

Short Communication

**Rescue of disabled infectious single-cycle (DISC) *Equine arteritis virus* by using complementing cell lines that express minor structural glycoproteins**

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Received 15 July 2004

Accepted 10 September 2004

***Equine arteritis virus (EAV)* contains seven structural proteins that are all required to produce infectious progeny. Alphavirus-based expression vectors have been generated for each of these proteins to explore the possibilities for their constitutive expression in cell lines. This approach was successful for minor glycoproteins GP<sub>2b</sub>, GP<sub>3</sub> and GP<sub>4</sub> and for the E protein. Subsequently, it was demonstrated that cell lines expressing these proteins could rescue EAV mutants that were disabled in the expression of the corresponding gene, resulting in the production of virus particles carrying the mutant genome. This system was particularly efficient for GP<sub>2b</sub>- and GP<sub>4</sub>-knockout mutants. Upon infection of non-complementing cells with these mutants, a self-limiting single cycle of replication was initiated, resulting in the expression of all but one of the viral proteins. These disabled infectious single-cycle (DISC) arteriviruses can also be used to express foreign sequences and are potentially useful in both fundamental research and vaccine development.**

*Equine arteritis virus (EAV)* is the prototype of the family *Arteriviridae* (order *Nidovirales*), which also includes *Lactate dehydrogenase-elevating virus*, *Porcine respiratory and reproductive syndrome virus (PRRSV)* and *Simian hemorrhagic fever virus* (reviewed by Snijder & Meulenberg, 2001). PRRSV and EAV cause economically important diseases in swine and horses, respectively, particularly in Western Europe, North America and Asia. Conventional live-attenuated and killed vaccines are used to reduce losses caused by PRRSV and EAV (reviewed by Snijder & Meulenberg, 2001). Live vaccines induce long-lasting protection, but do not completely prevent reinfection with wild-type (wt) virus or virus transmission. Furthermore, adverse effects of vaccination with a live PRRSV vaccine were reported, probably due to reversion to virulence of the vaccine virus (reviewed by Nielsen *et al.*, 2001). In this respect, killed arterivirus vaccines are safer, but they are less efficacious in the induction of protection. Recently developed systems for arterivirus reverse genetics (de Vries *et al.*, 2000; Meulenberg *et al.*, 1998; Nielsen *et al.*, 2003; van Dinten *et al.*, 1997) are of great value for the development of improved vaccines, including marker vaccines that would allow discrimination between vaccinated and infected animals.

Arterivirus particles (Fig. 1a) are 50–60 nm in diameter and contain a plus-sense RNA genome of 12.7–15.7 kb. The RNA is packaged by a small nucleocapsid protein (N) into a putatively icosahedral core structure that is surrounded by an envelope containing six viral membrane proteins (reviewed by Snijder & Meulenberg, 2001). As in other arteriviruses, the main envelope proteins of EAV are the unglycosylated membrane protein M and the major glycoprotein GP<sub>5</sub>, which also contains major determinants for the induction of neutralizing antibodies. GP<sub>5</sub> and M are present in the virion as a covalently linked heterodimer (de Vries *et al.*, 1995). In addition, virus particles contain four minor envelope proteins, the small unglycosylated envelope protein (E) (Snijder *et al.*, 1999) and three minor envelope glycoproteins named GP<sub>2b</sub>, GP<sub>3</sub> and GP<sub>4</sub>, which were recently demonstrated to exist as a covalently associated heterotrimer in the virion (Wieringa *et al.*, 2003). The genome (Fig. 1b;

den Boon *et al.*, 1991) contains a large replicase gene (ORFs 1a and 1b) that is followed by partially overlapping genes (ORFs 2a, 2b, 3, 4, 5, 6 and 7), which encode the seven structural proteins in the order E-GP<sub>2b</sub>-GP<sub>3</sub>-GP<sub>4</sub>-GP<sub>5</sub>-M-N. As in other nidoviruses, expression of these proteins depends on the synthesis of a nested set of subgenomic mRNAs.

