Adaptive mutation in nuclear export protein allows stable transgene expression in a chimaeric influenza A virus vector

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The development of influenza virus vectors with long insertions of foreign sequences remains difficult due to the small size and instable nature of the virus. Here, we used the influenza virus inherent property of self-optimization to generate a vector stably expressing long transgenes from the NS1 protein ORF. This was achieved by continuous selection of bright fluorescent plaques of a GFP-expressing vector during multiple passages in mouse B16f1 cells. The newly generated vector acquired stability in IFN-competent cell lines and in vivo in murine lungs. Although improved vector fitness was associated with the appearance of four coding mutations in the polymerase (PB2), haemagglutinin and non-structural (NS) segments, the stability of the transgene expression was dependent primarily on the single mutation Q20R in the nuclear export protein (NEP). Importantly, a longer insert, such as a cassette of 1299 nt encoding two Mycobacterium tuberculosis Esat6 and Ag85A proteins, could substitute for the GFP transgene. Thus, the inherent property of the influenza virus to adapt can also be used to adjust a vector backbone to give stable expression of long transgenes.

INTRODUCTION

The delivery and expression of foreign genes by viral vectors can serve a number of different medical purposes including: (i) the development of new diagnostic tests and screening procedures using viral vectors expressing reporter genes (Heaton et al., 2013; Manicassamy et al., 2010; Ozawa et al., 2011; Pena et al., 2011; Rimmelzwaan et al., 2011), (ii) the construction of armed oncolytic viruses (Kittel et al., 2005; Wolschek et al., 2011) and (iii) the generation of vector-based vaccines against pathogens that cannot be cultivated or attenuated (García-Sastre & Palese, 1995; He et al., 2007; Langley et al., 2010; Sereinig et al., 2006).

Influenza A virus potentially has a number of advantages as a viral vector due to its ability to induce a strong humoral and cell-mediated immune response upon mucosal administration, the absence of any DNA intermediate life-cycle stages and a ubiquitously expressed viral entry receptor (García-Sastre, 2000; García-Sastre & Palese, 1995; Neumann & Kawaoka, 2002; Neumann et al., 2002). Multiple serological subtypes enable prime–boost repetitive immunizations. Moreover, genetically engineered attenuated vaccine viruses have been found to be stable and safe when applied to animals or humans for vaccination purposes (Pena et al., 2011; Song et al., 2007; Wachek et al., 2010; Wang et al., 2008). However, the small size of the viral genome segments ranging from 890 to 2341 nt and a high mutation rate of the genome significantly hamper the development and production of stable influenza virus vaccine vectors for medical purposes.

Despite its smallest size within the influenza virus genome, the non-structural (NS) segment appeared to
be the most feasible target for the expression of foreign sequences. This segment encodes the non-structural IFN-antagonizing protein (NS1) and the nuclear export protein (NEP), the latter encoded by a spliced mRNA. As the NEP is highly conserved among influenza viruses, it appears to have little tolerance to manipulation. In contrast, the NS1 protein can be truncated, providing space for insertions of foreign sequences (Egorov et al., 1998; Ferko et al., 2001). We previously succeeded in constructing a stable influenza A virus vector by replacing the entire NS1 sequence by various ORFs encoding cytokines (Wolschek et al., 2011). As the size of the NS1 truncation is inversely proportional to the level of attenuation in IFN-competent systems (Egorov et al., 1998; García-Sastre et al., 1998), vectors completely lacking the NS1 are replication defective in IFN-competent hosts. This limits their application as a potent vaccine or as an effective oncolytic vector, as replication-competent viruses appear to be more effective for these purposes (Muster et al., 2004; Talon et al., 2000).

Several strategies have been described to successfully express chimaeric ORFs such as GFP by influenza A viruses that contain full-sized or partially truncated NS1 proteins (Heaton et al., 2013; Kittel et al., 2004; Manicassamy et al., 2010; Pena et al., 2013). A partial deletion of NS1 might be of specific value for a vector, as this modification attenuates the virus by enhancing the immune response and has been shown to be a valuable strategy by which to obtain oncolytic viruses (Muster et al., 2004).

Previously, we constructed vectors encoding only the N-terminal half of the NS1 protein, which appeared to be replication deficient in vivo and showed a rapid loss of the transgene when passaged in IFN-competent cells or murine lungs, limiting their suitability for medical applications (Ferko et al., 2001; Kittel et al., 2004). To improve the tolerance of potential influenza virus vectors to maintain insertions, we exploited the intrinsic property of the influenza virus genome to efficiently adapt to a new host or cultivation conditions (Neumann et al., 2002; Rimmelzwaan et al., 2011) by mutational changes. A previously obtained low-level GFP-expressing influenza vector (Kittel et al., 2005) was optimized by a continuous selection procedure of harvesting the brightest fluorescent plaques during virus passages in Vero and B16f1 cell lines. As a result, we obtained a viral vector that not only expressed high levels of GFP but also retained stable expression of the transgene in cell lines and in murine lungs. Moreover, the GFP insert could be replaced by other transgenes such as a cassette of the Mycobacterium tuberculosis protective antigens, namely Esat6–Ag85A with an overall length of 433 aa. It is noteworthy that the latter recombinant NS segment spanned 2147 nt corresponding to the size of polymerase genes and was still effectively packaged, maintained and expressed during multiple passages of the virus in cell culture.

