Alternative base pairs attenuate influenza A virus when introduced into the duplex region of the conserved viral RNA promoter of either the NS or the PA gene

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The development of plasmid-based rescue systems for influenza virus has allowed previous studies of the neuraminidase (NA) virion RNA (vRNA) promoter to be extended, in order to test the hypothesis that alternative base pairs in the conserved influenza virus vRNA promoter cause attenuation when introduced into other gene segments. Influenza A/WSN/33 viruses with alternative base pairs in the duplex region of the vRNA promoter of either the polymerase acidic (PA) or the NS (non-structural 1, NS1, and nuclear export, NEP, -encoding) gene have been rescued. Virus growth in MDBK cells demonstrated that one of the mutations, the D2 mutation (U–A replacing G–C at nucleotide positions 12–11), caused significant virus attenuation when introduced into either the PA or the NS gene. The D2 mutation resulted in the reduction of PA- or NS-specific vRNA and mRNA levels in PA- or NS-recombinant viruses, respectively. Since the D2 mutation attenuates influenza virus when introduced into either the PA or the NS gene segments, or the NA gene segment, as demonstrated previously, this suggests that this mutation will lead to virus attenuation when introduced into any of the eight gene segments. Such a mutation may be useful in the production of live-attenuated viruses.

INTRODUCTION

Influenza A virus is a member of the Orthomyxoviridae. It has eight single-stranded, negative-sense RNA gene segments (Lamb & Krug, 2001; Palese, 1977) that code for 11 proteins (Chen et al., 2001). Virion RNA (vRNA) is the template for both mRNA and complementary RNA (cRNA) production. cRNA serves as the template for the synthesis of more vRNA molecules. Synthesis of all three influenza virus RNA species (vRNA, mRNA and cRNA) is catalysed by the influenza virus RNA-dependent RNA polymerase. The polymerase consists of three subunits: the polymerase basic proteins 1 (PB1) and 2 (PB2) and the polymerase acidic protein (PA) (reviewed by Fodor & Brownlee, 2002; Lamb & Krug, 2001; Portela et al., 1999).

The terminal 13 and 12 nt of the 5’ and 3’ ends, respectively, are conserved in all eight vRNA gene segments and are known to form the vRNA promoter (reviewed by Fodor & Brownlee, 2002). The vRNA promoter is a partially duplex structure formed by base-pairing between nt 11′–13′ of the 5’ end with nt 10–12 of the 3’ end, which is conserved in all eight gene segments. Adjacent to the conserved base pairs are the segment-specific base pairs that extend the duplex by an additional 1 to 5 bp, depending on the segment (Desselberger et al., 1980; Robertson, 1979; Skehel & Hay, 1978). Following the observation that a stem–loop structure in both the 5’ and the 3’ termini is important for promoter function, a corkscrew conformation has been proposed (Brownlee & Sharps, 2002; Flick et al., 1996; Leahy et al., 2001).

Base pairing of nt 11′–13′ of the 5′ end of the vRNA promoter with nt 10–12 of the 3′ end of the vRNA promoter was found to be critical for efficient virus replication in MDBK cells (Fodor et al., 1998). However, it was possible to generate influenza viruses in which the conserved base pairs in the duplex region of the NA gene were replaced by alternative base pairs. The D2 mutation (G–C→U–A at nt positions 12′–11) was found to attenuate the virus significantly. Furthermore, it significantly reduced the NA mRNA and protein levels in virus-infected cells but did not dramatically affect replication (Fodor et al., 1998). The NA D2 virus was also found to be attenuated severely in mice and could be used to elicit protective immunity against a wild-type infection (Solorzano et al., 2000).

The development of plasmid-based techniques for the rescue of influenza viruses (Fodor et al., 1999; Neumann...
et al., 1999) has enabled promoter studies to be extended to other gene segments. In the present study, we test the hypothesis that alternative base pairs in the conserved influenza virus vRNA promoter cause attenuation when introduced into either segment 3 (PA gene) or segment 8 (NS1 and NEP-coding gene).

