Flaviviruses cause a variety of diseases, including encephalitis and haemorrhagic fevers. The genus *Flavivirus* contains (i) viruses that are transmitted by mosquitoes or ticks (arthropod-borne) and (ii) viruses with no known vector (NKV) (Lindenbach & Rice, 2001). Many flaviviruses have evolved unique strategies to adapt to the special requirements of their specific target cells (reviewed recently by Fernandez-Garcia et al., 2009). Permissivity to flavivirus infection is determined by many different host-cell factors that may either be required for efficient infection of mammalian and arthropod cells (host susceptibility factors, HSFs) or restrict infection in certain cells (host resistance factors, HRFs) (Krishnan et al., 2008; Sessions et al., 2009). For example, >40 candidate HSFs have been identified that are required for growth of the mosquito-borne dengue virus (DENV) type 2 in arthropod, but not in mammalian, cells (Sessions et al., 2009). It is largely unknown which viral determinants are responsible for host tropism and vector specificity, i.e. (i) why do certain flaviviruses infect mosquitoes and others only ticks and (ii) why are NKV flaviviruses not able to infect either ticks or mosquitoes? The ability or inability of flaviviruses to infect cultured cells derived from mosquitoes reflects the natural vector–virus relationship. Whilst the mosquito cell line C6/36 is highly susceptible to infection with mosquito-borne viruses such as yellow fever virus (YFV) and DENV, these cells are not infectable with several tick-borne viruses, or with the NKV viruses Apoi virus or Modoc virus (MODV) (Lawrie et al., 2004; Leyssen et al., 2002). In general, the inability of NKV flaviviruses to infect mosquito cells may possibly be due to a failure of the virus to gain entry into the cell, or to a post-entry event. To study whether the viral envelope (glyco)proteins determine the (in)ability of flaviviruses to infect mosquito cells, we monitored the replication of (i) two mosquito-borne flaviviruses [YFV strain 17D (YFV-17D) and DENV type 2 New Guinea strain (DENV2)], (ii) an NKV flavivirus (MODV) and (iii) two chimeric flaviviruses derived from them, which consist of either the YFV-17D or the DENV2 genome in which the prM+E region or the AnchC+prM+E region was replaced by the corresponding region of MODV, respectively.

MODV was obtained from the ATCC (VR-415), YFV-17D was derived from clone pACNR-FLYF17Da (Bredenbeek et al., 2003; Molenkamp et al., 2003), DENV2 from clone pDVWS601 (Gualano et al., 1998; Pryor et al., 2001) and the chimeric viruses MOD/YFV and MOD/DENV2 from clones pACNR-MOD/YFV (Charlier et al., 2003) and pDVWS-MOD/DENV2 (K. Dallmeier, N. Charlier, A. Davidson, S. J. Kaptein & J. Neyts, unpublished data), respectively. Virus replication was monitored in Vero-B or C6/36 cells over a period of 8 days. Cell monolayers were infected with 10^5 p.f.u. of either virus at 37 °C (Vero-B cells) and at 28 °C (C6/36 cells) in 25 cm² culture flasks, and washed after 2 h incubation. Cell-culture medium was

Replication of not-known-vector flaviviruses in mosquito cells is restricted by intracellular host factors rather than by the viral envelope proteins

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Chimeric yellow fever virus 17D (YFV-17D) and dengue virus type 2 (DENV2) carrying the surface proteins of Modoc virus (MODV), a not-known-vector (NKV) flavivirus, replicated efficiently in mammalian (Vero-B) and mosquito (C6/36) cells, whereas MODV failed to replicate in mosquito cells. Transfection of C6/36 cells with MODV RNA did not result in virus replication; however, transfection of these mosquito cells with YFV-17D or DENV2 RNA did. The inability of NKV viruses (such as MODV) to infect and replicate in arthropod cells is thus not determined by the viral envelope, but by a post-entry event.

Three supplementary figures are available with the online version of this paper.
harvested either every day or every second day and titrated for infectious virus content on Vero-B cells (Fig. 1a, b). Viral RNA loads were determined by specific real-time quantitative RT-PCR (qRT-PCR) using RNA extracted from 140 μl cell-culture supernatant (QIAamp Viral RNA kit; Qiagen) (Figs 1c, d and 2), as reported previously (Leyssen et al., 2001; Charlier et al., 2004; Alen et al., 2009).