RESULTS

Two-step selection of a virus with high and stable GFP expression

First, we optimized a previously obtained NS1–GFPStSt vector expressing GFP from a separate ORF, arranged by the insertion of an overlapping stop–start codon sequence downstream of the aa 124 position of the NS1 protein (Kittel et al., 2005). The expression of GFP by this vector was at the border of the detection limit. However, we noted the occasional appearance of single plaques with bright fluorescence in Vero cells, and these were picked for selection of a naturally occurring high-level GFP-expressing variant. After 10 rounds of plaque purification, we obtained the ‘original’ NS116–GFP/O vector that was able to form brightly fluorescent as well as non-fluorescent plaques at a ratio of 1:1. Sequence analysis of the GFP-expressing clone revealed a deletion in the NS1 segment, upstream of the GFP insert, resulting in a frame shift and loss of the stop codon leading to the formation of an NS1–GFP fusion protein. The new virus NS116–GFP/O contained only the N-terminal 104 aa of NS1 fused to GFP via a stretch of 12 aa derived from frame 2 of the NS segment (Fig. 1a). Additionally, NS1–GFP contained 29 aa at its C-terminal end derived from the multiple cloning site of the initial plasmid vector. When tested on IFN-competent B16f1 mouse melanoma cells, this virus displayed only limited growth not exceeding 4.5 log TCID50 ml−1 (Fig. 1b) and weak fluorescence (Fig. 1c).

To generate a virus that expressed high levels of GFP and that also grew well in IFN-competent cells, a second optimization step was performed in B16f1 cells. This included 15 rounds of plaque purification of the NS116–GFP/O virus, selecting the brightest fluorescent plaques. As a result, the ‘adapted’ vector NS116–GFP/A started to grow to a titre of 7 log TCID50 ml−1 in B16f1 cells (Fig. 1b), showing uniform bright fluorescence of the infected cells (Fig. 1c). As stated above, the parental GFPStSt induced such bright plaques only occasionally. It should be noted that, similar to the GFPStSt virus (Kittel et al., 2005), both the NS116–GFP/O and the NS116–GFP/A viruses grew at titres of up to 7 log TCID50 ml−1 in Vero cells.

The genetic stability of both viral vectors was compared by assessing the ratio between GFP-positive and the total number of visible plaques after five consecutive passages of each virus in B16f1 or Vero cells. Whereas the NS116–GFP/O virus could form only 44 and 17 % GFP-positive plaques in B16f1 and Vero cells, respectively, the adapted vector NS116–GFP/A showed 97 and 98 % GFP-positive plaques in these cells (Fig. 2a).

Growth and genetic stability in vivo

Next, we investigated whether adapted NS116–GFP/A virus also acquired genetic stability in vivo. Infection of mice revealed that both the original and adapted viruses could
grow in murine lungs at titres of up to 6 log TCID\textsubscript{50} ml\textsuperscript{-1} and were cleared equally at day 6 after infection. Both viruses were attenuated in comparison with PR8 wt strain as they were cleared more rapidly (Fig. 2b). Moreover, whereas the wt virus is characterized by an LD50 of 4.1 log TCID\textsubscript{50}, the vector still lacked a lethal activity at 7 log TCID\textsubscript{50}. The genetic stability of the vectors after replication in murine lungs was assessed by comparison of titres (TCID\textsubscript{50} ml\textsuperscript{-1}) calculated by counting GFP-positive or cytopathic effect (CPE)-positive wells. Whereas the NS\textsubscript{116}–GFP/O virus gradually lost GFP expression during replication in murine lungs (more than a 2 log reduction in GFP-positive titre versus total titres on day 4), the NS\textsubscript{116}–GFP/A virus stably expressed the transgene until the

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**Fig. 1.** (a) Schematic representation of recombinant NS segment structures NS1–GFPStSt (above) and NS\textsubscript{116}–GFP (below) viruses. NS1 represents 125 or 104 aa of the ORF from NS1, whilst the NEP ORF was expressed via splicing as indicated. The light blue box represents the 12 aa shared between NS1 and NEP, the black box represents non-translated parts, the light grey box represents nucleotides encoding a random 12 aa fused to GFP (SLYQNGPGDHGL) and the dark grey box represents coding sequence derived from the initial GFP cloning vector for 29 aa; ncr is the non-coding region. Nucleotide positions are indicated at the top. (b) Growth kinetics of chimaeric viruses in B16f1 and Vero cells. Cells were infected with NS\textsubscript{116}–GFP/O or NS\textsubscript{116}–GFP/A virus at an m.o.i. of 0.01. At the indicated time points post-infection (p.i.), supernatant was collected and virus titre was determined by TCID\textsubscript{50} assay on Vero cells. (c) Expression of GFP by chimaeric viruses. B16f1 cells were infected with each virus at an m.o.i. of 0.01 and images were taken at 48 h p.i. under a fluorescence microscope.
end of replication on day 6 (Fig. 2b). Comparing total versus GFP + titres of this vector indicated that only a minimal amount viral progeny (<0.3 log TCID50 ml−1) lost transgene expression.

