

Generation of recombinant lentogenic Newcastle disease virus from cDNA

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Recombinant lentogenic Newcastle disease virus (NDV) of the vaccine strain Clone-30 was reproducibly generated after simultaneous expression of antigenome-sense NDV RNA and NDV nucleoprotein, phosphoprotein and RNA-dependent RNA polymerase from plasmids transfected into cells stably expressing T7 RNA polymerase. For this purpose, the genome of Clone-30, comprising 15 186 nt, was cloned and sequenced prior to assembly into a full-length cDNA clone under control of a T7 RNA polymerase promoter. Recombinant virus was amplified by inoculation of transfection supernatant into the allantoic cavity of embryonated specific-pathogen-free (SPF) chicken eggs. Two marker restriction sites comprising a total of five nucleotide changes artificially introduced into noncoding regions were present in the progeny virus. The recombinant NDV was indistinguishable from the parental wild-type virus with respect to its growth characteristics in cell culture and in embryonated eggs. Moreover, an intracerebral pathogenicity index of 0.29 was obtained for both viruses as determined by intracerebral inoculation of day-old SPF chickens, proving that the recombinant NDV is a faithful copy of the parental vaccine strain of NDV.

Introduction

Newcastle disease (ND) is a highly contagious disease of many avian species that can lead to substantial losses in the poultry industry worldwide. The aetiological agent of the disease, Newcastle disease virus (NDV), which has been designated avian paramyxovirus 1 (Alexander, 1997), produces a disease of varying severity in poultry, depending on virus strains and host species infected. NDV strains are grouped according to the severity of clinical signs that they cause in chickens. While lentogenic strains cause only a mild respiratory disease, mesogenic strains produce disease with moderate mortality. Velogenic strains cause severe respiratory disease, in combination with visceral haemorrhages, or neurological signs, and mortality up to 100% in chickens (Alexander, 1989, 1997; Hanson & Brandly, 1955). In order to protect domestic poultry against ND, vaccination is practised worldwide using

inactivated as well as live vaccines containing lentogenic strains, such as Hitchner B1, La Sota and Clone-30.

Since several live vaccines currently available are associated with mild disease signs and weight loss, introduction of an improved live vaccine is greatly desired by the poultry industry. Development of improved attenuated vaccines is currently facilitated by the availability of powerful recombinant DNA technologies. These techniques were initially applicable only to DNA viruses and later to positive-strand RNA viruses. Very recently, however, the large group of negative-strand RNA viruses, to which NDV belongs, also became amenable to genetic engineering (reviewed by Palese *et al.*, 1996; Conzelmann, 1998).

NDV is a member of the genus *Rubulavirus* in the family *Paramyxoviridae* (Rima *et al.*, 1995) and contains a non-segmented single-stranded RNA genome of negative polarity. The NDV genome encodes six structural proteins, namely, the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin–neuraminidase (HN) and the RNA-dependent RNA polymerase (L). Apart from the six structural proteins, NDV was also shown to edit its P gene mRNA and to encode two additional gene products, de-

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signed V and W proteins (Steward *et al.*, 1993). As for all other members of the order *Mononegavirales*, the genomic RNA of NDV, together with the NP, P and L proteins, forms the ribonucleoprotein (RNP) complex, a structure that serves as template for the viral RNA polymerase. Therefore, in contrast to many DNA viruses and positive-strand RNA viruses, it is not possible to initiate an infectious cycle of negative-strand RNA viruses by simple transfection of cells with naked genomic nucleic acid. Only after intracellular reconstitution of an RNP complex entirely from cDNA is recovery of recombinant viruses possible. A system based on cotransfection of a plasmid expressing full-length antigenomic RNA together with three other plasmids encoding viral N, P, and L proteins under control of the phage T7 RNA polymerase promoter, which resulted in the recovery of recombinant viruses, was first developed for rabies virus (Schnell *et al.*, 1994), and subsequently for other members of the *Mononegavirales* (for a recent review see Conzelmann, 1998). Most of the recovery systems described to date utilize recombinant vaccinia virus as a source of bacteriophage T7 RNA polymerase (Schnell *et al.*, 1994; Collins *et al.*, 1995; Garcin *et al.*, 1995; Lawson *et al.*, 1995; Whelan *et al.*, 1995; Baron & Barrett, 1997; Durbin *et al.*, 1997; He *et al.*, 1997; Hoffmann & Banerjee, 1997). A modification of the initial protocol was introduced by replacing vaccinia virus with cell lines stably expressing T7 RNA polymerase. This approach was successfully applied to recover attenuated or slowly growing recombinant measles virus and bovine respiratory syncytial virus (Radecke *et al.*, 1995; Buchholz *et al.*, 1999), thus circumventing difficult and time-consuming purification steps of a newly generated recombinant virus.

