The PB1 segment of an influenza A virus H1N1 2009pdm isolate enhances the replication efficiency of specific influenza vaccine strains in cell culture and embryonated eggs

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Influenza vaccine strains (IVSs) contain the haemagglutinin (HA) and neuraminidase (NA) genome segments of relevant circulating strains in the genetic background of influenza A/PR/8/1934 virus (PR8). Previous work has shown that the nature of the PB1 segment may be a limiting factor for the efficient production of IVSs. Here, we showed that the PB1 segment (PB1Gi) from the 2009 pandemic influenza A virus (IAV) A/Giessen/06/2009 (Gi wt, H1N1pdm) may help to resolve (some of) these limitations. We produced a set of recombinant PR8-derived viruses that contained (i) the HA and NA segments from representative IAV strains (H3N2, H5N1, H7N9, H9N2); (ii) the PB1 segment from PR8 or Gi wt, respectively; and (iii) the remaining five genome segments from PR8. Viruses containing the PB1 Gi segment, together with the heterologous HA/NA segments and five PR8 segments (5+2+1), replicated to higher titres compared with their 6+2 counterparts containing six PR8 segments and the equivalent heterologous HA/NA segments. Compared with PB1PR8-containing IVSs, viruses with the PB1Gi segment replicated to higher or similar titres in both cell culture and embryonated eggs, most profoundly IVSs of the H5N1 and H7N9 subtype, which are known to grow poorly in these systems. IVSs containing either the PB1Gi or the cognate PB1 segment of the respective specific HA/NA donor strain showed enhanced or similar virus replication levels. This study suggests that substitution of PB1PR8 with the PB1Gi segment may greatly improve the large-scale production of PR8-derived IVSs, especially of those known to replicate poorly in vitro.

INTRODUCTION

Influenza viruses belong to the family Orthomyxoviridae and are classified into three genera termed Influenza virus A, B and C. Among these, influenza A viruses (IAVs) pose the greatest threat to human and animal health. They contain eight segments of negative-sense ssRNA (Ritchey et al., 1976; Shaw & Palese, 2013). The eight genomic segments encode at least 10 viral proteins, comprising the three subunits of the viral RNA polymerase complex [polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1) and polymerase acidic protein (PA)], the surface glycoproteins [haemagglutinin (HA) and neuraminidase (NA)], the nucleoprotein (NP), the matrix protein (M1), the ion channel protein (M2), the non-structural protein 1 (NS1) and the nuclear export protein (NEP). IAVs are genetically diverse, with new strains rapidly emerging by point mutations introduced by the low-fidelity viral RNA replicase (potentially resulting in antigenic drift if changes occur in the HA and/or NA coding sequences and affect important antigenic epitopes) or by genetic reassortment of genomic segments (potentially leading to antigenic shift if antigenically distant HA and NA segments are incorporated). Minor and major antigenic changes in the HA and NA proteins of circulating IAV are important driving forces for the emergence of new strains causing epidemics or even major pandemics (Neumann et al., 2009; Peng et al., 2014; Smith et al., 2009; Yoon et al., 2014). Based on their antigenic properties, HA and NA proteins are classified into different types (H1–18 and N1–11) including those of the recently isolated bat-origin IAV (H17N10 and H18N11) (Tong et al., 2012, 2013).

Vaccination is considered the first line of defence against emerging seasonal IAV. Influenza vaccine strains (IVSs) have been developed for inactivated and live-attenuated vaccines. Traditionally, vaccines have mainly been generated...
by natural reassortment between the predominant seasonal strain (against which antibodies need to be raised to achieve sufficient protection) and a high-yield egg-adapted acceptor strain, A/Puerto Rico/8/1934 (H1N1, PR8). To isolate the desired high-yield reassortant IVS (HYR-IVS) that expresses the HA and NA of the seasonal strain, extensive genetic and antigenic screening methods are required (Ozaki et al., 2004). More recently, plasmid-based reverse genetics systems have been developed. They allow faster production of recombinant IVSs because they do not depend on time-consuming natural reassortment and screening processes (Hoffmann et al., 2002; Schickli et al., 2001; Subbarao et al., 2003). Typically, genetically engineered IVSs produced by plasmid-based reverse genetics methods are based on the embryonated egg- or Vero cell-adapted PR8 strain (Hu et al., 2015) and contain the two segments encoding the major viral antigens – HA and NA – of the circulating reference IAV. In some cases, more than two segments of the circulating (donor) strain are incorporated in the PR8-based IVS. IVSs suitable for large-scale manufacturing are required to replicate efficiently and to produce high amounts of HA per egg and/or viral particles with high HA content (Jing et al., 2013). An alternative approach to embryonated eggs, specific mammalian cell lines that have been licensed for vaccine production by regulatory agencies (e.g. Vero cells) are increasingly being used for the production of IVSs (Hess et al., 2012; Hu et al., 2015; Huang et al., 2015; Naruse et al., 2015). In many cases, however, PR8-based reassortants containing HA and NA segments from circulating strains fail to grow efficiently in embryonated eggs and/or cell culture, providing a significant obstacle to efficient large-scale vaccine production (Abt et al., 2011; Compans et al., 1972; Wanitchang et al., 2010).

