Arch Virol (2007) 152: 1241–1249
DOI 10.1007/s00705-007-0961-x
Printed in The Netherlands

Archives of Virology

Generation of a velogenic Newcastle disease virus from cDNA and expression of the green fluorescent protein

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Received October 19, 2006; accepted February 19, 2007; published online March 29, 2007

Summary

Newcastle disease virus (NDV) is a pathogen that is important in the poultry industry worldwide. Specifically, the virulent (velogenic) NDV is a particular threat because it has now occurred frequently worldwide. The outbreaks caused by highly virulent NDV in waterfowl and especially in goose flocks, have led to greater concern in recent years as aquatic birds were previously resistant to most virulent NDV strains from chickens. The molecular determinants of host tropism, virulence and emergence of NDV isolated from diseased goose flocks are poorly understood. In the present study, we rescued a highly virulent NDV isolated from a goose using the reverse genetics approach. Infectious virus was successfully generated by cotransfection of a full-length cDNA clone of the NDV strain ZJ1 with helper plasmids. The recombinant NDV was indistinguishable from the parental wild-type virus with respect to its growth kinetics in cell culture as well as its biological properties. A recombinant NDV expressing green fluorescent protein (GFP) was generated, and GFP was subsequently detected in cells and various organs from the infected chickens.

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Introduction

Newcastle disease (ND) is a highly contagious disease of many avian species that has caused substantial losses in the poultry industry around the world [1–3]. The causative agent is Newcastle disease virus (NDV), which has been classified as a member of the newly defined genus *Avulavirus* in the family *Paramyxoviridae* according to the ICTV report [8, 14]. The results of standard pathotyping assays, which utilize inoculation of embryonated chicken eggs and live chickens, are the basis for classifying NDV as velogenic (highly virulent), mesogenic (moderately virulent), or lentogenic (low virulent) [2].

NDV has a negative-sense, single-stranded RNA genome of ∼15 kb that contains six genes ordered 3′-NP-P-M-F-HN-L-5′, which encode the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the RNA-dependent RNA polymer-

ase (L) [15]. Additionally, proteins (designated V and W) may be produced by an RNA-editing event that occurs during transcription of the P gene [23].

The genomic RNA of NDV, together with the NP, P and L proteins, forms the ribonucleoprotein (RNP) complex, a structure that serves as a template for the viral RNA polymerase. Therefore, recovery of recombinant viruses is possible only after intracellular reconstitution of an RNP complex. Different research groups have reported generation of infectious NDV strains, such as LaSota, Clone30, Beaudette C, Hitchner B1 and Herts/33, based on cotransfection of a plasmid containing a full-length cDNA clone together with three helper plasmids encoding NP, P and L proteins using reverse genetic manipulation [6, 12, 16, 18, 21].

Although effective live or inactivated ND vaccines are currently available, NDV remains an ongoing threat to commercial flocks. Aquatic birds are usually resistant to even the most virulent NDV strains that affect chickens. However, outbreaks caused by NDV in waterfowl have been reported in various regions of South and East China since the late 1990s [13, 20]. Spread of viruses in goose flocks of China has raised concern about their emergence, characteristics and evolution. A number of virulent NDV strains have been isolated from goose flocks by our laboratory and some of them have been characterized both pathotypically and genotypically [13]. The results revealed that most of the isolates are velogenic and belong to the genotype VII [13]. Additionally, we reported the entire genome of NDV strain ZJ1 isolated from an outbreak in geese [9]. In the present study, we constructed a genomic cDNA clone of NDV ZJ1. Infectious highly virulent goose NDV was successfully generated for the first time by cotransfection of the full-length cDNA clone and helper plasmids derived from NDV LaSota into BSR-T7/5 cells. The rescued virus was indistinguishable from the wild-type ZJ1 with respect to virulence and growth characteristics in cell culture. The recombinant virus contains a nucleotide sequence marker as a result of the cloning procedure which makes it distinguishable from the wild-type virus. A GFP-expressing recombinant NDV was additionally generated, and the expression of GFP was detectable in cells and organs of infected chickens. The established reverse genetics system provides a powerful tool for the analysis of NDV genomic functions and the development of novel NDV vaccine candidates.

