

Establishment of a Rescue System for Canine Distemper Virus

UTA GASSEN, FERGAL M. COLLINS, W. PAUL DUPREX, AND BERT K. RIMA*

School of Biology and Biochemistry, Medical Biology Centre, The Queen's University of Belfast, Belfast BT9 7BL, Northern Ireland

Received 25 April 2000/Accepted 10 August 2000

***Canine distemper virus* (CDV) has been rescued from a full-length cDNA clone. Besides *Measles virus* (MV) and *Rinderpest virus*, a third morbillivirus is now available for genetic analysis using reverse genetics. A plasmid p(+)-CDV was constructed by sequential cloning using the Onderstepoort vaccine strain large-plaque-forming variant. The presence of a T7 promoter allowed transcription of full-length antigenomic RNA by a T7 RNA polymerase, which was provided by a host range mutant of vaccinia virus (MVA-T7). Plasmids expressing the nucleocapsid protein, the phosphoprotein, and the viral RNA-dependent RNA polymerase, also under control of a T7 promoter, have been generated. Infection of HeLa cells with MVA-T7 and subsequent transfection of p(+)-CDV plus the helper plasmids led to syncytium formation and release of infectious recombinant (r) CDV. Comparison of the rescued virus with the parental virus revealed no major differences in the progression of infection or in the shape and size of syncytia. A genetic tag, consisting of two nucleotide changes within the coding region of the L protein, has been identified in the rCDV genome. Expression by rCDV of all the major viral structural proteins has been demonstrated by immunofluorescence.**

Canine distemper virus (CDV) is an enveloped virus with a monopartite negative-stranded RNA genome. Together with *Measles virus* (MV) and *Rinderpest virus*, it belongs to the morbilliviruses which form a serologically closely related genus in the family *Paramyxoviridae*. CDV primarily affects dogs, but infections of other terrestrial carnivores, in both captivity and the wild, have been reported (1, 2, 18, 21, 22, 25, 26, 29, 33, 36). The mortality rates associated with CDV infection vary among susceptible species and range from 0% in domestic cats to 50% in dogs and 100% in ferrets. One of the currently available vaccines (Onderstepoort strain) efficiently protects dogs, but it is insufficiently attenuated for other species, and high levels of mortality can occur due to its remaining virulence (10). Thus, there is a need to develop more attenuated vaccines for CDV to fully protect susceptible animals.

A rescue system for CDV would provide the means to study attenuating effects of defined mutations and might subsequently facilitate generation and examination of new vaccines. Mutations that cause persistence of the virus could also be determined. This system would be useful in gaining a better understanding of CDV infection which can more easily be studied in cell culture and animal models by coexpression of additional reporter genes (e.g., the enhanced green fluorescent protein) from the recombinant viral genome (19, 20).

The CDV genome is 15,690 nucleotides (nt) in length and consists of a short 3' leader region and six genes encoding the N nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H) and large (L) proteins (4, 5, 7, 16, 32, 37, 42). They are separated by intergenic regions of 3 nt and are followed by a short 5' trailer region. The nonstructural proteins V and C are encoded within the P gene. V is expressed by cotranscriptional RNA editing, and C is expressed from an overlapping reading frame (8, 12). The genome of paramyxoviruses does not consist of a naked RNA molecule. In many

members of the subfamily *Paramyxovirinae*, 6 nt are likely to be tightly associated with N protein (9, 30). The exact structure of these ribonucleoprotein complexes is not known but it has recently been suggested for vesicular stomatitis virus that the N protein binds to the sugar-phosphate backbone of the RNA, exposing the bases to the outside. As a consequence, the RNA of the *Mononegavirales* may be transcribed without dissociation from the nucleoprotein (27). The phosphoprotein and the RNA-dependent RNA polymerase are also associated with the RNP. Paramyxovirus genomic or antigenomic RNA as for all *Mononegavirales* cannot function as mRNA. Thus, to initiate an infectious cycle by introducing genome analogues into the cell, all viral proteins involved in transcription and replication have to be provided *in trans*. Since the generation of infectious rabies virus from a cDNA clone in 1994 (40), several other negative-stranded RNA viruses have been rescued using a number of different approaches. To date two morbilliviruses, namely MV (35) and *Rinderpest virus* (3), have been recovered from cDNA. These viruses are closely related and distinct from CDV and phocine distemper virus (PDV) in terms of genome length and sequence homology (6, 17, 24, 34, 41).

In this study, we have established a rescue system for CDV. We generated a full-length cDNA clone of the CDV strain Onderstepoort [large plaque-forming variant (OND-LP)] and the helper plasmids encoding N, P, and L proteins. Recombinant virus (rCDV) was recovered from cell cultures transfected with all four plasmids. Immunofluorescence and a genetic tag identified rCDV. The growth characteristics of rCDV were compared with the original CDV strain.

MATERIALS AND METHODS

Cells and viruses. Vero cells were maintained in BHK medium supplemented with 8% newborn calf serum. HeLa cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. All media and sera were obtained from Life Technologies/Gibco BRL. For transfections the cells were seeded into six-well trays and grown to approximately 80% confluence. For immunofluorescence Vero cells were grown on glass coverslips (diameter, 13 mm) to 100% confluence and infected with rCDV at a multiplicity of infection (MOI) of 0.1. For the growth analysis Vero cells were grown in 25-cm² flasks to 100% confluence and infected with rCDV or CDV Onderstepoort (OND-LP) at an MOI of 0.1. Virus was removed after an incubation of 2 h, and new medium was added. The samples of cell-associated and cell-free virus at time point 0 were collected immediately after addition of new medium and, after that, every 4 h. For a 50%

* Corresponding author. Mailing address: School of Biology and Biochemistry, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Rd., Belfast BT9 7BL, Northern Ireland, United Kingdom. Phone: 44 2890 335858. Fax: 44 2890 236505. E-mail: b.rima@qub.ac.uk.