Here, we report the cDNA cloning and sequencing of the entire NDV genome as well as the establishment of a system that allows the genetic manipulation of NDV. We show that the growth characteristics and pathogenicity of the recombinant NDV are indistinguishable from parental virus, confirming its authenticity. This recovery system, which is based on a vaccine strain of NDV, not only provides the possibility for experimental investigation of NDV molecular biology, but also serves as a basis for the development of improved marker/vector vaccines.

Methods

■ **cDNA synthesis and assembly of a full-length clone.** NDV strain Clone-30 (a vaccine strain derived from the lentogenic NDV strain La Sota, provided by Intervet International, Boxmeer, The Netherlands) was purified from 50 ml of allantoic fluid with a titre of 10^{10} egg-infectious doses (EID₅₀)/ml. After clarification of the allantoic fluid, NDV was sedimented by ultracentrifugation (1.5 h, 4 °C, 150 000 g), and viral RNA was isolated by guanidinium isothiocyanate extraction and subsequent centrifugation through a CsCl cushion (Sambrook *et al.*, 1989). cDNA to genomic RNA was generated by two specifically primed cDNA syntheses (first strand reaction, Time Saver cDNA synthesis kit, Pharmacia) with primers P1F (Clone-30 nt 1–21) and P5F (Clone-30 nt 8326–8346). A third first strand reaction was carried out with primer

P7F (Clone-30 nt 12736–12754) (Fig. 1) using SuperScript II RNase H⁻ Reverse Transcriptase (Gibco-BRL). Subsequently, the reaction mixes were incubated with 2 units RNase H (Biozym) for 20 min at 37 °C. After heat inactivation (5 min, 70 °C), the first strand cDNA was purified by phenol–chloroform extraction and ethanol precipitation. Each first strand cDNA was redissolved in a total of 20 µl H₂O. Subsequently, PCR was carried out on 1 µl of the first strand cDNA using the Expand High Fidelity (HF) PCR system (Boehringer Mannheim) with primer pair P2FMluI (5' TGTGAATC**ACGCGT**GCGAGCCCGA 3', Clone-30 nt 68–91, *MluI* site underlined, nts differing from the Clone-30 consensus sequence in bold) and P3R (5' GACAAGCG**GAAGAGCC**ATGCAA**ACTTGGCTGTG** 3', Clone-30 nt 8911–8890, synthetic adapter containing a *SapI* recognition site, underlined, and additional nucleotides in italics) to obtain fragment N1 (Clone-30 nt 1–8911) and primer pair P5F (Clone-30 nt 8326–8346) and P6R (Clone-30 nt 12919–12900) for the generation of fragment N2 (Clone-30 nt 8326–12919) (Fig. 1). Fragment N3 (Clone-30 nt 12736–15058) was generated by HF-PCR using primer pair P7F (Clone-30 nt 12736–12754) and P8RMluI (5' **GTATAATTA**AAATCA**ACGCGT**ATACAA 3', Beaudette C nt 15058–15033; *MluI* site underlined, nts differing from the Clone-30 consensus sequence in bold) from a cloned NDV fragment (nt 12736–15073) which had been obtained previously by RT-PCR. The terminal sequences of the genomic RNA were determined by polyadenylation of RNA and amplification of the 3' end, and amplification of the 5' end using the 5'-RACE protocol (Gibco BRL) as described previously (Mundt & Müller, 1995). Specific oligonucleotides were deduced to amplify PCR fragments which include leader and trailer, respectively (Fig. 1), with primers P4FT7 (5' CTGA**AGCTT**GTAA**TACGACTCA**TATAGGGACCAAACAGAGAATCCGTAAG 3', *HindIII* site underlined, T7 promoter and three additional G residues in bold, Clone-30 nt 1–21) and P2RMluI (5' TCGGGCTCGCA**ACGCGT**GATTACACCT 3', Clone-30 nt 91–65), and P8FMluI (5' TTGTAT**ACGCGT**TGATTTTATCATATTATG 3', Clone-30 nt 15033–15062) and P9R (5' TACTGCAGGG**GAAGACG**TACCAAACAAAGATTTGGTGAA 3', synthetic adapter, in italics, containing *PstI*/*BbsI* recognition sites underlined, Clone-30 nt 15186–15166) by HF-PCR, introducing two artificial *MluI* sites (underlined, nt 76, NP gene noncoding region, and nt 15039, L gene noncoding region) by mutation of five nucleotides (bold) (Clone-30 nt 76 T to A, nt 79 A to C, nt 15039 T to A, nt 15041 T to G and nt 15042 T to C). In a multi-step cloning procedure, a complete NDV antigenome expression plasmid (pflNDV-1) was assembled in the *SmaI* site of pX8δT (Schnell *et al.*, 1994) from cloned PCR fragments described above using naturally occurring restriction sites and the artificial *MluI* sites as shown in Fig. 1.

