Expression vectors and delivery systems
Editorial overview

Garry P Nolan* and Allan R Shatzman†

Addresses
*Department of Molecular Pharmacology and Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA; e-mail: gnolan@cmgm.stanford.edu
URL: http://www-leland.stanford.edu/group/nolan
†SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, PO Box 1539, King of Prussia, PA19406, USA;
e-mail: allan-r-shatzman@sbprd.com

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Introduction

While evolution has provided an embarrassment of biological diversity for humans to explore, we still remain primitive in our abilities to effectively translate current understandings to advances in biomedicine or industrial biotechnology. Progress has come largely through an ability to manipulate genes, and gene regulatory units, in the test tube by recombinant DNA practice. Return of such modified genes to living cellular test tubes has provided blueprints and toolkits for further explorations. A growing understanding of heterologous cellular systems (viral, bacterial, fungal, insect and mammalian) has identified fascinating similarities that beg us to move entire biochemical systems from one disparate organism to another. Indeed, most interesting are the exploitable differences that exist across organismal boundaries. This drives us to refine our knowledge of how best to manipulate these differences for biomedical or industrial applications.

Gene delivery and biochemical control of gene products constitute rate-limiting steps in this progress. For instance, the first demonstration of DNA as transferable genetic material (Haemophilus influenzae) is over 50 years old. Prior to that, the first purposeful use of a viral DNA-transfer device for therapeutic benefit (cowpox virus) is a century old. Only in the past two decades have we had reasonably effective control over the indivisible units of inheritance. More recently the means have been developed to apply this knowledge to such broadly different, though related, arenas as gene therapy, transgenic animals or plants, large-scale production of industrially or medically important proteins and biomaterials, or the pursuit of pure knowledge.

This issue of *Current Opinion in Biotechnology* covers two ends of a rapidly coalescing spectrum. The general fields explored are, firstly, gene delivery systems and, secondly, synthesis of materials we can effectively express. The 'why' of such delivery and expression will be left to the needs of the beholder. An objective of this issue is to contrast delivery systems against advances in expression of interesting biomaterials, be those materials natural, designed, or purposefully evolved *in statio*. The expectation is that workers at either end of the spectrum might find answers to their interests near the field interfaces.

Viral delivery vehicles – sincere forms of flattery

It is perhaps not surprising that the most efficient means for delivering genes to target cells — mammalian cells or otherwise — exploits the ruthless efficiency of viral vehicles. Generically, viral delivery systems are either short-term or long-term in their expression of the delivered gene material [1]. Designation as short or long-term is related to the particulars of the life cycle of the virus species being employed for delivery. With lytic viruses, such as baculovirus (insect cells), adenovirus (mammalian cells) or alphaviruses (dual mammalian/insect host range), viral gene expression is short-term, usually detrimental to the host cell, and generally concludes when the virus has utilized all available cellular resources. Usually, such viruses shut down host cellular metabolism (such as use of host cellular messenger RNAs) to maximize viral exploitation of the cell's resources. For short-term, high-level expression of genes of interest this cannibalization of cellular resources can be favorable to the researcher, as potent expression of proteins in bioreactors dedicated to rapid protein production minimizes costs. Baculovirus use in insect cells in culture, reviewed in three articles in this issue (Pfeifer, pp 518–521; Bouvier *et al*., pp 522–527; Jarvis, Kawar and Hollister, pp 528–533), still stands as one of the most efficient means for expressing a variety of proteins in eukaryotic cells (explored more thoroughly in the next section).

