Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 12750–12752, October 1998

Commentary

RNA virus vectors: Where are we and where do we need to go?

Peter Palese

Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029

The ability to genetically engineer animal viruses has dramatically changed our understanding of how these organisms replicate and has allowed the construction of vectors to direct the expression of heterologous proteins in different systems. The small DNA-containing viruses were in the forefront of the recombinant DNA revolution, which started in the 1970s. In fact, transfected DNA molecules of SV40 (approximately 5,000 bp in length) allowed the first rescue of defined viral mutants (1). Subsequently, the molecular engineering of herpes simplex viruses (genome of approximately 150 kbp) and of vaccinia viruses (genome of approximately 190 kbp) represented major breakthroughs for modern virology (2–4). After this seminal work in the early 1980s, techniques were developed to specifically alter the genomes of adenoviruses, adeno-associated viruses (5), and many other DNA-containing viruses. Most recently, it has become feasible to rescue human cytomegalovirus (6) and Epstein–Barr virus (7) by cotransfecting into cells cosmids containing overlapping fragments of the respective viral genomes. Thus, extraordinary progress has been made in harnessing the genomes of DNA viruses to (*i*) help us understand the structure function relationships of the viral components and to (*ii*) generate mutants and recombinant viruses expressing foreign proteins. Similar success was brought to the retrovirus field by the study of myriads of novel constructs of RNA tumor viruses. In addition, retrovirus-based vectors have been shown to express foreign genes in animals over periods of weeks and months (5).

Equally exciting has been the progress in the development of genetic engineering methods for nonretroviral RNA viruses. The paper by Rice and coworkers in this issue of the *Proceedings* (8) is the most recent example of a highly imaginative approach to using RNA viruses (or components of RNA viruses) to express foreign genes. The foundation of the system described here goes back to 1987 when Rice *et al.* (9) first succeeded in making infectious Sindbis virus from a full-length cDNA clone. Sindbis virus is an alphavirus containing a 12-kb, single-stranded, positive-sense, capped and polyadenylated RNA genome. When the genomic (naked) RNA of the virus is introduced into cells, infectious virus forms. Two avenues were developed to express foreign proteins from Sindbis virus constructs: (*i*) A self-replicating, self-limiting replicon was made by replacing the genes for the virion structural proteins with that of a reporter gene (10). (*ii*) A chimeric virus was made by introducing a subgenomic promoter, which drives the expression of the heterologous protein (11, 12). These replicons/vectors amplify to high levels and thus will kill the transfected/infected cells. The present paper describes an ingenious way to circumvent the cytopathic properties of these vector systems.

Agapov *et al.* (8) developed noncytopathic Sindbis virus replicons that replicate in BHK cells and express large amounts of foreign proteins. This was done by transfecting Sindbis virus replicons, which express a puromycin *N*-acetyltransferase into cells growing in the presence of puromycin. Cells that can survive (and divide) in the presence of the drug carry replicons, which are not cytotoxic and express the puromycin-inactivating enzyme. One replicon selected by Agapov *et al.* (8) happens to have a mutation in the nsP2 protein, a component of the RNA replicase. Cell clones transfected with such a replicon expressing β-galactosidase maintain high expression levels after 10 cell passages (in $>90\%$ of the cells). Superb molecular biology by Agapov *et al*. (8) has led to second generation bipartite vectors (DI/replicon systems), which are also noncytopathic and express levels as high as 30 μg of a foreign protein per 10^6 cells.