Efficient IAV replication is linked to optimal activities and interactions of the viral polymerase complex (and its subunits and co-factors) (Ngai et al., 2013; Wille et al., 2013). Previous studies of HYR-IVSs produced by natural reassortment between circulating strains and PR8 have shown that the PB1 segment of many HYR-IVSs was derived from the circulating strain and, apparently, provided a significant growth advantage over IVSs that contained the PR8 PB1 segment (PB1PR8) (Fulvini et al., 2011; Ramanunnair et al., 2013). This growth advantage was observed for both type A and type B HYR-IVSs (Abt et al., 2011; Compans et al., 1972; Le et al., 2015; Wanitchang et al., 2010). Furthermore, substitution of specific residues within the PB1PR8 protein (G180E, S216G, S361R, Q621R and N654S), which are known to be involved in enzyme activity or protein interactions with the other polymerase proteins, were found to be beneficial (Plant et al., 2012; Ulmanen et al., 1981).

The data obtained in the current study suggest that the PB1 segment of a 2009 pandemic IAV isolate (PB1GI) may enhance the polymerase activities and replication efficiencies of a wide range of recombinant reassortant PR8-derived viruses. We found that PB1GI was able to support efficient replication in cell culture and embryonated eggs of genetically engineered reassortant IVSs containing HA and NA segments from genetically diverse viruses representing the H3N2, H5N1, H7N9 and H9N2 subtypes. Furthermore, a comparison of recombinant viruses containing either the PB1 segment of the donor strain or the PB1GI segment revealed that recombinant reassortant IVSs of the H5N1 IAV and H7N9 subtype, respectively, that contained the PB1GI segment replicated at least equally efficiently as their viral counterparts containing the cognate PB1 segment of the respective donor virus.

RESULTS

PB1GI affects polymerase activity and replication efficiency of PR8

Possible effects on virus replication caused by replacements of specific segments in wt PR8 were studied by characterizing a set of recombinant IAVs. PR8 and 7 + 1 reassortant PR8 viruses in which the PB2, PA or NP segment of PR8 was replaced with the equivalent genome segment of influenza virus A/Giessen/6/2009 (GI) were found to replicate to similar titres (data not shown). By contrast, substitution of PR8 PB1 (PB1PR8) with GI PB1 (PB1GI) resulted in a virus with a higher growth rate in MDCK-II cells at 12 and 24 h post-infection (p.i.) as determined by the number of f.f.u. ml⁻¹ (Fig. 1a) and haemagglutination units (HAU) ml⁻¹ (Fig. 1b). Primer extension data (Fig. 1c) further suggested that levels of NP-specific vRNA, mRNA and cRNA were increased in cells infected with PR8-PB1GI compared with PR8 wt at early time points p.i., suggesting that increased viral titres and HA content of reassortant PR8-PB1GI were associated with an increase in viral RNA accumulation [including viral mRNA, cRNA and viral genomic RNA (vRNA)].

Furthermore, we observed that a 6 + 2 IVS, which contained the HA and NA segments of GI wt along with six PR8-derived segments (PR8-HA/NA/GI), replicated extremely poorly and was only detected after one passage post-rescue using reverse transcription (RT)-PCR performed with RNA from cell-free supernatant of the infected MDCK-II cells (Fig. 2c, d). In contrast, the 5 + 3 IVSs PR8-HA/NA/PB1GI which additionally contained the PB1GI segment, replicated very efficiently in different systems and could easily be detected by RT-PCR and classical HA assay (Fig. 2). Suggesting that PB1GI is beneficial for the production of a PR8-based IVS that contains the HA/NA/GI segments. In agreement with previous findings (Wanitchang et al., 2010), PR8-HA/NA/PB1GI replicated efficiently in MDCK-II cells (Fig. 2a) and Vero cells (Fig. 2b) and in embryonated eggs at 24 h (Fig. 2c, f) and 36 h (Fig. 2g, h) p.i.

