

Nonstructural Protein L* Species Specificity Supports a Mouse Origin for Vilyuisk Human Encephalitis Virus

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ABSTRACT Vilyuisk human encephalitis virus (VHEV) is a picornavirus related to Theiler's murine encephalomyelitis virus (TMEV). VHEV was isolated from human material passaged in mice. Whether this VHEV is of human or mouse origin is therefore unclear. We took advantage of the species-specific activity of the nonstructural L* protein of theiloviruses to track the origin of TMEV isolates. TMEV L* inhibits RNase L, the effector enzyme of the interferon pathway. By using coimmunoprecipitation and functional RNase L assays, the species specificity of RNase L antagonism was tested for L* from mouse (DA) and rat (RTV-1) TMEV strains as well as for VHEV. Coimmunoprecipitation and functional assay data confirmed the species specificity of L* activity and showed that L* from rat strain RTV-1 inhibited rat but not mouse or human RNase L. Next, we showed that the VHEV L* protein was phylogenetically related to L* of mouse viruses and that it failed to inhibit human RNase L but readily antagonized mouse RNase L, unambiguously showing the mouse origin of VHEV.

IMPORTANCE Defining the natural host of a virus can be a thorny issue, especially when the virus was isolated only once or when the isolation story is complex. The species *Theilovirus* includes Theiler's murine encephalomyelitis virus (TMEV), infecting mice and rats, and Saffold virus (SAFV), infecting humans. One TMEV strain, Vilyuisk human encephalitis virus (VHEV), however, was isolated from mice that were inoculated with cerebrospinal fluid of a patient presenting with chronic encephalitis. It is therefore unclear whether VHEV was derived from the human sample or from the inoculated mouse. The L* protein encoded by TMEV inhibits RNase L, a cellular enzyme involved in innate immunity, in a species-specific manner. Using binding and functional assays, we show that this species specificity even allows discrimination between TMEV strains of mouse and of rat origins. The VHEV L* protein clearly inhibited mouse but not human RNase L, indicating that this virus originates from mice.

KEYWORDS Theiler's murine encephalomyelitis virus, Vilyuisk human encephalitis virus, L* protein, mitochondria, RNase L, species specificity, natural host

Defining the natural host species of a virus is often hampered by the fact that an evolutionarily well-adapted virus tends to lose virulence toward its natural host (1, 2). In the process of coevolution, many DNA viruses integrated host genes into their own genome. This feature can provide hints to trace the original host species of a given virus (3). On the other hand, RNA viruses have a limited coding capacity and therefore do not generally integrate host-derived genes. In this case, host prediction can be aided by phylogenetic proximity to viruses of known species origin. In addition, species-specific activities of certain viral proteins can help to identify the original host. For instance, Theiler's murine encephalomyelitis virus (TMEV) (or Theiler's virus) encodes the accessory L* protein that inhibits host RNase L, a well-characterized effector enzyme of the interferon pathway (4, 5), through direct protein-protein interactions (6). Remarkably,

