Complete Genome Sequence of Fer-de-Lance Virus Reveals a Novel Gene in Reptilian Paramyxoviruses

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The first recorded epidemic in reptiles caused by a paramyxovirus occurred in a Swiss serpentarium in 1972 (16), resulting in the isolation of Fer-de-Lance virus (FDLV) from a Fer-de-Lance viper (Bothrops atrox) (16). Since that time, over 40 paramyxovirus-like agents have been isolated from various reptile species and described in the literature (1, 2, 14, 17). These viruses have hemagglutination and neuraminidase activity, and in reptile cell lines they replicate at 28 to 30°C, causing cytopathic effects including syncytia, cytoplasmic inclusion bodies, and lysis. Serological studies indicate that many of these agents are antigenically related (3, 10, 26, 48, 50). Clinically, these viruses are serious pathogens causing epidemics with high mortalities in captive reptile populations in snake farms and zoological collections (14, 27). Infected animals show respiratory signs of severe proliferative pneumonia, sometimes accompanied by nervous disorders and pancreatic necrosis. Although the prevalence of paramyxoviruses in wild reptiles is uncertain, the viruses have been isolated from wild hosts (30) and described as potential agents of emerging infectious disease that may threaten wildlife health (11, 28). Despite this history, there has been no genetic characterization of these viruses until recent publications describing partial genome sequences from other reptile paramyxoviruses until recent publications describing partial genome sequences from other reptile paramyxoviruses (2, 17, 30, 33). The FDLV genome sequence described here is the first complete genome sequence of a reptile paramyxovirus, FDLV, has been determined. The genome is 15,378 nucleotides in length and consists of seven nonoverlapping genes in the order 3’ N-U-P-M-F-HN-L 5’, coding for the nucleocapsid, unknown, phospho-, matrix, fusion, hemagglutinin-neuraminidase, and large polymerase proteins, respectively. The gene junctions contain highly conserved transcription start and stop signal sequences and tri-nucleotide intergenic regions similar to those of other Paramyxoviridae. The FDLV P gene expression strategy is like that of rubulaviruses, which express the accessory V protein from the primary transcript and edit a portion of the mRNA to encode P and I proteins. There is also an overlapping open reading frame potentially encoding a small basic protein in the P gene. The gene designated U (unknown), encodes a deduced protein of 19.4 kDa that has no counterpart in other paramyxoviruses, and has no similarity with sequences in the National Center for Biotechnology Information database. Active transcription of the U gene in infected cells was demonstrated by Northern blot analysis, and bicistronic N-U mRNA was also evident. The genomes of two other snake paramyxovirus genotypes were also found to have U genes, with 11 to 16% nucleotide divergence from the FDLV U gene. Pairwise comparisons of amino acid identities and phylogenetic analyses of all deduced FDLV protein sequences with homologous sequences from other Paramyxoviridae indicate that FDLV represents a new genus within the subfamily Paramyxovirinae. We suggest the name Ferlaviridae for the new genus, with FDLV as the type species.

The RNA genome of approximately 15,500 nucleotides (nt) in the order 3’ N-P-M-F-HN-L 5’, indicating the nucleocapsid, phospho-, matrix, fusion, hemagglutinin-neuraminidase, and large polymerase proteins, respectively. The gene junctions contain highly conserved transcription start and stop signal sequences and tri-nucleotide intergenic regions similar to those of other Paramyxoviridae. The FDLV P gene expression strategy is like that of rubulaviruses, which express the accessory V protein from the primary transcript and edit a portion of the mRNA to encode P and I proteins. There is also an overlapping open reading frame potentially encoding a small basic protein in the P gene. The gene designated U (unknown), encodes a deduced protein of 19.4 kDa that has no counterpart in other paramyxoviruses, and has no similarity with sequences in the National Center for Biotechnology Information database. Active transcription of the U gene in infected cells was demonstrated by Northern blot analysis, and bicistronic N-U mRNA was also evident. The genomes of two other snake paramyxovirus genotypes were also found to have U genes, with 11 to 16% nucleotide divergence from the FDLV U gene. Pairwise comparisons of amino acid identities and phylogenetic analyses of all deduced FDLV protein sequences with homologous sequences from other Paramyxoviridae indicate that FDLV represents a new genus within the subfamily Paramyxovirinae. We suggest the name Ferlaviridae for the new genus, with FDLV as the type species.

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dase, and large polymerase protein genes, respectively. Beyond this basic similarity, however, there is diversity in overall genome length, presence or absence of overlapping open reading frames (ORFs) within these invariant genera, genome terminal sequences, length and sequence of intergenic and untranslated regions, transcription regulatory signals, hexamer phasing positions of transcription units, and editing strategies for P gene expression. This variation, along with phylogenetic analyses, has resulted in the recent establishment of the new genera Henipavirus, including HeV and NIV (8, 19, 57, 59), and Avutavirina, including avian rubulaviruses with Newcastle disease virus (NDV) as the type species (12, 51). Thus, there are now five recognized genera in the subfamily Paramyxovirinae.

Among these efforts to broaden and improve the taxonomy within the mammalian and avian Paramyxoviridae, we now present the first complete genome sequence of a paramyxovirus from a reptile host. This sequence has revealed a novel transcription unit potentially encoding a 19.4-kDa protein between the N and P/V genes and a mosaic of other features that suggest that reptile paramyxoviruses will likely comprise another new genus within the subfamily Paramyxovirinae.

MATERIALS AND METHODS

Genome sequencing. The FDLV strain was obtained from the American Type Culture Collection (ATCC VR-895) and propagated in the IgH2 iguana heart cell line (ATCC CCL-108; Rockville, Md.) at 28°C (2). Construction of a random-primer cDNA library to purified viral genomic RNA has been described previously (2). Clones with significant alignment to paramyxovirus sequences were identified by searches of the National Center for Biotechnology Information (NCBI) database. Primers for reverse transcription (RT)-PCR amplification across gaps not covered by the cDNA clones were designed by using terminal sequences of clones containing N, P, F, HN, and L gene sequences. PCR amplification and sequencing of products were as described (2).