TABLE 1. Oligonucleotides used in cloning of p(+)-CDV and pEMC-N, -P, and -L

Fragment/protein	Primer	Sequence (5' to 3') ^a	Internal RE site ^b
N	emcn-1	CCT ACC Acc ATG GCT AGC CTT CTT AAA AGC	<i>NcoI</i>
	emcn-3	GGA CCT tGa TCa TAA GTT TTT TAT AAT GAG	<i>BclI</i>
P	emcp-1	CCC Tcc ATG GCA GAG GAA CAG GCC TAC CAT	<i>NcoI</i>
	emcp-2	GTC CTA AGa TcT TTA TAA TTG CTT TTA AGC	<i>BglII</i>
L	CDVL Nco I F	CTTTTAGCCATGGACTCTGTAT	<i>NcoI</i>
	CDVL BssH II R	TGGGGAAGCGCGGAGTGACG	<i>BssHIII</i>
	CDVL BssH II F	TCACTCGCGCGCTTCCCCAAGTAGTAGATA	<i>BssHIII</i>
	CDVL Aat II R	AGGCCTTGACGTGACGAATTATTTCTTAGAGG	<i>AatII</i>
	CDVL Aat II F	CAGAAAGACGTCAAGGCCTCAGAATTAAGTC	<i>AatII</i>
	CDVL Eco 47 III R	CTAATCAGAGCGCTATAACCTAATAATTTGAACC	<i>Eco47III</i>
FL1	CDV-FL-MluT7	tca tgc acg cgt taa tac gac tca cta taA CCA GAC AAA GTT GGC TAA	<i>MluI</i>
	CDV-FL-Kpn I rev	agt caa tgg cgc gcC ATT TCC TTC GGA ATA	<i>BssHIII</i>
FL2	CDV-FL-Kpn I for	TGG AAT ACG ATG TGA TCA TGT TTA	
	CDV-FL-Sal I rev	tgg cgc gcC TGG GTT AAT GTC GAC ATT TG	<i>BssHIII/SalI</i>
FL3	CDV-FL-Sal I for	CAA ATG TCG ACA TTA ACC CAG	<i>SalI</i>
	CDV-FL-Spe I rev	agt caa tgg cgc gcA CCT GTT GGC TTG CTA	<i>BssHIII</i>
FL4	CDV-FL-Spe I for	TTA GCT TCG CTT CTA GGA ATC TCA	
	CDV-FL-Afl II rev II	agt caa tgg cgc gcC TGA GGA GAC TGC CAA	<i>BssHIII</i>
FL5	CDV-FL-Afl II for	TAG GGA ACG CCC TTA AGA AAC T	<i>AflIII</i>
	CDV-FL-Hpa I rev II	agt caa tgg cgc gcG ACA TCT CTA TCT CTA	<i>BssHIII</i>
FL6	CDV-FL-Hpa I for II	TGA ACT CCG GAT GGC TTA CCA TTC	
	CDV-FL-Swa I rev	agt caa tgg cgc gcG ATT GTA CCT GAG GAA	<i>BssHIII</i>
FL7	CDV-FL-Swa I for	TCA TGC ATC TCC TAT CAT CAG AAA	
	CDV-FL-Aos I rev	agt caa tgg cgc gcC TAT GTA TGG CAC CCT	<i>BssHIII</i>
FL7/8	CDV-FL-Swa I for	TCA TGC ATC TCC TAT CAT CAG AAA	
	CDV-FL-Aoc I rev	agt caa tgg cgc gcT GAG CCT CTT CTA AGA TAT GT	<i>BssHIII</i>
FL9	CDV-FL-SgrA I-tag	TTG TCT TCA ACC ACC GGC GAT TCG AAC ACC GT	<i>SgrAI/tag; Csp45I</i>
	CDV-FL-Avr II rev	agt caa tgg cgc gcT GTT ATA ATA ACA TCT TG	<i>BssHIII</i>
FL10	CDV-FL-Aoc I for	CTG ACA TAC CTA AGG AGA GGC TCA	
	CDV-FL-F9 rev	gcg cgc cca gcc ggc gcc agc gag gag gct ggg acc atg ccg gcc ACC AGA CAA AGC TGG GTA TGA	<i>BssHIII/NarI</i>
Spacer	5'-spacer	cgc cag tgc ggc gcc gca tag	<i>NarI(oh), NotI, BssHIII (oh)</i>
	3'-spacer	ggt cag ccg ccg ggc tat cgc gc	<i>BssHIII (oh), NotI, NarI (oh)</i>

^a Capital letters represent CDV sequences and lower case non-CDV sequences. Restriction endonuclease recognition sites are underlined.

^b The genetic tag is indicated in bold. oh, overhang.

tissue culture-infective dose, which was performed using standard methods, Vero cells were grown in 96-well trays until confluent. For phase-contrast microscopy, a confluent monolayer of Vero cells was infected with rCDV at an MOI of 0.5. When cytopathic effects (CPEs) were visible, cells were formalin fixed and stained with methylene blue. Both viruses, OND-LP and rCDV, were propagated in Vero cells. Cells and viruses were grown at 37°C under 5% CO₂.

Plasmid constructions. All cloning procedures were performed following standard protocols. PCR amplifications were carried out using *Pwo* (Boehringer) or *Taq* DNA polymerases (Life Technologies). PCR products were first cloned into pGEM-T (Promega) and then subcloned into pEMC vectors or pBS SK II (Stratagene). The vector backbone pEMC, used for cloning of coding sequences of CDV N, P, and L proteins, has been described elsewhere (35). The plasmids pEMC-Na, pEMC-Pa, and pEMC-La, which code for MV N, P, and L proteins, pCDV(-):CAT, and p107MV(-):CAT, were a kind gift from M. A. Billeter, University of Zürich.

The P gene of MV was excised from pEMC-Pa using restriction enzymes *NcoI* and *BamHI*. The coding sequences of the CDV N and P proteins were then inserted into this vector backbone. The sequence coding for the CDV N protein was amplified by PCR with primers emcn-1 and emcn-3 from first-strand cDNA and that for the CDV P gene was amplified with primers emcp-1 and emcp-2 (Table 1). Both PCR products were digested with *NcoI* and *BclI* or *NcoI* and *BglII*, respectively, to generate overhangs compatible with those present in the pEMC backbone and cloned into the vector. Due to an *NcoI* site within the coding region of the N protein (nt 1,144), a ligation with two inserts had to be performed.

The vector pEMC-La was used as the backbone for cloning of the CDV L-coding sequence. An *Eco47III* site was introduced by site-directed mutagenesis close to the end of the coding sequence. Using this site and the *NcoI* site at the start of the open reading frame, the coding sequence was removed and the so-called L-cassette, a small multiple-cloning site (46 nt), containing recognition sites for *BssHIII* and *AatII* and maintaining the sites for *NcoI* and *Eco47III*, was inserted. The coding sequence of the CDV L protein was amplified from first-strand cDNA in three separate steps using primers CDVL Nco I F and CDVL BssH II R, CDVL BssH II F and CDVL Aat II R, and CDVL Aat II F and CDVL Eco 47 III R. The products were subsequently cloned into the pEMC cassette vector using the designated restriction enzymes to form pEMC-L CDV

(F. M. Collins, U. Gassen, W. P. Duprex, M. D. Baron, and B. K. Rima, unpublished data).

The full-length cDNA clone p(+)-CDV was generated in 12 sequential cloning steps using the plasmid pBS SK II (Stratagene) as the vector backbone (Fig. 1A). Fragments FL1 through FL10 coding for the T7 promoter and the full-length antigenome were generated by PCR using the primers given in Table 1. The forward (for) primers generally did not contain restriction sites unless otherwise stated. They were complementary to areas upstream of enzyme recognition sites, stated in primer names, which were used for cloning of the respective PCR products. The reverse (rev) primers all contained a *BssHIII* recognition site and were complementary to sequences downstream of the recognition site required for the next cloning step. The fragments were sequentially cloned into the growing plasmid by using unique endonuclease recognition sites within the CDV genome and the artificially introduced *BssHIII* site (Fig. 1B). The primer CDV-FL-F9 rev contained a portion of the δ -ribozyme sequence up to the *NarI* recognition site. The rest of the δ -ribozyme and the full T7 terminator sequences were excised from p(+)-MV using *NarI* and *NotI* and introduced into p(+)-CDV, which was cleaved with the same two enzymes within a new multiple-cloning site introduced into the plasmid prior to this last cloning step (Fig. 1C).

