Cardiovirus leader proteins are functionally interchangeable and have evolved to adapt to virus replication fitness

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The leader (L) proteins encoded by picornaviruses of the genus Cardiovirus [Theiler’s murine encephalomyelitis virus (TMEV) and Encephalomyocarditis virus (EMCV)] are small proteins thought to exert important functions in virus–host interactions. The L protein of persistent TMEV strains was shown to be dispensable for virus replication in vitro, but crucial for long-term persistence of the virus in the central nervous system of the mouse. The phenotype of chimeric viruses generated by exchanging the L-coding regions was analysed and it was shown that the L proteins of neurovirulent and persistent TMEV strains are functionally interchangeable in vitro and in vivo, despite the fact that L is the second most divergent protein encoded by these viruses after the L* protein. The L protein encoded by EMCV and Mengo virus (an EMCV strain) shares about 35 % amino acid identity with that of TMEV. It differs from the latter by lacking a serine/threonine-rich C-terminal domain and by carrying phosphorylated residues not conserved in the TMEV L protein. Our data show that, in spite of these differences, the L protein of Mengo virus shares, with that of TMEV, the ability to inhibit the transcription of type I interferon, cytokine and chemokine genes and to interfere with nucleocytoplasmic trafficking of host-cell proteins. Interestingly, analysis of viral RNA replication of the recombinant viruses raised the hypothesis that L proteins of TMEV and EMCV diverged during evolution to adapt to the different replication fitness of these viruses.

INTRODUCTION

The family Picornaviridae is composed of small, non-enveloped, single-stranded, positive-sense RNA viruses. Their genome encodes a large polyprotein that undergoes autoproteolytic processing. Of the viral proteins that are formed from the polyprotein, four capsid proteins (VP1, VP2, VP3 and VP4) and six proteins involved in the replication cycle (2B, 2C, 3A, 3B, 3C and 3D) are structurally and functionally well-conserved among picornaviruses. In contrast, some accessory proteins, such as leader (L), L* and 2A (partly), that are not primarily required for genome replication have diverged much more, probably to acquire functions related to the specificity of virus–host interaction (Agol, 2002; Palmenberg & Sgro, 2002). The L protein encoded by cardioviruses, including Theiler’s murine encephalomyelitis virus (TMEV) and Encephalomyocarditis virus (EMCV), does not exhibit significant similarity to L proteins of other picornaviruses. It notably lacks the protease motifs found in the L protein of aphthoviruses (Devaney et al., 1988).

TMEV is responsible for infections of the central nervous system (CNS) of the mouse (Theiler & Gard, 1940). Neurovirulent strains (GDVII and FA) of the virus induce an acute, lethal necrotizing encephalitis. Persistent strains like DA and BeAn provoke a chronic demyelinating disease considered as a model for multiple sclerosis. The latter strains have the striking ability to persist and multiply lifelong in the CNS in the face of a strong and specific immune response (Brahic et al., 2005; Lipton, 1975).

The L protein of TMEV is a small, acidic protein of 76 aa organized into three domains (Fig. 1): (i) an N-terminal atypical (CHCC) zinc-finger domain that was shown to bind Zn\(^{2+}\) ions (Chen et al., 1995), (ii) an acidic domain and (iii) a C-terminal Ser/Thr-rich domain.

The L protein is dispensable for replication of DA and GDVII viruses in BHK-21 cells (Calenoff et al., 1995; Kong et al., 1994; Michiels et al., 1997). However, this protein was shown to be important for persistence of the DA strain in the CNS (van Pesch et al., 2001), suggesting a function of this protein at the host–pathogen-interaction level.

In agreement with this idea, the L protein of the persistent virus DA was found to inhibit transcriptional activation of type I interferon (IFN) genes (van Pesch et al., 2001) and of other host genes that are typically activated by viral infection, such as that encoding the RANTES (regulated upon...
activation normal T-cell expressed and secreted) chemokine (S. Delhaye, B. Michel, C. Ricour & T. Michiels, unpublished observations).

The L protein of the DA virus was also shown to trigger the subcellular redistribution of cytoplasmic and nuclear cellular proteins (Delhaye et al., 2004). It notably affects nucleocytoplasmic trafficking of IFN regulatory factor-3 (IRF-3), a transcription factor crucial for early transcriptional activation of IFN genes (Yoneyama et al., 1998), and of polypyrimidine tract-binding protein (PTB), a nuclear protein that was shown to bind the internal ribosome entry site (IRES) on the viral RNA genome (Kaminski et al., 1995).