PB1GI enhances the replication of PR8-derived IVSs with diverse HA/NA subtypes

To study further the potential impact of PB1GI on the replication efficiency of IVSs containing HA/NA segments from different subtypes, we generated a set of reassortant IVSs containing the PB1 segment of the respective donor GI strain.
with the genome structure 5(PR8) + 2(HA/NA) + 1(PB1Gi). The following recombinant viruses were produced: (i) an H3N2 subtype IVS (PR8-HA/NAVict-PB1Gi); (ii) an H5N1 subtype IVS (PR8-HA/NAKAN-PB1Gi); (iii) an H7N9 subtype IVS (PR8-HA/NAAnhui-PB1Gi); and (iv) an H9N2 subtype IVS (PR8-HA/NABeijing-PB1Gi). The replication characteristics of the resulting viruses were compared with those of the corresponding recombinant 6 + 2 reassortant IVSs that contained the wt PR8 PB1 segment. At 24 and 36 h p.i., the 5 + 2 + 1 IVSs PR8-HA/NAVict-PB1Gi (H3N2) and PR8-HA/NAAnhui-PB1Gi (H7N9) viruses replicated with similar kinetics in MDCK-II cells compared with their 6 + 2 counterparts (Figs 3a, g and 4). In embryonated eggs, only the 5 + 2 + 1 virus of PR8-HA/NABeijing-PB1Gi (H9N2) replicated higher titres (particularly at later time points p.i.), while there was no major growth difference for the 5 + 2 + 1 and 6 + 2 variants of the H3N2 subtype virus. By contrast, in Vero cells, both 5 + 2 + 1 IVSs replicated more efficiently than their 6 + 2 counterparts, even though the overall yield for both viruses remained below that obtained in MDCK-II cells and embryonated eggs (Figs 3b, h and 4). Even more striking growth differences were observed for PR8-HA/NAKAN-PB1Gi (H5N1) and PR8-HA/NAAnhui-PB1Gi (H7N9). For both viruses, the 5 + 2 + 1 IVSs were found to replicate significantly more efficiently than their 6 + 2 counterparts in all systems used [MDCK-II and Vero cells (Fig. 3c–f) and embryonated eggs (Fig. 4)]. The combined data led us to conclude that the additional insertion of the PB1Gi segment provided a significant growth advantage in recombinant PR8-derived IVSs containing heterologous HA/NA segments, while it was neutral in a few other cases.

**Similar HA content per viral particle in PR8-based 5 + 2 + 1 and 6 + 2 IVSs**

Given that virus neutralization requires antibodies that recognize specific epitopes on the HA protein, a high HA content per virion allows to increase the overall amount of produced vaccines (Cobbin et al., 2013). We therefore sought to rule out the possibility that enhanced replication of 5 + 2 + 1 IVSs resulted in virus particles with reduced HA content. Allantoic fluids collected from embryonated eggs infected with either 5 + 2 + 1 or 6 + 2 IVSs at 36 h p.i. were concentrated by ultracentrifugation and the HA titre of the resuspended virus preparations were determined. Next, the HA protein content of 10 000 HAU (in relation to the viral NP) was determined for H3N2, H5N1, H7N9 and H9N2 IVSs (6 + 2 and 5 + 2 + 1) by Western blotting using appropriate HA- and NP-specific antibodies. The data revealed comparative HA/NP ratios for each pair (6 + 2 and 5 + 2 + 1) of IVSs (Table 1), suggesting that the increased replication conferred by the PB1Gi segment in PR8-derived IVSs with different HA/NA subtypes did not affect the HA content of the respective 5 + 2 + 1 viruses. To confirm this point, the HAU/f.f.u. ratios in allantoic fluid samples collected from embryonated eggs (n=5) inoculated with 5 + 2 + 1 IVSs and the corresponding 6 + 2 counterparts were determined. The results showed that the inclusion of PB1Gi in 5 + 2 + 1 IVSs only slightly increased the HAU/f.f.u. ratio by less than onefold (Table 2). Collectively, these data indicated that the inclusion of PB1Gi can result in a higher HA yield of PR8-based 5 + 2 + 1 IVSs by improving the growth rate rather than by improving the HA content per viral particle.

**Effect of PB1Gi on the replication/transcription activity of the viral polymerase of PR8-based IVSs in MDCK-II cells**

To compare viral RNA accumulation in cells infected with 5 + 2 + 1 and 6 + 2 IVSs, MDCK-II cells were infected with H5N1 and H7N9 subtype 5 + 2 + 1 IVSs and their 6 + 2 counterparts (m.o.i.=3). At 2, 4, 6 and 8 h p.i., the mRNA, cRNA and vRNA levels were determined by
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(a) MDCK-II

(b) Vero

(c) HA segment

(d) NA segment

(e) Egg, 24 h.p.i.

(f) Egg, 24 h.p.i.

(g) Egg, 36 h.p.i.

