The genus *Morbillivirus* includes a number of important human (*Measles virus, MeV*), livestock (*Rinderpest virus, RPV; Peste-des-petits-ruminants virus, PPRV*) or other animal (*Canine distemper virus, CDV*) pathogens. In some cases, growth or handling of the live viruses requires very high level containment, which can lead to problems and restrictions when preparing material where correctly folded surface glycoproteins are required, for example for use in serological ELISAs, and the glycoproteins are only folded properly in the virus, or a virus-like particle of some kind. We have sought to establish a system by which the virus growth could become completely dependent on a helper cell line, thereby making the production, shipment and use of the resultant virus entirely biosafe. One possible way that this might be done is by removing the coding sequence for an essential viral protein and providing that protein *in trans* in a modified cell line. We show here that this is possible for this group of viruses.

Morbilliviruses, like other paramyxoviruses, are non-segmented negative-strand RNA viruses. They have six genes (or transcription units), encoding, respectively, the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), the two envelope glycoproteins (F and H) and the viral RNA polymerase (L). The P gene also gives rise to two non-structural or accessory proteins, V and C, which play a variety of roles in modulating host immune responses and the dynamics of virus replication (e.g. Baron & Barrett, 2000; Nakatsu et al., 2008; Nanda & Baron, 2006; Ohno et al., 2004; Palosari et al., 2003; Parks et al., 2006; Takeuchi et al., 2003; Tober et al., 1998), but are not essential proteins in the virus life cycle, since recombinant morbilliviruses lacking either or both proteins have been made which grow in cell culture (Baron & Barrett, 2000; Radecke & Billeter, 1996; Schneider et al., 1997). When selecting a viral gene to delete, we took into consideration that recombinant forms of MeV lacking their M protein have been made which can grow in cell culture, albeit very inefficiently (Cathomen et al., 1998). Removing either one of the glycoproteins would not prevent viral replication, only limit its spread. The P protein was selected as the viral protein to provide *in trans* as it acts as a subunit of the polymerase, as well as working with the N protein in encapsidation of the genome, and is thus completely indispensable in the virus life cycle. In addition, the interactions of P with N and L have been found to be largely virus specific (Brown et al., 2005), meaning that the efficient replication of a virus depends on the presence of its own P protein. Recombination has not been observed naturally in the order *Mononegavirales*, so there is no way for the virus to recover a deleted gene, even if it co-infected a cell along with a wild-type version of the virus.

Methods for making recombinant versions of morbilliviruses have been established for some time (Baron & Barrett, 1997; Gassen et al., 2000; Radecke et al., 1995), although the rescue of recombinant PPRV was only recently achieved (Hu et al., 2012). Since our initial aim was to prepare virus-like material with the surface glycoproteins of PPRV for use in a competition ELISA that is used to screen for anti-PPRV antibodies (Anderson & McKay, 1994), and since there was no working system to make recombinant PPRV available at the time we initiated this project, we used an existing chimeric virus in which the outer structure (M, F and H proteins) are from PPRV while the core replication machinery (N, P and L) and the promoters are from RPV, RPV_PPRM FH...
We then attempted the recovery of the various P-deleted virus-like particles (VLPs). Although viruses lacking expression of V and C are viable (Baron & Barrett, 2000; Radecke & Billeter, 1996; Schneider et al., 1997), we could not recover RPV_PPRMFH-P−. We were able to observe replication of both P−C− and P−VC− from the appearance of the viral H protein in the transfected cells. However, only the latter construct grew well enough to spread through the culture and give rise to titratable progeny stock. Multistep growth curves showed that the P−VC− VLPs grew reasonably well on VDS-P cells, but nevertheless slower and to lower titre than observed for the P+ virus on VDS cells (Fig. 2a); no growth of the P−VC− VLPs was seen on normal VDS cells (Fig. 2b). Immunofluorescence showed that much higher levels of viral protein accumulated when cells (with or without extra P protein) were infected with the P+ virus than when the P-VLPs were growing on VDS-P cells (Fig. 2b). When we prepared viral antigen for use in the diagnostic cELISA, harvested by the standard methods of freeze–thawing, centrifugation and sonication (Anderson et al., 1990) from VDS-P cells infected with the P−VC− VLPs, at the stage when cytopathic effect was advanced, the antigen preparations did not contain sufficient viral protein to react strongly with the mAb, and so could not be used to replace the virus preparations used as standard. These observations suggested that the levels of P protein provided by the cell line are too low to support the normal replication and assembly of the virus genome. Indeed, staining for P protein in cells infected with P+ virus showed the accumulation of much higher levels of this protein than seen in the cell line (Fig. 2b). The relatively low level of P protein in the helper cell system may also explain why only the VC+ VLP was fully replication competent, despite the evidence that neither V nor C are normally essential. P and V are thought to act as chaperones in the assembly of the nucleocapsid (Spehner et al., 1997; Tober et al., 1998), and
it is likely that, at low concentrations of P, the requirement for V increases. Similarly, while the morbillivirus C protein is not essential, its absence does decrease viral replication (Baron & Barrett, 2000; Radecke & Billeter, 1996); combining the deleterious effects of low P levels with those of the absence of C is presumably too much for the viral replication machinery to overcome.

