Marburg virus (MARV) and the closely related Ebola virus (EBOV) are enveloped, non-segmented, negative-strand (NNS) RNA viruses belonging to the family Filoviridae. Filoviruses cause severe disease in humans and non-human primates, and the 2013–2016 outbreak of EBOV in West Africa showed the potential of filoviruses to cause large-scale epidemics associated with high fatality rates (Acharya, 2014; Hartman et al., 2010).

The MARV genome encodes seven viral structural proteins among which the viral surface glycoprotein GP and the viral matrix protein VP40, together with host-derived lipids, form the viral envelope, while NP, VP35, VP30, VP24 and L encapsidate the viral RNA forming the filamentous nucleocapsid (Becker et al., 1998; Bharat et al., 2011; Feldmann et al., 1991). The enzymatically active subunit (L) of the MARV polymerase comprises 2331 aa (Mühlberger et al., 1992). L, together with the MARV protein VP35, forms the RNA-dependent RNA polymerase complex which is essential for transcription and replication of the viral genome (Mühlberger et al., 1998). Based on the sequence alignment of MARV L and polymerases of other NNS RNA viruses, six conserved sequence regions have been identified, designated domains I–VI (Mühlberger et al., 1992), which are believed to constitute various enzymatic activities of L protein (Liang et al., 2015; Poch et al., 1990; Sidhu et al., 1993; Stec et al., 1991). Domain III of MARV L is anticipated to represent the RNA polymerase active site. This site contains a GDNQ/E motif, which is highly conserved of NNS RNA viruses (Magoffin et al., 2007; Mühlberger et al., 1992; Poch et al., 1990). The insertion of mutations within or in close proximity to the GDNQ/E motif has been shown to abolish polymerase activity of rhabdo- and paramyxoviruses (Magoffin et al., 2007; Malur et al., 2002; Noton et al., 2012; Schnell & Conzelmann, 1995; Sleat & Banerjee, 1993; Smallwood et al., 2002). Remarkably, three mutations in the MARV polymerase L (S741C, D758A and A759D)-located N- and C-terminal of the GDNQ/E motif (residues 744–747) have been detected in a MARV which became lethal for guinea pigs after sequential passages (Lofts et al., 2007). This observation suggested that mutations in the active site of RNA-dependent RNA polymerase not only impair, but might improve the activity of viral polymerase complex in a species-specific manner.

In this study, we analysed the influence of mutations S741C, D758A and A759D on polymerase function in human and guinea pig cells. Currently, no specific antibodies are available which allow detection of MARV L. Therefore, to control the expression of L and constructed mutants, the coding region of mCherry (AY678264) was inserted via a unique BstBI restriction site into the L gene. The mCherry sequence is hence localized to a homologous region. Insertion of mCherry-L in a homologous region did not impair the function of EBOV L protein (Hoennen et al., 2012). Similarly, the reporter gene activity mediated by mCherry-tagged MARV L was also only slightly reduced in comparison to the untagged L protein (Fig. 1b).
Mutants of L containing the previously reported guinea pig-lethal MARV aa changes (Lofts et al., 2007) were constructed by site-directed mutagenesis of the L gene resulting in either single aa substitutions S741C (L$_1$), D758A (L$_2$) and A759D (L$_3$), or combinations of double substitutions (L$_{1,2}$, L$_{1,3}$, L$_{2,3}$) and a triple mutant (L$_{1,2,3}$). Details of the cloning procedure are available upon request.