Little is known about the activity of the L protein from neurovirulent TMEV strains. It shares about 85% amino acid identity with the L protein of persistent strains and the three domains defined in the protein are conserved (Fig. 1). Despite this high identity, the L protein is the second most divergent protein between TMEV and EMCV/Mengo virus (35% amino acid identity). The EMCV/Mengo virus L protein contains the atypical zinc finger and the acidic domain. In contrast, it lacks the Ser/Thr-rich C-terminal domain present in the TMEV L. The L protein of EMCV/Mengo virus has been shown to be phosphorylated on residues (framed) Thr47 (Zoll et al., 2002) and possibly Tyr41 (Dvorak et al., 2001). The second group of viruses in the genus Cardiovirus is composed of EMCV strains and of Mengo virus, a slightly divergent strain of EMCV (Duke et al., 1990). The L proteins of EMCV and Mengo virus are almost identical (Fig. 1) and will be referred to as the EMCV/Mengo virus L protein. The L protein is one of the most divergent protein between TMEV and EMCV/Mengo virus (35% amino acid identity). The EMCV/Mengo virus L protein lacks the Ser/Thr-rich C-terminal domain and the EMCV/Mengo virus L protein has been reported to be phosphorylated on residues (framed) Thr47 (Zoll et al., 2002) and possibly Tyr41 (Dvorak et al., 2001).

The relationship between the functions of the L proteins of TMEV and EMCV/Mengo virus is not clear. The EMCV/Mengo virus L protein was reported to induce a shut-off of cell protein synthesis (Zoll et al., 1996) that is possibly taking place at the translational level, to modulate IRES activity (Hoffman & Palmenberg, 1996), to inhibit IFN production (Zoll et al., 1996) and to increase ferritin synthesis (Zoll et al., 2002).

The aim of this work was to test to what extent the L proteins of cardioviruses are functionally interchangeable. Therefore, we constructed and analysed chimeric viruses derived from the persistent TMEV DA strain and expressing the L protein of the neurovirulent TMEV GDVII strain or that of Mengo virus. L proteins from persistent and neurovirulent TMEV strains appeared to be fully interchangeable in vivo and in vitro. L proteins from TMEV and Mengo virus share the ability to inhibit transcriptional activation of cytokine and chemokine genes and to promote nucleocytoplasmic redistribution of host proteins. Interestingly, our observations suggest that L proteins of cardioviruses diverged to fit the replication kinetics of the different viruses.

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**Fig. 1.** Alignment of L protein sequences from TMEV and EMCV strains. L sequences shown in the alignment are from TMEV strains DA (GenBank accession no. M20301) (Ohara et al., 1988), Vilyusik (M94868) (Pritchard et al., 1992), GDVII (M20562) (Pevear et al., 1988), BeAn (M16202) (Pevear et al., 1987), Yale (U33047) (Michiels et al., 1995), NGS910 (AB90161) (Ohsawa et al., 2003), from EMCV (NC001479) (Duke et al., 1992) and from Mengo virus (L22089) (Duke et al., 1990). Asterisks indicate residues affected by the L cys mutation. The L proteins of TMEV strains share about 85% identity and contain three conserved domains: an atypical (CHCC) N-terminal zinc-finger domain, an acidic domain and a C-terminal Ser/Thr-rich domain (conserved residues of the three domains are outlined). The L protein of EMCV/Mengo virus and that of TMEV share about 35% identity. The zinc-binding and acidic domains are conserved. In contrast, the L protein of EMCV/Mengo virus lacks the Ser/Thr-rich C-terminal domain. The EMCV/Mengo virus L protein has been reported to be phosphorylated on residues (framed) Thr47 (Zoll et al., 2002) and possibly Tyr41 (Dvorak et al., 2001).
**METHODS**

**Viruses and cells.** Viruses used in this study (Table 1) were produced as described previously (van Pesch et al., 2001) by transfection of BHK-21 cells with viral RNAs transcribed in vitro from the corresponding infectious cDNA clones: pTMDA1 (McAllister et al., 1989; Michiels et al., 1997), pKJ6 (Jnaoui & Michiels, 1998), pTM598, pTM659 (van Pesch et al., 2001), pSPA10, pSPA11, pSPA12, pSPA13, pSPA24 and pSPA28 (this work). Viruses derived from these plasmids (named DA1, KJ6, TM598, TM659, etc.) were titrated on BHK-21 cells by a standard plaque assay. BHK-21 and L929 cells were cultured as described previously (van Pesch et al., 2001).

