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Rescue of the prototypic Arenavirus LCMV entirely from plasmid

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Abstract

We document a helper-independent reverse genetics system for rescuing infectious arenaviruses from cloned cDNAs. We constructed plasmids containing full-length cDNAs of the antigenomic (ag) L and S segments of the Armstrong (ARM) strain of the prototypic Arenavirus lymphocytic choriomeningitis virus (LCMV) flanked at their 5′- and 3′-termini by the T7 RNA polymerase (T7RP) promoter and ribozyme sequences, respectively. These plasmids directed intracellular synthesis of viral L and S ag RNA species in cells expressing plasmid-supplied T7RP. Co-expression of plasmid-supplied LCMV trans-acting factors, nucleoprotein (NP) and polymerase (L), resulted in replication and expression of L and S ag and genome RNA species, and generation of LCMV infectious progeny termed rT7/LCMV. The recombinant rT7/LCMV was unequivocally identified based on a genetic tag introduced in the recombinant S segment. In addition, rT7/LCMV exhibited growth and biological properties predicted for an ARM-like LCMV. To our knowledge, this is the first documented Arenavirus rescue, as well as of an ambisense negative strand (NS) RNA virus, entirely from cloned cDNAs. Our results extend the use of reverse genetic approaches for DNA-mediated virus rescue to all known virus families with NS RNA genome.
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Keywords: Rescue; Lymphocytic choriomeningitis virus (LCMV); Arenavirus; Reverse genetics

Introduction

Arenaviruses merit significant attention both as tractable model systems to study acute and persistent viral infections,(de la Torre and Oldstone, 1996; Oldstone, 2002; Zinkernagel, 2002) and as clinically important human pathogens including several causative agents of severe, often lethal, hemorrhagic fever (HF), like Lassa fever virus (LFV) (Buchmeier et al., 2001; Geisbert and Jahrling, 2004; McCormick and Fisher-Hoch, 2002; Peters, 2002). In addition, mounting evidence indicates that the prototypic Arenavirus lymphocytic choriomeningitis virus (LCMV) might be a neglected human pathogen of clinical significance (Buchmeier and Zajac, 1999; Jahrling and Peters, 1992). Therefore, it is important to develop better antiviral strategies to combat pathogenic arenaviruses, a task that would be facilitated by a better understanding of the Arenavirus molecular and cell biology.

LCMV is an enveloped virus with a bisegmented negative single-stranded RNA genome and a life cycle restricted to the cell cytoplasm (Buchmeier and Zajac, 1999; Buchmeier et al., 2001; Meyer et al., 2002; Salvato, 1993; Southern, 1996). Each genomic RNA segment, L (ca 7.3 kb) and S (ca 3.4 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by an intergenic region (IGR) with a predicted folding of a stable hairpin structure. The S RNA encodes the viral glycoprotein precursor, GPC, (ca 75 kDa) and the nucleoprotein, NP, (ca 63 kDa), whereas the L RNA encodes the viral RNA dependent RNA polymerase (RdRp, or L polymerase) (ca 200 kDa), and a small RING finger protein Z (ca 11 kDa). The NP and L coding regions are transcribed into a genomic complementary mRNA, whereas the GPC and Z coding regions are translated from genomic sense mRNAs that are transcribed using as templates the corresponding antigenome RNA species. NP is the most abundant viral protein and encapsidates viral genomes and antigenomic replicative intermediates. The viral glycoprotein precursor GPC is posttranslationally cleaved by the SKI-1/S1P cellular protease to yield the two mature virion glycoproteins GP-1 (40–46 kDa) and GP-2 (35 kDa) (Beyer et al., 2003; Pinschewer et al., 2003a, 2003b). Correct processing of GPC is required for

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the production of infectious virions (Beyer et al., 2003; Kunz et al., 2003; Pinschewer et al., 2003a, 2003b), which bud from the plasma membrane (Dalton et al., 1968; Murphy et al., 1970). Non-covalently associated GP1/GP2 complexes make up the spikes on the virion envelope, and mediate virus interaction with the host cell receptor and subsequent virus cell entry via receptor mediated endocytosis (Cao et al., 1998; Kunz et al., 2001, 2002). Arenavirus L proteins contain the characteristic conserved A, B, C and D motifs within the proposed catalytic domain III of the L polymerases of NS RNA viruses (Poch et al., 1989). Using mutation-function assays, we confirmed the key role on Arenavirus polymerase activity of the highly conserved amino acid residues within motifs A and C and provided both genetic and biochemical evidence that oligomerization of L is required for its function (Sanchez and de la Torre, 2005), a parallel finding to that reported for the L protein of paramyxoviruses (Smallwood and Moyer, 2004; Smallwood et al., 2002). The small (11 kDa) RING finger protein Z (Salvato et al., 1989, 1992) functions as the Arenavirus counterpart of the matrix protein found in many NS RNA viruses (Perez et al., 2003, 2004; Strecker et al., 2003). Additional roles of Z in the Arenavirus life cycle have been proposed based on its interaction with several host cell proteins (Borden et al., 1997, 1998; Campbell Dwyer et al., 2000), and its ability to inhibit RNA synthesis mediated by the virus polymerase (Cornu and de la Torre, 2001, 2002; Cornu et al., 2004; Hass et al., 2004; Lopez et al., 2001).

The inability to genetically manipulate the virus genome has hampered studies aimed at a detailed understanding of the Arenavirus molecular and cell biology. As with other negative strand (NS) RNA viruses, the template of the LCMV polymerase is exclusively a nucleocapsid consisting of the genomic RNA tightly encapsidated by the virus NP, which associated with the virus polymerase proteins forms a ribonucleoprotein (RNP) complex. This RNP is active in transcription and replication and is the minimum unit of infectivity (Conzelmann, 2004; Garcia-Sastre and Palese, 1993; Neumann et al., 2002; Tordo et al., 1992). In contrast to positive-stranded RNA viruses, deproteinized genomic and antigenomic RNAs of negative strand RNA viruses cannot function as mRNAs and are not infectious. Thus, generation of biologically active synthetic NS viruses from cDNA will require trans complementation by all viral proteins involved in virus replication and transcription. These considerations have severely hindered the application of recombinant DNA technology to the genetic analysis of these viruses. However, during the last decade, following the pioneering work of Palese's group (Luytjes et al., 1989), significant progress has been made in this area and for many negative strand RNA viruses, short model genomes could be encapsidated and expressed either by infectious helper viruses or by plasmid-encoded proteins. This approach has revolutionized the analysis of cis-acting sequences and trans-acting proteins required for virus replication, transcription, maturation and budding (Conzelmann, 2004; Kawaoka, 2004; Neumann et al., 2002). Moreover, it has allowed the generation and rescue of infectious viruses entirely from cloned cDNAs for members of most families of NS RNA viruses (Conzelmann, 2004; Kawaoka, 2004; Neumann et al., 2002; Schneider et al., 2005), which has provided investigators with novel and powerful approaches for the investigation of viral pathogenesis. In addition, these developments have also paved the way for engineering these viruses for vaccine and gene therapy purposes.

