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Short Communication

Influence of insertion site of the avian influenza virus haemagglutinin (HA) gene within the Newcastle disease virus genome on HA expression

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Members of the order *Mononegavirales* express their genes in a transcription gradient from 3′ to 5′. To assess how this impacts on expression of a foreign transgene, the haemagglutinin (HA) of highly pathogenic avian influenza virus (HPAIV) A/chicken/Vietnam/P41/05 (subtype H5N1) was inserted between the phosphoprotein (P) and matrix protein (M), M and fusion protein (F), or F and haemagglutinin–neuraminidase protein (HN) genes of attenuated Newcastle disease virus (NDV) Clone 30. In addition, the gene encoding the neuraminidase of HPAIV A/duck/Vietnam/TG24-01/05 (subtype H5N1) was inserted into the NDV genome either alone or in combination with the HA gene. All recombinants replicated well in embryonated chicken eggs. The expression levels of HA-specific mRNA and protein were quantified by Northern blot analysis and mass spectrometry, with good correlation. HA expression levels differed only moderately and were highest in the recombinant carrying the HA insertion between the F and HN genes of NDV.

Newcastle disease is a highly contagious infection of many avian species that causes substantial economic losses in the poultry industry worldwide. The causative agent is Newcastle disease virus (NDV), a negative-strand RNA virus belonging to the genus *Avulavirus* within the family *Paramyxoviridae* (Fauquet *et al.*, 2005) of the order *Mononegavirales*. Its genome is 15 186 nt long and encodes six structural proteins: the nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin–neuraminidase protein (HN) and the RNA-dependent RNA polymerase (L). The additional proteins V and W are transcribed from the P gene by an RNA editing mechanism (Steward *et al.*, 1993). Based on its pathogenicity in chickens, NDV is categorized into lentogenic, mesogenic and velogenic pathotypes. The virulence of an NDV strain is determined by the activation cleavage of the F protein by cellular proteases (Nagai *et al.*, 1976). A polybasic amino acid stretch between aa 112 and 116 of the F protein characterizes highly virulent NDV, whereas NDV of low virulence exhibits only single basic amino acids at this site (Collins *et al.*, 1993; Glickman *et al.*, 1988; Millar *et al.*, 1988; Toyoda *et al.*, 1987). To protect poultry from Newcastle disease, vaccination is practised worldwide using either inactivated or live vaccines based on lentogenic viruses such as NDV La Sota or Clone 30, which are applied by spray or drinking water.

The generation of recombinant NDV (rNDV) by reverse genetics (Krishnamurthy *et al.*, 2000; Peeters *et al.*, 1999; Römer-Oberdörfer *et al.*, 1999) has made NDV amenable to genetic manipulation. Insertion of foreign genes into the NDV genome has resulted in the development of bivalent vector vaccines. For this purpose, efficient expression of an immunogenic foreign protein by the NDV vector is required. Expression levels of genes of non-segmented negative-strand RNA viruses are regulated primarily by their position relative to the single promoter, and also by *cis*-acting sequences located at the beginning and end of each gene. For vesicular stomatitis virus, it has been shown that the amounts of mRNA and protein decrease following the order of transcription from the 3′-proximal to the 5′-distal end of the viral genome forming a transcription gradient (Wertz *et al.*, 2002; Whelan *et al.*, 2004). To assay whether this also applied to foreign transgenes, Zhao & Peeters (2003) inserted the gene encoding secreted alkaline phosphatase (SEAP) into different intergenic regions of the NDV genome and showed that SEAP activities were highest when the transgene was inserted between the NDV M and F genes, and not between the more 3′ insertion site between P and M. These results did not correlate with the proposed transcription gradient described for *Mononegavirales*.

Development of NDV vector vaccines expressing avian influenza virus (AIV) immunogens has become a promising option to simultaneously fight two important virus diseases of poultry. The negative-strand RNA genome of influenza A viruses (family *Orthomyxoviridae*) consists of eight segments coding for 11 proteins (Chen *et al.*, 2001; Palese & Shaw, 2007). The surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) exist in different