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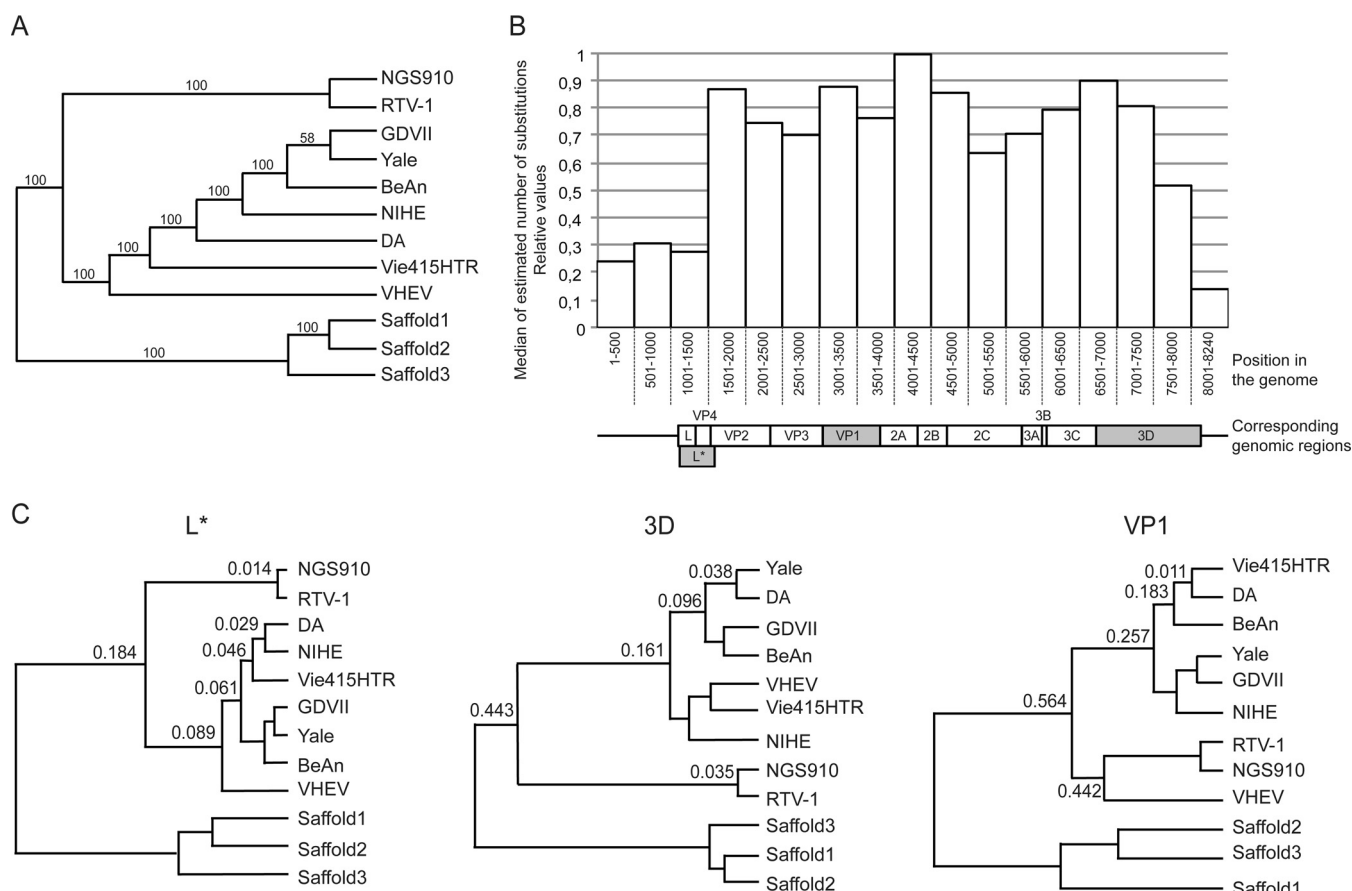


FIG 1 Phylogenetic analysis of VHEV and TMEV isolates. (A) Phylogenetic analysis of full-length TMEV genomic sequences from rat strains (NGS910 and RTV-1), mouse strains (GDVII, Yale, BeAn, NIHE, DA, and Vie415HTR), and VHEV. Human Saffold virus sequences of genotype 1, 2, and 3 isolates were used as an outgroup. Numbers refer to node confidence (clade credibility values). (B) Relative mutation rate as a function of the position in the genome. The corresponding genomic regions are represented below the graph. (C) Relative mutation rate of individual cDNA sequences coding for the L*, 3D, and VP1 proteins. Relative mutation rates are indicated at representative nodes.

this protein acts in a species-specific fashion, as L* from the DA strain of TMEV, which was isolated from mice, inhibits mouse but not human RNase L or its orthologues from other species such as chicken, horse, dog, or even rat (6).