We have described a reverse genetic system for LCMV that is suitable for the investigation of cis-acting signals and trans-acting factors involved in virus RNA synthesis and gene expression, as well as viral assembly and budding (Cornu and de la Torre, 2001, 2002; Lee et al., 2000, 2002; Perez and de la Torre, 2003; Perez et al., 2003; Pinschewer et al., 2003a, 2003b, 2005; Sanchez and de la Torre, 2005). Similar systems have been now developed for LFV (Hass et al., 2004) and the New World Arenavirus Tacaribe virus (TV) (Lopez et al., 2001). In addition, we have documented the generation of a recombinant LCMV (rLCMV/VSVG), where the glycoprotein of vesicular stomatitis virus (VSVG) substituted for the LCMV GP (Pinschewer et al., 2003a, 2003b, 2004). This rLCMV was generated by intracellular reconstitution of a recombinant LCMV S RNP via reverse genetics, and infection with LCMV helper virus. Production of infectious LCMV, but not of rLCMV/VSVG, required the correct processing of LCMV GPC by the cellular protease S1P, which facilitated the selection of rLCMV/VSVG by using S1P deficient cells. The isolated rLCMV/VSVG has provided us with a powerful tool to facilitate the isolation of LCM viruses containing engineered rS segments. For this, cells instructed to express the rS RNP of interest are infected with rLCMV/VSVG, and the virus progeny is subjected to selection with a neutralizing antibody to VSV G to eliminate the helper rLCMV/VSVG. This approach however requires several rounds of selection, and is limited to the rescue of LCMV carrying recombinant S segments.

Here, we document the rescue of infectious LCMV, Armstrong strain (LCMV-ARM), entirely from plasmid, without the need of using a helper virus. Transfection of cells with plasmids that directed T7RP-mediated intracellular synthesis of L and S antigenomic (Lag and Sag) RNA species, together with pol-II expression plasmids for the T7RP and the viral trans-acting factors L and NP permitted the rescue of a rLCMV called rT7/LCMV. Production of rT7/LCMV was readily detected 72 h after transfection, which was followed by a rapid increase in virus production reaching titers of 10^7 PFU/ml. The rT7/LCMV was unequivocally identified based on a genetic tag introduced in the recombinant S segment. In addition, rT7/LCMV exhibited growth and biological properties predicted for an ARM-like LCMV including: (1) induction of lethal choriomeningitis (LCM) following intracranial inoculation (ic) of adult mice, (2) induction of robust T-cell responses and (3) ability to establish persistence upon infection of newborn mice. Our findings extend the use of reverse genetic approaches for DNA-mediated rescue of members of all known families with NS RNA genome.

Results

Transcription and replication of antigenomic segments of LCMV

We generated constructs pT7-L(+)HR and pT7-S(+)HR (Fig. 1A) by inserting full-length cDNAs of the L and S, respectively, segments of LCMV-ARM between the T7RP promoter (pT7) and an LCMV-specific hairpin ribozyme. Both L and S full-length cDNAs were obtained via RT-PCR using RNA isolated from purified LCMV-ARM virions, and cloned in an anti-genomic (ag) polarity with respect to the T7p. The S cDNA could be amplified in a single step, whereas the complete L cDNA was amplified in two PCR products, one containing the ag 5′-end and the L ORF, and the other containing a C-part of the L ORF together with the L IGR, the Z ORF and the ag 3′-end. Both PCR products overlap for sequences located within the C-terminus of the L ORF to facilitate the generation of a full-length L cDNA via ligation of the two initial PCR