antigenic variations (H1–H16 and N1–N9), which are used to classify influenza A virus into various subtypes (Fouchier *et al.*, 2005; Webster *et al.*, 1992). A further classification into low pathogenic (LP), notifiable low pathogenic (NLP) and highly pathogenic (HP) pathotypes is made according to their ability to elicit disease (Alexander, 1997). Outbreaks of HPAIV are caused exclusively by the H5 and H7 subtypes. Both also have the potential to cause zoonotic infections in humans. Therefore, the control of HPAIV infections is essential for the poultry industry and to prevent human infections. Currently available influenza vaccines, based on inactivated whole-virus preparations or attenuated live virus, do not allow differentiation between infected and vaccinated animals (the DIVA principle; reviewed by Fuchs *et al.*, 2009). This problem could be overcome by vector vaccines. The successful use of NDV as a vector for the expression of AIV HA has been described in different studies, where the foreign gene was integrated between the P and M genes (DiNapoli *et al.*, 2010; Ge *et al.*, 2007; Nakaya *et al.*, 2001; Park *et al.*, 2006) or the F and HN genes (Schröer *et al.*, 2009; Veits *et al.*, 2006). In both cases, the recombinant viruses conveyed protection against NDV, as well as against influenza infection of the corresponding subtype. However, an improved virus vaccine that results in higher antibody levels against AIV after immunization is desirable. Recently, it was shown that co-immunization with infectious laryngotracheitis virus (ILTV) recombinants expressing H5 and N1 further increased the efficacy of an ILTV vector vaccine (Pavlova *et al.*, 2009). Enhanced protection against lethal influenza virus infection has also been achieved by other vector vaccines co-expressing HA and NA (Chen *et al.*, 1999; Johansson, 1999; Qiao *et al.*, 2003).

In this study, we created different NDV/AIV recombinants to analyse whether transcription of the foreign gene followed the NDV transcription gradient (Lamb & Kolakofsky, 2001; Sakai *et al.*, 1999; Wertz *et al.*, 1998). If so, insertion of the AIV H5 gene in a more 3′-proximal position should result in higher protein yields. Therefore, H5 of HPAIV A/chicken/Vietnam/P41/05 (H5N1) was inserted into the NDV genome between the P and M, M and F, or F and HN genes. In two additional recombinants, N1 of HPAIV A/duck/Vietnam/TG24-01/05 (H5N1) was inserted between the F and HN genes alone or together with an H5 insertion between P and M (Fig. 1). All rescued viruses were characterized *in vitro*, and the expression levels of AIV HA and NA were determined by Northern blot analysis. HA protein expression was also quantified by mass spectrometry.

Lentogenic vaccine strain Clone 30 (GenBank accession no. Y18898) served as the NDV backbone for all recombinant viruses. Sequences for HA were based on HPAIV strain A/chicken/Vietnam/P41/05 (H5N1) (GenBank accession no. AM183672) and for NA on HPAIV A/duck/Vietnam/TG24-01/05 (H5N1) (GenBank accession no. AM183678). The sequence of H5 was identical to that of the previously described HA of recombinant NDVH5Vm (Römer-Oberdörfer *et al.*, 2008). Recombinant NDVH5VmPM was generated by insertion of the amplified H5 gene of NDVH5Vm using primers with *Mlu*I sites, and gene end as well as gene start signal sequences. The amplified fragment consisted of an *Mlu*I site, the gene end signal sequence of the NDV P gene, nucleotide T as an intergenic region, the gene start signal sequence of the NDV HN gene, the non-coding region of the NDV HN gene, the ORF of AIV H5, the non-coding region of the NDV HN gene and another *Mlu*I site. The NDV backbone was altered by the introduction of an artificial single *Mlu*I site in front of the P gene end signal sequence after back mutation of known artificial *Mlu*I sites of the rNDV genome (Römer-Oberdörfer *et al.*, 1999). NDVH5VmMF was obtained by insertion of the H5 gene flanked by *Mlu*I sites into a newly created single *Mlu*I site in front of the gene end signal sequence of M using an NDV genome without other artificial *Mlu*I sites. NDVN1FHN was generated by substitution of the H5Vm ORF by the N1 ORF using *Nco*I and *Afl*II sites. As the AIV N1 ORF contains an *Nco*I site, insertion was carried out in two steps. The resulting N1 ORF sequence differed by 1 nt from the sequence in GenBank as a result of the newly generated *Nco*I site, which was used for cloning as described previously (Veits *et al.*,