Chloramphenicol acetyl transferase (CAT) assays. Transfections were performed following the same protocol as for rescue (below). Plasmids were used in the following amounts: 1.2 μ g of pEMC-N, 1.2 μ g of pEMC-P, 0.4 μ g of pEMC-L, and 1.0 μ g of pCDV(-):CAT or p107MV(-):CAT. At 20 h posttransfection (p.t.), crude cell extracts were generated and tested for CAT activity using standard protocols (Promega Protocols and Applications Guide). Incubation time for the enzyme assay was set to 2 h. The substrate [¹⁴C]chloramphenicol (50 mCi/mmol) was obtained from Amersham. Radioactivity of the butyrylated chloramphenicol products was measured in a liquid scintillation counter (LKB).

Virus rescue. Upon reaching a confluence of approximately 80% in six-well trays, HeLa cells were incubated in 2 ml of Optimem (Life Technologies) for 30 min. The cells were infected with the host range mutant of vaccinia virus Ankara expressing T7 polymerase (MVA-T7) at an MOI of 0.5 for 30 min. Prior to this infection, 8 μ l of Lipofectin was mixed and incubated with 92 μ l of Optimem following the supplier's instructions (Life Technologies). Plasmids [1.5 μ g of pEMC-N, 1.5 μ g of pEMC-P, 0.5 μ g of pEMC-L, and 5.0 μ g of p(+)-CDV] were mixed into 100 μ l of Optimem. The plasmid mixtures were then carefully pipet-

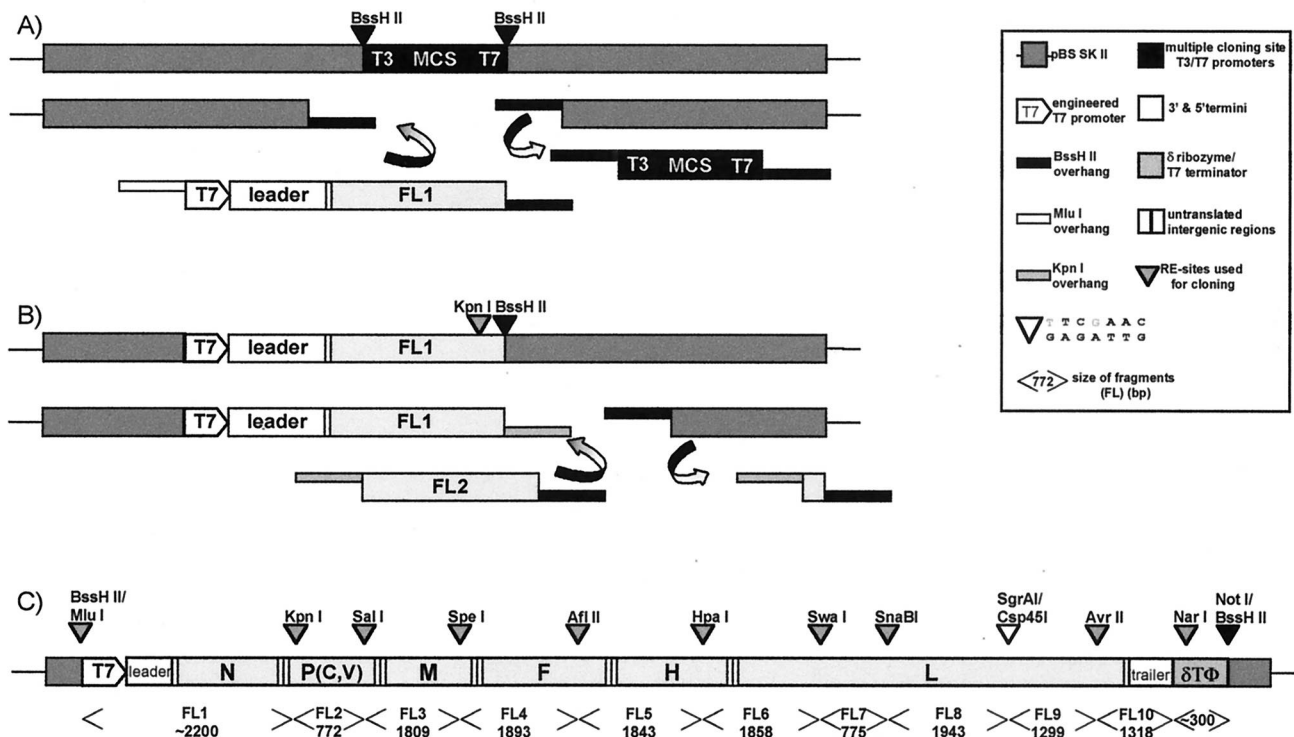


FIG. 1. Schematic representation of p(+)-CDV: cloning procedure and full-length cDNA clone. (A) The multiple cloning sites of pBS SK II and adjacent T3/T7 promoters were excised from the plasmid with *BssH*III. The remaining plasmid then served as backbone for construction of p(+)-CDV. The first cDNA coding for the T7 promoter, viral leader sequence, and mainly the N protein was ligated into the vector. The restriction enzyme *Mlu*I was used to block one *BssH*III recognition site during the cloning procedure (see panel C). (B) *BssH*III was used throughout the construction of p(+)-CDV to cut at the 3' ends. (C) The full-length clone of 18,743 nt was generated in 12 separate cloning steps. The restriction sites used for cloning are indicated. As the genetic tag, a *Csp45I* site was introduced into the coding sequence of the L protein. The δ ribozyme and T7 terminator sequences were required for generation of RNAs of exact length. The representation is not to scale.

ted into the Lipofectin. MVA-T7 was removed from the cells, and plasmids in Lipofectin were added to the HeLa cells. The cells were incubated with an additional 2 ml of Optimem at 37°C for 16 h to recover from the procedure before the transfection medium was exchanged with normal growth medium. At 48 h p.t., 5×10^4 Vero cells per well were added. At 3 days p.t., 1 ml of the supernatant was removed and added to a confluent monolayer of Vero cells in a 75-cm² flask. At 6 days p.t., when the infection had progressed well, cells and supernatants were harvested and stored at -70°C.

Immunofluorescence. Vero cells were grown on glass coverslips and transfected as described above, with 1.2 μ g of pEMC-N or pEMC-P to detect expression of either protein, or infected with rCDV at an MOI of 0.1. At 24 h postinfection (p.i.) or p.t., cells were fixed in ice-cold acetone and stored at -20°C. The primary serum of a patient with subacute sclerosing panencephalitis (SSPE) or monoclonal antibodies (MAbs) anti-N 2.78 and anti-P 2.98, obtained from N. Duffy, and anti-M 2.47, anti-F IC5, and anti-H 2.80, obtained from A. Trudgett (both from The Queen's University of Belfast), were added in appropriate dilutions. Secondary fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse or rabbit anti-human antibodies (DAKO) were diluted 1:40 in phosphate-buffered saline. The procedure was performed using standard protocols. The nuclei were counterstained with propidium iodide (120 ng/ μ l), and the coverslips were mounted on slides with Citifluor (Amersham). The indirect fluorescence of recombinant viral proteins was examined under a confocal microscope equipped with a krypton-argon laser (Leica).