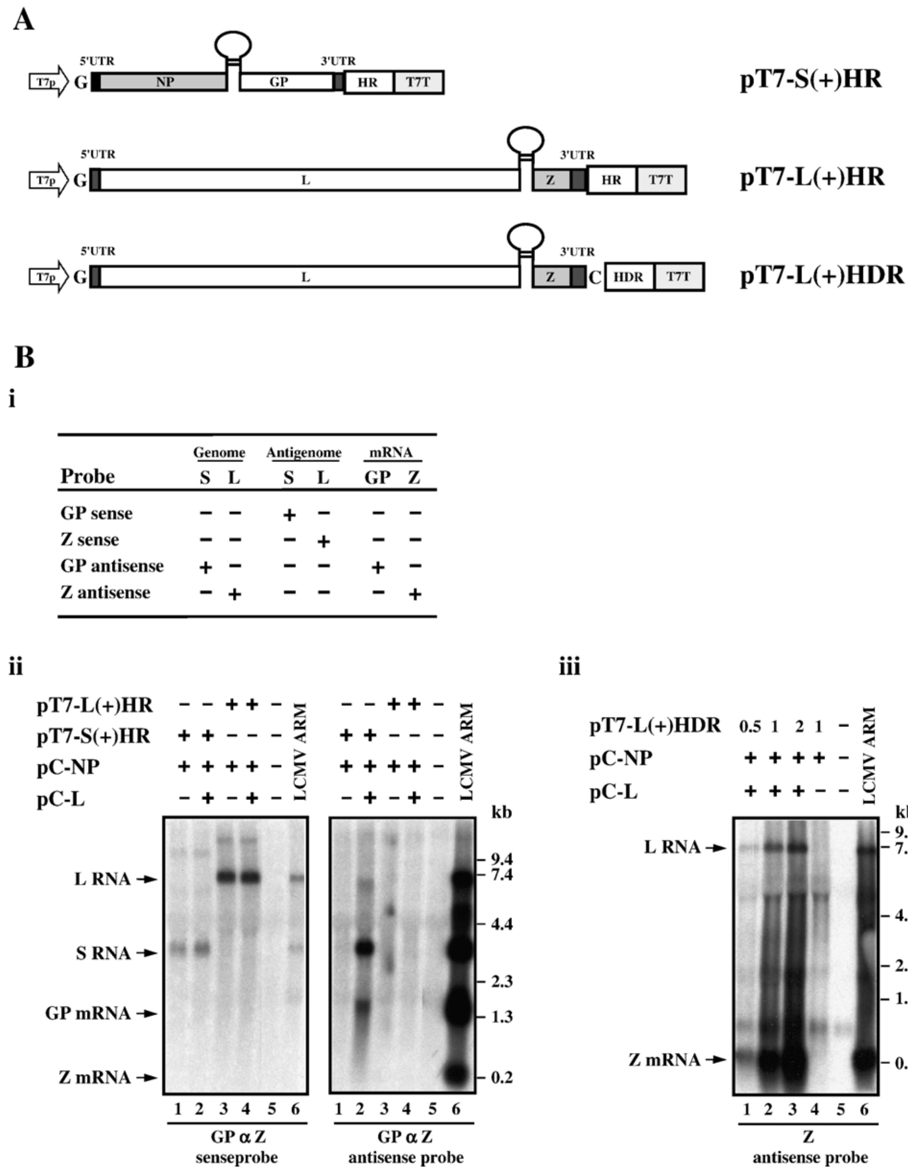

Fig. 1. LCMV polymerase mediated replication and transcription of plasmid supplied ag S and L RNA species. (A) Schematics of plasmids encoding Sag and Lag RNA species. Plasmid pT7-S(+)HR and pT7-L(+)HR contain the following elements: (1) a truncated T7RP promoter (pT7) lacking two G residues at its 3′-end, (2) the complete sequence of the S (pT7-S(+)HR) or L (pT7-L(+)HR) RNAs in antigenomic polarity with respect to the pT7, (3) the sequence of an LCMV-specific hairpin ribozyme (HR), and (4) the sequence of T7 terminator (T7T). Plasmid pT7-L(+)HDR is a modification of pT7-L(+)HR where an additional C residue was introduced after the 3′UTR, followed by hepatitis delta riboenzyme (HDR) sequence instead of the HR. (B) Analysis of transcription and replication by the virus polymerase of plasmid supplied Sag and Lag RNA species. BHK-21 cells ($2.5 \times 10^5/cm^2$) were transfected with pC-T7 (1 μg), pT7-L(+)HR (1 μg) or pT7-L(+)HDV (using the indicated amounts), pT7-S(+)HR (0.4 μg), pC-NP (0.4 μg) and pC-L (0.5 μg). Forty-eight-hour post transfection total cell RNA was isolated and equal amounts of each sample were analyzed by Northern blot hybridization using appropriate [^{32}P] rUTP labeled RNA probes to detect the Lg, Lag, Sg and Sag RNA species, as well as GP and Z mRNAs. (Bi) Predicted RNA species detected by Northern Blot hybridization using the indicated strand-specific probes. (Bii) The GP and Z antisense probes detected the Sag RNA and GP mRNA derived from pT7-S(+)HR , but failed to detect the Lag RNA and Z mRNA derived from pT7-L(+)HR. (Biii) The Z antisense probe readily detected the Lag RNA and Z mRNA derived from pT7-L(+)HDR.

fragments. We encountered significant difficulties to determine a defined nucleotide sequence for the L IGR (nucleotides 359 to 567, genome polarity). This was only possible by using a thermo-stable RT to conduct RT-PCR at higher temperature and in the presence of 5% DMSO. The corresponding PCR product was cloned and nucleotide sequence of multiple clones determined. We observed genetic heterogeneity among independent clones, but no evidence of dominance of a specific sequence. Several clones harbored large deletions that corresponded to stem loop structures deleted during the amplification or during the sequencing reaction by polymerase slippage. Others have also reported deletions in the L IGR of LCMV (Salvato et al., 1989; Shimomaye and Salvato, 1989) and LFV (Vieth et al., 2004). Based on our sequence data, we derived a consensus sequence for the L IGR (GenBank AY894816) that differed from the previously published (Salvato et al., 1989), but identical to one recently determined by Grande-Perez et al. (Grande-Perez et al., 2005), and the one independently determined by Whelan's group (GenBank AY894816).

The use of a truncated T7RP promoter, containing a single G, directed T7RP-mediated synthesis of the L and S ag RNA species with the correct 5′-termini including the non-templated G residue found at the 5′-termini of Arenavirus genome and antigenome RNA species. The generation of the authentic viral 3′-termini was mediated by an LCMV-specific hairpin ribozyme (HR) inserted downstream to the 3′-UTR of the L and S ag RNA, followed by a T7 terminator motif (T7T).

We first examined whether these constructs could direct intracellular synthesis of S and L ag RNA species that could be correctly encapsidated by plasmid supplied NP to generate a nucleocapside template that could be replicated and transcribed by the intracellularly reconstituted virus polymerase. We observed that plasmid-supplied T7RP directed intracellular synthesis of Sag and Lag RNA efficiently, and the Sag RNA was subsequently replicated and transcribed by the virus polymerase as determined by Northern blot using strand-specific riboprobes (Fig. 1B). In contrast, the lack of hybridization signal with the Z antisense riboprobe indicated that T7RP produced an Lag RNA that did not serve as an efficient template for the generation of L genomic (Lg) RNA, and subsequent Z mRNA synthesis, by the LCMV polymerase (Fig. 1Bii). We reasoned that inefficient RNA self-cleavage mediated by the HR resulted in low levels of correctly processed 3′-termini of the Lag RNA, which interfered with encapsidation or polymerase recognition, or both. To attempt overcoming this problem, and based on our previous findings (Perez and de la Torre, 2003), we generated the construct pT7-L(+)HDV (Fig. 1A), where the HDV substituted for the HR ribozyme. The Lag RNA produced via T7RP contained an extra C at its 3′-end to improve the HDV-mediated Lag RNA processing (Perez and de la Torre, 2003). This new construct directed the intracellular synthesis of a Lag RNA that was efficiently replicated into Lg RNA by the virus polymerase (Fig. 1Biii).

Rescue of LCMV from plasmid

We next attempt to rescue LCMV from plasmid (Fig. 2A). For this, we transfected BHK-21 cells with plasmids expressing the Sag and Lag RNA species together with pC-L and pC-NP, as well as pC-T7, using optimized conditions that recreated ratios of L/S G RNA and relative levels of GP and Z mRNA observed in LCMV-infected cells (MOI = 0.1) at 48 h p.i. Three days post transfection, we subcultured the cells to prevent the characteristic deterioration of BHK-21 cells when left as confluent monolayers for extended period of time. We examined tissue culture supernatant (TCS) collected at days 3, 7 and 10 post transfection for the presence of infectious LCMV by infecting fresh monolayers of BHK-21 cells and detection 36 h later of viral antigen by IF (Fig. 2B). Infectious virus was readily detected at 72 h after transfection, and titers grew up rapidly to 10^7 PFU/ml. Similar results were obtained in several independent experiments.