To assess the expression and ability of the L mutants to interact with the main components of the MARV replication complex (Becker et al., 1998; Mühlberger et al., 1998), we performed immunofluorescence analysis of human Huh-7 and guinea pig 104C1 cells transfected with all plasmids required for mini-genome replication and transcription (Mühlberger et al., 1998). Briefly, cells were transfected with a plasmid encoding a MARV-specific mini-genome carrying the Renilla luciferase reporter gene under T7 promoter, the plasmids encoding NP, VP30, VP35, T7 DNA-dependent RNA polymerase, and one of the L constructs. It has been shown previously that single expression of MARV L results in diffuse distribution of the protein while upon co-expression with nucleocapsid proteins NP and VP35 MARV L relocates to perinuclear inclusions due to VP35-mediated interaction with NP (Becker et al., 1998). The singly expressed mCherry-tagged MARV L (L) was also diffusely distributed in the cytoplasm (Fig. 1c(i)), and relocalized into perinuclear inclusions only upon co-expression with NP and VP35 (Fig. 1c(ii–v)). As shown in Fig. 1(d), all L constructs were expressed and located in the perinuclear NP-induced inclusions, indicating that none of the mutations destroyed the interactions between L and the components of MARV replication complex in human and guinea pig cells. In addition, no obvious differences in autofluorescence signals of the analysed constructs were detected (Fig. 1d).

To analyse the polymerase activity of L mutants, the mini-genome reporter assay was used (Mühlberger et al., 1999). Human HEK293 and guinea pig 104C1 were transfected with all plasmids required for mini-genome replication and transcription (see above). Additionally, a pGL4 plasmid encoding firefly luciferase under control of the cellular RNA polymerase II promoter served for normalization of transfection efficiency. The reporter gene activity values in the cell lysates were measured 48 h post-transfection (p.t.) using the Dual Luciferase assay (Promega). The results obtained with L were set to 100 %. The assay was performed in triplicate with samples from three independent experiments. The S741C (L$_1$) mutant resulted in a 2.6-fold increase of polymerase activity in human cells, and a 4.5-fold increase in guinea pig cells (Fig. 2a, b). Surprisingly, L$_2$ and L$_3$, as well as combinations of the substitutions (L$_{1,3}$, L$_{2,3}$ and L$_{1,2,3}$), showed almost total abrogation of polymerase activity in both human and guinea pig cells. Thus, although the L mutants containing D758A and A759D substitutions were able to interact with NP and VP35, they exhibited no polymerase activity. The activity of L$_{1,2}$ was also decreased, but its activity was 4.8-fold higher in guinea pig cells than in human cells.

The severe inhibitory effect of D758A and A759D mutations on polymerase activity in both human and guinea pig cells raised the question of whether other factors might restore the polymerase activity required for virus replication specifically in guinea pig cells. Since temperature influences the polymerase activity of L proteins of NNS RNA viruses (Brown et al., 2011), and the body temperature of guinea pigs is higher (39.5 °C) than that of humans (Terril & Clemmons, 1998), we hypothesized that polymerase activity of the severely affected L mutants might be restored at a higher temperature. Therefore, cells transfected as previously described were incubated at 39.5 °C for 48 h before lysis. The polymerase activity of L$_1$ at 39.5 °C was 3.4- and 5.1-fold higher in human and guinea pig cells, respectively, as compared to L, indicating that the L$_1$ mutant preserved the enhanced activity at a higher temperature in both species (Fig. 2c, d). The reporter gene activity of the L$_{1,2}$ mutant was lower at 39.5 °C than at 37 °C in cells of both species, suggesting that the activity of L is impaired by mutation. However, the difference in the activity of the L$_{1,2}$ mutant in guinea pig and human cells was preserved at 39.5 °C. None of the other L mutants showed enhancement of polymerase activity at higher temperatures (Fig. 2c, d), indicating that temperature can not relieve the inhibitory effect of D758A and A759D mutations on polymerase activity.

To prove that the inhibitory effect of D758A and A759D substitutions on polymerase activity is not caused by the presence of mCherry in the L constructs, we analysed the polymerase activity of L mutants not tagged by mCherry (Fig. 2e, f). The reporter gene activities of the mutants were similar to the mCherry-containing mutants of L (compare Fig. 2a, b and d, e). Taken together, all mutants of L containing D758A or A759D substitutions showed extremely low reporter activity, independently of the presence of mCherry.