**METHODS**

Plasmids. The eight influenza A/WSN/33 virus vRNA-encoding plasmids (pPOLI-PB1-RT, pPOLI-PB2-RT, pPOLI-PA-RT, pPOLI-HA-RT, pPOLI-NP-RT, pPOLI-NA-RT, pPOLI-M-RT, pPOLI-NS-RT) were generated as described previously (Fodor et al., 1999). Briefly, vRNAs are expressed by transcription from a truncated form of the human polymerase I promoter and then an exact 3′ end is generated by ribozyme cleavage. Mutations were introduced into the vRNA-encoding plasmids using standard PCR mutagenesis techniques. pcDNA-PB2, pcDNA-PB1, pcDNA-PA and pcDNA-NP protein expression plasmids are pcDNA3 (Invitrogen)-based plasmids and were generated as described previously (Fodor et al., 2002).

Virus rescue. Rescued viruses were generated using a 12 plasmid rescue system, essentially as described originally (Fodor et al., 1999) but with some modifications which have also been described elsewhere (Fodor et al., 2002).

Sequencing the mutated genes of transfected viruses. TRIzol reagent (Invitrogen) was used according to the manufacturer’s instructions to isolate total RNA from MDBK cells infected at an m.o.i. of 1. vRNA was sequenced by standard methods. Details and primers are available from the authors on request. Segment 3 (PA gene) was sequenced fully in the PA-recombinant viruses. Segment 8 (NS1- and NEP-encoding genes) was sequenced fully in the NS-recombinant viruses. No mutations, other than the desired promoter mutations, were detected.

Growth curves. Briefly, as described previously (Fodor et al., 1998), near-confluent MDBK cells in 3-cm diameter dishes were infected with transfected viruses at an m.o.i. of 0·001 and at various time-points after infection, virus particles in the medium were titrated by plaque assay in MDBK cells.

**RESULTS**

Rescue of influenza viruses with alternative base pairs in either segment 3 or segment 8 vRNA promoters and their growth in MDBK cells

We generated viruses with the D1, D2 and D3 mutations (Fig. 1) in the vRNAs of either segment 3 (PA gene) or segment 8 (NS gene) using the plasmid-based rescue system of the PA vRNA, and the internal control primer, targeting PB2 vRNA (described above). Reverse transcription reactions were incubated at 50°C for 90 min, analysed on 6% denaturing polyacrylamide gels and quantified by phosphorimager analysis. To compare the levels of vRNA, mRNA and cRNA in NS D2 or PA D2 virus-infected cells with wild-type virus-infected cells, the product of the experimental primer was normalized to the internal control. The product of mutant virus-infected cells was expressed as a percentage of wild-type.

Western blot analysis of proteins in virus-infected cells. MDBK cells were infected with NS D2, PA D2 or wild-type viruses, as described for primer-extension analysis. At 12 or 24 h p.i., cells were harvested and resuspended in SDS-PAGE loading buffer. The cells were boiled and analysed by SDS-PAGE on 12% polyacrylamide gels. To ensure an equal amount of protein was loaded for each sample, the amount of protein in each lane was quantified using a Bradford assay (Pierce). Membranes (Amersham Pharmacia) were probed with either a polyclonal NS1 antibody (kindly donated by P. Palese, Mount Sinai School of Medicine, New York, USA) or a monoclonal PA antibody, PA-2 (kindly donated by A. Portela, Agencia Española del Medicamento, Madrid, Spain) (Barcena et al., 1994; Ochoa et al., 1995). Bound antibodies were detected by standard methods.

**Fig. 1.** vRNA promoter sequences. (A) Representation of the conserved sequences of the influenza A virus vRNA promoter in the corkscrew conformation. Base pairs are indicated with a line. Conserved base pairs in the double-stranded region involving both the 5′ and the 3′ ends of the RNA segment are boxed. Numbering of residues starts from both the 3′ and the 5′ ends. N, Segment-specific base pairs. The U₃ polyadenylation site is also shown. (B) Base pairs of D1-, D2- and D3-transfectant viruses in the conserved double-stranded region. Mutated base pairs are highlighted.
(see Methods; Fodor et al., 1999; Neumann et al., 1999). The growth properties of the viruses in tissue culture were analysed in MDBK cells (Fig. 2). The NS D1 (Fig. 2A) and PA D1 (Fig. 2B) viruses that contain the D1 mutation (11', 10 A–U→C–G; Fig. 1) were indistinguishable from wild-type in MDBK cells. However, the NS D2 (Fig. 2A) and PA D2 (Fig. 2B) viruses that contain the D2 mutation (12', 11 G–C→U–A; Fig. 1) were attenuated but to different extents. The NS D2 virus (Fig. 2A) was attenuated severely, reaching a maximum titre of approximately 3 log lower than wild-type. The PA D2 virus (Fig. 2B) reached a maximum titre similar to wild-type but exhibited a decrease in growth kinetics, taking up to 12 h longer to reach maximum titre. The NS D3 (Fig. 2A) and PA D3 (Fig. 2B) viruses that contain the D3 mutation (13', 12 G–C→A–U; Fig. 1) both showed signs of slight attenuation in MDBK cells. The virus titres of the NS D3 virus were reproductibly lower than wild-type by 0.5–1 log (Fig. 2A) and the virus titres of the PA D3 virus were always marginally lower than wild-type (Fig. 2B).