Previously, by making gene knockouts in a full-length cDNA clone, we have shown that expression of each of the EAV structural proteins is required for the production of infectious progeny virus (Molenkamp *et al.*, 2000). A more detailed analysis of cells transfected with these knockout mutants revealed that they segregate into two groups (Fig. 1c): cells transfected with mutants lacking expression of GP<sub>5</sub>, M or N do not release significant amounts of viral proteins into the medium, whereas cells transfected with knockout mutants for the minor envelope proteins E, GP<sub>2b</sub>, GP<sub>3</sub> or GP<sub>4</sub> secreted large amounts of GP<sub>5</sub>, M and N. A detailed analysis of this phenomenon, published elsewhere (Wieringa *et al.*, 2004), revealed that these three proteins are incorporated into non-infectious, subviral particles that contain the viral genome and are undistinguishable from wt virus in terms of buoyant density and morphology. Thus, it was concluded that the GP<sub>2b</sub>-GP<sub>3</sub>-GP<sub>4</sub> complex is not required to form the arterivirus 'core particle', but is critically involved, possibly in conjunction with the E protein, in virion infectivity, possibly through a role in receptor binding and/or virus entry (Wieringa *et al.*, 2004).

Arterivirus mutants that are defective in the production of infectious progeny open new possibilities for vaccine development. Their genetic debility will render them capable of only a single cycle of replication, thus resulting in a self-limiting infection and preventing spread to other animals. Disabled virus mutants that produce non-infectious, (sub)viral particles during their abortive cycle of replication may be particularly powerful vaccine candidates, as described above for the EAV minor structural protein knockouts (Fig. 1c and Wieringa *et al.*, 2004). Upon entry into a susceptible cell, such EAV mutants will replicate their genome, express the full repertoire of non-structural proteins, generate subgenomic mRNAs, express all but one of the structural proteins and release significant amounts of defective particles that contain major antigens of the virus. Such a vaccine would stimulate both the humoral and cellular arms of the immune system and would offer possibilities for development of carrier vaccines, e.g. by incorporation of foreign sequences into one of the major structural proteins.

The large-scale production of such 'disabled infectious single-cycle' (DISC) arteriviruses will require *trans*-complementing cell lines that can mediate the packaging of the disabled genome into an infectious virus particle by providing the missing structural protein. To investigate the possibility of expressing EAV structural proteins constitutively, we employed SinRep19 (Agapov *et al.*, 1998), an RNA-based expression vector that is based on the genome of the alphavirus *Sindbis virus* (Sin). Essentially, this vector is an attenuated RNA replicon that expresses a selectable marker from one subgenomic RNA and a gene of choice from its second subgenomic RNA. SinRep19 replicons expressing different EAV structural protein genes were engineered by cloning PCR-amplified genes between the *Mlu*I and *Sph*I sites of the vector. *In vitro*-transcribed vector RNA was transfected into BHK-21 cells, which support both Sin and EAV replication. A vector expressing green fluorescent protein (SinRep-GFP; Agapov *et al.*,

1998) was used as a positive control. After 24 h, puromycin selection was applied to select for cells containing the RNA replicon. Although expression of the GP<sub>5</sub>, M and N proteins could initially be confirmed by immunofluorescence microscopy, positive cells did not divide and cell lines expressing these proteins could not be established. In contrast, cells transfected with replicons expressing E, GP<sub>2b</sub>, GP<sub>3</sub>, GP<sub>4</sub> or GFP were found to become puromycin-resistant and cell division, albeit slightly delayed, was readily observed. To assess the stability of this type of RNA replicon-based cell line, the GP<sub>4</sub>-expressing cell line was passaged for over 3 months. The consensus sequence of the GP<sub>4</sub> gene was determined directly from an RT-PCR product and was found to be unchanged, indicating that the majority of cells still expressed the wt GP<sub>4</sub> protein.