RNA isolation, cDNA synthesis, PCR, and automated sequencing. For viral RNA isolation, Vero cells were infected with OND-LP or rCDV at an MOI of 0.1. The cells were harvested in guanidium-isothiocyanate solution, and RNA was isolated using CsCl-gradient centrifugation (38). The total RNA was stored in 70% ethanol at -70°C. For first-strand cDNA synthesis, 1 μ g of total RNA was used following the protocol supplied with AMV reverse transcriptase (Promega). The cDNA synthesis was primed with an oligo-dT₍₁₂₋₁₈₎ primer or a primer complementary to base pairs 15,657 through 15,690, for cloning of the full-length cDNA p(+)-CDV or CDV-FL-Avr II rev (Table 1), respectively, for amplification of the area carrying the genetic tag. PCR reactions were carried out following protocols supplied with *Taq* (Life Technologies) or *Pwo* (Boehringer) DNA polymerases. Cycling conditions were adjusted to templates and primers by varying the standard set-up by prolonging elongation time up to 2.5 min or increasing annealing temperature from 50 to 55°C.

The plasmids pEMC-N, -P, and -L were partially sequenced to confirm correct

ligation of cDNAs into the pEMC backbone. The plasmid p(+)-CDV was fully sequenced using 61 primers annealing to sequences approximately 250 bp apart. The region containing the genetic tag was directly sequenced from first-strand cDNA generated from rCDV-RNA using a primer complementary to nt 12,991 through 13,011 close to the tag. Sequencing reactions were performed following the ABI Prism sequencing kit instructions (PE Applied Biosystems; this kit was suitable for automated sequencing with a PE Applied Biosystems 373A sequencer).

Nucleotide sequence accession number. The CDV insert sequence of plasmid p(+)-CDV is accessible under GenBank accession no. AF 305419.

RESULTS

Construction of plasmids expressing recombinant CDV N, P, and L proteins. For successful rescue of most negative-stranded RNA viruses in the *Paramyxoviridae* and *Rhabdoviridae*, the N, P, and L proteins have to be provided and expressed from different plasmids to the vector coding for the full-length genome (13, 40). We generated three plasmids, pEMC-P, pEMC-N, and pEMC-L, to code for the respective proteins of CDV. The successful cloning of the coding sequences was verified by restriction endonuclease analysis and determination of nucleotide sequences of the respective plasmids (data not shown).

We first used specific antibodies to verify expression of both the N and the P proteins in transfected Vero cells. CDV N protein expression was detected by using a cross-reactive MV antiserum from an SSPE patient. The P protein was detected with an MAb raised against PDV, which cross-reacts with CDV. In both cases the viral protein was indirectly visualized with an FITC-labeled secondary antiserum. The results are shown in Fig. 2. The FITC fluorescence was clearly visible as a

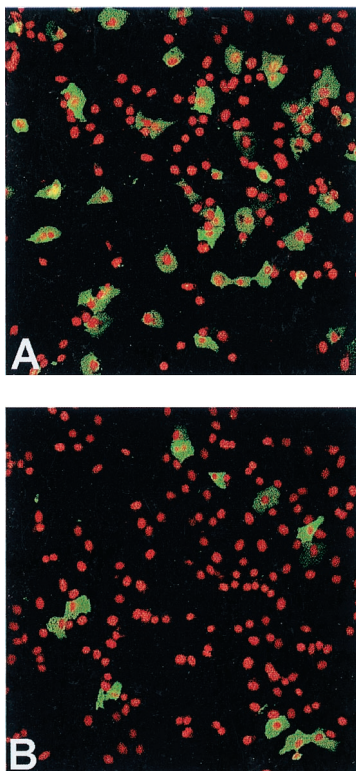


FIG. 2. Recombinant N and P proteins visualized by immunofluorescence. Vero cells (10^5) were transfected with 1.2 μ g of pEMC-N or pEMC-P, acetone fixed at 24 h p.i. and subjected to immunofluorescence. (A) N protein was indirectly visualized with SSPE antiserum and FITC-labeled mouse anti-human secondary antibody. (B) Phosphoprotein expression was examined using an anti-PDV P primary and FITC-labeled rabbit anti-mouse secondary antibody. Nuclei were counterstained with propidium iodide (red). Photographs were taken with a confocal laser microscope (magnification, $\times 160$).

green stain in cells transfected with the N or P plasmids (Fig. 2A and B). Nontransfected cells were detectable due to the red propidium iodide counterstain of their nuclei. These results indicated that N and P proteins were expressed in an antiserum-recognizable form from the respective plasmids, but no conclusions could as yet be drawn about their functionality.

The construction of the plasmid coding for the CDV L protein was performed in several steps. Three PCR products were generated and subsequently cloned into the vector backbone pEMC-cassette. Into the coding sequence of the designated hinge regions of the L protein, the restriction sites *Bss*III and *Aat*II were introduced. This allows the three domains of morbillivirus L proteins to be exchanged (32). Between domain I and domain II, the alteration caused two amino acid changes ($L_{626/27}$, VS to RA). The mutations introduced into the second hinge region were silent. Again the successful cloning was monitored by endonuclease restriction analysis and sequencing of the intermediate plasmids and the final construct pEMC-L (CDV) (data not shown).

Because no antiserum or MAb to CDV L protein is currently available, its expression could not be assessed by means of immunofluorescence.

Rescue of minigenome constructs. We tested the functionality of N, P, and L proteins by assessing their ability to rescue minigenome constructs (14) which contain the CAT reporter gene in antisense. The only viral sequences present are the leader and trailer regions of the genome. We cotransfected the plasmids encoding the N, P, and L proteins with such a mini-

replicon. Rescue events were monitored by assaying of CAT protein expression in crude cell extracts. Unfortunately, we found that active protein was produced from pCDV(-):CAT in transfected HeLa and Vero cells in the absence of pEMC-L. This meant that pCDV(-):CAT could not be used to test activity of N, P, and L proteins. To overcome this problem, we tested for functionality of the proteins using p107MV(-):CAT as a minireplicon. Unlike pCDV(-):CAT, the MV version of the plasmid did not give rise to CAT activity by itself (300 cpm) or when one of the helper plasmids was omitted. CAT activity was equally high when cells were transfected with a combination of CDV N, P, and L plasmids (22,500 cpm) as with MV-derived plasmids (23,100 cpm). Recombinant N, P, and L proteins were deemed to be functional and could be used in CDV rescue.

Construction of p(+):CDV. The cDNA clone p(+):CDV was generated using genomic RNA from the vaccine strain CDV OND-LP. A strategy was chosen using unique endonuclease recognition sites within the genomic sequence of CDV. This reduced the risk of introducing changes, which might impair growth of any recombinant virus. A plasmid of 18,743 kb was generated with a T7 promoter directly adjacent to the viral leader sequence, the antigenome of CDV followed by δ -ribozyme and T7 terminator sequences for correct termination of RNA molecules (Fig. 1C). After each cloning step, the resulting plasmids were screened for correct uptake of the new fragment and sequenced across the cloning site. The complete p(+):CDV was sequenced because *Taq* DNA polymerase was used for the majority of PCR amplifications. We confirmed that no major sequence changes had taken place. In addition, sequencing confirmed the correct construction of the plasmid and presence of the genetic tag within the coding region of L. Two more mutations were detected after comparison with published CDV Onderstepoort sequences (4, 5, 7, 16, 32, 37). One nt exchange was found in the M-F intergenic at nt position 4,724 (T to A), and one was detected within the coding region of the L at position 9,067 (A = T, L13 E = V).