We took advantage of this remarkable species specificity of L* to further characterize another member of the *Theilovirus* species whose origin is uncertain: Vilyuisk human encephalitis virus (VHEV). This virus was isolated as a putative causative agent of endemic encephalitis episodes occurring in the Yakuts population near the Vilyui river in Siberia (7). VHEV was recovered from a mouse inoculated with cerebrospinal fluid of a patient presenting with chronic encephalitis. However, whether the virus originated from the human sample or was a contaminant of the inoculated animal used to recover the virus is still an open question (7).

RESULTS

VHEV L* is phylogenetically related to L* of mouse viruses. We first subjected both genomic and polyprotein sequences of VHEV and rodent theiloviruses to phylogenetic analysis. The protein tree is identical to the very robust genomic tree shown in Fig. 1A. These trees show a clear separation of the rat and mouse clades, where the sequence of VHEV is monophyletic with those of the mouse viruses. Significant differences in evolutionary rates between the different genes were observed. The lowest nucleotide substitution rate was measured for L* and the overlapping open reading frame (ORF), while higher rates were observed for the VP1 region, the 2A region, and part of the 3D region (Fig. 1B). In good agreement with a previous analysis by Liang

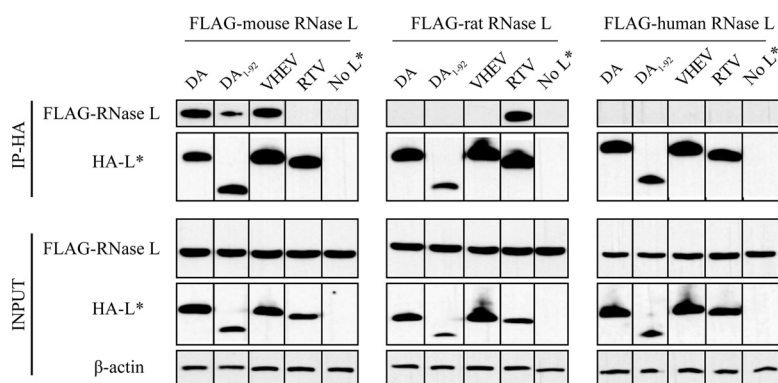


FIG 2 Species-specific interaction between L* and RNase L. Shown are data from coimmunoprecipitation of FLAG-tagged mouse (left), rat (middle), and human (right) RNase L proteins with HA-tagged L* proteins from TMEV strain DA (mouse), RTV-1 (rat), or VHEV. DA₁₋₉₂ L* is a truncated L* protein expressed from a gene with a stop codon introduced at codon 93. An empty vector that did not express L* (No L*) was used as a negative control. Top panels show FLAG and HA detection after immunoprecipitation (IP) of HA. Bottom panels show the detection of HA, FLAG, and β-actin in cell lysates (Input). Reproducible results were obtained in 3 independent experiments.

et al. (8), individual gene trees (Fig. 1C) show that L* and 3D cDNAs of VHEV cluster with the mouse clade, whereas the VP1 protein of VHEV considerably diverges from the mouse clade and clusters with the rat VP1s. Relative nucleotide substitution rates as a function of the position in the genome were measured by cutting the 12 aligned genomes in blocks of 500 nucleotides. For each of the resulting 17 subalignments, the corresponding phylogenetic tree was inferred, and the relative substitution rate was calculated (Fig. 1B). Due to an additional constraint by having overlapping L and L* genes, the mutation rate in this part of the virus genome has been decreased considerably.

Specificity of L*-RNase L interactions. We next aimed to study the functional species specificity of L* proteins from various TMEV isolates, including VHEV and RTV-1, a strain isolated from rats. For this purpose, L*-coding regions from these strains and from mouse strain DA were cloned into pTM945, a lentiviral vector allowing the coexpression of the cloned gene with that of mCherry. L*-coding sequences were also tagged with an N-terminal hemagglutinin (HA) epitope and subcloned into pTM898, a lentiviral vector allowing the coexpression of the cloned gene with the Neo resistance gene.