These results with the P-deleted morbillivirus contrast with those obtained with a similar strategy used to create a helper cell-dependent filovirus, Ebola virus (Halfmann et al., 2008). In that case, the authors deleted the viral VP30 gene and provided this protein in trans. VP30 is not the exact equivalent of the paramyxovirus P protein, being dispensable for viral RNA replication (Mühlberger et al., 1999) and, while required for replication and packaging of the whole virus (Volchkov et al., 2001), the optimal level of VP30 appears to be much lower than for other nucleoprotein components (Mühlberger et al., 1999). Perhaps because of this lower requirement for VP30, the VP30-deleted Ebola virus replicated at a similar rate and to similar titres as the wild-type virus. To use this technique for the production of PPRV antigen, it is apparent that either higher levels of the P protein must be expressed in the cell line or a viral protein selected that is required at lower levels during viral replication. We are currently creating cell lines expressing the PPRV L protein, since all members of the order Mononegavirales show a transcription gradient from the promoter-proximal 3' end of the genome (N gene) to the distal end (L gene), and the L protein is the therefore the least highly expressed of the viral proteins. Only low levels of the L proteins should be required to support normal viral replication. Indeed, overexpression of L can decrease replication (Baron & Barrett, 1997).

Using the P−VC+ construct, we were able to confirm that the VLPs were completely dependent on the helper cell line. No viral protein was seen in VDS cells even 7 days after infection with P−VC+ (Fig. 3a). Real-time PCR was able to detect the small amount of genome RNA left on cells during the infection stage (Fig. 3b), and detected a strong production of new viral RNA when VDS-P cells were infected with the P−VC+ VLPs, but no production of new genomes was observed when normal VDS cells were used, even when infection was allowed to proceed over an extended period (Fig. 3b). Similarly, viral mRNA was only observed when the VLPs were used to infect VDS-P cells, not in VDS cells (Fig. 3c). Putting the VLPs through several blind passages on VDS cells showed no recovery of viral transcription (Fig. 3e, f), just the gradual dilution of the initial amount of viral genome that adhered to the cell, and a failure to produce viral mRNA, showing that there was no trace contaminant of P protein-expressing virus. The gene-deleted virus is therefore completely restricted in its replication to the helper cell line, and such constructs could be considered when production of viral proteins or VLPs has to be carried out for viruses that otherwise require high levels of containment, for growth or transport or both.

http://vir.sgmjournals.org 1197
Fig. 3. Helper cell dependency of VLP growth. (a) VDS and VDS-P cells were infected with P^VC^+ VLPs at an m.o.i. of 0.01 and fixed at 7 days p.i. The cells were stained as described in Fig. 2(b), except that the secondary antibodies were AlexaFluor568 anti-mouse IgG and AlexaFluor488 anti-rabbit IgG. Bars, 25 μm. (b–d) VDS and VDS-P cells were infected with P^VC^+ VLPs at an m.o.i. of 0.005. Cells were harvested immediately, and at 8 and 13 days p.i. and total RNA purified using the Qiagen RNeasy kit. cDNA was reverse transcribed from 100 ng RNA using either (b) random primers or (c, d) the poly(A)-specific primer (T)_{16}VN; the relative amount of viral RNA (genome + mRNA) (b) or viral mRNA (c) was determined using RPV N gene-specific primers (sequence provided on request). The mRNA for ribosomal protein L13A (d) was used as an internal control. (e–g) The P^VC^+ VLPs were blind passaged three times (p1, p2 and p3) in VDS cells for 7 days each passage, and total cellular RNA prepared after each passage. The relative amount of (e) total viral RNA, (f) viral mRNA and (g) internal control L13A mRNA was determined in each RNA preparation as described above. RNA from the first passage of the VLPs in VDS-P cells was used as a positive control. RFU, Relative fluorescence units.
References

Anderson, J. & McKay, J. A. (1994). The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes. *Epidemiol Infect* **112**, 225–231.

Anderson, J., McKay, J. A. & Butcher, R. N. (1990). The use of monoclonal antibodies in competitive ELISA for the detection of antibodies to rinderpest and peste des petits ruminants viruses. In *Seromonitoring of Rinderpest throughout Africa: Phase One Proceedings of the Final Research Coordination Meeting of the IAEA Rinderpest Control Projects, Cote d’Ivoire 19–23 November 1990* IAEA-TECDOC-623. Vienna: International Atomic Energy Agency.

Baron, M. D. & Barrett, T. (1997). Rescue of rinderpest virus from cloned cDNA. *J Virol* **71**, 1265–1271.

Baron, M. D. & Barrett, T. (2000). Rinderpest viruses lacking the C and V proteins show specific defects in growth and transcription of viral RNAs. *J Virol* **74**, 2603–2611.

Baron, M. D., Foster-Cuevas, M., Baron, J. & Barrett, T. (1999). Expression in cattle of epitopes of a heterologous virus using a recombinant rinderpest virus. *J Gen Virol* **80**, 2031–2039.