Sequences of the 3′ and 5′ genomic termini were determined by rapid amplification of cDNA ends (5′ RACE; Gibco BRL) with primers designed from sequences near the FDLV genomic termini. For 3′-end analysis, total RNA from FDLV-infected IgH2 cells was used as a template for amplification with three FDLV N gene-specific primers (GSP) that bind to the N gene mRNA or anti-genome of FDLV: GSP1, 5′-CCATTGCGAAAATCTC-3′; GSP2, 5′-AACCT CATTATCGGGTATG-3′; and GSP3, 5′-ATGCACTGACCTGAGATG-3′. FDLV genomic RNA extracted from infected cell culture supernatant was used to amplify the 5′ end of the genome by using three FDLV L gene primers: GSP1, 5′-TATTTAGACAAGATGAC-3′; GSP2, 5′-CAGAGCTTGGACTAAGGC-3′; and GSP3, 5′-CTAAAAGACATACCCCTGCG-3′. PCR products were purified from a low-melt agarose gel (Wizard kit; Promega) and sequenced.

In the complete genome sequence, 85.6% of the nucleotide sites were determined in multiple cDNA clones or PCR fragments, and 14.4% of the sequence was determined by the sequence of a single cDNA clone. Thus, a minor portion of the genome sequence may contain quasispecies sequence microheterogeneity relative to the consensus of the original FDLV template population. Sequence data were analyzed with MacVector 6.0 and AssemblyLIGN 1.0.9 software (Oxford Molecular Group). Phylogenetic analyses were carried out with the neighbor-joining and parsimony programs in the PAUP* version 4.0b software package (53). Consensus trees were derived from 1,000 bootstrapped replicates of the data sets, and bootstrap values above 70 were considered significant (23).

Analysis of P/V mRNA editing. Confluent IgH2 cells were infected with FDLV at a high multiplicity of infection and allowed to replicate at 28°C for 3 days. Polyadenylated mRNA was extracted (Micro-Fast Track 2.0; Invitrogen) and copied in a RT reaction mixture (13) containing antisense primer 5′-ACACCTC TCCGACAGCA-3′ and 200 U of Moloney murine leukemia virus RT (USB) enzyme. The CDNA was treated with 0.7 mg per ml of RNase A for 15 min at 37°C and heated to 95°C for 2 min before being added to a 50-μl single-round PCR containing 50 pmol of the antisense primer described above, 50 pmol of sense primer 5′-CCGGTGCAAAGCCGGA-3′, and 2.5 units of Taq DNA polymerase (USB). Reaction conditions were as described (2), with an annealing temperature of 50°C and a final extension step at 72°C for 7 min. The 336-bp product was purified (StrataPrep PCR kit; Stratagene) and cloned by using the Perfectly Blunt pSTBlue-1 Cloning kit (Novagen). Clones were screened by the rapid colony PCR procedure and sequenced as above.

Northern blot analysis of U gene transcription. Total RNA (TotALLY RNA; Ambion Inc.) or mRNA (Micro-FAST Track 2.0) was extracted from IgH2 cells 2 to 3 days postinfection with a high multiplicity of FDLV infection or from uninfected IgH2 cells (3 × 106 cells each). Radioactive 32P-labeled single-stranded RNA probes specific for FDLV U ("unknown") and N gene sequences were created with the Lign/Scribe kit protocols (Ambion Inc.). Initial templates for the probes were PCR products representing a 382-bp region of the U gene (nt 1670 to 2057 of the FDLV genome) and a 369-bp region of the N gene (nt 535 to 903). Single-round PCRs were performed as described above using 5′-ATG TCAACCCCCATCAA-3′ and 5′-CCACCTGGTATCAACCA-3′ primers for the U gene and 5′-GGTCAAACAGGCTGATG-3′ and 5′-TCTGAAAGAA ACGAGTGAAG-3′ primers for the N gene. Denaturing gel electrophoresis, blotting, hybridization at 68°C, and autoradiography were as described (Northern-Max; Ambion).

Determination of U gene sequences from other snake paramyxoviruses. Snake paramyxovirus isolates Gono-GER85 and Bti-Ca98 were originally from Germany and California, respectively, and have been described (2). These viruses were grown in IgH2 cells at 28°C and used as templates for a first round of RT-PCR (2) with the sense primer 5′-CTATAGGCCAGAAAATGG-3′ and antisense primer 5′-CTTGTTAGGTTGTGAAAC-3′ to amplify a 1,450-bp region surrounding the U gene. A second-round PCR was performed with primers 5′-GGCTATAGCTGGCATGTC-3′ and 5′-AAGGCTTGCTGTTCG AAA-3′ to yield a 777-bp amplification product. The second-round primers were used to sequence the purified PCR products (StrataPrep; Stratagene).

Nucleotide sequence accession number. The complete FDLV genome sequence has been deposited in the GenBank database under the accession number AF141760.

RESULTS

Cloning of FDLV genomic RNA. Several initial attempts to amplify regions of purified FDLV genomic RNA by using degenerate primers designed to anneal with conserved sequences from mammalian and avian paramyxoviruses failed to generate products by RT-PCR (data not shown). Therefore, a randomly primed cDNA library was generated with FDLV genomic RNA as the template. Twenty-five clones were identified that had insert sequences with significant similarity to paramyxovirus N, P, F, HN, or L gene sequences in the NCBI database. The complete sequences of these clones represented 57% of the final FDLV genome sequence. A total of eight RT-PCR fragments, comprising 35% of the final sequence, were then generated and analyzed to fill the gaps between the cDNA clones. Rapid amplification of cDNA ends was used to determine 704 nt at the 3′ genome terminus and 519 nt at the 5′ terminus.

FDLV transcription regulatory signals and genomic termini. The complete FDLV genome is 15,378 nt in length. This length is very similar to the uniform size range that has been described for most paramyxovirus genomes (37, 38), and it is distinct from the unusually long genomes described recently for HeV and NIV (19, 59), and the FDLV genome is evenly divisible by six, so it is consistent with the rule of six described for members of the subfamily Paramyxovirinae (5, 34).