(h) Egg, 36 h.p.i.
Fig. 2. Replication efficiency of the generated 5 + 3 (PR8-HA/NA/PB1Gi) and 6 + 2 (PR8-HA/NA/Gi) recombinant IVSs expressing swine-origin influenza virus HA/NA/PB1. (a, b) After transfection of 293T cells with the relevant plasmids, the supernatant of the transfected cells was passaged onto MDCK-II cells for 72 h. Next, an aliquot (500 μl) from the MDCK-II cells was passaged onto MDCK-II cells (a) and Vero cells (b) in triplicate. Virus replication in the two cell cultures was determined at the indicated time points p.i. by a focus assay. ***P<0.001; NS, not significant. (c, d) RT-PCR and PCR (control) to detect HAGi (c) and NAGi (d) segments of the recombinant 6 + 2 and 5 + 3 IVSs. Both segments were positively detected by RT-PCR (but not PCR) performed with RNA extracted from cell-free supernatant of MDCK-II cells inoculated with 500 μl supernatant obtained from 293T cell culture, which was transfected with eight bidirectional plasmids to generate such viruses. Mr, peqGOLD DNA Ladder Mix (100–10000 bp). (e–h) In parallel, 100 μl cell-free supernatant from transfected 293T cells of each virus was injected into 11-day-old embryonated chicken eggs (n=10). Allantoic fluids (n=5 for each time point) were collected at 24 h (e, f) and 36 h (g, h) p.i. Virus titres were determined by focus assay (e, g; f.f.u. ml⁻¹) and HA assay (f, h; HAU ml⁻¹). The results showed that the inclusion of PB1Gi facilitated the generation and replication of recombinant IVSs expressing HAGi and NAGi. The horizontal bar represents the mean and error bars indicate SD.

primer extension as described previously (Fodor et al., 1998). The data revealed a significant increase of viral mRNA and, to a lesser extent, of vRNA in cells infected with two representative 5 + 2 + 1 IVSs compared with their 6 + 2 IVSs counterparts (Fig. 5). The data were consistent with the results presented above and supported our conclusions on the superior replication efficiency of the 5 + 3 IVSs, respectively. For the H5N1 subtype IVSs, virus replication characteristics of 5 + 3 versus 6 + 2 IVSs (Abt et al., 2010). Similarly, the production of a high-yield IVSs against an H5N1 IAV strain was greatly improved by incorporating homologous PB1 or M segments in the PR8-IVS (5 + 3) genome (Abt et al., 2011). These observations for genetically engineered IVSs were consistent with previous experiences in classical vaccine production. Many high-yield IVSs produced by natural reassortment obtained the PB1 segment of the circulating strain rather than the PB1PR8 segment (Sugiyama et al., 2009), but there are also examples in which the indigenous PB1 of the circulating strain was not beneficial for producing a more efficiently replicating IVS (Arranz et al., 2012; Hemerka et al., 2009). Therefore, it would be highly desirable to identify a ‘generic’ (broadly or even universally applicable) PB1 segment that may replace the PB1PR8 segment to overcome possible restrictions of replication observed for many 6 + 2 IVSs (Abt et al., 2011; Sugiyama et al., 2009; Wanitchang et al., 2010). Here, we explored the capability of the PB1Gi segment to support the replication of different recombinant PR8-based 5 + 2 + 1 IVSs. Such a beneficial effect has been reported previously for the PB1 segment of A/Nonthaburi/102/2009(H1N1) (PB1Nonthaburi) when inserted into the PR8-based 5 + 3 IVS against the 2009 pandemic H1N1 IAV, which led to improved replication in embryonated eggs (Wanitchang et al., 2010). Apparently, the effect of PB1Nonthaburi was specific for 5 + 3 reassortants harbouring the homologous HA and NA, and PB1Nonthaburi did not improve the replication of PR8 with wt HA and NA (Wanitchang et al., 2010). Contrary to this earlier study, the PB1Gi protein was found to significantly increase the replication efficiency of a reassortant PR8. The PB1Nonthaburi and PB1Gi amino acid sequences differ at only two positions (PB1Nonthaburi versus PB1Gi: N61T and V325I). As residue 61 is part of a domain (aa 1–78) that is known to be involved in interactions with the PA C-terminal domain, it seems possible