What are some of the characteristics (advantages) of RNA virus-based vector systems as described by Agapov *et al.*? (*i*) An *in vitro* application is the use of these rapidly selectable noncytopathic RNA vectors for cell culture studies. After efficient RNA transfection by electroporation, puromycin selection is imposed, and a population of cells expressing the gene of interest is derived in a matter of days. This should be extremely helpful for a variety of biological investigations, as highlighted by the use of the system to trans-complement viral functions, like the NS1 replicase component of yellow fever virus (13) (*ii*) These replicon-based vectors may express heterologous proteins to high levels for prolonged periods and thus serve as excellent vaccine delivery systems. (*iii*) They do not contain a complete complement of all viral genes and thus no infectious particles are produced which could result in a more generalized infection spreading to other tissues. These vectors may thus meet stringent safety concerns. (*iv)* Because the vector component derived from the virus is small and does not express structural proteins, the host immune response to the vector is likely to be limited. Thus, the long-term expression of foreign proteins in a noncytotoxic manner may blow new wind into the sails of our gene therapy enterprise. (*v*) It is unlikely that this RNA virus vector (or for that matter other RNA virus-based systems) would cause cell transformation. Because these RNA viruses lack a DNA phase, there is no concern about unwanted integration of foreign sequences into chromosomal DNA.

Although long-term expression of foreign genes may be desirable for certain medical applications, other purposes are better approached using RNA vectors, which are self-limiting. Pioneering work with another alphavirus, Venezuelan equine encephalitis virus (VEE), by Johnston and coworkers (14) has shown that self-limiting replicon vectors based on the sequence of a live attenuated strain of VEE induce protective immunity against influenza in animals. Furthermore, alphavirus expression vectors, which are cytolytic (and thus suicidal), may enhance the armamentarium of DNA vaccines. Administration to mice of plasmids that transcribe self-amplifying alphavirus RNA replicons has been highly effective in inducing an enhanced immune response against a variety of infectious agents (15, 16).

Other positive sense RNA viruses such as poliovirus also have been successfully engineered to express foreign genes/epitopes (17–19). These vectors, although limited in their ability to express large foreign sequences, share advantages with other RNA virus vectors in that they don't appear to

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Table 1. Designer RNA virus/vector systems

Positive sense (ref.)	Negative sense (ref.)
Sindbis virus/replicons (8, 12, 13)	Influenza virus (26–31)
VEE virus (14, 24)	Rabies virus (32)
Semliki Forest virus (16)	Vesicular stomatitis virus (33–35)
Poliovirus (18, 19, 25)	Respiratory syncytial virus (36)
Kunjin virus (39)	Sendai virus (37)
	SV5 (38)

The above represents a partial list of positive and negative sense RNA viruses, which have been genetically engineered to express foreign proteins. Some of the constructs give rise to infectious (attenuated) viruses, others form noninfectious replicons, which are restricted to replication (and expression of the foreign gene) in the transfected/infected cell. The Sindbis virus replicon system described in Agapov *et al*. (8) is noninfectious as well as noncytopathic and thus allows long-term expression following division of cells. For negative sense RNA viruses with segmented genomes (influenza viruses) and with nonsegmented genomes (rabies, vesicular stomatitis, respiratory syncytial, SV5, and Sendai viruses) effective expression systems have been obtained by inserting the foreign gene as a bicistronic element or by adding an additional transcriptional unit into the respective viral genome. Retrovirus-based systems are not included in the present discussion and expression systems based on double-stranded RNA viruses have not been yet described.

(down) modulate the immune system as do many large DNA viruses, including poxviruses and herpes viruses (20). Thus, they may induce highly protective immune responses which may include the strengthening of mucosal defenses.

Finally, there is the large family of negative strand RNA viruses which is now amenable to genetic engineering. Again, these RNA viruses with segmented or nonsegmented genomes do not in general modulate the immune system. In fact, some members are extraordinarily good inducers of cellular (CTL) and humoral immune responses in humans. Also, the fact that they have no DNA phase adds to their potential safety for use in humans. Finally, negative strand RNA viruses do not show measurable rates of homologous RNA recombination, which contributes to the stability and safety of these expression vectors. Expression of foreign proteins/antigens has now been shown for genetically engineered influenza viruses, rabies virus, vesicular stomatitis virus, measles, respiratory syncytial, SV5, and Sendai viruses (for recent reviews, see refs. 21–23). Many of these efforts are aimed at expressing antigens of viruses, bacteria or parasites for which we presently have no (or inadequate) vaccines available. The next step would be for viral vectors to express multiple foreign proteins for use against multiple disease agents. Such single constructs could serve as universal vaccines. Efforts in many laboratories are underway to characterize these novel constructs and to expand our knowledge of how these chimeric viruses/vectors replicate and how they interact with the host.