A preliminary complementation experiment was performed by using the previously described EAV knockout mutants for E, GP<sub>2b</sub>, GP<sub>3</sub> and GP<sub>4</sub> expression (2ako, 2bko, 3ko and 4ko, respectively; Table 1). In these constructs, gene expression was prevented by mutagenesis of the translation initiation codon. A disadvantage of these mutations was the fact that they could revert readily to the wt sequence, resulting in the production of wt virus. In particular, the ORF2a- and ORF3-knockout mutations were found to be prone to reversion (data not shown; Table 1). Thus, following the first indications for successful complementation (Fig. 2a), improved knockout mutants for ORFs 2b, 3 and 4 were engineered by adding an internal deletion in these genes to the mutation that inactivated translation initiation. Care was taken not to disturb the regulatory RNA sequences that are involved in subgenomic RNA synthesis (Pasternak *et al.*, 2001). As the 5'- and 3'-proximal parts of ORFs 2b, 3 and 4 each overlap with neighbouring genes (Fig. 1b), we chose to make these internal deletions in the central part of each gene, which does not overlap with another ORF. The sequences deleted from ORFs 2b, 3 and 4 corresponded to nt 9956–10088, 10507–10562 and 10798–10933 of the viral genome, respectively, and a *Hind*III restriction site was engineered at the position of each of the deletions. At the protein level, these mutations resulted in truncation of GP<sub>2b</sub> after Asp-45 and in-frame deletions from Val-68 to Asn-86 in GP<sub>3</sub> and from Phe-34 to Leu-78 in GP<sub>4</sub>.

When transfected into normal BHK-21 cells (or cells containing the SinRep–GFP control replicon), the improved GP<sub>3</sub> and GP<sub>4</sub> mutants (3ko $\Delta$ 3 and 4ko $\Delta$ 4) were found to be fully competent in genome replication and subgenomic RNA synthesis (data not shown). Unfortunately, the improved GP<sub>2b</sub> knockout mutant (2bko $\Delta$ 2) was found to be replication-deficient in repeated experiments, for unclear reasons. As expected, in normal BHK-21 cells, mutants 3ko $\Delta$ 3 and 4ko $\Delta$ 4 did not produce infectious progeny and the signal observed by immunofluorescence assay disappeared after the death of single-transfected cells at 48–72 h post-transfection (h.p.t.). Plaque assays using supernatant from these transfected cell cultures did not result in a single plaque, even at low dilutions.

When transfected into cell lines carrying the appropriate SinRep replicon (SinRep–ORF3 or SinRep–ORF4), successful complementation was evident from rapid spread of the infection. At 36–48 h.p.t., cytopathogenic effect (CPE) affecting the whole cell culture was observed readily with mutants 3ko $\Delta$ 3 and 4ko $\Delta$ 4. Culture supernatants were now anticipated to contain

DISC viruses, i.e. infectious virus particles containing the full complement of structural proteins, but carrying a mutant genome with a debilitating deletion in ORF3 or ORF4. Upon infection of non-complementing cells, they should engage in an abortive single cycle of replication. To prove this point, (dilutions of) culture supernatants were used to infect normal BHK-21 cells and, in immunofluorescence assays, they were indeed found to produce single-cell infections without spread of the infection to neighbouring cells (data not shown). However, upon infection of the corresponding complementing cell line with the same inocula, rapid virus spread and CPE were observed, confirming the dependence of the DISC virus on the *trans*-complementation provided by the cell line. These results were extended and confirmed in plaque assays (Fig. 2b; Table 1). As in the case of cells that were transfected with the corresponding mutant full-length RNAs (Fig. 1c), cells infected with DISC viruses lacking expression of one of the minor structural proteins were found to produce non-infectious, subviral particles containing the GP<sub>5</sub>, M and N proteins (data not shown; Fig. 1c).

Theoretically, the internal deletions in the 3ko $\Delta$ 3 and 4ko $\Delta$ 4 viruses could be repaired during replication in the complementing cell line by RNA recombination involving the SinRep replicon RNA carrying the intact copy of the same gene. This might result in a subpopulation of virions containing a wt, instead of mutant, EAV genome. By using undiluted material and low dilutions of culture supernatants (from different time points after transfection) to infect normal BHK-21 cells or SinRep–GFP-containing cells (Fig. 2b), we screened extensively for such potential recombinants in immunofluorescence and plaque assays, but could not find any indications for infectious recombinants.

