

Recovery of a Virulent Strain of Newcastle Disease Virus from Cloned cDNA: Expression of a Foreign Gene Results in Growth Retardation and Attenuation

Sateesh Krishnamurthy, Zhuhui Huang, and Siba K. Samal¹

Virginia–Maryland Regional College of Veterinary Medicine, University of Maryland, College Park, Maryland 20742

Received May 23, 2000; returned to author for revision July 11, 2000; accepted August 30, 2000

A recombinant mesogenic NDV strain, Beaudette C, and an engineered recombinant NDV expressing an additional gene were generated entirely from cloned cDNAs. For this purpose, a full-length cDNA clone of the virus genome, represented in eight different subgenomic fragments, was assembled in a transcription plasmid between a T7 RNA polymerase promoter and a hepatitis delta virus ribozyme sequence. Infectious NDV could be generated in the cells infected with recombinant vaccinia virus, which expressed T7 RNA polymerase, by simultaneous expression of antigenome-sense NDV RNA from the full-length plasmid and NDV NP, P, and L proteins from cotransfected plasmids. Recombinant virus was then amplified and recovered, either after inoculation of transfection supernatant into the allantoic cavity of embryonated specific-pathogen-free eggs or after further passage in cell culture. Characterization of the recombinant NDV showed similarities in growth and pathogenicity to that of the parental wild-type virus. By using this system, a recombinant NDV containing a foreign gene encoding chloramphenicol acetyltransferase (CAT) was generated. To do this, the CAT transcription cassette containing the CAT open reading frame, flanked by NDV gene start and gene end sequence motifs, was inserted into the region between the HN and L genes of the full-length cDNA. This construct was then used in the generation of a recombinant NDV expressing CAT protein. The CAT gene was maintained stably for at least eight passages without any detectable loss of the gene from the recombinant. Generation of the recombinant virus, however, was associated with reduced plaque size, slower replication kinetics, and more than 100-fold decrease in yield. In addition, the virus showed an increase in mean death time for eggs and a lower intracerebral pathogenicity index in day-old chicks, implicating attenuation of the recombinant virus. Thus, introduction of an additional gene into the NDV genome represents a method to achieve growth retardation and attenuation. These results also indicate that NDV can be engineered to express foreign protein stably and can be manipulated in the future for use as a vaccine vector. © 2000 Academic Press

INTRODUCTION

Newcastle disease is a highly contagious and economically most important disease of poultry. The disease also inflicts many other avian species. The disease varies in degree of severity, ranging from mild, inapparent infection to severe disease, causing 100% mortality (Alexander, 1997). Based on the severity of the disease in chickens, the causative agent of the disease, Newcastle disease virus (NDV), is divided into three pathotypes: (1) the *lentogenic* strains, which cause mild inapparent infection; (2) the *mesogenic* strains, which cause mild respiratory symptoms with low mortality; and (3) the *velogenic* strains, which are highly virulent and cause high mortality. Vaccination has been the widely used method of prevention of this disease. Though vaccination with live lentogenic NDV strains has largely controlled the disease, a better, stable, and completely apathogenic vaccine is desirable to ensure complete efficacy and

safety. Apart from the importance of producing a better vaccine, NDV serves as an excellent model for studying viral pathogenesis because the virulence of NDV can be measured on a quantitative basis in chick embryos and young chickens. Therefore, it is important to design methods for genetically modifying NDV and to use the engineered virus for purposes of studying pathogenesis and constructing a better vaccine virus.

NDV is a member of the genus *Rubulavirus* of the family *Paramyxoviridae* (Murphy *et al.*, 1995) and contains nonsegmented single-stranded negative-sense RNA as the genome. The genome of NDV is 15,186 nucleotides long (de Leeuw and Peeters, 1999; Krishnamurthy and Samal, 1998; Phillips *et al.*, 1998) and contains six genes, which encode nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (HN), large protein (L), and finally, V and W proteins, which are produced by RNA editing during P gene transcription (Millar and Emerson, 1988; Steward *et al.*, 1993). One aspect of NDV and other negative-strand RNA viruses is that naked RNA alone is not infectious. Other components of the ribonucleoprotein complex (RNP), namely NP, P, and L proteins, are essential to initiate the first round of RNA synthesis

¹To whom correspondence and reprint requests should be addressed at University of Maryland, VA-MD Regional College of Veterinary Medicine, 8075 Greenmeade Drive, College Park, MD 20742. Fax: (301) 935-6079. E-mail: ss5@umail.umd.edu.

and establish infection. So the current technique of genetic manipulation of negative-strand RNA viruses by reverse genetics involves cotransfection, into permissive cells, of plasmids expressing full-length RNA and NP, P, and L proteins, all under the control of T7 promoter. This results in reconstitution of the RNP complex inside the cell and recovery of the virus. The source of the T7 RNA polymerase is either a recombinant vaccinia virus expressing the gene or a cell line constitutively expressing the polymerase. Using this approach of recovery, rabies virus was the first nonsegmented negative-stranded RNA virus to be recovered entirely from cDNA (Schnell *et al.*, 1994). Since then, other members of nonsegmented negative-strand RNA viruses have been recovered from cDNA: Sendai virus (Garcin *et al.*, 1995; Kato *et al.*, 1996), human respiratory syncytial virus (RSV) (Collins *et al.*, 1995), vesicular stomatitis virus (VSV) (Lawson *et al.*, 1995; Whelan *et al.*, 1995), SV5 (He *et al.*, 1997), measles virus (Radecke *et al.*, 1995), parainfluenza virus 3 (Durbin *et al.*, 1997a; Hoffman and Banerjee, 1997), rinderpest virus (Baron and Barrett, 1997), bovine respiratory syncytial virus (Buchholz *et al.*, 1999), and, recently, lentogenic NDV strain La Sota (Oberdorfer *et al.*, 1999; Peeters *et al.*, 1999) and mumps virus (Clarke *et al.*, 2000).

In this report, we describe the recovery of a mesogenic strain of NDV (Beaudette C) entirely from cDNA. Mesogenic strains like Beaudette C are ideal for studying pathogenesis because their intermediate virulence creates a possibility to attenuate or increase their virulence further by reverse genetics. In this study, the recovered virus was found to be biologically and antigenically similar to the parental wild-type virus. Thus, recovery of a mesogenic strain of NDV would provide a means of studying the molecular biology and pathogenesis of NDV.

Additionally, we report here the recovery of infectious recombinant NDV encoding a foreign protein, chloramphenicol acetyltransferase (CAT). The CAT coding sequence, flanked by NDV gene start and gene end sequence motifs, was inserted between the HN and L genes of the full-length NDV cDNA. We observed in the recovered virus a delayed growth, a decreased yield, and a decreased virulence to embryonated chicken eggs and day-old chicks. In addition, expression of the CAT protein by the recombinant NDV was found to be highly stable. These results suggested the ability to insert foreign genes into the genome of NDV for coexpression and to use NDV as an attenuated vaccine vector.

RESULTS

Construction of cDNA to NDV genome

Construction of the NDV cDNA clone encoding the complete 15,186-nucleotide (nt) NDV genome of strain Beaudette C was made possible by generating eight individual cDNA fragments that were cloned sequentially

into the low-copy-number plasmid pBR322/dr (Fig. 1 and Table 1). Cloning of these fragments positioned the NDV cDNA between the T7 promoter and the hepatitis delta virus (HDV) ribozyme sequence. The resulting NDV cDNA in the plasmid pNDVf1 was a faithful copy of the NDV genome, except for the following nucleotide changes from the published sequence (Chambers *et al.*, 1986a,b): T5464C, A7915G, C10167T, A10877C, and C12239T. Three of these nucleotide differences resulted in amino acid (aa) changes: nt 7915 in the HN gene, resulting in change of amino acid threonine to alanine (aa position 502); nt 10167 in the L gene, resulting in change of amino acid threonine to methionine (aa position 596); and nt 10877 in the L gene, resulting in change of amino acid methionine to leucine (aa position 833). These changes were present in all the viral genomic RNAs that were isolated from three different stocks of NDV strain Beaudette C and subjected to reverse transcription coupled PCR (RT-PCR) and sequencing. Also, two genetic tags were introduced in the cDNA to positively identify the recombinant virus: nt positions 6294 and 6295 at the F/HN intergenic region were replaced (CG to GC) to create an *Mlu*I restriction site, and nucleotide positions 8357, 8358, and 8359 at the HN/L intergenic region were replaced (CAT to ACC) to create an *Age*I site. The biologically derived parental strain Beaudette C and NDV cDNA contained a double frameshift (nts 12239 and 12327) in the L gene, resulting in differences of 28 amino acids (aa 1287–1316 of the L protein) from the published sequence of NDV strain Beaudette C (Yusoff *et al.*, 1987). This change, however, is in accordance with the published sequence of NDV strain La Sota Clone-30 (Oberdorfer *et al.*, 1999). To facilitate transcription of T7 RNA polymerases, 3G residues were included before the NDV leader sequence. An antigenome (+)-sense RNA transcript was produced by transcription of NDV cDNA.