Where do we go from here? Agapov *et al.* (8) have shown for the first time that a virus/vector expression system which was thought to be lytic can be modified so that it becomes noncytopathic and still effectively expresses (long-term) a foreign protein. Could the Sindbis virus system become an attractive alternative to—or have significant advantages over—retrovirus-based expression vectors? Since Sindbis virus replicons amplify only in the cytoplasm and get distributed into the dividing cells without affecting the chromosomal make-up, many safety concerns could be satisfied. Together with lytic RNA virus vectors, the noncytopathic Sindbis replicon approach provides us with a spectrum of expression vehicles, which should be extraordinarily helpful in the future. The next step should be the widespread testing and characterization of these constructs in animals with the objective of rapidly applying the newly discovered knowledge to the treatment of human diseases. Unfortunately, approval for use in patients often does not keep pace with the progress in the molecular biology.

Gene therapy includes not only the permanent expression and/or replacement of a specific gene but also the delivery of a gene for a limited time only. Attempts to treat cancer by the temporal expression of anti-angiogenic factors or the induction of immune responses to tumor-specific antigens via viral vectors would fall into this category. Progress in this area will depend also on the ability to target the vectors to specific cells, including dendritic cells, bone marrow cells, and/or organ-specific cells.

Where may success be closest at hand? Although the challenges in developing safe and effective vaccines against infectious agents are daunting, the argument could be made that the extraordinary bench achievements in recent years justify optimism. For example, would the administration of alphavirus-based vaccine vectors, which express surface and internal components of HIV, afford partial protection against the development of AIDS? We may rightfully hope that the induction of specific CTL and humoral immune responses by live RNA virus vectors will have prophylactic as well as therapeutic effects against AIDS. Would alphavirus and other RNA virus vaccine vectors expressing malarial antigens provide a sufficiently protective immune response so that this dreadful disease could be modulated? The course of deadly new diseases and the continuing impact of infectious agents that have been with us for a long time may now be altered because we have learned how to coerce viruses into helping us fight these diseases. The future is already here.

1. Goff, S. P. & Berg, P. (1976) *Cell* **9,** 695–705.
2. Post, L. E., Mackem, S. & Roizman, B. (1981) *Cell* **24,** 555–565.
3. Panicalli, D. & Paoletti, E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4927–4931.
4. Mackett, M., Smith, G. L. & Moss, B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7415–7419.
5. National Academy of Science Colloquium on Genetic Engineering of Viruses and Viral Vectors (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11287–11425.
6. Kemble, G., Duke, G., Winter, R. & Spaete, R. (1996) *J. Virol.* **70,** 2044–2048.
7. Tomkinson, B., Robertson, E., Yalamanchili, R., Longnecker, R. & Kieff, E. (1993) *J. Virol.* **67,** 7298–7306.
8. Agapov, E. V., Frolov, I., Lindenbach, B. D., Prágai, B. M., Schlesinger, S. & Rice, C. M. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 12989–12994.
9. Rice, C. M., Levis, R., Strauss, J. H. & Huang, H. V. (1987) *J. Virol.* **61,** 3809–3819.
10. Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M. & Huang, H. V. (1989) *Science* **243,** 1188–1191.
11. Raju, R. & Huang, H. V. (1991) *J. Virol.* **65,** 2501–2510.
12. Hahn, C. S., Hahn, Y. S., Braciale, T. J. & Rice, C. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2679–2683.
13. Lindenbach, B. D. & Rice, C. M. (1997) *J. Virol.* **71,** 9608–9617.
14. Pushko, P., Parker, M., Ludwig, G. V., Davis, N. L., Johnston, R. E. & Smith, J. F. (1997) *Virology* **239,** 389–401.
15. Driver, D. A., Polo., J. M., Belli, B. A., Banks, J. A., Hariharan, M. & Dubensky, T. W. (1998) *Curr. Res. Mol. Therapeut.* **1,** 510–517.
16. Berglund, P., Smerdou, C., Fleeton, M. N., Tubulekas, I. & Liljeström, P. (1998) *Nat. Biotechnol.* **16,** 562–565.
17. Racaniello, V. R. & Baltimore, D. (1981) *Science* **214,** 916–918.