Discussion

Despite significant technical advances over the past few years, the rapid development of new IVSs suitable for the respective season and the large-scale production of many millions of vaccine doses remains a formidable challenge for vaccine manufacturers. Unfortunately, additional technical problems such as poor replication of specific reassortants derived from the commonly used PR8 strain may further delay the production of sufficient amounts of vaccine in time, as it was the case with the 6 + 2 IVSs containing the HA/NA of the 2009 pandemic strain (H1N1 2009pdm; previously called swine-origin influenza virus). This particular 6 + 2 IVS replicated only inefficiently in cell culture and embryonated eggs, and therefore it failed to produce sufficient amounts of viral antigen for large-scale vaccine manufacturing. The problem could be solved by including the indigenous PB1 segment of the H1N1 2009pdm strain in the IVS (Wanitchang et al., 2010). Therefore, it would be highly desirable to identify a ‘generic’ (broadly or even universally applicable) PB1 segment that may replace the PB1PR8 segment to overcome possible restrictions of replication observed for many 6 + 2 IVSs (Abt et al., 2011; Sugiyama et al., 2009; Wanitchang et al., 2010). Here, we explored the capability of the PB1Gi segment to support the replication of different recombinant PR8-based 5 + 2 + 1 IVSs. Such a beneficial effect has been reported previously for the PB1 segment of A/Nonthaburi/102/2009(H1N1) (PB1Nonthaburi) when inserted into the PR8-based 5 + 3 IVS against the 2009 pandemic H1N1 IAV, which led to improved replication in embryonated eggs (Wanitchang et al., 2010). Apparently, the effect of PB1Nonthaburi was specific for 5 + 3 reassortants harbouring the homologous HA and NA, and PB1Nonthaburi did not improve the replication of PR8 with wt HA and NA (Wanitchang et al., 2010). Contrary to this earlier study, the PB1Gi protein was found to significantly increase the replication efficiency of a reassortant PR8. The PB1Nonthaburi and PB1Gi amino acid sequences differ at only two positions (PB1Nonthaburi versus PB1Gi: N61T and V325I). As residue 61 is part of a domain (aa 1–78) that is known to be involved in interactions with the PA C-terminal domain, it seems possible
Fig. 3. Effect of PB1Gi on the replication kinetics of 5+2+1 and 6+2 IVSs for H3N2 (a, b), H5N1 (c, d), H7N9 (e, f) and H9N2 (g, h) subtypes in MDCK-II (a, c, e, g) and Vero (b, d, f, h) cells, respectively. IVSs were used to inoculate cells in triplicate at an m.o.i. of 0.01. Samples of supernatants (200 μl) were harvested at 24 and 36 h p.i. The virus load in the collected aliquots was then quantified by focus assay (f.f.u. ml⁻¹). *P<0.05; **P<0.01; ***P<0.001; NS, not significant (P>0.05).
that the difference in the replication efficiencies observed for PR8 reassortants containing PB1Nonthaburi or PB1Gi is linked to one of these residues (González et al., 1996).

Similar to PR8-HA/NA
Nonthaburi, PR8-HA/NA
Gi replicates very poorly. In previous trials (discussed by Wanitchang et al., 2010) to generate 6 + 2 IVSs against 2009 pandemic H1N1, it was also shown that such recombinant 6 + 2 IVSs were slow growing and that at least three serial passages in embryonated eggs were needed to acquire possible adaptive mutations (such as D225G, Q226R or S186P in the HA segment) to allow efficient replication. The positive RT-PCR signal of cell-free supernatant from MDCK-II cells infected with the supernatant of plasmid-transfected 293T cells (used to generate the recombinant 6+2 and 5+3 IVS viruses) indicated that, in principle, the HA/NA
Gi segments of this recombinant PR8-HA/NA
Gi virus are not incompatible with the other PR8 segments but result in a very low

Fig. 4. Impact of PB1Gi on the replication kinetics of 5+2+1 and 6+2 IVSs of H3N2, H5N1, H7N9 and H9N2 subtypes in embryonated chicken eggs. Specific-pathogen-free eggs (n=10) were inoculated individually with 100 f.f.u. of reassortant viruses in 200 μl PBS. F.f.u. (a, c) and HAU (b, d) titres of individual eggs inoculated with the different IVSs determined at 24 (a, b) and 36 h p.i. (c, d) (n=5 for each time point) are shown. Due to early mortality of some embryos, the titres of six eggs infected with PR8-HA/NA
Vict-PB1Gi, PR8-HA/NA
Bei and PR8-HA/NA
Bei-PB1Gi, were analysed after 24 h. Therefore, the HAU and f.f.u. titres of the remaining embryonated eggs (n=4) were determined at 36 h p.i.
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Table 1. Relative HA1/NP ratios of different 6+2 and 5+2+1 IVSs

<table>
<thead>
<tr>
<th>Virus</th>
<th>IVSs</th>
<th>HA1/NP ratio*</th>
</tr>
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<tbody>
<tr>
<td>H3N2 (VICT)</td>
<td>PR8-HA/NAVICT</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NAVICT-PB1Gi</td>
<td>0.61 ± 0.01</td>
</tr>
<tr>
<td>H5N1 (KAN)</td>
<td>PR8-HA/NAKAN</td>
<td>1.05 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NAKAN-PB1Gi</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td>H7N9 (Anhui)</td>
<td>PR8-HA/NAAnhui</td>
<td>3.80 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NAAnhui-PB1Gi</td>
<td>4.66 ± 1.04</td>
</tr>
<tr>
<td>H9N2 (Beij)</td>
<td>PR8-HA/NABe</td>
<td>1.07 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NABe-PB1Gi</td>
<td>1.08 ± 0.06</td>
</tr>
</tbody>
</table>

*Viral particles in the collected allantoic fluids were precipitated using ultracentrifugation and viral proteins (HA, NP) of 10 000 HAU of each IVS were detected by Western blotting (n=3) and analysed using Quantity One 1-D analysis software.