Finally, to demonstrate the potential for expression of heterologous sequences by DISC EAV, the GFP reporter gene was inserted at the site of the internal ORF4 deletion in mutant 4ko $\Delta$ 4, which should result in its expression from subgenomic mRNA<sub>4</sub>. When transfected into SinRep–ORF4 cells, the 4ko $\Delta$ 4–GFP construct produced DISC virus titres that were comparable to those of mutant 4ko $\Delta$ 4 (data not shown). Cells infected with 4ko $\Delta$ 4–GFP DISC virus indeed expressed the GFP reporter gene, underlining the potential for the expression and transfer of foreign (marker) sequences by using this approach.

In summary, our SinRep19-based cell lines expressing EAV minor structural proteins constitute the first *trans*-complementation system for arterivirus gene function. Probably aided by the relatively small amounts of the minor structural proteins that are required for assembly of infectious arterivirus particles, the level of complementation achieved with the alphavirus vectors (virus yields up to approx.  $10^7$  p.f.u. ml<sup><MIN>1</sup>, only one log lower than those of the wt virus) can be considered quite efficient. Obviously, the use of other expression systems might further improve this result.

Although arteriviruses are clearly unrelated to the DNA-containing herpesviruses, this work provides an interesting parallel with the prior engineering of DISC herpesviruses that were used in studies on gene therapy (e.g. Rees *et al.*, 2002; Todryk *et al.*, 1999 and references therein) and herpesvirus vaccine development (e.g. Boursnell *et al.*, 1997; McLean *et al.*, 1994, 1996). Likewise, DISC arteriviruses are potentially useful in vaccine development. In addition,

the *trans*-complementing cell lines described here will be useful to study the molecular biology of the arterivirus minor structural proteins. Recent studies on arterivirus assembly have made it clear that this unique set of proteins probably plays a key role in virus infectivity and entry (Wieringa *et al.*, 2003, 2004).

### Acknowledgements

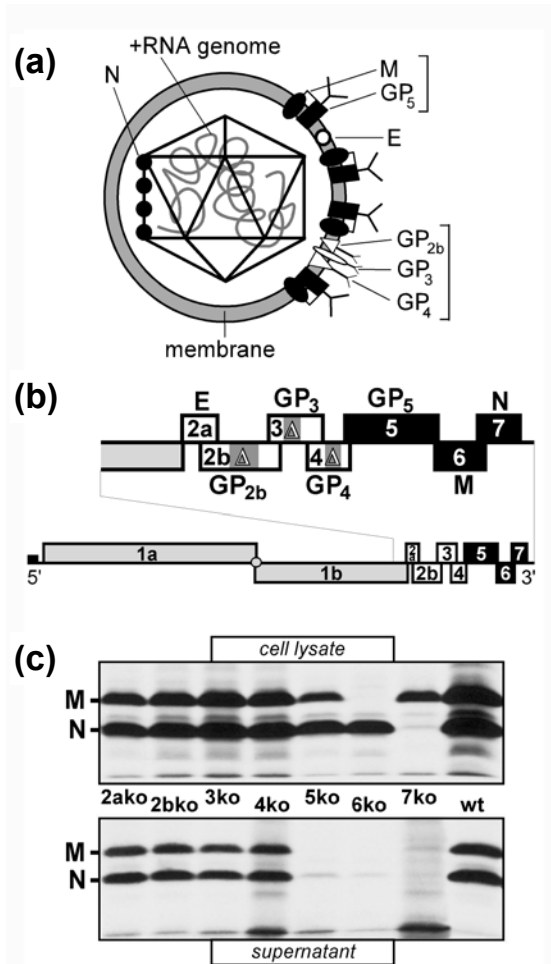
We thank Dr Charles Rice and coworkers for providing the pSinRep19 expression system, Dr Peter Bredenbeek for general advice on the use of alphavirus-based expression vectors and Drs Roeland Wieringa, Twan de Vries and Peter Rottier for sharing EAV-related reagents and research data. This research was supported financially (in part) by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (NWO-CW) and the Netherlands Technology Foundation (STW).