■ **Construction of expression plasmids.** For the construction of NP, P and L expression plasmids, the open reading frames of NP (nt 122–1791), P (nt 1887–3254) and L (nt 8381–15051) were cloned into pCite 2a (Novagen). For this purpose, HF-PCR fragments were generated with the respective translation start codon contained in an *NcoI* or *AflIII* adapter. These fragments were transferred into the expression plasmid pCite 2a in the correct open reading frame.

■ **Sequencing.** Initial PCR fragments of Clone-30 and the assembled full-length cDNA clone as well as support plasmids encoding NDV NP, P and L proteins were sequenced using an automated sequencer (LiCor, MWG Biotech). Sequences were analysed using Wisconsin package version 9.1 (Genetic Computer Group, Madison, WI, USA).

■ **Transfection and recovery of recombinant NDV.** Transfection experiments were done using BHK 21 cells, clone BSR T7/5, stably expressing the phage T7 RNA polymerase (Buchholz *et al.*, 1999). Cells grown to 80% confluency in 32 mm diameter dishes were transfected with a total amount of 10 µg DNA (5 µg pflNDV-1, 2 µg pCiteNP, 2 µg pCiteP and 1 µg pCiteL) (Superfect transfection kit, Qiagen). Supernatants

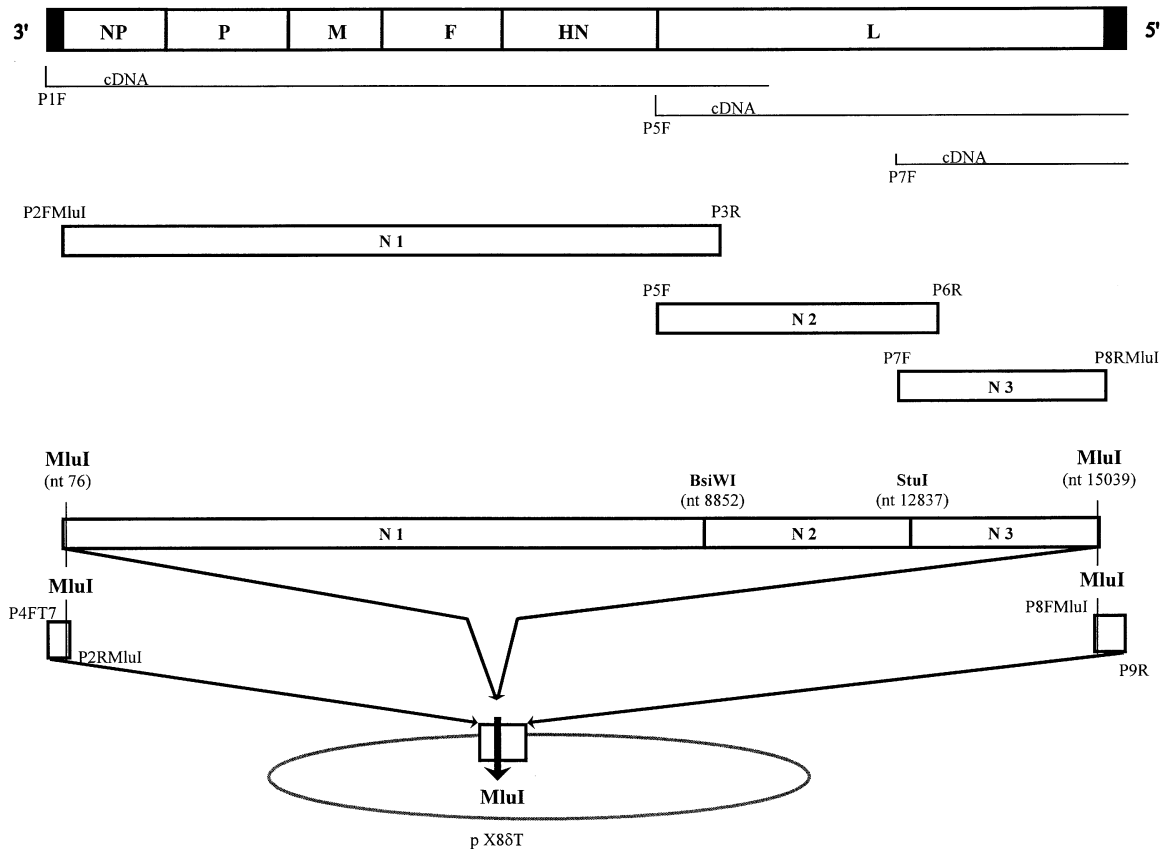


Fig. 1. Construction of full-length plasmid pINDV-1 expressing the Clone-30 antigenome (not drawn to scale). Schematic presentation of the NDV genome (top) and first strand cDNA (below). Oligonucleotide primers used for cDNA synthesis and PCR are indicated. Open boxes represent cloned RT-PCR products N1, N2 and N3, which were sequenced entirely and assembled using the cleavage sites indicated. These were transferred into plasmid X88T using artificial *MluI* sites created in the NP and L gene noncoding regions.

and cell monolayers were harvested at various times after transfection, cleared by centrifugation, and a volume of 200 μ l was inoculated into the allantoic cavity of 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs to amplify the recovered recombinant virus. The allantoic fluid was harvested 5 days after inoculation and tested for haemagglutinating activity (HA) (CEC, 1992).

