Recovery of an arenavirus entirely from RNA polymerase I/II-driven cDNA

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The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) was first isolated in the 1930s (1, 2). Ever since, it has served as a primary workhorse of viral immunologists for almost a century, and it has served as an important model for studying basic principles of arenavirus molecular biology. Its negative-stranded bisegmented RNA genome has, however, posed a major obstacle to attempts at manipulating the infectious virus by reverse genetic techniques. Here, we report the recovery of infectious lymphocytic choriomeningitis virus (the immunosuppressive strain clone 13) entirely from cDNA. Intracellular transcription of the short and the long viral genome segment from polymerase (pol) I-driven vectors and coexpression of the minimal viral-transacting factors NP and L from pol II-driven plasmids resulted in the efficient formation of infectious virus with genetic tags in both genome segments. The cDNA-derived viruses behaved identically to wild-type virus in both cell culture and infected mice. Importantly, they caused a chronic infection and suppressed the adaptive immune response to an unrelated third-party virus. This technology provides an important basis for investigating viral determinants of persistent infection and immunosuppression. In addition, our findings demonstrate that pol I/II-based vector systems may represent an efficient alternative strategy for the recovery of cytoplasmic negative-strand RNA viruses from cDNA.

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Abbreviations: LCMV, lymphocytic choriomeningitis virus; S segment, short segment; L segment, long segment; GP, glycoprotein; NP, nucleoprotein; MG, model genome; pol, polymerase; VSV, vesicular stomatitis virus; G13, wild-type G13; ARM, Armstrong; IGR, intergenic region; L-IGR, L segment-IGR; RT, reverse transcription; RSV, réovirus; SFFV, simian foamy virus; SFFV-A, wild-type SFFV-A; G13-A, Armstrong strain G13; T7, transposable element T7; T7 RNA pol, RNA polymerase; VSVG, ref. 15). In a reverse reassembly process, the VSVG-expressing S segment of this virus could be exchanged for a cDNA-derived S segment of interest, a strategy that was vastly facilitated by the potent selective capacity of VSV-neutralizing antibodies. Accordingly, viruses generated by this method have allowed us to study the role of the viral GP in liver disease (A.B., D. Merkler, E. Horvath, L. Bestmann, and D.D.P., unpublished work) and GP-based strategies to attenuate arenaviruses for use as live vaccines (A.B., N. U. Gerber, D. Merkler, E. Horvath, J.C.d.l.T., and D.D.P., unpublished work). Yet the arenavirus L segment harbors key determinants of the viral phenotype (16) that could not be studied in a reverse genetic manner. Here, we report that the intracellular expression of the LCMV RNA genome from pol I-driven vectors, combined with pol II-driven coexpression of the minimal transacting factors NP and L, resulted in the efficient recovery of infectious LCMV entirely from cDNA. This finding shows that pol I-driven systems may represent an efficient

infectious virus with genetic tags in both genome segments. The cDNA-derived viruses behaved identically to wild-type virus in both cell culture and infected mice. Importantly, they caused a chronic infection and suppressed the adaptive immune response to an unrelated third-party virus. This technology provides an important basis for investigating viral determinants of persistent infection and immunosuppression. In addition, our findings demonstrate that pol I/II-based vector systems may represent an efficient alternative strategy for the recovery of cytoplasmic negative-strand RNA viruses from cDNA.

immunosuppression | lymphocytic choriomeningitis virus | reverse genetics | negative-strand RNA virus

The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) was first isolated in the 1930s (1, 2). Ever since, it has served as a primary workhorse in the fields of immunology and viral pathogenesis, contributing substantially to the development of multiple key concepts in both disciplines (reviewed in ref. 3). Among its many interesting aspects, LCMV has been particularly instrumental in the investigation of antiviral tolerance and virus-induced immunosuppression in persistent infection. Moreover, this virus may represent a neglected human pathogen (4). Over the years it has been found that, in addition to LCMV, a substantial number of other arenaviruses are widely distributed in the rodent populations of the entire world. Some of them, e.g., Lassa fever virus, a close relative of LCMV, and the New World viruses Junin, Machupo, and Guanarito, are of particular interest because they can cause hemorrhagic fevers with high lethality when accidentally transmitted to humans (5).