18. Andino, R., Silvera, D., Suggett, S. D., Achacoso, P. L., Miller, C. J., Baltimore, D. & Feinberg, M. B. (1994) *Science* **265,** 1448–1451.
19. Basak, S., McPherson, S., Kang, S., Collawn, J. F. & Morrow, C. D. (1998) *J. Interferon Cytokine Res.* **18,** 305–313.
20. Ploegh, H. L. (1998) *Science* **280,** 248–253.
21. García-Sastre, A. (1998) *TibTech* **16,** 230–235.
22. Palese, P., Zheng, H., Engelhardt, O. G., Pleschka, S. & García-Sastre, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11354–11358.
23. Roberts, A. & Rose, J. K. (1998) *Virology* **247,** 1–6.
24. Caley, I. J., Betts, M. R., Irlbeck, D. M., Davis, N. L., Swanstrom, R., Frelinger, J. A. & Johnston, R. E. (1997) *J. Virol.* **71,** 3031–3038.
25. Barclay, W., Li, Q., Hutchinson, G., Moon, D., Richardson, A., Percy, N., Almond, J. W. & Evans, D. J. (1998) *J. Gen. Virol.* **79,** 1725–1734.
26. Li, S. Q., Schulman, J. L., Moran, T., Bona, C. & Palese, P. (1992) *J. Virol.* **66,** 399–404.
27. García-Sastre, A., Muster, T., Barclay, W. S., Percy, N. & Palese, P. (1994) *J. Virol.* **68,** 6254–6261.
28. Castrucci, M. R., Hou, S., Doherty, P. C. & Kawaoka, Y. (1994) *J. Virol.* **68,** 3486–3490.
29. Muster, T., Ferko, B., Klima, A., Purtscher, M., Trkola, A., Schulz, P., Grassauer, A., Engelhardt, O. G., García-Sastre, A. & Palese, P. (1995) *J. Virol.* **69,** 6678–6686.
30. Miyahira, Y., García-Sastre, A., Rodriguez, D., Rodriguez, J. R., Murata, K., Tsuji, M., Palese P., Esteban, M., Zavala, F. & Nussenzweig, R. S. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3954–3959.
31. Staczek, J., Gilleland, H. E., Jr., Gilleland, L. B., Harty, R. N., García-Sastre, A., Engelhardt, O. G. & Palese, P. (1998) *Infect. Immun.* **66,** 3990–3994.
32. Mebatsion, T., Schnell, M. J., Cox, J. H., Finke, S. & Conzelmann, K. K. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7310–7314.
33. Johnson, J. E., Schnell, M. J., Buonocore, L. & Rose, J. K. (1997) *J. Virol.* **71,** 5060–5068.
34. Whelan, S. P., Ball, L. A., Barr, J. N. & Wertz, G. T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8388–8392.
35. Roberts, A., Kretzschmar, E., Perkins, A. S., Forman, J., Price, R., Buonocore, L., Kawaoka, Y. & Rose, J. K. (1998) *J. Virol.* **72,** 4704–4711.
36. Bukreyev, A., Camargo, E. & Collins, P. L. (1996) *J. Virol.* **70,** 6634–6641.
37. Hasan, M. K., Kato, A., Shioda, T., Sakai, Y., Yu, D. & Nagai, Y. (1997) *J. Gen. Virol.* **78,** 2813–2820.
38. He, B., Paterson, R. G., Ward, C. D. & Lamb, R. A. (1997) *Virology* **237,** 249–260.
39. Khromykh, A. A. & Westaway, E. G. (1997), *J. Virol.* **71,** 1497–1505.