■ **Indirect immunofluorescence assay (IFA).** Transfected cells were fixed with 3% paraformaldehyde at various times after transfection. To screen for the presence of infectious NDV, an IFA was performed, using monoclonal antibody HN-10 (Werner *et al.*, 1999) directed to NDV HN protein and FITC-conjugated goat anti-mouse IgG F(ab)₂ (DAKO).

■ **Virus growth.** QM7 cells (3.5×10^5 per 35 mm dish) were infected with wt Clone-30 or recombinant NDV at an m.o.i. of 1 and incubated at 37 °C in minimum essential medium supplemented with 10% foetal calf serum in a 5% CO₂ atmosphere. Supernatants and infected cells were frozen at the indicated times. Titrations were done in duplicate in microwell plates. Briefly, 100 μ l of tenfold serial virus dilutions were added to 10⁴ cells per well. After 48 h of incubation, the cells were fixed with 3% paraformaldehyde, and the 50% tissue culture infective dose (TCID₅₀) was determined by IFA using rabbit hyperimmune serum to NDV and FITC-conjugated swine anti-rabbit IgG F(ab)₂ (DAKO). To analyse virus growth in embryonated eggs, the allantoic cavity of 10-day-old embryonated SPF chicken eggs was inoculated with 7×10^4

TCID₅₀/0.2 ml recombinant and parental Clone-30 virus, respectively. Allantoic fluid was harvested and pooled at the indicated times. Titrations were done in duplicate on QM7 cells as described above.

■ **RT-PCR.** RNA from allantoic fluid was prepared using the Purescript RNA isolation kit (Biozym). RT-PCR was performed using primers P1 (Clone-30 nt 1–26) and P10R (Clone-30 nt 639–620) to amplify a fragment including the newly introduced *MluI* site in the noncoding region of the NP gene. Similarly, a fragment containing the *MluI* site in the noncoding region of the L gene was amplified using primers P10F (Clone-30 nt 14757–14775) and P9R (see above). The RT-PCR was essentially performed as described (Oberdörfer & Werner, 1998).

■ **Determination of the intracerebral pathogenicity index (ICPI).** The ICPI of the recombinant NDV as well as of the parental wild-type virus was determined following intracerebral inoculation of 1-day-old SPF chickens with 8.0 log₁₀ EID₅₀ virus per chicken. The appearance of clinical signs and mortality was scored for 8 days as described by Alexander (1989).

