Biochemical and Biophysical Research Communications **264,** 1–5 (1999)
Article ID bbrc.1999.1474, available online at http://www.idealibrary.com on IDEAL®

Protection of MLV Vector Particles from Human Complement

Sabine Breun,* Brian Salmons,† Walter H. Günzburg,*,[1] and Jörg G. Baumann*
**Institute of Virology, University of Veterinary Sciences, Veterinärplatz 1, A-1210 Vienna, Austria; and*
†Bavarian Nordic Research Institute GmbH, Fraunhoferstrasse 18b, D-82152 Martinsried, Germany

Received August 27, 1999

Murine cell-derived MLV vector particles usually are highly sensitive to human complement-mediated lysis. Expression of the human complement inhibitor CD59 on murine packaging cells resulted in partial protection of these cells from lysis caused by human complement proteins. Furthermore, CD59 was incorporated into MLV vector particles released by these packaging cells, leading to an improved resistance of the virions against human complement-mediated inactivation.

Most mammals carry a functional α-galactosyltransferase gene, resulting in protein modification and expression of Gal(1-3)Gal carbohydrate (α-Gal) epitopes on the cell surface. Old world monkey, ape and human cells lack these sugar residues due to mutations in the α-galactosyltransferase gene [1, 2]. Up to 1% of circulating IgG in human blood interacts with these epitopes, probably as a result of permanent activation by crossreacting epitopes on enterobacteria [3, 4]. Enveloped viruses budding from non-primate cells incorporate α-Gal modified proteins along with other cell surface proteins and recently it has been shown that retroviruses derived from non-human cells are sensitive to human complement due to the presence of such α-Gal modified proteins in their envelopes [5–9]. The inactivation of retroviral vectors by complement is a major obstacle for *in vivo* applications [10–12]. Some enveloped viruses such as herpes simplex virus, vaccinia virus and herpesvirus saimiri have evolved complement escape mechanisms based on the expression of viral complement regulatory proteins [13–15]. Alternatively enveloped viruses emerging from primate cells can incorporate membrane-attached complement inhibitors which include CD59 (Protectin, HRF20, MIRL), CD55 (DAF) and CD46 (MCP1) [16]. Amongst these viruses are human cytomegalovirus (HCMV), human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and human T-lymphotropic virus (HTLV-I) [17–19]. All of these viruses show resistance to human complement [17, 20–22]. It has been demonstrated that the human complement regulator CD59, which inhibits the formation of the membrane attack complex, gives better protection against the complement system than CD46 or CD55 in the case of HIV-1 [22]. Based on these findings, we tried to confer resistance to complement mediated inactivation upon murine leukaemia virus (MLV) particles budding from a murine cell line. For this purpose an expression plasmid for human CD59 was constructed (Fig. 1).

The packaging cell line PALSG/S, based on murine PA317 packaging cells [26] and stably transfected with the retroviral vector pLXSNEGFP [27], was transfected with pZeoSVCD59 and after zeocin selection (190 μg/ml) two resistant populations (PALSG/S-CD59.1 and PALSG/S-CD59.2, each consisting of more than 300 independent colonies) were obtained. The presence and activity of CD59 on the cell surface was tested in a complement lysis assay (Fig. 2) using fresh human serum. Human serum was obtained as a pool from three healthy donors. Treatment with 100 μl human serum (Fig. 2A, left) resulted in a lysis of more than 50% of parental PALSG/S cells (white bars). In contrast only 25% lysis was observed in the case of PALSG/S-CD59.1 (dotted bars) and PALSG/S-CD59.2 cells (grey bars). A more pronounced effect is visible after treatment with 400 μl human serum (Fig. 2A, right). 90% of the parental PALSG/S cells were lysed (white bars), whereas the PALSG/S-CD59.1 and PALSG/S-CD59.2 cells only showed 50% (dotted bars) and 30% (grey bars) lysis, respectively.

As expected human HeLa cells (black bars) were completely protected, since these cells lack α-Gal epitopes. The increase in cell number observed with HeLa cells (to about 120% of growth without human serum) might be due to growth factors contained in the human serum. A similar experiment was performed using parental PA317 cells as well as PA317

[1] To whom correspondence should be addressed. Fax +43-1-25077-2390. E-mail: walter.guenzburg@vu-wien.ac.at.

