Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 8759–8760, October 1993

Commentary

High titers of retrovirus (vesicular stomatitis virus) pseudotypes, at last

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In a recent issue of the *Proceedings*, Burns *et al.* (1) presented evidence that they can make very high titer stocks of a pseudotype virus whose genome is derived from the Moloney retrovirus and whose envelope is the G protein of vesicular stomatitis virus (VSV). As expected, the virus has the broad host range characteristic of VSV. For example, unlike Moloney virus, it is able to infect hamster cell lines. Less predictable, but strikingly, the pseudotype also infects fish cell lines including a zebrafish line. The titers of virus reported are a spectacular 10^9 colony-forming units/ml. This stunning technical leap—a leap of six to seven orders of magnitude in titer over previous reports for this virus—may have important applications in human gene therapy. It also has important implications for basic research. The report by Burns *et al.* (1) comes more than 20 years after the discovery that retroviruses and VSV can swap envelope proteins (2), and it appears after years of unsuccessful efforts by various laboratories and biotech companies to produce high titers of retroviral pseudotypes bearing VSV G protein on their surface.

It has long been known that when two viruses infect the same cell, the proteins coded by one viral genome can sometimes package the genome of the other. This process, called phenotypic mixing, is hardly surprising when the viruses are closely related. However, phenotypic mixing can also occur between viruses that belong to completely different taxonomic groups. In 1972 Zavada (2) discovered that phenotypic mixing occurs between retroviruses and VSV, the prototype rhabdovirus.

VSV is a lytic virus that replicates in the cytoplasm, has a single negative-stranded RNA genome of ≈11,000 nt, a bullet-shaped virion surrounded by a membrane, and the ability to shut off host transcription and translation on its way to killing its host cell (see ref. 3). Retroviruses have a positive-strand RNA genome of 8–10,000 bases, replicate via a DNA intermediate that must integrate into the host genome to be efficiently transcribed, and in most cases, do not kill the cells they infect (see ref. 4). Both viruses release progeny virions by budding.

Retroviruses use specific cell surface proteins as receptors to get into cells and attach to the receptors via their envelope glycoproteins. This interaction is very often species specific, and in some cases even tissue specific. As a result, retroviruses usually have a quite limited host range. In contrast, VSV has a notoriously broad host range, infecting everything from human to insect cells. It is thought that the virus must use a universal membrane component to get into cells, possibly phospholipid (5). VSV G glycoprotein is responsible for the viral host range.

When VSV and a typical mouse or chicken retrovirus infect the same cell, the progeny are a mixture of viruses that include parental types and pseudotypes. The latter, the result of phenotypic mixing, (*i*) contain a retroviral genome but the G protein and, hence, have the expanded host range of VSV, (*ii*) contain a VSV genome and the envelope protein and, hence, have the restricted host range of the coinfecting retrovirus, or (*iii*) have a mixture of envelope proteins derived from both viruses (2, 6, 7). Before molecular cloning was possible, the only tools available for separating the various types of particles emerging from a mixed infection were neutralizing antisera and temperature-sensitive mutants of VSV that could prevent replication of particles with a VSV genome (2, 6–9). Both methods were messy and it was difficult to obtain either pure or high titer stocks of pseudotypes. Molecular cloning offered new ways to solve the problems.

Molecular cloning of viral genomes and the development of retroviral packaging lines lead to the recent report by Burns *et al.* (1). Packaging cell lines are engineered to synthesize all the proteins needed to make and release infectious retroviral virions (proteins encoded by the viral *gag*, *pol*, and *env* genes) (10, 11). The cells are then provided with a viral genome, designed to be the only RNA molecule in the cell with the capability of being packaged efficiently into virus particles. To meet this requirement, an RNA molecule needs a packaging sequence, termed psi (ψ), which in the case of Moloney virus is contained within a few hundred nucleotides near the 5′ end of the RNA (10, 12). The other requirement to be a successful viral genome are sequences needed for reverse transcription and sequences required for integration of the resulting proviral DNA. These include a primer binding site and sequences at and near the termini of the RNA that become the long terminal repeats in the double-stranded DNA copy of the viral RNA (see ref. 4). The rest of the RNA, up to about 10,000 bases worth, can encode anything at all, including, for example, a gene to correct cystic fibrosis, sickle cell anemia, etc. The particles released from packaging cell lines are infectious but defective, since they lack *gag*, *pol*, and *env* genes. They can infect cells just once, integrating a copy of their genes into the host cell genome. It is these particles that are potentially so useful for gene therapy.