The FDLV genome sequence contained six putative gene junction sequences that were highly conserved within FDLV and with known gene junctions of other paramyxoviruses (Fig. 1). The FDLV transcription start signals consisted of a 10-base sequence that was strictly conserved for five of the seven genes, and only a single base varied for the other two genes. The consensus of these FDLV transcription start signals was most similar to the consensus start signal for HeV, but similarity was
A. Fer-de-Lance Virus Genome

The FDLV genome begins with a 5'-leader sequence (Fig. 2A). The length of the 3' leader is exactly conserved with all other known paramyxoviruses (38). The 5' leader length is within the 25- to 58-nt range seen in the Respirovirus, Morbillivirus, Rubulavirus, and Henipavirus genera. The FDLV leader and trailer sequences are exactly complementary for the first 18 nt, and if a 2-base gap is allowed in the alignment, then 30 out of the 36 bases in the 5' trailer can form complementary base pairs with the 3' leader (Fig. 2A). The sequence of the first 12 to 13 nt at the 3' ends of paramyxovirus genomes has been found to be genus specific, and it is considered critical as a promoter element for the viral RNA-dependent RNA polymerase (38). The 3' terminal sequence of FDLV is highly conserved with the Henipavirus genus and also with the Respirovirus and Morbillivirus genera (Fig. 2B).

FDLV genome organization. The gene junction sequences of FDLV defined seven discrete, nonoverlapping transcription units. Analysis of the ORFs indicated that five of the transcription units (the first and the fourth through seventh) each contained a single large ORF capable of encoding a protein that could be identified by database similarity searches as one of the known invariant paramyxovirus proteins: N, M, F, HN, and L. The second transcription unit encoded a small protein that had no discernible similarity to any sequence in the NCBI database. The gene and its putative protein product were designated U. The ORF in the third transcription unit was less than half the length of the gene and encoded a protein with similarity to paramyxovirus V proteins. A second ORF in an overlapping reading frame encoded a protein with similarity to the carboxyl terminus of paramyxovirus P proteins. Thus, the third ORF is the paramyxovirus P/V gene. The FDLV genome was thus determined to contain seven genes in the order 3'-N-U-P/V-M-F-HN-L-5' (Fig. 1). The presence of an additional gene between the N and P/V genes is a unique feature not seen in any other paramyxovirus.

A summary of the features of the seven FDLV genes and their deduced proteins is provided in Table 1. The total coding percentage for the FDLV genome is 92%, which is exactly the percentage found in all Paramyxovirinae genera except henipaviruses, which have 82 to 84% (59). Subunit hexamer phasing positions for the start sites of the six FDLV genes that are common to all Paramyxovirinae are shown in Table 2. The pattern of the FDLV gene start sites is unique relative to the patterns for other paramyxoviruses, which have been shown to be genus specific within the Paramyxovirinae (19, 34, 59). The FDLV pattern is most similar to that of the morbilliviruses MeV and Rinderpest virus, matching at four out of the six start sites. As has been noted previously (34), position 5 is not used as the start site for any paramyxovirus gene, including those of FDLV.

FDLV structural genes. The N protein of paramyxoviruses is an abundant virion protein that encapsidates the viral RNA to form the nucleocapsid. The FDLV N gene transcription unit contains a single large ORF coding for a 471-amino-acid (aa) protein with a calculated size of 51.3 kDa (Table 1). This size is slightly smaller than the currently known range of paramyxovirus N proteins, which is 489 to 553 aa (38). The first AUG codon for this ORF is in strong context for translation initiation (35), and the next AUG is 327 nt downstream, suggesting that the first AUG is the most likely start site. Pairwise align-
ments indicated that the FDLV N protein has relatively low levels of amino acid identity with the N proteins of viruses in the Paramyxovirinae subfamily, ranging from 21 to 27%, and it has even less identity with HRSV in the Pneumovirus subfamily (Table 3). Alignment of the FDLV N protein sequence with N proteins of 16 other paramyxoviruses clearly showed that the amino-terminal 85% of the protein (aa 1 to 398) was relatively well conserved, while the carboxy-terminal 15% (aa 399 to 471) had almost no similarity (data not shown). This pattern is consistent with previous descriptions in which the C-terminal 20% of paramyxovirus N proteins is poorly conserved and contains most of the phosphorylation and antigenic sites (38). Within the conserved domain of the N proteins, the Y260 and F324 residues that have been reported to be conserved in all Paramyxovirinae are present in FDLV as Y253 and F317, respectively. These amino acids are important for RNA binding and N-N protein self-assembly, respectively (40). The FDLV F317 is also the start of a larger conserved motif found near the middle of all paramyxovirus N proteins, F-X4-Y-X4-SYAMG (38). In FDLV this motif is present with a single amino acid difference, as F-X4-Y-X4-SFAMG.

FIG. 2. FDLV genomic termini. Complementarity between the FDLV 3' and 5' genomic termini is shown in panel A, with shading indicating complementary base pairs and dashes indicating deletions in the alignment. Comparisons of the FDLV 3' terminal 20 nt with 3' terminal sequences of other paramyxoviruses is shown in panel B, with shading indicating identical conserved residues. Lowercase letters indicate bases that do not form complementary base pairs between the 3' and 5' genomic ends of each virus (note that no genomic trailer sequence is available for MenV). Numbers of bases matching the FDLV 3' 20-nt sequence are shown to the right of each virus terminal sequence, followed by the GenBank accession number source for each sequence. Abbreviations: PIV3, parainfluenza virus 3; CDV, canine distemper virus; SV5, simian virus 5; VSV, vesicular stomatitis virus.

| TABLE 1. FDLV genome transcription units and deduced protein products |
|-----------------------------|-----------------------------|-----------------------------|
| **FDLV gene** | mRNA features (nt) | Deduced protein features (aa) |
| | Length | 5' UTR | ORF | 3' UTR | Length | Calculated Mr | pI |
| N | 1,550 | 55 | 1,413 | 82 | 471 | 51.3 | 5.4 |
| U | 604 | 14 | 501 | 89 | 167 | 19.4 | 10.0 |
| P/V (V)* | 1,433 | 30 | 681 | 722 | 227 | 25.0 | 5.8 |
| P/V (P)* | 1,435 | 30 | 1,287 | 118 | 429 | 46.7 | 5.2 |
| P/V (I)* | 1,434 | 30 | 480 | 927 | 161 | 17.4 | 4.9 |
| M | 1,310 | 26 | 1,053 | 231 | 351 | 38.5 | 9.7 |
| F | 1,794 | 63 | 1,635 | 96 | 545 | 58.4 | 5.5 |
| RN | 1,938 | 29 | 1,692 | 217 | 564 | 61.6 | 4.9 |
| L | 6,640 | 24 | 6,543 | 73 | 2,181 | 247.0 | 6.2 |

* U gene features shown assume initiation of translation at the first AUG codon. The actual codon for initiation has not been determined (see text).