0006-291X/99 $30.00

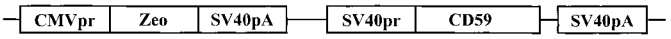

FIG. 1. Expression plasmid pZeoSVCD59. The plasmid pZenNeoCD59, that carries the CD59 cDNA [23], was digested with *Xho*I (Promega) and the resulting fragment of 1.2 kbp was ligated into the vector pZeoSV (Invitrogen) that had been linearized with *Xho*I and then dephosphorylated using calf intestinal phosphatase (Promega). After butanol precipitation [24], the ligated DNA was used to transform *E. coli* DH10B (Gibco) using electroporation [25]. Transformed colonies were selected on zeocin (25 μg/ml, Invitrogen) and plasmid DNA was isolated. Correct orientation of the insert was confirmed by sequence analysis using primers in the CD59 gene as well as the cloning vector. This plasmid, designated pZeoSVCD59 carried zeocin resistance under transcriptional control of the CMV immediate early promoter (CMVpr) and the CD59 cDNA under SV40 control (SV40pr).

cells transfected with the construct pZeoSVCD59 (populations PA317CD59.1 and PA317CD59.2). Treatment with 100 μl human serum (Fig. 2B, left) resulted in lysis of about 50% PA317 cells (white bars), but no detectable lysis of the two populations expressing CD59 (dotted and grey bars). Increasing serum to 400 μl resulted in lysis of about 70% of PA317 cells (white bars), whereas only 25% lysis occurred with population PA317CD59.1 (dotted bars) and no complement effect was seen with PA317CD59.2 (grey bars), indicating that the stable vector producing PALSG/S cells are more prone to complement mediated lysis than PA317 cells. Transfection of the murine packaging cells with the expression plasmid