Rat RNase L cDNA was cloned from rat embryos by using Gateway technology and transferred to the pcDNA3 expression vector. It was then expressed in transfected HeLa-M cells, and the activity of the RNase was verified by 2'-5' oligoadenylates (2-5A) transfection and analysis of RNA degradation (not shown).

For interaction studies, HA-L* and FLAG-RNase L were coexpressed in 293T cells by transfection of the plasmid constructs. After cell lysis, HA-L* was immunoprecipitated by using an anti-HA antibody, and FLAG-RNase L was detected by Western blotting using an anti-FLAG antibody (Fig. 2). As expected, L* of DA readily interacted with mouse RNase L but not with either human or rat RNase L. A truncated DA L* carrying a stop codon at position 93 (DA-L*₁₋₉₂) displayed a weak interaction. L* of RTV-1 interacted only with rat RNase L, confirming the rat origin of this strain. No cross-species interaction occurred, even between mouse and rat. VHEV L*, like DA L*, interacted with mouse RNase L but not with rat or human RNase L.

VHEV L* inhibits overexpressed mouse but not human RNase L. The functional inhibition of RNase L activity by L* was studied in transfected HeLa-M cells, which have minimal endogenous (human) RNase L activity (9). Cells were cotransfected with a plasmid coexpressing L* and mCherry and with a plasmid expressing L* and Neo in a 9-to-1 ratio so that most cells expressing RNase L would also express L*. Cells transfected with the RNase L expression plasmid were selected for 2 to 3 days with 1 mg per

