Lymphocytic choriomeningitis virus (LCMV), the prototypic arenavirus, is an enveloped virus with a bisegmented negative-strand (NS) RNA genome (9, 24). Each of the two-genome segments, designated L (ca. 7.2 kb) and S (ca. 3.4 kb), expresses two viral gene products using an ambisense coding strategy. The S RNA directs the synthesis of the nucleoprotein (NP) (ca. 63 kDa) and the GP-C enveloped glycoprotein precursor. NP, encoded in the antisense genome, is the most abundant viral protein and encapsidates viral genomes and antigenspecific replicative intermediates. GP-C, encoded in genome polarity, is posttranslationally cleaved by cellular subtilase S1P into the mature viral glycoproteins GP-1 (40 to 46 kDa) and GP-2 (35 kDa) (2, 28). Noncovalently associated GP1/GP2 complexes make up the spikes on the virion envelope and mediate virus interaction with the host cell receptor (11, 40). The L segment codes for the virus RNA-dependent RNA polymerase (RdRp) (L, ca. 200 kDa) (32) and a small (11-kDa) RING finger protein called Z (32, 33) that functions as the arenavirus counterpart of the matrix protein found in many NS RNA viruses (27). Additional roles of Z in the arenavirus life cycle have been proposed based on its interaction with several host cell proteins (3, 4, 10) and its ability to inhibit RNA synthesis mediated by the LCMV polymerase (13).

The RdRps of many NS RNA viruses consist of L, a multifunctional enzyme that appears to possess all of the enzymatic activities associated with RNA synthesis, and a virally encoded phosphoprotein cofactor (15). However, there is no evidence that such a cofactor is required for the formation of the arenavirus functional RdRp (21, 23). The determination of the crystal structures of the RdRps of three different viruses revealed common structural features despite a significant overall sequence divergence (1, 7, 22, 25). In addition, sequence alignments among more than 80 L proteins of NS RNA viruses have identified six conserved regions, designated domains I to VI, which were proposed to specify the essential functional features common to all L proteins (30). Within domain III are found the conserved A, B, C, and D motifs, which are thought to form the module containing the active site in RNA synthesis (29). These predictions are well supported by functional data obtained from mutational-analysis studies with several L proteins (12, 14, 16, 19, 34, 35, 37, 38, 43).

As with other RdRps, arenavirus L proteins, including those of LCMV, have the characteristic conserved A, B, C, and D motifs within domain III (29) (Fig. 1A). The development of arenavirus reverse-genetics systems has opened new avenues for studies aimed at determining the structure-function relationships of the arenavirus polymerase. Here we have used a LCMV minigenome (MG) rescue assay to investigate the role of the highly conserved A and C motifs in arenavirus polymerase activity.

Effect of mutations in motifs A and C within conserved domain III of the LCMV L protein on LCMV polymerase activity. To investigate the role of conserved amino acid residues in motifs A and C on the polymerase activity of the LCMV L protein, we mutated these residues to conserved and nonconserved amino acids (Fig. 1B). Motif A contains an aspartate (D) residue that is conserved in all RNA polymerases, and evidence indicates that this D residue appears to be essential for the catalytic activity of L polymerases (26). Motif C contains highly conserved amino acid residues that represent specific signatures of each major group of RNA viruses. Thus, the sequence GDD is conserved among polymerases of positive-strand (PS) RNA viruses, whereas GDN and SDD are...
conserved signature motifs for the L polymerases of nonsegmented NS (NNS) and segmented NS (SNS) RNA viruses, respectively.

We first confirmed all mutations by sequencing and subsequently analyzed their polymerase activities in vivo using an LCMV MG rescue assay (21). For this assay, BHK-21 cells (7 × 10⁵ cells per well in M6 plates) were infected (multiplicity of infection [MOI] = 3) with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase (VVT7) and subsequently transfected with plasmids encoding the LCMV MG and NP, together with wild-type (wt) or mutant L proteins under the control of the T7 promoter. Twenty-four hours later, cell extracts were prepared and analyzed for their levels of chloramphenicol acetyltransferase (CAT) activity. In this assay, the intracellularly T7-synthesized MG RNA is encapsidated by plasmid-supplied NP to generate the RNP template that can be replicated and transcribed by the intracellularly reconstituted virus polymerase. Therefore, expression of the MG, as determined by levels of CAT activity, is an accurate surrogate marker of the virus polymerase activity. Our results showed that all of the mutations introduced in motifs A and C resulted in L proteins devoid of polymerase activity, as determined by the lack of detectable CAT activity (Fig. 2A, lanes 1 to 10).