Materials and methods

Viruses and cells

NDV strain ZJ1 was isolated from an outbreak in geese by our laboratory and identified as velogenic (ICPI: 1.89) [13]. The virus was plaque purified on chicken embryo fibroblast (CEF) cells by three rounds and was grown in 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs (Nanjing Bioproducts Firm, China). BHK-21 cells, clone BSR T7/5, stably expressing the phage T7 RNA polymerase, developed by Buchholz et al. [5], was a gift from Dr. Zhigao Bu (Harbin Veterinary Institute, China). The cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS) and 1 mg/ml G418, as previously reported [5].

Assembly of the full-length NDV cDNA and recombinant plasmid containing the GFP gene

100 µl of the purified virus was used for RNA preparation. Viral RNA was extracted with Trizol LS Reagent (Gibco). The extracted RNA was subjected to reverse transcription (RT)-PCR using primers (Table 1) and the Expand High Fidelity (HF) PCR System (Roche, Germany) to generate fragments for construction of genomic cDNA clone (Fig. 1a). Six restriction enzymes sites (*Pin*AI, *Spe*I, *Mun*I, *Mlu*I, *Bsm*BI and *Bbs*I) within the genome sequence were chosen for construction of the full-length genomic cDNA of ZJ1. The *Spe*I site was introduced into the primer FLPF3 (Fig. 1a) to facilitate engineering of the NDV genome and to provide a genetic tag. In a multiple cloning procedure, full-length NDV cDNA, named TVT-FLNDV-ZJ1, was assembled in the plasmid TVT7R (0, 0) (a generous gift from Andrew Ball [11]) by using available restriction sites according to the strategy shown in Fig. 1a.

In order to insert three nonviral G nucleotides (nt) in the position between the T7 promoter and the virus genome sequence, TVT-FLNDV-ZJ1 was digested with *Nde*I, and the 6.5-kb fragment containing the TVT7R (0, 0) vector was recovered and self-ligated for mutagenesis. The mutagenesis was performed using the "quick change site-directed mutagenesis kit" (Stratagene) with primers MPT7YF (5′-CGA AATTAATACGACTCACTATA***GGG***ACCAAACAGAGAA TCTG-3′) and MPT7YR (5′-CAGATTCTCTGTTTGGT ***CCC***TATAGTGAGTCGTATTAATTTCG-3′). After that, the 6.5-kb fragment was reinserted, and the resulting full-length clone containing the three G nts was named NDV3GM122.

The GFP gene was obtained by PCR amplification from plasmid pEGFP (Clontech) using primers GFP1 (5′-ct**gggccc**t*ctagaaaaaatacgggtagaag*taccATGGTGAGCAAG

Table 1. Primers used in this study

Primer	Nucleotide sequence	Orientation	Position and enzymes
FLPF1	CGTCTCGTATAACCAAACAGAGAATCTGTGAGG	sense	1–22; *Bsm*BI
FLPR1	TTTACTAGTCCTTCTCCACTCCCATGTCAG	antisense	2951–2929; *Spe*I
FLPF2	GTTTGGTTGTGCTAACGTTTCATCCT	sense	2744–2766
FLPR2	GGAACTAGTGGCTCTCATCTGATCGAG	antisense	6202–6180; *Spe*I
FLPF3	CGGACTAGTAGAGCATGAATGCAGATAAGAGG	sense	6197–6225; *Spe*I
FLPR3	AAAACGCGTCATCTGATCTTACCTCTCTCGTT	antisense	10680–10657; *Mlu*I
FLPF4	CAAGTGACCCGACTGACTGTGATCTAT	sense	10461–10487
FLPR4	AGACAGATGGAAACAGCAGATCGTACT	antisense	13106–13080
FLPF5	GACACTAGTGGTCACACCAGCTTGCAGAT	sense	12981–13000; *Spe*I
FLPR5	GTAAGTGGTTTAGAGACAAACCACCCATCTCTGC	antisense	15170–15192; *Bsm*BI
NPF	CGGAATTCGAGCGCGAGGCCGAAGCTCGAA	sense	78–99; *Eco*RI
NPR	ATGTCGACCTGGGTGTTGTCGATCAGTAC	antisense	1585–1605; *Sal*I
PF	CCGCTCGAGGTGGATTAGGGTGAAGATGGCCACT	sense	1877–1901; *Xho*I
PR	GCCGTCGACAGTGACGGGAGCCTGTTATGAGT	antisense	3092–3113; *Sal*I
LF1	GGG TCTAGAAACCAACAAGAGGGAACAC	sense	8359–8377; *Xba*I
LR2	AA GTCGACGGTGACCAGCTTCTGTTCC	antisense	11766–11748 nt; *Sal*I
LF3	GCTCGCTGACGCTAGCAGATTATG	sense	11721–11744; *Nhe*I
LR4	AAAGTCGACAGTCATCAGTTAGGAAG	antisense	15046–15027; *Sal*I

The primers are listed 5′ to 3′. Locations of the primers are according to the released sequence of NDV strain ZJ1 (GenBank accession number: AF431744).