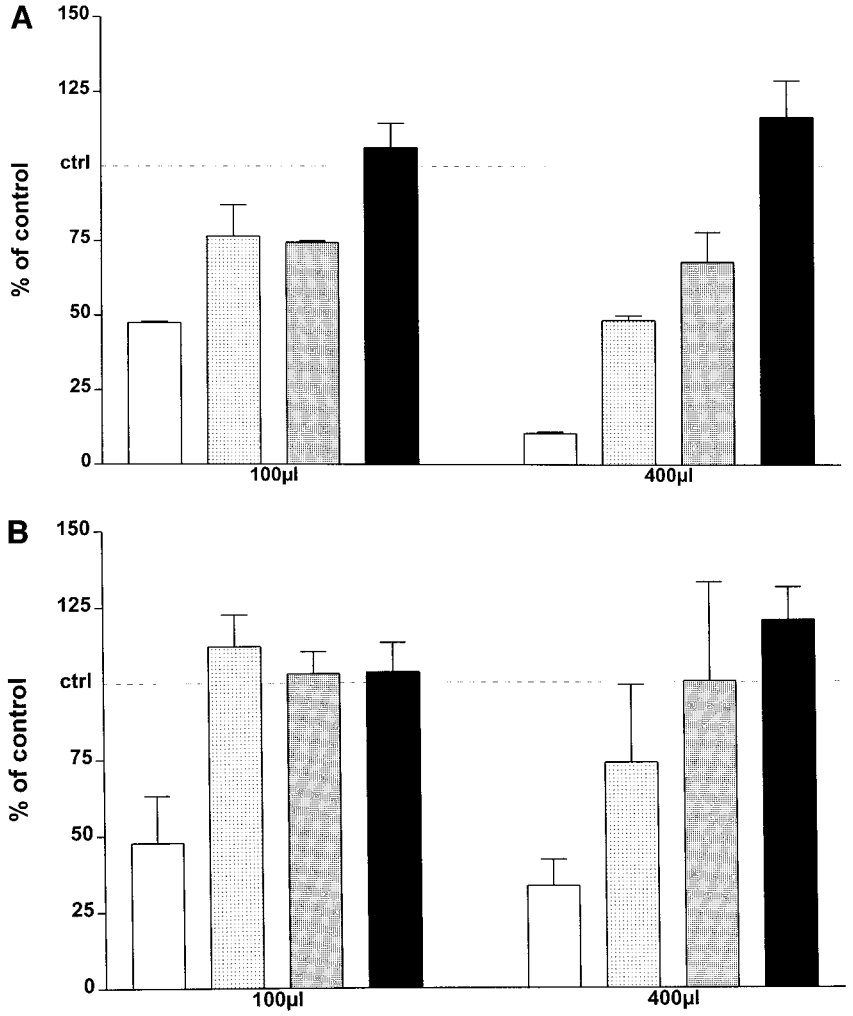

FIG. 2. Effect of human serum on packaging cells. 1×10^6 PALSG/S, PALSG/S-CD59.1, PALSG/S-CD59.2, and HeLa (human cervical carcinoma) cells were seeded in 10-cm dishes (Sarstedt) with 10 ml Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal calf serum (FCS, Gibco/BRL). On the following day the medium was replaced by 2 ml of fresh DMEM/10% FCS containing 0, 100 or 400 μl of human serum. Serum was allowed to act upon the cells for 5 h at 37°C, before it was diluted by addition of 6 ml DMEM/10% FCS. On the next day the cells were counted. The cell number obtained without human serum treatment was regarded as 100% (dotted line). (A) Results obtained with PALSG/S (white bars) and derived populations PALSG/S-CD59.1 (dotted bars), PALSG/S-CD59.2 (grey bars) and HeLa cells (black bars). In B there are populations with PA317 (white bars) and derived populations PA317CD59.1 (dotted bars), PA317CD59.2 (grey bars) and HeLa cells (black bars). Bars represent mean and standard error of the mean values of three independent experiments.

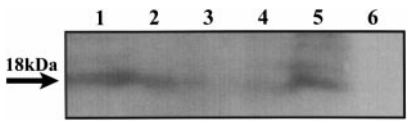

FIG. 3. Incorporation of CD59 into retroviral vector particles. The two populations of CD59 expressing PALSG/S cells as well as parental PALSG/S cells were allowed to grow to confluence in a tissue culture flask (185 cm^2, Nunc) before medium was replaced by fresh serum free medium and 24 h later the culture supernatant was filtered (0.45 μm pore size) and pelleted by ultracentrifugation (150000 $\times$ g, 4 h). Proteins were separated in a 12% SDS-PAGE (40 V, overnight). After transfer (2.5 mA/cm^2, 1 h) to a Nylon membrane (Hybond-P, Amersham) detection of CD59 was performed using the monoclonal antibody MEM-43 (Serotec, 1:250) and a secondary anti-mouse alkaline phosphatase conjugated antibody (Promega, 1:5000). Lanes 1 and 5 contain cell extracts from human K562 cells (1) and HeLa cells transfected with pZeoSV-CD59 (5), lanes 2 to 4 and 6 contain supernatants from PALSG/S cells transfected with either pZenNeoCD59 (2) or pZeoSV-CD59 (3, 4), as well as from parental PALSG/S cells (6).

pZeoSV-CD59 rendered them resistant to human complement mediated lysis suggesting that functional CD59 protein is present on the cell surface. Incorporation of CD59 into retroviral vector particles was assayed in a Western blot analysis of filtered cultured supernatant (Fig. 3). A band of 18 kDa corresponding to CD59 was detected in supernatant from PALSG/S cells transfected with pZenNeoCD59 [23] (Fig. 3, lane 2) and pZeoSV-CD59 (Fig. 3, lanes 3 and 4) as well as in cell extracts of the human K562 B-lymphocyte cell line (Fig. 3, lane 1) and HeLa cells transfected with pZeoSV-CD59 (Fig. 3, lane 5), whereas no detectable CD59 was found in supernatant from parental PALSG/S cells (Fig. 3, lane 6).

Finally the ability of virus-incorporated CD59 to protect the virions from complement inactivation was investigated. Therefore filtered virus supernatant of the packaging cells was incubated with human serum for one hour before infection of target cells. Titers of the CD59-protected virus relative to the unprotected parental PALSG/S derived virus are shown in Fig. 4. Addition of heat inactivated human serum (Fig. 4, IHS250) did not affect the titer significantly. With increasing amounts of human serum (HS50, HS100, HS250) the titer of vector virus derived from parental PALSG/S cells declined. With 250 μl of human serum (Fig. 4, HS250) the unprotected virus showed a strongly (about 4000 fold) decreased titer, whereas the virus from CD59 expressing packaging cells (dotted and grey bars) did not show such a dramatic drop in titer, equivalent to a 50- and 200-fold better protection than obtained with supernatant from the parental PALSG/S cells. Similar but less pronounced effects were observed with lower serum concentrations (HS50 and HS100, Fig. 4).

