

# Recombinant Newcastle disease virus as a viral vector: effect of genomic location of foreign gene on gene expression and virus replication

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Newcastle disease virus (NDV) was examined for its suitability as a vector for the expression and delivery of foreign genes for vaccination and gene therapy. A reporter gene encoding human secreted alkaline phosphatase (SEAP) was inserted as an additional transcription unit at four different positions in the NDV genome, between the NP and P, M and F, and HN and L genes and behind the L gene. Eight infectious recombinant NDV (rNDV) viruses, four in the non-virulent strain NDFL and four in the virulent derivative NDFLtag, were generated by reverse genetics. SEAP expression levels, replication kinetics and virus yield were examined. Replication kinetics of the rNDV viruses in primary chicken embryo fibroblasts showed that the insertion of an additional gene resulted in a delay in the onset of replication. This effect was most prominent when the gene was inserted between the NP and P genes. With the exception of the strain that carried the SEAP gene behind the L gene, all recombinant strains expressed high levels of SEAP, both in cell culture and in embryonated chicken eggs. In embryonated eggs, the rNDV viruses showed a 2·6- to 5·6-fold (NDFL) or 2·1- to 8·1-fold (NDFLtag) reduction in yield compared with the parent strains. These results show that foreign genes can be inserted at different positions in the NDV genome without severely affecting replication efficiency or virus yield.

Received 8 October 2002

Accepted 5 December 2002

## INTRODUCTION

Newcastle disease virus (NDV) is an important avian pathogen causing severe economic losses in the poultry industry worldwide. NDV strains can be classified as highly virulent (velogenic), intermediate (mesogenic) or non-virulent (lentogenic) on the basis of their pathogenicity for birds (Beard & Hanson, 1984). The molecular basis for this classification is mainly determined by the amino acid sequence of the protease cleavage site of the F protein and by the ability of cellular proteases to cleave the F protein of different pathotypes. The precursor glycoprotein F<sub>0</sub> must be cleaved into F<sub>1</sub> and F<sub>2</sub> for the progeny virus to be infectious. Lentogenic NDV strains, such as Hitchner B1 (Hitchner & Johnson, 1948) and La Sota (Goldhaft, 1980), are widely used as live vaccines against Newcastle disease. However, NDV live vaccines may still cause disease, depending on environmental conditions and the presence of complicating infections.

NDV is a non-segmented, negative-stranded RNA virus and a member of the genus *Rubulavirus* in the family *Paramyxoviridae*. Recently it was suggested that NDV should be assigned to a new genus within the subfamily *Paramyxovirinae* (de Leeuw & Peeters, 1999). The 15186-nucleotide-long genome (Krishnamurthy & Samal, 1998; Philips *et al.*, 1998; de Leeuw & Peeters, 1999) contains six

genes encoding the nucleocapsid (NP) protein, phosphoprotein and V protein (P/V), matrix (M) protein, fusion (F) protein, haemagglutinin-neuraminidase (HN) and large polymerase (L) protein (Millar & Emmerson, 1998). The NP protein binds to the genomic and antigenomic RNA to form a remarkably stable nucleocapsid core structure to which P and L proteins are attached (Lamb & Kolakofsky, 1996). This ribonucleoprotein (RNP) complex, rather than naked viral RNA, is the template for replication of further RNPs and the transcription of translatable free mRNAs. The NDV genes are tandemly linked in the order 3'-NP-P-M-F-HN-L-5' and are separated by junction sequences that consist of three elements, known as gene-end (GE), intergenic (IG) and gene-start (GS) sequences. The viral RNA polymerase complex is believed to enter the genomic RNA at a single 3' entry site and to transcribe the genome by a sequential start-stop mechanism. Thus, there is a gradient of mRNA abundance according to the position of the gene relative to the 3' end of the template (Abraham & Banerjee, 1976; Ball & White, 1976; Emerson, 1982; Iverson & Rose, 1982; Collins *et al.*, 1996; Lamb & Kolakofsky, 1996).

For most members of the *Paramyxovirinae*, including NDV, efficient replication is dependent on the genome length being a multiple of six. This requirement is known as the 'rule of six' (Calain & Roux, 1993). It is assumed that each NP subunit is in contact with exactly six nucleotides and that

this arrangement is required for efficient replication. A shift of the NP subunit other than 6n causes a shift in the position of the promoter resulting in improper initiation of replication (Egelman *et al.*, 1989; Murphy & Parks, 1997; Kolakofsky *et al.*, 1998; Peeters *et al.*, 2000).