**Genetic changes in NS116–GFP/A virus versus NS116–GFP/O virus**

Total genome sequencing of the NS116–GFP/A virus revealed only four coding mutations in the viral genome compared with the NS116–GFP/O virus. These mutations were found in ORFs encoding the polymerase proteins PB2, the haemagglutinin (HA), NEP and the chimaeric NS1–GFP (Table 1). Interestingly, the mutation in the NS1–GFP ORF (Y368H) was found in the plasmid-derived 29 aa tail downstream of the GFP sequence. All segments of the NS116–GFP/A virus were cloned into the bidirectional plasmid pHW2000 (Hoffmann et al., 2000), and were subsequently used to generate a complete synthetic NS116–GFP/A virus. This cloned virus had identical properties to the selected adapted variant with respect to viral growth and stability of GFP expression, providing further evidence that no other changes accounted for the observed different viral phenotype. It should be noted that the backbone of NS116–GFP/O contained 25 mutations compared with published influenza A [A/Puerto Rico/8/34/Mount

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**Fig. 2.** (a) Genetic stability of NS116–GFP/O and NS116–GFP/A viruses in vitro. The number of GFP-positive plaques formed by NS116–GFP/O or NS116–GFP/A virus on Vero or B16f1 cells was determined as a percentage of the total number of plaques (100 %) in a plaque assay. Results are shown as means ± SEM of three independent experiments. (b) Virus replication and genetic stability of NS116–GFP/O and NS116–GFP/A in vivo. BALB/c mice were infected intranasally under narcosis with the indicated viruses. On days 2, 4 and 6, the amount of virus in murine lungs was titrated using a limiting dilution assay on Vero cells. Viral titres (log10 TCID50 ml−1) were determined by estimating cytopathic effect (CPE +) and fluorescence (GFP +). Data represent means ± SEM from three independent experiments. (c) Growth kinetics of mutant viruses in B16f1 and Vero cells. Subconfluent monolayers of cells were infected with the indicated viruses at an m.o.i. of 0.01. At the indicated time points p.i., supernatant was collected and the number of virus particles was determined by a TCID50 assay on Vero cells. Data represent means ± SEM (n=3).
Sinai (H1N1) wt virus sequences (GenBank accession nos AF389115.1, AF389116.1, AF389117.1, AF389118.1, AF389119.1, AF389120.1, AF389121.1 and AF389122.1) (Table S1, available in JGV Online). Thus, these viruses can be regarded as influenza PR8-like viruses.

Influence of genetic changes on viral phenotype

To understand which of the genetic changes between the two viruses accounted for the differences in viral phenotype, we reverted each acquired mutation in the PB2 or NS segment of the NS116–GFP/A virus to the corresponding sequence of the NS116–GFP/O virus. The role of the HA in viral growth and stability was tested by exchanging the entire PR8-derived HA segment with the heterologous HA segment of the influenza A/New Caledonia/20/99 strain. The rescued viruses and their phenotypes are presented in Table 2.

With respect to growth, the NS116–GFP/A/NS1 368 and NS116–GFP/A/NEP20 viruses showed significantly decreased viral titres in both IFN-deficient Vero and IFN-competent B16f1 cells (Table 2, Fig. 2c). The mutation in the PB2 gene did not affect viral growth on Vero or on B16f1 cells. The exchange of the whole HA segment of the NS116–GFP/A (H1N1) virus with the New Caledonia strain HA (NS116–GFP/A/HA virus) led to a significant reduction in growth in both cell lines. Thus, both NS segment-related mutations as well as the origin of HA had an impact on growth restriction of viruses irrespective of the IFN status of the tested cell lines.

To study the influence of these mutations on the genetic stability of the transgene, the same viruses were analysed to determine the percentage of GFP-positive plaques compared with the total number of plaques after five passages in Vero and B16 cells. Surprisingly, only reversion of the mutation in the NEP (mutant virus NS116–GFP/A/NEP20) led to a significant reduction in the number of GFP-positive plaques (Table 2). For all the other revertants the stability of transgene expression was not significantly altered compared with the NS116–GFP/A (Table 2). The reversion of aa 535 in PB2 led to a tendency towards decreased stability but did not reach a statistically significant level.

Table 1. Nucleotide and amino acid changes of NS116–GFP/A virus in comparison with NS116–GFP/O virus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virus</th>
<th>Nt position</th>
<th>Nt</th>
<th>Aa</th>
<th>Aa position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>NS116–GFP/O</td>
<td>1632</td>
<td>G</td>
<td>535 M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS116–GFP/A</td>
<td>A</td>
<td></td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td>NS116–GFP/O</td>
<td>1467</td>
<td>G</td>
<td>479 G</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS116–GFP/A</td>
<td>A</td>
<td></td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>NEP</td>
<td>NS116–GFP/O</td>
<td>1128</td>
<td>T</td>
<td>368 Y</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NS116–GFP/A</td>
<td>C</td>
<td></td>
<td>H</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Maximal viral titres and stability of the adapted-change-reversion viruses