Table 2. HAU/f.f.u. ratios of different 6+2 and 5+2+1 IVSs

<table>
<thead>
<tr>
<th>Virus</th>
<th>IVS</th>
<th>HAU/f.f.u.*</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3N2 (VICT)</td>
<td>PR8-HA/NAVICT</td>
<td>3.41 × 10^{-5}</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NAVICT-PB1Gi</td>
<td>2.54 × 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>H5N1 (KAN)</td>
<td>PR8-HA/NAKAN</td>
<td>3.32 × 10^{-5}</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NAKAN-PB1Gi</td>
<td>2.02 × 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>H7N9 (Anhui)</td>
<td>PR8-HA/NAAnhui</td>
<td>2.43 × 10^{-4}</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NAAnhui-PB1Gi</td>
<td>1.43 × 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>H9N2 (Beij)</td>
<td>PR8-HA/NABe</td>
<td>5.17 × 10^{-5}</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>PR8-HA/NABe-PB1Gi</td>
<td>1.18 × 10^{-5}</td>
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*F.f.u. ml^{-1} and HAU ml^{-1} were determined for viral particles in the collected allantoic fluids at 36 h p.i.

It is largely accepted that PB1 of the 2009 pandemic H1N1 influenza virus was transferred from an avian to a human influenza virus in 1968 (Kawaoka et al., 1989) and subsequently introduced into swine influenza virus in 1998 (Plant et al., 2012). Several other amino acid differences were detected in both the N and C termini of PB1Gi. The functional implications of these differences remain to be investigated in detail but may be linked to specific interactions with other polymerase subunits (Binh et al., 2013; Zhao et al., 2015).

Concerning the basic question of whether the antigen yield, namely the HA yield, is enhanced in the IVSs carrying PB1Gi, it was shown previously that the PB1 segment can improve the replication of poorly growing (10^6 f.f.u. ml^-1) 6+2 vaccine strains (for example, PR8-based H5N1 and H7N9 subtypes). Although it has no effect or just slightly increases virus titres of several well-growing (10^8 f.f.u. ml^-1) vaccine strains (PR8-derived H3N2 and H9N2 subtypes), it does not impair virus replication. The variable degree of growth improvement is, in fact, not a surprising result regarding the overall variability of influenza virus replication.

Using comparative sequence analysis of the PB1 amino acid sequences of PR8 and Gi, we found that, in contrast to PB1_{PR8}, PB1_{Gi} harbours several amino acids that were reported previously to improve the replication of PR8 wt in eggs (G180E, S216G, S361R, Q621R and N654S) (Plant et al., 2012). Several other amino acid differences were detected in both the N and C termini of PB1Gi. The functional implications of these differences remain to be investigated in detail but may be linked to specific interactions with other polymerase subunits (Binh et al., 2013; Zhao et al., 2015) suggest that substitution of PB1_{PR8} with PB1_{Gi} greatly improves the replication of poorly growing (10^6 f.f.u. ml^-1) 6+2 vaccine strains (for example, PR8-based H5N1 and H7N9 subtypes).
This assumption was supported by our finding that PB1Gi can enhance the polymerase activity (especially the transcription activity) of 7 + 1 PR8 reassortant virus when compared with PR8 wt (Fig. 1), and of H7N9 and H5N1 type 5 + 2 + 1 IVSs when compared with the according 6 + 2 viruses (Fig. 5). The increased mRNA (not vRNA) amount in the presence of PB1GI is yet not understood and needs further investigations. Moreover, unlike PB1PR8, PB1Gi harbours a C129A change leading to a non-functional truncated PB1-F2 (11 aa), thereby probably abolishing PB1-F2-mediated apoptosis and virulence mechanisms (Chakrabarti & Pasricha, 2013). These and other possible functional implications deserve further studies. In this context, it is worth noting that swine-origin influenza virus expressing the complete PB1-F2 has been reported to cause cell death at very early time points p.i., which, in turn, leads to low virus yield (Chen et al., 2010). Nevertheless, further investigation is required to determine the underlying mechanism(s) by which PB1Gi improved the yield and characteristics of PR8-based IVSs compared with the isogenic PB1PR8.

Despite the fact that the inclusion of indigenous PB1 segments (5 + 3 IVSs) could occasionally help to improve the virus replication efficiency, it was shown in this study that PB1Gi (5 + 2 + 1 IVSs) could increase the overall virus yield more efficiently than the indigenous PB1 segment or at least lead to comparable virus production. The ability of indigenous PB1 to promote the growth kinetic of 5 + 3 IVSs is presumably dependent on its impact on the PR8 polymerase activity or possibly to direct interaction with homologous HA/NA segments. A surprising finding of our study was the ability of PB1Gi to improve the propagation of IVSs with different HA/NA subtypes using a specific constellation of polymerase genes. Taken together, the results presented here suggest that PB1Gi is an interesting candidate to displace PB1PR8 for efficient production of 5 + 2 + 1 IVSs against different highlighted IAV strains.