The P/V gene of FDLV has the potential to encode three proteins (V, P, and I) that are all amino coterminal for the first 158 aa.

| TABLE 2. Subunit hexamer phasing positions for gene start sites and P editing sites of selected Paramyxovirinae |
|-----------------------------|-----------------------------|-----------------------------|
| **Genus** | Virus(es) | N | P/V | M | F | HN | L | P edit |
| Unassigned | FDLV | 2 | 2 | 4 | 3 | 6 | 3 | 2 |
| Morbillivirus | MeV, RPV | 2 | 2 | 4 | 3 | 3 | 2 | 6 |
| Morbillivirus | CDV, DMV | 2 | 2 | 4 | 2 | 3 | 2 | 6 |
| Henipavirus | HeV | 2 | 3 | 4 | 4 | 3 | 5 |
| Unassigned | TPMV | 2 | 2 | 1 | 3 | 3 | 2 | 6 |
| Avulavirus | NDV | 2 | 4 | 4 | 3 | 6 | 1 |
| Respirovirus | hPIV3, bPIV3 | 2 | 1 | 1 | 1 | 1 | 2 | 2 |
| Respirovirus | SeV | 2 | 1 | 1 | 1 | 1 | 2 | 1 |
| Rubulavirus | MuV | 2 | 1 | 6 | 1 | 1 | 6 | 3 |
| Rubulavirus | SV5 | 2 | 1 | 1 | 2 | 1 | 6 | 3 |
| Rubulavirus | SV41 | 2 | 1 | 1 | 1 | 1 | 6 | 3 |

* Viruses are presented in order of similarity to FDLV hexamer phasing positions. Abbreviations (with GenBank accession numbers): RPV, rinderpest virus (X68311); DMV, dolphin morbillivirus (Z47758); hPIV3, human parainfluenza virus 3; bPIV3, bovine parainfluenza virus 3 (NC_002161); SV41, simian virus 41 (X64275). Genes unique to individual viruses are not included in the table but have the following hexamer phasing positions: 1 for the U gene of FDLV and 2 for the SH genes of MuV and SV5.
Morbillivirus, MeV, canine distemper virus, dolphin morbillivirus (DMV), and phocine distemper virus; for

has a role in blocking host interferon defense mechanisms (22, 44, 47). The

pressed from an alternative reading frame in the P gene and

HN.

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virus P genes encode only the P protein and do not edit the mRNA. NA, not applicable.

The P genes of nearly all members of the

boxy-terminal region of a paramyxovirus V protein (38, 54).

overall (Table 3), the carboxy-terminal 69 aa are extremely

c encoded after the RNA editing site.

The FDLV V and P proteins are, thus, amino coterminal for

The actual editing site sequence will be described in a separate

that is predicted to facilitate the insertion of G residues at nt

where the viral polymerase inserts nontemplated G residues

via edited mRNAs (38).

The highest percent amino acid identity observed for each protein is highlighted in bold; dashes indicate sequences that are not available. The HRSV and Ebola virus P genes encode only the P protein and do not edit the mRNA. NA, not applicable.

* V-C indicates the V protein-specific carboxy terminus encoded after the RNA editing site.

* A, attachment protein. The attachment proteins are as follows: for morbilliviruses, H; for HRSV-1, G; for Ebola virus, GP; and for FDLV and all other viruses, HN.

* Ebola virus is in the family Filoviridae and is included as an indicator of identities outside of the family Paramyxoviridae.

As reported for other paramyxovirus P proteins, the FDLV P protein is poorly conserved within the family, sharing only 12 to 18% amino acid identity (Table 3). At 429 aa, the size of the FDLV P protein is similar to the P proteins of rubulaviruses and avulaviruses, and it is shorter than the P proteins of respiroviruses, morbilliviruses, and henipaviruses (20, 56, 59). Features shared in common among most paramyxovirus P proteins are an acidic pI and a large number of potential phosphorylation sites for serine- or threonine-specific kinases (38). Accordingly, the FDLV P protein has a pI of 5.2, and there are 11 consensus recognition sites for casein kinase II and 6 sites for protein kinase C (32).

The complete FDLV V protein shares 13 to 25% amino acid identity with other paramyxovirus V proteins and is closest to rubulavirus V proteins (Table 3). In contrast, the V-specific carboxy-terminal region encoded after the editing site (aa 159 to 227) shares 34 to 51% amino acid identity with other paramyxoviruses, and the highest identity is with henipaviruses (Table 3). The V protein carboxy-terminal domain is the most conserved region among all paramyxovirus proteins. In the FDLV V protein carboxy-terminal domain, all seven cysteines known to be conserved among other paramyxovirus V proteins are present, forming the zinc finger domain (38, 34). Also in this domain, the invariant H-R-R-E motif is strictly conserved in the FDLV V protein, and the conserved W-C-N-P is present with a single amino acid change to F-C-N-P (38).