DISCUSSION

Our data demonstrate that CD59 is biologically functional after transfection of PA317 and PALSG/S cells with the pZeoSVCD59 construct, as shown by the protection of the cells from complement mediated lysis. Further, CD59 is incorporated into vector particles leading to a partial protection of the virions from complement mediated inactivation. Complement protection in the presence of 250 μl of human serum was 50 to 200-fold better than without CD59 in the packaging cells. The better protection of virus from population PALSG/S-CD59.2 resembles the observation made on the cellular level: the PALSG/S-CD59.2 cells were better protected against complement mediated lysis than PALSG/S-CD59.1 (Fig. 2A). This might be due to differences in expression levels of CD59 and/or α-Gal epitopes on the cell surface.

The influence of human serum on the infectivity of MLV particles produced from human cells was shown some years ago and is dependent on both the cell and virus type [10, 28]. The absence of α-Gal epitopes on the viral surface does not guarantee complement resistance, as particles released from human cells carrying different retroviral envelopes are still inactivated [10]. Indeed it is known that MLV surface proteins are able

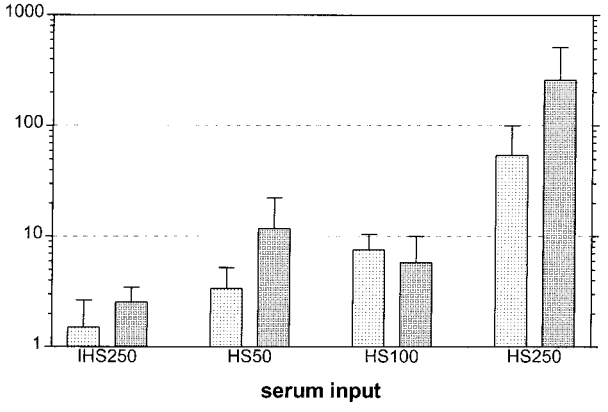

FIG. 4. Effect of human complement on vector titer. PALSG/S, PALSG/S-CD59.1 and PALSG/S-CD59.2 cells were grown to confluence in 10-cm dishes, before the medium was exchanged for 6 ml of fresh DMEM/10% FCS. On the next day the culture supernatant was filtered (0.45 μm pore size) and 100 μl were mixed with 50, 100 or 250 μl of undiluted human serum. Parallel assays were performed with 250 μl of heat inactivated (56°C, 30 min) human serum. After incubation for one h at 37°C, DMEM/10% FCS was added to a total volume of 2 ml, including Polybrene (8 μg/ml), before the supernatant was used to infect 1×10^6 HeLa cells as previously described and G418 resistant colonies determined [27]. The y-axis shows relative titers of CD59-protected virions compared to the titer of non-protected parental virus, which was regarded as 1. Data shown are from three independent experiments. From left to right factors are shown after treatment with 250 μl inactivated serum (IHS250) as well as treated with 50, 100 and 250 μl human serum (HS50, HS100, HS250). Supernatant coming from PALSG/S-CD59.1 is shown as dotted and PALSG/S-CD59.2 as grey bars.

to directly activate the complement cascade by binding of C1q [29, 30]. A number of different approaches have been used to overcome complement mediated inactivation, including soluble complement inhibitors [31], incubation with α-Gal [32] and co-administration with synthetic inhibitors [33]. Expression of human CD59 has been shown to confer protection against complement mediated lysis in transgenic mice [23] and the gene has been incorporated into retroviral vectors which have then been used to transfer complement resistance to target cells [34]. In these experiments human serum was used to select both the packaging cells as well as the transduced murine target cells [35]. Our findings extend these observations to the protection of retroviral vector particles against human complement system.

Even vectors emanating from murine fibroblasts, which are known to product high levels of α-Gal [36], can be significantly protected. As sensitivity to complement is conferred by viral and cellular factors, a combination of α-Gal negative cells and overexpression of one or more human complement regulators might lead to a complete protection against vector inactivation through complement. It has been shown that even complement sensitive retroviruses can infect primates [37, 38] and it might be expected that complement resistant viruses may achieve this even more easily. Since a number of biologically active endogenous murine, porcine and feline retroviruses have also been described in the past three years [39–44] that could potentially transcomplement or mobilize defective murine retroviral vectors, it may be advisable to base packaging cell lines on cells of human origin. Although a number of human endogenous viruses have been described these share little or no sequence homology with standard MLV based vectors, making the chance of recombination here extremely unlikely [45, 46]. Additionally, since the human endogenous retroviruses appear not to give rise to replication competent virus it would not be expected that they would be able to mobilize complement resistant replication defective MLV vector particles.