GGCGAGGAG-3′) and GFP2 (5′-tt**gggccc**tcTTACTTGTA CAGCTCGTCCATGCCGAGAGTGATCCC-3′) and cloned into pCR2.1 vector (Invitrogen, USA), resulting in plasmid pCRGFP. The *Apa*I site (show in bold) and NDV gene-start (GS) and gene-end (GE) signals (show in italics) were introduced. The fragment spanning position 2849–5223 of the ZJ1 genome was amplified using primers P3 (5′-GGG TGAAATGACGCTCAATAAACTCTC-3′) and P4 (5′-ATG GTCTCATCTGTGGCCCGAATACT-3′), cloned into the pCR2.1 vector and subsequently cloned into the plasmid pUC18 vector using *Hin*dIII and *Xba*I restriction enzymes, resulting in plasmid pUC*Age*I/*Psi*I. The *Apa*I site, which was located at position 3134 of the ZJ1 genome was utilized to insert the GFP gene. Accordingly, the *Age*I/*Psi*I fragment of TVT-FLNDV-ZJ1 was substituted by the *Age*I/*Psi*I fragment of pUC*Age*I/*Psi*I. The new full-length plasmid was designated pNDV/ZJ1GFP (Fig. 1b).

Recovery of recombinant NDV

NDV3GM122 and the helper plasmids, pCIneoNP, pCIneoP and pCIneoL, which were derived from NDV LaSota (provided by Ben Peeters [18]), in the amount of 1 μg each were cotransfected into BSR-T7/5 cells, which were cultured in 35-mm diameter dishes with DMEM-10% FCS for one day. Transfection was performed using a Superfect transfection kit according to the manufacturer's instruction (QIAGEN, Germany). The pCI-L plasmid was replaced with pCIneo in one cotransfection which served as the negative control. Three to five days later, the culture supernatants and cell monolayers were harvested and cleared by low-speed centrifugation, and a volume of 200 μl was used to inoculate the allantoic cavities of 10-day-old embryonated SPF chicken eggs to amplify the recovered virus LaZJ1R.

To generate GFP-expressing recombinant NDV, cotransfection was performed using pNDV/ZJ1GFP and helper plasmids derived from LaSota by the same method described above. Transfection supernatant was collected 3–5 days post-transfection and cleared by centrifugation, and a volume of 200 μl was used to inoculate the allantoic cavities of 10-day-old embryonated SPF chicken eggs to enable efficient virus propagation. Four successive passages in embryonated SPF chicken eggs were performed with 100 μl (10^4 $TCID_{50}$/ml) of the new recovered virus LaZJ1RGFP to confirm the genetic stability of the reporter gene.

Hemagglutination (HA), Hemagglutination inhibition (HI) and RT-PCR

The HA test was performed in a 96-well microtiter plate to test the HA titer of the allantoic fluids that were collected from the inoculated chicken eggs. For HA positive allantoic fluids, the HI titers were then determined using McAb 6B1, which is specific for the NDV HN protein. RNA from HA- and HI-positive allantoic fluid was extracted using a viral RNA isolation kit (Shanghai Sangon product, China). HF RT-PCR using primers YLSPEF (5′-TTTAGCTGGTGG CAATATGGAT-3′) and YLSPER (5′-GCAGTGTCACTGG GTGAATTGGGTT-3′) was then performed to amplify a ~2.1 kb fragment containing the introduced *Spe*I site in posi-