Another factor which might influence the activity of the L mutants was that, together with the three mutations in L, a point mutation, D184N, in VP40 was selected in guinea pig-lethal MARV (Lofts et al., 2007). Our previous study showed that the D184N mutation in VP40 negated its suppressive function on MARV polymerase activity specifically in guinea pig cells, and thus conveys a species-specific improved viral fitness (Koehler et al., 2015). We therefore presumed a functional co-selection of the mutations in VP40 and in L, and tested the function of L mutants in the presence of VP40 and VP40$_{D184N}$. Cells were transfected with the plasmids required for the mini-genome assay and, in addition, with a plasmid encoding either VP40 or VP40$_{D184N}$. Reporter gene activity of cell lysates at 24 h.p.t. showed that VP40 and VP40$_{D184N}$ inhibited the activity of both L and L$_1$ in human and guinea pig cells (Fig. 3), supporting published data on the inhibitory effect of EBOV and MARV VP40 on viral transcription/replication (Hoenen et al., 2010). In human cells, the inhibition of L was more pronounced in the presence of VP40$_{D184N}$ than in the presence of VP40 (6.3- and 4.8-fold, respectively, Fig. 3a). Likewise, L$_1$ was also more strongly inhibited by
Fig. 1. Characterization of guinea pig-lethal MARV L mutants. (a) Upper image shows the domain organization of wild-type MARV L (L\textsubscript{WT}) and a schematic presentation of conserved regions (CRI–CRVI). The position of the highly conserved GDNQ motif (bold letters) is indicated, as well as positions of the mutations (red letters) in guinea pig-lethal MARV L (L\textsubscript{1,2,3}). RdRp.
VP40

polymerase domain; Cap, capping domain; CD, connector domain; MT, methyltransferase domain; CTD, C-terminal domain. Lower image shows domain organization of mCherry-tagged L, position of the GDNQ motif, positions of guinea pig-lethal mutations (indicated by asterisks) and mCherry (red). (b) Polymerase activity of the untagged and mCherry-tagged L construct. HEK293 cells were transfected with plasmids encoding the proteins required for mini-genome assay (NP, VP35, VP30, T7 polymerase, MARVI mini-genome and pGL4) together with a plasmid encoding either L

Schnell & Conzelmann, 1995; Sleat & Banerjee, 1993; Brown et al., 2002). In the new host. To our knowledge, this is the first study showing that mutations located in close proximity to the active site of the L protein N-terminal of the GDNQ/E motif in L proteins of NNS RNA viruses have a positive effect on polymerase activity (Malur et al., 2002; Schnell & Conzelmann, 1995; Sleat & Banerjee, 1993; Smallwood et al., 2002).

The negative effects of D758A and A759D mutations on the function of L were astonishing since it was expected that all selected mutations supported high replication capabilities of rodent-lethal MARV (Lofts et al., 2007). Further studies are needed to clarify the influence of D758A and A759D mutations in the context of recombinant viruses.