Previously, we reported a reverse genetics system for generating infectious NDV from cloned full-length cDNA (Peeters *et al.*, 1999). This process involved the *in vivo* transcription by means of T7 RNA polymerase of cDNA-encoded antigenomic RNA and the simultaneous expression of NP, P and L from co-transfected plasmids. Later on, similar systems were described for other NDV strains (Römer-Oberdörfer *et al.*, 1999; Krishnamurthy *et al.*, 2000; Huang *et al.*, 2001). This made it possible to produce genetically modified NDV and to manipulate the NDV genome to insert foreign sequences, thus allowing the use of genetically altered NDV as a vector to deliver and express foreign genes for vaccination and gene therapy.

Here we report the insertion of a reporter gene encoding secreted alkaline phosphatase (SEAP) at four different positions distributed along the NDV genome – between NP and P, M and F, and HN and L genes and behind the L gene. This allowed us to compare the characteristics of recombinant NDV viruses containing the same additional transcription unit at different insertion positions along the entire NDV genome. Eight rNDV viruses were generated: four in strain NDFL and four in strain NDFLtag. The latter strain was previously generated by changing the amino acid sequence of the protease cleavage site of the F<sub>0</sub> protein from that of La Sota (GGRQGR ↓ L) to the consensus sequence of virulent NDV strains (GRRQRR ↓ F) by means of PCR mutagenesis (Peeters *et al.*, 1999). The characteristics of the different viruses as vectors to deliver and express foreign genes for vaccination and gene therapy were evaluated by examining SEAP expression levels, replication kinetics and virus yield.

## METHODS

**Cells and viruses.** Primary chicken embryo fibroblasts (CEF) cells were grown in the Glasgow modification of Eagle's medium containing 5% foetal calf serum (FCS) and 2% antibiotic mix. QM5 cells were maintained in medium 199 supplemented with 10% tryptose phosphate broth, 10% FCS and 2% antibiotic mix.

NDV strains NDFL, NDFLtag and other recombinant NDV viruses were grown in embryonated specific-pathogen-free (SPF) eggs. The fowlpox recombinant virus fpEFL7pol (Britton *et al.*, 1996) (hereafter called FPV-T7), which expresses T7 RNA polymerase, was grown on primary chicken embryo liver cells.

**Construction of a transcription cassette consisting of the GE, IG and GS sequences and the SEAP gene.** All sequences were determined using the PRISM Ready Reaction dye deoxy terminator cycle sequencing kit (Perkin Elmer). PCR amplifications were carried out with the proofreading *Pfu* DNA polymerase (Stratagene). Two oligonucleotides were designed for the GE, IG and GS sequences from the P–M junctions (5'-CGCGATCGATCGCGCCGCGGATTAAGAAAAAATACGGGTAGAAGATCGAAGCGCTGATAT-3';

5'-CGATATCAGCGCTTCGATCTTCTACCCGTATTTTTTCTTAATCGCGGCCGATCGATCG-3') and cloned into the *AccI* site in pUC19. The SEAP gene (Berger *et al.*, 1988) was recovered from plasmid pSEAP-Basic (Clontech) by digestion with *SphI* and *Clal* and cloned into pOLTV5, resulting in pOLTV553 (Peeters *et al.*, 2000). The SEAP gene was recovered from pOLTV553 by digestion with *Clal/XmnI*, treated with *E. coli* DNA polymerase Klenow fragment to make the ends blunt and cloned into the *EcoRV* site behind the GE–IG–GS sequences in pUC19. It is extremely convenient to have a selectable marker that can be used to select transformants in the final part of the construction of full-length cDNA. Therefore, the chloramphenicol resistance ( $Cm^R$ ) gene was recovered from plasmid pACYC184 (Chang & Cohen, 1978) by means of PCR and cloned in the *XbaI* site in front of the GE–IG–GS sequences. The integrity of the entire foreign sequence was confirmed by sequencing using the dideoxy chain termination method. The resulting plasmid was named pUC19/ES/SEAP/ $Cm^R$ .

**Construction of full-length recombinant cDNAs containing the SEAP gene at different positions.** Cloning of the full-length cDNA of NDV strain LaSota in a transcription vector yielding pNDFL+ and the construction of its derivative, pNDFLtag, has been described previously (Peeters *et al.*, 1999). Plasmids expressing the NDV NP, P and L proteins (pCIneoNP, pCIneoP and pCIneoL) under the control of a bacteriophage T7 RNA polymerase promoter have also been described (Peeters *et al.*, 1999).

Because appropriate restriction sites are lacking in the full-length cDNA in the pNDFL plasmids, direct insertion of the transcription cassette was not possible. Therefore, as a general strategy, a subgenomic fragment containing the selected insertion site was first recovered and cloned into a suitable vector. Next, a PCR fragment containing the  $Cm^R$ –GE–IG–GS–SEAP cassette was cloned into a unique restriction site in the non-coding region before the GE sequences of the preceding gene. Finally, the original subgenomic fragment was cloned back into the full-length cDNA of both pNDFL+ and pNDFLtag. All full-length rNDV cDNAs were designed to obey the rule of six (Kolakofsky *et al.*, 1998; Peeters *et al.*, 2000).