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Max. viral titre (log10 TCID50 ml–1)*</th>
<th>Stability: % GFP+ plaques/total plaques after five passages*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS116–GFP/O</td>
<td>4.5 ± 0.1†</td>
<td>73.5 ± 0.2†</td>
</tr>
<tr>
<td>NS116–GFP/A</td>
<td>4.0 ± 0.1†</td>
<td>80.0 ± 0.2†</td>
</tr>
<tr>
<td>NS116–GFP/A/NS1 368</td>
<td>3.5 ± 0.3†</td>
<td>61.2 ± 0.3†</td>
</tr>
<tr>
<td>NS116–GFP/A/NEP20</td>
<td>3.4 ± 0.4†</td>
<td>6.3 ± 0.4†</td>
</tr>
<tr>
<td>NS116–GFP/A/PB2 535</td>
<td>3.2 ± 0.2†</td>
<td>7.3 ± 0.3†</td>
</tr>
<tr>
<td>NS116–GFP/A/HA segment from NCd 4.7 ± 0.2†</td>
<td>94.6 ± 2.3†</td>
<td>98.0 ± 0.8†</td>
</tr>
</tbody>
</table>

*Data are presented as means ± SEM (n=3 for titre values, n=6 for stability values).
†P<0.05, two-tailed t-test, compared with NS116–GFP/A.
‡The whole segment was exchanged.
statistical significance ($P=0.25$). The exchange of the HA did not affect the stability of transgene expression, irrespective of the HA origin. We therefore reasoned that the mutation in the HA had little impact on genetic stability and we did not further analyse the impact of single nucleotide change in this segment. Thus, the acquired stability of the adapted vector was not related to an adjustment within chimaeric NS1–GFP and its capacity to antagonize the IFN system, but was possibly related to some function of the NEP.

**Involvement of NEP Q20R mutation in regulation of NEP splicing**

To understand how the NEP Q20R mutation can influence GFP expression, we analysed the expression of NEP and NS1–GFP viral proteins in B16f1 and Vero cells. Immunofluorescence analysis revealed in both cell lines an earlier appearance and a higher accumulation of the NEP after infection with virus NS116–GFP/O compared with the adapted virus NS116–GFP/A (Fig. 3a). Correspondingly, the NS116–GFP/O virus demonstrated a low GFP-specific signal, reflecting diminished synthesis of the NS1–GFP fusion protein, whereas the adapted vector NS116–GFP/A revealed a clearly visible high GFP expression. As the NEP is expressed via splicing, we next analysed the ratio of NEP versus the NS1–GFP mRNA by quantitative PCR (qPCR) on the nuclear and cytoplasmic fractions at 4 and 6 h post-infection (p.i.). This indicated that the ratio was shifted towards NS1–GFP mRNA by the adaptation. Interestingly, both revertants in the NS segment (NEP20 and NS1 368) led to an even higher NEP : NS1–GFP ratio compared with the original virus, indicating an individual role of mutations in the regulation of splicing. These observations were supported by immunoblotting analysis (Fig. S1), indicating that NS116–GFP/A expressed higher levels of GFP but lower levels of NEP when compared with the NS116–GFP/O virus. Revertant viruses NEP20 and NS1 368 also expressed much lower levels of NS116–GFP/O virus. Specifically, the NS1 368 virus revealed very low virus-specific protein expression, again suggesting specific roles of these mutations by themselves.

**Correlation of viral growth and stability with IL-6 induction**

It is well documented that the NS1 protein of influenza virus is responsible for antagonizing the innate immune response in infected cells (Garcia-Sastre et al., 1998; Palese et al., 1999). Thus, impairment of NS1 function by inserting a foreign sequence may modulate cytokine production during viral infection. We therefore tested the expression of several pro-inflammatory cytokines upon infection of B16f1 cells with viruses NS116–GFP/O and NS116–GFP/A, as well as with viruses containing reverted changes. We observed only minimal differences in virus-induced TNF, IFNs and monocyte chemotactic protein-1 among the different viruses. Moreover, there was no significant difference in the expression of mRNA specific for IFN-stimulatory gene 15 (ISG15), Mx1 or type I IFN as determined by qPCR. However, NS116–GFP/O induced a high level of IL-6 production (995 pg ml$^{-1}$), whereas NS116–GFP/A induced significantly less of this cytokine (266 pg ml$^{-1}$) ($P<0.05$, two-tailed $t$-test; Fig. 4). The measurement of IL-6 concentrations induced by the revertant viruses indicated that mutations in the NS segment could cause a dramatic increase in IL-6 induction, whereas the mutation in PB2 did not have any significant influence. Thus, the adapted vector acquired a better capacity to antagonize IL-6 in B16f1 cells, presumably due to increased production of NS1–GFP chimaeric protein (Fig. 4).

**Capacity of the selected vector to maintain longer inserts**

We then investigated whether the vector backbone of the NS116–GFP/A virus would also allow stable expression of longer foreign sequences. Thus, the sequence of GFP was replaced by those of the two tuberculosis (TB) vaccine antigens, Esat6 and Ag85A, which are considered important antigenic determinants for inclusion into novel TB vaccine candidates (Skeiky & Sadoff, 2006). The sequence of the Esat6 antigen (97 aa) was linked to the C-terminal end of the truncated NS1 protein, followed by the sequence of the Ag85A antigen (297 aa), the latter being preceded by a 2A autocleavage site (Percy et al., 1994) and a modified mouse IgK-derived signal peptide (Wolschek et al., 2011) (Fig. 5a). The virus NS116–Esat6Ag85A/A was successfully rescued in Vero cells.