In mammalian cells, several classes of lytic viruses have been modified for expression of proteins. One of the newer classes of lytic viruses being tamed for use is the positive strand RNA alphaviruses. Semliki Forest virus, emblematic of this class [2], is advantageous in that the titer of virus readily produced can reach 1,011 and is thus applicable to large-scale rapid production of proteins or use as vaccine delivery. The formidable obstacle for broadening the utility of the system is the short-term nature of expression of the introduced proteins, given that infection interminably results in death of the target cell within a few days. Steps are being taken to minimize this problem and these are reviewed in their respective articles (Garoff and Li, pp 464–469). The utility of other lytic viral classes, such as Herpes simplex (non-lytic under appropriate circumstances) and adenoviruses, have been reviewed elsewhere as gene therapy vectors [3,4]. The most important advance in adenoviral applications has been the wholesale stripping of viral genes from within the vector

using so-called 'gutless' adenoviral constructs [5]. This has enabled more room for delivered genetic material of interest and minimizes the delivery of toxic viral gene products to the host cell (which might be contra-indicated in a gene delivery situation in humans or animals).

Other viruses have a more cozy, committed relationship with the cells they infect. In such cases, the cell might not be unduly taxed by the presence of a stably resident virus genome. In general, viruses that establish themselves stably in the chromosomes of a cell, or reside as extrachromosomal DNA elements, have uses in long-term, mid-level production of protein or gene expression. Examples of stable viral delivery systems include chromosomally localized retroviruses and adeno-associated viruses, and Epstein-Barr virus (EBV) extrachromosomal replicons. In these cases, the utility is to produce a stable, long-term expression for functional studies or continued harvesting of biomaterial from infected cells.

Retroviruses have been the workhorse of stable delivery vectors since their introduction by Mulligan, Baltimore and colleagues [6] over fifteen years ago. These first vectors were based on Moloney Murine Leukemia Virus (MMuLV) and the study of numerous other retroviruses [7]. Capable of delivering genes to nearly any dividing mammalian cell type, retroviral genomes can accommodate up to eight kilobases of foreign genetic material. Their ease of creation has been remarkably attractive for investigators in gene therapy and biomedicine. With the introduction of the transient transfection production systems for retroviruses [8–10], high-titer virus production can now be accomplished in a few days using simple procedures. Currently, the most widely-applied variation is termed the Phoenix retroviral production system that is based on a highly transfectable subclone of 293 cells stably transformed with SV40 T antigen [11] and murine leukemia virus components. In our laboratory, we use this system regularly for delivery of representative complex libraries of expressed cDNAs [12] or short peptides to cells for pathway mapping and complementation cloning of apoptosis molecules, T-cell signaling and HIV-1 counter-regulatory components. With the introduction of pantropic envelope proteins, such as Vesicular Stomatitus virus glycoprotein [13], these retroviruses have been used to stably deliver genes to insect cells and other non-mammalian eukaryotes.

Perhaps the most significant advance in retroviral applications is the landmark work by Naldini *et al.* [14] demonstrating application of HIV-1 as a gene delivery vehicle and reviewed in this issue (Naldini, pp 457–463). Classified as a lentivirus, HIV-1 and related retroviruses have the unique capacity to infect non-dividing as well as dividing cells. Thus, primary cells become viable targets for delivery of genes for gene therapy, protein production or target validation in pharmaceutical searches. In several classic papers these vectors have shown stable long-term production of proteins in a variety of model organs, such as brain, optic nerves, and skin. Third and fourth generation HIV-1 vectors with vastly improved utility and safety features (i.e. lacking the capacity to produce wild-type HIV-1 virus) have now been created. Other non-primate lentiviruses without the psychological issues associated with HIV-1 use in the laboratory, but maintaining efficient abilities to infect non-dividing cells, have been created including those employing Feline Immunodeficiency virus ([15]; M Curran and GP Nolan, unpublished data), Equine Infectious Anemia Virus, and a goat-derived lentivirus (A Kingsman, personal communication). In a fascinating twist researchers have employed alphaviruses or adenoviruses to produce retrovirus packaging proteins or packagable genomic RNA [16–18]. These and related systems are likely to increase the utility of these viral delivery vehicles.