Genetic characterization of rescued LCMV

To confirm the identity of the rT7/LCMV rescued from cDNA we took advantage of a genetic tag introduced into the S cDNA. This tag consisted of a single silent mutation that eliminated an Eco NI restriction site present within the S of LCMV-ARM (Fig. 3A). We used RNA isolated from BHK-21

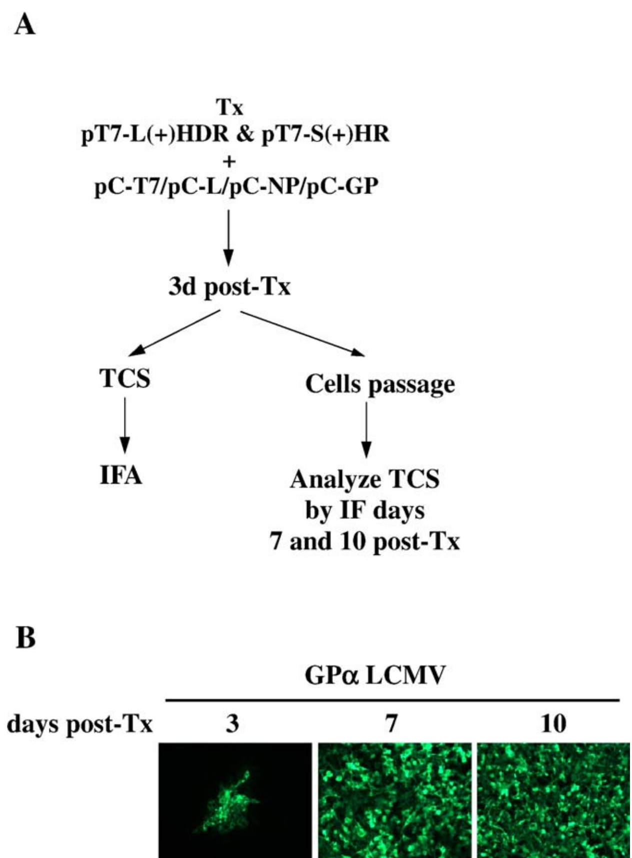

Fig. 2. Recovery of rT7/LCMV from cDNAS. (A) Schematic of the plasmid-based rescue system. BHK-21 cells (10^6) were transfected with pC-T7 (2 μg), pT7-L(+)HDR (2 μg), pT7-S(+)HR (1.3 μg), pC-NP (0.8 μg), pC-L (1 μg) and pC-GP (0.4 μg). Three days later cells were transfer into T75 to expand them. (B) Detection of infectious virus in the tissue culture supernatant (TCS) of transfected cells. TCS were collected at days 3, 7 and 10 after transfection and the presence of infectious virus examined by infection of BHK-21 cells and detection of viral antigen by IF at 36 h pi.

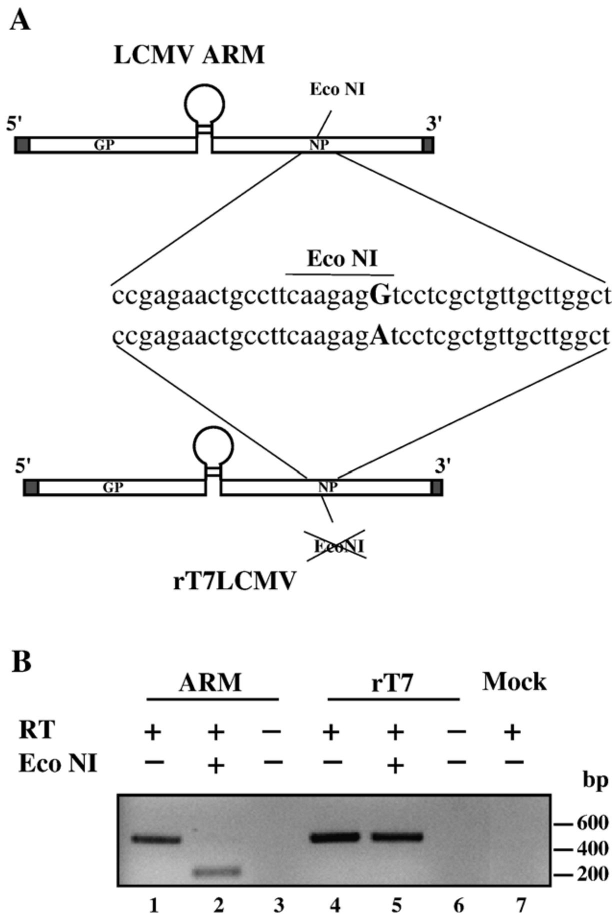

Fig. 3. Genetic characterization of rescued rT7LCMV. (A) Schematic of the genetic tag introduced within the S segment. The genetic tag consisted of a silent mutation (G to A) that resulted in the lost in the S segment of rT/LCMV of an *Eco*NI site present in LCMV-ARM. (B) Detection of the genetic tag in the S segment of rT7/LCMV. RNA was isolated from BHK-21 cells infected with the rescued rT7/LCMV and subjected to RT-PCR with primers to amplify a 520-bp fragment containing the genetic tag. PCR products were subjected to restriction analysis with *Eco*NI and the products analyzed by agarose (2%) gel electrophoresis. PCR fragments derived from RNA isolated from LCMV-ARM, but not from rT7/LCMV, infected cells were digested by *Eco*NI. Sequence analysis of the PCR products confirmed that PCR products derived from RNA isolated from LCMV-ARM or rT7/LCMV infected cells differed only at the predicted site where the genetic tag was introduced.

cells infected with either rT7/LCMV or LCMV-ARM to conduct RT-PCR to amplify a segment of 520 bp that contained the genetic tag. As predicted, PCR products derived from LCMV-ARM, but not from rT7/LCMV, infected cells were susceptible to digestion with Eco NI (Fig. 3B). Sequencing of the corresponding PCR products confirmed that a single change G to A in rT7/LCMV was responsible for the lost of the Eco NI present in LCMV-ARM.

Growth properties of rT7/LCMV in cultured cells

We next compared the growth properties in cultured cells of rT7/LCMV and LCMV/ARM. For this we infected BHK-21 cells (MOI = 0.1) with either rT7/LCMV or LCMV-ARM, and determined levels of viral RNA and virus titers at different times p.i. Both rT7/LCMV and LCMV/ARM exhibited similar kinetics and levels of RNA replication and transcription as determined by Northern blot hybridization using a NP DNA probe that hybridized to the S RNA (replication) and NP mRNA (transcription) (Fig. 4A). Likewise, both viruses had similar kinetics and production peaks of infectious progeny (Fig. 4B).

Biological properties of rLCMV

To further corroborate that the experimental procedures used for the rescue of rT7/LCMV did not result in unexpected phenotypic viral properties we compared rT7/LCMV and LCMV-ARM wt with respect to three well-characterized biological features of LCMV: (1) its ability to induce lethal lymphocytic choriomeningitis (LCM) upon intracranial (ic) inoculation of adult mice, (2) its ability to induce a robust viral-specific T-cell response upon inoculation (i.p.) of adult mice, which results in subsequent viral clearance, and (3) its ability to establish a persistent infection upon infection of newborn mice.

