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The Hemagglutinin of Canine Distemper Virus Determines Tropism and Cytopathogenicity

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Canine distemper virus (CDV) and measles virus (MV) cause severe illnesses in their respective hosts. The viruses display a characteristic cytopathic effect by forming syncytia in susceptible cells. For CDV, the proficiency of syncytium formation varies among different strains and correlates with the degree of viral attenuation. In this study, we examined the determinants for the differential fusogenicity of the wild-type CDV isolate 5804Han89 (CDV_{5804}), the small- and large-plaque-forming variants of the CDV vaccine strain Onderstepoort (CDV_{OS} and CDV_{OL}, respectively), and the MV vaccine strain Edmonston B (MV_{Edm}). The cotransfection of different combinations of fusion (F) and hemagglutinin (H) genes in Vero cells indicated that the H protein is the main determinant of fusion efficiency. To verify the significance of this observation in the viral context, a reverse genetic system to generate recombinant CDVs was established. This system is based on a plasmid containing the full-length antigenomic sequence of CDV_{OS}. The coding regions of the H proteins of all CDV strains and MV_{Edm} were introduced into the CDV and MV genetic backgrounds, and recombinant viruses rCDV-H_{5804}, rCDV-H_{OL}, rCDV-H_{Edm}, rMV-H_{5804}, rMV-H_{OL}, and rMV-H_{OS} were recovered. Thus, the H proteins of the two morbilliviruses are interchangeable and fully functional in a heterologous complex. This is in contrast with the glycoproteins of other members of the family *Paramyxoviridae*, which do not function efficiently with heterologous partners. The fusogenicity, growth characteristics, and tropism of the recombinant viruses were examined and compared with those of the parental strains. All these characteristics were found to be predominantly mediated by the H protein regardless of the viral backbone used.

Canine distemper virus (CDV) and *Measles virus* (MV) are closely related members of the *Morbillivirus* genus in the *Paramyxoviridae* family in the order *Mononegavirales* (29). The disease caused by CDV in susceptible animals, like dogs and ferrets, strongly resembles the course of MV infection in humans and is characterized by fever, rash, and leukocytopenia. CDV frequently spreads in the central nervous system and can lead to different neuropathological alterations (48).

The genome organizations of CDV and MV are very similar, with both consisting of single-stranded negative-sense RNA of 15,690 nucleotides (nt) (CDV vaccine strain Onderstepoort) or 15,894 nt (MV vaccine strain Edmonston B [MV_{Edm}]), respectively (30, 37). The genomic RNA that is tightly encapsidated by the nucleocapsid (N) protein serves as a template for transcription and replication by the viral polymerase (L) protein and its cofactor phosphoprotein (P). The N, P, and L proteins together with the viral RNA constitute the ribonucleoprotein (RNP) complex (36), which directs the sequential synthesis of capped and polyadenylated mRNAs from six transcription units or the replication of full-length encapsidated antigenomes (19). The viral envelope contains two integral membrane proteins, the fusion (F) and hemagglutinin (H) proteins, and a membrane-associated protein (matrix [M]), which mediates the contacts with the RNP (5). The H glycoprotein mediates the binding of the virus to the cell membrane, and the F protein executes the fusion of the two membranes, which enables the entry of the viral RNP into the cytoplasm (20). It is of interest that the amino acid sequence of the mature F protein shows about 4% variability among different CDV strains, which is in the range of variability of the other structural proteins, whereas the CDV H proteins vary by about 10%. F and H proteins of CDV and MV differ in 33 and 64% of their residues, respectively (3, 12, 13). This difference also manifests itself antigenically, which enables the discrimination of wild-type and vaccine strain H proteins with monoclonal antibodies (MAbs) (14, 34, 35).

A correlation between the proficiency of syncytium formation by certain CDV strains and their level of attenuation can be drawn: the more attenuated a strain is, the higher its fusogenicity is (7, 42, 47, 52). Therefore, the identification of factors that determine the extent of fusogenicity in vitro could give insights into the mechanism of virulence in vivo. It is known that the coexpression of CDV or MV F and H proteins is sufficient to induce fusion in Vero cells, but the determinants of fusogenicity remain to be determined (41).

Using a transient-expression system, we have identified the H protein as the major fusogenicity determinant. Furthermore, to assess the contribution of the H protein not only to the fusogenicity but also to the growth characteristics and tropism of CDV, we attempted to generate recombinant viruses with H-gene replacements. Toward this end, we cloned and sequenced the entire genome of the small-plaque-forming variant of CDV vaccine strain Onderstepoort (CDV_{OS}). We also established a reverse genetic system that allows the recovery of recombinant CDV; a similar system has recently been estab-

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lished using the large-plaque-forming variant of the vaccine strain Onderstepoort (CDV_{OL}) (11). We then examined the effects of the introduction of H genes originating from strains with different fusogenicity in the context of CDV_{OS} and MV_{Edm}.

MATERIALS AND METHODS

Cells and viruses. Vero cells (ATCC CCL-81) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS). 293 cells (ATCC CRL-1573) and DF1 cells (a kind gift of M. Federspiel) were maintained in the same medium with 10% FCS. DH 82 cells (ATCC CRL-10389) were cultured in Eagle's minimal essential medium with nonessential amino acids and 15% FCS. All tissue culture media as well as additions and FCS were purchased from Life Technologies. The wild-type CDV isolate 5804Han89 (CDV_{5804}), the CDV vaccine strains CDV_{OS} and CDV_{OL} (Institute of Virology, Veterinary School Hannover), and the MV strain MV_{Edm} were propagated in Vero cells. The wild-type CDV isolate originated from a dog that showed clinical signs of infection. The vaccine strain CDV_{OS} was the third passage of an Onderstepoort vaccine obtained by B. Liess in 1965. The sequence of the vaccine strain CDV_{OL} corresponds exactly to that of the Onderstepoort strain used by Sidhu et al. (37) (revised sequence, GenBank accession no. gi:3335048). The MV strain MV_{Edm} used in this study was recovered from plasmid p(+)MVNSe (39). Stocks of the host range mutant of vaccinia virus Ankara that expresses the T7 polymerase (MVA-T7) (43) were grown in the chicken fibroblast line DF1.

RT and establishment of consensus sequences. All cloning procedures were performed following standard protocols. To generate the CDV-based plasmids, total RNA was isolated from Vero cells infected with CDV_{5804}, CDV_{OS}, or CDV_{OL}. The reverse transcription (RT) reactions were performed using Superscript II RNase H^- Reverse Transcriptase (Gibco BRL) and random primers. The region of interest was then PCR amplified using the Expand High Fidelity PCR system (Roche Biochemicals) and specific primers. All PCR products were first cloned into TA Cloning vectors (Invitrogen) according to the manufacturer's protocol. These vectors contain additional restriction sites up- and downstream of the region in which the PCR product is ligated. At least three different clones were sequenced (ABI PRISM 377 DNA Sequencer; Perkin-Elmer Applied Biosystems) and compared to determine the consensus sequence. The alignment showed that the RT-PCR products had approximately one mismatch every 3 kb. Only clones that concurred completely with the consensus sequence were used for the cloning of the full-length plasmid. The comparison of this consensus sequence with the published sequence for another Onderstepoort strain (37) (revised sequence accession no. gi:3335048) revealed 103 nucleotide exchanges, of which 48 resulted in amino acid differences. Most of these changes occurred in the H and L protein (14 each) followed by the M and F protein (7 each). In the N and P proteins three differences each were observed.

