

An advance in liver-specific gene delivery

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One of the more intractable problems in gene therapy over the past decade has been the development of methods to allow the selective delivery of genes to the liver and their stable maintenance and expression therein. Hepatocytes (the principal parenchymal cell of the liver) are a much sought-after target of gene therapy because they play many unique roles in the physiology of the mammalian host (1). The liver is an important site of synthesis of many plasma proteins, including those of the complement and coagulation systems. Hepatocytes also synthesize a wide variety of enzymes involved in intermediary metabolism, and mutations affecting several of these produce clinical disorders that are attractive targets for liver-specific gene therapy (2). The liver also produces low density lipoprotein (LDL) receptors (3), and hepatic restoration of wild-type LDL receptors in hosts bearing mutant receptors ameliorates their hypercholesterolemia (4). Finally, of course, there are several important chronic viral infections of the hepatocyte (notably hepatitis B and hepatitis C) for which the liver-specific delivery of antiviral compounds (e.g., INF- α or other cytokines) might be of therapeutic value. In a recent issue of PNAS, Protzer *et al.* (5) report new progress in the development of liver-specific viral vectors based on the genomes of hepatitis B viruses and present data on the use of these vectors to deliver antiviral cytokines to hepatocytes.

The liver poses formidable obstacles to hepatocyte-specific gene delivery. Many liposomes that effectively deliver genes to other cell types do not efficiently target the hepatocyte, often winding up instead in the Kupffer cell, a hepatic macrophage. Partial success has been achieved in targeting hepatocytes by using DNA-containing complexes designed to interact with specific receptors on liver cell surfaces that mediate receptor-mediated endocytosis (reviewed in ref. 2). Virus-mediated delivery to hepatocytes has been equally problematic (6). Because most hepatocytes in the adult liver divide infrequently, they are poor targets for retrovirus-mediated gene transfer with murine leukemia virus-based vectors, which establish integrated proviruses only in dividing cells (7, 8). Adenovirus vectors

have been shown to efficiently deliver genes to cultured hepatocytes and to mouse liver cells *in vivo* (4), but current generations of adenoviral vectors excite an immune and inflammatory response that usually leads to the rapid elimination of transduced cells (9, 10). Newer generations of adenovirus vectors are being developed to reduce the expression of viral genes and thereby mitigate this problem (11, 12), though it is unclear whether it can be totally eliminated. Some success in liver targeting with adeno-associated viruses has been reported, but large doses of virions transduced fewer than 5% of hepatocytes (13).

For all of these reasons, virologists have long been interested in the possibility of using other viruses that are known to be liver specific as potential agents of hepatocyte-directed gene transfer. The leading candidate viruses in this regard are those of the hepatitis B virus family, also known as hepadnaviruses (hepatotropic DNA viruses). These viruses naturally display a high (though not absolute) degree of hepatotropism, with infection trained principally on the hepatocyte compartment (Kupffer cells, for example, are not infected). The hepadnaviral life cycle is not directly cytotoxic, and persistent infections frequently result (14). Viral replication proceeds via reverse transcription of an RNA intermediate, through a complex biochemical pathway that is now understood in great detail. Work in several laboratories over the past decade has identified the main viral proteins involved in replication and extensively mapped the cis-acting elements of the genome that are required to support viral DNA and RNA synthesis (see ref. 14 for review). These advances now have made it possible to design strategies to harness the hepadnaviral genome as a vector for liver-specific gene transfer.

Such strategies are conceptually straightforward and are based on paradigms earlier established in retroviral vectoring. The idea is to substitute one or more viral genes with the gene of interest; because virtually all hepadnaviral genes are essential for replication, the resulting chimera is replication defective and requires complementation *in trans* by a helper virus genome. As in retroviral vec-

toring, the helper virus in this case is one in which all viral coding regions are intact and expressed, but which bears a deletion in the viral packaging signal ϵ . This is a key cis-acting element required for incorporation of the genomic viral RNA into virus particles (15), where it can be reverse-transcribed. The helper, therefore, can provide all of the essential replication functions, but cannot itself be propagated as an infectious virus. Cotransfection of the chimeric genome and helper genome into a permissive cultured hepatoma cell results in the release of encapsidated chimeric progeny. These progeny then can be used to infect either primary hepatocytes *in vitro* or animal hosts *in vivo*. Because of the established species specificity of hepadnaviral infection, vectors for targeting cells of any given species must be developed from that species' corresponding hepadnavirus—human hepatitis B virus (HBV) for human hepatocytes, duck HBV (DHBV) for duck liver cells, etc. Unfortunately, the absence of a known murine hepadnavirus at present bars the application of this technology to that convenient small animal host.