The mutations detected in guinea pig-lethal MARV L are unique considering their position in L protein. All mutations in L polymerases of NNS RNA viruses recorded upon changes of virulence in a new host were located in or between conserved regions V and VI, as reported for members of Paramyxoviridae (Brown et al., 2011; Dortmans et al., 2011; Fujii et al., 2002; Heiden et al., 2014), Rhabdoviridae (Kim et al., 2014), Filoviridae (Ebihara et al., 2006) and Bornaviridae (Ackermann et al., 2007). This C-terminal component of L proteins of NNS RNA viruses, especially the region between domains V and VI, is highly variable, and considered as a domain of L which interacts with unique host cell transacting transcriptional cofactors (Dortmans et al., 2011; Poch et al., 1990; Sidhu et al., 1993). The mutations in guinea pig-lethal MARV L protein, however, affect the domain III close to the GDNQ/E motif, presumably directly modulating the processivity of viral RNA-dependent RNA polymerase (Liang et al., 2015). Hence, changes in the polymerase activity of L mutants are expected to be independent of direct interactions with host factors. Indeed, our study showed that changes in polymerase activity induced by the S741C substitution, or D758A and A759D substitutions, were similar in both species and species-specific differences were negligible. The eightfold increase in polymerase activity of the L mutant upon co-expression with VP40 at specifically in guinea pig cells (Fig. 3b). Indeed, the polymerase activity of L1 mutant was eightfold higher in guinea pig cells than in human cells upon co-expression with VP40 (34.8% in human cells and 279.5% in guinea pig cells, Fig. 3a, b). The expression controls showed that VP40 and VP40 were expressed at similar levels (Fig. 3c, d). The reporter gene activity of L mutants containing D758A and A759D substitutions was not recovered upon co-expression with VP40 (not shown).

Thus, our data suggest that a single S741C mutation in L acquired during passaging of MARV in guinea pigs improves the activity of L and might provide a replicative advantage in the new host. To our knowledge, this is the first study showing that mutations located in close proximity to the GDNQ/E motif in L proteins of NNS RNA viruses have a positive effect on polymerase activity (Malur et al., 2002; Schnell & Conzelmann, 1995; Sleat & Banerjee, 1993; Smallwood et al., 2002).

To summarize, our study showed that the S741C mutation located at the active site of the L protein N-terminal of the GDNQ/E motif (744–747 aa) increased polymerase activity in human and guinea pig cells. The enhanced polymerase activity of the S741C mutant was not temperature-sensitive, but was increased specifically in guinea pig cells upon co-expression with VP40, suggesting that a mutual effect of the S741C mutation in L protein and the D184N mutation in VP40 might provide MARV with a strong advantage for replication in guinea pigs. We think this work will be useful to further investigation of pathogenicity factors of MARV and will help to better understand the biology of negative-strand RNA virus polymerases.
Fig. 2. Influence of mutations in L on polymerase activity in human and guinea pig cells. Human (HEK293) and guinea pig (104C1) cells were transfected as described under Fig. 1(d). At 48 h p.t., cells were lysed and reporter gene activity (reflecting mini-genome transcription and replication) was measured at different temperatures. (a, b) Incubation at 37 °C. Lower panels show controls for protein expression. Cell lysates were analysed by SDS-PAGE and Western blotting using mCherry- and vinculin-specific antibodies. (c, d) Incubation at 39.5 °C. (e, f) Reporter gene activity mediated by L constructs without mCherry tag. Transfection of cells was performed as described above. The mCherry-tagged L constructs were replaced by L constructs lacking mCherry. The reporter gene activity values for L were set to 100%, black columns (HEK293 cells), grey columns (104C1 cells). Data are shown on the logarithmic scale (means and sd). **P<0.01, ***P<0.001.
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Fig. 3. The effect of VP40 and VP40\textsubscript{D184N} on polymerase activity of L\textsubscript{1} mutant in human and guinea pig cells. (a) Human (HEK293) and (b) guinea pig (104C1) cells were transfected as described in Fig. 1(d) and plasmids encoding either VP40 or VP40\textsubscript{D184N}. Where indicated, the L\textsubscript{1}-encoding plasmid (white columns) was replaced by an L\textsubscript{1}-encoding plasmid (grey columns). At 24 h p.t., cells were lysed and reporter gene activity was measured. Shown are the means and sd. *P<0.05, **P<0.01. (c, d) Western blot analysis of HEK293 or 104C1 cells transfected as described above. Cell lysates were analysed by SDS-PAGE and Western blotting using mCherry-, vinculin- and VP40-specific antibodies. VP40, lanes 3 and 6; VP40\textsubscript{D184N}, lanes 4 and 7.

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