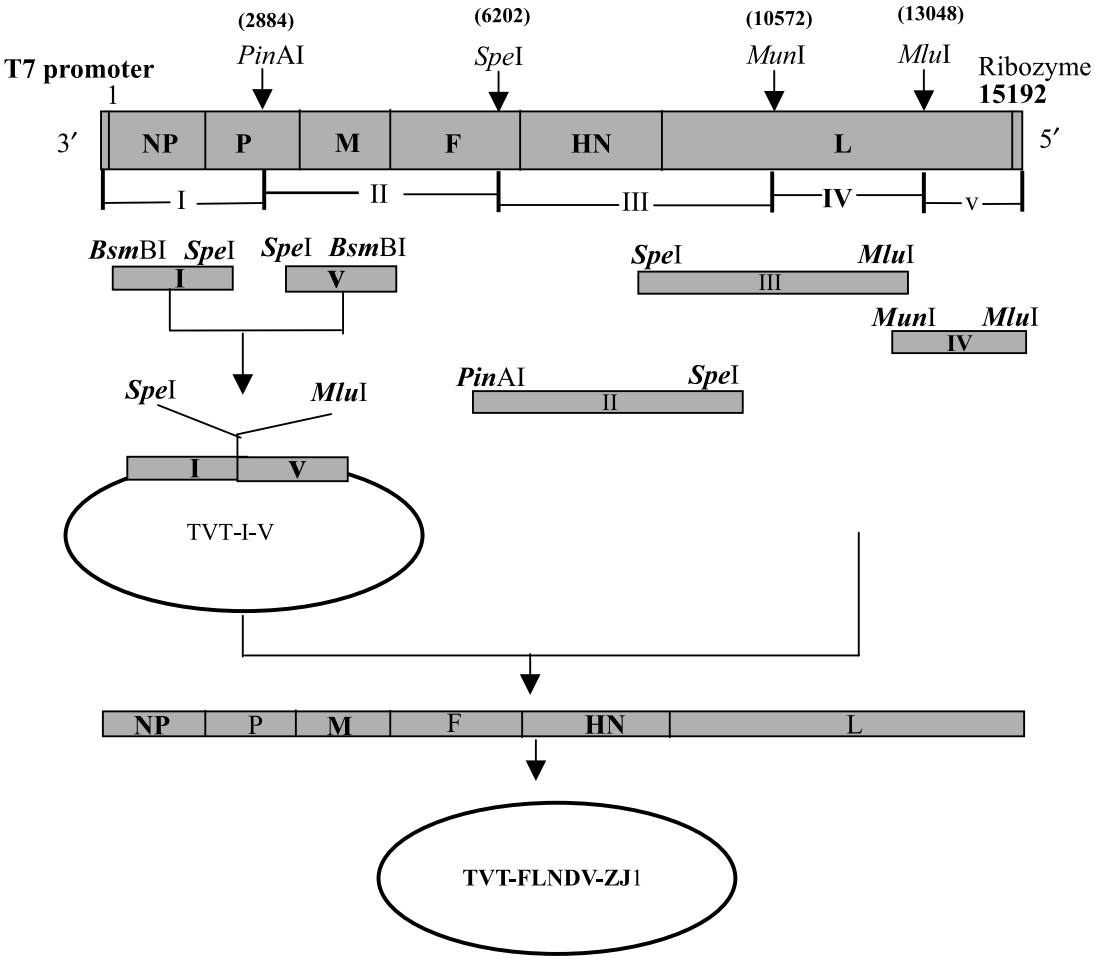


Fig. 1a. Construction strategy of the full-length plasmid TVT-FLNDV-ZJ1 expressing the ZJ1 antigenome (not drawn to scale). Schematic representation of the NDV genome and the restriction sites used for assembling the full-length cDNA clone. Black boxes represent cloned RT-PCR products, which were joined at the shared restriction sites and assembled in the transcription plasmid TVT7R (0, 0) by inserting the I and V cDNA fragments and then three others in the order III, IV and II. *Bsm*BI was used to clone I–V ligated fragment into the TVT7R (0, 0) vector. The unique *Mun*I site (not shown), which is close to the *Mlu*I site in fragment III, was used to insert fragment IV into recombinant TVT-I-V-III. The numbers above the restriction sites indicate the position of the respective restriction enzymes in the NDV genome. The bottom panel shows the final composition of the full-length cDNA of NDV ZJ1

tion 6202 of the full-length cDNA. The purified PCR product was sequenced directly in both directions by using the same sequencing technology mentioned above. The PCR products derived from wild-type ZJ1 and LaZJ1R were each digested with *Spe*I to detect the different pattern of digestion for differentiation.

Characterization of the recovered LaZJ1R virus

The pathogenicity of the recovered virus as well as the parental wild-type virus ZJ1 were determined using three *in vivo* tests of mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) according to the standard procedures [2]. The growth characteristics of the parental and recombinant viruses were assessed by a multistep growth assay on HEp-2 cells. HEp-2 cells were infected at a multiplicity of infection (MOI) of 0.01 and incubated at 37 °C in DMEM-10% FCS. Supernatants were harvested at eight-hour intervals and titrated by plaque assay in CEF cells. Each assay was repeated at least once, and results were reported as average from all assays.