Construction of expression plasmids and of a full-length DNA copy of the CDV_{OS} genome. The F and H genes of CDV_{5804}, CDV_{OS}, and CDV_{OL} were subcloned into the eukaryotic expression vector pCG (4), resulting in pCG-F_{5804}, pCG-H_{5804}, pCG-F_{OS}, pCG-H_{OS}, pCG-F_{OL}, and pCG-H_{OL}. Furthermore, the N, P, and L genes of CDV_{OS} were subcloned into the pTM1 vector (26), in which an internal ribosomal entry site is located downstream of the T7 promoter to ensure efficient translation of the RNA transcribed by the T7 polymerase, yielding pTM1-N_{OS}, pTM1-P_{OS}, and pTM1-L_{OS}.

A full-length cDNA clone of CDV_{OS} was generated by subcloning overlapping 2- to 3-kb fragments of the genome. Unique internal restriction sites within the genomic sequence as well as those externally provided by the TA cloning vector were used. The low-copy-number plasmid pBR322 was chosen as the backbone to avoid difficulties in propagating the large full-length DNA in bacteria, as described previously for another system (25). The cloning strategy involved the two-step generation of a vector that contained the T7 promoter followed by the CDV sequence up to a unique internal restriction site in the P gene (*Sal*I) and the last third of the L gene from the unique internal restriction site *Pin*AI with the adjacent hepatitis δ ribozyme and T7 termination signal. The preassembled intermediate part (nt 2962 to 14911) was then inserted into the *Sal*I and *Pin*AI sites of this vector, leading to the full-length antigenomic CDV cDNA clone pCDV3 (Fig. 1).

The correct connection between the T7 promoter and the leader region of the CDV antigenome was constructed by inserting the T7 promoter sequence directly into the forward primer, resulting in the subclone pCR2.1-T7NP_{OS}. The hepatitis δ ribozyme sequence followed by the T7 termination signal was amplified from p(+)MV (30) and attached to the trailer region of the CDV antigenome by overlap extension PCR (15) leading to pCR2.1-5′$Ribo_{OS}$. The overlapping subclones were generated as described above, resulting in pCR2.1-PM_{OS}, pCR2.1-F_{OS}, pCR2.1-FH_{OS}, pCR2.1-HL_{OS}, pCR2.1-$L1_{OS}$, and pCR2.1-$L2_{OS}$ (Fig. 1, top). The large intermediate fragment covering the 11,950-nt region between the P and L gene was assembled from these subclones.

Construction of full-length plasmids with exchanged H proteins. The MV plasmids p(+)MVNSe, pTM-EdN, pTM-EdP, pEMC-La, pCG-EdF, and pCG-EdH used in this study were a kind gift of M. Billeter (30, 33). To facilitate the construction of full-length CDV plasmids with exchanged H proteins, unique restriction sites were introduced in the 3′ (*Pac*I; nt 7046 to 7053) untranslated regions (UTRs) of the F open reading frame (ORF) and the 3′ (*Apa*I; nt 8927 to 8932) UTR of the H ORF by site-directed mutagenesis (Quick-Change Site Directed Mutagenesis Kit; Stratagene). The resulting plasmid was named pCDVII.

The H-protein genes of CDV_{5804}, CDV_{OL}, and MV_{Edm} were amplified from the pCG plasmids described above, using primers which introduced a *Pac*I site upstream and a *Apa*I site downstream of the respective H gene in a way that left the UTR unchanged and respected the rule of six (27). The PCR products were cloned into pCDVII, and the sequences were confirmed. The resulting plasmids were named pCDV-H_{5804}, pCDV-H_{OL}, and pCDV-H_{Edm}. The generation of pCDV-H_{Edm} required a two-step cloning procedure due to an internal *Apa*I site. The plasmid p(+)MVNSe was used as the backbone for the generation of the MV-based recombinants. It contains a unique *Pac*I site in the 3′ UTR of the F ORF and a *Spe*I site in the 3′ UTR of the H ORF. Consequently, the H-protein genes of CDV_{5804}, CDV_{OL}, and CDV_{OS} were amplified as described above using primers that introduced a *Pac*I site upstream and a *Spe*I site downstream of the respective H genes and cloned into p(+)MVNSe, resulting in p(+)MV-H_{5804}, p(+)MV-H_{OL}, and p(+)MV-H_{OS}. The generation of p(+)MV-H_{OL} required a two-step cloning procedure due to an internal *Spe*I site. The sequences were subsequently confirmed.

Transfections. For the fusion experiments, Vero cells were transfected with the different F- and H-coding plasmids using a molar ratio of 1:4. This ratio had been determined to be the most effective for fusion activity (data not shown). Lipofectamine 2000 (Gibco BRL) was used as a transfection reagent, following the protocol of the supplier. Briefly, cells were seeded in 24-well plates so that they reached about 80% confluence for transfection. For each well to be transfected, 1 μg of DNA was diluted in 50 μl of OptiMEM (Gibco BRL). Another 50 μl of OptiMEM containing 2 μl of Lipofectamine 2000 reagent was added to each well, and the mixture was incubated at room temperature for 30 min. Before the solution was added to the cells, the culture medium was removed and replaced with 0.5 ml of DMEM without serum. The fusion activity was evaluated 72 h after transfection. The size and number of syncytia were used to quantitate the fusion activity of the combination of F and H proteins.

Recovery of recombinant viruses. It has been shown previously that the polymerase complex of certain members of the family *Paramyxoviridae* can efficiently drive the replication of a minigenome with leader and trailer sequence of other members of the same subfamily (8, 51). Therefore, we used the MV and CDV plasmids coding for the N, P, and L proteins in parallel for our initial attempt to recover CDV from cDNA. In contrast to the leader and trailer sequences, which are highly conserved in CDV and MV, which is a possible explanation for their recognition by the heterologous polymerase complex in a minigenome system, the internal UTRs, which play an important role in the control of viral transcription are less homologous (36, 37). Nevertheless, both polymerase complexes led to the recovery of recombinant viruses with comparable efficiencies.