The FDLV P/V gene does not contain an ORF analogous to the C ORF of respiroviruses and morbilliviruses in an overlapping reading frame upstream of the editing site. However, there is a smaller ORF upstream of the editing site (nt 215 to 373 of the 1,433-nt P gene), that overlaps the large P/V ORF in the +1 reading frame (Fig. 3). This small ORF begins with an AUG that is not in optimal context for initiation, but the third codon is also an AUG, but in strong context. The ORF potentially encodes a 53-aa protein with a predicted Mr of 6.1 kDa and a strongly basic pI of 13.4. The protein is arginine rich and contains no acidic amino acids. A similar ORF encoding a
and with the hydrophobic tails of viral F and HN proteins (38). Structural protein in virions, and it associates with membranes for refer to the analogous ORF in FDLV as SB. ORF was designated SB, for small basic protein, and we there-

similar location within the HeV P/V/C gene (58). This HeV protein of 65 aa with a pI of 11.9 has been described in a sequence not previously noted are as follows: for parainfluenza virus 4, M55976; for SalV, AF237881. FIG. 3. FDLV P gene expression strategy and RNA editing site sequence, compared with other paramyxovirus P gene RNA editing sites. The horizontal scale at the top indicates the 1,433-nt P gene mRNA, and bars below indicate ORFs accessed in the three possible reading frames by ribosomal choice and RNA editing. In the editing site sequences, spacing between the 6 nt upstream, An, and Gn elements is to facilitate visual comparison (21, 38). Accession numbers for

fluenza virus 4, M55976; for SalV, AF237881. protein of 65 aa with a pI of 11.9 has been described in a similar location within the HeV P/V/C gene (58). This HeV ORF was designated SB, for small basic protein, and we therefore refer to the analogous ORF in FDLV as SB.

The M protein of paramyxoviruses is the most abundant structural protein in virions, and it associates with membranes and with the hydrophobic tails of viral F and HN proteins (38). The first AUG codon in the FDLV M gene is in strong context for translation initiation, and it begins an ORF that encodes a 351-aa protein (Table 1). The deduced FDLV M protein has a basic pI of 9.7, which is typical for other M proteins. By pairwise amino acid identity, it is closest to the M proteins of respirivoviruses, morbilliviruses, henipaviruses, and TPMV, with which it shares 34 to 37% identity (Table 3). There is less identity to rubulavirus, avulavirus, MenV, and TIV M proteins, and there is no identity with the M protein of the pneumovirus HRSV.

Paramyxovirus F proteins are type I integral membrane proteins that mediate viral penetration into host cells by fusion of the viral envelope with the cell plasma membrane. F proteins are synthesized as inactive precursors that are cleaved by cellular proteases to generate F1 and F2 fragments that are linked by disulfide bonds to create the active form of the protein. The FDLV F ORF encodes a 545-aa protein that is closest in sequence to that of TPMV, followed by respirivirus and henipavirus F proteins (Table 3). Hydrophobicity and transmembrane analyses confirm that the FDLV F protein has features of a type I integral membrane protein. These features include a putative signal sequence at the N-terminal 21 aa and a transmembrane domain of approximately 28 aa near the carboxy terminus (aa 492 to 520), followed by a carboxy-terminal hydrophilic tail of 24 aa (data not shown). There is also a 25-aa hydrophobic domain at aa 111 to 136 that is likely to represent the fusion peptide because it follows immediately after a multibasic consensus cleavage site, R-E-K-R, at aa 107 to 110. Morbilliviruses, rubulaviruses, avulaviruses, pneumoviruses, and some respirivoviruses (human parainfluenza virus 3) have a conserved multibasic F protein cleavage site with the consensus sequence R-X-R/K-R that is required for cleavage by the cellular protease furin (24). The presence of this consensus site in the FDLV F protein suggests that it is also cleaved by furin, as has been suggested (17). The FDLV F protein has three potential N-linked glycosylation sites, two in the subunit F1 and one in F2.

Paramyxovirus HN proteins are type II integral membrane proteins on the virion surface that mediate attachment to cell-surface receptor molecules. They are the major antigenic determinants of paramyxoviruses. The FDLV HN gene contains an ORF beginning with an AUG in strong initiation context, encoding a 564-aa protein. The FDLV HN is closest to respirivirus HN proteins and most distant from morbillivirus H proteins (Table 3). Analyses of hydrophobic and transmembrane domains indicate that the FDLV HN protein has features of a type II membrane protein. There is a hydrophobic 25-aa putative transmembrane domain near the amino terminus (aa 26 to 50) following an amino-terminal cytoplasmic tail of 25 aa (data not shown). There are two potential N-linked glycosylation sites, and the acidic pI of 4.9 is within the typical range of 4.8 to 5.5 for HN proteins of other Paramyxovirinae (38). The FDLV HN protein contains the sialic acid binding site motif N-R-K-S-C-S (aa 246 to 251) that is conserved in respirivoviruses and rubulaviruses (29) but is not present in morbilliviruses, henipaviruses, or MenV. This finding suggests the probability that FDLV uses a sialic acid-containing cellular receptor similar to respirivoviruses and rubulaviruses. The globular head domain of the FDLV HN protein also contains the same site motif of N-R-K-S-C-S (aa 246-251) of the seven neuraminidase active-site residues found to be conserved in HN proteins of respirivoviruses and rubulaviruses (39). These features are consistent with the known neuraminidase activity of FDLV virions (14).

The polymerase proteins of Paramyxoviridae, like the poly-
mersases of all viruses in the superfam-
ly Mononegavirales, are large proteins that form a complex with N and P proteins to carry out multiple enzymatic functions involved in viral trans-

TABLE 4. Pairwise percent amino acid identities of FDLV L protein domains and subdomains with those of other paramyxoviruses

<table>
<thead>
<tr>
<th>Virus (genus)</th>
<th>Entire L</th>
<th>% Identity by major domain[a]</th>
<th>II-III hinge</th>
<th>% Identity by subdomain in domain III</th>
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<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
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<td>SeV (Respirovirus)</td>
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<td>49</td>
<td>69</td>
<td>63</td>
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<td>69</td>
<td>62</td>
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<td>62</td>
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<td>32</td>
<td>50</td>
<td>46</td>
</tr>
<tr>
<td>NDV (Avulavirus)</td>
<td>26</td>
<td>31</td>
<td>39</td>
<td>46</td>
</tr>
<tr>
<td>HRSV (Pneumovirus)</td>
<td>18</td>
<td>20</td>
<td>31</td>
<td>30</td>
</tr>
</tbody>
</table>

[a] Major L protein sequence domains and subdomains in domain III are as defined by Poch et al. (46). Viruses are listed in order of their overall similarity to FDLV, and the highest percent amino acid identity observed for each L region is highlighted in bold.