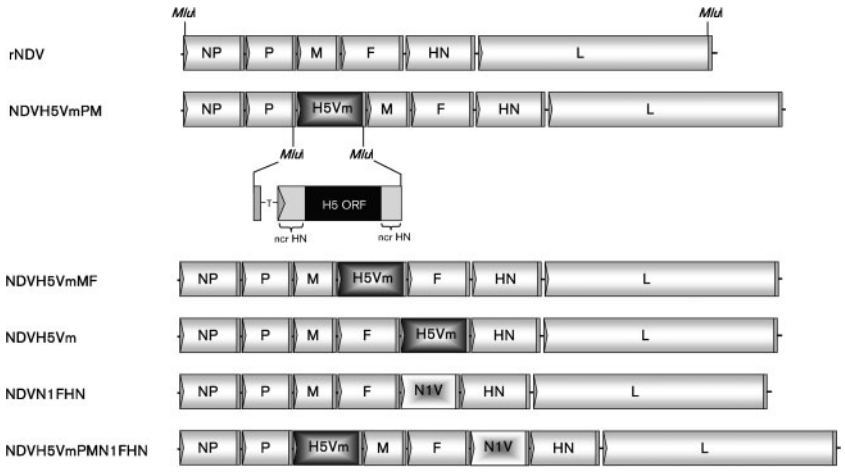

Fig. 1. Schematic representation of the genome organization of NDV and recombinants with insertion of the HA and/or NA genes of HPAIV H5N1. Transcription control signals are indicated by a triangle for the transcription start and a shaded rectangle for the transcription stop sequences. ncr, Non-coding region.

2006). NDVH5VmPMN1FHN expressing AIV HA and NA was constructed by substitution of the *Not*I–*Bsi*WI fragment of NDVH5VmPM by that of NDVN1FHN (Fig. 1). Recovery of infectious NDV from cDNA was performed on BSR-T7/5 baby hamster kidney cells, which stably express phage T7 RNA polymerase (Buchholz *et al.*, 1999). The *in vivo* replication of recovered virus was determined in 10-day-old specific-pathogen-free (SPF) chicken eggs, which were inoculated with 200 μl 10^3 $TCID_{50}$ rNDV ml^{-1}. Virus titres in allantoic fluid were determined on QM9 cells. All the generated NDV/AIV recombinants replicated well, independent of the AIV H5 insertion site, although with a delay in onset of replication compared with wild-type NDV Clone 30 (Fig. 2). All the generated NDV/AIV recombinants reached a final titre ($TCID_{50}$ ml^{-1}) of $10^{8.0}$–$10^{8.5}$ (Fig. 2), demonstrating that neither integration of the AIV H5 gene alone nor simultaneous integration of the H5 and N1 genes into the NDV genome had a detrimental effect on virus replication, despite the considerable size of the inserts of more than 3500 nt. Expression and cleavability of the HA protein of the NDV/AIV recombinants was determined by Western blot analysis. Proteins of virus-infected chicken embryo fibroblasts (CEFs) were separated by SDS-PAGE and transferred to nitrocellulose membranes followed by incubation with polyclonal monospecific rabbit serum directed against AIV H5 or AIV N1, and incubation with the respective HRP-labelled secondary antibody. Three proteins of approximately 70, 50 and 25 kDa were detected after incubation with the AIV H5-specific antiserum, corresponding to the uncleaved HA0 and the cleavage products HA1 and HA2 (Fig. 3a). The HA protein with a polybasic cleavage site expressed by the recombinants NDVH5VmPM, NDVH5VmMF, NDVH5Vm and NDVH5VmPMN1FHN

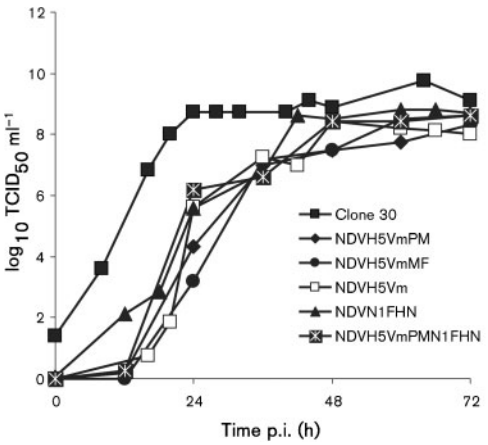

Fig. 2. Replication kinetics of wild-type NDV Clone 30 and recombinants expressing one or two AIV proteins in embryonated SPF chicken eggs. Eggs were infected with 200 μl 10^3 $TCID_{50}$ ml^{-1} of the indicated viruses and the titres of progeny virus in allantoic fluids were determined at the indicated time points by titration on QM9 cells, followed by indirect immunofluorescence analysis.