Results

Sequence determination of NDV Clone-30 and cDNA cloning

cDNA clones spanning the genomic sequence of NDV strain Clone-30 were generated from viral RNA by HF-PCR using a set of three NDV-specific primer pairs. The PCR

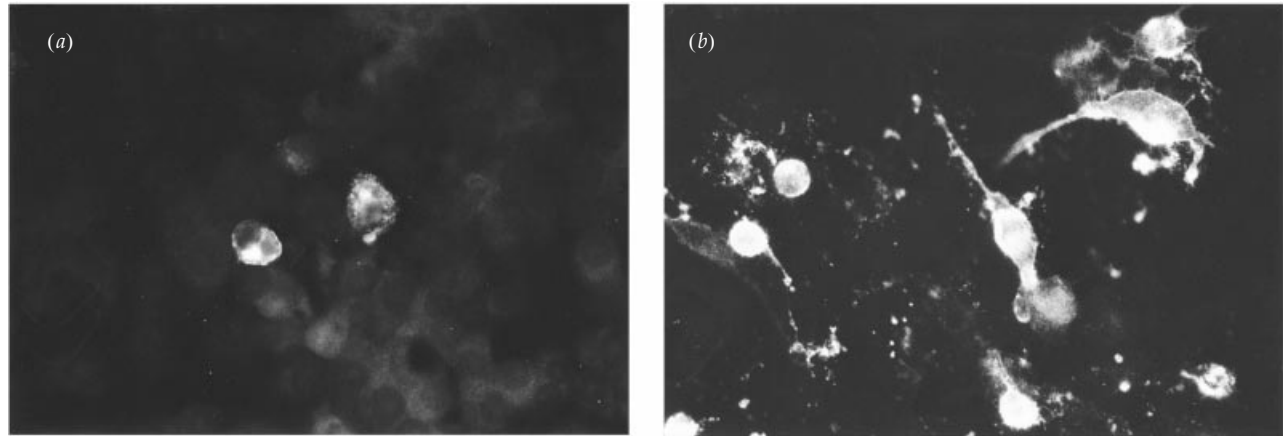


Fig. 4. Detection of recombinant NDV by indirect immunofluorescence using anti-HN monoclonal antibody HN-10. (a) BSR T7/5 cells 72 h after transfection with pflNDV-1 and NP, P and L expression plasmids. (b) QM7 cells 48 h after infection with 0.2 ml of a 10^{-1} dilution of HA-positive allantoic fluid, harvested 5 days after inoculation with BSR T7/5 culture supernatant obtained 72 h after transfection.

this region were similar to that of Clone-30, confirming a difference from the published sequences.

An NDV full-length plasmid allowing the expression of full-length NDV antigenome RNA was assembled from cDNA clones as shown in Fig. 1. This plasmid, termed pflNDV-1, was constructed in a way that a T7 promoter with three additional G residues is preceding the first nucleotide of the NDV leader region, and the last nucleotide of the trailer sequence is directly adjacent to an autocatalytic hepatitis delta virus ribozyme sequence. The NP, P and L expression plasmids contain an internal ribosomal entry site allowing cap-independent translation.

Recovery of infectious recombinant NDV from cDNA

For the generation of infectious NDV from cDNA, we used a vaccinia-virus-free system in which the T7 RNA polymerase was expressed in a cell line (BHK-21, clone BSR T7/5) stably transfected with the phage T7 RNA polymerase gene (Buchholz *et al.*, 1999). Dishes of subconfluent cells were transfected with plasmids pflNDV-1, pCiteNP, pCiteP and pCiteL, to achieve simultaneous intracytoplasmic expression of RNP proteins and full-length antigenome RNA. To test whether the process led to intracellular reconstitution of RNPs and initiation of an infectious cycle, transfected cells were first examined for expression of HN protein. Forty-eight hours post-transfection, very few single cells exhibited weak fluorescence, whereas at 72 h post-transfection few but clearly stained cells were detected (Fig. 4a). No positive signal was observed in untransfected controls. Since most host cells are not permissive for lentogenic NDV in cell-to-cell spread (Rott & Klenk, 1988) because they lack a trypsin-like protease necessary for cleavage of the F₀ protein, 200 μ l of supernatants that were collected 24, 48 and 72 h after transfection were

inoculated into the allantoic cavity of 10-day-old embryonated SPF chicken eggs. The allantoic fluid was harvested 5 days after inoculation and tested for HA. No HA was found in allantoic fluid from eggs inoculated with material harvested 24 and 48 h post-transfection, whereas after inoculation of supernatant harvested 72 h post-transfection we reproducibly found eggs positive with HA titres ranging from 2 to 2048. IFA was performed 48 h after inoculation of 200 μ l of a 10^{-1} dilution of HA-positive allantoic fluid into QM7 cells using monoclonal antibody HN-10 (Werner *et al.*, 1999), yielding a strong immunofluorescence staining of single cells, which is typical for lentogenic NDV (Fig. 4b), thus proving the recovery of NDV entirely from cDNA.