Comparison of the growth of the NS116–GFP/A virus with that of the NS116–Esat6Ag85A/A vector revealed that elongation of the NS segment up to 2144 nt in the viral vector did not significantly change viral titre in Vero or B16 cells (Fig. 5b). Most importantly, substituting GFP with the TB antigens led to a stable vector: reverse transcription-PCR (RT-PCR) analysis using NS-specific primers indicated that the chimaeric NS116–Esat6Ag85A/A segment was retained in size for at least 15 serial passages in Vero cells with high (Fig. 6a) or low (Fig. S2a) m.o.i. Sequence analysis of the NS116–Esat6Ag85A/A virus carried out after 15 passages in Vero cells confirmed the correct presence of foreign sequences within the NS1 ORF and did not reveal any compensatory mutations.

Immunofluorescence staining analysis confirmed that both TB antigens were expressed in infected cells after 15 passages of the virus in Vero cells. As expected, the intracellular localization of Esat6 antigen was predominantly nuclear due to the fusion to NS1, whereas the Ag85A antigen driven by the leader peptide accumulated in the Golgi compartment (Fig. 6b, Fig. S2b).

**DISCUSSION**

In this study, we showed that it is possible to generate a stable chimaeric influenza A virus vector by virtue of
adaptation of the virus to the insert. Importantly, the optimized vector, despite being adapted to a mouse melanoma cell line, retained the acquired genetic stability in the different hosts of monkey tissue culture cells and mice. The GFP insert in the NS1 ORF appeared to be dispensable and could be exchanged by a cassette of TB antigens spanning an overall length of 433 aa. It is noteworthy that the resulting recombinant NS segment containing 2147 nt corresponded to the size of polymerase genes. Despite this length, it was effectively packaged, maintained and expressed during multiple passages of the virus in cell culture.

Interestingly, the phenotypic difference in the two vectors (NS16-GFP/O and NS16-GFP/A) was associated with the appearance of only four mutations. Thus, adjusting the virus to high and stable expression of a transgene can be mediated by only a few genetic changes. It should be noted that the viral backbone of NS16-GFP/O has 25 mutations compared with the influenza A/PR8 wt virus. Therefore, the analysis of changes between the NS16-GFP/O and NS16-GFP/A vectors might be virus specific but might have different effects in the context of wt PR8 virus or other strains.

Most interestingly, the stability of the vector appeared to be regulated mainly by the single NEP20 mutation, which could suggest a direct influence of the NEP function in this context. The stability-promoting mutation was found within the nuclear export signal sequence of the NEP, which spans positions 12–21 (O’Neill et al., 1998). Mänz et al. (2012) recently showed that the mutation M161 in the nuclear export signal is required for host adaptation of an avian virus to human cells. The authors further suggested that the interaction of the NEP with the polymerase protein PB2 might improve the function of the polymerase in a host-dependent manner. The fact that the adapted virus revealed mutations in both NEP and PB2 might support a similar relevant interaction of the two proteins for the adaptation process of the viruses described here. In line with this argument, Bullido et al. (2001) showed that overexpression of NEP is able to inhibit polymerase function. However, we did not find any specific influence of the NEP mutation Q20R on viral-specific RNA synthesis regulation by qRT-PCR analysis (data not shown). Thus, the downregulation of the NEP observed in the adapted virus in combination with the mutational changes might provide a better host-range adaptation in mice and thus might account for the difference between the adapted and the original virus. Up to now, little has been known about mutations that affect viral genome stability. As we analysed a chimaeric virus, this NEP20 mutation might affect only the present construct and is not necessarily applicable to other chimaeric vectors such as those described by others (Heaton et al., 2013; Manicassamy et al., 2010; Pena et al., 2013) or wt viruses.

As B16f1 cells are IFN competent, the differences in growth of the adapted and original virus could have been due to a different sensitivity of the viruses to type I IFNs. However, we did not observe any differences in growth of the two viruses when pre-treated with human IFN in A549 cells, which are a standard model for IFN-sensitive cells (data not shown). Moreover, the adapted virus had no growth advantage compared with the NS16-GFP/O virus in other human IFN cell lines such as SK-MEL28 or A549 cells.

A hallmark of the poorly growing original vector NS16-GFP/O was the overproduction of NEP and a diminished synthesis of NS1–GFP product at the early time points of infection. The multifunctional NEP has also been implicated in the regulation of mRNA versus cRNA levels (Paterson & Fodor, 2012; Robb et al., 2010). In accordance with this, we found that both mutations in the NS segment – the NEP Q20R and the Y368H substitution in the non-functional C-terminal end of the NS1–GFP fusion protein – were involved in the regulation of NS1–GFP mRNA and protein synthesis. As single reversion of both changes severely affected viral growth in IFN-competent B16f1 cells, the synergistic effect of both mutations appeared to be relevant for effective viral function, NS1–GFP synthesis and related mRNA splicing in this segment. It is known that, in influenza virus-infected cells, the extent of splicing is regulated in a way such that the amounts of the spliced NEP mRNAs should not exceed around 10 % of those of the unspliced NS1 mRNAs (Alonso-Caplen et al., 1992). Accordingly, we found by qPCR analysis that higher expression of GFP correlated with a lower splicing efficacy. As the mutations of the NS1 aa 368 and NEP aa 20 are outside the splicing signals, we assume that secondary structure accounted for the difference. The regulation of splicing appears to be complex, as it is controlled by cis-acting sequences in NS1 mRNA but is also dependent on the efficiency of nucleocytoplasmic transport of unspliced NS1 mRNA (Alonso-Caplen et al., 1992). Therefore, we assumed that unspliced chimaeric NS1–GFP mRNA of the original virus might not have a proper cooperation with the cellular NEP adjusted to interact with the wt influenza NS mRNA (Read & Digard, 2010). However, we did not find nuclear retention of NS segment-encoded RNA in the qPCR analysis, ruling out such an effect.