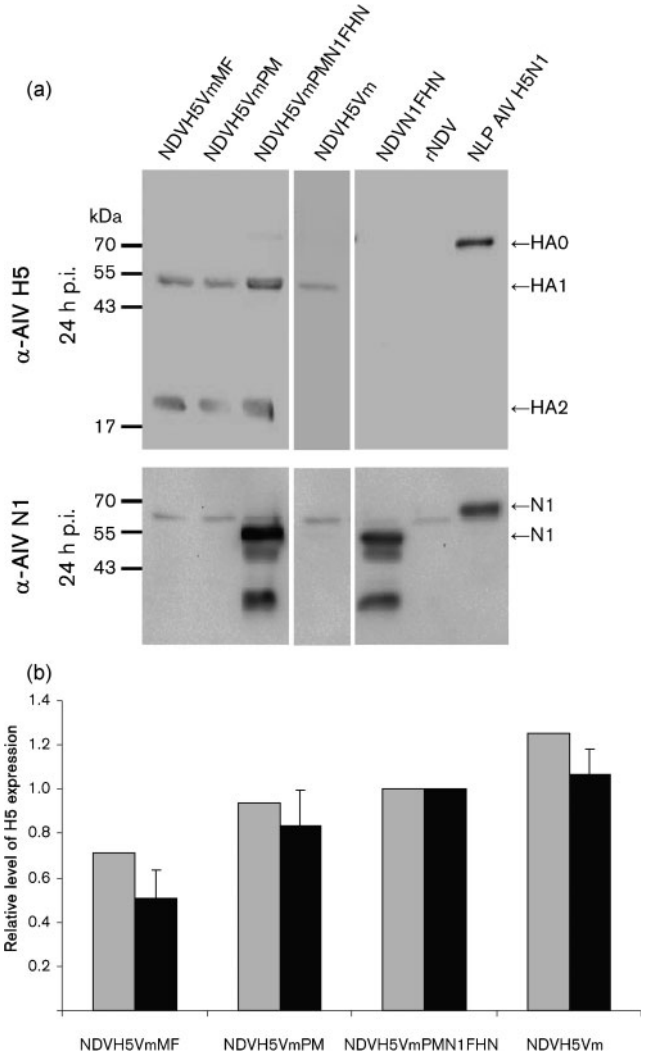

Fig. 3. (a) Western blot analyses of NDV recombinants. Proteins of infected CEFs were separated by SDS-PAGE and blotted onto nitrocellulose. Membranes were incubated with monospecific rabbit anti-AIV H5 serum and monospecific rabbit anti-AIV N1 serum. Proteins were detected by chemiluminescence after incubation with suitable HRP-labelled secondary antibodies. (b) Relative mRNA and protein expression levels of AIV H5 in infected DF-1 cells. mRNA was quantified by Northern blot analysis (shaded columns) and protein by quantitative mass spectrometry using the SILAC technique (filled columns). Error bars indicate the SD of three independent experiments. Protein and mRNA levels were normalized to the NDV P expression levels from the same samples.

was completely processed by cellular proteases. In contrast, the HA0 protein with a monobasic cleavage site expressed by NLPAIV H5N1 (A/common teal/Germany/Wv632/05), which was used as a control, remained mainly uncleaved (Fig. 3a). As expected, no HA protein was present in NDVN1FHN or rNDV. Expression of the N1 protein was confirmed for the recombinants NDVN1FHN, NDVH5VmPMN1FHN and NLPAIV H5N1 (A/common teal/Germany/Wv632/05) with an N1-specific antiserum (Fig. 3a). Size differences in the

N1 proteins expressed by the NDV recombinants (A/duck/Vietnam/TG24-01/05, GenBank accession no. AM183678) and wild-type AIV (A/common teal/Germany/WV632/05, GenBank accession no. AM913983.1) reflected differences in the lengths of the ORFs (450 vs 470 aa, respectively) and different numbers of potential glycosylation sites (three and seven, respectively).