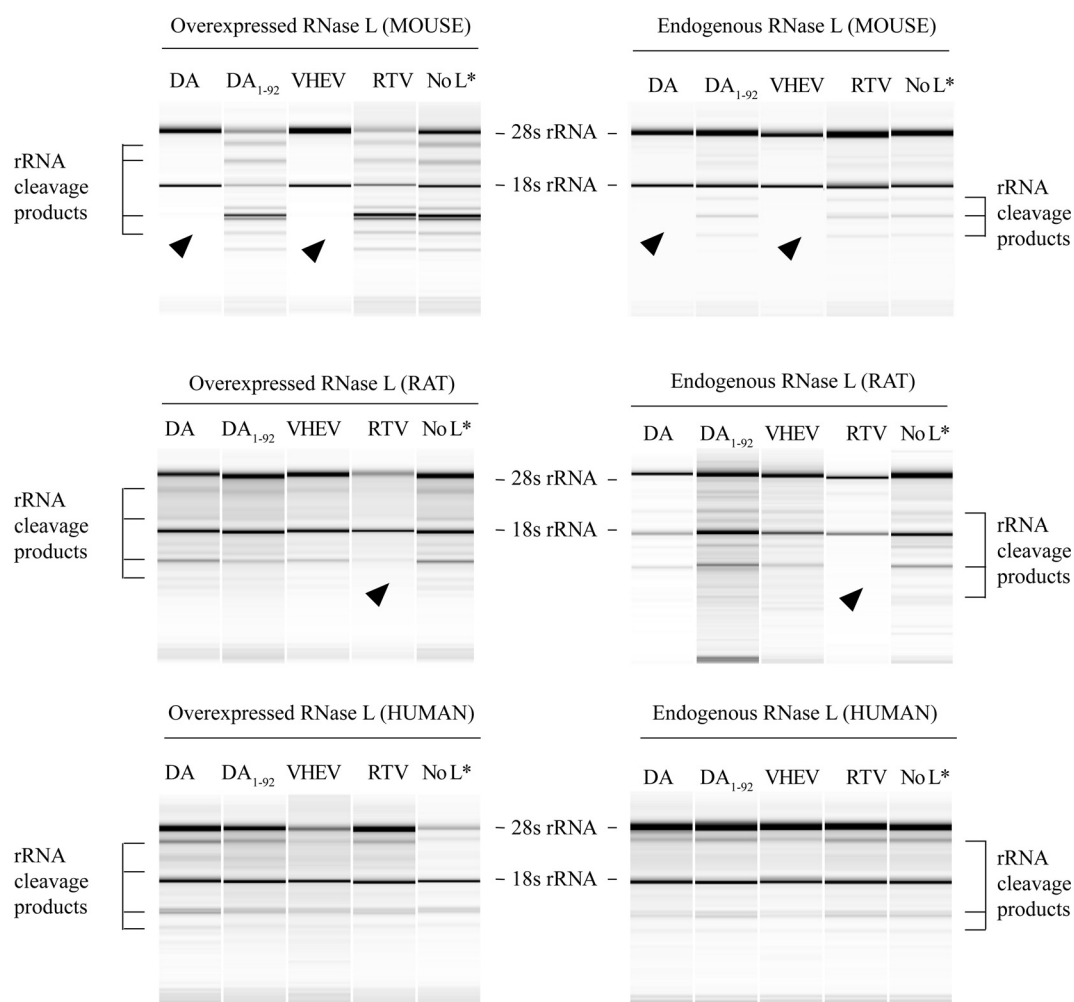


FIG 3 Inhibition of species-specific RNase L activity by L*. Analysis of RNase L-mediated RNA degradation was performed by capillary electrophoresis. RNA samples were extracted 7 h after 2-5A transfection. (Left) Data obtained after the overexpression of the indicated RNase L and L* proteins in HeLa-M cells. Similar data were obtained in 4 independent experiments. (Right) Data obtained after endogenous RNase L stimulation in L929 (mouse), NR8383 (rat), and HeLa (human) cells transduced with lentiviruses expressing the indicated L* proteins. Arrowheads point to samples where L* inhibited RNase L-mediated RNA degradation. Reproducible results were obtained from 3 independent experiments where RNase L was activated by either poly(I:C) ($n = 2$) or 2-5A ($n = 1$) transfection.

ml of G418, and flow cytometry analysis of mCherry fluorescence showed that more than 90% of selected RNase L-expressing cells also expressed L*. RNase L was then activated by the transfection of 2-5A. At 7 h posttransfection, total cell RNA was extracted and analyzed on RNA chips for RNA degradation (Fig. 3, left). RNase L activity inhibition correlated nicely with coimmunoprecipitation data, except for truncated DA L*₁₋₉₂, which retained some interaction but lost inhibitory potential. These data confirmed the species specificity of L*: the rat specificity of RTV-1 L* and the mouse specificity of VHEV L*.

Antagonism of endogenous RNase L. We next confirmed the antagonism of L* toward endogenously expressed RNase L. Therefore, human HeLa, mouse L929, and rat NR8383 cell populations were transduced and selected with G418 to stably express the HA-L* constructs. Confocal microscopy analysis showed that almost all cells expressed HA-L*, as expected, and that the VHEV and RTV-1 L* proteins partly colocalized with mitochondria, as was previously shown for DA L* (10, 11) (Fig. 4).

Transduced cells expressing L* were then transfected by 2-5A as described above. At 7 h posttransfection, total cell RNA was extracted and analyzed on RNA chips for rRNA degradation (Fig. 3, right). As in the overexpression experiment, L* proteins displayed