Recovery of infectious recombinant NDV from cDNA

A recombinant vaccinia virus-based transfection system was used to recover infectious recombinant NDV from cDNA. HEp-2 cells were infected with recombinant vaccinia virus (MVA/T7) capable of synthesizing T7 RNA polymerase. Simultaneously, the cells were transfected with the plasmid pNDVf1, along with plasmids encoding proteins of RNP complex, namely NP (pNP), P (pP), and L (pL). In a parallel transfection, plasmid pL was excluded in the experiment to serve as a negative control. Four days after transfection, the supernatant was used in either of two different ways to recover the virus. The supernatant was either injected into the allantoic cavities of 9-day-old embryonated eggs or amplified further in HEp-2 cells and DF1 cells (chicken embryo fibroblast cell line). The allantoic fluid of the eggs injected with the transfectant gave a positive hemagglutination (HA) titer

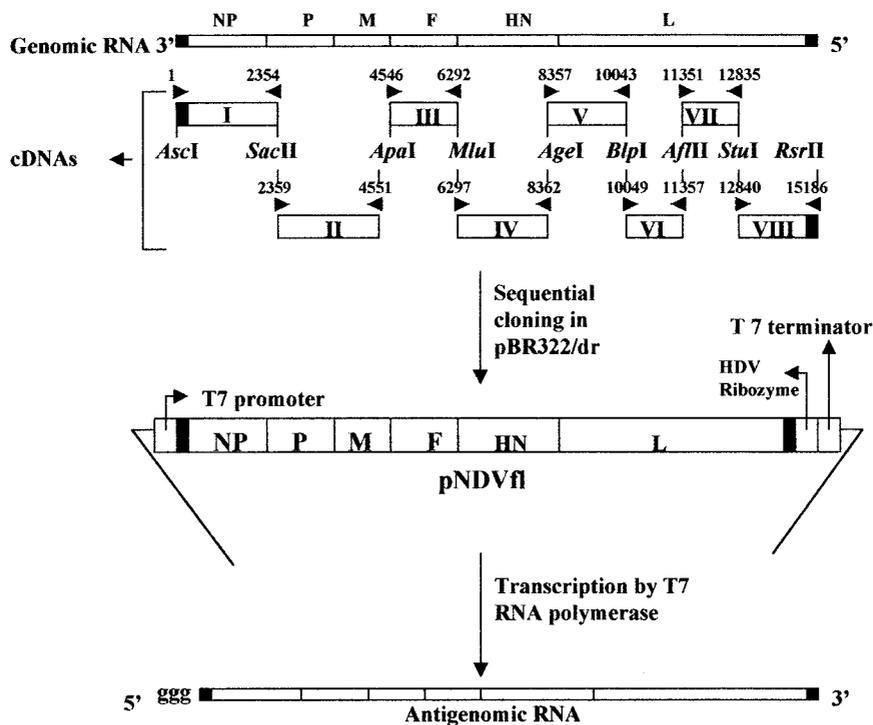


FIG. 1. Full-length NDV cDNA assembled in pBR322/dr from subgenomic cDNA fragments (I to VIII) that were generated by high-fidelity RT-PCR (not to scale). The blocked arrows indicate the primers used in RT-PCR. The numbers shown above the primers represent the position in the genome of the first nucleotide of the primer, represented 5' to 3'. Plasmid pBR322/dr is the modified form of plasmid pBR322, designed to include a 72-nucleotide oligonucleotide linker between the *EcoRI* and *PstI* sites, an 84-nucleotide HDV antigenome ribozyme sequence and a T7 RNA polymerase transcription termination signal. Transcription by the T7 RNA polymerase of the plasmid pNDVfl results in the NDV antigenomic RNA with 3 nonviral G residues at the 5' terminus.

ranging from 32 to 2048. The cell culture-amplified supernatant gave NDV titers slightly in excess of 10^4 plaque-forming units (PFU)/ml at the end of passage 1 and slightly in excess of 10^8 PFU/ml at the end of passage 2. Thus, the cotransfection method of rescue resulted in efficient recovery of NDV. After passage 2, the cell culture passaged virus was plaque purified to eliminate vaccinia virus and then individual plaques were used to inject 9-day-old embryonated eggs. No plaques were visualized nor HA titer quantified in the case of negative controls, further confirming the specificity of recovery of NDV from cDNA. The recovered virus was designated rNDV/BC, to distinguish it from the parental wild-type NDV (pNDV/BC). The allantoic fluid containing the recovered and amplified NDV was analyzed in a hemagglutination inhibition (HI) test by using NDV antiserum and NDV HN-specific MAbs 15C4, AVS, and 10D11. The reactivities with antiserum and monoclonal antibodies were similar for both the parental and recombinant strains of NDV (Table 2).

Immunological staining of cells infected with recovered virus

When DF1 cells were infected with the rNDV/BC, extensive fusion was observed and the cytopathic effect

(CPE) was as robust as that of pNDV/BC. Infected allantoic fluid or cell culture supernatant was used to produce plaques on DF1 cells. The plaques were then visualized by immunological staining, using either an NDV polyclonal sera or MAb against the NDV HN protein (Fig. 2). Specific staining of plaques indicated NDV replication and expression of NDV proteins. The plaque size was identical to that of the parental virus. No positive signals were observed in the case of negative controls.

Identification of genetic markers in the recovered virus

Two genetic markers (*MluI* and *AgeI*) were introduced in the full-length NDV cDNA clone to unambiguously identify the recovered virus. To identify these markers in the rNDV/BC, HA-positive allantoic fluid was subjected to RNA extraction and RT-PCR. Control experiments confirmed that the PCR products were dependent on reverse transcription. The RT-PCR product that was amplified to demonstrate the *MluI* site marker was subjected to restriction enzyme digestion with enzymes *MluI*. The second product that served to demonstrate the *AgeI* site marker was digested with the enzyme *AgeI*. Upon agarose gel electrophoresis of the digested products, the restriction enzyme patterns revealed the existence of

TABLE 1
Oligonucleotide Primers Used during Full-Length cDNA Synthesis and RT-PCR

| cDNA fragments | Primers | Order of cloning |
|----------------|---|------------------|
| I | + 5'ACTGGGGCGCGCCTAATACGACTCACTATAGGACCAACAGAGAATCCGTAAGTTAG3' - 5'AGACCCGCGGCTGGGTTGACTTCCCTG3' | 8 |
| II | + 5'AGACCCGCGGAAACAGCCAGG3' - 5'GCAGGGGCCCATCTTGCACCTAGAA3' | 7 |
| III | + 5'ACAGGGGCCCCAGACCTTCTACCAA3' - 5'ATCGACGCGTAGTTTTTCTAAACTCTC3' | 6 |
| IV | + 5'ATCGACGCGTTGTAGATGACCAAAG3' - 5'GCACACCGGTAGCTGTTTTGCCTTGTATC3' | 5 |
| V | + 5'GCACACCGGTAAATAGTACGGGTAGGACATG3' - 5'TTCAGCTTAGCGAAGATCCGTCCATTAAGT3' | 2 |
| VI | + 5'TTCAGCTAAGCTGACAAAGAAGTTAAGGAAGT3' - 5'AAGCCTTAAGAAACATGTTTGGGCTTGCAAC3' | 4 |
| VII | + 5'AAGCCTTAAGAAACATACGCAAAGAGTCT3' - 5'TCAGAGGCCTTCTTACTCTCAGATAATAGAG3' | 3 |
| VIII | - 5'TCAGAGGCCTTCTTACTCTCAGATAATAGAG3' 5'ATGCCGGACCGcgaggagggtggagatgccatgccgACCCACCAACAAGATTTGGTGAATAACAAG3' | 1 |

Note. The cDNA fragments correspond to the fragments in Fig. 1. T7 promoter sequences are marked in italic type, the virus-specific sequences are underlined, and restriction sites are marked in bold type. The partial HDV ribozyme sequence (24-nt) overhang is shown in lowercase. Orientation of the primer sequence is shown for sense (+) and antisense (-).

both genetic markers in rNDV/BC (Fig. 3, lanes 2 and 4). Nucleotide sequence analysis of cloned PCR products confirmed the sequences spanning the restriction site markers. This confirmed the rNDV/BC to be the recombinant derived from cloned full-length cDNA.

Growth characteristics of the recombinant virus

Replication in a multistep growth cycle for rNDV/BC and pNDV/BC was evaluated following infection of the virus in triplicate in DF1 cells at a multiplicity of infection (m.o.i.) of 0.005. The samples were collected at 6-h intervals for a period of 36 h. The viruses in the samples were later quantitated by plaque assays. Both the kinetics and the magnitude of replication of the two viruses were very similar, which indicated that the growth of rNDV/BC was not adversely affected by the genomic changes in the full-length NDV cDNA (Fig. 4).