*Mlu*I digestion of RT-PCR fragments confirmed that progeny virus was recombinant

The genome of the newly generated virus contains two artificially introduced *Mlu*I sites serving as genetic markers to identify recombinant NDV. In order to verify the presence of these genetic tags, RNA was prepared from the HA-positive allantoic fluids and subjected to RT-PCR. DNA fragments encompassing the region containing the artificial *Mlu*I sites in the noncoding region of NP and L genes of recombinant Clone-30 NDV were identical in size to the corresponding PCR products obtained from parental Clone-30 virus RNA (Fig. 5, lanes 5–8). Digestion of the RT-PCR products from recombinant NDV Clone-30 (rNDV) with *Mlu*I yielded fragments of 560 and 79 bp (Fig. 5, lane 10) and 282 and 164 bp (147 bp virus-specific, plus 17 bp primer-derived) (Fig. 5, lane 12), whereas the RT-PCR products of parental Clone-30 virus remained uncleaved (Fig. 5, lanes 9, 11). No PCR products were obtained if the reverse transcriptase was omitted (Fig. 5, lanes 1–4).

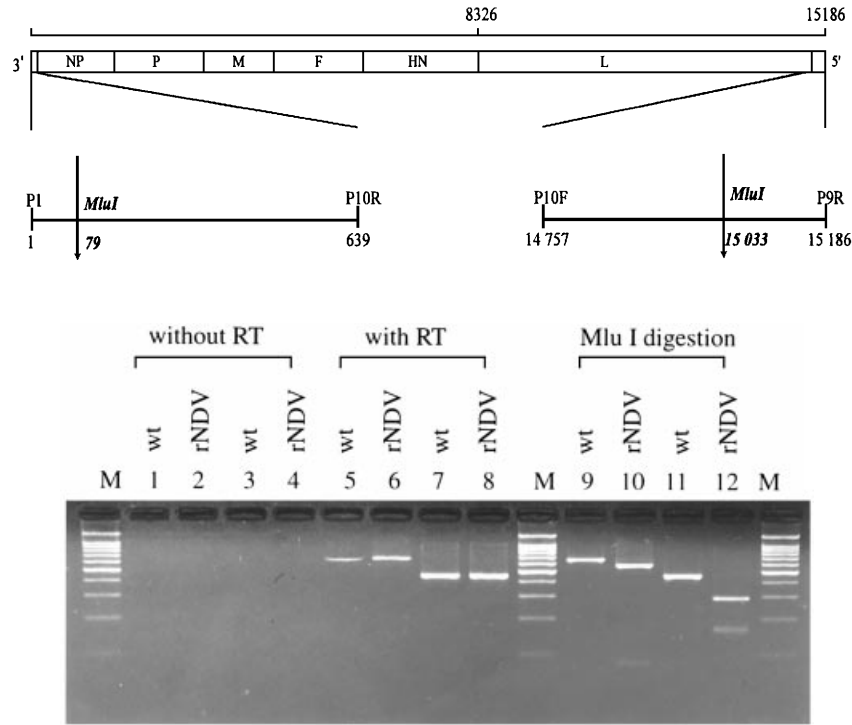


Fig. 5. RT-PCR of NDV RNA. RT-PCR products of RNA derived from standard (wt) or recombinant Clone-30 NDV (rNDV). PCR was performed using primers P1/P10R (lanes 1, 2, 5, 6, 9, 10) and P10F/P9R (lanes 3, 4, 7, 8, 11, 12). Products were separated in an agarose gel either undigested (lanes 4–8) or after digestion with *MluI* (lanes 9–12). M, 100 bp DNA ladder (Promega).