Infection of cells and animal experiment with LaZJ1RGFP

CEF and BSR-T7/5 cells were infected with LaZJ1GFP virus at an MOI of 0.01, respectively. The cell without infection was used as the negative control. Twenty-four hours

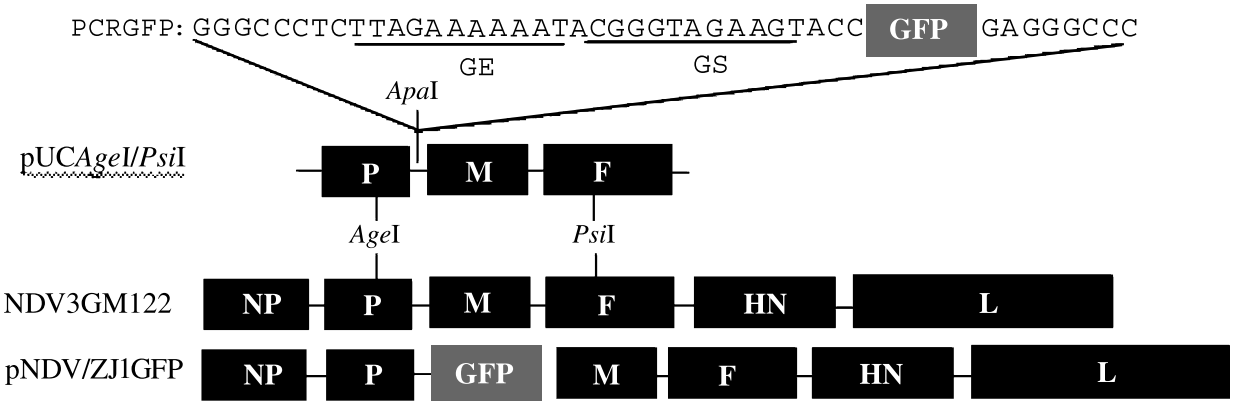


Fig. 1b. Construction of pNDV/ZJ1GFP. The GFP gene containing an *Apa*I site was amplified by PCR and cloned into the pCR2.1 vector. The fragment spanning position 2849–5223 of the ZJ1 genome was amplified and cloned into the pUC18 vector, resulting recombinant pUC*Age*I/*Psi*I. The GFP gene was then inserted into pUC*Age*I/*Psi*I using *Apa*I, and the resulting recombinant containing the GFP gene was excised with *Age*I and *Psi*I and used to replace the corresponding fragment in NDV3GM122

post-infection (p.i.), infected cells were examined using a reverse fluorescence microscope to detect the expression of GFP. Six 10-day-old SPF chickens were inoculated by the eye-drop and intranasal routes with the collected allantoic fluid of LaZJ1RGFP (10^4 $TCID_{50}$/ml virus solution, 100 µl/animal). In this experiment, three chickens inoculated with PBS served as infection control. All animals were monitored daily for clinical signs. Organ samples (brain, lung, trachea, cloaca, liver and kidney) were taken 4 days p.i., and GFP autofluorescence was visualized under a reverse fluorescence microscope.

Results

Construction of full-length cDNA of ZJ1 and recombinant plasmid containing the GFP gene

A recombinant TVT-FLNDV-ZJ1 virus from the full-length genomic cDNA of ZJ1 was obtained using the strategy shown in Fig. 1a. To increase the rescue efficiency of infectious virus from cDNA [12, 18, 21], three additional G's behind the T7 promoter were introduced, and the plasmid NDV3GM122, containing the full-length genome, was obtained. A second plasmid containing the full-length genome was constructed which also encoded the open reading frame of GFP. The insertion was done between the NDV genes P and M, resulting in the plasmid pNDV/ZJ1GFP. The genome sequences of both plasmids, pNDV3GM122 and pNDV/ZJ1GFP, follow the rule of six [19].

Generation of infectious recombinant NDV from full-length cDNA

Supernatants of transfections using the full-length clones and the helper plasmids from LaSota were inoculated into eggs, which resulted in HA-positive allantoic fluids, indicating that both newly generated NDV recombinants LaZJ1R and LaZJ1RGFP were replication-competent in embryonated chicken eggs. Bright fluorescence was detected in LaZJ1RGFP-infected CEF and BSR-T7/5 cells, demonstrating the expression of GFP (Fig. 3).