Rescue of CDV. The CDV rescue system was based on the MVA-T7-mediated rescue established for MV by Schneider et al. (39). After the functionality of N, P, and L proteins had been determined, the protocol for rescue was optimized. HeLa cells were chosen for the rescue experiments, which were set up in triplicate. Negative controls without pEMC-L were included. The effect of MVA-T7 on the cells was observed in control experiments without plasmids or Lipofectin. At 2 days p.t., the HeLa cells started to detach, and in order not to lose any of the transfected cells, Vero cells were added. The obvious effect of this supplement was that the HeLa cells reattached to the substrate, and both cell types settled to form a monolayer. At 3 days p.t., 1 ml of each supernatant was added to confluent monolayers of Vero cells. The next day syncytia were clearly visible in the six-well trays. Due to the different potentials of HeLa and Vero cells for fusion, the syncytia did not appear as defined round structures but were rather diffuse in nature. Syncytia also formed in the flask which contained the 3-day p.t. supernatant, and these showed normal progression over the next 2 days. Rescued virus (rCDV) was then propagated in Vero cells. Preliminary observations revealed no impairment in the progression of infection, and CPEs looked like normal syncytia caused by OND-LP infection (Fig. 3A). Control transfections without pEMC-L never gave rise to infectious virus, and CPE did not appear. In those HeLa cells which were infected only with MVA-T7, no CPE could be observed, other than that cells also started to detach at 2 days p.i. This indicated that the detachment of the HeLa cells at that time was a result of the MVA-T7 infection rather than of the trans-

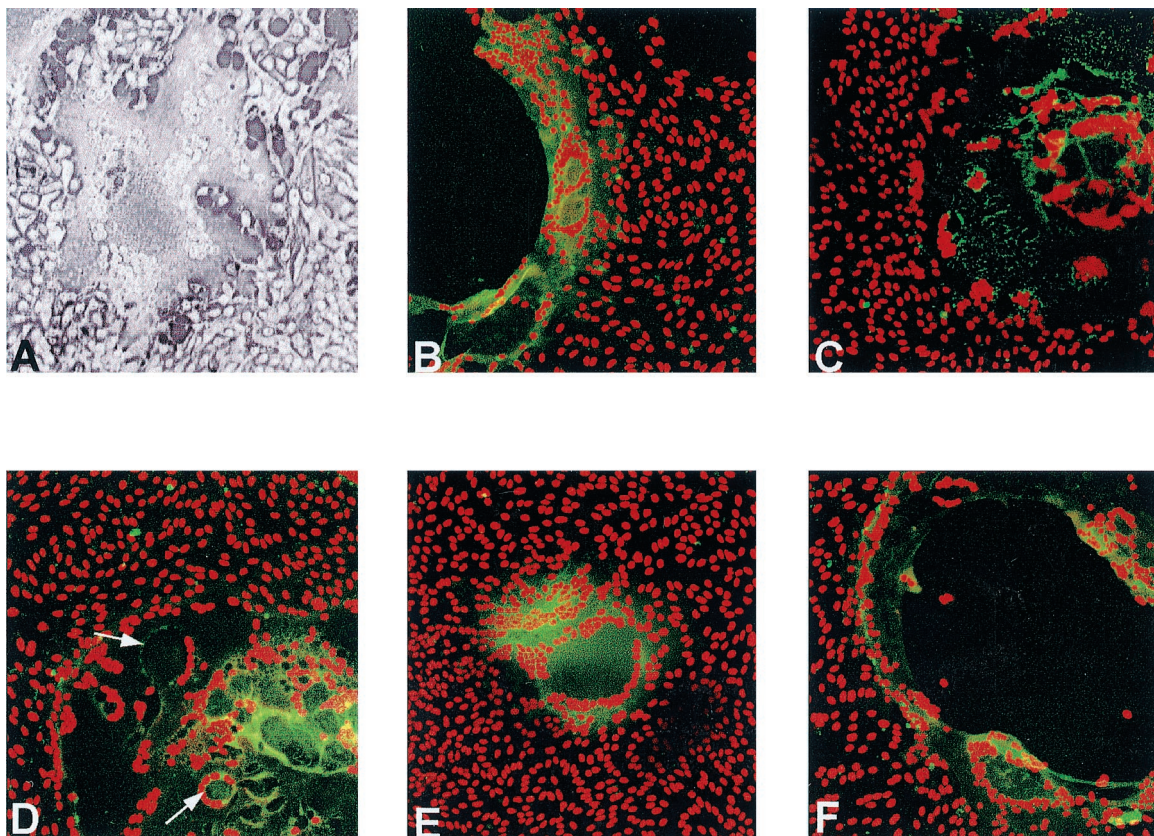


FIG. 3. Immunofluorescence of rCDV. Vero cells were infected with rCDV at an MOI of 0.1 and acetone fixed at 24 h p.i. when CPEs of different stages of progression were visible. Expression of viral proteins was detected with specific MAbs, and nuclei were counterstained with propidium iodide. Staining for the N (B), P (C), M (D), F (E), and H (F) proteins is shown by the green fluorescence of FITC-labeled rabbit anti-mouse antibodies. (A) Light-microscopic photograph of a syncytium in an infected native monolayer of Vero cells stained with methylene blue (magnification, $\times 160$).

fection procedure. The virus has been rescued successfully in every one of five attempts.

Immunofluorescence of rCDV. Indirect fluorescence with antibodies specific to all viral proteins except the L polymerase was used to characterize rCDV and to confirm that the infectious virus expressed all major structural proteins. Two MAbs (anti-F IC5 and anti-M 2.47) specifically recognized CDV proteins and did not cross-react with PDV or MV structures, while the N, P, and H MAbs cross-reacted with PDV and MV. Vero cells grown on coverslips were infected with passage-four rCDV at an MOI of 0.1 and fixed at 24 h p.i., when syncytia of different stages were visible. The green fluorescence of indirectly stained viral proteins was observed by confocal laser microscopy (Fig. 3). We demonstrated that N, P, M, F, and H proteins were expressed in infected cells.

Figure 3 also allowed us to document how syncytium formation progressed in the course of the rCDV infection. In Fig. 3E a syncytium had only started to form. A limited number of cells were involved, but nuclei had already moved to the border of the syncytium. A diffuse green immunofluorescence pointed to expression of viral F protein. In Fig. 3C and D, countless cells fused to form large syncytia which were typical for later stages of CDV infection and which started to disintegrate. The disintegration of syncytia started with formation of "bubbles," around which nuclei assembled (Fig. 3D, arrows), and finished with bursting of the whole structure. Holes in the cell monolayer were surrounded by recessed membranes and remaining nuclei (Fig. 3B and F).