Knowledge of how to make retroviral packaging cell lines suggested that one might be able to construct a line that would produce retroviral pseudotypes with VSV host range. In 1991, Emi, Friedmann, and Yee (13) took an important step in this direction. They showed conclusively that G protein was the only VSV-coded protein needed to make mouse retroviral pseudotypes with the VSV host range (13, 14). This result, and a similar finding by Hunter and coworkers (15) that influenza hemagglutinin protein can be used in place of the Rous sarcoma virus env glycoprotein, is interesting. How is G protein, or hemagglutinin protein, recruited into budding retroviral virions? In VSV particles, it has been thought that a matrix protein (M) binds the nucleocapsid and also the tail of the G protein to pull it into the budding particle. But G protein has no sequence similarity to retroviral envelope proteins, so it is unlikely that it interacts specifically with retroviral capsid proteins (14). In fact, how G or hemagglutinin, or even env glycoproteins, are recruited into retroviral virions remains unclear. The current hypothesis is that they may simply get in because they are not attached to anything else (15, 16). In any case, if G or hemagglutinin can be incorporated into retroviruses and determine their host range, who knows what other interesting molecules with interesting host ranges might also work?

Emi *et al.* (13) achieved Moloney (VSV-G) pseudotype titers no higher than 10^2 to 10^3 infectious units/ml. Thus, a remaining technical problem to make the

findings useful was to produce high titers of the virus. This technical problem turned out to be a bear whose solution is a tribute to the perseverence of Yee and Friedmann and their collaborators, as shown in the report of Burns *et al.* (1). To make high titers of the desired retroviral pseudotypes requires a cell that expresses large amounts of gag and pol proteins and large amounts of VSV G protein. The problem is that G protein is toxic to cells (presumably because it causes membrane fusion). To date, it has not been possible to obtain stable cell lines with the desired levels of production. Burns *et al.* (1) produce their pseudotypes in a transient assay.

Burns *et al.* (1) introduced a *gag–pol* coding construct into 293 cells, an exceptionally transfectable adenovirus-transformed human cell line (17), and selected a cell clone expressing high levels of the proteins. To make the desired pseudotypes, they transfect this line with a construct that encodes a Moloney virus based genome consisting of long terminal repeats, the VSV G gene (included so virus can spread among the 293 cells, although this has since turned out not to be necessary), and the gene for neomycin phosphotransferase. About 2 weeks after transfection, cells produce high titers of a virus that confers G418 resistance to hamster, fish, and insect cell lines, indicating that they have acquired the neomycin-resistance gene. The pseudotype presumably has a genome consisting of Moloney virus long terminal repeats flanking the VSV G and neomycin-resistance genes. Titers are 5×10^5 to 4×10^6 colony-forming units/ml. These are respectable titers for any retroviral packaging line and are spectacular for this particular pseudotype, but much higher titers of the pseudotypes were obtained by concentrating the virus from culture fluids (see ref. 18). Unlike many retroviruses that are quite fragile and whose envelope proteins often seem to fall off without provocation, these particles can take abuse. Upon first centrifugation, a stock of pseudotype virus retained about 95% of its titer, while a second pounding reduced it to about 70%. Using two centrifugations, Burns *et al.* (1) could obtain small volumes of stocks with titers exceeding 10^9 colony-forming units/ml.

The ability of the Moloney (VSV-G) pseudotype to infect hamster cells is not surprising since it has been known that an appropriate receptor gene confers Moloney virus infectability on these and most mammalian cells. More unpredictable, although not out of line with our knowledge of retroviral promiscuity, is the ability of the pseudotype to infect fish cells (1, 19). The result is not outlandish because most restrictions to cross-species retroviral infection occur at the level of cell surface receptors or much later, during transcription or synthesis and processing of virion proteins. Restrictions that affect synthesis or integration of proviral DNA are less common, and when they occur, may not be severe. In fact, the titer of the Moloney (VSV-G) pseudotypes is about 100-fold lower on fish cells than hamster cells. Whether this is a true host-range difference or is merely due to the lower temperature used to grow the fish cells remains to be seen.