The recombinant virus LaZJ1R was serially passaged up to four times in embryonated SPF chicken eggs. The hemagglutination of the rescued virus was specifically inhibited by McAb 6B1 in the HI test in each round of passage. In order to ensure unambiguously that the recovered virus LaZJ1R was derived from cotransfection rather than wild-type NDV contamination, we introduced a *Spe*I restriction site into the full-length cDNA. This served as a genetic tag for identification of the recovered virus. RT-PCR was performed to amplify the fragment encompassing the *Spe*I restriction site. As expected, the sequencing result showed the presence of the introduced *Spe*I site. Digestion of the RT-PCR product with *Spe*I confirmed the presence of the *Spe*I site in the amplified fragment of the rescued virus in contrast to the RT-PCR product of the wild-type virus, which was not digested (data

not shown). These results indicated the successful recovery of infectious NDV entirely from cDNA.

Biological characterization of the generated virus

The standard pathogenicity tests were conducted to determine the virulence of the recovered LaZJ1R and wild-type ZJ1 in terms of MDT, ICPI and IVPI. In the ICPI test, the appearance of clinical signs and mortality was scored for 8 days, as described by Alexander [2]. The results listed in Table 2 show that the ICPI scores of LaZJ1R and ZJ1 were 1.88 and 1.89, respectively (out of a maximum possible value of 2.0). Thus, both are velogenic viruses, in contrast to vaccine strains such as LaSota and Clone30, which exhibit ICPI values of 0–0.3 [3]. The virulence of ZJ1 and LaZJ1R was further determined by MDT in 10-day-old embryonated SPF chicken eggs. The values for MDT of LaZJ1R and ZJ1 were 54 and 52 hours, respectively (Table 2), and both were therefore categorized as velogenic strains. The IVPI values for the two viruses were 2.70 and 2.80, respectively (Table 2). Taken together, the results of the pathogenicity tests show that the recovered virus possessed characteristics similar to those of the parent NDV ZJ1.

To compare the growth of LaZJ1R with that of ZJ1, we analyzed the kinetics and final virus yield under multistep growth conditions on HEp-2 cells. HEp-2 cells were infected with either LaZJ1R or ZJ1 at an MOI of 0.01. Samples were harvested at eight-hour intervals to assess the virus titers by plaque assay on CEF cells. The results revealed that the kinetics and magnitude of replication for LaZJ1R were very similar to those of ZJ1 (Fig. 2). These results demonstrated that LaZJ1R retains biological properties and growth characteristics similar to those of the parental wild-type ZJ1.