TABLE 2
HI Titers of Antisera and MABs

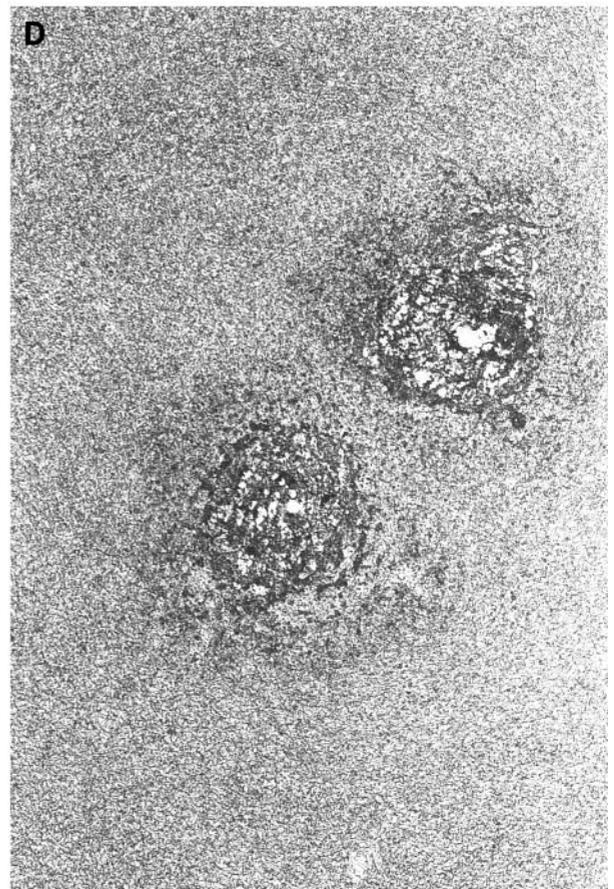
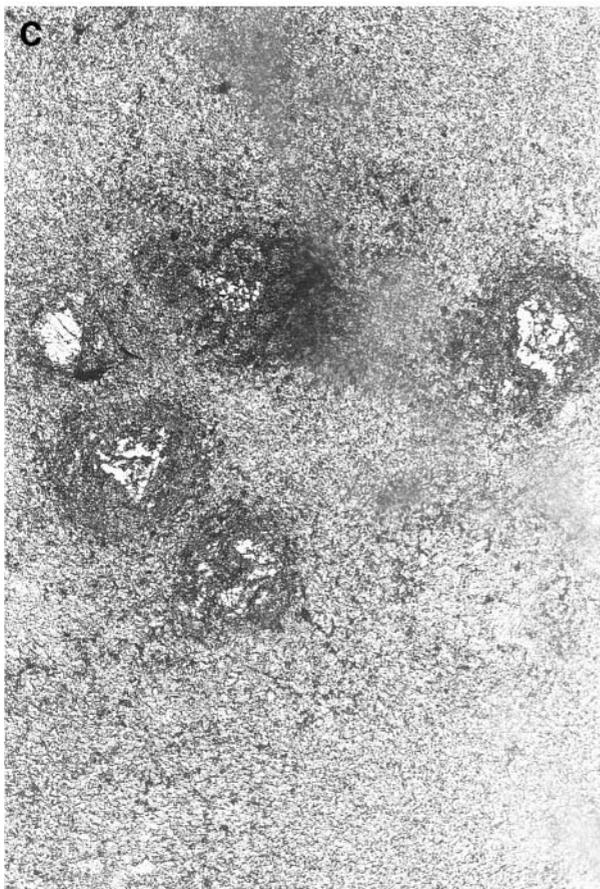
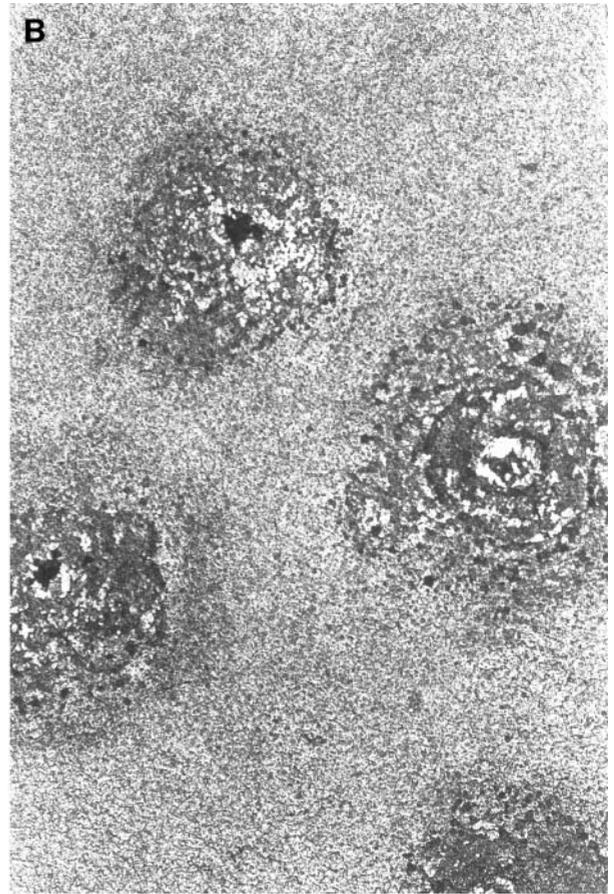
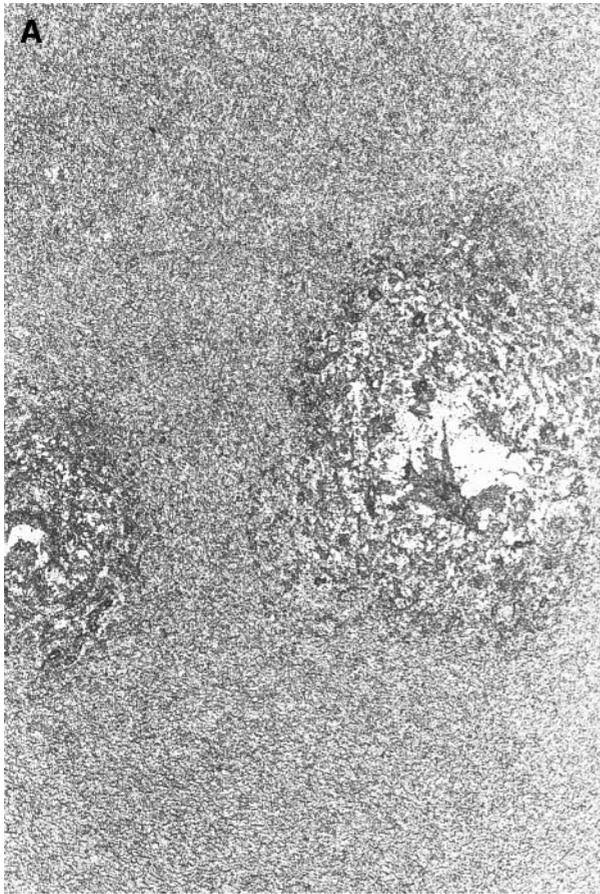
| Strain | Titer ^a | | | |
|---------|--------------------|---------|----------|-----------|
| | NDV serum | MAB AVS | MAB 15C4 | Mab 10D11 |
| pNDV/BC | 4096 | 8 | 4096 | 256 |
| rNDV/BC | 8192 | 32 | 8192 | 512 |

^a Expressed as the reciprocal of the highest dilution of serum or MAB that caused inhibition of hemagglutination. Results for control serum were negative.

Pathogenicity of the recombinant virus

One of the pathogenicity tests to determine the virulence of the original Beaudette C strain and of the newly generated recombinant Beaudette C strain was conducted by performing the mean death time (MDT) in eggs. To determine MDTs of the original and the recombinant Beaudette C strains, different dilutions of rNDV/BC and pNDV/BC were injected into the allantoic cavity of 9-day-old embryonated, specific-pathogen-free (SPF) chicken eggs and the mean death time for the minimum lethal dose to kill the eggs was recorded. The MDTs for rNDV/BC (62 h) and pNDV/BC (60 h) were very similar, indicating pathogenicity of infection in eggs is identical for both viruses (Table 3).

The test for assessing the virulence of NDVs in birds was conducted by determining the intracerebral pathogenicity index (ICPI) in day-old chicks. ICPI is an internationally accepted procedure for assessment of virulence of NDV isolates. The test is performed by inoculating the birds intracerebrally and scoring for the appearance of clinical signs and mortality. The observation and scoring are done every 24 h for a period of 8 days (Alexander, 1989). The ICPI is the mean score per bird per observation over the 8-day period. The most virulent NDV strains give indices close to 2.0, while avirulent viruses give values close to 0.0. In our experiments, the result of ICPI for pNDV/BC was 1.58 and for rNDV/BC was 1.63 (Table 3). These values are within the range expected for the wild-type Beaudette C and show that the recombinant NDV is similar in virulence and pathogenesis to the natural pathogen.



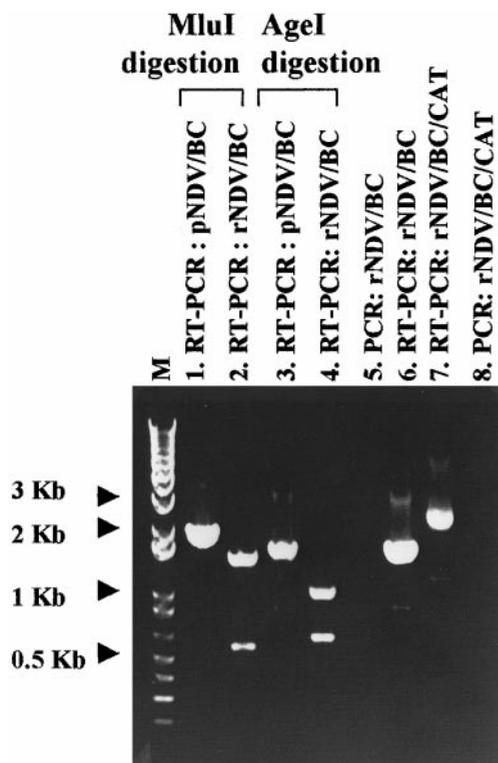


FIG. 3. Identification of genetic markers in the genome of rNDV/BC (lanes 1 to 5) and confirmation of the location and size of CAT gene in the genome of the recombinant rNDV/BC/CAT (lanes 6 to 8). RT-PCR was performed from the genomic RNA purified either from viruses amplified in embryonated chicken eggs (for demonstration of genetic markers) or from viruses in the cell culture supernatants (for confirmation of CAT gene location). The RT-PCR products corresponding to pNDV/BC and rNDV/BC (lanes 1 to 4) were both digested by *MluI* (lanes 1 and 2) or *AgeI* (lanes 3 and 4). The PCR/digested products were run on a 1% agarose gel in the presence of ethidium bromide. Lanes 5 and 8 refer to PCR without the RT step.