All of the above steps are straightforward, and the broad outlines of this approach have been clear to most hepadnavirologists for more than a decade. Indeed, work in several laboratories over this interval has vigorously pursued this possibility (16), but successes have been few. The biggest problem has been the fact that the tiny hepadnaviral genome (3 kb) is virtually blanketed with critical cis-acting elements—initiation sites for minus and plus strand DNA synthesis, promoter elements for multiple critical transcripts, and numerous sequences affecting RNA transport, processing, stability, and packaging. (Their many dispersed cis-acting elements—and the small size of the viral genome—also mean that hepadnaviruses never will be able to carry more than small cargoes of foreign genes.) In addition, unlike its retroviral counterpart, the viral reverse transcriptase acts preferentially *in cis* and is only inefficiently supplied in

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trans, for reasons that are still incompletely understood (17, 18). Because most substitutions in the genome will interrupt the reverse transcriptase coding region, their replication is also subject to this limitation.

Protzer *et al.* (5) report a major advance in hepadnaviral vectoring. Through patient and diligent screening of many recombinants, they have identified a region of the viral genome that evidently lacks important cis-acting sequences and therefore tolerates substitution with foreign DNA. This region coincides almost exactly with the coding region for the major viral envelope glycoprotein, S. Working principally with DHBV, the authors have created two recombinants, one bearing a green fluorescent protein marker and the other a type 1 IFN coding region. Cotransfection with the ϵ -deficient helper resulted in the production of stocks bearing around 10^8 viral genome equivalents per ml, and the resulting stocks efficiently infected primary duck hepatocytes *in vitro*. By 1 week postinfection, virtually all hepatocytes on the dish had been successfully infected, and the selectivity of wild-type infection was preserved—no Kupffer cells in the preparations displayed viral gene expression. Using their genetically marked viruses, the authors were able to prove what has long been suspected by hepadnavirologists based on *in vivo* experimentation—namely that prior infection with DHBV establishes a block to superinfection. However, this block is relative and not absolute: detectable superinfection does occur, but is reduced 20-fold in frequency from that observed in cultures that do not bear resident hepadnaviral genomes. These are important results with major implications both for the fundamental biology of hepadnaviruses and for hepatic vectorology.

That being said, many formidable obstacles remain. First, when used to infect whole animals rather than primary hepa-

toocytes in culture, only very small numbers of hepatocytes were transduced—approximately one in 10^5 in the avian system. Infection with vectors based on human HBV appears to be even more inefficient—in primary hepatocytes (a system in which virtually all cells become infected with DHBV vectors) fewer than 1% of cells appeared to be successfully transduced. One way to minimize the disadvantage of low transduction efficiency is to engineer the vector to express secretory molecules that can affect surrounding cells in a paracrine fashion. The expression of duck IFN by the DHBV chimera in ref. 5 affords an example of such an approach. Although this chimera was tested only in culture—a situation in which DHBV transduction efficiency is very high—it did effectively suppress wild-type DHBV coinfection. This raises the possibility that local delivery of IFN by HBV vectors could be useful in the therapy of chronic HBV infection *in vivo*; however, in such a setting, the delivery of IFN by the HBV vector will be further reduced by the relative block to superinfection discussed above. It remains to be seen whether future refinements in HBV vectoring will be able to overcome the formidable twin problems of low transduction efficiency and superinfection resistance.

Another important issue is that because the protocol for generating vector stocks involves cotransfection with a helper genome, there is potential for the production of wild-type virus by homologous recombination. Because in the case of HBV such a recombinant would have pathogenic potential, additional modifications will be required to eliminate this possibility. Finally, there is the issue of host immunity to the vector. The current generation of vectors continues to express the viral core protein and, in the case of the HBV recombinants, the viral X gene. Both of these proteins are known to induce cytotoxic T lymphocyte responses *in vivo* (19),

raising the possibility that the same inflammatory responses that have bedeviled adenoviral vectoring in the liver also may present themselves in hepadnaviral vectoring. (Because the expression of these proteins is not essential in this system, this problem should be easily circumvented by mutationally inactivating these coding regions.)

We are, therefore, still a long way from the routine use of hepadnaviruses in gene therapy. Nonetheless, the results of Protzer *et al.* (5) represent an important landmark in this long odyssey. They establish that the approach is possible in principle and define the major hurdles still to be cleared. But even if these viruses fail to fulfill their promise as vectors for *in vivo* therapy, the recombinants generated by Protzer *et al.* should remain enormously useful for experimental gene transfer in hepatocyte cultures and for the study of hepadnaviral infection. A major potential application concerns the definition of the entry pathway for these important viruses. Earlier work has established that at least two molecules are likely to be required to mediate DHBV entry. One of these, a cell surface carboxypeptidase, already has been identified and serves as the principal binding component for DHBV on the cell surface (20–22). However, expression of this protein is not sufficient to allow infection; presumably one or more coreceptors is/are required. By developing genetically marked hepadnaviruses, Protzer *et al.* have opened the door for gene transfer approaches to the identification of the coreceptor, much as has been achieved in recent years for HIV. Similarly, by developing a system in which superinfection-interference can be assayed and quantified, the authors have opened this interesting phenomenon to molecular investigation. The elucidation of the molecular details of these processes may well prove to be hepadnaviral vectoring's most important contribution to biology.

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