In order to insert the SEAP gene between the NP and P genes, plasmids pNDFL+ and pNDFLtag were digested with *SalI* and a 555 bp fragment (nt 1644–2199) was cloned into plasmid pUC19 and named pUC19*SalI*. The *BsrGI* restriction enzyme site (TGTACA) at position 1719 was chosen to insert the  $Cm^R$ –GE–IG–GS–SEAP cassette. Using two specific oligonucleotide primers carrying the recognition sequence for *BsrGI* and using plasmid pUC19/ES/SEAP/ $Cm^R$  as a template, a PCR fragment harbouring the  $Cm^R$ –GE–IG–GS–SEAP cassette was produced and cloned into the *BsrGI* site of pUC19*SalI*. After digestion with *SalI*, the fragment containing the SEAP cassette was cloned back into the full-length constructs pNDFL+ and pNDFLtag. Finally, the  $Cm^R$  gene was removed by *XbaI* digestion followed by self ligation. The resulting plasmids were called pHZ-SEAP/NP-P and pHZ-SEAP/NP-Ptag.

To insert the SEAP gene between the M and F genes, an oligonucleotide containing the recognition sequence for *ApaI* (5'-GATCATGGGCCCAT-3') was synthesized and cloned into the *BamHI* site of pOK12 in which two *BspHI* sites had previously been removed by digestion with *BspHI*, Klenow treatment and self ligation. The 2667 bp *ApaI*–*NotI* fragments (nt 2285–4952) of pNDFL+ and pNDFLtag were cloned into the *ApaI* and *NotI* sites of modified pOK12 resulting in pOK12*ApaI/NotI*. The *BspHI* site (nt 4427) of this plasmid was utilized to insert the  $Cm^R$ –GE–IG–GS–SEAP cassette. One extra *BspHI* site within the SEAP gene was removed by PCR mutagenesis. The  $Cm^R$ –GE–IG–GS–SEAP cassette was cloned into the *BspHI* site of pOK12*ApaI/NotI* and cloned back into pNDFL+ and pNDFLtag after

*ApaI* and *NotI* digestion. The  $\text{Cm}^{\text{R}}$  gene was removed as described above and the resulting plasmids were called pHZ-SEAP/M-F and pHZ-SEAP/M-Ftag.

To insert the SEAP gene between the HN and L genes, an oligonucleotide containing the recognition sequences for *SpeI* and *BsiWI* (5'-AATTACTAGTGGCCCGTACGC-3') was synthesized. It was cloned into the *EcoRI* site of pUC19, which introduced the *SpeI* and *BsiWI* sites in pUC19 and removed the original *EcoRI* site in vector pUC19. The 758 bp *SpeI*-*BsiWI* fragments (nt 8094–8852) of pNDFL+ and pNDFLtag were cloned into the *SpeI* and *BsiWI* sites of modified pUC19. The *EcoRI* site at nt 8236 was utilized to insert the  $\text{Cm}^{\text{R}}$ -GE-IG-GS-SEAP cassette. One extra *EcoRI* site within  $\text{Cm}^{\text{R}}$  gene had been previously removed by PCR mutagenesis. As above, the full-length plasmids carrying the SEAP gene between the HN and L genes were obtained and called pHZ-SEAP/HN-L and pHZ-SEAP/HN-Ltag.

To insert the SEAP gene behind the L gene, the strategy was somewhat different. Plasmids pNDFL+ ( $\text{Cm}^{\text{R}}$ ) and pNDFLtag( $\text{Cm}^{\text{R}}$ ) containing the  $\text{Cm}^{\text{R}}$  gene in the *BsiWI* site were digested with *AvrII* (nt 3568 and 14707) and the 6266 bp *AvrII* fragment containing the replication origin, kanamycin-resistance gene and part of the L gene was isolated and self-ligated to form a new plasmid named pAvr2. The *EcoRI* site at nt 15098 was used to insert the  $\text{Cm}^{\text{R}}$ -GE-IG-GS-SEAP cassette. The  $\text{Cm}^{\text{R}}$  gene was first removed by *XbaI* digestion and the new plasmid was designated pAvr2/ES/SEAP. After *AvrII* digestion, it was cloned back into the full-length constructs of pNDFL+ ( $\text{Cm}^{\text{R}}$ ) and pNDFLtag( $\text{Cm}^{\text{R}}$ ). Finally, the  $\text{Cm}^{\text{R}}$  gene was removed by *BsiWI* digestion followed by religation. The resulting plasmids were called pHZ-SEAP/L and pHZ-SEAP/Ltag.