The recombinant viruses were recovered using a MVA-T7-based system (33). 293 cells were infected with MVA-T7 with a multiplicity of infection (MOI) of 0.8 and seeded in six-well plates with a density of 10^6 cells per well. The calcium phosphate transfection was performed using the Profection mammalian transfection system (Promega). Four micrograms of the respective antigenomic plasmid and a set of three plasmids (2 μg of N-protein plasmid, 2 μg of P-protein plasmid, and 0.5 μg of L-protein plasmid in 10 mM Tris-HCl [pH 8.5]) from which the proteins of the viral polymerase complex of either CDV or MV are expressed were diluted in 175 μl of double-distilled water. Then, 25 μl of 2 M $CaCl_2$ was added to the solution followed by vortexing. This mixture was added dropwise to 200 μl of 2× HEPES-buffered saline (pH 7.1) while vortexing continuously. After incubation for 30 min at room temperature, the mixture was added dropwise to the cells. The supernatant was removed the next day, and the cells were maintained in DMEM with 10% FCS for 3 days. Since the infection of 293 cells does not lead to the formation of easily detectable syncytia, cells of each well were transferred to a 75-cm^2 dish in which Vero cells had been seeded at 50 to 60% confluency. The first syncytia could be seen between 7 and 10 days after transfection. Normally, for each virus three syncytia were picked and transferred onto fresh Vero cells in six-well plates. These infected cells were expanded into 75-cm^2 dishes. When the cytopathic effect (CPE) was pronounced, the culture

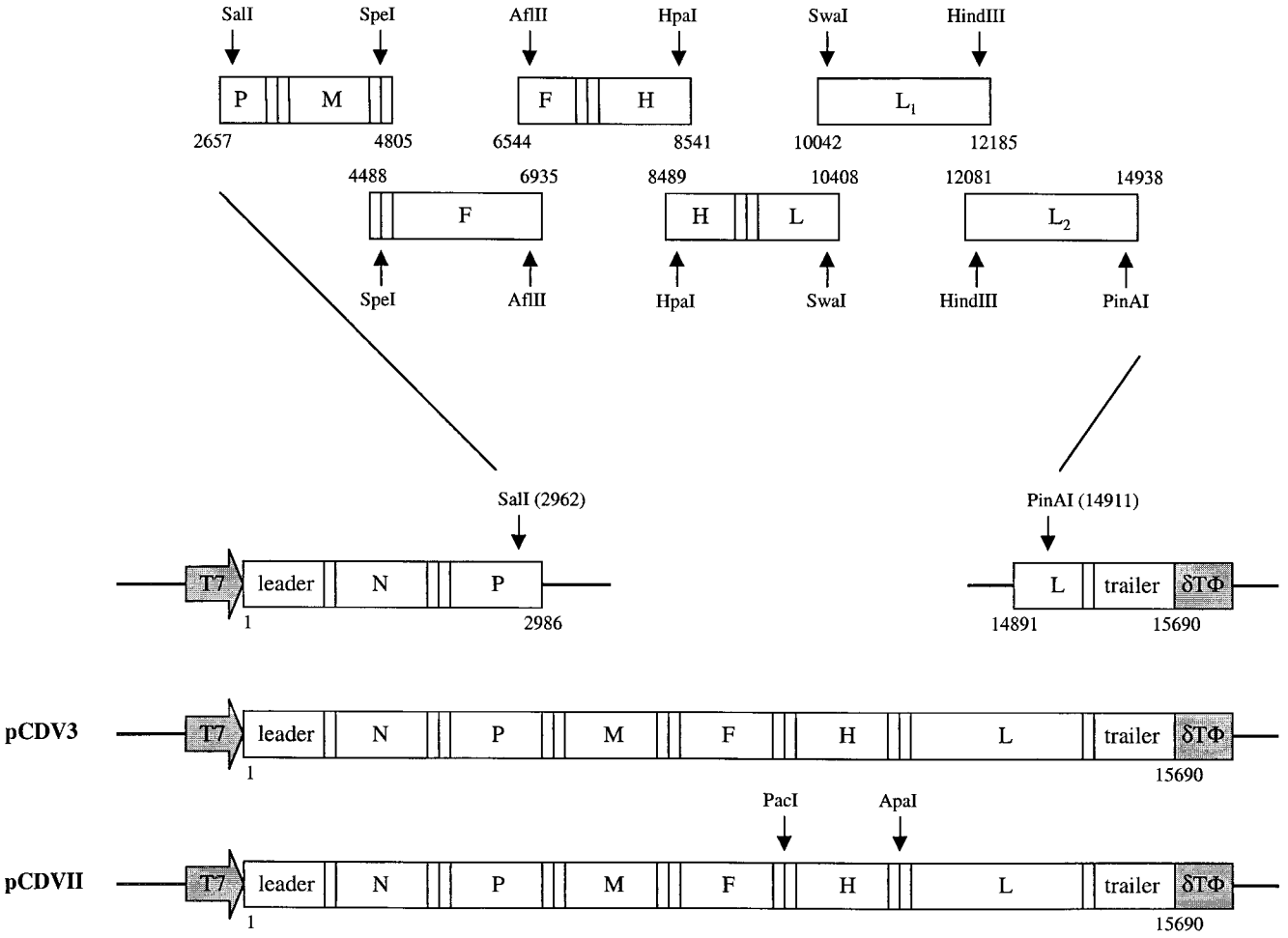


FIG. 1. Cloning strategy and structure of the CDV full-length cDNA plasmids. (Top) Schematic representation of the six fragments used to assemble the intermediate segment (11,950 nt) of pCDV. (Center) Intermediate vector. The T7 promoter (grey arrow), the CDV sequence from nt 1 to 2986 and from nt 14891 to 15690, and the hepatitis δ ribozyme and the T7 terminator (grey boxes) are shown. (Bottom) The two full-length plasmids pCDV3 and pCDVII. Two restriction sites (*Pac*I [nt 7046 to 7053] and *Apa*I [nt 8927 to 8932]) were introduced into the 3′ and 5′ UTRs of the H gene of pCDV3 by site-directed mutagenesis to generate pCDVII. The first and last nucleotide of each fragment (referring to the complete genome) are indicated. The drawing is not to scale. The pBR322 vector backbone (thick line), fragments of the CDV genome (boxes), untranslated intergenic regions (three vertical lines), and the approximate locations of restriction sites used (arrows) are indicated.

medium was replaced by 2 ml of OptiMEM (Gibco BRL) and the cells were scraped into the medium and subjected once to freezing and thawing. The cleared supernatants were used for all further analysis. Viruses with the wild-type CDV H protein did not display strong syncytium formation; nevertheless, when the overlaid cells were split once at a ratio of 1:4, foci were detected. Ten of these foci were picked for each virus and transferred onto fresh Vero cells for further propagation to secure the viruses. Between three and eight of these wells contained virus as confirmed by immunohistochemical staining. These viruses were expanded further to grow a virus stock.