**Construction of chimeric viruses.** The site-directed mutagenesis method of Kunkel (1985) was adapted in order to replace exactly the entire L-coding region of the DA1 virus by that of the GDVII strain. The synthetic oligonucleotide typically used to introduce the mutation was replaced by a denatured PCR fragment carrying the entire L region of virus GDVII flanked by DA1 sequences to allow annealing of the fragment to the plasmid to be mutated. This mutagenic PCR fragment was obtained by performing PCR with Pfu polymerase (Promega) on pTMGDVII (Tangy et al., 1989), using hybrid primers (TM484, TM485) bearing 3’ sequences complementary to the extremities of the GDVII L region and 5’ sequences matching the neighbouring DA1 sequence. Mutagenesis was performed on pTM410, a plasmid carrying nt 1–1730 of the DA1 cDNA (van Eyll & Michiels, 2000). The mutated L region was then subcloned in a plasmid carrying the full-length viral cDNA, either pTMDA1 (wild-type virus) or pKJ6, which is a pTMDA1 derivative carrying mutations in the capsid-coding region that enhance L929 cell infection. The recombinant plasmids obtained were called pSPA11 and pSPA10, respectively (Table 1; Fig. 2).

The same strategy was followed to construct derivatives in which codons 1 and 5 of the L* open reading frame (ORF) are AUG instead of ACG. In this case, the PCR fragment was amplified from plasmid pOV28 (van Eyll & Michiels, 2000).

To replace the L region in pTMDA1 by that of Mengo virus, the mutagenic PCR fragment was obtained by amplifying the L region of Mengo virus from pMC24 (Duke et al., 1990) with primers TM486 and TM487. Mutagenesis was performed on pTM410 and yielded pSPA6. The mutated fragment was subcloned from pSPA6 into pKJ6 to yield pSPA24.

We then constructed a derivative of pSPA14 carrying point mutations in codons −4 and −2 relative to the L/VP4 boundary (TTC→ATG, F→M; and ACA→CCA, T→P) to restore the 3C cleavage site of strain DA1. Therefore, pSPA6 was subjected to divergent PCR mutagenesis (Imai et al., 1991) with Pfu polymerase and primers TM534 and TM536 to yield pSPA20. A fragment containing the Mengo virus L-coding region with the restored 3C cleavage site was cloned back from pSPA20 in pKJ6 to give pSPA24.

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**Table 1. Characteristics of the DA1-derived viruses used in this study**

<table>
<thead>
<tr>
<th>Virus</th>
<th>L</th>
<th>L*</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA1</td>
<td>L&lt;sub&gt;DA&lt;/sub&gt;</td>
<td>wt</td>
<td>wt: Wild-type</td>
</tr>
<tr>
<td>TM598</td>
<td>L&lt;sub&gt;TM598&lt;/sub&gt;</td>
<td>wt</td>
<td>L*: Mutations disrupting the zinc finger of L without affecting L*</td>
</tr>
<tr>
<td>SPA11</td>
<td>L&lt;sub&gt;GDVII&lt;/sub&gt;</td>
<td>L*-ACG</td>
<td>L*-ACG: Codons 1 and 5 of L* (hybrid) are ACGs as in GDVII</td>
</tr>
<tr>
<td>SPA13</td>
<td>L&lt;sub&gt;GDVII&lt;/sub&gt;</td>
<td>L*-AUG</td>
<td>L*-AUG: Codons 1 and 5 of L* (hybrid) are AUGs as in DA1</td>
</tr>
<tr>
<td>SPA10</td>
<td>L&lt;sub&gt;Mengo&lt;/sub&gt;</td>
<td>/</td>
<td>Absence of L* ORF</td>
</tr>
<tr>
<td>SPA11</td>
<td>L&lt;sub&gt;Mengo&lt;/sub&gt;</td>
<td>/</td>
<td>L*: Mutation of SPA24</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Genome organization of chimeric viruses. Chimeric viruses SPA11 and SPA13 were derived from the wild-type DA1 virus. SPA10, SPA12 and SPA24 derive from KJ6 and have a capsid adapted to L929 cells. The 5’ non-coding region (5’NC) is represented by a line and coding regions by frames. Only the 5’ part of the genome is represented. SPA10, SPA11, SPA12 and SPA13 harbour the L-coding region of the neurovirulent GDVII strain (light grey) and thus encode hybrid L* proteins. Codons 1 and 5 of L* are ACG in SPA10/SPA11 (as in GDVII) and AUG in SPA12/SPA13 (as in DA1). SPA24 contains the Mengo virus L-coding region (dark grey). This virus does not encode an L* protein. The arrowhead symbolizes the mutations introduced in L<sub>Mengo</sub>, two and four residues upstream of the L/Vp4 boundary, to restore the 3C cleavage site of the parental DA1 virus. *Capsid adapted to L929 cells.
Finally, the L59 mutation (C19→R, P20→T and C22→R) was introduced in pSPA24 to disrupt the zinc-finger motif of the Mengo virus L in the chimeric construct. Mutagenesis was performed by divergent PCR amplification of pSPA20 with primers TM581 and TM582. The mutated L fragment was cloned in pKJ6 to yield pSPA28.