Arenavirus genomes consist of two single-stranded RNA segments, each of them encoding for two viral genes in ambisense orientation, separated by an intergenic region (IGR) that is predicted to fold into a secondary RNA structure of high stability. The short segment (S segment) expresses the viral surface glycoprotein (GP) and the nucleoprotein (NP) that encapsidates the viral genome and replicative intermediates (Fig. 1A). The long segment (L segment) carries the genes for the viral RNA-dependent RNA polymerase (pol) L and the small matrix protein Z. Despite their ambisense coding strategy, however, arenaviruses are negative-strand viruses, i.e., transcription of purified viral RNA into permissive cells does not initiate an infectious cycle, a fact that has long impeded the analysis of arenavirus molecular biology. Only relatively recently, a mini-

replicon system has been developed, allowing for the analysis of the viral cis-acting elements and transacting factors involved in transcription, replication, and particle formation (6–10), and similar systems have subsequently been described for Tacaribe virus and Lassa fever virus (11, 12). The LCMV reverse genetic system had initially been based on the intracellular expression of a viral model genome (MG) from T7 RNA pol (T7), combined with either T7-driven or pol II-driven coexpression of the minimal viral-transacting factors NP and L (6, 7). Owing to the virus’s cytoplasmic life cycle, T7 had been the obvious choice for expression of an LCMV genome analogue. For orthomyxoviruses with a nuclear life cycle, however, pol I-driven vectors had long been accepted as a powerful method for expressing MGs and later for the efficient recovery of infectious viruses entirely from plasmid (reviewed in ref. 13). Stimulated by findings made in the bunyavirus field (14), we have also documented that pol I-driven vectors represented an efficient alternative means for expressing LCMV MG RNAs (9). Pol I-derived MG RNA species were exported to the cytoplasm where they assembled with plasmid-supplied L and NP proteins into ribonucleoproteins (RNPs) that were active in cytoplasmic transcription and replication (9). This system could even be exploited to reconstitute a recombinant full-length S-segment RNP. Upon reassembly with helper virus as a source for the L segment, an infectious LCMV virus with an engineered S segment was recovered that expressed the GP of vesicular stomatitis virus (VSV) instead of the LCMV-GP (rLCMV/VSVG, ref. 15). In a reverse reassembly process, the VSVG-expressing S segment of this virus could be exchanged for a cDNA-derived S segment of interest, a strategy that was vastly facilitated by the potent selective capacity of VSV-neutralizing antibodies. Accordingly, viruses generated by this method have allowed us to study the role of the viral GP in liver disease (A.B., D. Merkler, E. Horvath, L. Bestmann, and D.D.P., unpublished work) and GP-based strategies to attenuate arenaviruses for use as live vaccines (A.B., N. U. Gerber, D. Merkler, E. Horvath, J.C.d.l.T., and D.D.P., unpublished work). Yet the arenavirus L segment harbors key determinants of the viral phenotype (16) that could not be studied in a reverse genetic manner. Here, we report that the intracellular expression of the LCMV RNA genome from pol I-driven vectors, combined with pol II-driven coexpression of the minimal transacting factors NP and L, resulted in the efficient recovery of infectious LCMV entirely from cDNA. This finding shows that pol I-driven systems may represent an efficient