Growth curves and immunohistochemical staining. Cells (8×10^5/well) were seeded into six-well plates and infected at a MOI of 0.01 with the respective viruses. All analyses were performed in duplicate. After 2 h of adsorption, the inoculum was removed and the cells were washed twice with medium and further incubated at 32°C. At various times after infection, supernatant and cell-associated virus were recovered separately and stored at −70°C. The 50% tissue culture infectious dose ($TCID_{50}$) of the samples was determined in Vero cells. For viruses that did not display sufficient syncytium formation to determine the $TCID_{50}$ visually, the plates were washed once with 0.3× phosphate-buffered saline (PBS) (Gibco BRL), pH 7.8, dried, and heat fixed for 7 h at 80°C. Immunohistochemical staining was performed, using the CDV H-protein-specific rabbit antiserum MC713 (CDV-Hcyt) (1:1,000 dilution), which was generated by immunizing a rabbit with a keyhole limpet hemocyanin-coupled peptide consisting of the 24 N-terminal residues of the CDV H protein. The peroxidase-conjugated donkey anti-rabbit immunoglobulin G antiserum (Amersham Pharmacia Biotech) was used as a secondary antibody and 3-amino-9-ethylcarbazole was the substrate (Biomeda Corp.).

Indirect immunofluorescence assay. Subconfluent Vero cells were infected with a MOI of 0.01 with the respective virus and incubated for 48 h at 37°C. The cells were then fixed with 2% paraformaldehyde, blocked with 0.5 M glycine, permeabilized with 0.1% Triton X-100, and incubated with the CDV P-protein-specific MAb CD/PX4 (1:200 dilution) (23) for 60 min at room temperature. This MAb recognizes an epitope that is conserved in CDV and MV. The staining was performed using a fluorescein isothiocyanate-conjugated mouse anti-mouse immunoglobulin G (Amersham Pharmacia Biotech).

Western blot analysis. Vero cells were seeded into six-well plates, simultaneously infected with a MOI of 0.01 with the respective virus, and incubated at 37°C until CPE was observed. Cells were washed twice with PBS before the addition of 0.5 ml of lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 8.0]) with complete protease inhibitor (Roche Biochemicals) to each well. After incubation for 30 min at 4°C, the lysates were cleared by centrifugation at 5,000 × *g* for 15 min at 4°C and the supernatant was mixed with an equal amount of 2× Laemmli sample buffer (Bio-Rad) containing 0.5% β-mercaptoethanol. The samples were incubated for 10 min at 95°C and subsequently fractionated on

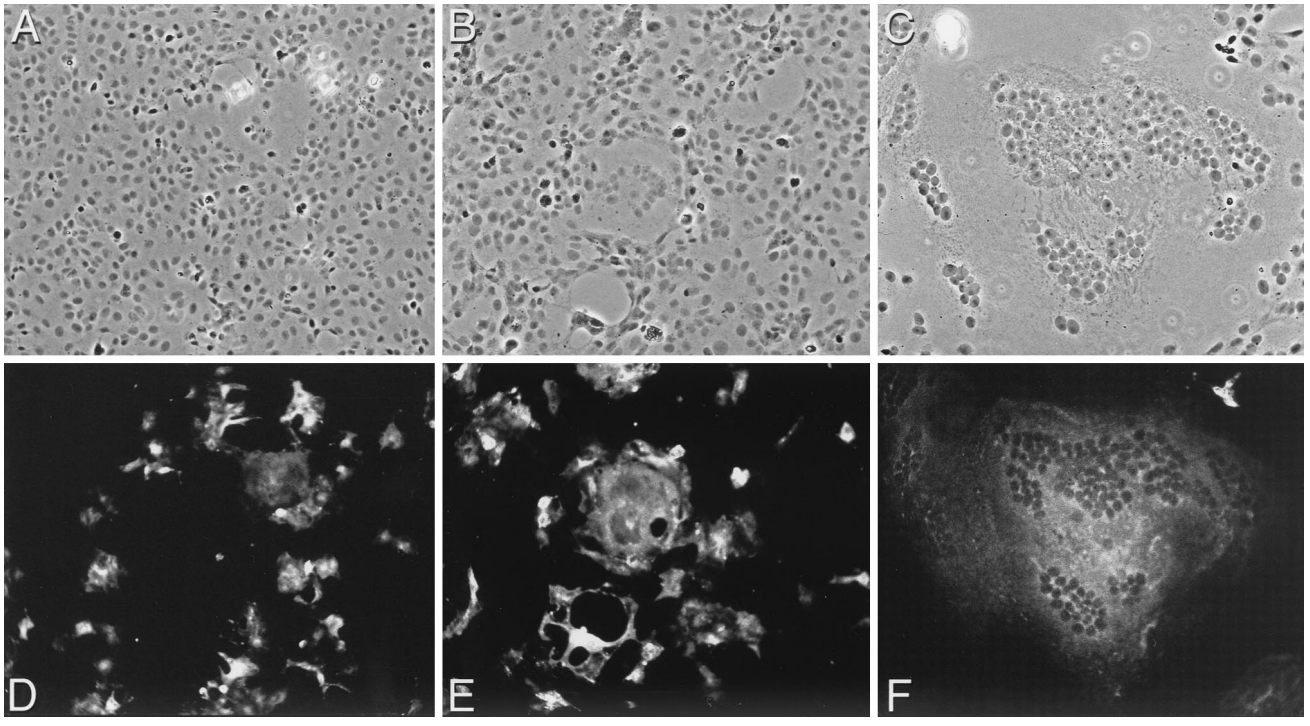


FIG. 2. Vero cells infected with different CDV isolates. CDV_{5804} (A and D) CDV_{OS} (B and E), and CDV_{OL} (C and F) were used. Cells were fixed with paraformaldehyde, permeabilized 48 h after infection with a MOI of 0.01, and observed by phase-contrast microscopy (A to C) or immunofluorescence staining (D to F). A MAb against the CDV P protein (CD/PX4) that recognizes an epitope conserved between MV and CDV was used.

7.5% (H protein) or 10% (F protein) SDS-polyacrylamide gels (Bio-Rad) and blotted on polyvinylidene difluoride membranes (Millipore). After the membranes were blocked with 1% blocking reagent (Roche Biochemicals) overnight, they were incubated with the following primary antibodies (1:10,000) for 2 h at room temperature: anti-Fcyt rabbit antipeptide serum that recognizes the 14 carboxy-terminal residues of the CDV and MV F protein (6) or a combination of anti-Hcyt rabbit antipeptide serum that recognizes the 14 amino-terminal residues of the MV H protein (6) and CDV-Hcyt. Following the incubation with a peroxidase-conjugated goat anti-rabbit immunoglobulin G antiserum, the membranes were subjected to enhanced chemiluminescence detection (Amersham Pharmacia Biotech).