Recovery and characterization of recombinant NDV encoding CAT protein

After we developed the system to recover infectious recombinant NDV from cloned cDNA, we decided to use reverse genetics to explore the potential of NDV to express a foreign gene. The introduction of the CAT open reading frame (ORF) flanked by NDV gene start and gene end sequence motifs in the location between the HN and L ORF of pNDVf1 resulted in the production of a cDNA encoding a longer antigenome of 15,882 nucleotides. The potential for this antigenomic RNA to be rescued into NDV particles and the ability of virus to sustain the production of additional mRNA and CAT protein stably were assessed. The strategy of construction of this cDNA is shown in Fig. 5. The scheme for recovering

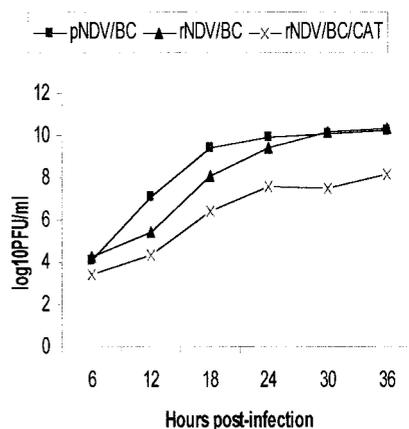


FIG. 4. Multistep growth curve for pNDV/BC, rNDV/BC, and rNDV/BC/CAT and viruses in DF1 cells. Cell monolayers in 25-cm² flasks were infected with 0.005 PFU/cell with three replicate flasks per virus. Samples were taken every 6 h for 36 h. The virus in the supernatant was titrated by plaque assay. The log titer is derived from the average of the virus titer.

infectious NDV was similar to that described previously, except that the transfectant supernatant was passaged to fresh cells and a total of eight serial passages were completed at intervals of 4 days, with each passage obtaining an m.o.i. of approximately 0.01. The recovery of NDV encoding CAT (rNDV/BC/CAT) was first assessed by the appearance of CPE on DF1 cells. The onset of CPE was delayed compared to onset in rNDV/BC, thus indicating a slower growth cycle. The plaque size of the CAT-containing recombinant was smaller, with an average diameter of 50–60% that of rNDV/BC virus (Fig. 2).

The presence of the CAT gene in the genome of recombinant was verified by RT-PCR amplification, with the oligonucleotide primers located in the coding region of HN [NDV (+)-sense primer corresponding to positions 7743 to 7765] and L [NDV (–)-sense primer corresponding to positions 9350 to 9372] genes. The result showed a single fragment on analysis of rNDV/BC and rNDV/BC/CAT RNAs (Fig. 3). The size of the PCR product corresponding to rNDV/BC/CAT RNA conformed well to the predicted size of 2325 nucleotides, which would include the size of the CAT transcription cassette (Fig. 3, lane 7). The presence of a single fragment on RT-PCR ruled out any population with partial loss of the CAT gene.

Since the rNDV/BC/CAT virus displayed delayed CPE, we investigated whether this virus exhibited slower growth than that of rNDV/BC. To do this, cells in triplicate were infected with either rNDV/BC or rNDV/BC/CAT at an m.o.i. of 0.005, and samples were taken at 6-h intervals and quantitated by plaque assays. Both the growth ki-

FIG. 2. Plaques produced by pNDV/BC (A), rNDV/BC/ (B), rNDV/BC/CAT (C), and rNDV/BC/(–)CAT (D) on DF1 cells. Infected cells overlayed with 0.9% methylcellulose were incubated for a period of 4 days. After this period, the plaques were visualized by immunological staining using a MAb against the NDV HN protein.

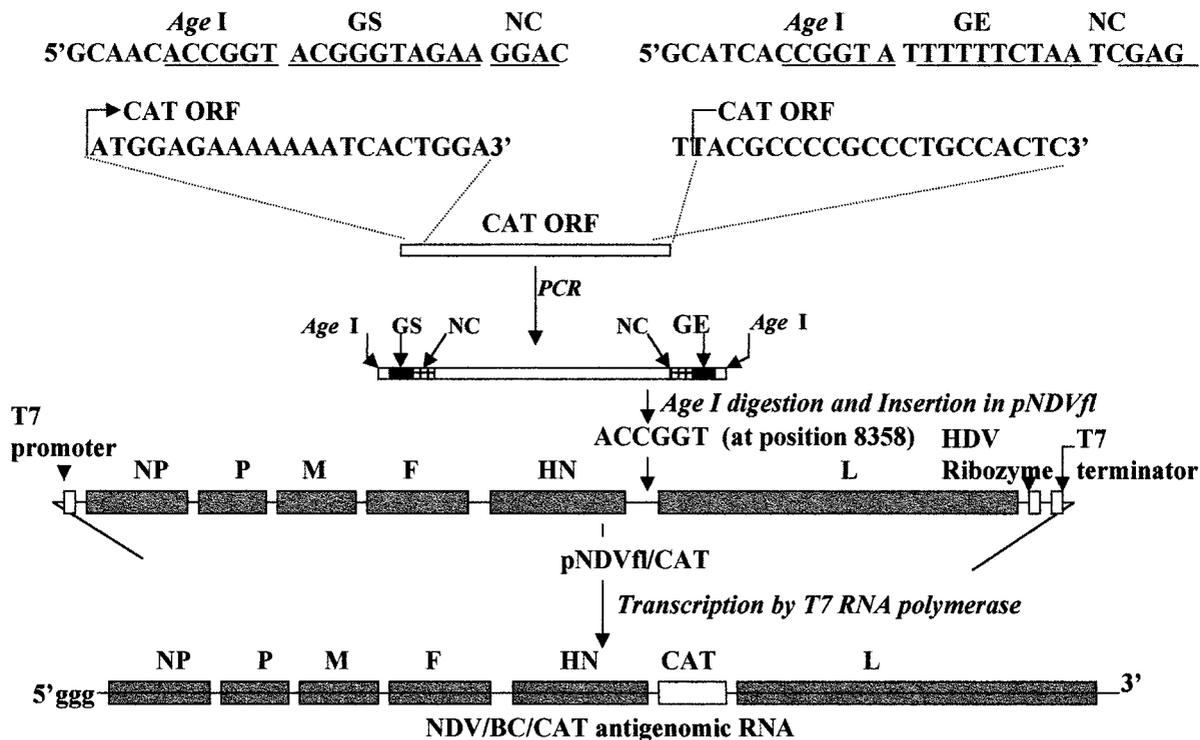


FIG. 5. Construction of pNDVfl/CAT by insertion of the CAT gene cDNA into HN and L intergenic region of the plasmid pNDVfl. The nucleotide sequences represent the oligonucleotide primer used for amplifying the CAT ORF. The resulting PCR product with the *AgeI* overhang on either end contained the gene start (GS) signal, noncoding (NC) sequence, and gene end (GE) signal. The nucleotide length of the construct was maintained as a multiple of six. The RNA encoded by pNDVfl/CAT included the three 5'-terminal nonviral G residues contributed by the T7 promoter.

netics and the final titer of rNDV/BC/CAT relative to that of rNDV/BC were lower (Fig. 4). The maximum titer achieved was more than 100-fold lower than that of rNDV/BC.

CAT enzyme expression and stability

To verify the expression of CAT protein from rNDV/BC/CAT, cell lysates from the transfectant and all of the eight serial passages were analyzed for CAT activity. Surprisingly, the transfectant lysate showed a high level of CAT expression, while the lysate from passage 1 showed no activity. This result can be explained by the fact that, after transfection, most of the CAT expression is probably mediated after the promiscuous transcription of the CAT gene either by one of the vaccinia virus polymerases or after the production of positive-sense RNA from the transfected plasmid by the T7 RNA polymerase. No CAT activity was detectable at passage level 1, probably because of the low titer of the virus. Efficient CAT expression was detectable in cells of passage level 2 and onward, indicating higher titer of the virus (Fig. 6 depicts CAT expression of P2 and P8 only). These results also show that the CAT gene was stable for at least eight passages. In addition to identifying the CAT enzyme activity by CAT assay, immunoprecipitation of cell lysates was conducted to characterize CAT protein synthesis. DF1 cells were labeled with [³⁵S]methionine and cell

lysates were analyzed by polyacrylamide gel electrophoresis, both before and after immunoprecipitation. Precipitation with anti-CAT antibodies revealed the presence of a single species of CAT polypeptide in rNDV/BC/CAT-infected cells, but not in rNDV/BC-infected cells (Fig. 7).

Analysis of mRNAs by Northern blot hybridization

A separate CAT transcription cassette in the genome of NDV should suggest the synthesis of an additional polyadenylated mRNA. The presence of this mRNA and the levels of expression of HN and L mRNA, the genes of

TABLE 3
MDT in Eggs and ICPI in Day-Old Chicks

| Strain | MDT ^a | ICPI ^b |
|----------------|------------------|-------------------|
| pNDV/BC | 60 h | 1.58 |
| rNDV/BC | 62 h | 1.63 |
| rNDV/BC/CAT | 75 h | 1.50 |
| rNDV/BC/(-)CAT | 63 h | ND ^c |

^a Lentogenic strains, MDT > 90 h; mesogenic strains, MDT = 60–90 h; velogenic strains, MDT < 60 h.

^b Lentogenic strains, ICPI < 0.7; mesogenic strains, ICPI = 0.7–1.6; velogenic strains, ICPI > 1.6.

^c ND, not determined.

which flank the CAT gene, were tested by Northern blot hybridization. The hybridization was performed on RNA from cells infected with rNDV/BC and rNDV/BC/CAT, each at passage level 6.

Hybridization of the mRNAs extracted from rNDV/BC/CAT-infected cells with the negative-sense CAT-specific riboprobe detected a single major band of the predicted size of CAT mRNA (Fig. 8, lane 2). There were no detectable shorter forms of CAT mRNA. Negative-sense riboprobes specific to HN and L genes were also used for hybridization of replicate blots. The levels of HN and L mRNAs were significantly less for rNDV/BC/CAT than for rNDV/BC (Fig. 8). Comparison between the relative amounts of various mRNAs was not possible because of the different probe used for each. However, densitometry scanning was used to determine the percentage of radioactivity present in each mRNA band of rNDV/BC and rNDV/BC/CAT. The values of mRNAs of rNDV/BC/CAT were 24% (HN) and 13% (L), considering a value of 100% for each of the mRNAs of rNDV/BC. This result implicated a lower amount of the viral mRNA accumulation in the cells infected with the slower-growing CAT-containing recombinant virus.