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Abbreviations: LCMV, lymphocytic choriomeningitis virus; S segment, short segment; L segment, long segment; GP, glycoprotein; NP, nucleoprotein; MG, model genome; pol, polymerase; VSV, vesicular stomatitis virus; GT13, clone 13; wtGT13, wild-type GT13; ARM, Armstrong; IGR, intergenic region; L-IGR, L segment-IGR; RT, reverse transcription; RSV, réovirus; SFFV, simian foamy virus; SFFV-A, wild-type SFFV-A; G13-A, Armstrong strain G13; T7, transposable element T7; T7 RNA pol, RNA polymerase; VSVG, ref. 15). In a reverse reassembly process, the VSVG-expressing S segment of this virus could be exchanged for a cDNA-derived S segment of interest, a strategy that was vastly facilitated by the potent selective capacity of VSV-neutralizing antibodies. Accordingly, viruses generated by this method have allowed us to study the role of the viral GP in liver disease (A.B., D. Merkler, E. Horvath, L. Bestmann, and D.D.P., unpublished work) and GP-based strategies to attenuate arenaviruses for use as live vaccines (A.B., N. U. Gerber, D. Merkler, E. Horvath, J.C.d.l.T., and D.D.P., unpublished work). Yet the arenavirus L segment harbors key determinants of the viral phenotype (16) that could not be studied in a reverse genetic manner. Here, we report that the intracellular expression of the LCMV RNA genome from pol I-driven vectors, combined with pol II-driven coexpression of the minimal transacting factors NP and L, resulted in the efficient recovery of infectious LCMV entirely from cDNA. This finding shows that pol I-driven systems may represent an efficient
alternative for the recovery of cytoplasmic negative-strand RNA viruses from cDNA. Moreover, the technology presented here and a simultaneously developed T7-driven system for the recovery of LCMV from cDNA (34) will provide powerful reverse genetic approaches for the study of the arenavirus immunobiology by using genetically engineered replication-competent viruses.

Results
We had observed that an LCMV L segment-based MG of ~7.6 kb could be expressed from a pol I-driven vector and yielded reporter gene activity when the viral-transacting factors were coexpressed (D.D.P. and J.C.d.l.T., unpublished work). This finding lent support to the hypothesis that pol I should be able to express not only a full-length S segment (15) but also the L segment (ref. 8 and D.D.P and J.C.d.l.T. unpublished work). Thus, we analyzed the Cl13 L segment 5' terminus by using a 5' RACE protocol that was based on A-tailing of first-strand cDNA with terminal deoxynucleotidyl transferase (for technical details see Supporting Text). This approach unambiguously revealed a G residue at position −1 on both the LCMV L-segment genome and antigenome (Fig. 1B). Different investigators (24–26) have unequivocally shown that the 3' end of the L-segment genome terminates on a G residue and not on a C (Fig. 1C and D). Thus, intramolecular annealing of the genome into a panhandle structure [the putative viral promoter (8), Fig. 1C] does not form a flush end as for most negative-strand RNA viruses (22). Also, this finding suggests that the 5' G of antigenomes found here represents a nontemplated base like in the S segment (ref. 22 and Fig. 1D). An analogous conclusion for the L-segment genome 5' G, although likely, however, awaits formal determination of its orientation, i.e., of the antigenome 3' end.

Based on this sequence information we assembled Cl13 S- and L-segment cDNAs containing the master consensus sequence and cloned them in genomic polarity under control of the mouse pol I promoter and terminator cassettes (14). Thereby we obtained the plasmids pI-S-Cl13(−) and pI-L-Cl13(−), respectively (Fig. 1E). pS-Cl13(−) was partially based on a previously published backbone [pS-BSm(−)] (15)] that contained a noncoding single-nucleotide tag, deleting an EcoNI site for discrimination from wild-type virus (Fig. 2C). In contrast, pL-Cl13(−) was assembled de novo and did not contain a genetic tag. A second construct was also generated [pL-Cl13(−)*, Fig. 1F] that differed from the wild-type Cl13 (wtCl13) sequence by a noncoding single-nucleotide transition in the Z ORF. Thereby an additional AvaII recognition site was introduced (Fig. 2C). The NP ORFs of ARM53b and Cl13 are identical, and hence the available pol II-driven expression plasmid pC-NP (7) could also be used here for intracellular coexpression of this viral protein. The previously published plasmid for intracellular ex-
expression of the ARM53b L ORF under control of pol II [pC-L (7)] differed, however, from our CI13 sequence at four coding positions, and hence a plasmid was generated (pC-CI13-L) expressing the CI13 L ORF (master consensus sequence) under control of the same pol II expression cassette (27).