All viruses, including the chimeric MOD/YFV and MOD/DENV2, were fully viable and replicated efficiently in Vero-B cells (Figs 1a, c and 2a). As expected, YFV-17D replicated efficiently in C6/36 cells, whereas the NKV flavivirus MODV did not replicate in these cells (Fig. 1b, d). Surprisingly, the MOD/YFV chimera replicated readily in C6/36 cells, although less efficiently than the parental YFV-17D (Fig. 1b, d). These findings were corroborated by the observation that the MOD/DENV2 chimera replicated efficiently in Vero-B as well as in C6/36 cells (akin to the parent virus DENV2, but unlike MODV) (Fig. 2b). Data from representative experiments are depicted in Figs 1 and 2. Comparable data were obtained in one (MODV, DENV2 and MOD/DENV2) or two (MODV, YFV and MOD/YFV) additional independent experiments (see Supplementary Figs S1 and S2, available in JGV Online). The somewhat reduced replication efficiency of MOD/YFV and MOD/DENV2 may possibly result to some extent from a less efficient cell attachment, internalization and/or endosomal fusion mediated by the MODV envelope. However, a general attenuation of chimeric flaviviruses has been reported (Pletnev et al., 1992; Pletnev & Men, 1998; Charlier et al., 2004). It was suggested that this may relate to as yet poorly understood incompatibilities within the artificially joined chimeric genomes, independent of changes in the virus entry pathways (Pugachev et al., 2004).

From the experiments described above, it appears that the block to productive MODV replication in insect cells (which should finally lead to release of infectious virus progeny) is not at the level of attachment and entry, but rather at a downstream post-entry stage of the virus life cycle. To study this hypothesis, Vero-B and C6/36 cells were transfected with 2 μg MODV RNA isolated from infectious particles (RNeasy Mini kit; Qiagen) using DMRIE-C Reagent (Invitrogen) according to the manufacturer’s instructions. On days 1 and 8 post-transfection, supernatants and cell pellets were collected and analysed for the presence of viral RNA by qRT-PCR. Transfection of Vero-B cells with MODV RNA resulted in an increase in viral RNA in the culture supernatant (Fig. 3). To confirm that the increase in viral RNA observed was due to virus replication, cells transfected in parallel were treated with the antiviral drug ribavirin (ICN) (at a concentration of 100 μg ml⁻¹), which indeed abrogated the production of virus progeny RNA. However, when C6/36 cells were transfected with the same amount of input MODV RNA, no increase in viral RNA levels (neither intracellular nor

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**Fig. 1.** Replication kinetics of MODV (circles), YFV-17D (crosses) and MOD/YFV (triangles) in Vero-B and C6/36 mosquito cells. (a, b) Infectious virus titre in culture supernatant of infected Vero-B or C6/36 cells. CCID₅₀, Median cell-culture infectious dose. (c, d) Increase in viral RNA in culture supernatant of infected Vero-B or C6/36 cells over a period of 8 days as detected by means of qRT-PCR. Deviations between higher viral RNA levels (c) than titres (a) observed in particular for YFV-17D result most probably from the detection of increasing amounts of immature virus particles and/or non-infectious viral RNA and correspond to a more pronounced cytopathic effect 6 and 8 days after infection observed for YFV-17D, but not the other viruses studied in parallel.
extracellular) was detected over time. The transfection assay was validated by transfecting C6/36 cells with RNA isolated from YFV-17D infectious particles. In that case, YFV-17D replication could be confirmed by an at least 10- to 30-fold increase in YFV-17D RNA over the ribavirin-treated controls as measured by qRT-PCR (see Supplementary Fig. S3, available in JGV Online). Moreover, YFV-17D-transfected C6/36 cultures exhibited an overt cytopathic effect after 8 days, whereas mock-transfected cultures appeared normal (not shown).

The host tropism of flaviviruses has been studied previously by using chimeric flaviviruses. Pletnev & Men (1998) reported the construction of two chimeric viruses that consisted of the DENV type 4 genome in which the prM+E genes had been replaced by the corresponding genes of Langat virus, a tick-borne flavivirus. Like our mosquito-borne/NKV flavivirus chimeras, this virus replicated in C6/36 cells, although less efficiently than the parent DENV type 4 (Pletnev & Men, 1998). A chimeric tick-borne encephalitis/dengue type 4 [TBE(ME)/DEN4] virus that contained the prM+E genes of TBEV replicated in mosquito cells (Pletnev et al., 1992), whereas TBEV did not. The results of these studies suggest that the envelope proteins of TBEV are not likely to restrict the replication of TBEV in mosquito cells. It remained to be determined whether the inability of the NKV flaviviruses to replicate in mosquito cells is the result of either (i) the inability of the virus to gain entry into the cell or (ii) an abortive infection once the virus has entered the cell. A number of putative cellular receptors (glycosaminoglycans, DC-SIGN, laminin receptor, BiP, αVβ3 integrin, Hsp70 and Hsp90, mannose receptor) for mosquito-borne flaviviruses have been identified by using different mammalian cell lines (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003; Chu & Ng, 2004; Jindadamrongwech et al., 2004; Thepparit & Smith, 2004; Reyes-Valle et al., 2005; Lee et al., 2006; Miller et al., 2008). Very little information is available about the entry of flaviviruses into insect cells. Two membrane proteins of mosquito (C6/36) cells that bind and mediate entry of West Nile virus (WNV), Japanese encephalitis virus (JEV) and DENV2, leading to productive infection, were identified (Chu et al., 2005). However, there is growing evidence that flaviviruses can use several alternative receptors for infectious entry (Acosta et al., 2009; Alen et al., 2009), which in turn may be highly dependent on the differentiation status of the respective target cells (Kwan et al., 2008).