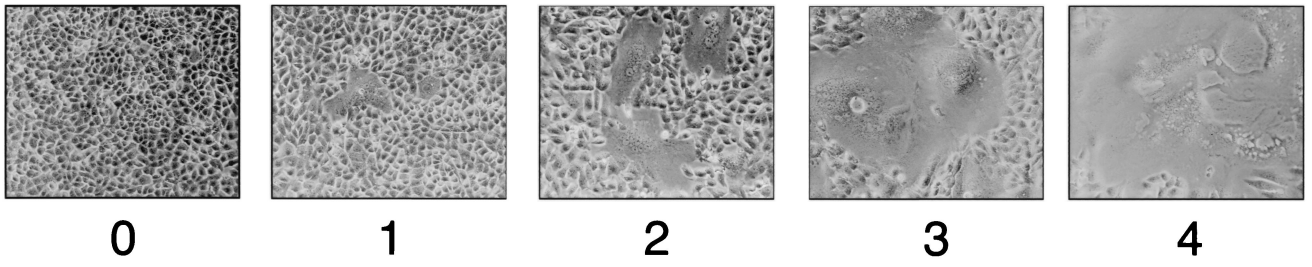
Nucleotide sequence accession number. The consensus sequence has been deposited in GenBank under accession no. AF 378705.

RESULTS

The H protein determines the efficiency of cell-cell fusion in a transient-expression assay. CDV strains fuse host cells with different efficiencies. Figure 2 illustrates the minimal CPE of the wild-type isolate CDV_{5804} (Fig. 2A and D), the intermediate fusion efficiency of the vaccine strain CDV_{OS} (Fig. 2B and E), and the strong fusion activity of CDV_{OL} (Fig. 2C and F). To identify the determinants of the fusion efficiency of these strains, the F and H-protein genes of CDV_{5804}, CDV_{OS}, and CDV_{OL} were reverse transcribed and cloned in the eukaryotic expression vector pCG. The expression of the gene products was analyzed by indirect immunofluorescence staining. We observed that cotransfection of the homologous F and H plasmids of MV and of the different CDV strains in Vero cells resulted in syncytium formation within 48 to 72 h after transfection. Based on this experimental setup, a scoring system was established to quantify fusion activity (Fig. 3). The extent of syncytium formation was scored between 0 (no fusion detectable) and 4 (complete fusion). Control immunoprecipitations of biotinylated double-transfected cells showed that similar amounts of the different proteins were available at the cell surface (data not shown).

Significant differences in fusion efficiencies were noticed. Cells cotransfected with MV H and F were completely fused and largely detached at the time of evaluation and therefore scored highest (score 4). The combination of MV H with the F protein of the different CDV strains led to a reduction in fusion activity (average scores of 1.75, 2.5, and 2). This confirms that the CDV and MV glycoproteins are able to complement each other when cotransfected in cells expressing an appropriate receptor (41). However, the fusogenicities of the MV H-CDV F combinations were reduced compared to those of the homologous MV proteins.

The most striking observation derived from the data presented in Fig. 3 was that the extent of fusion in the different combinations was determined mainly by the H protein. In the case of the CDV_{OL} H protein, fusion activity was independent of the coexpressed CDV F (average scores always 2.75), but the MV F protein led to a higher average score (3.5). The cotransfection of the CDV_{OS} H protein with all F proteins led to a moderate fusion activity with demarcated syncytia consisting of an average of 15 to 20 nuclei (average scores of 1.5, 1.75, 1.75, and 1.75). Coexpression of the H protein of CDV_{5804} with different F proteins produced only a few small syncytia often consisting of four to seven cells (average scores of 0.75, 0.75, 1, and 0.5). Thus, the combination of different CDV F and H



	H_{5804}	H_{OS}	H_{OL}	H_{Edm}
F_{5804}	1-1-0-1 (0.75)	2-1-2-1 (1.5)	3-2-3-3 (2.75)	2-1-2-2 (1.75)
F_{OS}	0-1-1-1 (0.75)	2-2-2-1 (1.75)	2-3-3-3 (2.75)	2-3-3-2 (2.5)
F_{OL}	1-1-1-1 (1)	1-2-2-2 (1.75)	3-3-3-2 (2.75)	2-2-2-2 (2)
F_{Edm}	1-0-1-0 (0.5)	2-2-1-2 (1.75)	3-4-3-4 (3.5)	4-4-4-4 (4)

FIG. 3. Fusion activity of different combinations of F and H proteins. Three days after transfection of Vero cells with plasmids expressing proteins of strains CDV_{5804}, CDV_{OS}, CDV_{OL}, and those of MV_{Edm} cells were observed by phase-contrast microscopy. The fusion activity was determined by using the standards shown at the top of the figure, with the score shown beneath each picture. The numbers at the bottom of the figure are the scores from four independent experiments (averages are shown in the parentheses).

proteins revealed that the H protein is the major determinant of the extent of fusion. The data obtained with isolated proteins (Fig. 3) reflected those obtained with the parental virus (Fig. 2).

Recovery and characterization of recombinant morbilliviruses. To confirm that the H protein of CDV is the major cytopathogenicity determinant, we attempted to transfer the corresponding genes in an otherwise identical genomic background. Therefore, a reverse genetic system for CDV was established as described in Materials and Methods based on CDV_{OS}. To facilitate the construction of recombinant viruses differing only in their H proteins, unique restriction sites were introduced upstream (*Pac*I) and downstream (*Apa*I) of the H ORF of the full-length plasmid pCDV3 (Fig. 1). The resulting plasmid pCDVII (Fig. 1) was used for all further experiments.

First, we verified that recombinant virus could be recovered from this plasmid. Indeed, a virus, designated $rCDV_{OS}$, was recovered; this virus was indistinguishable from CDV_{OS} by in vitro growth characteristics (data not shown). Then, we transferred the H genes of the two other CDV strains and of MV_{Edm} into pCDVII after producing PCR-generated *Pac*I-*Apa*I fragments of the respective genes. In that way, the full-length cDNAs of pCDVII-H_{5804}, pCDVII-H_{OL}, and pCDVII-H_{Edm} were generated. Moreover, the three CDV H genes were also introduced into the MV genomic clone p(+)MVNSe (39) by taking advantage of the unique restriction sites *Pac*I and *Spe*I that are located at the corresponding positions. The insertion of PCR-generated *Pac*I-*Spe*I fragments of the respective genes led to the plasmids p(+)MV-H_{5804}, p(+)MV-H_{OL}, and p(+)MV-H_{OS}. Subsequently, recovery of the recombinant viruses was attempted. Within 2 days after the transfer of the 293 cells transfected with pCDVII-H_{OL}, pCDVII-H_{Edm}, and p(+)MV-H_{OL} onto Vero cells, multiple syncytia were detected. Consistently, the recovery of $rCDV_{OS}$-H_{5804}, rMV-H_{OS}, and rMV-H_{5804} required that Vero cells overlaid by the transfected 293 cells be passaged once before infected foci could be identified.