As schematically depicted in Fig. 2A, cotransfection of BHK-21 cells with pC-NP, pC-CI13-L, pL-S-CI13(−)*, and either pL-CI13(−) or pl-L-CI13(−)* resulted within ~72–96 h in the recovery of high titers of infectious virus from the culture supernatant (Fig. 2B). Notably, this result was obtained at the first try by using standard lipofection procedures. Moreover, the recovery was highly reproducible i.e., from a total of >30 individual M6 tissue culture wells that were transfected in multiple experiments all but one yielded infectious virus, underscoring the high efficiency of the system. To ascertain the viral origin from cDNA we tested for the presence of the genetic tags in the viral genome. The virus obtained when transfecting pL-S-CI13(−)* and pl-L-CI13(−)* will be referred to as rCI13*, whereas the virus derived from pL-S-CI13(−)* and pl-L-CI13(−)* was named rCI13** to indicate the expected genetic tags in only one or both genome segments, respectively. Fresh BHK-21 cells were infected with rCI13*, rCI13**, or wtCI13 for comparison, and RT-PCRs were carried out to amplify viral RNA stretches spanning the expected genetic tags in the S and L segments (schematically depicted in Fig. 2C). All PCR products were RT-dependent, excluding residual plasmid contamination in the virus preparations that might have confounded the subsequent analysis (Fig. 2D). As expected, the S segment-derived amplification product of wtCI13 was sensitive to digestion with EcoNI, whereas the products derived from rCI13* and rCI13** were not. The L segment-derived RT-PCR products of wtCI13 and rCI13* yielded identical fragments when digested with Avall, whereas rCI13* yielded smaller ones as predicted (Fig. 2C). Hence, rCI13* and rCI13** were derived from the transfected parental plasmids.

Next, we compared the growth curves of rCI13* and rCI13** with wtCI13 in cell culture and found them to be indistinguishable (Fig. 3A). To also compare viral replication kinetics in vivo, C57BL/6 mice were infected i.v. with 2 × 10^6 plaque-forming units (PFU) of wtCI13, rCI13*, rCI13**, or ARM53b as a control virus that is unable to persist in adult mice. Upon infection with wtCI13, rCI13*, or rCI13**, high-titer viremia was established within 4 days, lasting for at least 29 days. In contrast, ARM53b was cleared from the blood within 8 days after infection. We also verified that the viral populations persisting in rCI13** and rCI13***-infected mice still carried the genetic tags. Viral RNA was extracted from the serum of mice at day 12 after infection, and RT-PCR products were tested by restriction digestion as outlined above. The analysis yielded the same result as shown in Fig. 2D for tissue culture infections, confirming that the genetic tags were stably expressed (Fig. 5, which is published as supporting information on the PNAS web site).

Persistent infection with CI13 is known to subvert both the LCMV-specific CTL response to immunodominant epitopes and the adaptive immune response to unrelated third-party infections (18, 28). To test whether the cDNA-derived viruses shared the ability of wtCI13 to cause such an acquired immunodeficiency syndrome, the above groups of mice and uninfected controls were challenged with 2 × 10^6 PFU of VSV i.p. on day 22 after primary infection (Fig. 4). Serum was collected on days 25 and 29 (i.e., days 3 and 7 after VSV challenge, respectively) to determine VSV-neutralizing antibody titers. ARM-infected mice mounted virtually unimpaired IgM responses on day 25, followed by isotype class switch and neutralizing IgG titers on

yielded the expected fragments upon digestion with Avall or EcoNI, respectively (Middle), and were fully RT-dependent (compare with −RT, Bottom). Single-headed arrow: undigested PCR product; double-headed arrow: digested fragments; feathered arrow: primer dimers.
day 29 that were in a similar range as in mice that had not undergone prior infection with LCMV (Fig. 4A). In marked contrast, these responses were suppressed to below detection levels in mice that were persistently infected with wtCl13, rCl13*, or rCl13**. The early IgM response (i.e., day 3 after VSV infection) is known to be T cell-independent, whereas isotype class switch and the production of IgG is CD4+ T cell-dependent, indicating that the cDNA-derived viruses rCl13* and rCl13** shared the ability of wtCl13 to suppress both of these immune functions. Moreover, persistent infection with one of these three viruses caused a marked suppression of the VSV-specific CD8+ T cell response, whereas ARM infection did not (Fig. 4B). As expected, persistent infection with wtCl13, rCl13*, or rCl13** also resulted in the exhaustion of the CD8+ T cell response to the immunodominant LCMV epitope NP396, whereas NP396-specific IFN-γ-producing CD8+ T cells were readily detected in ARM-infected mice (Fig. 4B). Taken together, the in vivo behavior of the cDNA-derived and genetically tagged rCl13* and rCl13** viruses was indistinguishable from wtCl13, validating our pol I-based reverse genetic system for the analysis of the virus–host relationship in adult persistent LCMV infection. Importantly, two viruses recovered from different L-segment expression plasmids (rCl13* and rCl13**) exhibited an identical phenotype. This is an additional indication for the high reproducibility of our reverse genetic system, a property that will be of paramount importance for future studies addressing the impact of individual point mutations on viral immunobiology.