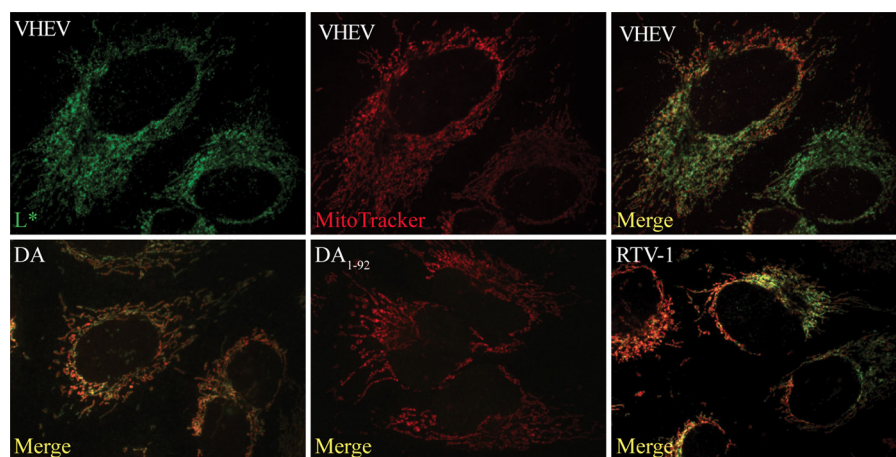


FIG 4 Mitochondrial localization of L* from VHEV and TMEV isolates in HeLa cells. HA-L* proteins from the indicated viruses were detected in transduced HeLa cells by immunofluorescence (green) using an anti-HA antibody. Mitochondria were labeled by incubating cells for 45 min with MitoTracker before processing for immunolabeling. (Top) L* immunofluorescence, MitoTracker, and merged confocal images for VHEV. (Bottom) Merged confocal images for L* from DA, DA₁₋₉₂ (not detectable in mitochondria), and RTV-1. Similar L* colocalizations with mitochondria were observed in L929 and NR8383 cell populations (not shown).

a strong species specificity, with RTV-1 L* being specific for rat RNase L and VHEV L* being specific for mouse RNase L.

DISCUSSION

Taken together, our data confirm the exquisite species specificity of TMEV L* protein activity. These data draw attention to the importance of studying the activity of nonstructural viral proteins in a homologous host-pathogen context. Our data confirm the rat origin of the TMEV RTV-1 strain and underline the striking functional divergence that occurred in the evolution of rat and mouse TMEV clades. In the case of VHEV, L* specificity unambiguously shows that the virus originates from mouse and suggests that this virus was a contaminant of mice that were inoculated with human samples, at the time of virus isolation. However, given the surprisingly divergent VP1 protein sequence of this virus, a recent cross-species jump from mouse to human cannot be totally excluded, even if VHEV VP1 does not cluster with VP1 of Saffold virus, the only documented human *Theilovirus*.

MATERIALS AND METHODS

Phylogenetic analysis. Multiple alignments were obtained by using ClustalX 2.1 (12) and saved in Nexus format. Phylogenetic analyses were carried out by Bayesian analysis using MrBAYES 3.2 (13). The general time reversible (GTR) model of evolution (14) was used for nucleotide sequences, and the Jones, Taylor, and Thornton (JTT) model of evolution (15) was used for protein sequences. To estimate Bayesian posterior probabilities, Markov chain Monte Carlo (MCMC) chains were run for 100,000 generations and sampled every 100 generations (burn-in, 25,000 generations). Nucleotide substitution rates of theiloviruses as a function of the position in the genome were calculated by cutting the 12 aligned genomes into blocks of 500 nucleotides using the SlidingBayes program (<https://rega.kuleuven.be/cev/avd/software/slidingbayes>) (16). For each of the resulting 17 subalignments, a phylogenetic tree under the relaxed-clock model was inferred, and the relative substitution rate from the time of the bifurcation of mouse and rat to the tips of the tree was calculated. Sequences were from either rat strains (NGS910 [GenBank accession no. [AB090161.1](#)] and RTV-1 [accession no. [EU542581.1](#)]), mouse strains (GDVII [accession no. [X56019.1](#)], Yale [accession no. [EU723238.1](#)], BeAn [accession no. [M16020.1](#)], NIHE [accession no. [HQ652539.1](#)], DA [accession no. [JX443418.1](#)], and Vie415HTR [accession no. [EU718733.1](#)]), VHEV (assembly of accession no. [M80888](#), [M94868](#), and [EU723237](#)), or human theiloviruses known as Saffold virus (Saffold1 [accession no. [NC_009448.2](#)], Saffold2 [accession no. [EU681176.2](#)], and Saffold3 [accession no. [EU681178.2](#)]).