Pathogenicity of rNDV/BC/CAT

The pathogenicity of CAT-expressing recombinant NDV in eggs was assessed by injecting different dilutions of virus into the allantoic cavity of 9-day-old embryonated SPF chicken eggs. The MDT was calculated as the time that is required for a minimum lethal dose to kill

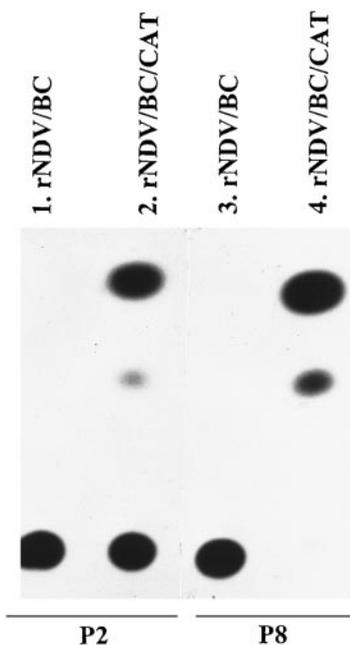


FIG. 6. CAT expression by rNDV/BC and rNDV/BC/CAT during passage 2 and passage 8. Cell lysates were analyzed for acetylation of [14 C]chloramphenicol by thin-layer chromatography.

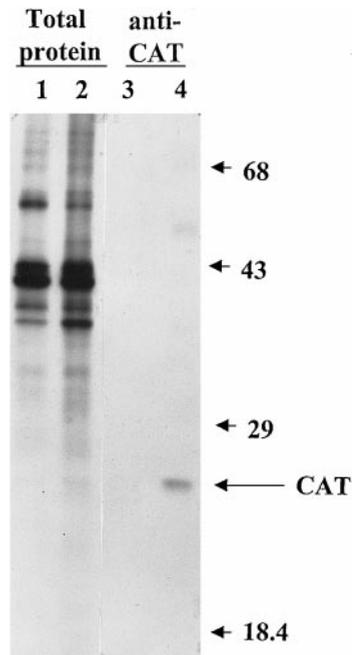


FIG. 7. Immunoprecipitation of CAT protein from cells infected with rNDV/BC/CAT virus labeled with 35 S-methionine. Proteins were immunoprecipitated with anti-CAT antibodies from lysates of cells infected with rNDV/BC (lane 3) and rNDV/BC/CAT (lane 4). Aliquots of total protein from lysates of rNDV/BC (lane 1) and rNDV/BC/CAT (lane 2) were analyzed in parallel. Numbers on the right are molecular weight in kilodaltons. The detection of CAT protein required longer exposure (36 h) of the autoradiograph than that of the total protein (15 h).

the eggs. The MDT for rNDV/BC/CAT was higher (75 h) than that for rNDV/BC (62 h) (Table 3). This implicates attenuation of virus pathogenicity for the CAT gene-containing recombinant NDV.

Similarly, assessment of pathogenicity in day-old chicks revealed a lower ICPI value for rNDV/BC/CAT (an

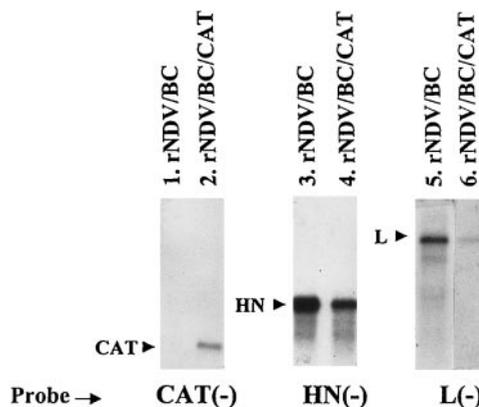


FIG. 8. Northern blot hybridization of intracellular CAT, HN, and L mRNAs encoded by rNDV/BC and rNDV/BC/CAT. Poly A (+) mRNAs were isolated from total intracellular RNA by oligo (dT) chromatography and were electrophoresed on formaldehyde-agarose gels. The gels were transferred to nitrocellulose membrane and probed with the negative-sense riboprobe.

ICPI value of 1.5) than that for rNDV/BC (ICPI of 1.63). Thus, introduction of an additional gene into the NDV genome might represent a method of attenuation of the virus to birds.

Growth and biological properties of CAT gene-deleted version of recombinant

To confirm that attenuation or decrease in growth of CAT gene-containing recombinant was due to the presence of an extra gene and not due to any extraneous mutation in the genome, we decided to recover and characterize the virus obtained by transfecting a CAT gene-deleted version of pNDVf1/CAT. To do this, pNDV/CAT was digested with restriction enzyme *AgeI*, to remove the CAT gene, and was religated back to obtain the plasmid containing the full-length NDV genome, but devoid of the CAT gene. Transfection of this plasmid led to the recovery of a recombinant virus (designated rNDV/BC(-)CAT), identical in growth characteristics and biological properties to the virus rNDV/BC. There was a reversion back to MDT (Table 3), plaque size (Fig. 2), and growth characteristics (data not shown) of rNDV/BC, implicating that growth impairment and attenuation of rNDV/BC/CAT resulted entirely from the inclusion of an additional gene.

DISCUSSION

In this report, we describe the recovery of an infectious mesogenic strain of NDV (strain Beaudette C) entirely from cDNA. Although La Sota, a lentogenic strain of NDV was recently recovered from cDNA (Oberdorfer *et al.*, 1999; Peeters *et al.*, 1999), this is the first report of the recovery of a mesogenic strain of NDV. Mesogenic strains are suitable candidates for the study of genetic manipulation that could result in more attenuated or more virulent viruses. In this study, a vaccinia-based system was used. This system, in which the recombinant vaccinia virus provides the T7 RNA polymerase to synthesize the antigenomic RNA from the full-length plasmid and the proteins NP, P, and L from the cotransfected plasmids, has also been used to recover several other nonsegmented negative-stranded RNA viruses. The problems associated with the presence of vaccinia virus in the recovery system are minimized, largely because the fast growth of NDV eliminates the need for early separation of vaccinia virus. HEp-2 cells were chosen for transfection because this human tissue-derived cell line is relatively more resistant to MVA-T7-induced cytopathology, is readily transfectable, and supports NDV growth. After transfection, we tested two different approaches to recovering the virus. In terms of virus titer generated, either passage of the virus from the transfection harvest in the egg or a chicken embryo-derived cell line proved highly efficient. NDV was recovered three of three times on numerous occasions by these methods.

Therefore, both these methods of recovery can be utilized to recover NDV particles entirely from cDNA.

Several members of paramyxoviruses replicate efficiently only if their genome nucleotide length is a multiple of 6 (Calain and Roux, 1993; Durbin *et al.*, 1997b; Murphy and Parks, 1997; Sidhu *et al.*, 1995). The natural genome length of NDV is a multiple of 6 (de Leeuw and Peeters, 1999; Krishnamurthy and Samal, 1998; Phillips *et al.*, 1998), but certain minigenome lengths other than the multiple of 6 have been found to replicate with measurable efficiency (our unpublished data). We, however, have maintained the full-length cDNA as a multiple of 6 to maximize the efficiency of recovery. Sequence analysis of the full-length NDV cDNA showed five nucleotide changes compared to the published sequence of NDV Beaudette C strain. These sequence changes resulted in one amino acid substitution in the HN protein and two amino acid substitutions in the L protein. We wanted to determine whether these nucleotide changes were the result of misincorporation during RT-PCR or were present in our parental NDV strain Beaudette C. Viral genomic RNAs isolated from three different stocks of NDV strain Beaudette C were subjected to RT-PCR with primers that specifically amplified the regions containing the changed nucleotides. The PCR fragments were cloned and several clones were sequenced. All clones derived from three different virus stocks showed the changes in the nucleotides compared to the published sequence. These results indicate that the substitution of five nucleotides found in our full-length NDV cDNA were present in our parental virus. Therefore, it is apparent that the wild-type NDV strain Beaudette C has accrued nonlethal point mutations during serial passages over the years. Additionally, introduction of two genetic markers resulted in a total of five nucleotide substitutions in the F/HN and HN/L intergenic regions of the full-length cDNA. These changes did not adversely affect the recovery of the virus. As expected, the three additional G residues adjacent to the T7 promoter did not inhibit the rescue of NDV. Removal of extra G residues from the end of the genome was previously documented in VSV (Pattnaik *et al.*, 1992) and NDV (Peeters *et al.*, 1999).

The successful recovery of NDV from cDNA clones will permit genetic manipulation of the entire genome of NDV. Recombinant NDVs can be generated to study structure-function relationships of individual viral genes and to analyze promoter elements and other noncoding sequences. One of the outcomes of these studies will lead to unraveling the molecular basis of NDV pathogenesis. NDV is an ideal model to study the pathogenesis of paramyxoviruses not only because NDV strains vary from mild to highly virulent but also because the pathogenesis experiments can be carried out directly in the natural host, chickens. Determination of the function of individual NDV proteins, especially F, HN, and V proteins, will be helpful to elucidate the molecular basis of NDV

pathogenicity. This understanding could lead to designing better vaccines for Newcastle and other paramyxovirus diseases.

One other application of reverse genetics would be to use the recombinant viruses to express additional proteins. In the last few years, several research groups used negative-strand RNA viruses to express foreign proteins (Baron *et al.*, 1999; Bukreyev *et al.*, 1996; Hasan *et al.*, 1997; He *et al.*, 1997; Luytjes *et al.*, 1989; Mebatsion *et al.*, 1996; Sakai *et al.*, 1999; Schnell *et al.*, 1996; Spielhofer *et al.*, 1998). The results of these studies showed that the expression levels of foreign proteins are quite high and foreign genes are very stable after many passages *in vitro* and *in vivo*. In the present study, the reverse genetics system was used to generate an NDV recombinant containing a foreign gene. A transcription cassette of bacterial CAT gene was inserted into the HN/L intergenic region of the full-length cDNA. The CAT gene was inserted in this region by analogy to schemes used for VSV and SV5 (He *et al.*, 1997; Schnell *et al.*, 1996). We chose the CAT gene for this study because of its small size and the existence of an excellent assay system for the enzyme. Flanked by NDV gene start and gene end signals, the CAT gene should be subjected to transcription and expression strategy similar to that of other NDV genes. We deduced that insertion of a new cistron downstream of the HN gene would have minimal effects on viral replication, perhaps reducing the L gene expression slightly in consequence of the polarity of transcription. The total genome length was maintained as a multiple of six nucleotides for efficient replication of the recombinant virus. CAT expression was observed in cell culture infected with the virus for each of the eight passages. Thus, the CAT gene was maintained stably in the NDV recombinant without any detectable loss of any part of the gene. The growth of the virus was delayed, however, and a maximum titer 100-fold lower than that of the wild-type recombinant was noted. A decrease in virus production resulting from the presence of an additional gene was also reported in other nonsegmented negative-strand viruses (Bukreyev *et al.*, 1996; Hasan *et al.*, 1997; Sakai *et al.*, 1999). The presence of the additional gene also resulted in an increase in MDT.