Why is the report by Burns *et al.* (1) so exciting? Retroviruses are powerful tools for research as well as gene therapy because of the efficiency, tidiness, and permanence with which they insert genes into susceptible cells. The wide host range and high titer of the Moloney (VSV-G) pseudotypes means that this powerful tool can now be brought to additional biological problems and organisms. For example, one might be able to perform lineage tracing in frogs, flies, or monkeys (see ref. 18), or possibly, a personal favorite, insertional mutagenesis in zebrafish. Zebrafish are currently the organism of choice for large-scale forward genetic screens to find genes important in all aspects of early vertebrate development and organogenesis (20, 21). At the virus titers reported by Burns *et al.* (1), it might be possible to infect many cells of a developing zebrafish embryo (there are only about 8000 cells at gastrulation) or many of the germ cells in older animals, and thus, to use retroviral insertional mutagenesis in place of current methods of chemical or γ-ray mutagenesis. Although there are no fish ES cells, germ-line chimeras can be made by transplanting primary cells from one blastula stage embryo to another (22), so another way to get virus into the germ line might be to infect donor cells before transplanting them. To what extent the speed of early zebrafish development relative to the time needed for synthesis and integration of proviral DNA will hinder these approaches in the absence of spreading virus is hard to predict, but fortunately, relatively easy to determine. As for human gene therapy, which motivated and partially supported the research, the broad host range of the Moloney (VSV-G) pseudotype would probably make it the retroviral particle of choice for tagging cells or introducing genes into cells that are susceptible to retroviral infection. These include cells of the hematopoietic lineages and probably any other dividing cell types. How useful the pseudotypes will be will depend in part on whether the desired therapeutic genes can be packaged in virus particles at the same high titers seen by Burns *et al.* (1).

Are there any concerns with these potentially very important results? In the initial study by Emi *et al.* (13), three methods were used to confirm the structure and behavior of the pseudotypes: host range (ability to confer G418 resistance); neutralization by anti-VSV serum; and Southern blot analysis of infected cells to confirm the presence of integrated proviral DNA. Burns *et al.* (1) use only the first method. In the case of the zebrafish cells particularly, it would have been reassuring to see molecular analysis confirming retroviral infection. [These studies were hindered by the inability of the particular cell line used to plate at low cell density (19).] As for the very high titers of virus reported, Burns *et al.* (1) took care to rule out a common cause of deceptively high titers, namely contamination with helper virus. Thus, the only concern that one might have about this paper is that, after such a long wait, it almost seems too good to be true.

1. Burns, J. C., Friedmann, T., Driever, *Proc. Natl. Acad. Sci. USA* **90**, 8033–8037.
2. Zavada, J. (1972) *J. Gen. Virol.* **125**, 183–191.
3. Wagner, R. R. (1991) in *Fundamental Virology*, eds. Fields, B. N. & Knipe, D. M., *et al.* (Raven, New York), pp. 489–503.
4. Weiss, R., Teich, N., Varmus, H. & Coffin, J., eds. (1982) *RNA Tumor Viruses, Molecular Biology of Tumor Viruses* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
5. Schlegel, R., Willingham, C. & Pastan, I. (1982) *J. Virol.* **43**, 871–875.
6. Zavada, J. (1972) *Nature New Biol.* **240**, 122–124.
7. Weiss, R., Boettiger, D. & Love, D. N. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 913–918.
8. Witte, O. N. & Baltimore, D. (1977) *Cell* **11**, 505–511.
9. Weiss, R. & Bennett, P. L. P. (1980) *Virology* **100**, 252–274.
10. Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33**, 153–159.
11. Danos, O. & Mulligan, R. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6460–6464.
12. Linial, M., Medeiros, E. & Hayward, W. S. (1978) *Cell* **15**, 1371–1381.
13. Emi, N., Friedmann, T. & Yee, J.-K. (1991) *J. Virol.* **65**, 1202–1207.
14. Rose, J. K. & Gallione, C. J. (1981) *J. Virol.* **39**, 519–528.
15. Dong, J., Roth, M. G. & Hunter, E. (1992) *J. Virol.* **66**, 7374–7382.
16. Perez, L. G., Davis, G. L. & Hunter, E. (1987) *J. Virol.* **61**, 2981–2988.
17. Graham, F. L., Smiley, J., Russel, W. C. & Nairn, R. (1977) *J. Gen. Virol.* **36**, 59–72.
18. Cepko, C. (1989) *Methods Neurosci.* **1**, 367–392.
19. Driever, W. & Rangini, Z. *In Vitro Cell Dev.*, in press.
20. Kimmel, C. B. (1989) *Trends Genet.* **5**, 283–288.
21. Rossant, J. & Hopkins, N. (1992) *Genes Dev.* **6**, 1–13.
22. Lin, S., Long, W., Chen, J. & Hopkins, N. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4519–4523.