Detection of the genetic tag of rCDV. The rescued virus was characterized to prove its distinctiveness from the parental Onderstepoort vaccine strain. During the cloning procedure, a genetic tag was introduced into the coding region of the L protein. We determined the presence of this artificial *Csp45I* recognition site by two different methods. The region containing the tag was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from RNA of passage-four virus with primers generating a 1,733-bp product. A PCR product of this size was separated by agarose gel electrophoresis, and following treatment with endonuclease *Csp45I*, two fragments 354- and 1,379-bp in size were visualized. RNA obtained from CDV Onderstepoort was amplified by RT-PCR and treated with *Csp45I* as a control. As expected the PCR product could not be cut using this enzyme. To ensure that no plasmid contamination was carried over from the transfections, rCDV RNA was directly used for PCR amplification without the initial RT step. No PCR product was obtained (Fig. 4A). Second, we sequenced the region containing the genetic tag directly from first-strand cDNA, derived from genomic RNA of the same virus stock. The results clearly showed that the two nucleotide exchanges were present in the genomic RNA of rCDV (Fig. 4B).

Growth kinetics of rCDV. In order to confirm the general observation that rCDV did not show any alterations in growth compared to the Onderstepoort vaccine strain, we analyzed a growth curve. Vero cells were infected at an MOI of 0.1, and supernatant and cell-associated virus was collected every 4 h.

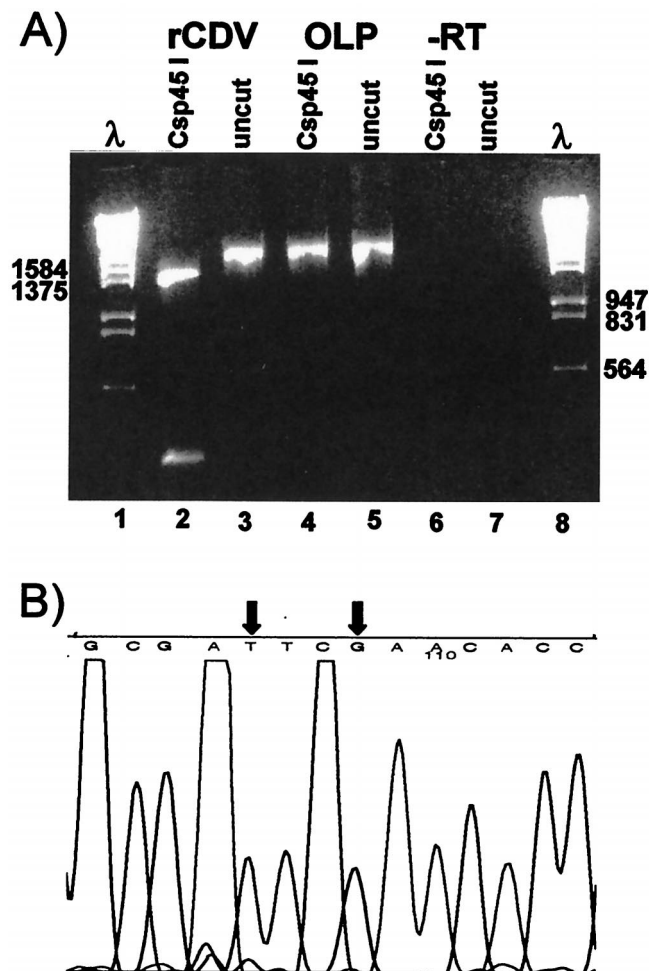


FIG. 4. Characterization of rCDV. Vero cells were infected with passage-four rCDV and harvested for RNA isolation when the monolayer was fused. First-strand cDNA was synthesized with AMV-RT and used either for sequencing or for PCR amplification of the area containing the genetic tag. (A) Shown is a photograph of a 1.5% agarose gel with separated PCR products either cut with endonuclease *Csp45I* or undigested. In lanes 1 and 8, molecular weight markers have been separated (λ *EcoRI/HindIII*). The cDNA of rCDV was digested with *Csp45I* (lane 2) or untreated (lane 3). RNA of CDV Onderstepoort was used for PCR amplification and separated either after treatment with *Csp45I* (lane 4) or after no treatment (lane 5). For lanes 6 and 7, RNA of rCDV was subjected to PCR without the RT step as a control for plasmid contaminations. Shown is a chromatogram of the sequence analysis of the area containing the genetic tag (B). Arrows indicate the two nt changes.

At 20 h p.i., CPE was clearly visible, and by 36 h p.i. the whole-cell monolayer was fused. After 52 h the cell monolayer started to detach, and it was no longer possible to separate virus in supernatant and cell-associated virus. Virus titers were estimated for each time point in triplicate (Fig. 5). The rescued virus grew to equivalent titers, and the growth kinetics showed no significant difference.

DISCUSSION

We have established a rescue system that allows the generation of infectious recombinant CDV from a full-length cDNA clone p(+)-CDV. Plasmids coding for the viral proteins N, P, and L, which are indispensable in replication and transcription, were constructed. Expression of N and P proteins was shown by means of immunofluorescence. The functionality of N, P,

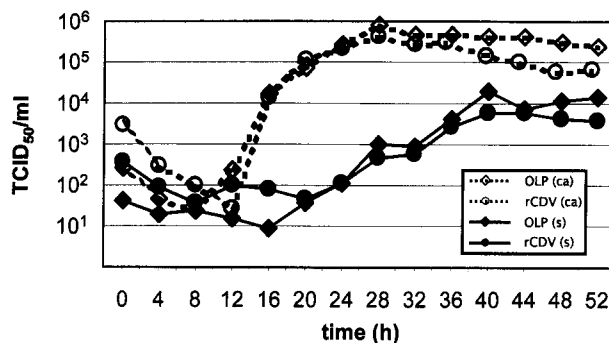


FIG. 5. Growth analysis of rCDV and CDV Onderstepoort separated into cell-associated virus and virus in supernatant. Vero cells were infected at an MOI of 0.1, and virus was collected every 4 h over a period of 52 h. Shown are the mean values of three separate 50% tissue culture-infective doses for CDV Onderstepoort and rCDV as virus in supernatant(s) and cell-associated virus (ca).

and L proteins was demonstrated in minirescue of CAT protein as a reporter. When the plasmid pCDV(-):CAT was used, it gave rise to CAT activity without the normally required L protein, indicating the presence of promoter-like elements. The same phenomenon was observed when using the plasmid pCDV(-):CAT-3, which lacks an extra stop codon at the end of the CAT open reading frame and as such does not conform to the rule of six (9). This indicated that RNA replication was probably not required to generate this enzyme activity. Hence, detailed studies of the CDV promoter would be difficult using this system. Because p107MV(-):CAT could be rescued with CDV helper proteins, we did not pursue the problem. Successful minirescue with p107MV(-):CAT confirmed that CDV N, P, and L were expressed as functional proteins and interestingly demonstrated that the helper proteins of one morbillivirus could be used to rescue the minigenome constructs of another. It has been shown for other members of the *Paramyxoviridae*, namely Sendai virus and bovine respiratory syncytial virus, that minigenome analogues can be rescued by closely related viruses (15, 46).