Interestingly, we found that the better growth of adapted NS16-GFP/A virus and related mutants was associated with significantly less induction of IL-6 in B16f1 cells. Interestingly, the NEP revertant, which had an unstable phenotype with poor growth, was actually associated with 50 % higher IL-6 levels than the NS16–GFP/O virus. It has recently been described that increased growth of influenza viruses correlates with a lower induction of cytokines of the innate immune system (Sutejo et al., 2012). On the other hand, high-level induction of cytokines can eliminate viral spread, a mechanism that is thought to disable the spread of avian influenza A viruses in humans. We thus hypothesize that the low-level IL-6 induction of the NS16–GFP/A virus compared with NS16–GFP/O virus might be a sign of better adaptation to the host cell,
(a) 6 8 12 16 (h.p.i.)

NS116–GFP/O

NS116–GFP/A

(b) 4 h.p.i. 6 h.p.i.

Nucleus

Proportion of total NS mRNA (%)

Cytoplasm

Proportion of total NS mRNA (%)

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resulting in reduced stimulation of the antiviral cellular response.

One requirement of a universally applicable influenza A virus vector is the possibility of exchanging the external proteins in order to secure prime–boost immunization protocols. Therefore, we regard it as important that exchange of the HA in NS116–GFP/A virus with the A/New Caledonia/20/99 H1 subtype did not affect stability but only growth. It should be noted that we also exchanged the HA segment with the corresponding segment of the influenza A/Duck/Singapore/97 H5 subtype. This virus grew up to a titre of 7 log TCID50 ml⁻¹ in B16f1 and Vero cells and expressed GFP at high levels in more than 95% of the plaques. Given the known role of the HA as a surface protein and the stable phenotype of two different HA reassortant vector prototypes, we therefore suggest that this segment has little impact on transgene stability.

Most importantly, the vector backbone maintained its stability when a different transgene such as IL-2 (unpublished data) or a cassette of two antigens, Esat6 and Ag85A derived from Mycobacterium tuberculosis, was inserted. Previously, we demonstrated that immunization with influenza A virus vectors expressing Esat6 antigen might be a potent vaccination strategy against TB (Sereinig et al., 2006). The insert capacity of a new vector backbone allowed us to include a second protective Ag85A antigen to induce a broader TB-specific immune response. In contrast to Esat6 protein, Ag85A is expressed by the conventional BCG vaccine, creating the possibility of performing prime–boost immunization studies utilizing a BCG–influenza vector immunization scheme. The results of pre-clinical efficacy studies have indicated the advantage of the above-mentioned vaccination strategy (A.-P. Shurygina, M. Stukova, O. Kiselev, M. Bergmann, A. Egorov, unpublished data).

In conclusion, we suggest that influenza A virus can be adapted for stable transgene expression. This technology should facilitate the development of influenza A virus vectors suitable for medical and veterinary applications.

**METHODS**

**Cell lines.** Mouse melanoma cell line B16f1 was maintained in Dulbecco's modified eagle medium/nutrient mixture F-12 (Gibco, Invitrogen,) supplemented with 10% heat inactivated FCS (Gibco) and 2 mM GlutaMAX I (Gibco). Monkey kidney epithelial (Vero) cells adapted to grow in serum-free medium were maintained in a serum-free OptiPro medium (Gibco). All cell lines were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

**Viruses.** The influenza virus A/Puerto Rico/8/34/NS1-125GF was kindly provided by C. Kittel (Institute of Applied Microbiology, Vienna, Austria). The virus was generated on the background of the A/PR/8/34 (H1N1) (PR8 wt) strain where the NS genomic segment was modified by inserting the GFP ORF after nt 400. This virus was adapted by serial passages to growth on B16f1 cells in the presence of 5 μg trypsin (Sigma-Aldrich) μl⁻¹. For the propagation of all viruses, Vero cells were infected at an m.o.i. of 0.01 and incubated in OptiPro medium containing 5 mg trypsin ml⁻¹ at 37 °C for 2 days. Virus titres were determined by a plaque assay or a TCID₅₀ assay. It should be noted that, corresponding to the parental NS–GFPStSt virus (Kittel et al., 2005), the NS₁₁₆–GFP viruses are influenza A/PR8/34-like viruses but they contained 22 amino acid changes compared with the A/PR8/34 wt sequence.