Both LCMV-ARM and rT7/LCMV induced lethal LCM with similar efficiency in infected (i.c.) adult B6 mice (Fig. 5A). Accordingly, both rT7/LCMV and LCMV/ARM reached similar infectious titers in brains and sera of infected mice (Fig. 5B). *Eco*NI digestion of RT-PCR products derived from RNA isolated from brains of infected mice confirmed the genetic identity of the viruses and showed that reversion of rT/LCMV to LCMV-ARM genotype was not required for development of lethal LCM (Fig. 5C). Likewise, adult B6 mice infected (i.p.) with either rT7/LCMV or LCMV/ARM developed CD8- and CD4-T cell responses of similar quality and magnitude (Fig. 6) that resulted in undetectable virus titers in serum at day 15 p.i. (not shown). Moreover, as documented extensively for LCMV, newborn B6 mice infected with rT7/LCMV developed a persistent infection as determined by the presence of infectious virus (Fig. 7A) and RNA (Fig. 7B) in brain and liver at day 21 p.i. Likewise, mice infected at birth with either LCMV-ARM or rT7/LCMV had similar serum viral load (Fig. 7A).

Discussion

The use of plasmids that direct intracellular synthesis of positive-sense cRNA (antigenomic species) rather than the negative-sense genomic vRNA appeared to facilitate the rescue of a variety of cytoplasmic NS RNA viruses (Conzelmann, 2004; Kawaoka, 2004; Neumann et al., 2002). The rationale behind this approach was that it would prevent potential hybrid arrest effects due to annealing between negative-sense vRNA and positive-sense mRNAs. In the case of viruses with an ambisense gene organization, including arenaviruses, both genome and antigenome RNA species contain sequences complementary to one of the viral mRNAs (Buchmeier et al., 2001; Meyer et al., 2002; Salvato, 1993; Southern, 1996), which could pose special problems for the cDNA-mediated rescue of these viruses. To minimize this potential obstacle for the cDNA-mediated rescue of infectious LCMV we used plasmids that expressed ag RNA species of

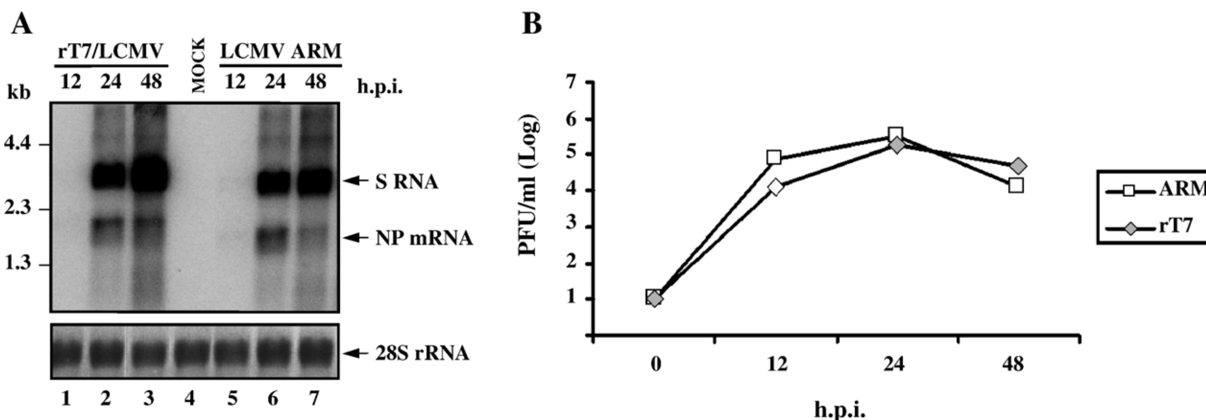

Fig. 4. Growth properties of rT7/LCMV. Vero cells were infected with LCMV ARM or rT7/LCMV (both at MOI = 0.1). TCS and RNA were harvested at 0, 12, 24, and 48 h p.i. (A) RNA was analyzed by Northern Blot hybridization using a ^{32}P-labeled NP DNA probe that hybridizes to both NP mRNA (transcription) and S RNA (replication). (B) Viral titers in TCS were determined by plaque assay.

the L and S segments. This prevented the annealing between plasmid derived ag L and S RNA species and the L and NP mRNAs. Notably, we observed similar rescue efficiencies when we used, instead of the Lag and Sag, the corresponding Lg and Sg constructs. Consistent with this, cell lysates of cells transfected with genomic or antigenomic L constructs containing the CAT reporter gene in lieu of the Z ORF, together with L and NP expressing plasmids, had similar levels of CAT activity (Fig. 8). This finding indicates that annealing between viral mRNAs and genome, or antigenome, RNA species do not appear to pose a significant problem for the rescue of Arenavirus, which is likely applicable to other ambisense NS RNA viruses.

A main hurdle for the rescue of LCMV from cDNAs was to obtain a correct functional IGR. We detected a large proportion of virion-associated L molecules with an altered IGR. Whether this finding reflects a biological property of the virus or a technical issue related to the RT-PCR experimental procedures used to obtain the L IGR remains to be determined. Notably, we consistently obtained the correct L IGR sequence when we used as template for the RT reaction a RNA generated by in vitro transcription of a plasmid DNA containing the cDNA of the IGR we (GenBank AY894816) and others (Grande-Perez et al., 2005) have recently documented. These results would suggest that Lg and Lag RNA species with altered IGR sequences are generated in virus-infected cells and might have functional correlates yet to be defined. Another obstacle we encountered was to establish experimental conditions leading to the correct L/S balance upon amplification by the intracellularly reconstituted LCMV polymerase. Our initial rescue attempts were based on the use of an LCMV-specific HR to generate the authentic 3′-termini of both Lag and Sag RNA species initially synthesized by T7RP. We found however that although the LCMV-specific HR was very active when present in the Sag RNA, it appeared to be mostly inactive in the context of the Lag RNA. This unexpected finding, specially considering the sequence similarity between the 3′-termini of the Sag and Lag RNA species resulted in undetectable levels of Lg RNA derived via replication by the virus polymerase of the T7RP-mediated input Lag RNA. Only substituting the HDR for the HR to generate the 3′-end of the L ag RNA could solve this problem.

Using the same Sag and Lag cDNAs, we have also established a pol-I/pol-II based rescue of rLCMV (described elsewhere). In this system, the authentic 5′- and 3′-termini of both Sag and Lag RNA species were created by the precise transcription initiation and termination sites of pol-I (Kawaoka, 2004; Neumann et al., 2002; Pinschewer et al., 2003a, 2003b). Both the T7RP and pol-I based rescue systems exhibited similar efficiencies (data not shown). These results are consistent with the presence of a correct IGR and 3′-termini sequences in the Lag RNA as key factors for the successful rescue of LCMV.