Table 2. Virulence of the parental wild-type and rescued viruses

Virus	MDT	ICPI	IVPI
ZJ1	54	1.89	2.70
LaZJ1R	52	1.88	2.80

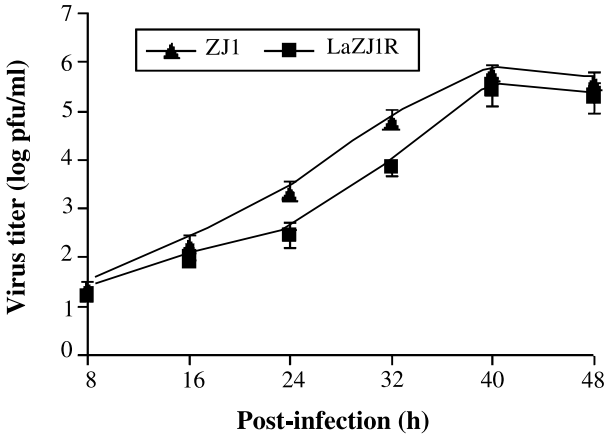
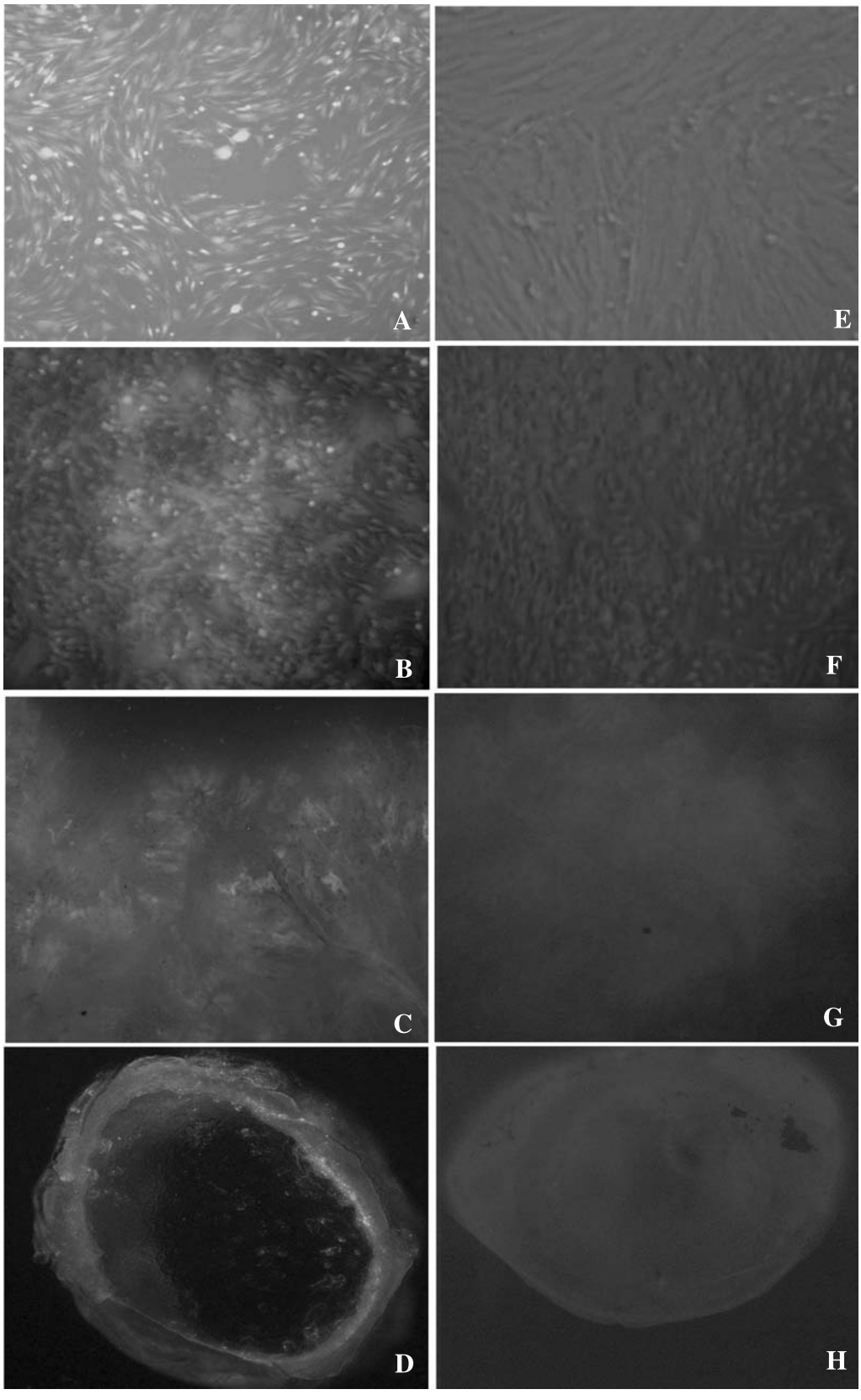


Fig. 2. Multistep growth curve of ZJ1 and LaZJ1R in HEp-2 cells. Cells were infected with the indicated NDV ZJ1 and the generated virus LaZJ1R at an MOI of 0.01. Samples were taken at 8-h intervals for 48 hours, and virus titers were determined by plaque assay in CEF cells. Values are averages from the results from independent experiments

Dissemination of GFP-expressing recombinant NDV in vivo

To determine the dissemination of the recombinant NDV *in vivo*, 100 µl of LaZJ1RGFP (10^4TCID$_{50}$/ml) was delivered by the eye-drop and intranasal routes to six 10-day-old chickens. Three days after inoculation, typical clinical signs of ND were observed in all chickens. In contrast, no clinical signs or gross lesions were observed during the experiment in those inoculated with PBS. All chickens were sacrificed at day 4 p.i. At necropsy, gross lesions were found in organs of the chickens inocu-