Cells. 293T (17), L929 (ECACC), HeLa (ATCC CCL2), and HeLa-M (9) cells were cultured in Dulbecco modified Eagle medium (catalogue no. BE12-604F; Lonza) containing 4.5 g/liter glucose and L-glutamine and supplemented with 10% fetal calf serum (FCS). NR8383 cells (ATCC) were cultured in Ham's F-12K medium (catalogue no. 21127; Gibco) supplemented with 15% fetal calf serum and 1% minimal essential medium (MEM) nonessential amino acids (catalogue no. 11140; Gibco). BHK-21 cells (ATCC) were cultured

TABLE 1 Expression plasmids used in this study^a

Vector	Tag	Expressed protein(s), species, or particularity	Type (parental vector)
L*-expressing vectors			
pMD01		L*-DA (IRES-mCherry)	Lentiviral (pTM945)
pMD09		L*-DA ₁₋₉₂ stop codon 93 (IRES-mCherry)	Lentiviral (pTM945)
pMD05		L*-Vilyuisk (IRES-mCherry)	Lentiviral (pTM945)
pMD06		L*-RTV-1 (IRES-mCherry)	Lentiviral (pTM945)
pMD11	N-term HA	L*-DA (IRES-Neo)	Lentiviral (pTM898)
pMD12	N-term HA	L*-DA ₁₋₉₂ stop codon 93 (IRES-Neo)	Lentiviral (pTM898)
pMD14	N-term HA	L*-Vilyuisk (IRES-Neo)	Lentiviral (pTM898)
pMD15	N-term HA	L*-RTV-1 (IRES-Neo)	Lentiviral (pTM898)
RNase L-expressing vectors			
pFS164		RNase L-Mouse	Plasmid (pcDNA3)
pMD22		RNase L-Rat	Plasmid (pcDNA3)
p-huRNaseL		RNase L-Human	Plasmid (pcDNA3)
pFS165	N-term FLAG	RNase L-Mouse	Plasmid (pcDNA3)
pMD27	N-term FLAG	RNase L-Rat	Plasmid (pcDNA3)
pFS183	N-term FLAG	RNase L-Human	Plasmid (pcDNA3)
Empty vectors			
pTM898		IRES-Neo	Lentiviral (pcDNA3)
pTM945		IRES-mCherry	Lentiviral (pcDNA3)

^aAll constructs allowed expression of the Neo (G418/Geneticin) resistance gene. N-term, N-terminal; IRES, internal ribosome entry site.

in Glasgow's minimum essential medium (catalogue no. 11710035; Gibco, Thermo Fisher Scientific) supplemented with 10% newborn calf serum and 2.95 g/liter tryptose phosphate broth. All media were supplemented with 100 U/ml penicillin and 10 µg/ml streptomycin.

Vectors. Expression vectors used in this study are listed in Table 1. Plasmids used for mouse and human RNase L expression were described previously by Sorgeloos et al. (6). L*-coding sequences from VHEV and RTV-1 (rat strain) were subcloned from a synthetic plasmid ordered based on sequences in GenBank (accession no. [M94868](#) and [EU542581.1](#)). Wild-type (WT) and truncated (L*₁₋₉₂) L*-coding regions of virus strain DA1 (mouse strain) were subcloned from preexisting constructs (18).

The lentiviral vectors pMD01, pMD05, pMD06, and pMD09 were obtained by cloning the L*-coding sequences between the BamHI and XbaI sites of pTM945, a lentiviral vector allowing the coexpression of the cloned gene with that of mCherry (19).

The lentiviral vectors pMD11, pMD12, pMD14, and pMD15 were obtained by PCR cloning of L*-coding sequences in pTM898 (6), a derivative of pCCL.sin.cPPT.hPGK.GFP.pre allowing the coexpression of the cloned gene with the Neo resistance gene (6). In these constructs, the PCR primers added a region coding for an N-terminal HA epitope (YPYDVPDYA) connected to the second residue of L* via an LLVST linker. Viral particles were pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV-G). Transduced cells were selected with G418 (1 mg/ml), and transgene expression was verified by indirect immunofluorescence and immunoblot analyses.