For all of the constructions, the fragment containing the mutated region that was transferred to full-length cDNA clones was sequenced to ensure that the PCR step did not introduce unexpected mutations. Characteristics of the recombinants are summarized in Table 1 and Fig. 2.

**RNA extraction and RT-PCR.** Total RNA was extracted from infected L929 cells or from mouse spinal cords by the technique of Chomczynski & Sacchi (1987). For reverse transcription, 1 µg DNase I-treated RNA (Shaw-Jackson & Michiels, 1999) was mixed with random hexamers (82 µM) in a volume of 10 µl, denatured for 5 min at 70 °C, cooled on ice and then incubated for 1 h at 42 °C in a total volume of 25 µl with 100 units RevertAid Hminus M-MulLV reverse transcriptase (MBI Fermentas), 500 µM each of the four dNTPs and 20 units RnaseOut (Invitrogen) in the reverse transcription buffer supplied. The reaction was ended by a 2 min step at 95 °C. Control RT-PCRs were performed without reverse transcriptase to rule out the possibility that PCR products were amplified from genomic DNA contamination.

Real-time PCR was performed in a final volume of 25 µl with 5 µl cDNA (diluted 40-fold), each primer at 400 nM and 12.5 µl of a 2× mix mainly prepared with the PCR core kit for SYBR Green I (Eurogentec). Primers used are shown in Table 2. Real-time PCR was conducted on iCycler or MyIQ Real-Time detection systems (Bio-Rad) for 15 s, 60 °C for 1 min) and a melt-curve analysis.

**Immunofluorescence assays.** L929 cells were grown on coverslips and infected with 1 p.f.u. virus per cell. At indicated time points, cells were fixed for 5 min (PTB) or 15–20 min (IFN-3 and VP1) with 4% pafomaldehyde in PBS and permeabilized for 5 min with 0.1% Triton X-100 in PBS.

To visualize IFN-3, cells were treated for 5 min with 0.45% H2O2 in PBS to inhibit endogenous peroxidase, washed in PBS and blocked for 1 h in TNB blocking solution (Perkin Elmer). Cells were incubated successively for 30 min with a rabbit polyclonal antibody (1:200 in TNB) directed against the IFN-3 protein (Zymed; ref. 51-3200), for 30 min with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Dako Cytomation; ref. P0448) diluted 1:200 in TNB and for 10 min in Fluorescein Tyramide amplification reagent (TSA kit; Perkin Elmer). To visualize PTB, cells were blocked for 1 h in TNB and incubated successively for 1 h with a mouse monoclonal anti-PTB antibody (Zymed; ref. 32-4800) diluted 1:50 in TNB and for 1 h with an Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (Molecular Probes; A-11017) diluted 1:400 in TNB. To detect viral antigen, cells were blocked for 1 h in goat serum (1:50 in PBS) and incubated for 1 h with a murine mAb F12B3 directed against the VP1 protein of TMEV (1:100 in a PBS/Twee 0-1% solution) and then for 1 h with an Alexa Fluor 594-conjugated goat anti-mouse immunoglobulin G (Molecular Probes; A-11032) diluted at 1:800 in a PBS/Twee 0-1% solution. Immunofluorescence assays were performed at room temperature. Three washes in PBS were done between all incubation steps. Coverslips were finally washed and mounted with Mowiol [10% [w/v] Mowiol 4-88 (Calbiochem; ref. 475904), 25% (w/v) glycerol, 0-1% (w/v) diazabicyclo-octane (Sigma; ref. D2522) in 100 mM Tris/HCl (pH 8.5)] for fluorescence microscopy.

**Infection of mice.** Three- to four-week-old female FVB/N mice were obtained from the animal facility of the University of Louvain. Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use. TMEV infections were initiated by intracranial injection of 40 µl serum-free medium containing 105 p.f.u. of the indicated virus. Control mice were injected with 40 µl serum-free culture medium.