Our data thus indicate that the inability of NKV flaviviruses to replicate in arthropod (mosquito) cells occurs at a post-entry stage, as (i) chimeric MOD/YFV or MOD/DENV2 encoding the envelope (glyco)proteins of MODV productively infects C6/36 cells without ribavirin corresponds to an increase from <50 to approximately 10,000 viral genome equivalents per cell at days 1 and 8 post-transfection, respectively.

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**Fig. 2.** Release of viral RNA in supernatants of Vero-B (a) and C6/36 mosquito (b) cells infected with MODV (□), DENV2 (▲) or MOD/DENV2 (■).

**Fig. 3.** Increase in MODV RNA levels (either intracellular or extracellular) in Vero-B and C6/36 cells that have been transfected with MODV RNA and that were further cultured in the absence (empty bars) or presence (filled bars) of ribavirin. An increase of intracellular viral RNA by more than two orders of magnitude as observed for MODV in Vero-B cells without ribavirin corresponds to an increase from <50 to approximately 10,000 viral genome equivalents per cell at days 1 and 8 post-transfection, respectively.

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detected (Fig. 3), as would be the case if the block affected the late stages of virus replication, such as virion assembly and egress. It is likely that differences in host protein profiles between mammalian and mosquito (and also tick) cells are responsible for the inability of NKV flavivirus RNA to replicate in insect cells. Further investigations are now required to determine whether the inability of NKV flaviviruses to replicate in insect cells is due to the lack of HSFs present in mammalian cells, or alternatively due to the presence of specific HRFs in insect cells. Cellular RNA-binding proteins have been shown to bind to the 3′ or 5′ non-coding region of the flavivirus genome (Ta & Vrati, 2000; De Nova-Ocampo et al., 2002; Li et al., 2002; Edgil et al., 2006; Kim & Jeong, 2006); some of these RNA-binding proteins have been shown to be necessary for efficient flaviviral translation and/or genome replication (reviewed recently by Paramajpe & Harris, 2010). In addition, members of the NF90/NFAR protein group were shown to regulate replication of the related pestiviruses by specific association with regulatory elements in the 5′ and 3′ untranslated regions (Isken et al., 2003), whilst several HSFs have been shown to regulate hepatitis C virus replication (Okamoto et al., 2006). However, interestingly, several (putative) RNA-binding proteins that allow DENV2 to replicate in Drosophila (dipteran) cells, but are dispensable as HSFs in human hepatoma cells (Sessions et al., 2009), have been identified. Moreover, recently the innate antiviral immune system of invertebrates, namely the RNA interference (RNAi) pathway of recognizing and silencing double-stranded viral RNA, has been shown to play a major role for the replication of DENV and WNV in their respective mosquito hosts (Brackney et al., 2009; Sanchez-Vargas et al., 2009). It is therefore tempting to speculate that vector-borne flaviviruses such as YFV and DENV developed specific means to counteract the innate antiviral reponse of their mosquito vectors (Sanchez-Vargas et al., 2009), as described for other RNA viruses infecting plants and insects (Voinnet, 2005; Marques & Carthew, 2007), whereas MODV as an NKV flavivirus is still fully prone to the antiviral action of RNAi.

It will be important to study which host-cell factors determine whether flaviviruses are able to replicate in cells of arthropod origin. The identification of HSFs required for NKV flavivirus replication may help to better understand what determines the vector capacity of natural arthropod vectors of emerging flaviviral pathogens, such as DENV, JEV and WNV. Likewise, the identification of sequences in the virus genome that are responsible for the recognition of such host-cell factors will provide important novel insight into the biology of flaviviruses.

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