Discussion

Unlike for all of the other known families of negative-strand RNA viruses (13, 29), arenaviruses have to our knowledge not previously been recovered entirely from cloned cDNA, and the same seems to be the case for bona fide ambisense viruses. With this study and a simultaneous study by Sanchez and de la Torre (34), these hurdles have been overcome. Here, we have exploited pol I-driven vectors for expression of the viral genome segments, a strategy that is against current practice for a cytoplasmic virus like LCMV. A T7-based rescue system, as exploited by A. B. Sanchez and J.C.d.I.T. (34), would have represented the obvious method of choice. It has been known for a long time, however, that viruses with a nuclear life cycle such as influenza A can be recovered by using either T7- or pol I-driven systems (13). As shown here, the same holds true for cytoplasmic viruses. This possibility had previously been suggested by pol I-based MG systems for bunyaviruses, filoviruses, and arenaviruses (9, 14, 30), but a complete pol I-based rescue of an infectious negative-strand virus with a cytoplasmic life cycle has to our knowledge not previously been reported. The rescue system of choice for a particular virus may therefore not depend only on its intracellular compartment of replication. The differential proneness of T7 or pol I to terminate prematurely at terminator-like sequences within a given viral genome may represent only one of many factors that may be difficult to predict but could substantially influence the efficiency of virus recovery. In addition, pol I-based systems render the use of a 3′ terminal ribozyme unnecessary.
because, unlike for T7, transcription termination at the pol I terminator is of high precision. Ribozymes, however, can be of limited cleavage efficiency requiring modifications on the transcript 3’ terminus (6). In favor of T7-driven systems speaks their functionality in virtually any cell type, whereas pol I expression cassettes are relatively species-specific, e.g., the vector used here operates almost exclusively in rodent cells. Furthermore, the use of a pol I/II-driven system bears the potential risk for unintentional splicing of the primary transcripts, which may prevent the successful application of our rescue strategy to specific arenaviruses or strains.

We have expressed viral RNA segments of genomic (−) polarity bearing the potential risk of hybrid arrest, i.e., the annealing of L- and NP-mRNAs with the unencapsidated genomic L- and S-segment RNAs, respectively. It had been proposed that this process could prevent ribonucleoprotein formation and thereby the initiation of the infectious cycle. The efficient recovery of virus by using genomic polarity transcripts does not exclude that hybrids form, but it suggests that hybrid arrest was not a major limiting factor in this setting. Preliminary data suggest, however, that virus can also be recovered when RNA of antigenomic polarity is expressed (data not shown). For future attempts at rescuing other arenaviruses from cDNA, it may be worth trying both polarities in parallel to enhance the likelihood that other potential pitfalls like terminator-like sequences in the transcript (see above) can be avoided.

The LCMV rescue system for Cl13 presented here and the T7-driven LCMV rescue system (34) should enable us to tackle a number of long-standing questions about the virus’s molecular biology, its relationship with the host cell, and the specific role of the four known viral genes in various models of viral pathogenesis (3, 16, 18, 19). Chronic infection with Cl13 represents one of the prototypical small-animal models for investigating basic mechanisms of T cell dysfunction in chronic infection and viral interference with antigen presentation as a substrate of virus-induced immunosuppression (18, 19). The ability for directed engineering of the entire LCMV genome will offer unique opportunities for investigating the viral determinants thereof and studying the immunological pressure exerted on the persisting virus. In addition, the methodology and vector combinations used here should allow for the rescue of other arenaviruses, including Lassa fever virus and the South American hemorrhagic fever viruses, from plasmid. This technology may accelerate the development and fine-tuning of live-attenuated arenavirus vaccines (16) and may substantially facilitate their safe production for use in endemic areas where they are urgently needed (5, 31).

