a dual gene therapeutic approach.

Besides its potential for assessing dual gene therapy and for the follow-up of patients with Parkinson's disease who are treated by gene therapy (*28*,*29*), the D_2R–FESP reporter system could be most useful as part of a dual gene-expression cassette. In this system, the therapeutic gene and the transmembranous receptor reporter gene are combined in a bicistronic sequential DNA sequence that is driven by the same upstream promoter.

Both genes are coupled and expressed at the same time and ideally at the same level using an internal ribosomal entry site (IRES). The IRES element permits the translation of two open reading frames from one messenger RNA: one reading frame encoding the recombinant protein of interest (therapeutic) and the other encoding the reporter protein (*30*). The amount of radiolabeled ligand binding to the reporter protein is a direct reflection of the amount of therapeutic gene protein transcribed. Bicistronic adenoviral vectors carrying the human D_2R and the human somatostatin type 2 receptor (hSSTr2) as therapeutic or reporter genes (or both) are currently being designed and tested in cells and animals (*31*,*32*).

The Na^+/I symporter (NIS) gene reporting and therapeutic system is another example of a membranous receptor–ligand reporter gene that could be extremely powerful not only for radioiodine therapy but also for transfected tumor localization and gene therapy assays. The complementary DNA encoding NIS has been recently isolated and characterized. The unique ability of this intrinsic membrane protein to transport I^- is now exploited to design vectors expressing the NIS gene in tumors for targeted radiotherapy (*33*,*34*).

The requisites for using a reporter gene in imaging are similar to those used in molecular biology with a few additions: The endogenous activity or concentration of the reporter gene is low and ideally is absent from the targeted cells. The expression of the reporter gene is stable and reflects the expression of the therapeutic gene. The substrate can be labeled with single photons or positrons (or both) suitable for SPECT and PET imaging. The substrate binds readily to the gene product as a receptor–ligand or crosses cell membranes readily; it is rapidly metabolized by the reporter gene product and effectively trapped in the transduced cells; it is specifically retained by transfected cells and reflects the activity of the transgene expression. The substrate should also be stable in the blood, cleared rapidly from the bloodstream, be nonimmunogenic, and have no or minimal side effects.

CONCLUSION

Reporter gene technology is used widely in molecular biology to monitor the cellular events associated with signal transduction and gene expression both in vitro and in cell experiments. Various reporter genes with easily measurable phenotypes are now well characterized and available. The principal advantages of these assays are their high sensitivity, reliability, convenience, and adaptability to large-scale measurements.

The use of reporter transgenes to dissect biologic issues in transgenic animals has been a relatively recent development. Reporter genes have been used to identify regulatory elements that are important for tissue-specific gene expression or for development, to produce in vivo models of cancer, to study in vivo mutagenesis, and as a tool in lineage analysis and for marking cells in transplantation experiments.

In vivo reporting systems such as the HSV1-tk, D_2R–FESP, hSSTr2, and NIS are now being developed to image in vivo and monitor noninvasively the level, duration, and location of transgene expression. With advances in this technology and in detection methods, it is likely that clinicians will, in the future, be able to assess and correlate the targeting specificity and transduction of therapeutic genes with clinical outcomes.

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