Though long considered the poor cousin in the stable delivery field, owing primarily to its limited packaging size, adeno-associated viruses are coming to the fore as understanding is developed of their ability to integrate into chromosomal DNA. The utility of these viruses is their ability to integrate into non-dividing and dividing cells and the capacity to produce heterologous protein at high titer. It now appears that the rate-limiting step for stable integration into cells for these virions is the requirement for establishing a double-stranded intermediate prior to integration (these viruses package and deliver as a linear, single-stranded, snap-back terminated DNA genome). Provision of a double-stranded DNA form suitable for integration can take as long as two weeks in certain cell types. Evidence exists for their integration into a limited set of sites in host chromosomes. This, and related subjects on the exciting advances with adeno-associated viruses are covered in this issue (Rabinowitz and Samulski, pp 470–475).

Thus, integrative viruses have come a long way since their introduction; however, all seem to suffer from the influence of regulatory variations dependent upon their lack of true site-specific integration. Thus, each viral integration site can have significant variation in expression of the introduced gene. Furthermore, down-regulation of the introduced genes by epigenetic mechanisms is a significant, further obstacles that some have tried to overcome with insulator elements or other regulatory regimes. To overcome the most blockades to control many investigators have explored a variety of inducible expression systems. Perhaps the most widely applied is the bacterial tetracycline regulatory system developed originally by Gossen and Bujard [19] and exploited in retroviruses by others [20,21]. Significant modifications to the system have resulted in a variety of highly controllable expression systems that can be transported into cells with retroviruses, by standard electroporation, or by other DNA introduction methods. Rossi and Blau (pp 451–456) review this system and complementary approaches using bifunctional crosslinking ligands.

Not to be left out, but maintaining a cool distance from the attendant difficulties of integration into chromosomal

DNA, are episomal elements that stably reside in the nucleus, but replicate during the cells cycle and partition into daughter cells efficiently. Best exploited to date are the EBV vectors developed by Calos and co-workers [22] (Sclimenti and Calos, pp 476–479). Perhaps the most interesting feature of these vectors is that their heterologous promoters resident on the episome are not influenced by any chromosome resident regulatory events. Thus, studies of promoter regulation might be best carried out on EBV vectors wherein there are no apparent dominant effects of vector sequences upon the promoter of interest (GP Nolan, MP Calos, unpublished data). Of course, yeast and bacterial expression systems have long exploited episomal systems for controllable expression in the use of plasmids with replication origins and selection markers.

Future progress in viral vectors is likely to be in areas of targeted vector delivery to specific cell subsets in the body, higher titer production, and an eventual ability to efficiently assemble viruses *in vitro* without intervening assembly in living cells. Steps along these lines in the delivery of naked DNA, or DNA complexed with lipids or other cell uptake enhancing devices are in progress and discussed in this issue (Scherman *et al*., pp 480–485). As DNA transposons have now been shown to work in a variety of cell types, including mammalian cells [23] they will probably be merged with other DNA delivery vehicles in the near future. For example, integrative mechanisms that exploit high titer delivery of DNA by non-integrating adenoviruses have been married with newer TC1/mariner transposon systems (Fletcher, GP Nolan, unpublished data) with the hope of rapidly delivering large genes to chromatin. The variety of what can be delivered, from proteins to catalytic or regulatory RNA ribozymes (covered by Welch, Barber and Wong-Staal, pp 486–496) is diverse. So, it is clear that exciting and significant advances are being made along many fronts for delivery of genetic material to target cells.