To confirm the identity of the recombinant viruses, their F and H glycoproteins were characterized by Western blot analysis. The different H proteins can be distinguished by their size and migration pattern (Fig. 4). On this gel system, the H protein of the MV_{Edm} strain migrates as a sharp protein band

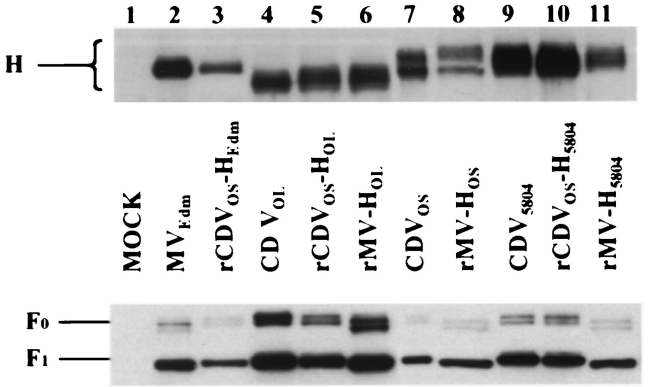


FIG. 4. Western blot analysis of the H and F proteins of the parental and recombinant viruses. Vero cells were infected with a MOI of 0.01 and harvested when CPE was advanced. Proteins were separated by reducing SDS-polyacrylamide gel electrophoresis (7.5% for the H protein; 10% for the F protein) and blotted onto polyvinylidene difluoride membranes. The membranes were incubated with the anti-Fcyt rabbit antipeptide serum to detect the F proteins or with a mixture of the anti-Hcyt and anti-CDV-Hcyt rabbit antipeptide serum, respectively, to detect the H proteins.

of about 80 kDa (17), which corresponds to the migration pattern of the H protein of $rCDV_{OS}$-H_{Edm} (Fig. 4, compare lanes 2 and 3). This method can be used for the identification of the different CDV H proteins as well. The H protein of CDV_{OL} migrates faster than the MV H protein (lane 4), as do the H proteins of the two recombinant viruses $rCDV_{OS}$-H_{OL} and rMV-H_{OL} (lanes 5 and 6). The CDV_{OS} H protein has one additional potential glycosylation site at position Asn-456 and migrates slower than the H_{OL} protein, with a characteristic pattern of bands (lane 7). The H protein of the recombinant virus with a MV backbone, rMV-H_{OS}, displays the same migration pattern (lane 8). The H protein of CDV-H_{5804} is three residues longer than the respective CDV_{OS} and CDV_{OL} proteins and has seven potential glycosylation sites (13), of which an unknown number are functional, resulting in a complex pattern of bands (lane 9). The H proteins of the recombinant viruses $rCDV_{OS}$-H_{5804} and rMV-H_{5804} migrated similarly (lanes 10 and 11). Thus, all the H proteins had the expected characteristics. The CDV F_1 proteins and their precursor F_0 (Fig. 4, lanes 3 to 5, 7, and 9 to 10) migrated slightly slower than the MV F_1 and F_0 proteins (Fig. 4, lanes 2, 6, 8, and 11), confirming the identity of the viral backbone (Fig. 4). Moreover, sequence analysis of the H genes after RT-PCR indicated that no point mutations had occurred compared to the parental genes.

The H protein determines the CPE and tropism of the recombinant viruses. We analyzed whether the origin of the H protein determines the extent of cell fusion. CDV strains can be distinguished by their fusion activity in Vero cells (Fig. 5C, E, and H). Our transient-expression-based functional fusion test indicated that the H protein may have a decisive influence on the CPE (Fig. 3). The availability of recombinant viruses differing only in their H protein allowed us to detect possible effects of other genes on cell-cell fusion. After the cells were infected by the recombinant viruses $rCDV_{OS}$-H_{OL} and $rCDV_{OS}$-H_{5804}, fusion activities similar to those observed for the strains that donated the H proteins were observed (Fig. 5F and I). Moreover, the CDV-MV recombinants rMV-H_{OS}, rMV-H_{OL}, and rMV-H_{5804} also displayed fusion activities similar to that of the H-protein donor strain (Fig. 5D, G, and J). Furthermore, the recombinant CDV with the MV H protein ($rCDV_{OS}$-H_{Edm}) caused a CPE similar to that of MV, as shown in Fig. 5A and B. These results demonstrate that the H proteins determine the extent of cell-cell fusion not only in a transient-expression assay but also in the context of a viral infection.

We then examined whether the tropism of the recombinant viruses is determined by their H genes. It is known that parental MV and CDV grow on many primate and certain canine cell lines with comparable efficiencies (21, 41). After confirming this observation in Vero cells (data not shown), we compared the growth of the recombinant viruses with that of the parental strains in DH 82 cells, a canine macrophage cell line in which only CDV grows to high titers.

Multiple wells of DH 82 cells were infected with a MOI of 0.01 of each virus. Over a period of 5 days, cells from two wells were lysed daily, and the titers of the cell-associated virus were determined. The comparative growth analysis is shown in Fig. 6. Viruses were grouped according to the origin of their H proteins: from top to bottom MV_{Edm}, CDV_{OS}, CDV_{OL}, and CDV_{5804}. The two viruses with the MV_{Edm} H protein, the parental MV_{Edm} and the recombinant $rCDV_{OS}$-H_{Edm} strains, grew slowly and to low titers (Fig. 6A). The two strains with the CDV H_{OS} protein, the parental strain CDV_{OS} and rMV-H_{OS}, grew with a very similar kinetics to titers near 10^6 (Fig. 6B). For the two other H proteins, H_{OL} and H_{5804}, not only the parental strain and the MV-based strain were available, but a recombinant virus with the backbone of CDV_{OS} was also available. Of the viruses with H_{OL}, CDV_{OL} was the fastest growing, reaching a titer that approached 10^7 4 days after infection (Fig. 6C). The H_{OL} viruses with another CDV backbone had a slightly slower growth kinetics but reached a similar titer 1 day later. The H_{OL} with the MV backbone was the slowest and reached a titer more than 10 times lower than that of the parental strain. These results suggest that genes other than H do have an effect on the propagation of morbilliviruses in these cells. The viruses with the CDV H_{5804} (Fig. 6D) reached lower titers than those with the H_{OL} protein: again, the parental strain reached the highest titer.

These data demonstrate that the H protein not only determines the fusion activity of a virus but also strongly influences its growth characteristics. The replacement of the H protein of CDV_{OS} with that of CDV_{OL} led to a 10-fold increase in titer, whereas the insertion of the CDV_{5804} H protein caused a >10-fold decrease.

DISCUSSION

The fusion activity of CDV strains ranges from low for wild-type viruses to high for attenuated vaccine strains (7, 14, 42, 47). It was suggested that the increase in fusion activity correlates with attenuation. In this study we present evidence that the H protein is the major determinant of fusion activity. This conclusion is supported by experiments based on the transient expression of the F and H proteins and on the production viruses expressing the H proteins of different strains in an otherwise identical context.