**Effect of the D2 mutation on vRNA levels in virus-infected MDBK cells**

Of the three alternative base pairs tested, the D2 mutation caused the greatest attenuation in both the NS gene (Fig. 2A) and the PA gene (Fig. 2B). Therefore, to study the effects of the D2 mutation further, we compared vRNA levels in virus-infected MDBK cells using a primer-extension assay (Fig. 3). On visual inspection, Fig. 3 shows that the transcripts derived from the D2-mutated genes are in lower yield than the corresponding wild-type transcripts. Phosphorimage analysis was used to quantify the differences. Only the 9–12 h time-points were compared, as radioactivity at the 7 and 8 h time-points was insufficient for accurate quantification. To enable a direct comparison between the NS vRNA levels in NS D2 and wild-type virus-infected cells, the NS vRNA levels for both viruses were normalized using an internal control (see Methods and Fig. 3 legend). The normalized NS vRNA levels in NS D2 virus-infected cells were expressed as a percentage of the normalized NS vRNA levels in wild-type virus-infected cells (Fig. 4A). Averaging the values at the different time-points, the NS vRNA levels in NS D2 virus-infected cells were approximately 70% of wild-type (Fig. 4A). A similar analysis of PA vRNA levels in PA D2 virus-infected cells (Fig. 4B) indicated that the PA vRNA levels in PA D2 virus-infected cells were, on average, approximately 41% of wild-type. A Student t-test showed that each time-point was significantly different from wild-type for both the NS D2 virus (Fig. 4A) and the PA D2 virus (Fig. 4B), with all P values <0.01. A further Student t-test comparing the NS D2 with the PA D2 vRNA levels suggested that the difference...
between the 70 and 41% vRNA levels detected for the NS D2 and PA D2 virus-infected cells, respectively, was statistically significant ($P < 0.01$). Therefore, the PA vRNA appeared to be reduced by the D2 mutation more than the NS vRNA.

Effect of the D2 mutation on mRNA and cRNA levels in virus-infected MDBK cells

To clarify further the effects of the D2 mutation on influenza virus RNA, we used primer-extension analysis to study mRNA and cRNA levels in virus-infected MDBK cells (see Methods). One primer can be used to detect both mRNA and cRNA of the same gene, as both RNAs are positive sense (Fodor et al., 1998). Fig. 5 shows a significant decrease in the mRNA-derived transcripts in both NS D2 (Fig. 5A) and PA D2 (Fig. 5B) virus-infected cells compared to wild-type virus-infected cells. However, levels of cRNA appear to be unaffected (Fig. 5). The difference in mRNA and cRNA levels in NS D2 and PA D2 virus-infected cells from wild-type virus-infected cells was quantified (Fig. 6). The mRNA produced from the D2-mutated gene was, on average, approximately 39 and 33% of wild-type in NS D2 (Fig. 6A) and PA D2 (Fig. 6B)
virus-infected cells, respectively. Student t-tests showed that the amount of mRNA at each time-point was significantly different from wild-type in both the NS D2 (Fig. 6A) and the PA D2 (Fig. 6B) virus-infected cells, with all P values < 0.01. However, cRNA levels in cells infected with either mutant virus did not differ significantly from wild-type (Fig. 6C, D). Primer-extension data are summarized in Table 1.