We also assessed the virulence of the virus to birds by determining its ICPI. This procedure of determining virulence is highly sensitive and reproducible and is the only method currently accepted by the Office of International Epizootics for pathotyping NDV isolates. A lower ICPI for the CAT-expressing recombinant (a value of 1.5) than for the wild type recombinant (a value of 1.63) proved that the inclusion of the CAT gene in the NDV genome results in attenuation. Recently, we recovered NDV recombinant-expressing green fluorescent protein (GFP), with the GFP gene inserted between the F and HN genes of the full-length cDNA clone (unpublished data). Our initial results show that this recombinant also exhib-

its reduced growth in cell culture and a decreased virulence to eggs. This finding indicates that any gene introduced as an additional transcriptional unit in the NDV genome is likely to be associated with the reduced growth and attenuation of the recombinant virus. An implication of all these results is that NDV can be engineered to express foreign protein stably and can be manipulated in the future for use as a vaccine vector.

Several factors need to be addressed regarding expression levels, size of the foreign gene, and attenuation. It is necessary to explore the functional attributes of the intergenic and noncoding regions of NDV. This knowledge should lead to finer insertion strategies for the foreign gene, thereby achieving a balance between the expression level of the foreign or viral genes and the attenuation level of the virus. As shown for other viruses, the expression level of the foreign gene in NDV should be high when placed near the 3' end of the genome, because of the polarity of transcription (Sakai *et al.*, 1999; Wertz *et al.*, 1998). Additionally, the upper size limit of the foreign gene that can be inserted into the NDV genome needs to be investigated, to achieve expression of multiple/larger genes. Although the upper size limit of the insertion that negative-strand RNA viruses can accommodate is not known, results from several studies indicated that these viruses can tolerate large size inserts. In Sendai virus, a 3.2-kb foreign gene (Sakai *et al.*, 1999) was inserted and expressed efficiently. Multiple genes, adding up to 4.2 kb of RNA, were introduced into recombinant VSVs (Roberts and Rose, 1998).

An important application of this work will be in the development of NDV as a vaccine vector for the expression of protective antigens of other avian pathogens. Several characteristics of NDV already suggest its suitability as a vaccine vector for avian pathogens: (1) Live NDV vaccines are widely used in poultry with proven track records of efficacy and safety. (2) NDV naturally infects the respiratory tract, leading to the induction of both mucosal and systemic immunity. Therefore, NDV would be a suitable vector for antigens of agents infecting respiratory tract and other organs. (3) NDV does not show measurable rates of recombination. Therefore, NDV strains cannot create new variants. (4) NDV replicates independently of nuclear functions and thus cannot integrate into cellular DNA that can result in cellular transformation or viral persistence. The last three features confer advantages over the live recombinant vaccine vectors derived from pox and herpes viruses. Additionally, pox and herpes virus vectors express the foreign proteins in combination with a large repertoire of proteins encoded by their large-size genomes (Moss and Flexner, 1987). In contrast, the foreign protein in NDV is expressed with only seven other proteins. For the generation of specific immune responses in vaccine applications, it will be advantageous to have only a limited number of proteins expressed. All these factors favorably

augur the use of NDV as a vaccine vector. Techniques of reverse genetics should augment this use significantly.

In summary, we have described the successful recovery of a mesogenic strain of NDV from positive-sense transcripts of a cDNA clone. Availability of a functional cDNA clone should be useful for basic studies of NDV molecular biology and pathogenesis. In addition, we have demonstrated that the NDV genome can accept and maintain an additional gene. An important application of this work will be in the development of NDV as an attenuated vaccine vector for the delivery of heterologous viral proteins.

MATERIALS AND METHODS

Cells and viruses

HEp-2 and DF1 cells were grown and maintained in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal calf serum. NDV strain Beaudette C was obtained from The National Veterinary Services Laboratory (Ames, IA). The virus was grown in 9-day-old embryonated SPF chicken eggs by injecting the virus in the allantoic cavity. Two days later, the allantoic fluid was harvested and clarified, and the virus was purified as described previously (Kingsbury, 1966).

cDNA synthesis and construction of NDV full-length plasmid

Viral RNA was extracted from the purified virus by the use of TRIzol (Life Technologies, Gaithersburg, MD) according to manufacturer's protocol. A total of eight cDNA fragments, spanning the entire NDV genome, were generated by RT-PCR (Fig. 1). Specifically, each cDNA fragment was primed by a positive-sense oligonucleotide primer (Table 1), which carried a recognition sequence for a restriction enzyme that was unique or present two or more times in the genome. The primer corresponding to the fragment I had a T7 promoter overhang 3' to the *Ascl* restriction site, and the primer corresponding to fragment VIII had a 24-nt 3'-end sequence overhang of HDV ribozyme sequence. Also, the positive-sense oligonucleotide primer corresponding to fragment IV was modified at nt positions 6294 and 6295 to create an *MluI* site tag (at the F/HN intergenic region), and the negative-sense oligonucleotide primer corresponding to fragment V was modified at nt positions 8357, 8358, and 8359 to create an *AgeI* site tag (at the HN/L intergenic region).

The first-strand cDNA was synthesized using Thermo-script RT-PCR kit (Life Technologies) according to the manufacturer's protocol. Subsequently, PCR was performed using high-fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA) in a reaction containing the primer used in the RT reaction and the corresponding negative-sense oligonucleotide primer (Table 1). The RT-PCR product was then digested with the respective restriction

enzymes and ligated to the plasmid pBR322/dr (Fig. 1). The fragments were ligated sequentially in the order shown in Table 1. Plasmid pBR322/dr was constructed by modification of plasmid pBR322. The modification included a backbone of 72-nt linker between the *EcoRI* and *PstI* sites, and an HDV 84-nt antigenome ribozyme sequence and T7 RNA polymerase transcription termination signal downstream of the polynucleotide linker. After ligation into the plasmid, each fragment was sequenced completely by the dideoxy chain termination method (Sanger *et al.*, 1977). The resulting NDV full-length expression plasmid was termed pNDVf1.

Construction of expression plasmids

cDNA fragments bearing the open reading frame (ORF) of NP, P, and L genes were generated by RT-PCR. The NP gene was cloned in the plasmid pGEM 7Z (pNP) between *EcoRI* and *BamHI* sites. The cloned gene was sequenced completely by the dideoxy chain termination method. The P and L genes were cloned in an expression plasmid, which has an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) downstream of the T7 RNA polymerase promoter and makes use of the translation start codon contained in *NcoI* site of IRES. The cloned genes were sequenced to entirety by the dideoxy chain termination method (Sanger *et al.*, 1977).

Construction of full-length plasmid containing CAT gene

The restriction enzyme site of *AgeI* at position 8358 of pNDVf1 was utilized to insert the CAT gene between the ORF of HN and the L gene (Fig. 5). The ORF encoding the CAT protein was engineered to contain NDV gene start and gene end sequences. Specifically, the positive-sense oligonucleotide primer used to amplify the CAT gene by PCR was modified to contain the gene start sequence and a noncoding region of 4 nt downstream of the *AgeI* site and upstream of the CAT-specific sequence. The negative-sense oligonucleotide primer containing the *AgeI* site overhang included a noncoding region of 5 nt, and the gene end sequence for the CAT gene apart from the CAT-specific sequence. PCR was performed by the use of high-fidelity *Pfu* DNA polymerase (Stratagene). The PCR-amplified fragment was inserted at the *AgeI* site of pNDVf1 and the resulting plasmid was designated pNDVf1/CAT. The integrity of the entire foreign sequence was confirmed by sequencing using the dideoxy chain termination method (Sanger *et al.*, 1977). This plasmid thus resulted in a 40-nt intergenic region between HN and the CAT gene and a 14-nt intergenic sequence between CAT and the L gene. The insertion of 696 nucleotides of foreign sequence (a nucleotide multiple of six) resulted in the plasmid encoding an antigenome of 15,882 nucleotides.

Transfection and recovery of recombinant NDV

The HEp-2 cells were chosen for transfection experiments because these cells are permissive for NDV, transfect efficiently, and are resistant to CPE of vaccinia virus strain MVA. Specifically, cells grown to 80% confluency in six-well plates were washed twice with Opti-MEM just before transfection. The cells were then transfected with 5 μ g pNDV, 2.5 μ g pNP, 1.5 μ g pP, and 0.5 μ g of pL in a volume of 0.2 ml of Opti-MEM/well. The transfection was carried out with Lipofectamine Plus (Life Technologies), according to the manufacturer's instructions. Along with the transfection mixture, 1 focus-forming unit per cell of recombinant vaccinia virus (MVA/T7) expressing T7 RNA polymerase was added in a volume of 0.8 ml Opti-MEM/well. Twelve hours later, the medium was replaced with 3 ml Opti-MEM containing 2% fetal bovine serum (FBS) and 40 μ g of cytosine arabinoside/ml. Four days after transfection, the medium was harvested, the cell debris was pelleted by low-speed centrifugation, and the supernatant was used for infecting a fresh batch of HEp-2 cells in 25-cm² flasks. The virus in the supernatant was allowed to adsorb for 6 h and was then replaced with 5 ml of D-MEM containing 2% FBS and 40 μ g/ml cytosine arabinoside. After one additional passage in DF1 cells (at a passage interval of 4 days) the supernatant was plaque purified. Plaque purification was performed on DF1 cells and individual plaques were used to inject 9-day-old embryonated chicken eggs through the allantoic route. Alternatively, 100 μ l supernatant from transfection was directly used to inject 9-day-old embryonated chicken eggs. Two days later, the eggs were chilled for a period of 12 h, after which the allantoic fluid was harvested. The virus in the allantoic fluid was amplified by further passage in eggs.