**Rescue of recombinant NDV strains.** Rescue of rNDV/SEAP was performed as previously described (Peeters *et al.*, 1999). Briefly, CEF cells or QM5 cells were transfected with 1  $\mu\text{g}$  full-length cDNA constructs together with 0.4  $\mu\text{g}$  plasmid pCIneoNP, 0.2  $\mu\text{g}$  pCIneoP and 0.2  $\mu\text{g}$  pCIneoL or pCIneo (negative control) using 6  $\mu\text{l}$  FuGene6 (Roche). After incubation for 3–5 days, the culture supernatants were harvested, passed through a 0.2  $\mu\text{m}$  filter and inoculated into the allantoic cavities of 9- to 11-day-old embryonated SPF chicken eggs. The monolayer was tested for infective centres using an immunoperoxidase monolayer assay (Wensvoort *et al.*, 1986) with monoclonal antibody 8E12A8C3 (ID-Lelystad) against the F protein. Seven days after inoculation, the allantoic fluids were harvested and the presence of virus was determined by a rapid plate haemagglutination (HA) test using chicken erythrocytes. A positive haemagglutination reaction indicated that virus was present in the allantoic fluid of inoculated eggs.

In order to eliminate the FPV-T7 helper virus, virus-containing allantoic fluid was first passed through a 0.45  $\mu\text{m}$  filter and then through a 0.2  $\mu\text{m}$  filter. Subsequently, the allantoic fluid was diluted by 10-fold serial dilutions from  $10^{-1}$  to  $10^{-10}$  and inoculated into SPF eggs. After 5 days, HA and SEAP tests were performed and eggs from the highest dilution that were both HA- and SEAP-positive were used for another egg passage. High-titre recombinant virus stocks were prepared in SPF eggs and stored at  $-20^{\circ}\text{C}$ .

**RNA isolation and RT-PCR.** Viral RNA was isolated from allantoic fluid using a High Pure viral RNA kit (Roche). Viral RNA was used for RT-PCR directly after elution in nuclease-free water. Viral cDNA was prepared using Superscript reverse transcriptase (Gibco BRL) and primer 3'UIT, as described previously (de Leeuw & Peeters, 1999). The RT products were then amplified by PCR using different pairs of primers to generate specific fragments covering the SEAP gene.

**Single-step growth curves.** Replication of the recombinant NDV/SEAP viruses under single-step growth conditions was examined in

CEF cells. Only the recombinant viruses containing the SEAP gene insertion in strain NDFLtag were used because they were able to replicate in cell culture. (Strains derived from strain NDFL cannot replicate in cell culture because the cells lack the appropriate proteases to activate the fusion protein.) CEF cells in 24-well plates were infected with each virus at an m.o.i.  $\geq 10$ . At 0, 8, 12, 24, 36, 48 and 72 h post-infection, a sample of the supernatant was harvested. The amount of virus in the supernatant was determined by end-point titration on CEF cells and was expressed as mean  $\log_{10}$  of the  $\text{TCID}_{50} \text{ ml}^{-1}$ .

**Quantification of SEAP expression levels.** CEF cells in six-well plates were infected with each virus at an m.o.i.  $\geq 10$ . A sample of the supernatant was harvested at 0, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h post-infection. SEAP expression was measured in disposable 96-well plates using the Phospha-Light chemiluminescent reporter assay for secreted alkaline phosphatase kit as described by the supplier (Tropix). Chemiluminescence was measured using a liquid scintillation counter (Wallac 1450 Microbeta PLUS).

To determine SEAP expression levels in eggs, 9- to 11-day-old embryonated SPF chicken eggs were inoculated with each recombinant virus (both the NDFL and NDFLtag derivatives) at a 50% egg infectious dose ( $\text{EID}_{50}$ ) of 100. After 4 days, aliquots of harvested allantoic fluids were assayed for SEAP expression in disposable 96-well plates as described above. SEAP expression was quantified with a standard curve using serial dilutions of SEAP protein ( $3 \mu\text{g ml}^{-1}$ , supplied in the assay kit).

**Quantification of virus yield.** The allantoic cavities of 9- to 11-day-old embryonated SPF chicken eggs were infected with 100  $\text{EID}_{50}$  of each virus. After 4 days, the allantoic fluid was harvested and the amount of virus was determined by end-point titration on CEF cells.