**NS1 and PA protein levels in virus-infected cells**

To determine if reduced mRNA levels (Figs 3 and 4) resulted in reduced protein levels, we used Western blot analysis to study the amount of NS1 and PA protein in virus-infected cells. At 24 h p.i., as expected, both the amount of NS1 protein in NS D2 virus-infected cells (Fig. 7A) and the amount of PA protein in PA D2 virus-infected cells (Fig. 7B) were dramatically reduced. However, to be able to compare directly the decrease observed for the NS1 mRNA (Fig. 6A) with the corresponding decrease in NS1 protein levels, we performed Western blot analysis at 12 h p.i. (Fig. 7C). The yield of NS1 at 12 h p.i. was estimated to be between 8 and 16 times lower than in cells infected with wild-type virus (Fig. 7C). PA protein levels could not be measured at 12 h p.i. because of lack of sensitivity of the Western blots for PA.

**DISCUSSION**

The development of a plasmid-based rescue system (Fodor et al., 1999; Neumann et al., 1999) has enabled us to extend previous work on mutations in the duplex region of the conserved influenza virus vRNA promoter of the NA gene (Fodor et al., 1998). In this study, we have tested the hypothesis that alternative base pairs in the duplex region of the conserved influenza virus vRNA promoter can be used to attenuate influenza viruses when introduced into other gene segments. We have tested the effect of three alternative base pairs, D1, D2 and D3 (Fig. 1), in the NS and PA genes. The growth properties of all viruses were determined in MDBK cells (Fig. 2) and were compared to previous findings for the viruses with the same mutations in the NA gene (Fodor et al., 1998). Growth properties of NS D1 and PA D1
viruses in MDBK cells were indistinguishable from wild-type, supporting the previous finding in the NA gene that the D1 double mutation at positions 11' and 10 does not affect virus growth (Fodor et al., 1998). The D3 mutation in the NS and PA genes also had a very similar effect to that observed in the NA gene. Consistently, the D3 viruses produced titres very similar to, but slightly lower than, wild-type. The decrease in titre is particularly evident for the NA D3 virus (Fodor et al., 1998) and the NS D3 virus (present study). When the results of the D1, D2 and D3 mutations in the NA-transfectant viruses were reported (Fodor et al., 1998), the possibility of a slight attenuation in the growth of the NA D3 virus in MDBK cells was not considered to be significant, as the difference in growth properties from wild-type was very small, i.e. less than 0.5 log difference in maximum titres. However, as this result is now reproduced in the NS and albeit to a lesser extent, in the PA gene, we propose now that it is likely that the D3 mutation can be used in any influenza virus gene to cause a slight attenuation in virus growth. This hypothesis is supported by follow-up work on the growth of the NA-transfectant viruses in mice, where the attenuation of the NA D3 virus was more marked than that in tissue culture (Solorzano et al., 2000).

As observed for the NA-transfectant viruses, the D2 mutation, which replaces a G–C base pair with a U–A base pair at positions 12' and 11 of the vRNA promoter, caused the greatest degree of attenuation of the three mutations tested in both the NS and the PA genes (Fig. 2). Interestingly, the severity of attenuation varied in the two genes tested. The NS D2 virus was most affected, reaching maximum titres 3 log lower than wild-type. The PA D2 virus was least affected, eventually producing titres similar to wild-type but taking up to 12 h longer to reach this maximum titre. The reason for these differences is unknown. One possibility is that the D2 mutation is having a different effect on the different genes because adjacent cis-acting elements, perhaps non-coding regions, modulate promoter activity (Bergmann & Muster, 1996; Zheng et al., 1996). Another possibility is that the D2 mutation has the same effect on both genes but that the virus can tolerate reduced levels of PA protein more readily than reduced levels of NS1 or NEP proteins. To distinguish between these possibilities, we analysed the effect of the D2 mutation on, firstly, levels of vRNA, mRNA and cRNA and, secondly, on levels of NS1 and PA protein in virus-infected cells.

A comparison of the levels of vRNA, mRNA and cRNA in virus-infected cells, as determined by primer-extension analysis (% of wild-type)