Brown, D. D., Collins, F. M., Duprex, W. P., Baron, M. D., Barrett, T. & Rima, B. K. (2005). Rescue of mini-genomic constructs and viruses by combinations of morbillivirus N, P and L proteins. *J Gen Virol* **86**, 1077–1081.

Cathomen, T., Mrkic, B., Spehner, D., Drillien, R., Naef, R., Pavlovic, J., Aguzzi, A., Billeter, M. A. & Cattaneo, R. (1998). A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation in the brain. *EMBO J* **17**, 3899–3908.

Cattaneo, R., Kaelin, K., Baczko, K. & Billeter, M. A. (1989). Measles virus editing provides an additional cysteine-rich protein. *Cell* **56**, 759–764.

Gassen, U., Collins, F. M., Duprex, W. P. & Rima, B. K. (2000). Establishment of a rescue system for canine distemper virus. *J Virol* **74**, 10737–10744.

Halfmann, P., Kim, J. H., Ebihara, H., Noda, T., Neumann, G., Feldmann, H. & Kawaoia, Y. (2008). Generation of biologically contained Ebola viruses. *Proc Natl Acad Sci U S A* **105**, 1129–1133.

Hu, Q., Chen, W., Huang, K., Baron, M. D. & Bu, Z. (2012). Rescue of recombinant peste des petits ruminants virus: creation of a GFP-expressing virus and application in rapid virus neutralization test. *Vet Res* **43**, 48.

Mahapatra, M., Parida, S., Baron, M. D. & Barrett, T. (2006). Matrix protein and glycoproteins F and H of Peste-des-pets-ruminants virus function better as a homologous complex. *J Gen Virol* **87**, 2021–2029.

Mühlberger, E., Weik, M., Volchkov, V. E., Klenk, H. D. & Becker, S. (1999). Comparison of the transcription and replication strategies of Marburg virus and Ebola virus by using artificial replication systems. *J Virol* **73**, 2333–2342.

Nakatsu, Y., Takeda, M., Ohno, S., Shirogane, Y., Iwasaki, M. & Yanagi, Y. (2008). Measles virus circumvents the host interferon response by different actions of the C and V proteins. *J Virol* **82**, 8296–8306.

Nanda, S. K. & Baron, M. D. (2006). Rinderpest virus blocks type I and type II interferon action: role of structural and nonstructural proteins. *J Virol* **80**, 7555–7568.

Ohno, S., Ono, N., Takeda, M., Takeuchi, K. & Yanagi, Y. (2004). Dissection of measles virus V protein in relation to its ability to block alpha/beta interferon signal transduction. *J Gen Virol* **85**, 2991–2999.

Palosasaari, H., Parisien, J. P., Rodriguez, J. J., Ulane, C. M. & Horvath, C. M. (2003). STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. *J Virol* **77**, 7635–7644.

Parks, C. L., Witko, S. E., Kotash, C., Lin, S. L., Sidhu, M. S. & Udem, S. A. (2006). Role of V protein RNA binding in inhibition of measles virus minigenome replication. *Virology* **348**, 96–106.

Radecke, F. & Billeter, M. A. (1996). The nonstructural C protein is not essential for multiplication of Edmonston B strain measles virus in cultured cells. *Virology* **217**, 418–421.

Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Döttch, C., Christiansen, G. & Billeter, M. A. (1995). Rescue of measles viruses from cloned DNA. *EMBO J* **14**, 5773–5784.

Schneider, H., Kaelin, K. & Billeter, M. A. (1997). Recombinant measles viruses defective for RNA editing and V protein synthesis are viable in cultured cells. *Virology* **227**, 314–322.

Spehner, D., Drillien, R. & Howley, P. M. (1997). The assembly of the measles virus nucleoprotein into nucleocapsid-like particles is modulated by the phosphoprotein. *Virology* **232**, 260–268.

Takeuchi, K., Kadota, S. I., Takeda, M., Miyajima, N. & Nagata, K. (2003). Measles virus V protein blocks interferon (IFN)-alpha/beta but not IFN-gamma signaling by inhibiting STAT1 and STAT2 phosphorylation. *FEBS Lett* **545**, 177–182.

Tatsuo, H. & Yanagi, Y. (2002). The morbillivirus receptor SLAM (CD150). *Microb Immunol* **46**, 135–142.

Tober, C., Seufert, M., Schneider, H., Billeter, M. A., Johnston, I. C., Niewiesk, S., ter Meulen, V. & Schneider-Schaulies, S. (1998). Expression of measles virus V protein is associated with pathogenicity and control of viral RNA synthesis. *J Virol* **72**, 8124–8132.

Volchkov, V. E., Volchkova, V. A., Mühlenberger, E., Kolesnikova, L. V., Weik, M., Doinik, O. & Klenk, H. D. (2001). Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytopotoxicity. *Science* **291**, 1965–1969.

von Messling, V., Springfeld, C., Devaux, P. & Cattaneo, R. (2003). A ferret model of canine distemper virus virulence and immuno-suppression. *J Virol* **77**, 12579–12591.