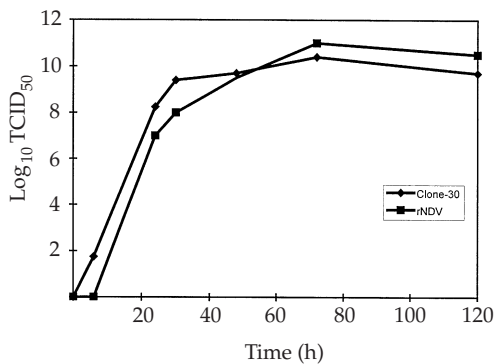


Fig. 6. Growth of NDV Clone-30 and rNDV. Growth curves of parental NDV Clone-30 and rNDV after inoculation into embryonated SPF chicken eggs.

Recombinant NDV is indistinguishable in its biological properties from parental Clone-30

The biological properties of the newly generated rNDV were compared to parental Clone-30 by determination of growth characteristics in tissue culture and in embryonated chicken eggs. These results showed that neither recombinant nor wild-type NDV Clone-30 can be propagated well in tissue culture (data not shown). In embryonated chicken eggs, in contrast, both viruses propagated efficiently and yielded similar

titres of 10^{11} /ml for rNDV and $10^{10.4}$ /ml for parental virus (Fig. 6).

To compare the pathogenicity of rNDV and of parental virus, the ICPI of both viruses was determined by scoring of clinical signs and mortality as described by Alexander (1989). Identical values of 0.29 out of a maximum possible value of 2 were obtained for both viruses, demonstrating that the rNDV is a faithful copy of the parental Clone-30.

Discussion

A system allowing the recovery of lentogenic NDV entirely from cDNA was established. Recombinant NDV, derived from the vaccine strain Clone-30, was generated after transfection of cells stably expressing phage T7 RNA polymerase with plasmids allowing simultaneous intracytoplasmic expression of antigenomic NDV RNA, and NP, P and L proteins. The recombinant NDV, which showed biological features indistinguishable from the parental virus, possesses two genetic tags that were introduced into the cDNA copy.

Recovery systems for recombinant negative-strand RNA viruses are based on intracytoplasmic reconstitution of the RNP complex, which represents the template for the viral polymerase, and is the prerequisite needed to start an infectious cycle (reviewed by Palese *et al.*, 1996; Conzelmann, 1998). The expression systems most widely used depend on infection of

cells with recombinant vaccinia virus (vTF-7-3 or MVA) (reviewed by Palese *et al.*, 1996; Conzelmann, 1998), providing T7 RNA polymerase needed for expression of RNA and proteins from transfected plasmids. However, this entails several problems: (i) the cytopathic effect caused by vaccinia virus interferes with recovery of slow growing viruses; (ii) newly recovered virus has to be separated from vaccinia virus by filtration (Schnell *et al.*, 1994), by passage on cells that are not permissive for the vaccinia virus strain (Collins *et al.*, 1995) or by vaccinia virus inhibitors (Whelan *et al.*, 1995); (iii) vaccinia virus may cause RNA recombination during recovery (Garcin *et al.*, 1995). For the successful generation of recombinant negative-strand RNA viruses of slow-growing species like measles virus (Radecke *et al.*, 1995) and bovine respiratory syncytial virus (BRSV) (Buchholz *et al.*, 1999), a vaccinia-virus-free recovery system is crucial. We therefore used a cell line stably expressing T7 RNA polymerase, which has proven to be suited for recovery of attenuated recombinant BRSV mutants (Buchholz *et al.*, 1999).

Lentogenic NDV strains possess an amino acid motif at the cleavage site of the precursor glycoprotein F₀ which requires cleavage by a trypsin-like host cell protease to be transformed into the fusogenic active form. The cell line BSR T7/5 used for recovery is derived from BHK-21 cells, which do not support efficient propagation of lentogenic NDV. Like its biologically derived parent, recombinant Clone-30 cannot be propagated to high titres in this and other cell lines because virions containing uncleaved F glycoprotein lack full infection capacity (Rott & Klenk, 1988). Plasmid-derived intracellular reconstitution of Clone-30 RNPs led to the onset of a replicative cycle in BSR T7/5 cells, with few infected cells that could be demonstrated in IFA. Clarified supernatants of transfected cells were inoculated into the allantoic cavity of embryonated chicken eggs, possessing proteases of trypsin-like specificity, which reproducibly led to amplification of recombinant Clone-30. Thus, a system for recovery of recombinant lentogenic NDV was established that combines a cellular T7 expression system and a virus propagation step allowing amplification of few primary infectious recombinant virions originating from single recovery events.