**METHODS**

**Cell lines and plasmids.** MDCK-II, 293T and Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen) supplemented with 100 IU penicillin ml⁻¹/100 μg streptomycin ml⁻¹ (Pen/Strep) and 10 % FBS. The plasmids carrying the eight gene segments of the high-yield PR8 (H1N1) virus were kindly supplied by Richard Webby at St Jude Children’s Research Hospital, TN, USA (Hoffmann et al., 2000). The vRNA segments PB2, PB1, PA and NP of influenza virus A/Giessen/6/2009 (Gi wt, H1N1); the HA and NA vRNA segments of influenza virus A/Victoria/3/1975 (Vic, H3N2) and A/Chicken/Beijing/2/1997 (Bei, H9N2); and the HA, NA and PB1 vRNA segments of A/Thailand/1(KAN-1)/04 (KAN, H5N1) were kindly provided by Prof. Richard Webby, St Jude Children’s Research Hospital, Memphis, TN, USA.

**Fig. 5.** Replication and transcription activities of the viral polymerase from 5 + 2 + 1 IVSs of H5N1 (a) and H7N9 (b) subtypes and their 6 + 2 counterparts in MDCK-II cells by primer extension analysis. The analysis was done with isolated mRNA, cRNA and vRNA (NP segment) at 2, 4, 6 and 8 h p.i. from MDCK-II cells infected with the different IVSs (m.o.i. = 3). The results shown are derived from the quantitative analysis of three independent experiments (n = 3) and the significance was investigated using two-way ANOVA. Results are shown as means ± SD. **P < 0.01; ***P < 0.001.
and A/Anhui/1/2013 (Anhui, H7N9) were cloned into pMP100B as described previously (Mostafa et al., 2013).

**RNA extraction and primer extension.** Confluent MDCK-II cells seeded in 35 mm dishes were infected with recombinant wt or reassortant PR8 (PR8 or PR8-PB1cd) or with recombinant IVSs expressing the HA and NA of H5N1/KANI or H7N9/Anhui in the genetic background of PR8 (6+2) or together with the PB1Gi in the PR8 background (5+2+1) at an m.o.i. of 3. The cells were harvested and total RNA was extracted using Trizol reagent (Invitrogen) as described previously (Mostafa et al., 1998; Wang et al., 2010). The gene-specific DNA primer used for the PR8 NP mRNA and cRNA was 5’-TGATGGAAGTGCAAGACCA-3’ and for rRNA was 5’-TCAAAGGGGGGTCACTTCA-3’. Transcription products were separated on 6% polyacrylamide gels containing 7 M urea, and detected and quantified using a Typhoon 9200 instrument (GE Healthcare). To test the significance of the data, a two-tailed one-sample t-test was used.

**Rescue of recombinant viruses.** Using the eight-plasmid reverse genetics system employing either pHW2000 or our own previously developed vector pMP100B, reassortant and recombinant viruses were generated as described previously (Hoffmann et al., 2000, 2001; Mostafa et al., 2013). Briefly, 8 μg plasmid DNA (1 μg per plasmid) was transfected into a co-culture of 293T/MDCK-II cells with TransIT2020 (Mirus) (2 μl per 1 μg plasmid DNA to be transfected). Cells were then incubated for 8 h at 37 °C, 5% CO₂. Afterwards, the transfection mixture was replaced with 1 ml Opti-MEM containing Pen/Strep and 0.2% bovine albumine (BA), and the cell cultures were incubated overnight at 37 °C, 5% CO₂. Subsequently, an additional 1 ml Opti-MEM containing Pen/Strep, 0.2% BA and 2 μg TPCK-treated trypsin (Sigma-Aldrich) ml⁻¹ was added and the cells were further incubated for 48 h. A volume of 500 μl of each transfection was harvested to inoculate fresh MDCK-II cells, which were incubated for 48 h at 37 °C, 5% CO₂. Thereafter, the virus-containing supernatant was harvested and stored at −80 °C until further use.

**RT-PCR versus PCR detection of PR8-HA/NAGi and PR8-HA/NA/PB1Gi.** The viral RNA was extracted from a 200 μl sample of cell-free supernatant of infected MDCK-II cells, inoculated with 500 μl supernatant from 6+2 (PR8-HA/NAcd)- or 5+3 (PR8-HA/NA/PB1cd)-transfected 293T cells, using a RNeasy Protect kit (Invitrogen) according to the manufacturer’s instructions. The HA and NA segments of Gi wt were then detected in separate reactions using SuperScript III One-Step RT-PCR Platinum Taq High Fidelity (Invitrogen) as described previously (Mostafa et al., 2015). The primers used for the H1 HA segment detection were PAN-H1-F (5’-CTCGTGCTATGGGGCGATTCA-3’) and PAN-H1-R (5’-TTGC-AATCGTGGACTGGTGT-3’), resulting in an expected product size of 308 bp. For the N1 NA segment, the PAN-N1-F (5’-TCCCCTTGAGATCGAGAAC-3’) and PAN-N1-R (5’-AAGACACCCCCGG-TGGATTG-3’) primers were used resulting in an expected amplicon length of 544 bp. To confirm that the extracted RNA was not contaminated with transfected plasmids, the extracted RNA was directly subjected to PCRs using the pre-defined primer sets for HA and NA and Phusion High-Fidelity PCR Master Mix with H/F Buffer (Thermo Fisher Scientific). Briefly, 5 μl extracted RNA was mixed with 2.5 μl each primer (50 pmoles) and 25 μl 2× Phusion Master Mix with H/F buffer. The total volume was adjusted to 50 μl using nuclease-free water (Ambion), followed by denaturation at 98 °C for 2 min, PCR amplification (35 cycles: 98 °C/30 s for denaturation, 58 °C/30 s for annealing and 72 °C/2 min for extension) and then final extension at 72 °C for 10 min.