The H proteins of CDV and MV are functionally interchangeable. Only certain combinations of the glycoproteins of *Paramyxoviridae* support efficient fusion in transient-expression experiments (16, 18). As for the combination of the MV and CDV glycoproteins, different observations have been reported. Wild et al. (50) have used a transient-expression system based on a vaccinia virus recombinant expressing MV H and plasmids expressing the F protein of MV, CDV, and hybrids thereof. They observed that the CDV F protein is unable to functionally interact with MV H unless a 45-amino-acid cystine-rich MV F-protein segment is exchanged for the homologous CDV F region. On the other hand, using another transient-expression system, Stern et al. (41) observed that the CDV and MV H proteins are functionally interchangeable in a cell fusion test provided that an appropriate cellular receptor is available. We have confirmed the second observation, and in addition we have observed that the fusion support efficiency of MV H is reduced when it interacts with any of the three CDV F proteins (Fig. 3). This is consistent with precise lateral interactions between the F and H oligomers being necessary to ensure high fusion activity.

In spite of suboptimal interactions at membrane fusion, recombinant viruses expressing heterologous F and H proteins

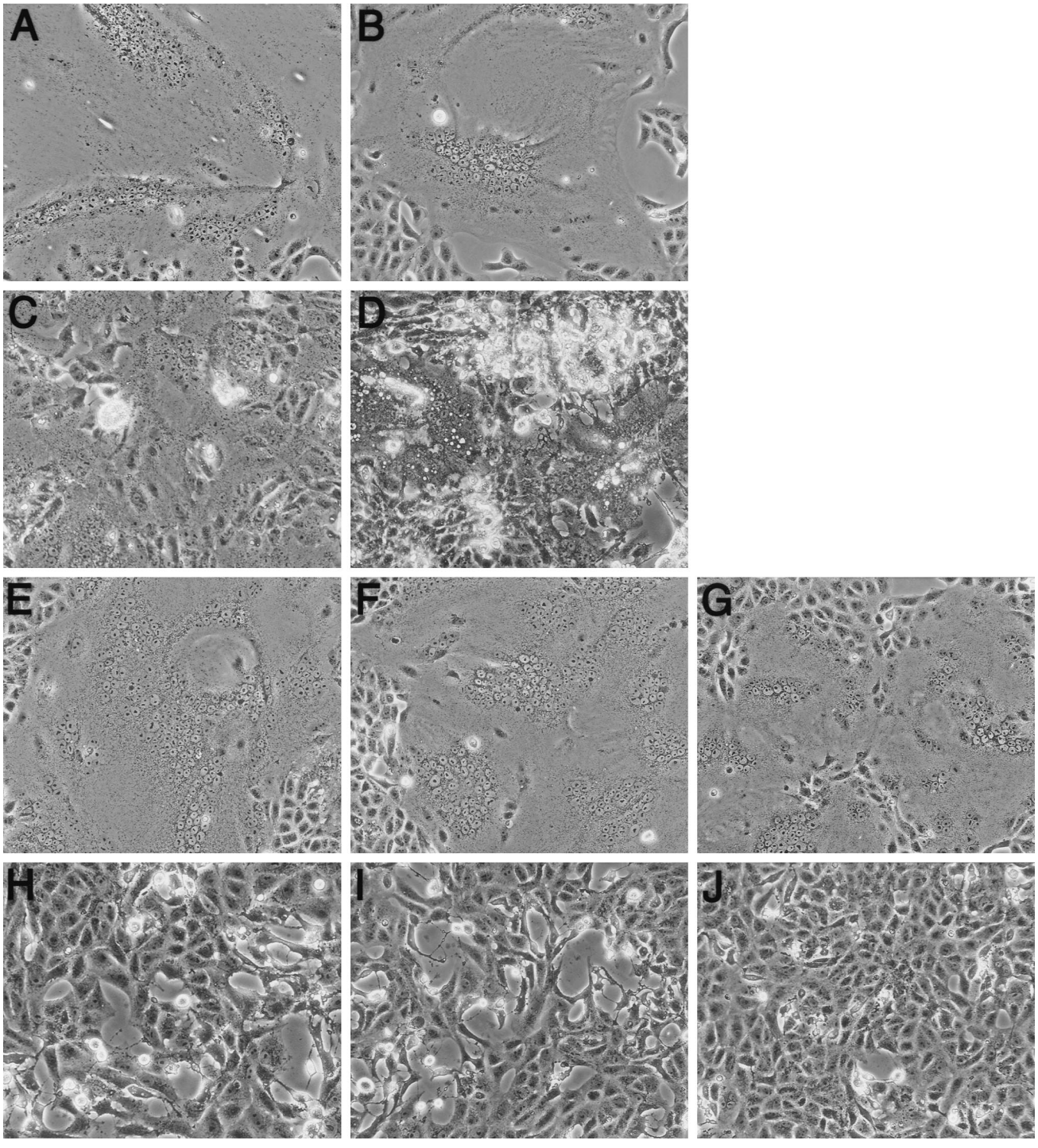
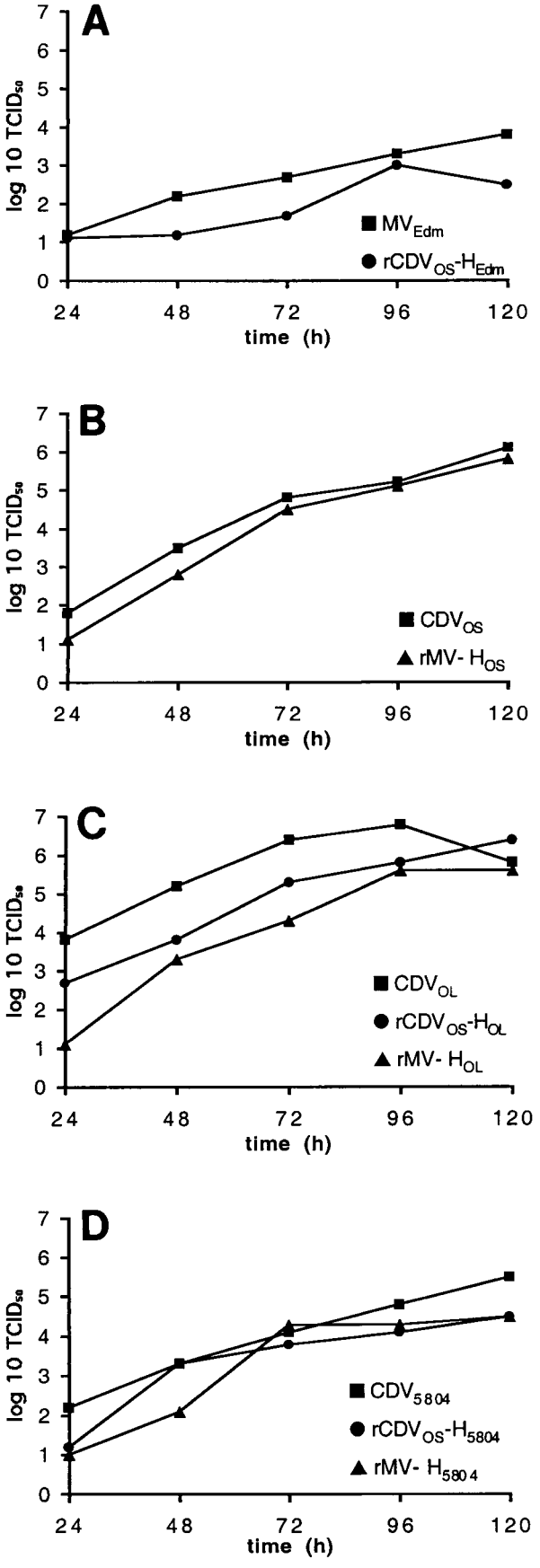


