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 embryos ated chicken eggs. In embryos ated eggs, the rNDV viruses showed a 2-6-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.

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 virulence (lentogenic) on the basis of their pathogenicity for birds (Beard & Hanson, 1984). The molecular basis for this virulence 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 F0 must be cleaved into F1 and F2 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 (Krishnamurty & 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 antigenic 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 RNP s and the transcription of translatable free mRNAs. The NDV genomes 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

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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 NDFLtag was 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 (CmR) gene was recovered from plasmid pACYC184 (Chang & Cohen, 1978) by means of PCR and cloned in the Xbal 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/CmR.

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 CmR–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 pUC19SalI. The BsrGI restriction enzyme site (TGTACA) at position 1719 was chosen to insert the CmR–GE–IG–GS–SEAP cassette. Using two specific oligonucleotide primers carrying the recognition sequence for BsrGI and using plasmid pUC19/ES/SEAP/CmR as a template, a PCR fragment harbouring the CmR–GE–IG–GS–SEAP cassette was produced and cloned into the BsrGI site of pUC19SalI. After digestion with SalI, the fragment containing the SEAP cassette was cloned back into the full-length constructs pNDFL+ and pNDFLtag. Finally, the CmR gene was removed by Xbal digestion followed by self ligation. The resulting plasmids were named pHZ-SEAP/NP-P and pHZ-SEAP/NP-tag.

To insert the SEAP gene between the M and F genes, an oligonucleotide containing the recognition sequence for Apal (5’-GATCATGGGCCCCAT-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 Apal–NotI fragments (nt 2285–4952) of pNDFL+ and pNDFLtag were cloned into the Apal and NotI sites of modified pOK12 resulting in pOK12Apal/NotI. The BspHI site (nt 4427) of this plasmid was utilized to insert the CmR–GE–IG–GS–SEAP cassette. One extra BspHI site within the SEAP gene was removed by PCR mutagenesis. The CmR–GE–IG–GS–SEAP cassette was cloned into the BspHI site of pOK12Apal/NotI and cloned back into pNDFL+ and pNDFLtag after

**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 grown in the Glasgow modification of Eagle’s medium containing 5% foetal calf serum (FCS) and 2% antibiotic mix. QM5 cells were grown in medium 199 supplemented with 10% tryptose phosphate broth, 10% FCS and 2% antibiotic mix. QM5 cells were maintained in medium 199 supplemented with 10% tryptose phosphate broth, 10% FCS and 2% antibiotic mix. QM5 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 pELFL7pol (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’-CGCGATCGATGCGCCGCCGGCGATAGAAAGATCGAAGCCCCGTATAT-3’; 5’-CGATATCGCGCTCCTCGATCTCCACCGTGATATTGTTCATTATCGCGCCGCCCATCAGATCGGCGGAG-3’). The resulting plasmid was named pUC19/ES/SEAP/CmR.

Construction of full-length recombinant cDNAs containing the SEAP gene at different positions.

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 pUC19SalI. The BsrGI restriction enzyme site (TGTACA) at position 1719 was chosen to insert the CmR–GE–IG–GS–SEAP cassette. Using two specific oligonucleotide primers carrying the recognition sequence for BsrGI and using plasmid pUC19/ES/SEAP/CmR as a template, a PCR fragment harbouring the CmR–GE–IG–GS–SEAP cassette was produced and cloned into the BsrGI site of pUC19SalI. After digestion with SalI, the fragment containing the SEAP cassette was cloned back into the full-length constructs pNDFL+ and pNDFLtag. Finally, the CmR gene was removed by Xbal digestion followed by self ligation. The resulting plasmids were named pHZ-SEAP/NP-P and pHZ-SEAP/NP-tag.
Apal and NotI digestion. The CmR 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'-AATTACTAAGTGGCCGTCGC-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 CmR–GE–IG–GS–SEAP cassette. One extra copy of modified pUC19 was used for RT-PCR directly after elution in nuclease-free water. Viral RNA was isolated from allantoic fluid using a High Pure viral RNA kit (Roche). Viral RNA was used to insert the CmR–GE–IG–GS–SEAP cassette. The CmR 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 pNDFLtag(CmR) and pNDFLtag(CmR) were digested with AvrII and self-ligated to form a new plasmid named pAvr2. The EcoRI site at nt 15098 was used to insert the CmR–GE–IG–GS–SEAP cassette. The CmR gene was first removed by Xbal digestion and the new plasmid was designated pAvr2/ES/SEAP. After AvrII digestion, it was cloned back into the full-length constructs of pNDFL+ (CmR) and pNDFLtag(CmR). Finally, the CmR gene was removed by BsiWI digestion followed by ligation. 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 or QM5 cells were transfected with 1 µg full-length cDNA constructs together with 0·4 µg plasmid pCIneoNP, 0·2 µg pCIneoP and 0·2 µg pCIneoL or pCIneo (negative control) using 6 µl FuGene6 (Roche). After incubation for 3–5 days, the culture supernatants were harvested, passed through a 0·2 µm 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 µm filter and then through a 0·2 µm 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°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. ≥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 log10 of the TCID50 ml−1.

Quantification of SEAP expression levels. CEF cells in six-well plates were infected with each virus at an m.o.i. ≥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 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 (EID50) 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 µg 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 EID50 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 (TAAGAAAAATACGGGTAGAA) 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 antigenic cDNA; Peeters et al., 1999) at four different positions, yielding pHZ-SEAP/NP-P, pHZ-SEAP/M-F, pHZ-SEAP/
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 EID₅₀ 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
100 EID$_{50}$ 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 & Billetter, 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,
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 behind the L gene than when it was inserted between the NP and P, M and F, and HN and L 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 then 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.

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