Genome analogues of members of the paramyxovirus genus replicate efficiently only if the total genome length is a multiple of six, a requirement which is called the 'rule of six' (Calain & Roux, 1993; reviewed by Kolakofsky *et al.*, 1998). All NDV genomes determined to date (Krishnamurthy & Samal, 1998; de Leeuw & Peeters, 1999; Phillips *et al.*, 1998) comprise 15186 nts, which is a multiple of six. This feature strongly suggests that the paramyxovirus 'rule of six' also applies to NDV. Recombinant NDV could reproducibly be recovered using the approach described above, though the recovery events, as determined by IFA, were more than tenfold lower as compared to recovery events of recombinant BRSV when using the identical system (U. Buchholz, unpublished results). Like other negative-strand RNA virus rescue systems, full-

length NDV and BRSV antigenome expression plasmids contain three additional G residues adjacent to the T7 promoter. These three G residues were shown to enhance the T7 polymerase-driven transcription (Pattnaik *et al.*, 1992). BRSV is a member of the pneumovirus genus, and thus does not obey the 'rule of six'. One reason for the fewer recovery events observed in transfected dishes might be the need for trimming the extra G residues from the 5' antigenome end of NDV genome, so that the genome length remains a multiple of six. Trimming of extra G residues from the end of the genome is well documented for vesicular stomatitis virus, a rhabdovirus, although genome length in this case does not follow the 'rule of six' (Pattnaik *et al.*, 1992). In the case of Sendai virus, it was reported that minigenomes containing the P editing site were constructed that did not obey the rule of six. After passage, they contained corrective nucleotide insertions or deletions in the P editing site (Kolakofsky *et al.*, 1998). Therefore, RT-PCR of the P editing site was done from egg-passaged recombinant NDV; subsequent sequencing revealed that the P editing site was unaltered (not shown). Thus, antigenomic RNAs or RNPs that should undergo a trimming process might give rise to lower rescue efficiency compared to RNAs that might be encapsidated and maintained as biologically active RNPs even with extra G residues.

The complete sequence of Clone-30 was highly similar to the sequence of La Sota (de Leeuw & Peeters, 1999), with the exception of the L gene which contains a double frameshift resulting in a 28 aa difference located in block V (Poch *et al.*, 1990). Block V represents a cluster of highest amino acid conservation among the members of the *Mononegavirales*, which includes one stretch of five highly conserved amino acids (Poch *et al.*, 1990). In contrast to the published NDV sequences of La Sota (de Leeuw & Peeters, 1999) and Beaudette C (Krishnamurthy & Samal, 1998), the sequences determined for Clone-30, for our La Sota isolate, and for strain Komarov contain the conserved block V motif common to members of *Mononegavirales* (Poch *et al.*, 1990; Fig. 3). Moreover, recombinant Clone-30 containing the determined sequence was indistinguishable from its biologically derived parent, and similar in its properties to NDV strain La Sota.

The *in vitro* and *in vivo* characterization of recombinant NDV revealed a phenotype identical to parental virus, thus proving that the mutations artificially introduced had no biological effect, and moreover, that the recombinant system now available for NDV yields a faithful copy of the biologically derived virus.

By using the NDV recombinant system, defined mutants can be designed enabling the experimental investigation of virus-host interactions and of the molecular basis of NDV pathogenesis. Characterization of recombinant NDVs with altered F protein cleavage sites will be helpful to elucidate the molecular basis of pathogenicity. Determination of the function of individual NDV proteins as well as of additional gene products, particularly V and W proteins that are synthesized as

a result of P gene mRNA editing, will certainly be facilitated by the availability of this system. The evidence showing a correlation between Sendai virus pathogenicity and V protein expression (Kato *et al.*, 1997) makes investigation in this area of NDV research appealing. Apart from giving insight into NDV pathogenesis, the above approaches will be useful for the design of safe and effective vaccines against this devastating poultry disease. Moreover, as shown for other negative-strand RNA viruses, it will be possible to use lentogenic NDV as a vaccine vector by expressing additional proteins to confer protection against other respiratory and intestinal pathogens of poultry.

Note added during revision. We acknowledge the successful recovery of another strain of NDV (Peeters *et al.*, 1999) which was reported after submission of this article.

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