Antibody staining of plaques

The plaques produced by the recovered virus were stained by NDV-specific antibodies. The assay was performed by using an overlay consisting of Opti-MEM medium supplemented with 2% fetal bovine serum and 0.9% methyl cellulose (reagents from MCB, Norwood, OH). After incubation for 4 days at 37°C, the overlay was removed and the monolayer was fixed with 80% methanol. The plaques were then incubated with either NDV chicken polyclonal antisera or monoclonal antibody specific to NDV HN. This was followed by incubation with either goat anti-chicken IgG or sheep anti-mouse IgG, both tagged with horseradish peroxidase. Plaques were then stained by reaction with substrate.

RT-PCR and demonstration of genetic markers

RT-PCR was performed on the RNA extracted from recombinant virus. Specifically, recombinant virus grown in the allantoic cavity of five 9-day-old embryonated eggs was

harvested, clarified, and purified as described previously (Kingsbury, 1966). RNA was extracted from one-third of the purified virus by TRIzol (Life Technologies) and then subjected to first-strand cDNA synthesis using the ThermoScript RT-PCR kit (Life Technologies). One of the reactions, which served to demonstrate the *Mlu*I marker (at position 6293 of the genome), was performed using the synthetic oligonucleotide primer that bound to the F gene sequence: 5'-GATGTGTAACCCCCCGGGTAT-3' (positions 5742 to 5763 in the full-length cDNA). The negative-sense oligonucleotide primer for PCR was derived from the HN gene sequence: 5'-CTCTTCATAGTCCCTATACATTC-3' (positions 7721 to 7743 in the full-length cDNA). The second reaction was designed to demonstrate the *Age*I marker (at position 8358 of the genome) and was primed with an oligonucleotide primer that hybridized to the HN gene sequence: 5'-CAATGGCCTTCACTCGGCCAGGT-3' (positions 7743 to 7765 in the full-length cDNA). The negative-sense oligonucleotide primer for PCR was derived from the L gene sequence: 5'-GCTATATCATTGGGGAGGA-3' (positions 9354 to 9372 in the full-length cDNA). The RT-PCR product was then subjected to restriction enzyme digestion: the first product with enzyme *Mlu*I, the second product with enzyme *Age*I. The restriction patterns were then demonstrated by agarose gel electrophoresis. A parallel experiment was conducted on the parental strain of Beaudette C as control.

RT-PCR was also performed to demonstrate the location of the CAT gene insert in the recombinant NDV expressing the CAT protein. The virus in the supernatant of passage 8 was utilized for extraction of viral RNA. Specifically, PEG 8000 was added to the supernatant at a final concentration of 10% and allowed to mix at 4°C for 3 h. The mixture was then centrifuged at 4000 rpm in a Beckmann GPKR centrifuge (GH3.7 rotor) for 15 min. The supernatant was discarded and the pellet was resuspended in a small amount of D-MEM. RNA was extracted from the suspension by TRIzol (Life Technologies) and subjected to first-strand cDNA synthesis using the ThermoScript RT-PCR kit (Life Technologies). The reaction was performed using the oligonucleotide primer, 5'-CAATGGCCTTCACTCGGCCAGGT-3', which is positive-sense and represents positions 7743 to 7765 in the full-length cDNA. PCR was then performed by using the above-mentioned oligonucleotide and a negative-sense oligonucleotide primer 5'-GCTATATCATTGGGGAGGA-3', representing positions 9354 to 9372 in the full-length cDNA. These two primers flank the CAT gene and part of the HN and L genes. The recombinant NDV without the CAT gene insertion in the genome was analyzed in parallel.

CAT assays and passage of recombinant NDV encoding CAT

CAT expression was assayed for a total of eight passages. At the end of transfection and first passage the entire media supernatant was used for passing the virus

to fresh DF1 cells in 25-cm² flasks. But at the end of every other passage, one-thirteenth of the media supernatant was used for passing to fresh DF1 cells. A total of eight serial passages were performed at a passage interval of 4 days. The cell pellets were utilized for the CAT assay and RNA isolation. For the CAT assay, the cell pellets were lysed by three freeze/thaw cycles and one-tenth of the lysed pellet was analyzed by thin-layer chromatography for the ability to acetylate [¹⁴C]chloramphenicol (Gorman *et al.*, 1982).

Isolation of poly (A)⁺ RNA and Northern blot hybridization

The virus from passage level 6 was quantitated by plaque assay and 10 m.o.i. of the virus was used to infect DF1 cells in a 25-cm² flask. After 24 h, the cell pellet was collected by centrifugation, then resuspended in a few drops of D-MEM; total RNA was extracted with TRIzol reagent (Life Technologies) as recommended by the manufacturer. The total RNA was utilized to extract poly (A)⁺ RNA by using the poly(A) tract mRNA isolation system IV kit (Promega, Madison, WI). mRNA samples (each representing one-tenth of one T-25 cell) were analyzed by electrophoresis on 1.5% agarose gels containing 0.44 M formaldehyde, run at 110 V for 2 h. The gel was then transferred to a nitrocellulose membrane (pore size, 0.2 μ m) using the Turbo-Blot apparatus (Schleicher & Schuell, Keene, NH) with a transfer buffer containing 3 M NaCl and 8 mM NaOH. The RNA was crosslinked to the membrane by the UV crosslinker (Stratagene). The membrane was then subjected to prehybridization for 8 h at 65°C in 6 \times SSC containing 0.1% SDS, 5 \times Denhardt's solution, and 0.5 mg/ml of sheared salmon sperm DNA. Hybridization was performed overnight in the same solution after addition of the appropriate 2 \times 10⁶ dpm of [³²P]CTP-labeled negative-sense probe. The probe was synthesized by *in vitro* transcription of linearized plasmid pGEM7Z containing either the complete gene of CAT (cloned between the *Xba*I and *Bam*HI sites of the plasmid) or the partial gene of HN or L (cloned between the *Xba*I and *Hind*III sites of the plasmid), all under the control of T7 RNA polymerase promoter. Washing of the membrane after hybridization was done in 10 \times SSC-1% SDS at 65°C for 1 h. A second washing was done in 0.1% SSC and 0.1% SDS at 65°C for 1 h. Blots were then exposed overnight to X-ray film. The density of the hybridized bands was estimated with a densitometer (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation and polyacrylamide gel electrophoresis

DF1 cell monolayers in a 25-cm² flask were infected with rNDV/BC or rNDV/BC/CAT at 10 PFU/cell. At 6 h postinfection, the cells were starved in a methionine-free medium (Life Technologies) for 30 min. At the end of the

starvation period, 50 μ Ci of [³⁵S]methionine (1000 Ci/mmol; Amersham, Arlington Heights, IL) per ml was added to the flask. Twelve hours later the labeled cells were washed three times with cold phosphate buffer saline and lysed in 0.3 ml of cold radioimmunoprecipitation assay (RIPA) buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.0% Triton X-100]. The lysates were clarified by centrifugation at 4°C. The lysate was then incubated at room temperature with 5 μ l of purified CAT-specific polyclonal rabbit antibody (5 Prime \rightarrow 3 Prime, Boulder, CO) for a period of 4 h. At the end of this period, 50 μ l of *Staphylococcus aureus* protein A (Life Technologies) was added and the samples were shaken at 4°C for 1 h. The immune complexes were collected by centrifugation at 16,000 *g* for 30 s. The complex was washed twice with solution I (0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 5 mM EDTA) and twice with solution II (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 25 mM EDTA). The pellet was resuspended in 30 μ l of sample buffer and then boiled for 3 min prior to loading on a 15% polyacrylamide gel. Following electrophoresis, the gels were fixed for 1 h in 30% methanol and 10% acetic acid, dried, and exposed to X-ray film.

Pathogenicity test in eggs

MDT in eggs was determined for each virus, to assess the pathogenicity in eggs. A series of 10-fold dilutions of infected allantoic fluid were made in sterile PBS, and 0.1 ml of each dilution was inoculated into the allantoic cavity of each of the five 9-day-old embryonated SPF chicken eggs. The eggs were stored at 37°C and examined four times daily for 7 days. The time that each embryo was first observed dead was recorded. The highest dilution at which all eggs died was considered the minimum lethal dose. The MDT was recorded as the mean time in hours for the minimum lethal dose to kill the embryos. The MDT has been used to classify NDV strains into velogenic (taking under 60 h to kill), mesogenic (taking between 60 and 90 h to kill), and lentogenic (taking more than 90 h to kill).

Pathogenicity test in birds

ICPI was used to determine the virulence of wild-type and recombinant NDVs in day-old chicks. For each ICPI test, 15 one-day-old chicks (10 birds for test and 5 birds for control) were used. These birds were from SPF flocks that were known to be free of antibodies to NDV. The inoculum consisted of fresh infective allantoic fluid with a HA titer of >2⁴ (1/16) for the test birds and allantoic fluid from normal uninfected 12-day-old embryonated chicken eggs for control birds, both inocula being diluted 1/10 in sterile PBS. The inoculation was done using a 27-gauge needle, attached to a 1-ml stepper syringe

dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were inoculated intracerebrally by inserting the needle up to the hub into the left or right rear quadrant of the cranium. The birds were observed and scored every 24 h for a period of 8 days. The scoring and determination of ICPI were done according to the method described by Alexander (1989).