The relative H5- and N1-specific mRNA levels of the NDV/AIV recombinants were determined by Northern blot analysis. Total RNA was isolated from the virus-infected CEFs, separated in denaturing agarose gels, transferred to nylon membranes and hybridized with ^{32}P-labelled antisense RNA specific for AIV H5, AIV N1 or NDV P. The signals were quantified by radioluminography using a FLA-3000 scanner (Fujifilm) and Advanced Image Data Analyser software (Raytest) for image analysis. The relative mRNA levels of HA and NA were calculated by normalization of their absolute signals to the P-specific signal in the same sample. For comparison of the different viruses, the relative mRNA levels of H5 as well as of N1 were again normalized to the level expressed by NDVH5VmPMN1FHN, which was set to 1.0. H5 mRNA expression of NDVH5Vm was 25 % higher compared with NDVH5VmPMN1FHN, whereas the H5 mRNA expression of NDVH5VmPM and NDVH5VmMF reached 94 and 71 % of the level found for NDVH5VmPMN1FHN (Fig. 3b). As expected, insertion of AIV H5 in a more 3′-proximal position than N1 led to a decrease in N1-specific mRNA. N1 mRNA expression from NDVH5VmPMN1FHN amounted to 53 % of that observed for NDVN1FHN (data not shown).

Relative HA protein expression levels in the NDV/AIV mutants were assayed by a combination of two-dimensional (2D) electrophoresis and quantitative mass spectrometry using the stable isotope labelling with amino acids in cell culture (SILAC) technique (Ong *et al.*, 2002) with some modifications (Skiba *et al.*, 2008). This procedure is highly precise and has been used for the quantification of cellular proteins in proteome studies but also to determine expression levels of viral proteins (Skiba *et al.*, 2008, 2010). In preceding 2D electrophoretic analyses with cell extracts from uninfected and NDV-infected cells and cells infected with one of the recombinants, HA, the NDV-specific P and a number of cellular proteins were located in 2D gels and identified by mass spectrometry and in 2D Western blots. For the quantification experiment, extracts from cells infected with NDVH5VmMF, NDVH5VmPM and NDVH5Vm were mixed with an extract from deuterium-labelled cell cultures infected with NDVH5VmPMN1FHN as an internal standard. The mix was separated by 2D electrophoresis and the relative HA levels were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. For the calculation of HA-specific relative mRNA levels, the relative expression level of the HA protein was normalized to the expression level of NDV P, which was determined in the same manner from P samples isolated from the same gel. The relative HA protein levels were again normalized to the internal standard, recombinant NDVH5VmPMN1FHN, which was set to 1.0. As shown in Fig. 3(b), levels of HA mRNA and protein expression correlated well, indicating that HA mRNA and protein synthesis are tightly linked. Maximum HA protein expression was observed after infection with recombinant NDVH5Vm, which was approximately twofold higher than after infection with recombinant NDVH5VmMF in which the weakest expression was observed. As mentioned above, Zhao & Peeters (2003) constructed a panel of NDV recombinants expressing the reporter gene encoding SEAP in different positions within the genome. The expression levels of SEAP were highest after insertion between the M and F genes, and lowest after insertion between NP and P. In our study, we detected the highest mRNA and protein levels of AIV H5 for NDV recombinants with the H5 insertion between F and HN. Although Zhao & Peeters (2003) did not test the insertion site between the F and HN genes, their results as well as ours are in contrast to expression levels of NDV proteins, which decrease from the 3′-proximal to the 5′-distal end of the viral genome (Lamb & Kolakofsky, 2001; Sakai *et al.*, 1999; Wertz *et al.*, 1998). As the foreign H5 transgene of all our recombinants was identical, differences in H5 mRNA and protein level were caused by the different insertion sites within the NDV genome.

Nevertheless, the observed differences in H5 expression levels of our NDV recombinants were moderate, indicating that transgenes can be inserted at different positions in the NDV genome without severely affecting the replication capacity of the recombinant virus. This result is consistent with the results of Zhao & Peeters (2003) for expression of SEAP.

In summary, we showed that NDV can accommodate HA, NA or both AIV glycoproteins, increasing the virus genome by more than 3500 nt, without significantly affecting virus replication. The *in vitro* expression levels of AIV H5 mRNA and protein were to some extent influenced by the location of the insertion site of the transgene within the NDV genome. Transgenic HA expression levels were highest when the HA gene was inserted between the NDV F and HN genes. However, even then they were only twice as high as the lowest expression level detected in our assay. Animal experiments will be needed to demonstrate whether these moderate differences in HA protein expression have an effect on the immune response after vaccination and protection of immunized chickens against HPAIV.

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