The lack of CAT activity associated with lysates from cells transfected with the mutant polymerases might be a consequence of the altered expression or stability of the mutated L proteins. However, results from Western blot analysis showed...
that all of the mutant L proteins were expressed in amounts similar to that of wt L (Fig. 2A), indicating that none of the mutations had a significant effect on protein expression or stability. Another concern would be that the experimental approach we used for the mutagenesis of L might systematically introduce additional unnoticed mutations that could affect polymerase function. To address this issue, we used the same experimental procedures to introduce mutations at amino acid position 1079 that converted the sequence found in LCMV strain Armstrong (ARM) L into that found in Cl-13, a variant of ARM that exhibits a distinct immunosuppressive phenotype in vivo (6). As predicted, these changes resulted in a functional L polymerase as determined by levels of MG-derived CAT activity (Fig. 2A, lanes 13 and 14). These findings indicated that technical reasons related to the mutagenesis approach used were not responsible for the lack of polymerase activity of the mutant L proteins. Notably, amino acid substitutions K1079N and K1079Q found in Cl-13 L proteins caused 5 (K1079Q)- and 10 (K1079N)-fold reductions in virus polymerase activity (Fig. 2B).

Effect of mutations in motifs A and C on RNA synthesis mediated by the LCMV L polymerase. L polymerases of NS RNA viruses perform two distinct biosynthetic processes, namely, replication and transcription of the genome RNA. Mutations affecting either one, or both, of these activities would affect levels of MG-derived CAT activity. We therefore determined levels of CAT mRNA and anti-MG (aMG) RNA species to assess the effects of the introduced mutations on MG transcription and RNA replication, respectively. For this determination, we infected cells with VVT7 (MOI = 3) and transfected them with the indicated L-expressing plasmids together with the LCMV MG and NP plasmids. RNA was isolated 24 h later and analyzed by Northern blotting using a CAT antisense riboprobe to detect both CAT mRNA (transcription) and aMG RNA (replication) species. Consistent with the CAT activity results (Fig. 2A), levels of both CAT mRNA and aMG RNA species were undetectable by Northern blotting for all RNA samples from cells transfected with L polymerases carrying mutations within the A and C motifs (Fig. 2C, lanes 1 to 11, bottom). These results indicated that mutations within motifs A and C affected both RNA replication (aMG) and transcription (CAT mRNA) mediated by the LCMV polymerase similarly. Hybridization of the same membrane to a CAT sense probe confirmed that levels of MG RNA generated via T7-mediated transcription and subsequent processing, via self-cleaveage of the hepatitis delta virus ribozyme, were similar for all samples (Fig. 2C, top). This result allowed us to exclude the idea that the differences in levels of MG RNA available for

For the indicated samples, chloramphenicol conversion was quantified by phosphorimager analysis. The percentage of conversion obtained in the absence of L was considered to be nonspecific background. This background conversion value was subtracted from the conversion values obtained for each sample. (C) Effect of mutant L proteins on LCMV MG RNA replication and transcription. BHK-21 cells were infected with VVT7 and transfected as in panel A. Twenty-four hours later, total cellular RNA was isolated and equal amounts of each sample were analyzed by Northern blot hybridization using the indicated CAT strand-specific RNA probes.
template formation contributed to the differences in levels of MG expression.