Fig. 3. GFP expression *in vivo* and *in vitro*. CEF and BSR-T7/5 cells were infected with LaZJ1RGFP at an MOI of 0.01. Twenty-four hours p.i., the expression of GFP in CEF (**A**) and BSR-T7/5 (**B**) cells was detected using a reverse fluorescence microscope. A ten-day-old SPF chicken was inoculated with 1×10^3 TCID$_{50}$ LaZJ1RGFP. Four days p.i., the kidney (**C**) and trachea (**D**) were taken, and the expression of GFP was detected using a reverse fluorescence microscope. Negative control cells, BSR-T7/5 (**E**) and CEF (**F**), and organs, kidney (**G**) and trachea (**H**), are also shown


A
E
B
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C
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lated with the recovered virus, especially in lung, kidney, and brain. Strong and specific expression of GFP was detected in the kidney and trachea (Fig. 3). However, less intense fluorescence of GFP was detected in other organs.

Discussion

Recently, the generation of recombinant NDV by reverse genetics techniques has been described [12, 18, 21]. This manipulation has facilitated the generation of a recombinant vaccine vector based on NDV. NDV has significant advantages over many other vaccine vectors and provides an efficient vector system for the delivery of protective antigens of other avian pathogens [10, 12, 24]. Recombinant NDV expressing an H5-subtype avian influenza virus (AIV) hemagglutinin (HA) was shown to be effective in protecting chickens against clinical disease after challenge with a lethal dose of velogenic NDV or highly pathogenic AIV [24].

To date, most of the rescued NDVs have been either lentogenic or mesogenic strains, and there has been no report of the recovery of field-isolated velogenic NDV. Additionally, all of the rescued viruses so far were from chicken isolates. This study describes the recovery of highly virulent recombinant NDV strain ZJ1, isolated from an outbreak in geese, from a cDNA clone. This was accomplished by using an approach in which cDNA-encoded antigenomic RNA is synthesized by means of T7 RNA polymerase in cells that were simultaneously cotransfected with NP, P, and L helper plasmids derived from NDV LaSota. The recombinant NDV expressing the GFP gene was generated accordingly.

The homology of strains ZJ1 and LaSota is low [9]. The successful recovery of infectious virulent NDV ZJ1 using helper plasmids from LaSota demonstrates that the RNP complex consisting of the ZJ1 genome and support proteins of LaSota is recognized by the polymerase complex of LaSota.

The established reverse genetics approach allows the generation of a genetically altered NDV, which can be used as a vector to deliver and express foreign genes for vaccination and gene therapy [12, 25]. It has been shown that NDV can serve as a highly effective vaccine vector for protection against other pathogens and for gene transfer to tumor cells [4, 10, 16, 24]. In the present study, we inserted the GFP gene between the P and the M gene of the full-length cDNA clone NDV3GM122, and we successfully rescued GFP-expressing recombinant NDV, which was confirmed by the expression of GFP in BSR-T7/5 and CEF cells (Fig. 3). In infected chickens, we detected the fluorescence of GFP in collected organ samples of brain, lung, trachea, cloaca, liver and kidney 4 days p.i. The result showed that GFP fluorescence was brighter in trachea and kidney than in other organs (Fig. 3). We repeated the work and obtained very similar results. The difference in the fluorescence intensity was postulated to be associated with the route of inoculation. It was reported that GFP-expressing lentogenic recombinant NDV infected various organs when day-old SFP chickens were inoculated by unilateral intracerebral administration [25]. The widespread infection of organs was presumed to be a result of the hematogenous viral dissemination.

Successive passages in embryonated chicken eggs were performed with the recovered virus before infection of chickens. The genetic stability of the additional gene in the recombinant NDV genome is indicated by the detection of GFP in organs, and this demonstrates the suitability of velogenic NDV ZJ1 for the development of vector vaccines. Additionally, GFP fluorescence is a convenient marker for identification of infected cells [7]. Therefore, the rescued recombinant NDV bearing GFP may be used as a reliable indicator of NDV infection *in vivo* and facilitates systematic examination of NDV dissemination and pathogenesis. In summary, our study indicates that virulent NDV stain ZJ1 can be used as a vaccine vector to stably express heterologous proteins effectively.

Acknowledgments

We are grateful to Ben Peeters for providing the plasmids pCIneoNP, pCIneoP and pCIneoL and many helpful suggestions. We thank Andrew Ball for providing the plasmid TVT7R (0, 0) and Zhigao Bu for providing BHK-21 (clone BSR T7/5) cells. This work was supported by the China National Natural Science Foundation (grant number: 39893290) and by the China Ministry of Science and Technology (grant number: "973"; G19990199).

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