Rat RNase L cDNA cloning. Rat RNase L cDNA was cloned into pDONR207 from rat embryo samples (kindly provided by J. N. Octave, Institute for Neuroscience, Université Catholique de Louvain [UCL], Brussels, Belgium) using Gateway technology. The sequence of the cloned rat RNase L gene fully matched the genomic sequence (GenBank accession no. [AC_000081.1](#) [Sprague-Dawley strain]). Rat RNase L cDNA was transferred to pTM977, a pcDNA3 derivative carrying a Gateway cassette, yielding pMD22. RNase L was then flanked with an N-terminal FLAG epitope by PCR cloning using a primer containing the FLAG epitope-coding sequence. The plasmid expressing FLAG-RNase L was called pMD27. Both untagged and FLAG-tagged RNase L clones were active as, when they were expressed in HeLa-M cells, they triggered RNA degradation upon 2-5A transfection (not shown).

Transfections. Plasmid DNA was transfected by using the TransIT LT-1 transfection reagent according to the manufacturer's protocols (catalogue no. 11668019; Mirus).

For poly(I:C) and 2-5A transfections, cells plated into 24-well plates were transfected with 0.3 µg/ml poly(I:C) (Amersham-Pharmacia) or 2-5A (10 to 40 µM equivalent ATP, kindly provided by R. Silverman) using 2 µl Lipofectamine 2000 transfection reagent according to the manufacturer's protocols (catalogue no. 11668019; Invitrogen).

Flow cytometry. Cells were dissociated with trypsin-EDTA and suspended in phosphate-buffered saline (PBS) containing 5% FCS and 0.5% paraformaldehyde. Data acquisition was done with an LSR Fortessa flow cytometer (BD Bioscience) using FACSDiva software.

RNA isolation and RNA chips. Total cell RNA was isolated according to methods reported previously by Chomczynski and Sacchi (20). RNA degradation was assessed by running RNA samples on RNA nano 6000 microfluidics chips run on a 2100 bioanalyzer (Agilent Technologies).

Coimmunoprecipitation assays. Coimmunoprecipitation assays were conducted on transfected 293T cells, as previously described (6), except that protein A/G magnetic beads (catalogue no. 88803; Pierce) were used instead of A/G Ultralink resin. HA-L* proteins were immunoprecipitated with anti-HA antibody (catalogue no. MMS101-P; Covance), and immunoprecipitated proteins were detected by using SDS-PAGE and immunoblot analysis with anti-HA (catalogue no. MMS101-P; Covance) and anti-FLAG (catalogue no. M8823; Sigma-Aldrich) antibodies. As a control, the total cell lysate corresponding to 10% of the input used for immunoprecipitation was also analyzed by immunoblotting.

Immunofluorescence. Cells were grown on poly-L-lysine-coated coverslips. For mitochondrial staining, cells were incubated for 45 min, before fixation, with 200 nM MitoTracker Red CMXRos (catalogue no. M7512; Molecular Probes). Cells were fixed for 5 min with 4% paraformaldehyde in PBS and permeabilized for 5 min with 0.1% Triton X-100 in PBS. Unspecific antigens were then blocked for 1 h by using Tris-NaCl blocking buffer (TNB) (Perkin-Elmer). Cells were then incubated for 1 h with anti-HA primary antibody (catalogue no. MMS-101P; Covance) at a dilution of 1:500 in TNB. Cells were intensively washed with 0.01% Tween 20 in PBS and incubated for 1 h with species-matched Alexa Fluor-conjugated secondary antibodies (catalogue no. A-11029; Invitrogen) at a dilution of 1:400 in TNB. Coverslips were mounted onto slides with Mowiol and analyzed by fluorescence microscopy using a spinning-disk confocal microscope equipped with an AxioCam MRm camera (Zeiss). The intensity, contrast, and color balance of images were evenly equilibrated by using Zen (Zeiss).

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