The rT7/LCMV differed from LCMV/ARM at the genome site predicted by the genetic tag introduced into the recombinant S segment. This genetic difference should not result in phenotypic differences between these two viruses. Finding such differences would have suggested that our reverse genetics approach for the rescue of rT7/LCMV might be prone to incorporate additional lesions in the viral genome, which would question its use as a tool for the generation of rLCMV with a defined genetic makeup for investigation of the Arenavirus biology. Our results, however, showed that both rT7/LCMV and LCMV-ARM exhibited the same kinetics and levels of RNA replication and transcription, as well as similar kinetics and production peaks of infectious progeny. Likewise, the rT7/LCMV exhibited all the biological properties predicted for an ARM-like virus. These results argue against unwanted artifacts associated with the rescue of infectious LCMV from cDNA, and support the view that it should be now possible to generate recombinant LCM viruses with predetermined specific mutations and analyze their phenotypic expression in its natural host, the mouse. LCMV is an important model to study both acute and persistent viral infection, as well as virus–host balance and associated disease, and a variety of basic concepts in immunology and viral pathogenesis have been developed using the LCMV model (Buchmeier and Zajac, 1999;

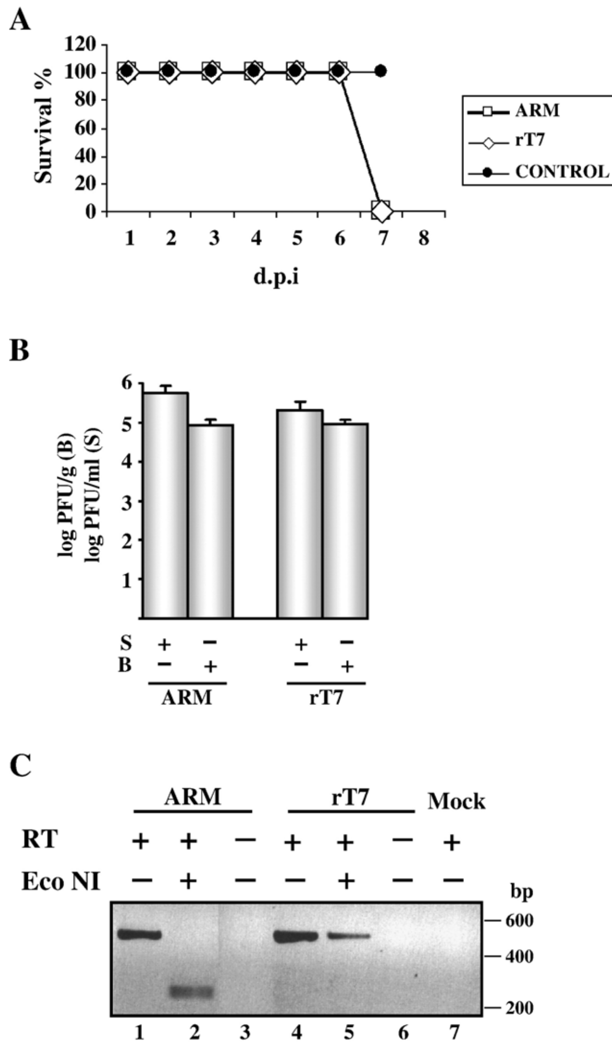

Fig. 5. Induction of lethal LCM upon ic inoculation of mice with rT7/LCMV. B6 mice were injected (ic) with 10^3 PFU of LCMV-ARM or rT7/LCMV, or with virus diluent (control). (A) Virus induced mortality. Both LCMV-ARM and rT7/LCMV induced lethal LCM with the same efficiency, with 100% ($N = 8$/group) of the inoculated mice dying at day 7 p.i. (B) Virus titers in brain and serum of infected mice. Brain homogenates and sera from infected mice were collected at day 5 p.i. and examined for the presence of infectious virus by plaque assay. Titers correspond to the average and SD ($N = 3$/group). (C) Detection of rT7/LCMV RNA in brain. Brain RNA was isolated from LCMV-ARM and rT7/LCMV-infected mice at 5 dpi and analyze by RT-PCR to detect the presence of the genetic tag within the S segment. PCR product derived from RNA isolated from brain tissue of LCMV-ARM, but not rT7/LCMV, infected mice contained, as predicted an *Eco*NI site.

Buchmeier et al., 2001; Oldstone, 2002; Salvato, 1993; Zinkernagel, 2002). In addition, LCMV provides investigators with an excellent system to study basic aspects of the molecular and cell biology of clinically important human pathogens including Lassa fever virus (LFV) and other HF arenaviruses. The ability to generate recombinant LCM viruses with predetermined specific mutations and analyze their phenotypic expression in its natural host, the mouse, would significantly contribute to the elucidation of the molecular mechanisms underlying LCMV–host interactions including virus persistence and associated disease.

Materials and methods

Cells and viruses

BHK-21 cells were maintained in high-glucose Dulbecco's Eagle medium (DMEM) supplemented with 10% heat-inactivated (55 °C for 30 min) fetal calf serum (FCS), 2 mM L-glutamine, 1× tryptose phosphate broth (Life Technologies), 1 mM sodium pyruvate, and 0.5% glucose. Vero E6 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and 10 mM HEPES. Infections with LCMV were done using a plaque isolate of strain Armstrong (ARM) (Lee et al., 2000). Virus titers were determined by plaque assay as described (Dutko and Oldstone, 1983).

Plasmids

Plasmids pC-L, pC-NP, pC-GP expressing the polymerase (L), nucleoprotein (NP), and glycoprotein (GP) gene products of LCMV, as well as pC-T7 expressing the T7RP have been described (Lee et al., 2000, 2002; Perez et al., 2003). Plasmids pT7-L(+)HR and pT7-S(+)HR allowed for T7 RNA polymerase (T7RP)-mediated intracellular synthesis of L and S, respectively, antigenomic (ag) RNA species of LCMV ARM. The Lag and Sag sequences in pT7-L(+)HR and pT7-S(+)HR, respectively, are their 5′- and 3′-termini flanked by a truncated T7RP promoter, containing a single G, and an LCMV-specific hairpin ribozyme (HR) (Xing and Whitton, 1992), respectively. The truncated T7RP promoter permitted the generation by T7RP of Lag and Sag RNA species containing at their 5′termini the non-template G characteristically found at the 5′ ends of arenavirus genomes and antigenomes, whereas self cleavage of the LCMV-specific HR facilitated the generation of antigenome L and S RNAs with concert 3′-termini. Plasmid pT7-L(+)HDV was generated by substituting sequence of the hepatitis delta virus (HDV) ribozyme for that of the HR to generate the concert 3′-end of the Lag RNA. To improve the HDV ribozyme self-processing, we added an extra C residue to the 3′-end of the plasmid encoded Lag RNA (Perez and de la Torre, 2003; Perrotta and Been, 1991).

We used a three-way PCR mutagenesis approach to introduce a genetic tag in to the S segment. This tag involved a single silent nucleotide substitution to eliminate an *Eco*NI site present in the S sequence of ARM.