**Table 2. Sense and antisense primers used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM484 (s)</td>
<td>TATGGACACTATGGGCTTGCAAACA</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>TM485 (as)</td>
<td>TTTCCCCTGGGTTCCATGACA</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>TM486 (s)</td>
<td>ATTACATTGACATAGCTGGCTACAACCATGGA</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>TM487 (as)</td>
<td>CAGATGAGAGGGGTTCTTGGTCTGGAACA</td>
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</tr>
<tr>
<td>TM534 (s)</td>
<td>CATCACACCCTCCATGCAA</td>
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<tr>
<td>TM536 (as)</td>
<td>GAGCCACAAGGAAAACGCTCT</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>TM581 (s)</td>
<td>AAAAGCTGCCGGCCCTTACAATACA</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>TM582 (as)</td>
<td>GTGCCGTTCCTCAAGAGTCTAGGAAT</td>
<td>Mutagenesis</td>
</tr>
<tr>
<td>TM650 (s)</td>
<td>AGCCCTGCGTGACGGGAACC</td>
<td>IFN-α4</td>
</tr>
<tr>
<td>TM651 (as)</td>
<td>CAGCAAGTGGTGACGGAAGAG</td>
<td>IFN-α4</td>
</tr>
<tr>
<td>TM425 (s)</td>
<td>ACTCTCCGCTGCTGTGGCTA</td>
<td>RANTES</td>
</tr>
<tr>
<td>TM426 (as)</td>
<td>CCCACTCTTCTCTGGTGG</td>
<td>RANTES</td>
</tr>
<tr>
<td>TM427 (s)</td>
<td>ATGAAACAGGGTGATCCCTCC</td>
<td>IFN-β</td>
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<tr>
<td>TM428 (as)</td>
<td>AGGAGCTCCTGACATTTCCGAA</td>
<td>IFN-β</td>
</tr>
<tr>
<td>TM421 (s)</td>
<td>AGAGGGGAAATCTGCGTGTAC</td>
<td>β-Actin</td>
</tr>
<tr>
<td>TM422 (as)</td>
<td>CAATAGTGATGACCTGGGCGT</td>
<td>β-Actin</td>
</tr>
</tbody>
</table>

*a*, Sense; *as*, antisense.
†IFN-β primers were from Petro (2005).
RESULTS

Construction of chimeric TMEV viruses

In order to test whether the L proteins of neurovirulent and persistent TMEV strains were functionally interchangeable, we constructed a chimeric virus (called SPA11) in which the L-coding region of the GDVII neurovirulent strain (L<sub>GDVII</sub>) was substituted for that of the DA1 persistent strain (Table 1; Fig. 2). TMEV strains have a unique feature among the picornaviruses in that their genome possesses an alternative ORF encoding a protein called L* (Kong & Roos, 1991; Roos et al., 1989) that is important for viral persistence (Ghadge et al., 1998; van Eyll & Michiels, 2002). The ORF encoding L* overlaps the main ORF in the L–VP4–VP2 regions. Thus, the chimeric DA1 virus harbouring the L-coding region of virus GDVII is expected to code for a hybrid L* protein (Fig. 2). In virus GDVII, the entire L* ORF is conserved, but codons 1 and 5 of the L* ORF are ACG and not AUG as in the DA strain. The presence of these ACG codons was shown to reduce substantially (but not completely) the amount of L* protein produced. Thus, to avoid any bias linked to variation in L* protein expression, we constructed an additional chimeric virus carrying the L-coding region of the GDVII strain, in which the AUG codons 1 and 5 of the L* ORF are restored. This virus was called SPA13.

To study the phenotype of the viruses in L929 cells, we also constructed SPA10 and SPA12, derivatives of SPA11 and SPA13, respectively, that contain capsid mutations known to enhance infection of L929 cells (Jnaoui & Michiels, 1998) (Table 1). Chimeric viruses produced from these clones had titres and plaque size in BHK-21 cells similar to those of their parental viruses (data not shown). Moreover, SPA10 and SPA12 exhibited the same replication kinetics as their parental viruses (data not shown). Chimeric virus SPA11 and SPA13, constructed SPA11 and SPA13, respectively, that contain capsid mutations known to enhance infection of L929 cells (Jnaoui & Michiels, 1998) (Table 1). Chimeric viruses produced from these clones had titres and plaque size in BHK-21 cells similar to those of their parental viruses (data not shown). Moreover, SPA10 and SPA12 exhibited the same replication kinetics as their parental viruses (data not shown).