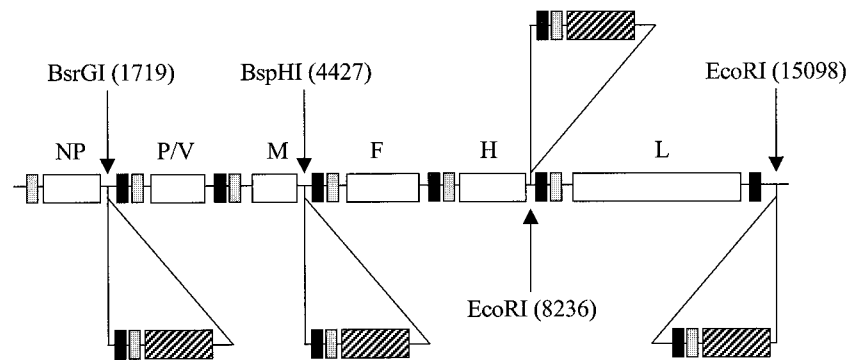
## RESULTS

### Design of the transcription cassette consisting of the GE, IG and GS sequences and the SEAP gene

The negative-stranded RNA genome of NDV contains six genes in the order 3'-NP-P-M-F-HN-L-5'. Comparison of the GE, IG and GS sequences located in the gene junctions shows that these elements are highly conserved, except for the IG sequences, which vary in size from 1 to 47 nucleotides (de Leeuw and Peeters, 1999). A transcription cassette was designed that consisted of the GS, IG and GE sequences from the original P-M junction sequence (TAAGAAAAAATACGGGTAGAA) and the SEAP open reading frame. In order to avoid differences that could affect the translation efficiency, the sequences between the GS sequences and the ATG start codon of the SEAP gene were kept the same. The cassette was inserted before the GE sequence of the preceding gene, except for the L gene where it was inserted behind the GE sequence (Fig. 1).

### Generation of full-length recombinant cDNAs

The SEAP transcription cassette was inserted into plasmid pNDFL+ (containing full-length NDV La Sota antigenomic cDNA; Peeters *et al.*, 1999) at four different positions, yielding pHZ-SEAP/NP-P, pHZ-SEAP/M-F, pHZ-SEAP/L



**Fig. 1.** Diagram showing the genomic organization of the recombinant NDV/SEAP strains. Black boxes indicate conserved gene-end (GE) sequences. Shaded boxes indicate conserved gene-start (GS) sequences. Hatched boxes represent the SEAP gene. Restriction sites that were used to insert the SEAP expression cassette are indicated. Numbers between parentheses represent the nucleotide positions in the NDV genome (de Leeuw & Peeters, 1999).

HN-L and pHZ-SEAP/L. In addition, the SEAP transcription cassette was also inserted at the same positions into pNDFLtag (encoding a virulent version of NDV La Sota; Peeters *et al.*, 1999), yielding pHZ-SEAP/NP-Ptag, pHZ-SEAP/M-Ftag, pHZ-SEAP/HN-Ltag, pHZ-SEAP/Ltag. Details of these constructions are given in Methods and Fig. 1. The introduction of the transcription cassette resulted in plasmids encoding a recombinant NDV/SEAP antigenome of 16872 nucleotides.

### Rescue of recombinant NDV strains

The co-transfection system described previously (Peeters *et al.*, 1999) was used to rescue infectious recombinant NDV from the recombinant cDNAs described above. Rescue of the rNDV/SEAP viruses was monitored 3–5 days after transfection by immunological staining of fixed monolayers using a monoclonal antibody against the F protein. At least 30–50 positive cells were detected in all of the transfection experiments, indicating that genome replication and expression of viral proteins occurred in these cells. Expression of the F protein was not detected when pCIneoL was replaced by pCIneo in the co-transfection experiments.

The supernatant of the transfected monolayers was harvested and injected into the allantoic cavities of 9- to 11-day-old embryonated SPF eggs. After incubation for 7 days, the allantoic fluid was harvested and analysed using HA and SEAP assays. Allantoic fluid that was both HA- and SEAP-positive was used for another egg passage. After elimination of the FPV-T7 helper virus, virus stocks were prepared in embryonated SPF eggs. The recovered recombinant viruses were designated rNDV/SEAP/NP-P, rNDV/SEAP/M-F, rNDV/SEAP/HN-L, rNDV/SEAP/L, rNDV/SEAP/NP-Ptag, rNDV/SEAP/M-Ftag, rNDV/SEAP/HN-Ltag and rNDV/SEAP/Ltag. Each rNDV/SEAP virus was characterized in respect of the presence and position of the SEAP insert using RT-PCR and sequence analysis (data not shown).

### Growth characteristics of recombinant viruses

The growth characteristics of the recombinant NDV/SEAPtag viruses were examined in a single-step growth cycle in CEF cells (Fig. 2). The kinetics of replication of the four recombinant viruses showed that insertion of the SEAP gene resulted in a delay in the onset of replication. However, after 72 h, virus titres reached a level that was only 1.8- to 3.2-fold lower than that of the parent strain NDFLtag.