(31, 46). These domains include major conserved domains I to VI, more highly conserved subdomains A to D within domain III, and the highly variable hinge region between conserved domains II and III. Pairwise alignments of the FDLV L protein with L proteins of other paramyxoviruses show a pattern that generally conforms to this pattern (Table 4). Domain II is the best conserved of the major domains, and domain VI is the least conserved, showing little identity above the level seen throughout the entire L protein. The hinge region is uniformly low in amino acid identity, as expected. Within domain III, subdomain IIIA is the most conserved, while subdomain IIID is not conserved above the overall level of identity for domain III. Within domain III, the highly conserved G-D-N-Q sequence that is present in nearly all Mononegavirales is present in FDLV. This sequence is unlike the variant G-D-N-E found in HeV and NiV (19, 59) and in TPMV (GenBank accession no. AF079780). The FDLV domain VI contains a putative ATP-binding site motif, K-X21-A-E-G-S-G, that is identical to the ATP-binding site sequences of rubulaviruses and avulaviruses. This sequence differs from the ATP-binding site sequences of respiroviruses, morbilliviruses, henipaviruses, and pneumoviruses by one to two residues (19).

Novel U gene structure, transcription, and diversity. The FDLV U gene is 604 nt long and contains a 501-nt ORF starting at nt 24. The first AUG codon is in poor context for initiation, and there is a second AUG 72 nt downstream that is in strong context. It has not been established which codon is the actual initiator of translation. Depending on whether the first or second AUG codon is used, the U ORF encodes a protein of 167 or 143 aa, with predicted sizes of 19.4 or 16.6 kDa. Both putative proteins are basic, with pIs of 9.6 to 10.0. They are unusually rich in tryptophan (3.6 to 4.2% relative to 0.47 to 1.1% in the other FDLV proteins). There is only one other AUG codon in the U gene, 67 nt downstream of the transcription start. This codon is in poor initiation context, but if it were expressed from the U mRNA by ribosomal choice, it would encode an 82-aa protein with a calculated M₀ of 9.6 kDa and a pl of 5.2.

Northern blot analysis of RNA from FDLV-infected cells demonstrated active transcription of U gene mRNA at the anticipated size of approximately 600 nt (Fig. 4). Simultaneous analysis of N gene mRNA transcription as a positive control indicated the expected N mRNA at approximately 1.5 kb, and due to the nearly identical sizes of the U- and N-specific radioactive probes, the difference in intensity on the two blots suggested that U mRNA was substantially more abundant than...
To assess the presence and genetic diversity of U genes in other snake paramyxoviruses, FDLV sequences were used to design RT-PCR primers for amplification of the entire U gene transcription unit, along with short flanking sequences of the N and P genes. Two additional snake paramyxovirus isolates, Gono-GER85 and Biti-CA98, were selected for characterization because partial HN and L gene sequences suggested that they represent two viral genotypes different from FDLV (2). The isolates were obtained from the host species Gonosoma oxycephala and Bitis atropos, which are members of two different major families of snakes. RT-PCR amplification of genomic RNA from Gono-GER85 and Biti-CA98 generated products of the expected size for the U gene. Sequence analysis confirmed that these two additional snake paramyxoviruses also have U genes in their genomes. Each U gene contained an intact transcription unit of 604 nt bounded by transcription start and stop sequences identical to those of the FDLV U gene (Fig. 1). The flanking N gene transcription stop site, P gene start site, and trinucleotide intergenic regions were also identical to those of FDLV (Fig. 1).

The U genes of Gono-GER85 and Biti-CA98 each contained a single ORF with a 3' untranslated region (UTR) of 89 nt, but the 5' UTRs upstream of the ORFs were 14 and 23 nt, respectively. The ORFs thus encoded proteins of 167 and 164 aa, respectively, with predicted sizes of 19.2 to 19.3 kDa and basic pI values of 9.3 to 9.6. These proteins have 6 to 13% amino acid diversity, and if the U proteins start with the first AUG codon, they diverge by 11 to 19%. In either case, FDLV is more closely related to Gono-GER85 than to Biti-CA98, as indicated previously by characterization of partial HN and L gene sequences (2). The level of nucleotide divergence observed among these three snake paramyxovirus U genes (11 to 16%) was similar to the divergence previously observed for their partial HN genes (11 to 18%) and partial L gene sequences (12 to 20%) (2), indicating that the U genes are not more variable than other snake paramyxovirus genes. A virus-specific protein with an apparent size of 16 to 17 kDa has been observed in Western blots of purified Gono-GER85 virion proteins probed with polyclonal antisera against the virus (J. Franke and W. Ahne, unpublished results). Although the identity of this protein has not yet been investigated, its size suggests it may be either the U protein or the P gene I protein (Table 1).

**P gene RNA editing site and editing activity.** Comparisons of paramyxovirus RNA editing site sequences and experimental mutagenesis studies have indicated that insertion of G residues during RNA editing occurs by a stuttering process and that a short *cis*-acting sequence of 6 nt just upstream of the AnGn site modulates the overall frequency of mRNA editing and the number of nucleotides inserted (21). The FDLV editing site and its upstream regulatory sequence are shown in Fig. 3B, along with the editing sites of several other paramyxoviruses. The FDLV editing site is unique among all paramyxoviruses in having a U residue within the An run. This is significant because present RNA editing models hypothesize that RNA ed-

![FIG. 5. Alignment of the putative U protein sequences from three different snake paramyxovirus species. The top line presents the full-length amino acid sequence of the deduced FDLV U protein, and in the two lines below, dots indicate amino acids identical to the FDLV sequence in the U protein sequences of Gono-GER85 (Gono) and Biti-CA98 (Biti). Only differences from the FDLV sequence are shown. Note that the Biti-CA98 sequence does not start at the same position as the sequences of the other two genes. The arrow indicates the conserved second methionine residue in all three sequences, which may be the actual start of translation (see text).](image-url)
discussed above, an alternative mechanism involving insertion of one or two Gs at the editing site occurs when the nascent mRNA realigns by slipping back one or two positions along a "slippery" sequence containing the An run, resulting in hybrids that are nearly as stable as the nonslipped hybrid (21). For FDLV, slippage of nascent mRNA by one or two positions would result in hybrids with mismatched A:A and U:U base pairs that are not stable. Thus, the FDLV editing site does not appear to be as slippery as other paramyxovirus editing sites. The 6 bases upstream of the FDLV AmGn sequence are also unique among paramyxoviruses, so that although FDLV uses the same editing strategy as rubulaviruses, the editing site is not discernibly more similar to rubulaviruses than to other paramyxovirus genera.