Primers sequences and detailed protocols used for the generation of the plasmid constructs are available upon request.

DNA transfection

Cells ($2×10^5$/per well) were seeded into 12-well plates, and transfected with different amount of DNA using lipofectamine 2000 at 2.5 μl/μg DNA.

For rescue of virus, BHK-21 cells were transfected using the Amaxa nucleofection technology (Amaxa, Koeln, Germany). Briefly, BHK-21 cells (10^6 cell per transfection) were resuspended in 100 μl of solution L (nucleofector kit) and mixed with cDNA. The mixture was transferred to the provided

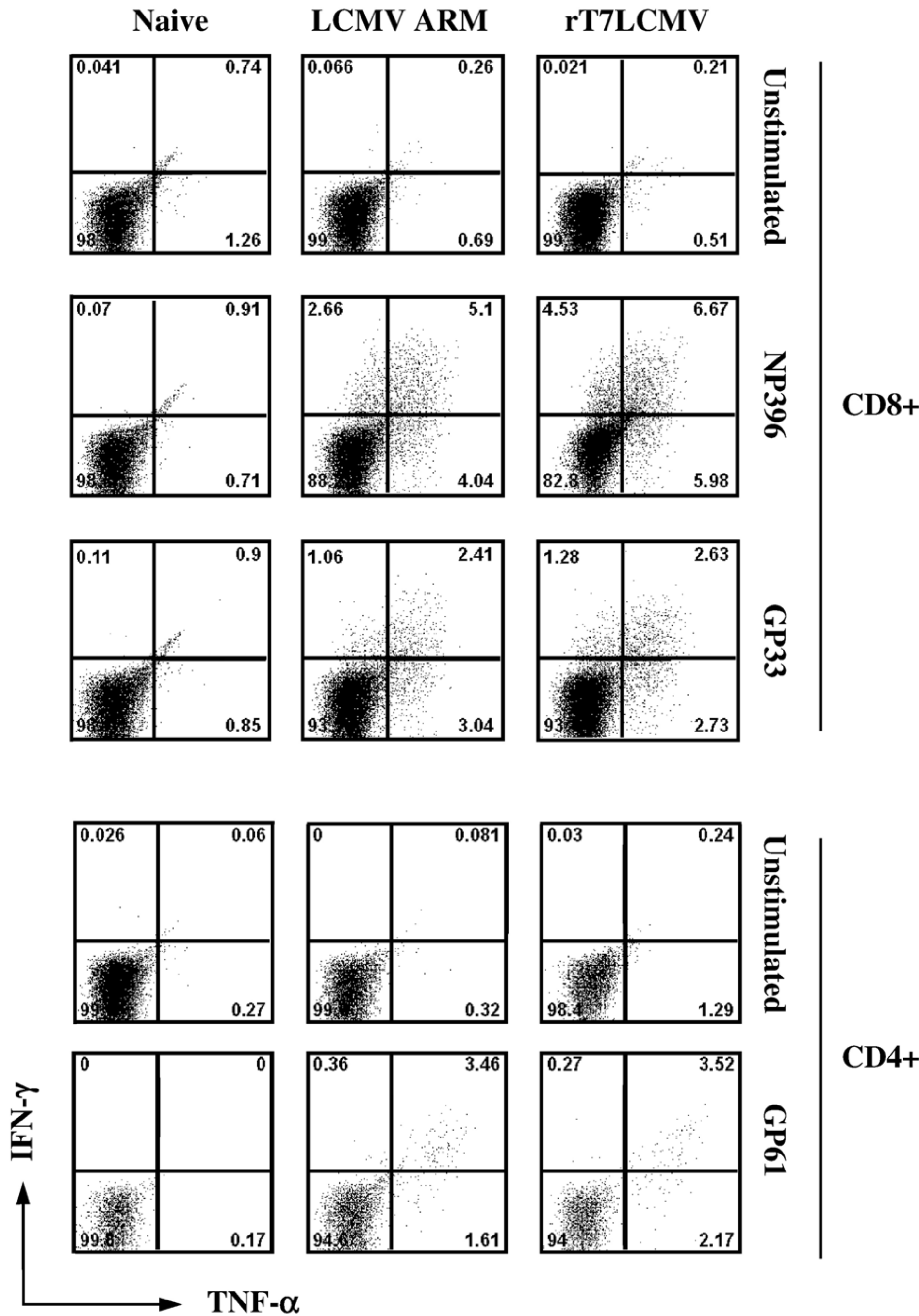

Fig. 6. Induction of T-cell responses. Adult B6 mice were injected intraperitoneally with LCMV ARM or rT7/LCMV (2×10^5 PFU). Eight days after infection, spleenocytes were prepared and stimulated for 5 h in vitro with virus specific peptides for NP (aa 396–404), GP (aa 33–41 and aa 61–80). Cells were then stained for CD8, CD4 and intra-cellular IFN-γ and TNFa. Values in the dot plots indicate the percentage of CD8 and CD4 T cells that were positive for IFN-γ and TNF-a. Both LCMV-ARM and rT7/LCMV induced similar T-cell responses. Data represent average values (N = 3/group).

electroporation cuvette and nucleofected using nucleofector program A-31 with an Amaxa Nucleofector apparatus. Immediately after nucleofection, cells were transferred into M6 wells containing pre-warmed 37 °C culture medium.

Detection of viral antigen by indirect immunofluorescence (IF)

Cells were grown onto coverslips placed on the bottom of the wells of an M24 plate. Cells were washed once with PBS and fixed in acetone/methanol (1:1) for 5 min at room temperature. After several washes with PBS and a blocking step with 10% normal goat serum in PBS for 30 min at room temperature, cells were incubated for 1 h at room temperature with a guinea pig polyclonal serum to LCMV. After several washes with PBS-0.1% Triton X-100, samples were incubated for 45 min at room temperature with a fluorescein isothiocyanate (FITC)-labeled goat anti-guinea pig immunoglobulin G (IgG). After extensive washes with PBS-0.1% Triton X-100, coverslips were mounted