FIG. 5. CPEs in Vero cells infected with the parental and recombinant viruses. Vero cells were photographed 48 h after infection with a MOI of 0.01. Parental and recombinant viruses carrying the same H protein are shown in the same row: MV_{Edm} [recovered from p(+)MVNSe] with $rCDV_{OS}$-H_{Edm} (A and B), CDV_{OS} with rMV-H_{OS} (C and D), CDV_{OL} with $rCDV_{OS}$-H_{OL} and rMV-H_{OL} (E, F, and G), and CDV_{5804} with $rCDV_{OS}$-H_{5804} and rMV-H_{5804} (H, I, and J).

were recovered. These viruses reached similar titers with similar kinetics compared to those of viruses with homologous proteins. Thus, membrane fusion efficiency at virus entry or afterwards is not rate limiting in the context of infections of cultured cells. Moreover, the interactions of H with the RNP and the M protein must be compatible between the MV and CDV systems. This fact is remarkable. The construction of recombinants of two other morbilliviruses, rinderpest (RPV) and peste des petits ruminants (PPRV), with different combinations of the glycoproteins was attempted, but no virus was



recovered when only one of the glycoproteins was exchanged (9). A recombinant RPV with both heterologous PPRV glycoproteins was recovered but had strongly impaired growth characteristics. Moreover, substitution of both glycoproteins has been the strategy of choice for the production of recombinants between parainfluenza virus type 3 (PIV3) and 2 (PIV2) (45); recombinants between PIV3 and PIV1 were obtained only when the ectodomains of both glycoproteins were selectively exchanged (44). MV and CDV have a divergence of 64% between their H proteins compared to 50% between RPV and PPRV and 51% between PIV3 and PIV1. It is therefore surprising that the MV and CDV envelope proteins are functionally interchangeable.

The H protein, its receptors, and attenuation. Different CDV strains grow efficiently in several cell types of different species (22, 24), a characteristic which our recombinant viruses with CDV H do maintain. This indicates that the H protein is the major determinant of viral tropism and suggests that this protein recognizes and attaches to a conserved and ubiquitous cell surface component or to a few different cellular proteins (32).

On the other hand, MV grows efficiently almost exclusively in primate cells; the ubiquitous protein CD46 and the B- and T-cell-specific protein SLAM have been identified as MV receptors (10, 28, 46). One exception to the "primate cell only" rule for efficient MV replication are certain canine cells: MV grows to high titers in MDCK cells (22) and in the thymic canine cell line Cf2Th (data not shown). These data suggest that MV enters these cells through another receptor, which may not be canine CD46 (22). Not all canine cell lines express this receptor: in the macrophage cell line DH 82, three CDV strains, but not MV_{Edm}, grow to high titers. Similarly, our recombinant viruses with MV H grew in canine DH 82 cells to titers about 1,000 times lower than those of the corresponding viruses with a CDV H protein.

Factors other than the H protein and its cellular receptor may influence virus growth. Generally, viruses with CDV background reached slightly higher titers than viruses with MV background in the early stages of the time course, which is consistent with the characteristics of the parental viruses. These differences may be due to the other viral proteins and their interactions with cellular factors.

In CDV, a correlation between the proficiency of syncytium induction and the level of attenuation has been drawn, linking high fusogenicity to attenuation. The recombinant viruses produced in this study, which differ exclusively in their H proteins,

FIG. 6. Time course of cell-associated virus production in DH 82 cells infected with parental and recombinant viruses. DH 82 cells were infected with a MOI of 0.01, and virus titers were determined by 50% end-point dilution at the indicated times postinfection. The titers represent the average of at least two experiments. Parental viruses were compared with recombinant viruses carrying the same H protein, respectively: MV_{Edm} [recovered from p(+)MVNSe] with $rCDV_{OS}$-H_{Edm} (A), CDV_{OS} with rMV-H_{OS} (B), CDV_{OL} with rMV-H_{OL} and $rCDV_{OS}$-H_{OL} (C), and CDV_{5804} with rMV-H_{5804} and $rCDV_{OS}$-H_{5804} (D). The respective parental viruses are indicated by solid squares, the recombinant viruses with a MV backbone are indicated by solid triangles, and the recombinant viruses with a CDV backbone are indicated by solid circles.

will allow testing of this correlation in a natural host (40, 49). It is interesting to note that the H protein of the wild-type strain CDV_{5804} has more potential glycosylation sites than the H proteins of the other two strains and has a higher apparent molecular weight which is consistent with increased oligosaccharide addition. It is conceivable that oligosaccharides on the CDV H protein may influence the strength of the interactions with cellular receptors. Alternatively, without significantly altering receptor binding, the oligosaccharides could influence the extent of viral propagation by altering the fusion efficiency of the F-H protein complex expressed on infected cells. Attenuated viruses passaged in cultured cells may have been selected for partial loss of these oligosaccharides.

Recombinant *Paramyxoviridae* as vaccines. It was consistently observed that the unmodified viral strains reached higher titers than the recombinant viruses (Fig. 6). For example, the parental strain CDV_{OL} reaches higher titers than rCDV-H_{OL} and rMV-H_{OL} (Fig. 6C). Similarly, in other recombinant *Paramyxoviridae* in which certain genes have been exchanged with those of related viruses, slower replication kinetics and lower viral titers have been observed (2, 44, 45).

Attenuation of currently available CDV vaccines is insufficient for highly susceptible animals. Vaccination of dogs with MV leads to partial immunity against subsequent challenge with CDV because of cross-reactivity (1). It is conceivable that a CDV H protein in a MV background could result in the induction of neutralizing antibodies against the homologous H protein in dogs without the risk of retained virulence. In humans, recombinant MV with a CDV H protein could be less affected than available attenuated MV strains by maternal antibodies, which is a major problem for the vaccination of 6- to 12 month-old children (31, 38).

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