### Kinetics of SEAP expression by recombinant viruses

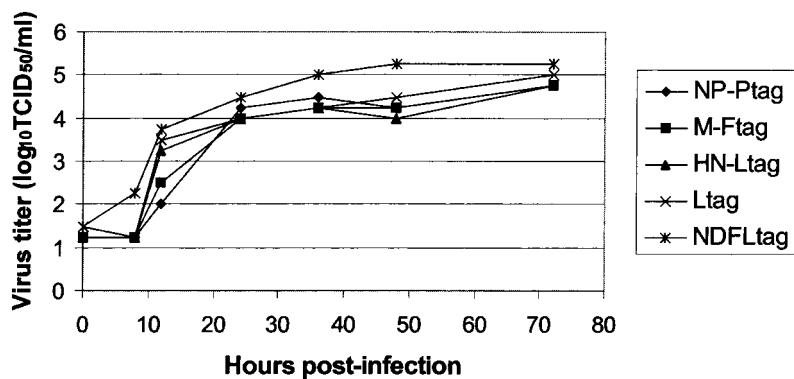
Fig. 3 shows expression of the SEAP gene after infection of CEF cells with the different recombinants. With the exception of strain rNDV/SEAP/Ltag, all strains expressed high levels of SEAP. No significant differences in the kinetics of SEAP expression were obvious in cells infected with strains rNDV/SEAP/NP-Ptag, rNDV/SEAP/M-Ftag and rNDV/SEAP/HN-Ltag.

### Quantification of SEAP activity in SPF chicken eggs

In order to compare the expression levels in embryonated eggs, 9-day-old SPF chicken eggs were infected with each virus at an EID<sub>50</sub> of 100. After incubation for 4 days, allantoic fluids were harvested and SEAP assays were carried out (Fig. 4). Allantoic fluids from eggs infected with wild-type NDFL and NDFLtag were analysed in parallel as negative controls. The SEAP activities were highest in the allantoic fluids of SPF chicken eggs infected with rNDV/SEAP/M-F, followed by rNDV/SEAP/HN-L and rNDV/SEAP/NP-P. SEAP activities in eggs infected with rNDV/SEAP/L were 150- to 500-fold lower. No SEAP activity could be detected in uninfected eggs or in eggs infected with NDFL or NDFLtag.

### Virus yield

Virus yield was determined after growth for 3 days in 9- to 11-day-old embryonated SPF chicken eggs inoculated with



**Fig. 2.** Single-step growth curve for rNDV/SEAP/NP-Ptag, rNDV/SEAP/M-Ftag, rNDV/SEAP/HN-Ltag, rNDV/SEAP/Ltag and pNDFLtag in CEF cells. Cell monolayers were infected with each virus at an m.o.i.  $\geq 10$ . Samples were taken at 0, 8, 12, 24, 36, 48 and 72 h post-infection and the virus titre in the supernatant was determined. The data represent the mean of three titrations.

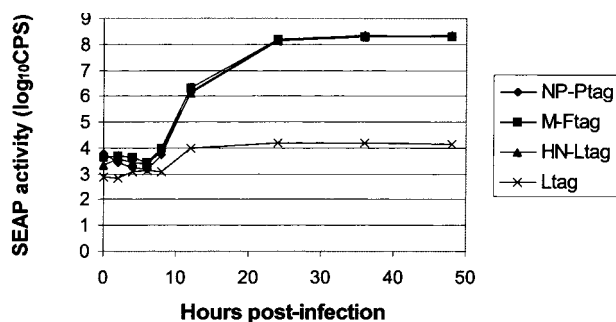
100 EID<sub>50</sub> of virus. The recombinant viruses showed a 2.6- to 5.6-fold (NDFL) or 2.1- to 8.1-fold (NDFLtag) reduction in yield compared with the parent strains (Fig. 5).

## DISCUSSION

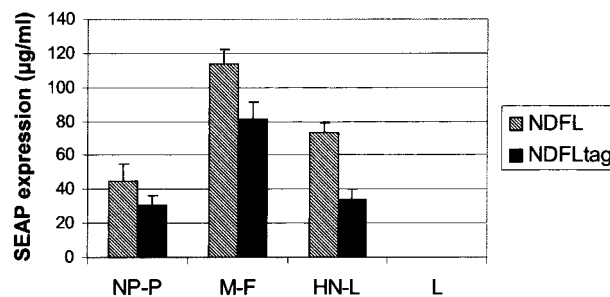
Genetic engineering of negative-stranded RNA viruses has allowed the construction of recombinant viruses that express foreign proteins. Expression of foreign proteins by several members of the *Paramyxoviridae* has been reported (Bukreyev *et al.*, 1996; Mebatsion *et al.*, 1996; He *et al.*, 1997; Johnson *et al.*, 1997; Roberts *et al.*, 1998; Baron *et al.*, 1999; Singh & Billeter, 1999; Bailly *et al.*, 2000; Buchholz *et al.*, 2000; Durbin *et al.*, 2000; Krishnamurthy *et al.*, 2000; Huang *et al.*, 2001; Nakaya *et al.*, 2001). In most of these cases, the foreign gene was inserted at a specific position in the viral genome and no systematic attempts were made to examine the effects of the position of the foreign gene on expression levels and virus replication.