Mutants SD-GN (SDD to GDN) and S1323G (SDD to GDD) (Fig. 1B) recreated the conserved residues found in motif C of L polymerases of RNA viral NNS and PS, respectively (41). However, both types of mutations resulted in L proteins with undetectable levels of polymerase activity. The change from SDD to GDN in L of the SNS RNA bunyavirus also resulted in a nonfunctional virus polymerase (19). Concordant findings have been reported for the L protein of rabies virus (RV), where mutations changing the GDN (NNS) to SDD (SNS) or to GDD (PS) within motif C resulted in a nonfunctional RV polymerase (34). Likewise, changes in the residue preceding the DD cores of the Qbeta bacteriophage (18) and influenza virus PB1 polymerases (2) resulted in enzymes without transcriptional activity. Also, the mutation GDN to GDD in the L protein of the prototypic mononegavirus vesicular stomatitis virus resulted in a virus polymerase still exhibiting 25% activity as measured in an in vitro transcription assay (35). The reasons for the different effects of the same mutation on the RV and vesicular stomatitis virus L proteins remain to be determined, but it cannot be ruled out that they reflect differences in the experimental assays used to assess polymerase activity.

LCMV polymerase activity in cells coexpressing nonfunctional mutant and wt L proteins. Intragenic complementation has been documented for the L genes of several NS RNA viruses (31, 36). Consistent with this, direct L-L physical interaction has been demonstrated for the paramyxoviruses Sendai virus (36) and parainfluenza virus type 3 (PIV3) (39), and
evidence indicates that in these cases, L oligomerization is required for polymerase activity. We therefore examined whether a similar finding also applied to the LCMV L protein. For this we examined MG activity in cells cotransfected with a variety of pair combinations of nonfunctional L proteins containing mutations within the A and C motifs. None of the pair combinations of L mutant proteins tested resulted in detectable levels of polymerase activity as determined by MG-derived CAT activity (data not shown). This finding is consistent with results reported for the Sendai virus L protein, where intragenic complementation was not observed among mutants with mutations within domain III (38).

As an alternative approach to examining the possible L-L genetic interaction, we assessed whether mutant L proteins exhibited a dominant negative (DN) phenotype when coexpressed together with wt L. This approach has been successfully used to examine the oligomeric structures of recombinant NMDA receptors (20). For this examination, we cotransfected cells with a fixed amount (250 ng) of wt polymerase and increasing amounts (0 to 250 ng) of representative motif A and C mutants (not shown). To assess virus polymerase activity in cells cotransfected with wt- and mutant-L-expressing plasmids (at a ratio of 1:1), levels of MG-associated CAT activity (Fig. 3A) were quantified with a phosphorimager (Fig. 3B). The four mutants assayed at position 1182 in motif A exhibited a strong dose-dependent inhibitory effect on wt polymerase activity, a finding characteristic of mutants with a DN phenotype. Notably, the polymerase activity of wt L was reduced 20- to 30-fold when cotransfected with motif A mutant plasmids at a 1:1 ratio (Fig. 3, compare lane 2 with lanes 5 through 8). We observed similar results with motif C mutants with the mutations D1324A, D1324N, SD-GN, and S1323G (Fig. 3, lanes 9, 11, 13, and 14). In contrast, D1324E and D1324R motif C mutants did not appear to have a DN phenotype (Fig. 3, lanes 10 and 12).

The results obtained with proteins with mutations at position 1182 in motif A of the L protein were suggestive of the L-L interaction being required for LCMV polymerase activity. Nevertheless, the outcome of this type of experiment, and hence our interpretation, can be significantly influenced by competition between wt and mutant L proteins for RNA templates or other factors required for LCMV polymerase activity. We could rule out this concern based on the observed L dose response of MG expression. Levels of MG-derived CAT activity increased in response to increasing amounts (150 to 500 ng) of wt L plasmid DNA. This suggests that the levels of total L protein produced by the amount of L plasmid DNA (250 ng) used in the cotransfection assays was below that required to create a situation where template or cellular factors required for LCMV polymerase become limiting factors.