### References

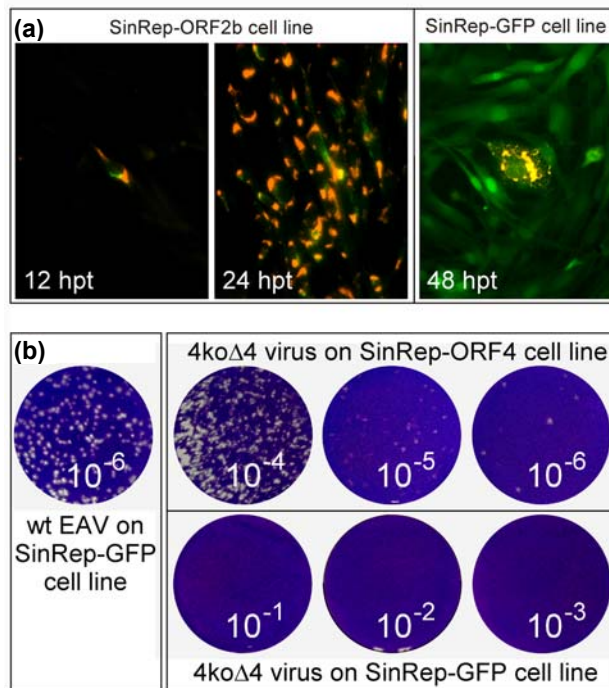
- Agapov, E. V., Frolov, I., Lindenbach, B. D., Prágai, B. M., Schlesinger, S. & Rice, C. M. (1998).** Noncytopathic Sindbis virus RNA vectors for heterologous gene expression. *Proc Natl Acad Sci U S A* **95**, 12989–12994.
- Boursnell, M. E. G., Entwisle, C., Blakeley, D. & 9 other authors (1997).** A genetically inactivated herpes simplex virus type 2 (HSV-2) vaccine provides effective protection against primary and recurrent HSV-2 disease. *J Infect Dis* **175**, 16–25.
- den Boon, J. A., Snijder, E. J., Chirnside, E. D., de Vries, A. A. F., Horzinek, M. C. & Spaan, W. J. M. (1991).** Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. *J Virol* **65**, 2910–2920.
- de Vries, A. A. F., Chirnside, E. D., Horzinek, M. C. & Rottier, P. J. M. (1992).** Structural proteins of equine arteritis virus. *J Virol* **66**, 6294–6303.
- de Vries, A. A. F., Post, S. M., Raamsman, M. J. B., Horzinek, M. C. & Rottier, P. J. M. (1995).** The two major envelope proteins of equine arteritis virus associate into disulfide-linked heterodimers. *J Virol* **69**, 4668–4674.
- de Vries, A. A. F., Glaser, A. L., Raamsman, M. J. B., de Haan, C. A. M., Sarnataro, S., Godeke, G.-J. & Rottier, P. J. M. (2000).** Genetic manipulation of equine arteritis virus using full-length cDNA clones: separation of overlapping genes and expression of a foreign epitope. *Virology* **270**, 84–97.
- McLean, C. S., Erturk, M., Jennings, R., Ní Challanáin, D., Minson, A. C., Duncan, I., Boursnell, M. E. G. & Inglis, S. C. (1994).** Protective vaccination against primary and recurrent disease caused by herpes simplex virus (HSV) type 2 using a genetically disabled HSV-1. *J Infect Dis* **170**, 1100–1109.
- McLean, C. S., Ní Challanáin, D., Duncan, I., Boursnell, M. E. G., Jennings, R. & Inglis, S. C. (1996).** Induction of a protective immune response by mucosal vaccination with a DISC HSV-1 vaccine. *Vaccine* **14**, 987–992.