Inhibition of IFN and RANTES gene transcription and perturbation of nucleocytoplasmic trafficking by the L protein of GDVII

To test whether the L protein of the neuroviral GDVII strain shares, with that of the DA strain, the ability to block the transcription of cytokine and chemokine genes, we compared the expression of IFN-α4, IFN-β and RANTES genes in L929 cells infected for 9 h with 1·5 p.f.u. of viruses KJ6 (L<sub>DA</sub>), SPA10 (L<sub>GDVII/L<sup>−ACG</sup></sub>), SPA12 (L<sub>GDVII/L<sup>−AUG</sup></sub>) and TM659 (L<sub>DA</sub>) per cell (Fig. 3). Immunolabelling of VP1 viral antigen in infected cells confirmed that the proportion of cells infected with the different viruses was similar (typically >90%; data not shown).

Transcription of these cytokine and chemokine genes, analysed by real-time RT-PCR, was upregulated strongly in cells infected with the L<sub>DA</sub> mutant virus (TM659), but not in cells infected with the parental KJ6 virus or in cells infected with the SPA10 and SPA12 viruses that express the L protein of virus GDVII. Under the same conditions, variation between the amounts of viral RNA was <1 C<sub>T</sub> (twofold) (data not shown). These results indicate that the L proteins of neuroviral and persistent TMEV strains share the ability to inhibit the transcription of cytokine and chemokine genes.

The L protein of the DA strain has also been reported to interfere with nucleocytoplasmic trafficking of nuclear and cytoplasmic cellular proteins, such as PTB and IRF-3 (Delhaye et al., 2004). To test whether this function was also conserved for the L<sub>GDVII</sub> protein, we used immunofluorescence to analyse the subcellular distribution of endogenous PTB and IRF-3 proteins in L929 cells infected with the different constructs. Redistribution of PTB and IRF-3 was clear in cells infected for 6 h 30 min with viruses KJ6, SPA10 and SPA12, but not in mock-infected cells or in cells infected with TM659, the L<sup>ACG</sup> mutant of KJ6 (Fig. 4). Again, VP1 immunolabelling, performed 9 h after infection, showed the same proportion of infected cells for TM659 and for the other viruses.

Thus, in vitro, the L protein of virus GDVII displayed all of the functions described for the L protein of the persistent viral strain.

L<sub>GDVII</sub> can substitute functionally for L<sub>DA</sub> during infection of mice

Previous data showed that the L protein is an important persistence determinant of the DA1 virus (van Pesch et al., 2001). Persistence ability involves a subtle interplay between the virus and the host and might require additional, as-yet-undescribed functions of the L protein. We thus analysed whether L<sub>GDVII</sub> could functionally replace the L<sub>DA</sub> protein in vivo. For this purpose, FVB/N mice were infected with 10<sup>5</sup> p.f.u. of either the wild-type DA1 virus, the L<sup>ACG</sup> mutant TM598 or the chimeric viruses SPA11 and SPA13. Viral persistence was examined by comparing, by real-time RT-PCR, the amounts of viral RNA present in the spinal cord of infected mice 45 days after infection (Fig. 5). As expected from previous studies (van Pesch et al., 2001), viral load at that time point was about 10,000 times lower for the L<sup>ACG</sup> mutant, TM598, than for the parental DA1 virus. In contrast, chimeric viruses SPA11 and SPA13 persisted as well as the DA1 virus, showing that the L protein of the neuroviral GDVII strain could functionally replace that of the DA strain. To rule out contamination of the viral stocks or selection of revertant viruses during infections in vivo, we amplified by RT-PCR and sequenced the L-coding regions of the viruses present in the RNA samples prepared from the spinal cords 45 days after infection. In all cases, the identity of the viruses was confirmed.

These data show that the L<sub>DA</sub> and L<sub>GDVII</sub> proteins are functionally interchangeable in vitro and in vivo. Interestingly, both SPA11 and SPA13 chimeric viruses persisted readily in the CNS, in spite of the fact that they expressed hybrid L* proteins. Furthermore, these viruses persisted...
irrespective of whether they had ACG or AUG codons to initiate translation of the L* ORF.

**Construction of chimeric TMEV viruses expressing the L protein of Mengo virus**

We next tested whether the L proteins of TMEV and EMCV/Mengo virus could differ in their ability to block transcription of cytokine and chemokine genes and to cause nucleocytoplasmic-trafficking perturbation. Therefore, we constructed a chimeric virus derived from KJ6 (DA1 virus adapted to L929 cells) in which the L-coding region was replaced by that of Mengo virus. However, the chimeric virus obtained, called SPA14, failed to produce detectable plaques in BHK-21 cells. *In vitro* translation studies suggested a defect of polyprotein processing by viral protease 3C at the level of the L/VP4 boundary (data not shown). To circumvent this problem, we constructed a new recombinant possessing the L-coding region of Mengo virus in which amino acids −4 and −2 relative to the L/VP4 boundary were converted back to those found in the parental TMEV sequence. This virus, called SPA24 (Fig. 2), was infectious and produced plaques similar in size to those of KJ6. A derivative of SPA24 bearing the L57* mutation in the Mengo virus L protein was constructed and called SPA28 (Table 1).