A novel cloning strategy was used to assemble the plasmid p(+)-CDV, which may find wider applications. Instead of cloning entire genes and having to manipulate the nt sequences in order to generate the plasmid, unique restriction endonuclease recognition sites within the viral genome were used such that the plasmid did not contain any sequence changes except for the genetic tag. Additionally, the endonuclease recognition site *Bss*HIII was introduced into reverse primer sequences used to amplify cDNAs to allow sequential assembly of p(+)-CDV. Sequence analysis of p(+)-CDV confirmed that the tag, 2 nt changes which do not cause any amino acid changes, had been inserted as designed and revealed two additional mutations. The first is situated in the noncoding region between M and F genes. The second mutation, which causes an amino acid exchange, was detected at the start of the L-coding sequence. This region is highly conserved among *Paramyxoviridae* (32). These mutations did not appear to have a detrimental effect on the growth of rCDV. The recombinant virus replicated normally in tissue culture and showed no differences when compared to the parental virus, as was confirmed by analysis of the growth kinetics of rCDV and OND-LP. The Onderstepoort vaccine strain causes mortality of up to 100% in ferrets (10, 43). It will be interesting to see whether rCDV has the same potential.

The T7 polymerase in our rescue system for CDV was de-

livered by MVA-T7, a derivative of the modified vaccinia virus Ankara. This host cell-restricted, highly attenuated mutant does not produce progeny in most mammalian cell lines and therefore does not interfere with recovery of rCDV after rescue (44). The system has previously been used to rescue MV to the same efficiency (39) as other systems (23, 31, 40, 45) with the vaccinia virus vTF7-3, which requires discrimination between the rescued viruses and the vaccinia virus. The efficiency of recovery of rCDV is similar to that of the other morbillivirus systems, with about 1 to 2 rescue events per 10^6 transfected cells. One limiting factor in rescue systems using T7 polymerase for expressing the antigenome transcripts can be overcome by introducing G residues at transcription start sites. This approach has been used in rescue of, e.g., rabies virus, vesicular stomatitis virus, and Sendai virus because T7 polymerase initiates more efficiently at multiple G residues (23, 40, 45). We used the normal viral sequence starting with ACCA, as was done before with MV and *Rinderpest virus* (3, 35).

One potential problem in rescue experiments involving a poxvirus is the homologous recombination catalyzed by vaccinia virus proteins between plasmids coding for the genome and the helper plasmids (23). In the reported work, the helper plasmids N and P were constructed using the same RNA of OND-LP, and hence recombination would not have had any impact. The tag within the coding region of the L protein of p(+)-CDV and the mutations present in the helper plasmid pEMC-L could have been exchanged by homologous recombination. However, this was not detected in this region in the rescued virus. In future work involving defined mutations in N, P, or L genes, which confer a replicative disadvantage on the virus, it has to be considered that recombination revertants might be recovered preferentially.

For verification that the rescued virus was a morbillivirus and specifically rCDV, we performed immunofluorescence studies with specific antisera. The MAbs that we used were cross-reactive with PDV, MV, and CDV proteins except for the anti-M and anti-F antibodies, which exclusively bind to the respective CDV proteins (N. Duffy, 1995. M.Sc. thesis, The Queen's University of Belfast, Belfast, Northern Ireland; A. Trudget, personal communication). The staining for N, P, M, F, and H proteins was positive. As M-protein-deficient MV (11) as well as respiratory syncytial virus, which does not express SH and G proteins (28), can be propagated in cell culture, it was important to confirm that rCDV expressed all of the major viral structural proteins.

CDV is now the third morbillivirus available for detailed analysis applying reverse genetics. We plan to introduce the green fluorescent protein into the cDNA clone to study the infection of the virus in cell culture and in an animal model as this has already been proven to be very useful in MV (19, 20). The rescue system may also facilitate studies on determinants of persistence in cells and animals and attenuating mutations in a natural host such as the ferret.

ACKNOWLEDGMENTS

We thank Martin A. Billeter and his colleagues for the generous donation of plasmid vectors.

This work was financed by the Biotechnology and Biological Sciences Research Council (grant 81/S08496). Fergal M. Collins was supported by an ESF postgraduate student training award.

REFERENCES

- Alexander, K. A., and M. J. Appel. 1994. African wild dogs (*Lyacon pictus*) endangered by a canine distemper epizootic among domestic dogs near the Masai Mara National Reserve, Kenya. *J. Wildl. Dis.* **30**:481-485.
- Appel, M. J. G., C. Reggiardo, B. A. Summers, S. Pearce-Kelling, C. J. Maré, T. H. Noon, R. E. Reed, J. N. Shively, and C. Örvell. 1991. Canine distemper virus infection and encephalitis in javelinas (collared peccaries). *Arch. Virol.* **119**:147-152.
- Baron, M. D., and T. Barrett. 1997. Rescue of rinderpest virus from cloned cDNA. *J. Virol.* **71**:1265-1271.
- Barrett, T., D. K. Clarke, S. A. Evans, and B. K. Rima. 1987. The nucleotide sequence of the gene encoding the F protein of canine distemper virus: a comparison of the deduced amino acid sequence with other paramyxoviruses. *Virus Res.* **8**:373-386.
- Barrett, T., S. B. Shrimpton, and S. E. Russell. 1985. Nucleotide sequence of the entire protein coding region of canine distemper virus polymerase-associated (P) protein mRNA. *Virus Res.* **4**:367-372.
- Barrett, T., S. M. Subbarao, G. J. Belsham, and B. W. Mahy. 1991. The molecular biology of morbilliviruses. In D. W. Kingsbury (ed.), *The paramyxoviruses*, p. 83-102. Plenum Press, New York, N.Y.
- Bellini, W. J., G. Englund, C. D. Richardson, S. Rozenblatt, and R. A. Lazzarini. 1986. Matrix genes of measles virus and canine distemper virus: cloning, nucleotide sequences, and deduced amino acid sequences. *J. Virol.* **58**:408-416.
- Bellini, W. J., G. Englund, S. Rozenblatt, H. Arnheiter, and C. D. Richardson. 1985. Measles virus P gene codes for two proteins. *J. Virol.* **3**:908-919.
- Calain, P., and L. Roux. 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J. Virol.* **67**:4822-4830.
- Carpenter, J. W., M. J. Appel, R. C. Erickson, and M. N. Novilla. 1976. Fatal vaccine induced canine distemper virus infection in black-footed ferrets. *J. Am. Vet. Med. Assoc.* **169**:961-964.
- Cathomen, T., B. Mrkic, D. Spehner, R. Drillien, R. Naef, J. Pavlovic, A. Aguzzi, M. A. Billeter, and R. Cattaneo. 1998. A matrix-less measles virus is infectious and elicits extensive cell fusion: consequences for propagation in the brain. *EMBO J.* **17**:3899-3908.
- Cattaneo, R., K. Kaelin, K. Bazko, and M. A. Billeter. 1989. Measles virus editing provides an additional cysteine rich protein. *Cell* **56**:759-764.
- Conzelmann, K. K. 1996. Genetic manipulation of non-segmented negative strand RNA viruses. *J. Gen. Virol.* **77**:381-389.
- Conzelmann, K. K., and M. Schnell. 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. *J. Virol.* **68**:713-719.
- Curran, J. A., and D. Kolakofsky. 1991. Rescue of Sendai virus DI genome by other parainfluenza viruses: implications for genome replication. *Virology* **164**:168-176.
- Curran, M. D., D. K. Clarke, and B. K. Rima. 1991. The nucleotide sequence of the gene encoding the attachment protein H of canine distemper virus. *J. Gen. Virol.* **72**:443-447.
- Curran, M. D., D. O'Loan, B. K. Rima, and S. Kennedy. 1990. Nucleotide sequence analysis of phocine distemper virus reveals its distinctness from canine distemper virus. *Vet. Rec.* **127**:430-431.
- Duignan, P. J., C. House, M. T. Walsh, T. Campbell, G. D. Bossart, N. Duffy, P. J. Fernandes, B. K. Rima, S. Wright, and J. R. Geraci. 1995. Morbillivirus infection in manatees. *Mar. Mammal Sci.* **11**:441-451.
- Duprex, W. P., S. McQuaid, L. Hangartner, M. A. Billeter, and B. K. Rima. 1999. Observation of measles virus cell-to-cell spread in astrocytoma cells by using a green fluorescent protein-expressing recombinant virus. *J. Virol.* **11**:9568-9575.
- Duprex, W. P., S. McQuaid, B. Roscic-Mrkic, R. Cattaneo, C. McCallister, and B. K. Rima. 2000. In vitro and in vivo infection of neural cells by a recombinant measles virus expressing enhanced green fluorescent protein. *J. Virol.* **74**:7972-7979.
- Follmann, E. H., G. W. Warner, J. F. Evermann, and A. J. McKeirnan. 1996. Serological evidence of morbillivirus infection in polar bears (*Ursus maritimus*) from Alaska and Russia. *Vet. Rec.* **138**:615-618.
- Forsyth, M. A., S. Kennedy, S. Wilson, T. Ethatov, and T. Barrett. 1998. Canine distemper virus in a Caspian seal. *Vet. Rec.* **143**:662-664.
- Garcin, D., T. Pelet, P. Calain, L. Roux, J. Curran, and D. Kolakofsky. 1995. A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus. *EMBO J.* **14**:6087-6094.
- Haas, L., and T. Barrett. 1996. Rinderpest and other animal morbillivirus infections: comparative aspects and recent developments. *Zentralbl. Veterinärmed.* **43**:411-420.
- Haas, L., H. Hofer, M. East, P. Wohlsein, B. Liess, and T. Barrett. 1996. Canine distemper virus infection in Serengeti spotted hyaenas. *Vet. Microbiol.* **49**:147-152.
- Harder, T. C., M. Kenter, H. Vos, K. Siebelink, W. Huisman, G. Van Amerongen, C. Örvell, T. Barrett, M. J. Appel, and A. D. Osterhaus. 1996. Canine distemper virus from diseased large felids: biological properties and phylogenetic relationships. *J. Gen. Virol.* **77**:397-405.
- Izeni, F., F. Baudin, D. Blondel, R. W. Ruigrok. 2000. Structure of the RNA inside the vesicular stomatitis virus nucleocapsid. *RNA* **6**:270-281.
- Karron, R. A., D. A. Buonagurio, A. F. Georgiu, S. S. Whitehead, J. E. Adamus, M. L. Clements-Mann, D. O. Harris, V. B. Randolph, S. A. Udem, B. R. Murphy, and M. S. Sidhu. 1997. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV

- subgroup B mutant. *Proc. Natl. Acad. Sci. USA* **94**:13961–13966.
29. **Kennedy, S.** 1998. Morbillivirus infections in aquatic mammals. *J. Comp. Pathol.* **119**:201–225.
 30. **Kolakofsky, D., T. Pelet, D. Garcin, S. Hausmann, J. Curran, and L. Roux.** 1998. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *J. Virol.* **72**:891–899.
 31. **Lawson, N. D., E. A. Stillman, M. A. Whitt, and J. K. Rose.** 1995. Recombinant vesicular stomatitis virus from DNA. *Proc. Natl. Acad. Sci. USA* **92**:4477–4481.
 32. **Mcllhatton, M. A., M. D. Curran, and B. K. Rima.** 1997. Nucleotide sequence analysis of the large (L) genes of phocine distemper virus and canine distemper virus (corrected sequence). *J. Gen. Virol.* **78**:571–576.
 33. **Morell, V.** 1994. Serengeti's big cats going to the dogs. *Science* **264**:1664.
 34. **Örvell, C., and H. Sheshberadaran.** 1991. Phocine distemper virus is phylogenetically related to canine distemper virus. *Vet. Rec.* **129**:267–269.
 35. **Radecke, F., P. Spielhofer, H. Schneider, K. Kaelin, M. Huber, C. Dötsch, G. Christiansen, and M. Billeter.** 1995. Rescue of measles virus from cloned cDNA. *EMBO J.* **14**:5773–5784.
 36. **Roelke-Parker, M. E., L. Munson, C. Packer, R. Kock, S. Cleaveland, M. Carpenter, S. J. O'Brien, A. Pospischil, R. Hofmann-Lehmann, H. Lutz, G. L. M. Mwamengele, M. N. Mgasa, G. A. Machange, B. A. Summers, and M. J. G. Appel.** 1996. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). *Nature* **379**:441–445.
 37. **Rozenblatt, S., O. Eizenberg, R. Ben-Levy, V. Lavie, and W. J. Bellini.** 1985. Sequence homology within morbilliviruses. *J. Virol.* **53**:684–690.
 38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 39. **Schneider, H., P. Spielhofer, K. Kaelin, C. Dötsch, F. Radecke, G. Sutter, and M. A. Billeter.** 1997. Rescue of measles virus using a replication-deficient vaccinia-T7 vector. *J. Virol. Methods* **64**:57–64.
 40. **Schnell, M. J., T. Mebatsion, and K. K. Conzelmann.** 1994. Infectious rabies virus from cloned cDNA. *EMBO J.* **13**:4195–4203.
 41. **Sharma, B., E. Norrby, M. Blixenkron-Møller, and J. Kövamees.** 1992. The nucleotide and deduced amino acid sequence of the M gene of phocine distemper virus (PDV): the most conserved protein of morbilliviruses shows a uniquely close relationship between PDV and canine distemper virus. *Virus Res.* **23**:13–25.
 42. **Sidhu, M. S., W. Husar, S. D. Cook, P. C. Dowling, and S. A. Udem.** 1993. Canine distemper terminal and intergenic non-protein coding nucleotide sequences: completion of the entire CDV genome sequence. *Virology* **193**:66–72.
 43. **Stephensen, C. B., J. Welter, S. R. Thaker, J. Taylor, J. Tartaglia, and E. Paoletti.** 1997. Canine distemper virus (CDV) infection of ferrets as a model for testing morbillivirus vaccine strategies: NYVAC- and ALVAC-based CDV recombinants protect against symptomatic infection. *J. Virol.* **71**:1506–1513.
 44. **Sutter, G., M. Ohlmann, and V. Erfle.** 1995. Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. *FEBS Lett.* **371**:9–12.
 45. **Whelan, S. P. J., L. A. Ball, J. N. Barr, and G. T. W. Wertz.** 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc. Natl. Acad. Sci. USA* **92**:8388–8392.
 46. **Yunus, A. S., S. Krishnamurthy, M. K. Pастey, Z. Huang, S. K. Khattar, P. L. Collins, and S. K. Samal.** 1999. Rescue of a bovine respiratory syncytial virus genomic RNA analog by bovine, human and ovine respiratory syncytial viruses confirms the “functional integrity” and “cross-recognition” of BRSV *cis*-acting elements by HRSV and ORSV. *Arch. Virol.* **144**:1977–1990.