Materials and Methods

Plasmids, Cells, and Transfections. The pC-NP plasmid has been described (7, 8). For generation of pC-L-C13, the C13 L ORF was amplified by RT-PCR in three separate fragments that were merged for insertion into the pC backbone (27), analogously to a strategy described for Lassa fever virus (ref. 12 and see Fig. 6, which is published as supporting information on the PNAS web site). pI-S-C13(−)• was generated by inserting a C13-GP cDNA into the pBS-Bsm(−) backbone that expresses under the pol I LCMV S-segment cDNA with a linker instead of the GP ORF (15). pL-L-C13(−) is based on the same pol I vector as pI-S-C13(−)• (14) and was assembled in a multistep cloning procedure from RT-PCR fragments with master consensus sequence (for details of the cloning strategy see Fig. 6; primers used for cloning of the L-segment constructs are listed in Table 1, which is published as supporting information on the PNAS web site). The AvaII site in the Z ORF was introduced in a standard two-way PCR approach. Additional primer sequences and PCR conditions are available on request. BHK-21 cells were transfected by using Lipofectamin (5 μl/μg DNA; Invitrogen).

Viruses, Viral Immunofocus Assay, and Identification of Genetic Tags. The ARM-derived variant C13 (17) was obtained from Rafi Ahmed (Emory Vaccine Center, Atlanta), and passage 5 was used for RT-PCR cloning and mouse experiments. ARM53b was obtained from Michael Buchmeier (The Scripps Research Institute). LCMV titers were determined in a NP-specific immuno-focus assay as described (32).

For identification of the genetic tags, total cellular RNA was extracted from virus-infected cells by using Trizol (Invitrogen) or from mouse serum by using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). RT was carried out with random hexamers by using Superscript II RT pol (Invitrogen), and Taq pol (NEB, Beverly, MA) was used for PCR with gene-specific primers 5’-GCATTGCTGGTGCTAGCTTA-3’ and 5’-CAATGAGTTGTACTAACGGC-3’ for the S segment or with primers 5’-TATGAGACAGAAGGTGCGACACGCCGGGATCTAAGGCGTTTAG-3’ (unpaired overhang in italics) and 5’-CTTGGTAGGAGGTGAGAGG-3’ for the L segment. EcoNI or AvaII digestion of the PCR products was performed without prior purification, which explained occasionally incomplete digestion.

Mice and Animal Experiments. C57BL/6 mice were bred at the Institut für Laboratoriumsmedizin am Universitäts-Spital Zürich and housed under specific pathogen-free conditions for the experiments. All animal studies were carried out with authorization by the Kantonale Veterinarmedizin and in accordance with Swiss law for animal protection.

Intracellular Cytokine Assay and VSV Neutralization Assays. Peptide stimulation and intracellular staining for IFN-γ have been described (33). Anti-CD8α-phycocerythrin and anti-IFN-γ-allophycocyanin were purchased from Pharmingen. VSV neutralization assays were carried out as described (32). Neutralizing IgG was measured by 2-mercaptoethanol resistance because secondary antibodies could not be used (32). Neutralizing activity exceeding the 2-mercaptoethanol-resistant fraction by 4-fold or more was considered IgM.

Viral Sequence Analysis. Sequencing protocols are outlined in detail in Supporting Text. Briefly, Cl13 virion RNA was purified, and cDNA was generated by using specific primers by using superscript II RT pol (Invitrogen) or Omniscript RT pol (Qiagen) for the L-IGR. PCR was carried out with PFU Turbo (Stratagene) or Amplitaq Gold (PerkinElmer) for the L-IGR. PCR products were either sequenced directly or first cloned into the PGEMT vector (Promega). The S-segment terminal sequences were determined by a RNA circulation-based protocol. For 5’ RACE analysis of the L-segment termini, protocols were adapted from the Invitrogen kit manual, 3’ RACE System for Rapid Amplification of cDNA Ends.

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