**L proteins from TMEV and Mengo virus share the ability to inhibit transcription of IFN genes and to promote nucleocytoplasmic redistribution of proteins**

We compared the influence of the L proteins of the TMEV DA strain and of Mengo virus on the transcription of the IFN-α4, IFN-β and RANTES genes. Therefore, L929 cells were infected for 9 h with KJ6 (LDA) and SPA24 (LMengo) and with the corresponding L57* mutants (TM659 and SPA28, respectively). *In vitro* translation studies suggested a defect of polyprotein processing by viral protease 3C at the level of the L/VP4 boundary. Data presented in Fig. 3 show a strong transcriptional upregulation of these genes in cells infected with the TM659 and SPA28 L57* mutants. In contrast, both the parental KJ6 virus and the chimeric SPA24 virus expressing the L protein of Mengo virus abrogated the transcriptional activation of these genes almost completely.

We further tested whether the L protein of Mengo virus, like that of TMEV, could interfere with nucleocytoplasmic trafficking of cellular proteins. Therefore, we performed immunofluorescence assays with anti-PTB and anti-IRF-3 antibodies on cells infected for 6 h 30 min with KJ6, TM659, SPA24 and SPA28 (Fig. 4). At that time point, the L protein of Mengo virus was as efficient as that of the DA1 virus to trigger subcellular redistribution of the nuclear protein PTB and of the cytoplasmic protein IRF-3. The L57* mutation suppressed this effect for both the TMEV and the Mengo virus L proteins.

Thus, in spite of the sequence divergences found between the cardiovirus L proteins, these proteins shared the ability to inhibit the transcription of cytokine and chemokine genes and to trigger nucleocytoplasmic redistribution of host-cell proteins.

**Impaired replication of the recombinant virus expressing the Mengo virus L protein**

Real-time RT-PCR was performed to compare viral RNA replication of the recombinants in infected L929 cells (Fig. 6). We observed that replication of SPA24, the
recombinant expressing the Mengo virus L protein, was far less efficient (about 9.5 and 36 times, respectively) at 6 and 9 h post-infection than that of KJ6. In contrast, SPA28, which expresses the Mengo virus L597 mutant protein, replicated even slightly better than KJ6. This suggests that the replication defect observed for SPA24 results from the activity of the Mengo virus L protein and does not merely reflect a defect in the processing of the chimeric polyprotein.

We did not detect such a difference of replication efficiency between KJ6 and the chimeric viruses expressing the L protein of GDVII (data not shown). However, we observed a modest (two to eight times) and transient, but reproducible, difference between the replication levels of KJ6 and the corresponding Lcys mutant (TM659). The virus expressing a mutated L protein transiently replicated faster than the wild-type virus ($P < 0.01$ in a paired $t$-test done for five independent infection experiments).

**Cardiovirus L proteins are functionally interchangeable**

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**Fig. 4.** L proteins of TMEV and EMCV/Mengo virus perturb the nucleocytoplasmic trafficking of cellular proteins. L929 cells were infected with 1 p.f.u. KJ6, TM659, SPA10, SPA12, SPA24 or SPA28 per cell or were mock-infected. IRF-3 and PTB were detected by immunofluorescence 6 h 30 min after infection. (a) IRF-3 immunolabelling: IRF-3 is a cytoplasmic protein in mock-infected cells and is partially redistributed (white arrows) to the nucleus in cells infected with viruses expressing wild-type L proteins from DA (KJ6), GDVII (SPA10 and SPA12) and Mengo virus (SPA24), but not in cells infected with LDA5 or L597 Mengo mutant viruses (TM659 and SPA28). (b) PTB immunolabelling: PTB is a nuclear protein in mock-infected cells. The destruction of the zinc finger of the L protein in TM659 and SPA28 prevents the redistribution of PTB from the nucleus to the cytoplasm (white arrows) observed in cells infected with KJ6, SPA10, SPA12 or SPA24.