- Meulenbergh, J. J. M., Bos-de Ruijter, J. N. A., van de Graaf, R., Wensvoort, G. & Moormann, R. J. M. (1998).** Infectious transcripts from cloned genome-length cDNA of porcine reproductive and respiratory syndrome virus. *J Virol* **72**, 380–387.
- Molenkamp, R., van Tol, H., Rozier, B. C. D., van der Meer, Y., Spaan, W. J. M. & Snijder, E. J. (2000).** The arterivirus replicase is the only viral protein required for genome replication and subgenomic mRNA transcription. *J Gen Virol* **81**, 2491–2496.
- Nielsen, H. S., Oleksiewicz, M. B., Forsberg, R., Stadejek, T., Bøtner, A. & Storgaard, T. (2001).** Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J Gen Virol* **82**, 1263–1272.
- Nielsen, H. S., Liu, G., Nielsen, J., Oleksiewicz, M. B., Bøtner, A., Storgaard, T. & Faaberg, K. S. (2003).** Generation of an infectious clone of VR-2332, a highly virulent North American-type isolate of porcine reproductive and respiratory syndrome virus. *J Virol* **77**, 3702–3711.
- Pasternak, A. O., van den Born, E., Spaan, W. J. M. & Snijder, E. J. (2001).** Sequence requirements for RNA strand transfer during nidovirus discontinuous subgenomic RNA synthesis. *EMBO J* **20**, 7220–7228.
- Rees, R. C., McArdle, S., Mian, S., Li, G., Ahmad, M., Parkinson, R. & Ali, S. A. (2002).** Disabled infectious single cycle-herpes simplex virus (DISC-HSV) as a vector for immunogene therapy of cancer. *Curr Opin Mol Ther* **4**, 49–53.
- Snijder, E. J. & Meulenbergh, J. J. M. (2001).** Arteriviruses. In *Fields Virology*, 4th edn, pp. 1205–1220. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott, Williams & Wilkins.
- Snijder, E. J., van Tol, H., Pedersen, K. W., Raamsman, M. J. B. & de Vries, A. A. F. (1999).** Identification of a novel structural protein of arteriviruses. *J Virol* **73**, 6335–6345.
- Todryk, S., McLean, C., Ali, S., Entwistle, C., Boursnell, M., Rees, R. & Vile, R. (1999).** Disabled infectious single-cycle herpes simplex virus as an oncolytic vector for immunotherapy of colorectal cancer. *Hum Gene Ther* **10**, 2757–2768.
- van der Meer, Y., van Tol, H., Krijnse Locker, J. & Snijder, E. J. (1998).** ORF1a-encoded replicase subunits are involved in the membrane association of the arterivirus replication complex. *J Virol* **72**, 6689–6698.
- van Dinten, L. C., den Boon, J. A., Wassenaar, A. L. M., Spaan, W. J. M. & Snijder, E. J. (1997).** An infectious arterivirus cDNA clone: identification of a replicase point mutation that abolishes discontinuous mRNA transcription. *Proc Natl Acad Sci U S A* **94**, 991–996.
- Wieringa, R., de Vries, A. A. F., Post, S. M. & Rottier, P. J. M. (2003).** Intra- and intermolecular disulfide bonds of the GP<sub>2b</sub> glycoprotein of equine arteritis virus: relevance for virus assembly and infectivity. *J Virol* **77**, 12996–13004.
- Wieringa, R., de Vries, A. A. F., van der Meulen, J. & 7 other authors (2004).** Structural protein requirements in equine arteritis virus assembly. *J Virol* (in press).

**Fig. 1.** EAV particle and genome structure. (a) Schematic representation of the EAV particle, showing an isometric nucleocapsid and an envelope containing six viral membrane proteins, including a GP<sub>5</sub>-M heterodimer and a GP<sub>2b</sub>-GP<sub>3</sub>-GP<sub>4</sub> heterotrimer. (b) EAV genome organization (bottom) and an enlarged view (top) of the 3'-proximal quarter of the genome, which encodes the seven structural proteins. Internal deletions made in ORFs 2b, 3 and 4 (see text) are marked with Δ. (c) Release of major structural proteins by cells transfected with EAV knockout mutants lacking expression of proteins E, GP<sub>2b</sub>, GP<sub>3</sub> or GP<sub>4</sub>. BHK-21 cells were transfected with previously described knockout mutants (Molenkamp *et al.*, 2000) for expression of one of the seven structural proteins or with a wt control. Protein synthesis in transfected cells was labelled with <sup>35</sup>S-methionine/cysteine from 9 to 12 h.p.t. Cell lysates were used for immunoprecipitation (de Vries *et al.*, 1992) with rabbit antisera that recognize the EAV M and N proteins (top). Using the same antisera, supernatant from <sup>35</sup>S-labelled transfected cells was screened for the release of EAV M and N proteins. Cells transfected with knockout mutants lacking expression of any of the four minor structural proteins (E, GP<sub>2b</sub>, GP<sub>3</sub> or GP<sub>4</sub>) were found to release large amounts of major structural proteins. A detailed analysis, published elsewhere (Wieringa *et al.*, 2004), revealed that these proteins are incorporated into non-infectious, subviral particles that contain the viral genome.