ACKNOWLEDGMENTS

We are grateful to Dr. Peter L. Collins for many invaluable discussions of the work. We thank Peter Savage and Daniel Rockemann for excellent technical assistance. This work was supported by U.S. Department of Agriculture Grant 98-35204-6427.

REFERENCES

- Alexander, D. J. (1989). Newcastle disease. In "A Laboratory Manual for the Isolation and Identification of Avian Pathogens" (H. G. Purchase, L. H. Arp, C. H. Domermuth, and J. E. Pearson, Eds.), 3rd ed., pp. 114–120. American Association of Avian Pathologists, Kennett Square, PA.
- Alexander, D. J. (1997). Newcastle disease and other avian *Paramyxoviridae* infections. In "Diseases of Poultry" (B. W. Calnek, Ed.), 10th ed., pp. 541–569. Mosby-Wolfe/Iowa State Univ. Press, Ames.
- Baron, M. D., and Barrett, T. (1997). Rescue of Rinderpest virus from cloned cDNA. *J. Virol.* **71**, 1265–1271.
- Baron, M. D., Cuevas, M. F., Baron, J., and Barrett, T. (1999). Expression in cattle of epitopes of a heterologous virus using a recombinant rinderpest virus. *J. Gen. Virol.* **80**, 2031–2039.
- Buchholz, U. J., Finke, S., and Conzelmann, K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J. Virol.* **73**, 251–259.
- Bukreyev, A., Camargo, E., and Collins, P. L. (1996). Recovery of infectious respiratory syncytial virus expressing an additional foreign gene. *J. Virol.* **70**, 6634–6641.
- Calain, P., and Roux, L. (1993). The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J. Virol.* **67**, 4822–4830.
- Chambers, P., Millar, N. S., Bingham, R. W., and Emmerson, P. T. (1986a). Molecular cloning of complementary DNA to Newcastle disease virus, and nucleotide sequence analysis of the junction between the genes encoding the haemagglutinin-neuraminidase and the large protein. *J. Gen. Virol.* **67**, 475–486.
- Chambers, P., Millar, N. S., and Emmerson, P. T. (1986b). Nucleotide sequence of the gene encoding the fusion glycoprotein of Newcastle disease virus. *J. Gen. Virol.* **67**, 2685–2694.
- Clarke, D. K., Sidhu, M. S., Johnson, J. E., and Udem, S. A. (2000). Rescue of mumps virus from cDNA. *J. Virol.* **74**, 4831–4838.
- Collins, P. L., Hill, M. G., Camargo, E., Grosfeld, H., Chanock, R. M., and Murphy, B. R. (1995). Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. *Proc. Natl. Acad. Sci. USA* **92**, 11563–11567.
- de Leeuw, O., and Peeters, B. (1999). Complete nucleotide sequence of Newcastle disease virus: Evidence for the existence of a new genus within the subfamily *Paramyxovirinae*. *J. Gen. Virol.* **80**, 131–136.
- Durbin, A. P., Hall, S. L., Siew, J. W., Whitehead, S. S., Collins, P. L., and Murphy, B. R. (1997a). Recovery of infectious human parainfluenza virus type 3 from cDNA. *Virology* **235**, 323–332.
- Durbin, A. P., Siew, J. W., Murphy, B. R., and Collins, P. L. (1997b). Minimum protein requirements for transcription and RNA replication of a minigenome of human parainfluenza virus type 3 and evaluation of the rule of six. *Virology* **234**, 74–83.
- Garcin, D., Pelet, T., Calain, P., Roux, L., Curran, J., and Kolakofsky, D. (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.
- Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**, 1044–1051.
- Hasan, M. K., Kato, A., Shioda, T., Sakai, Y., Yu, D., and Nagi, Y. (1997). Creation of an infectious recombinant Sendai virus expressing the firefly luciferase gene from the 3' proximal first locus. *J. Gen. Virol.* **78**, 2813–2820.
- He, B., Paterson, R. G., Ward, C. D., and Lamb, R. A. (1997). Recovery of infectious SV5 from cloned DNA and expression of a foreign gene. *Virology* **237**, 249–260.
- Hoffman, M. A., and Banerjee, A. K. (1997). An infectious clone of human parainfluenza virus type 3. *J. Virol.* **71**, 4272–4277.
- Kato, A., Sakai, Y., Shioda, T., Kondo, T., Nakanishi, M., and Nagai, Y. (1996). Initiation of Sendai virus multiplication from transfected cDNA or RNA with negative or positive sense. *Genes Cells* **1**, 569–579.
- Kingsbury, D. W. (1966). Newcastle disease virus RNA. I. Isolation and preliminary characterization of RNA from virus particles. *J. Mol. Biol.* **18**, 195–203.
- Krishnamurthy, S., and Samal, S. K. (1998). Nucleotide sequences of the trailer, nucleocapsid protein gene and intergenic regions of Newcastle disease virus strain Beaudette C and completion of the entire genome sequence. *J. Gen. Virol.* **79**, 2419–2424.
- Lawson, N. D., Stillman, E. A., Whitt, M. A., and Rose, J. K. (1995). Recombinant vesicular stomatitis virus from DNA. *Proc. Natl. Acad. Sci. USA* **92**, 4477–4481.
- Luytjes, W., Krystal, M., Enami, M., Pavin, J. D., and Palese, P. (1989). Amplification, expression and packaging of a foreign gene by influenza virus. *Cell* **59**, 1107–1113.
- Mebatsion, T., Schnell, M. J., Cox, J. H., Firk, S., and Conzelmann, K. K. (1996). Highly stable expression of a foreign gene from rabies virus vector. *Proc. Natl. Acad. Sci. USA* **93**, 7310–7314.
- Millar, N. S., and Emmerson, P. T. (1988). Molecular cloning and nucleotide sequencing of Newcastle disease virus. In "Newcastle Disease" (D. J. Alexander, Ed.), pp. 79–97. Kluwer Academic, Boston.
- Moss, B., and Flexner, C. (1987). Vaccine virus expression vectors. *Annu. Rev. Immunol.* **5**, 305–324.
- Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A., and Summers, M. D. (1995). "Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses." Springer-Verlag, New York.
- Murphy, S. K., and Parks, G. D. (1997). Genome nucleotide lengths that are divisible by six are not essential but enhance replication of defective interfering RNAs of the paramyxovirus simian virus 5. *Virology* **232**, 145–157.
- Oberdorfer, A., Mundt, E., Mebatsion, T., Buchholz, U., and Mettenleiter, T. C. (1999). Generation of recombinant lentogenic Newcastle disease virus from cDNA. *J. Gen. Virol.* **80**, 2987–2995.
- Pattnaik, A. K., Ball, L. A., LeGrone, A. W., and Wertz, G. W. (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**, 1011–1020.
- Peeters, B. P. H., De Leeuw, O. A., Koch, G., and Gielkens, A. L. J. (1999). Rescue of Newcastle disease virus from cloned cDNA: Evidence that cleavability of the fusion protein is a major determinant for virulence. *J. Virol.* **73**, 5001–5009.
- Phillips, R. J., Samson, A. C. R., and Emmerson, P. T. (1988). Nucleotide sequence of the 5' terminus of Newcastle disease virus and assembly of complete genomic sequence: Agreement with the rule of six. *Arch. Virol.* **143**, 1993–2002.
- Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dotsch, C., Christiansen, G., and Billeter, M. A. (1995). Rescue of measles viruses from cloned DNA. *EMBO J.* **14**, 5773–5784.

- Roberts, A., and Rose, J. K. (1998). Recovery of negative-strand RNA viruses from plasmid DNA: A positive approach revitalizes a negative field. *Virology* **247**, 1–6.
- Sakai, Y., Kiyotami, K., Fukumara, M., Asakawa, M., Kato, A., Shioda, T., Yoshida, T., Tanaka, A., Hasegawa, M., and Nagai, Y. (1999). Accommodation of foreign genes into the Sendai virus genome: Sizes of inserted genes and viral replication. *FEBS Lett.* **456**, 221–226.
- Sanger, F., Nicklen, J. E., and Coulson, A. R. (1977). DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
- Schnell, M. J., Buonocore, L., Whitt, M. A., and Rose, J. K. (1996). The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. *J. Virol.* **70**, 2318–2323.
- Schnell, M., Mebatsion, T., and Conzelmann, K. K. (1994). Infectious rabies viruses from cloned cDNA. *EMBO J.* **13**, 4195–4203.
- Sidhu, M. S., Chan, J., Kaelin, K., Spielhofer, P., Radecke, F., Schneider, H., Masureker, M., Dowling, P. C., Billeter, M. A., and Udem, S. A. (1995). Rescue of synthetic measles virus minireplicons: Measles genomic termini direct efficient expression and propagation of a reporter gene. *Virology* **208**, 800–807.
- Spielhofer, P., Bachi, T., Fehr, T., Christiansen, G., Cattaneo, R., Kaelin, K., Billeter, M. A., and Naim, H. (1998). Chimeric measles viruses with a foreign envelope. *J. Virol.* **72**, 2150–2159.
- Steward, M., Vipond, I. B., Millar, N. S., and Emmerson, P. T. (1993). RNA editing in Newcastle disease virus. *J. Gen. Virol.* **74**, 2539–2547.
- Wertz, G. W., Perepelitis, V. P., and Ball, L. A. (1998). Gene rearrangement and attenuated expression and lethality of a nonsegmented negative strand RNA virus. *Proc. Natl. Acad. Sci. USA* **95**, 3501–3506.
- Whelan, S. P., Ball, L. A., Barr, J. N., and Wertz, G. (1995). Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc. Natl. Acad. Sci. USA* **92**, 8388–8392.
- Yusoff, K., Millar, N. S., Chambers, P., and Emmerson, P. T. (1987). Nucleotide sequence analysis of the L gene of Newcastle disease virus: Homologies with Sendai and vesicular stomatitis virus. *Nucleic Acids Res.* **15**, 3961–3976.