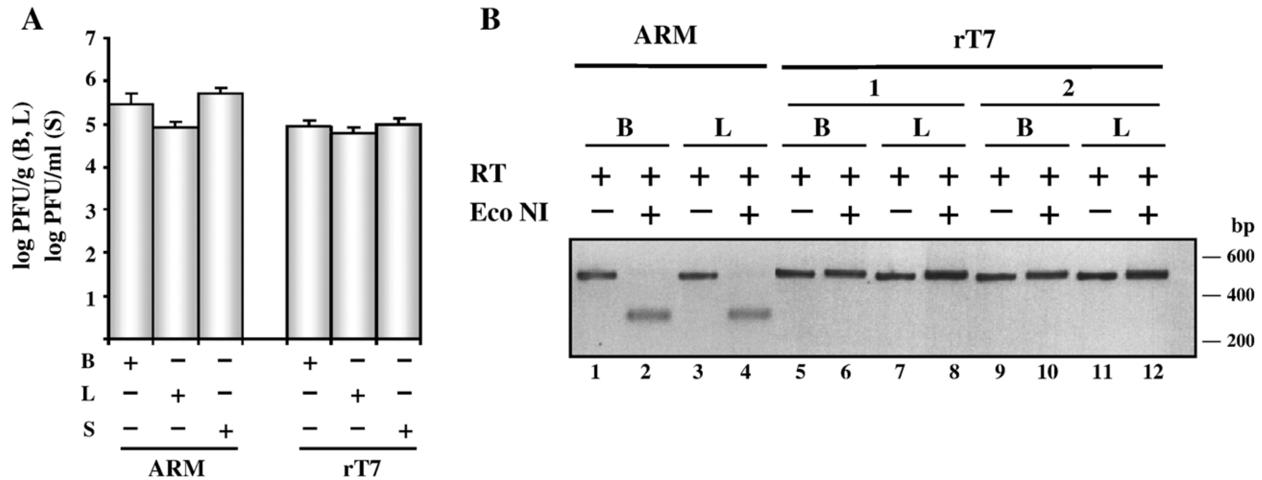

Fig. 7. Persistent infection of rT7/LCMV in mice. Newborn B6 mice were inoculated (ic) with rT7/LCMV (10^3 PFU). (A) Levels of infectious virus were determined for brain, liver and serum at 21 days p.i. by plaque assay. (B) Viral RNA in brain and liver, and the presence of the genetic tag within the S segment of rT7/LCMV, was determined by RT-PCR and digestion with *Eco*NI.

using Mowiol (10% Mowiol (Hoechst), 24% glycerol, 0.1 M Tris–HCl pH 8.5 and 2.5% 1,4-diazobicyclo{2.2.2}-octane (DABCO)) (Osborn and Weber, 1982) and analyzed by fluorescence microscopy. Slides were digitized by using Adobe Photoshop and Canvas software.

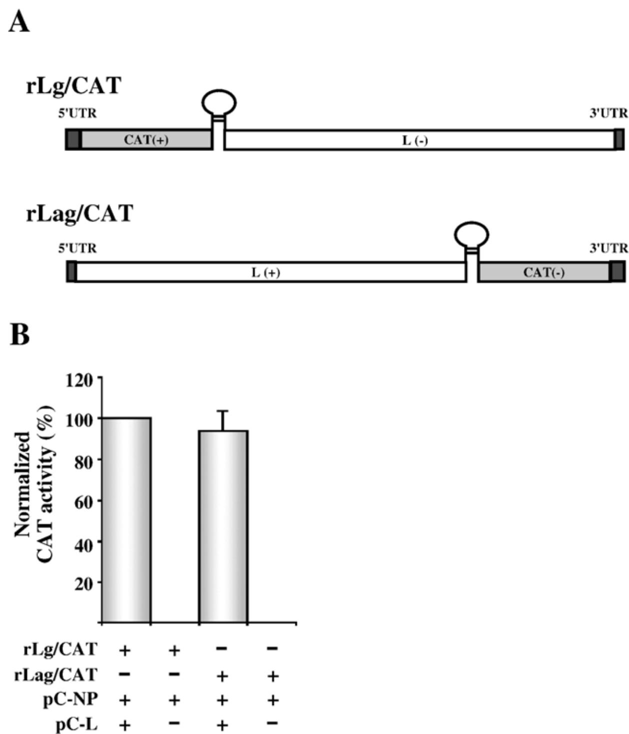

Fig. 8. (A) Schematic of recombinant genome (rLg/CAT) and antigenome (rLag/CAT) L segments containing the CAT ORF in lieu of the Z ORF. (+) and (−) refer to the polarity, sense (+) and antisense (−) of the CAT and L ORFs. (B) CAT activity associated with virus polymerase mediated expression of rLag/CAT and rLag/CAT constructs. BHK-21 cells were transfected with the indicated combinations of plasmids and 48 h later cell lysates were prepared for CAT assay. CAT activities were normalized by assigning a value of 100% to the CAT activity derived from rLg/CAT expression. CAT activity in the absence of pC-L was below 0.2%. Values correspond to the average and SD of three independent experiments.

RNA analysis by Northern blotting

RNA was isolated from cells by using TriReagent (Molecular Research Center, Cincinnati) according to the manufacturer's instructions. RNA was analyzed by northern blot hybridization using ^{32}P-labeled strand specific probes were generated using appropriated PCR fragments using Lin'Scribe kit (Ambion).

Detection of the genetic tag within the S segment

RNA was isolated from cells and B6 mice brain tissue infected with either LCMV ARM or rT7/LCMV using TriReagent (Molecular Research Center, Cincinnati) according to the manufacturer's instructions. RNA was reverse transcribed using SuperScript II RT and random hexamer primers (Invitrogen), and the corresponding cDNA amplified using *Pfu* Turbo polymerase (Stratagene) and specific primers to amplify a 520 bp NP fragment containing the genetic tag. Digestion of PCR products distinguish between ARM (*Eco*NI+) and rT7/LCMV (*Eco*NI−) NP derived PCR products.

Mice

Newborn and 8 weeks B6 mice were obtained from the rodent breeding colony at The Scripps Research Institute (La Jolla, Calif.). Intracranial inoculation of mice was done as described (Oldstone et al., 1982).

Flow cytometric analysis

Spleen single-cell suspensions were obtained by homogenizing the spleens through a mesh. Cells were stimulated with specific peptides from LCMV. Cells were incubated for 10 min with a rat mAb to CD16/32 to block Fc receptors and then with

the primary antibodies for 20 min on ice. Splenocytes were stained for cell surface antigens, using anti-CD8 antibody conjugated with Pacific Blue (Caltag), anti-CD4 antibody conjugated with allophycocyanin (APC-Cy7) (BD PharMingen) and anti-CD3 conjugated with peridin chlorophyll protein (PerCP-Cy5.5) (BD PharMingen), for 20 min on ice in PBS containing 1% (vol/vol) FBS and 0.1% (wt/vol) NaN_3. After, cells were washed two times, fixed, and permeabilized in 0.1% (wt/vol) saponin in HBSS. Intracellular cytokine staining was accomplished with incubation of antibodies to IFN-γ phycoerythrin (PE) and TNFa-fluorescein isothiocyanate (FITC) for 30 min on ice in 0.1% (wt/vol) saponin, 1% FBS PBS, followed by two washes and resuspension of cells in 1% (vol/vol) FBS, 0.1% (wt/vol) NaN_3 PBS, as described (Homann et al., 1998). Cells were acquired on a Digital LSR II (Becton Dickinson) and analyzed and FlowJo (Treestar) software.

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