**Fig. 5.** Persistence in the CNS of TMEV strains expressing LGDVII. Real-time comparative PCR was used to compare the amounts of viral RNA present in the spinal cord of FVB/N mice (groups of four mice) infected for 45 days with DA1, SPA11 and SPA13 or with the L597 mutant TM598. RNA levels detected were normalized to the amounts of $\beta$-actin for each sample. Histograms show the mean±sd of relative cycle-threshold differences ($D_{CT}$). Corresponding copy-number ratios between the L597 mutant and wild-type virus are indicated.
It is noteworthy that recombinants carrying the LGDVII-neurovirulent strain yet-undescribed functions, are shared by the L protein of the DA L protein, possibly including as-yet-unknown functions that were not tested here. Our study shows that the L protein of a neurovirulent TMEV strain can functionally replace that of the DA strain in vitro. Time comparative RT-PCR, were normalized to the amounts of β-actin for each sample. Histograms show the mean ± SD of relative cycle-threshold differences (ΔCt). Ratios between (i) KJ6 and TM659 RNA levels and (ii) SPA24 and SPA28 RNA levels are indicated. Graphs show the data from one experiment performed in triplicate. Several independent experiments done with at least two different virus productions confirmed these data.

DISCUSSION

L proteins of TMEV strains
Our study shows that the L protein of a neurovirulent TMEV strain in functionally replace that of the DA strain in vitro and in vivo. The fact that the recombinant virus carrying the L protein from the neurovirulent strain can persist in the CNS of infected mice suggests strongly that all of the functions of the DA L protein, possibly including as-yet-undescribed functions, are shared by the L protein of the neurovirulent strain.

It is noteworthy that recombinants carrying the LGDVII coding region persisted in the CNS of infected mice, despite the fact that they express a hybrid L* protein (Fig. 2). Protein L* was reported to enhance macrophage infection in vitro (Ghadge et al., 1998; Takata et al., 1998; van Eyll & Michiels, 2000). The primary role of L* in vivo has not yet been elucidated, but this protein was shown to be an important persistence determinant of the virus. The fact that SPA11 and SPA13 persisted readily in the CNS of infected mice shows that at least the N-terminal part of the L* protein is functionally interchangeable between neurovirulent and persistent TMEV strains. In addition, persistence of SPA11, which has an L* ORF starting with ACG codons instead of AUG codons, confirms our previous observation suggesting that L* can be expressed from the ACG codon at low but sufficient levels for function (van Eyll & Michiels, 2002).

L proteins of cardioviruses share the property to block transcriptional activation of cytokine and chemokine genes and to trigger nucleocytoplasmic redistribution of host proteins

In spite of sequence differences, our data show that the L protein of Mengo virus shares, with that of TMEV, the ability to block transcriptional upregulation of IFN-α, IFN-β and RANTES genes. The role played by the Mengo virus L protein to counteract the innate immune response had already been described and was suggested to take place at the translational level. Here, we show that this function takes place at the transcriptional level. Our work shows that the EMCV/Mengo virus L protein also causes early redistribution of nuclear and cytoplasmic cellular proteins. Whether transcriptional inhibition of cytokine genes and nucleocytoplasmic inhibition are linked or independent activities of the L proteins is not known. However, our data show that point mutations disrupting the zinc finger of the L protein affect both activities. Influence of the Mengo virus L on the phenotype of TMEV could not be tested in vivo, due to the absence of the L* ORF in the recombinant. Our data do not rule out the possibility that the L proteins of TMEV and EMCV/Mengo virus differ in as-yet-unknown functions that were not tested here.

Influence of cardiovirus L proteins on viral RNA replication
We observed that the replication level of the L575 TMEV mutant in L929 cells was slightly but reproducibly higher than that of the wild-type virus. The difference was transient and most apparent at 6 h post-infection. Our interpretation is that, by interfering with host-gene transcription and/or with nucleocytoplasmic trafficking, the L protein interferes with a host function that participates in virus replication. This detrimental effect of L is probably largely compensated, in the long run, by the beneficial effects resulting from IFN antagonism and other functions of L.

The SPA24 virus expressing the Mengo virus L protein had a strong deficit in virus replication compared with the parental KJ6 virus (about 50-fold less viral RNA 9 h after infection). Yet, influence of this recombinant on PTB trafficking was faster than that of KJ6. This suggests that the L protein of Mengo virus might be more active than that of TMEV, in agreement with the fact that replication of Mengo virus is faster than that of TMEV. Thus, there would be a co-evolution between L protein efficacy and virus replication kinetics. In the case of the TMEV recombinant expressing the Mengo virus L, activity of L would be detrimental to virus replication by acting too strongly or too early. In contrast, SPA28, which contains the L575 mutation in the Mengo virus L protein, replicated as well as, or even better than, KJ6.

Thus, our observations suggest that the L proteins of cardioviruses evolved to adapt to the different replication fitnesses of these viruses.
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