To confirm the location and activity of the putative FDLV editing site, mRNA from FDLV-infected cells was amplified by RT-PCR using primers flanking the editing site in a protocol specific for mRNA-sense templates. The resulting PCR product was cloned, and a 240-nt sequence surrounding the putative editing site was determined for 37 individual clones. Relative to the six Gs encoded directly from the genomic sequence, 24 clones had no insertion, 2 clones had a single G, 10 clones had a two-G insertion, and 1 clone had a three-G insertion. This finding confirms the location of the FDLV editing site and the capacity of the FDLV P gene to encode V, P, and I proteins by RNA editing. Table 5 shows a comparison of the relative proportions of mRNA encoding each of the three possible protein products in infected cells for paramyxoviruses that use a rubulavirus-like editing strategy (i.e., encode V protein as the primary transcript and edit by insertion of two Gs to encode P protein). Among paramyxoviruses that encode P proteins from the primary transcript, SeV generates an average of 62% P and 31% V mRNA in infected cells (56), and MeV edits to generate approximately equal proportions of P and V mRNA both in cell culture and in vivo (6).

Among the 37 clones sequenced to characterize the FDLV RNA editing frequency, 1 clone contained a deletion of a single A residue 59 nt upstream of the RNA editing site. In an independent experiment, one clone was found with an insertion of 20 As at this same site (data not shown). Other than the editing site itself, there were no other sequence differences found in any of the other clones. The A deletion and insertion occurred within a run of five As in the sequence 5'-AUGG(A)5C-3' (mRNA-sense), which is similar to the consensus FDLV transcription stop signal 5'-UAAG(A)5C-3'. Thus, these variants may be the result of an aberrant polyadenylation reaction, as suggested by Cataneo et al. (6), who observed an insertion of 19 residues after a stretch of five identical residues in an untranslated portion of the MeV F mRNA.

**Phylogenetic analyses.** The deduced amino acid sequences of the full-length N, P, M, F, HN, and L genes of FDLV were used to infer phylogenetic relationships with other Paramyxoviridae by using Ebola virus as an outgroup. Representative trees illustrating the relationships observed are shown in Fig. 6. The N protein phylogeny shows FDLV separate from all other known paramyxovirus species and genera, branching from near the trunk of the tree between the Rubulaviruses, Henipaviruses, and Morbilliviruses genera. The P, M, and F protein trees are similar in showing FDLV branching from the trunk, but the identities of the nearest branches vary (data not shown). In the HN and L protein trees, by both neighbor-joining and parsimony analyses, FDLV appears in a position basal to the Respirovirus genus but close to the trunk of the trees (Fig. 6B). Thus, phylogenetic analyses of HN and L suggest that, although distant, FDLV is most closely related to the respiroviruses. However, trees of the different proteins are not consistent, indicating variously that FDLV is most distant from respiroviruses (N and P proteins), rubulaviruses and avulaviruses (M, F, and L proteins), or morbilliviruses (HN protein). All of the trees clearly show FDLV as an independent branch not closely related to any known Paramyxovirus genus or species.

**DISCUSSION**

Despite the global prominence of reptiles as one of the five major classes of vertebrates, they have received relatively little attention as viral hosts. The viruses described from reptiles include members from six DNA virus families and from nine RNA virus families including Paramyxoviridae, Rhabdoviridae, Bunyaviridae, Retroviridae, Caliciviridae, Togaviridae, Picornaviridae, Flaviviridae, and Reoviridae (14). However, the seventh report of the ICTV includes only 12 DNA viruses and 9 RNA viruses from reptiles (37), and the majority of the viruses are not assigned to any genus. The first full-length genome sequence determined for a reptile virus was that of an endogenous retrovirus from pythons, which was reported in 2002 and could not be classified into any of the established retrovirus genera (25). To the best of our knowledge, the FDLV genome described here is the second complete genome sequence of any virus from a reptile host.

The FDLV genome consists of seven genes that potentially encode 10 proteins, including the six invariant proteins found in all paramyxoviruses, three accessory proteins (V, I, and SB) from the P gene, and the unique U protein. Characterization of the FDLV genome sequence has revealed several features in common with all Paramyxoviridae studied to date. The genome organization contains the six invariant paramyxovirus genes in the standard order found in all paramyxovirus species (37, 38). The sizes of the FDLV genes and deduced proteins are all either within the range for known Paramyxoviridae or slightly smaller. The pattern of amino acid conservation among FDLV proteins and homologous proteins in other paramyxoviruses, from most conserved to least, is V-carboxyl domain > L > M, F > N, and HN > V > P (Table 3). This order is exactly the relative order of conservation described among proteins of

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of clones</th>
<th>% mRNA encoding the following protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDLV</td>
<td>37</td>
<td>68 (0, +3) 5 (+1) 27 (+2)</td>
<td>This study</td>
</tr>
<tr>
<td>MenV</td>
<td>35</td>
<td>77 (0) 3 (+1) 20 (+2)</td>
<td>4</td>
</tr>
<tr>
<td>MuV</td>
<td>54</td>
<td>76 (0, +3) 2 (+4) 22 (+2, 5)</td>
<td>43</td>
</tr>
<tr>
<td>SalV</td>
<td>20</td>
<td>90 (0, +5) 5 (+4) 5 (+5)</td>
<td>49</td>
</tr>
<tr>
<td>SV5</td>
<td>22</td>
<td>55 (0) 0 (0) 45 (+2)</td>
<td>54</td>
</tr>
<tr>
<td>TIV</td>
<td>60</td>
<td>45 (0) 5 (+1) 50 (+2)</td>
<td>9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Viruses are presented in order of similarity to FDLV editing proportions. Numbers in parentheses indicate number of G residues inserted at the editing sites of various clones.
known *Paramyxoviridae* in the most recent ICTV report (37). In addition, the 55-nt 3′ leader sequence length, complementarity between the 3′ and 5′ genomic termini, transcription start and stop sequences, and pattern of conserved and divergent domains within the L protein are features FDLV shares with all other paramyxoviruses. Within the *Paramyxoviridae* family, the FDLV gene order, genome length in a multiple of 6 nt, lack of an M2 protein, evidence for expression of multiple proteins from the P gene locus, conserved V-carboxyl terminal domain, and uniformly low amino acid identities with HRSV proteins (Table 3) indicate conclusively that FDLV is not in the subfamily *Pneumovirinae* but is a member of the subfamily *Paramyxovirinae*.