**Viral growth kinetics in cell cultures and embryonated eggs.** Confluent monolayers of Vero and MDCK-II cells (3.5 cm dishes) were inoculated in triplicate with the reassortant viruses at an m.o.i. of 0.01 for 1 h at 37 °C to allow virus adsorption. The inoculum was then removed, the cell monolayer washed with PBS containing 1 mM MgCl₂, 0.9 mM CaCl₂ (PBS2⁺) to remove unbound virus, and 2 ml DMEM containing Pen/Strep, 0.2% BA and 1 μg TPCK-treated trypsin (infection medium) ml⁻¹ was added. At 24 and 36 h p.i., aliquots (200 μl) of the virus-containing supernatants were collected and replaced with 200 μl fresh infection medium. Viruses were determined using a standard haemagglutination assay (WHO, 2009) and focus assay (Mostafa et al., 2013). Embryonated eggs (n=10) were infected with 100 f.f.u. per egg of the appropriate IVS (6+2 or 5+2+1 type) diluted in 100 μl PBS2⁺. Allantoic fluids were collected at 24 and 36 h p.i. and infectious virus titres (f.f.u. ml⁻¹) and haemagglutination titres (HAU ml⁻¹) were determined.
Ultracentrifugation and Western blot analysis. Allantoic fluids collected for the different IVs were clarified from cell debris by ultracentrifugation at 8000 × g for 5 min. Viral particles in the semi-purified cell culture supernatant (30 ml) were pelleted through a sucrose cushion. A volume of 30 ml of the virus preparation was carefully pipetted on top of a 20% sucrose solution (12 ml) and then centrifuged using a Beckman SW28 rotor at 28,000 r.p.m. for 2 h at 4°C. The pellet was resuspended in 500 μl PBS and virus titres were determined by haemagglutination assay. An aliquot of 30 μl of each virus preparation containing 40,000 HAU was mixed with 10 μl 4 × SDS sample buffer [40% glycerol, 240 mM Tris/HCl (pH 6.8), 8% SDS, 0.04% bromophenol blue and 5% β-mercaptoethanol] and incubated for 5 min at 95°C. An aliquot of 10 μl of each sample (corresponding to 10,000 HAU) was then separated on pre-cast gradient NuPAGE Novex 4–12% Bis/Tris protein gels (Invitrogen) and transferred onto Immobilon-FL PVDF membranes (Merck Millipore). Following protein transfer, the PVDF membrane was blocked using blocking buffer [1 × TBS (pH 7.6) containing 5% non-fat dry milk] for 1 h at room temperature. The membrane was washed once for 5 min using washing buffer [1 × TBS (pH 7.6)/0.05% Tween]. Viral HA and NP proteins were detected using mAbs specific for the IAV NP (Abcam) and subtype-specific mAbs that recognize influenza A virus H3N2 (Abcam), H5N1 (Abcam), H7N9 (Sino Biological) and H9N2 (Sino Biological). Antibodies were used at a 1 : 2000 dilution in blocking buffer. After 1 h, the membrane was washed three times for 5 min each with washing buffer. Next, secondary antibodies, goat anti-mouse IRDye 680 (LI-COR) or goat anti-rabbit IRDye 800 (LI-COR) each diluted to 1 : 10,000 in blocking buffer containing 0.01% SDS, were added. Following incubation in the dark for 1 h, the membrane was washed three times (5 min each) with washing buffer and once with 1 × TBS, and proteins were visualized using an Odyssey Infrared Imaging System and the application software package (LI-COR).

Statistical analysis. Statistical tests and graphical data presentations were performed using GraphPad Prism 5.0 software (GraphPad Software). Significances were determined by repeated-measures ANOVA with Bonferroni’s post-hoc test or a one-way ANOVA followed by Tukey’s post-hoc test or a two-tailed unpaired t-test with Welch’s correction. All data are presented as mean ± SD.

Biosafety. All experiments using infectious virus were performed in accordance with German biosafety regulations pertaining to the propagation of influenza viruses. All experiments involving (complete-genome) IAVs were performed using Biosafety Level 2 and 3 containment laboratories approved for such use by the local authorities (RP, Giessen, Germany).

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