**Construction of plasmids.** The viral RNA of the NS₁₁₆–GFP/O and NS₁₁₆–GFP/A viruses were isolated from 300 μl of the supernatant of infected cells using a QiAamp Viral RNA Mini kit according to the manufacturer's instruction (Qiagen). cDNAs were synthesized using Uni12 primer (5’-AGCAAAAGCAGG-3’) (Hoffmann et al., 2001) and Superscript II reverse transcriptase (Promega) according to the manufacturer's protocol. The cDNAs were then amplified by PCR using segment-specific primers, and nucleotide sequence analysis of PCR products was performed by GeneArt (Invitrogen). All eight cDNA segments of the NS₁₁₆–GFP/A virus were cloned individually into the bidirectional plasmid pHW2000 containing the RNA polymerase I/RNA polymerase II system (Hoffmann & Webster, 2000; Hoffmann et al., 2000).

**Generation of viruses by reverse genetics.** Vero cells (1 x 10⁶) were transfected with 0.5 μg of each of eight pHW2000 plasmids encoding a single NS₁₁₆–GFP/A virus genome segment using the Nucleofector technique (Amaxa; Lonza), according to the Amaxa Cell Line Nucleofector kit V manufacturer's instructions. To generate the NS₁₁₆–GFP/A/NEP20, NS₁₁₆–GFP/A/NS1 368, NS₁₁₆–Esat6Ag85A/A and NS₁₁₆–GFP/A/PB2 535 viruses, we used the appropriate pHW-NS₁₆₈ or pHW-PB2 plasmid encoding modified PB2 and NS segments. For generation of the NS₁₁₆–GFP/A/H/A NC virus, a plasmid encoding HA of A/New Caledonia/20/99 was used. After

![Fig. 3. Differences in viral protein and mRNA synthesis of the chimaeric viruses. (a) Intracellular localization of NEP (red) and GFP (green) proteins. B16f1 or Vero cells were infected with NS₁₁₆–GFP/O or NS₁₁₆–GFP/A virus at an m.o.i. of 2. NEP localization was assessed by an indirect immunofluorescence assay with anti-NEP antibody at the indicated times p.i. (b) Quantification of mRNA levels of NS1 and NEP. B16f1 cells were infected at an m.o.i. of 2 and mRNA was isolated at 4 and 6 h p.i. from the nucleus or the cytoplasm, and levels of viral mRNA for NS1 and NEP were analysed by quantitative PCR.](image-url)

![Fig. 4. Total level of IL-6 in the supernatant of B16f1 cells infected with the indicated viruses. Cells were infected at an m.o.i. of 2. At 48 h p.i., the level of IL-6 in the supernatant was determined by ELISA. Data represent means ± SEM of triplicate determinations.](image-url)
96 h, the transfected cells were examined for GFP expression and the presence of CPE. When the CPE reached 70%, the supernatant was collected and the viral progeny were passaged twice in Vero cells and the titre of the stock was determined by plaque assay.

**Determination of multicycle virus replication.** To evaluate virus propagation in the indicated cell lines, a subconfluent monolayer of the cells was infected with the relevant virus at an m.o.i. of 0.01 and the supernatant was collected six times p.i. at 12 h intervals. The virus titre (log₁₀ p.f.u. ml⁻¹) was determined by TCID₅₀ assay and the titre is presented as mean ± SEM (n=3).

**Fig. 5.** (a) Schematic representation of the recombinant NS segment structure expressing antigens Esat6 and Ag85A of *Mycobacterium tuberculosis*. The light blue box represents the 12 aa shared between the NS1 and NEP proteins, the light grey box represents nucleotides encoding a random 12 aa, fused to nucleotides encoding the Esat6 antigen followed by an autoproteolytic 2A cleavage site, and the yellow box represents the nucleotide sequence of the modified mouse IgK-derived signal peptide; ncr is the non-coding region. Nucleotide positions are indicated at the top. The presence of NEP Q20R mutation is indicated by *. (b) Growth kinetic of chimaeric NS₁₁₆–Esat6Ag85A/A virus on B16f1 and Vero cells. Cells were infected at an m.o.i. of 0.01. Viral titres were determined by TCID₅₀ assay and the titre is presented as mean ± SEM (n=3).

**Fig. 6.** Genetic stability of the transgene expression of chimaeric NS₁₁₆–Esat6Ag85A/A virus. (a) RT-PCR analysis of the chimaeric NS/Esat6Ag85A segment in virus NS₁₁₆–Esat6Ag85A/A after 2 (P2) and 15 (P15) passages in Vero cells. Viral RNA was isolated from supernatant infected cells at 72 h p.i.; cDNA was synthesized using Uni12 primer and was used as a template for PCR. The control used was pHW plasmid encoding NS₁₁₆–Esat6Ag85A. mr, Marker; pl, plasmid DNA; wt, wt virus A/PR8/34. (b) Expression of Esat6 and Ag85A antigens in infected Vero cells. Cells were infected with NS₁₁₆–Esat6Ag85A/A virus at an m.o.i. of 2. After 15 passages, the cells were fixed and stained at 16 h p.i. using anti-Esat6 (green) or anti-Ag85A (red) antibody.
Genetic stability of the viruses. B16f1 and Vero cells were seeded on 12-well plates at a concentration 0.4 x 10^6 cells per well and on the next day were infected with the appropriate viruses at an m.o.i. of 0.01. After 45 min of incubation at room temperature, the viral inoculum was removed and the cells were overlaid with 1.5 ml OptiPRO medium containing 5 mg trypsin ml^-1. At 72 h p.i., the supernatant was collected, diluted at a ratio of 1:100 with OptiPRO medium and a new monolayer of either B16f1 or Vero cells was infected. This procedure was repeated five times. A plaque assay was then performed and the number of GFP-positive and -negative plaques was counted.