**Physical interaction of LCMV L proteins.** The strong DN effect exhibited by the majority of L mutants on the polymerase activity of wt L protein provided us with genetic evidence in support of the L-L interaction being required for the activity of the LCMV polymerase. Consequently, we sought direct evidence that in these cases, L oligomerization is required for polymerase activity. We therefore examined whether a similar finding also applied to the LCMV L protein. For this we examined MG activity in cells cotransfected with a variety of pair combinations of nonfunctional L proteins containing mutations within the A and C motifs. None of the pair combinations of L mutant proteins tested resulted in detectable levels of polymerase activity as determined by MG-derived CAT activity (data not shown). This finding is consistent with results reported for the Sendai virus L protein, where intragenic complementation was not observed among mutants with mutations within domain III (38).

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dence of whether the LCMV L protein also has, as with Sendai virus and PIV3 L proteins, the property of forming oligomeric structures. For this purpose, we generated LCMV L proteins containing two different tags, hemagglutinin (HA) and Flag, at their C termini. We first verified that both L-HA and L-Flag could be expressed to levels similar to those of wt L (Fig. 4A). As predicted, L-Flag was detected by the anti-Flag antibody but not the anti-HA antibody, and conversely, L-HA was detected by the anti-HA antibody but not the anti-Flag antibody (Fig. 4A). Moreover, both L-HA and L-Flag retained wt levels of polymerase activity as determined by their ability to support MG-derived CAT activity in the LCMV MG rescue assay (Fig. 4B). We then used a communoprecipitation assay to examine whether L-HA and L-Flag interacted physically. For this assay, we prepared cytosolic extracts from cells transfected with either each tagged L protein alone or the tagged L proteins together. An analysis of total cell lysates by Western blotting using an antibody to HA showed that L-HA was detected when expressed either alone or together with L-Flag, whereas L-Flag expressed alone was not detected by the antibody to HA (Fig. 4C). We then used aliquots of cell extracts for communoprecipitation with the anti-Flag M2 affinity gel freezer (A-2220; Sigma). Immunoprecipitated samples were analyzed by Western blot analysis using an antibody to HA (Fig. 4C). L-HA was detected in samples immunoprecipitated from lysates prepared from cells expressing both L-Flag and L-HA but not in the case of cells expressing only L-HA or L-Flag proteins. This result indicated the formation of L-HA/L-Flag complex in cells expressing both proteins, thus providing biochemical evidence of an L-L interaction. As with the Sendai virus L protein, the LCMV L-L interaction appears to require cotranslation of the interacting proteins based on the observation that L-Flag and L-HA did not communoprecipitate if these proteins were provided by mixing lysates of cells transfected individually with each protein (data not shown).

Our findings demonstrate that the sequence SDD characteristic of motif C of SNS RNA viruses, as well as the presence of the highly conserved D residue within motif A of L proteins, is strictly required for the polymerase activity of the LCMV RdRp. Sequence comparison of arenavirus L proteins (42) strongly suggests that these findings are likely to be common features of all arenavirus L proteins. The genetic evidence provided by the strong DN phenotype associated with many of the mutants examined for motifs A and C of the LCMV L protein, together with biochemical data from communoprecipitation studies, strongly suggests that formation of L-L complex is required for the polymerase activity of LCMV L protein. This finding extends to the arenavirus observations previously documented for the paramyxoviruses Sendai virus and human PIV3 (39).

Arenaviruses include clinically important human pathogens that cause severe hemorrhagic fever (HF), such as the Lassa fever virus and the South American HF viruses (17). Moreover, because of the severe morbidity and high mortality it causes, the lack of immunization and an effective treatment, and its ease of introduction into a susceptible population, Lassa fever virus is included in category A of potential bioterrorism microbial weapons (5, 8). Therefore, the development of novel effective antiviral approaches to combat HF arenaviruses is important. Evidence suggests that mammalian cells are devoid of enzymes with strong similarities to the viral L proteins. Hence, it might be feasible to target these viral enzymes without significantly compromising the physiology of infected cells. The evidence that the L proteins of the paramyxoviruses Sendai virus and human PIV3, and now also the prototypic arenavirus LCMV, appear to function as oligomeric structures raises the possibility of developing antiviral strategies aimed at disrupting the formation of L-L complexes, which is predicted to abrogate virus RNA synthesis. A detailed characterization of the structural and functional domains of the arenavirus L proteins will help to develop such antiviral approaches to combat pathogenic arenaviruses.

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