Within the subfamily, various features of the FDLV genome comprise a mosaic with regard to similarity to known genera. The 3′ terminal genomic sequence and transcription start and stop sequences are most similar to henipaviruses, but the genome length and 5′ trailer size are unlike henipaviruses. The trinucleotide intergenic regions are similar to henipaviruses, morbilliviruses, and respiroviruses. The ATP-binding motif in the L protein is identical to that of rubulaviruses and avulaviruses. The ICTV states that amino acid sequence relationships are the basis of the placement of viruses into genera within the family *Paramyxoviridae* (37). On this basis, none of the FDLV proteins shares enough amino acid identity with known paramyxovirus proteins to be considered within any of the known genera (Table 3). The FDLV P protein shares enough amino acid identity with known paramyxovirus proteins to be considered within any of the known genera (Table 3). The FDLV N and P proteins in the 3′ portion of the genome share highest amino acid identity with henipaviruses and morbilliviruses, respectively, while the M, F, HN, and L proteins in the 5′ region of the genome share highest identity with respiroviruses (Table 3). Within the P gene, the accessory V protein has highest identity to rubulaviruses, but the conserved V-carboxyl domain is most like henipaviruses (Table 3). Phylogenetic analyses of the FDLV proteins also indicate that FDLV is not consistently more closely related to any known paramyxovirus genus or species than to others (Fig. 6). The HN and L protein phylogenies suggested that FDLV was slightly closer to respiroviruses than to other genera. Despite the overall lack of relatedness between the proteins of FDLV and rubulaviruses, the FDLV P gene coding capacity and expression strategy are identical to those of the rubulaviruses. This feature is reminiscent of SalV, which was recently described as follows: “The noted similarity to the rubulaviruses is based only on a common editing mechanism and evidence for any direct phylogenetic relationship is unsupported at this time” (49). The fact that this statement is also true for FDLV, and that FDLV and SalV are not closely related, suggests that perhaps a switch between a respirovirus-like and a rubulavirus-like editing mechanism has occurred independently more than once during the evolution of the *Paramyxoviridae*.

Other features of the FDLV P gene raised two interesting observations. Lamb and Kolakofsky (38) have recently proposed a nomenclature for the amino-terminal modules of *Paramyxovirinae* P proteins upstream of the editing site. In this nomenclature, respirovirus and morbillivirus P proteins all have a P-amino 1 module, typified as being approximately 300 aa, with an acidic pI of about 5.5. Rubulaviruses and all other paramyxoviruses are suggested to have a P-amino 2 module, which is shorter and basic in nature, with a length of about 170 aa and a pI of about 10.5. The FDLV P protein amino-terminal module is 156 aa, but it has an acidic pI of 4.8. An analysis of several other paramyxoviruses revealed that NDV, HeV, NiV, TPMV, TiV, MenV, and SalV all have acidic P protein amino-terminal modules with pI values of 4.3 to 5.7, similar to FDLV (data not shown). Thus, a basic pI is not a consistent feature if these viruses are all considered to have P-amino 2 modules.

The second interesting observation involved the FDLV P

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**FIG. 6. Phylogenetic analyses of N and L proteins of FDLV and other Paramyxovirinae.** Trees shown are neighbor-joining distance trees with branch lengths accurately representing genetic distance. Bootstrap values above 70 from 1,000 resampled data sets were overlaid on the trees, and Ebola virus, from the family *Filoviridae*, was used as an outgroup. Viral species within the six established *Paramyxovirus* genera are indicated in shaded ovals. MenV and TiV were recently described as probable new members of the genus *Rubulavirus* (4, 49) and were thus included within the genus oval when they fell within a cluster defined by accepted species in the genus.
gene SB ORF. As noted by Wang et al. (58), the P gene SB ORF described in HeV, and now also in FDLV, is similar to ORFs reported to encode small basic proteins of 55 to 67 aa in the P genes of two rhabdoviruses (36, 52) and the filoviruses Marburg (15). The protein product of one of the rhabdovirus SB ORFs has been detected in virus-infected cells (52). Examination of other paramyxovirus P gene sequences for the potential to encode a protein of 30 to 100 aa with a pI over 10.0 identified 22 potential SB ORFs in a total of 13 different paramyxovirus species (data not shown). Based on size and position within the P gene, the potential SB ORFs most similar to the FDLV SB were present in HeV, MeV, simian virus 5 (SV5), and NDV. Each of these viruses has an ORF upstream of the P gene-editing site that encodes a protein of 50 to 65 aa, with predicted pIs of 11.9 to 13.4. Thus, potential to encode an SB protein is a relatively ubiquitous feature of P genes of viruses in the Mononegavirales superfamily.

Unique features of FDLV not found in any other Paramyxovirus genera include the pattern of the hexamer phasing positions of the gene start sites and the sequence of the RNA editing site, both of which have been described as being genus specific within the family Paramyxoviridae. The most notable unique feature is the presence of the novel U gene between the N and P genes. The U gene was shown to be present, with 11 to 16% nucleotide diversity, in three different snake paramyxovirus sequences. This finding suggests that the U gene is most likely a feature common to all reptilian paramyxoviruses. Due to this presence of the novel U gene and the lack of similarity with known Paramyxovirus genera as detailed above, we suggest that the snake paramyxoviruses should comprise a new genus within the subfamily Paramyxovirinae, in the family Paramyxoviridae. We suggest the name Ferlavirus for this new genus, to indicate Fer-de-Lance virus as the type species.

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