It has been shown that expression levels of paramyxovirus proteins are reduced following the order of transcription

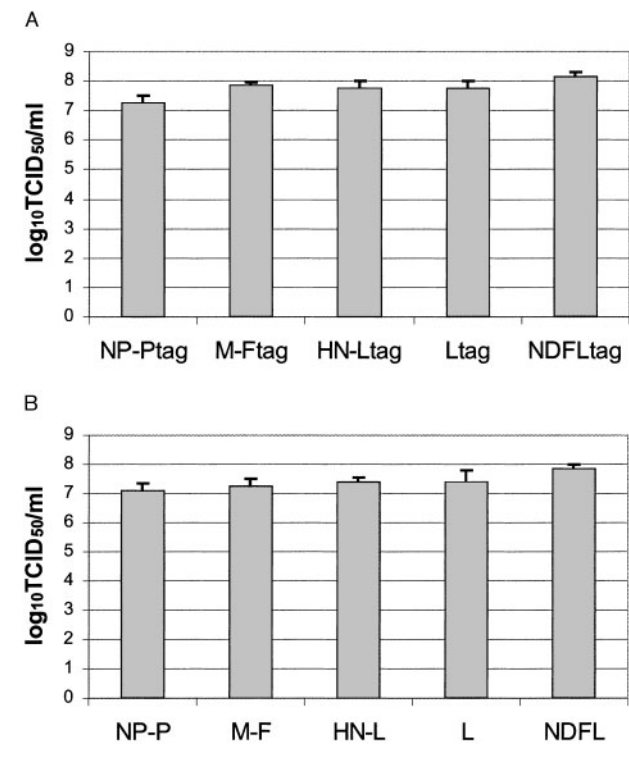
from the 3' proximal end to the 5' distal end of the viral genome (Lamb & Krug, 1996; Sakai *et al.*, 1999; Wertz *et al.*, 1998). To examine whether this is also true for an inserted foreign gene and to examine the effect of the genomic location of the foreign gene on gene expression and virus replication, we constructed recombinants in which the SEAP reporter gene was inserted at four different positions along the NDV genome. In all cases the SEAP gene was preceded by regulatory GE and GS sequences that were identical to those preceding the M gene. Furthermore, to minimize differences that could affect the efficiency of translation, the sequence from the GS sequence up to the ATG start codon of the SEAP gene was kept identical in all recombinants. It has been shown that differences in the sequence of the GE and GS elements may have profound effects on gene expression. Using a recombinant simian virus 5 that contained the green fluorescent protein (GFP) between the HN and L genes, He *et al.* (1997) showed that there were large differences in the relative expression levels of GFP depending on the transcription initiation signals used. Initiation of transcription of the GFP gene by the NP-V/P junction sequences resulted in high expression levels,



**Fig. 3.** Comparison of SEAP expression levels of rNDV/SEAP/NP-Ptag, rNDV/SEAP/M-Ftag, rNDV/SEAP/HN-Ltag and rNDV/SEAP/Ltag in cell culture. CEF cell monolayers were infected with each virus at an m.o.i.  $\geq 1$ . Samples were taken at 0, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h post-infection. A sample of supernatant was harvested and assayed using the Phospho-Light chemiluminescent reporter assay for secreted alkaline phosphatase kit as described by the supplier (Tropix).



**Fig. 4.** Quantification of SEAP activity of rNDV viruses in embryonated eggs. Embryonated SPF chicken eggs were inoculated with each rNDV virus at 100 TCID<sub>50</sub>. After four days, aliquots were harvested and assayed for SEAP expression. The amount of SEAP that was secreted into the allantoic fluid was quantified using a standard curve. The data are given as the average from three samples. Bars indicate standard deviations.



**Fig. 5.** Average recombinant virus yield. The allantoic cavities of 9- to 11-day-old embryonated SPF chicken eggs were inoculated with 100 EID<sub>50</sub> of the indicated recombinant virus. Allantoic fluid was harvested after 4 days of incubation. The amount of virus was determined by end-point titration as described in Methods. The data represent the average titre from three different samples. Bars indicate standard deviations. (A) Recombinant viruses with the SEAP gene insertions in strain NDFL. (B) Recombinant viruses with the SEAP gene insertions in strain NDFLtag.

whereas transcription initiation by a second copy of the HN-L junction sequence resulted in low expression levels.