ACKNOWLEDGMENTS

We thank Peter J. Cowan for the plasmid pZenNeo CD59 and Stefano Indraccolo for PALSG/S and K562 cells. We also thank Karin Aichner for taking blood samples from the donors, Hermann Unger for help with blood preparation and Francoise Rouault for technical discussions. This work was supported in part by grants from the Bayerische Forschungsstiftung "FORGEN" program and a three-month "Forschungsbeihilfe" to S.B. S.B. thanks Professor R. Kahmann (LMU Munich) for giving her the opportunity during her studies at the University of Munich to do this work in Vienna.

REFERENCES

1. Galili, U., Shohet, S. B., Kobrin, E., Stults, C. L. M., and Macher, B. A. (1988) *J. Biol. Chem.* **263,** 17755–17762.
2. Larsen, R. D., Rivera-Marrero, C. A., Ernst, L. K., Cummings, R. D., and Lowe, J. B. (1990) *J. Biol. Chem.* **265,** 7055–7061.
3. Galili, U., Macher, B. A., Buehler, J., and Shohet, S. B. (1985) *J. Exp. Med.* **162,** 573–582.
4. Galili, U., Mandrell, R. E., Hamadeh, R. M., Griffiss, J. M., and Shohet, S. B. (1988) *Infect. Immun.* **56,** 1730–1737.
5. Repik, P. M., Strizki, J. M., and Galili, U. (1994) *J. Gen. Virol.* **75,** 1177–1181.
6. Galili, U., Repik, P. M., Anaraki, F., Mozdzanowska, K., Washko, G., and Gerhard, W. (1996) *Vaccine* **14,** 321–328.
7. Welsh, R. M., O'Donnell, C. L., Reed, D. J., and Rother, R. P. (1998) *J. Virol.* **72,** 4650–4656.
8. Rother, R. P., Fodor, W. L., Springhorn, J. P., Birks, C. W., Setter, E., Sandrin, M. S., Squinto, S. P., and Rollins, S. A. (1995) *J. Exp. Med.* **182,** 1345–1355.
9. Takeuchi, Y., Porter, C. D., Strahan, K. M., Preece, A. F., Gustafsson, K., Cosset, F.-L., Weiss, R. A., and Collins, M. K. L. (1996) *Nature* **379,** 85–88.
10. Takeuchi, Y., Cosset, F.-L., Lachmann, P. J., Okada, H., Weiss, R. A., and Collins, M. K. L. (1994) *J. Virol.* **68,** 8001–8007.
11. Russell, D. W., Berger, M. S., and Miller, A. D. (1995) *Hum. Gene Ther.* **6,** 635–641.
12. Shimizu, K., Miyao, Y., Tamura, M., Kishima, H., Ohkawa, M., Mabuchi, E., Yamada, M., Hayakawa, T., and Ikenaka, K. (1995) *Jpn. J. Cancer Res.* **86,** 1010–1013.
13. McNearney, T. A., Odell, C., Holers, V. M., Spear, P. G., and Atkinson, J. P. (1987) *J. Exp. Med.* **166,** 1525–1535.
14. Kotwal, G. J., Isaacs, S. N., McKenzie, R., Frank, M. M., and Moss, B. (1990) *Science* **250,** 827–830.
15. Rother, R. P., Rollins, S. A., Fodor, W. L., Albrecht, J. C., Setter, E., Fleckenstein, B., and Squinto, S. P. (1994) *J. Virol.* **68,** 730–737.
16. Hourcade, D., Holers, V. M., and Atkinson, J. P. (1989) *Adv. Immunol.* **45,** 381–416.
17. Spear, G. T., Lurain, N. S., Parker, C. J., Ghassemi, M., Payne, G. H., and Saifuddin, M. (1995) *J. Immunol.* **155,** 4376–4381.
18. Marschang, P., Sodroski, J., Wurzner, R., and Dierich, M. P. (1995) *Eur. J. Immunol.* **25,** 285–290.
19. Montefiori, D. C., Cornell, R. J., Zhou, J. Y., Zhou, J. T., Hirsch, V. M., and Johnson, P. R. (1994) *Virology* **205,** 82–92.
20. Hoshino, H., Tanaka, H., Miwa, M., and Okada, H. (1984) *Nature* **310,** 324–325.
21. Banapour, B., Sernatinger, J., and Levy, J. A. (1986) *Virology* **152,** 268–271.
22. Saifuddin, M., Hedayati, T., Atkinson, J. P., Holguin, M. H., Parker, C. J., and Spear, G. T. (1997) *J. Gen. Virol.* **78,** 1907–1911.
23. Somerville, C. A., Kyriazis, A. G., McKenzie, A., Allison, J., Pearse, M. J., and D'Apice, A. J. (1994) *Transplantation* **58,** 1430–1435.
24. Thomas, M. R. (1994) *Biotechniques* **16,** 988–990.
25. Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988) *Nucleic Acids Res.* **16,** 6127–6145.
26. Miller, A. D., and Buttimore, C. (1986) *Mol. Cell Biol.* **6,** 2895–2902.
27. Klein, D., Indraccolo, S., von Rombs, K., Amadori, A., Salmons, B., and Günzburg, W. H. (1997) *Gene Ther.* **4,** 1256–1260.
28. Cosset, F.-L., Takeuchi, Y., Battini, J. L., Weiss, R. A., and Collins, M. K. L. (1995) *J. Virol.* **69,** 7430–7436.
29. Bartholomew, R. M., Esser, A. F., and Muller-Eberhard, H. J. (1978) *J. Exp. Med.* **147,** 844–853.
30. Cooper, N. R., Jensen, F. C., Welsh, R. M. J., and Oldstone, M. B. (1976) *J. Exp. Med.* **144,** 970–984.