Deliver with haste – but assembly required

Irrespective of our ability to deliver genes stably, transiently, or efficiently, once provisioned to target cells the hard part is expressing sufficient levels and purifying the material of interest from unwanted byproducts. For instance, obtaining functional protein is often more an art than following a recipe. Bouvier *et al*. (pp 522–527) review the production of a medicinally interesting class of proteins, G protein-coupled receptors (GPCRs), using baculovirus vectors and show how viral particles shed from infected cells have been used to co-purify expressed proteins of interest. As well, given the multi-subunit nature of the GPCR signaling apparatus, high-yield production of intact multi-component signaling structures will be important for biophysical determinations and drug development. One of the major drawbacks of baculovirus expression systems — that of inappropriate mammalian post-translational modifications — is addressed by Jarvis, Kawar and Hollister (pp 528–533) in their demonstrations of glycosylation pathway engineering in insect cells to endow them with mammalian glycosylation pathways. This type of engineering will be a critical consideration in the near future for expression of proteins with lowered antigenicity or appropriate post-translational (glycosylation-dependent) function. Yeast can also be effectively exploited as tools for understanding and expressing proteins in heterologous contexts. Though further afield from an evolutionary view than insect cell expression, yeast combine ease of use with extraordinary genetic and biochemical knowledge of their internal signaling systems. Thus, engineering of GPCR signaling systems, or other studies of signaling proteins, can be studied in relative isolation in yeast (Reiländer and Weiß, pp 510–517).

Whereas high-level protein expression can often be achieved, production of appropriately folded proteins remains difficult in a variety of systems. For example, in *Escherichia coli* the most common difficulty is the formation of inclusion bodies composed mostly of the expressed protein in complex with *E. coli* cellular components. Though there is a degree of enrichment of the desired protein, it is often complexed in a denatured form that is unusable for biochemical applications. Overcoming this difficulty, such as with important members of the nuclear hormone receptor family (Mossakowska, pp 502–505), seems to require co-expression with their normal cognate binding partners or ligands. Thus, a variety of techniques have been developed to renature or refold proteins that are expressed under such circumstances (Lilie, Schwartz and Rudolph, pp 497–501). In addition, a little used but fascinating application of prokaryotic mutants lacking peptidoglycan cell walls — so called L-form bacteria (Gumpert and Hoischen, pp 506–509). In these strains, the formation of the formidable bacterial barriers of the glycan cell wall are removed, along with associated proteases and other processing enzymes. Thus, proteins secreted from these cells are afforded immediate release to the media for purification. In addition, because inclusion bodies can form due to nonspecific complexing with the ompA bacterial surface protein, such cell wall-less mutants have utility to minimize problems with losing expressed proteins to inclusion bodies.

Finally, expression in the absence of living cells can also be applied for the formation of complex proteins and assembled structures. Notably, bioreactors with cellular extracts containing the ribosomal translation machinery of erythrocytes or bacterial cells or yeast have been employed to generate significant amounts of expressed proteins. Steady-state systems are explored by Plückthun and co-workers (pp 534–548) for large-scale production of proteins in relatively defined conditions. Interestingly, this technology has been employed for combinatorial generation of proteins *in vitro* using pseudo-genetic selection systems. Perhaps most notably, *in vitro* translation systems afford the opportunity to use non-natural amino acids for protein production. This will be of extreme importance in the future creation of novel enzymatic capabilities when such non-natural substitutions are made at reactive centers of enzymes.

Conclusions

To anyone working in the field of delivery of genes or expression of biomaterials a mercenary calculation is always a requirement for success. The most robust system(s) advertised by any partisan is no guarantee for the gene that you next desire to express. The most successful laboratories and industrial concerns have adopted two or three of the more common systems (baculovirus, Chinese hamster ovary cells, retroviral, yeast or *E. coli*), become expert in those technologies, and applied them to advantage. The articles in this issue include several of the more successful delivery or expression systems with frank discussions of their strengths and weaknesses. The articles also discuss overcoming of obstacles by simple modifications of procedures. Even the lowly *E. coli* was coaxed into new, more compliant capacities as in the case of the L-form bacteria. It is probably that viruses will be soon engineered with broader or more controllable attributes. From the other side progress will obviously be made in the *de novo* creation of synthetic virus-like particles that will eventually be bought as kits for efficient delivery to the cells of your choice. Soon enough we will learn to regulate genes as we desire and have suitable control over the form of their expression. Though we cannot achieve all these goals at present, the foundation is laid and the stepping stones of the path are obvious. In our past manipulation of the genetics and biochemistry of cells and organisms for biomedical or industrial applications, what has become most fascinating has been our mimicry and exploitation of natural host processes to deliver and express genes. We expect this trend to continue.

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