Fifteen serial passages of NS116-Esat6Ag85A/A in Vero cells at a high m.o.i. were performed as described above. In order to passage the virus at a low m.o.i., a limiting dilutions method was used. Vero cells were seeded on 48-well plates at a concentration 1 x 10^5 cells per well and the subconfluent monolayer was infected the next day with a 10-fold dilution of the second passage of NS116-Esat6Ag85A/A virus. At 72 h p.i., supernatant from wells with the highest dilution of the virus and showing CPE was collected and used as stock for the next round of passaging. After 15 serial passages of NS116-Esat6Ag85A/A virus at high or low m.o.i., viral RNA was isolated from the supernatant of infected cells and cDNA of NS116-Esat6Ag85/A was synthesized as described above. Amplification of the fragment was performed using PCR with the sense primer Len/134 (5'-AGCAAAAGCAGGG-TGACAAAG-3') and the antisense primer NS843 (5'-CTCTTGTGT-TCACTTCAAAAT-3') (Egorov et al., 1998). The pHW2000-NS116-Esat6Ag85A plasmid was used as a positive control. PCR products were separated by electrophoresis in 1% agarose gel and visualized using Gel-Red (Biotium) and the molecular masses were compared. A Quick-Load 100 bp DNA Ladder (BioLabs) was used to estimate the molecular masses of DNA fragments.

qPCR. In order to quantify viral mRNA of the NS1 and NEP proteins as well as ISG15, IFN-β, IFN-α and Mx1, qPCR was performed. Cells (1 x 10^6) were infected with NS116-GFP/O, NS116-GFP/A, NS116-GFP/A/NEP20 or NS116-GFP/A/NSI 368 virus at an m.o.i. of 3. Cytoplasmic and nuclear mRNA was purified at 4 and 6 h p.i. using an RNaseasy Mini kit (Qiagen). DNA was digested with DNase I (Invitrogen) at 50°C for 60 min. The obtained cDNA was quantified using a Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Life Sciences) by incubating with SuperSignal West Femto Substrate (Thermo Scientific). Whole-cell extracts were resolved by 15% SDS-PAGE and immunoblotting was performed as described elsewhere (Sereing et al., 2006). The following primary antibodies were used: mouse anti-NE mAb (diluted 1:5000; Millipore), rabbit anti-GFP mAb (diluted 1:1000; Santa Cruz) and rabbit anti-NE polyclonal antibody (kindly provided by AVIR Green Hills Biotechnology, Vienna, Austria). After washing, membranes were incubated for 1 h at room temperature with the corresponding HRP-conjugated secondary antibodies: HRP-conjugated goat anti-mouse IgG (diluted 1:10000; Bio-Rad) and HRP-conjugated goat anti-rabbit IgG (diluted 1:5000; Cell Signalling). The immunoblots were visualized by incubating with SuperSignal West Femto Substrate (Thermo Scientific).

Immunofluorescence assay. Vero and B16f1 cells were infected at an m.o.i. of 2 and at the indicated time points were fixed for 15 min using 3.7% formaldehyde (Sigma-Aldrich) and washed twice with PBS, followed by permeabilization with 0.25% Triton X-100 for 30 min and blocking with 1% BSA (Sigma-Aldrich) in PBS with 0.3 M glycine for 30 min. The cells were then incubated with rabbit anti-NEP antibody at a dilution of 1:1000 for 2 h at room temperature, followed by washing with twice with PBS. The cells were then incubated for 1 h with secondary anti-rabbit antibody conjugated to Alexa Fluor 546 (Life Technologies). Signals were visualized by using Carl Zeiss LSM 700 confocal microscope.

After 15 serial passages of chimaeric NS116-Esat6Ag85A/A virus, Vero cells were infected at an m.o.i. of 2. At 6 h p.i. 1 µl GolgiPlug (BD Biosciences) ml^-1 was added to inhibit cell protein transport. After 10 h, the cells were fixed and permeabilized as described above and stained with primary rabbit anti-Esat6 polyclonal antibody (Thermo Scientific) or chicken anti-Ag85A (Abcam) polyclonal antibody, followed by incubation with secondary anti-rabbit antibody conjugated to Alexa Fluor 488 or anti-chicken antibody conjugated to Alexa Fluor 555 (Life Technologies). In cases where the virus was passaged at a low m.o.i., primary mouse anti-NE mAb (Millipore) antibody was used, followed by incubation with secondary anti-mouse antibody conjugated to Alexa Fluor 633 (Life Technologies).

Virus replication and stability in murine lungs. Six-week-old BALB/c mice (n=4 per group) were infected intranasally with 1 x 10^6 p.f.u. NS116-GFP/O virus, NS116-GFP/A virus or PR8 wt virus per animal under ether anaesthesia. On days 2, 4, and 6, the mice were sacrificed by cervical dislocation; the lungs were removed aseptically and homogenized in 1 ml OptiPRO medium with a rotor homogenizer. The virus yield in homogenates was determined by TCID50 assay in Vero cells with evaluation of the presence of CPE and GFP expression.

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