We examined the replication kinetics of the different recombinants in a single infection cycle in CEF cells (Fig. 3). This analysis showed that the onset of replication of the recombinants was somewhat delayed compared with the parent strain. The delay was more prominent when the SEAP gene was inserted between the NP and P genes than when it was between the M and F or HN and L genes (Fig. 2). In paramyxoviruses, the relative level of the mRNAs is primarily determined by the rate at which the polymerase complex reinitiates transcription at the GS element of the distal gene after termination at the GE element of the proximal gene. Thus, insertion of a foreign gene between two viral genes may affect the relative abundance of the mRNAs of the flanking genes. If the relative amount of the corresponding gene product is critical for transcription or replication, then insertion of the foreign gene between these genes will have a more pronounced biological effect than insertion between genes whose products do not interact. It

has indeed been shown that the ratio of the NP to P protein may be critical for efficient replication of paramyxoviruses. In vesicular stomatitis virus, an N:P protein stoichiometry of between 1:1 and 2:1 has been shown to be optimal for supporting efficient replication and encapsidation, while ratios substantially above or below this range had a negative effect on replication (Pattnaik & Wertz, 1990). Thus, insertion of the SEAP gene between NP and P may result in a reduction of P gene expression and consequently in a change in the ratio of the NP to P proteins, which might compromise the replication ability of the recombinant virus.

Protein levels and stoichiometry may also be affected by mRNA stability. Insertion of the SEAP gene results in a reduction of the size of the 3' non-translated region of the gene preceding the SEAP gene. This may differentially affect the stability of the corresponding mRNA and thus it may also affect replication kinetics. Finally, it may be expected that the increase in total genome size has a negative effect on replication. Our data indicate that the latter effect is limited since strains rNDV/SEAP/L and rNDV/SEAP/Ltag showed a reduction in yield of only 2.1- to 2.6-fold (Fig. 5). In these strains, the SEAP gene is located behind the GE sequence of the L gene and thus does not interfere with transcription of the preceding gene. Furthermore, in these strains, the size and sequence of the 3' non-translated region of the L gene mRNA is not affected.

Quantification of SEAP gene expression by the different recombinants in CEF cells showed high expression levels when the SEAP gene was inserted between the NP and P, M and F, and HN and L genes. In contrast, very low levels were expressed when the SEAP gene was located behind the L gene (Fig. 3). The latter finding is not surprising in view of the fact that the L gene is already expressed at relatively low levels compared with the other viral genes (Lamb & Krug, 1996; Wertz *et al.*, 1998; Sakai *et al.*, 1999). Furthermore, the fact that the L gene of these constructs contains two GE sequences may also negatively affect the expression level of the downstream SEAP gene.

In embryonated eggs, SEAP expression levels at 4 days after inoculation were 2-3 times lower when the SEAP gene was located between the NP and P genes than when it was located between the M and F genes (Fig. 4). This again suggests that insertion of the SEAP gene between the NP and P genes has a negative effect on transcription and/or replication. This seems to be supported by the observation that strains rNDV/SEAP/NP-P and rNDV/SEAP/NP-Ptag also show the largest reduction in virus yield (Fig. 5).

A reduction in replication rate seems to be a general characteristic of recombinant paramyxoviruses that carry an additional foreign gene. When the foreign gene is inserted at the 3' end before the NP gene, this may affect the absolute level but not the relative level of the viral mRNAs. Similarly, if the foreign gene is inserted at the 5' end behind the L gene, the relative level of the viral mRNAs will not be affected. Therefore, due to interference with the normal transcriptional

gradient, the effect may be expected to be more prominent when the gene is inserted in the gene junctions than when it is expressed from the most 3' proximal or 5' distal locus. It has been reported that insertion of the CAT gene between the HN and L genes in NDV resulted in a reduction in yield of more than 100-fold (Krishnamurthy *et al.*, 2000). Furthermore, expression of the CAT gene was much lower when it was located between the HN and L genes than when it was located before the NP gene (Huang *et al.*, 2001). Comparison of these results with ours suggests that, in the construct of Krishnamurthy *et al.*, changes in spacing between the GE and GS elements and the sequence context of these elements before the L gene are responsible for the observed differences.

In conclusion, our results show that NDV can serve as a viral vector to express and deliver foreign genes by insertion at different positions without severely affecting replication efficiency or virus yield.

## ACKNOWLEDGEMENTS

We thank Olav de Leeuw, Helmi Fijten and Amra Hajdo-Milasinovic for excellent technical assistance. This research was sponsored by Lohmann Animal Health GmbH & Co. KG, Cuxhaven, Germany.

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