31. Rother, R. P., Squinto, S. P., Mason, J. M., and Rollins, S. A. (1995) *Hum. Gene Ther.* **6,** 429–435.
32. Rollins, S. A., Birks, C. W., Setter, E., Squinto, S. P., and Rother, R. P. (1996) *Hum. Gene Ther.* **7,** 619–626.
33. Miyao, Y., Ikenaka, K., Kishima, H., Tamura, M., Nakamura, K., Kurumi, M., Hayakawa, T., and Shimizu, K. (1997) *Hum. Gene Ther.* **8,** 1575–1583.
34. Hayashi, S., Isobe, K., Emi, N., Yokoyama, I., Okada, H., Nakashima, I., and Takagi, H. (1996) *Eur. Surg. Res.* **28,** 440–446.
35. Hayashi, S., Emi, N., Okada, H., Nagasaka, T., Yokoyama, I., and Takagi, H. (1998) *Gene Ther.* **5,** 282–285.
36. Takeuchi, Y., Liong, S. H., Bieniasz, P. D., Jager, U., Porter, C. D., Friedman, T., McClure, M. O., and Weiss, R. A. (1997) *J. Virol.* **71,** 6174–6178.
37. Gallagher, R. E., Schrecker, A. W., Walter, C. A., and Gallo, R. C. (1978) *J. Natl. Cancer Inst.* **60,** 677–682.
38. Donahue, R. E., Kessler, S. W., Bodine, D., McDonagh, K., Dunbar, C., Goodman, S., Agricola, B., Byrne, E., Raffeld, M., Moen, R., *et al.*, (1992) *J. Exp. Med.* **176,** 1125–1135.
39. Miller, A. D., Bonham, L., Alfano, J., Kiem, H. P., Reynolds, T., and Wolgamot, G. (1996) *J. Virol.* **70,** 1804–1809.
40. Patience, C., Simpson, G. R., Colletta, A. A., Welch, H. M., Weiss, R. A., and Boyd, M. T. (1996) *J. Virol.* **70,** 2654–2657.
41. Patience, C., Takeuchi, Y., and Weiss, R. A. (1997) *Nat. Med.* **3,** 282–286.
42. Akiyoshi, D. E., Denaro, M., Zhu, H., Greenstein, J. L., Banerjee, P., and Fishman, J. A. (1998) *J. Virol.* **72,** 4503–4507.
43. Baumann, J. G., Günzburg, W. H., and Salmons, B. (1998) *J. Virol.* **72,** 7685–7687.
44. Martin, U., Kiessig, V., Blusch, J. H., Haverich, A., von der Helm, K., Herden, T., and Steinhoff, G. (1998) *Lancet* **352,** 692–694.
45. Leib-Mösch, C., Brack-Werner, R., Werner, T., Bachmann, M., Faff, O., Erfle, V., and Hehlmann, R. (1990) *Cancer Res.* **50,** 5636s–5642s.
46. Wilkinson, D. A., Mager, D. L., and Leong, J.-A. C. (1994) *in* The Retroviridae (Levy, J. A., Ed.), Vol. 3, pp. 465–535. Plenum, New York.