**Fig. 2** Characterization of DISC EAV mutants. (a) Analysis of replication of EAV knockout mutants by immunofluorescence staining. Cell cultures were transfected (at low efficiency) with the knockout mutant (2bko) for GP<sub>2b</sub> expression. Full-length RNA was transfected into the complementing SinRep–ORF2b cell line, which expresses the gene inactivated in this virus mutant, or into a GFP-expressing control cell line. In the complementing cell line (left panels), rapid virus spread from initially transfected cells to neighbouring untransfected cells was observed, resulting in complete CPE by 48 h.p.t. Cells were double-labelled for expression of replicase subunit nsp3 (red) and structural protein GP<sub>5</sub> (green), as described previously (van der Meer *et al.*, 1998). In the GFP-expressing control cell line (right panel), only single-transfected cells were observed up to 48 h.p.t., after which these cells died and the signal faded. Spread of the infection to untransfected cells was not observed. Labelling for expression of replicase subunit nsp3 (red) and the GFP signal (green) is shown. (b) Plaque assays illustrating the rescue of a DISC EAV (mutant 4koΔ4) on a complementing cell line (SinRep–ORF4 cell line) and the absence of plaque formation on a non-complementing cell line. At 48 h.p.t., supernatant was harvested from SinRep–ORF4 cells transfected with the 4koΔ4 mutant. Serial dilutions of this DISC EAV material were used for plaque assays on the complementing SinRep–ORF4 cell line (top row) and, at low dilutions, the SinRep–GFP control cell line (bottom row), to test for the generation of revertants/recombinants. The 4koΔ4 DISC virus only produced plaques on the complementing cell line. Plaques were somewhat smaller than those of wt virus (left panel) and, in the case of the 4koΔ4 mutant, virus titres were about 10-fold lower than those for wt virus (see Table 1).



**Table 1.** Infectivity titres produced by EAV knockout mutants used in this study

Mutant	Mutation(s)*	Titre (p.f.u. ml <sup>-1</sup> ) on:		Remarks
		Appropriate complementing cell line	SinRep–GFP control cell line	
2ako	Initiation codon AUG→AUA	2×10 <sup>7</sup>	2×10 <sup>7</sup>	Efficient reversion to wt virus
2bko	Initiation codon AUG→ACG	1×10 <sup>7</sup>	<20	
3ko	Initiation codon AUG→GUG	3×10 <sup>6</sup>	3×10 <sup>4</sup>	Partial reversion to wt virus
4ko	Initiation codon AUG→ACG	5×10 <sup>7</sup>	<20	
2bkoΔ2	Initiation codon AUG→ACG Internal deletion in ORF2b*	–	–	Replication-negative
3koΔ3	Initiation codon AUG→GUG Internal deletion in ORF3*	5×10 <sup>5</sup>	<20	
4koΔ4	Initiation codon AUG→ACG Internal deletion in ORF4*	2×10 <sup>7</sup>	<20	
EAV211	–	2×10 <sup>8</sup>	2×10 <sup>8</sup>	Wild-type control

\*See text and Molenkamp *et al.* (2000) for details.

†Titres were determined by using supernatant harvested from transfections of the appropriate complementing cell line at 48 h.p.t.; serial dilutions of this material were used for side-by-side plaque assays on the same, complementing cell line and a non-complementing SinRep–GFP control cell line; means of two independent experiments are given.