<table>
<thead>
<tr>
<th>Virus</th>
<th>vRNA</th>
<th>mRNA</th>
<th>cRNA</th>
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<tbody>
<tr>
<td>NS D2*</td>
<td>70</td>
<td>39</td>
<td>94</td>
</tr>
<tr>
<td>PA D2*</td>
<td>41</td>
<td>33</td>
<td>99</td>
</tr>
<tr>
<td>NA D2†</td>
<td>100</td>
<td>&lt;13</td>
<td>100</td>
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*Data for NS D2 and PA D2 taken from this study using a mean of the different time-points.
†Data for NA D2 taken from Fodor et al. (1998).
were apparently not affected in cells infected with any of the three D2-mutated viruses. One possibility that could explain why the decrease in vRNA levels did not result in significantly decreased cRNA levels is that the total amount of vRNA in virus-infected cells is in excess to the amount of vRNA required to act as a template for cRNA synthesis. Therefore, the modest decrease in vRNA levels observed in the D2-mutated virus-infected cells may not have been sufficient to significantly affect cRNA synthesis. It should be noted, however, that we cannot exclude the possibility that the D2 mutation caused a small reduction in cRNA levels below our detection limits. Whilst the general effect of the D2 mutation on vRNA, mRNA and cRNA levels was the same in each of the three genes, the magnitude of the effect showed some variation among the different genes. The mRNA was reduced to the same level in NS D2 and PA D2 virus-infected cells but was reduced to a greater extent in NA D2 virus-infected cells (Table 1). The magnitude of the effect of the D2 mutation on vRNA production also differed. In this study, we show that the vRNA levels in NS D2 and PA D2 virus-infected cells are reduced, whereas it was reported previously that the vRNA levels in NA D2 virus-infected cells were not affected (Table 1). It is possible that the NA D2 virus may also produce slightly less vRNA than wild-type, as, when it was characterized previously (Fodor et al., 1998), a small reduction may not have been considered significant. It should also be noted that, whilst the vRNA levels in NS D2 and PA D2 virus-infected cells are statistically different from one another (70 and 41%, respectively; Table 1), the difference is still small.

A possible explanation for the variations observed in the effect of the D2 mutation in the different genes (Table 1) is that transcription and replication are influenced by nucleotides outside the conserved vRNA promoter. The non-conserved, non-coding nucleotides that are adjacent to the conserved termini differ in both length and sequence between the genes and have been shown to affect both transcription and replication (Bergmann & Muster, 1996; Zheng et al., 1996). One possible mechanism by which they could affect transcription is by controlling the efficiency of polyadenylation. It has been reported previously that the D2 mutation interferes with polyadenylation of mRNA (Fodor et al., 1998). Differences in the non-conserved nucleotides in the NA, NS and PA genes may modulate any effect on polyadenylation caused by the D2 mutation and therefore, result in differing overall polyadenylation efficiencies. We attempted to characterize the effect of the D2 mutation on the polyadenylation of the viruses presented in this study but were unsuccessful due to very low titres of the NS D2 virus.

To test if the observed decrease in mRNA levels (Table 1) resulted in a decrease in protein levels, we performed Western blots on protein samples from virus-infected cells (Fig. 7). As expected, both NS1 protein in NS D2 (Fig. 7A) and PA protein in PA D2 (Fig. 7B) virus-infected cells were reduced dramatically. The amount of NS1 protein in NS D2 virus-infected cells was estimated at between 8 and 16 times lower than wild-type (Fig. 7C). Although some caution should be taken in directly comparing values obtained from two very different experimental techniques, this suggests that NS1 protein levels are reduced to a greater extent than may be directly expected by the decrease in mRNA (Table 1). Whilst we cannot rule out an effect of the D2 mutation on translation (see below), the difference in NS1 protein and mRNA levels may be linked to the properties of the NS1 protein. The NS1 protein is known to play a key role in the inhibition of host gene expression (reviewed by Fodor et al., 2002; Qiu et al., 1995), 3'-end processing (Nemeroff et al., 1998; Shimizu et al., 1999) and nuclear export of
cellular mRNAs (Chen & Krug, 2000; Chen et al., 1999; Qian et al., 1994; Qiu & Krug, 1994). In addition, NS1 enhances the translation of viral mRNAs (de la Luna et al., 1995; Enami et al., 1994). Specifically, it has been proposed to recruit the eukaryotic translation initiation factor 4GI to viral mRNAs (Aragon et al., 2000). Consequently, the initial decrease in NS1 protein levels that would result directly from the reduction in mRNA may be enough to prevent an efficient inhibition of host gene expression. Competition for translation of viral transcripts with host transcripts would result in decreased translation of all viral proteins. Western blot analysis supports this hypothesis, as not only are the NS1 protein levels dramatically reduced in NS D2 virus-infected cells but PA protein levels are also reduced (Fig. 7B, compare lanes 2 and 4).

It should be noted that the D2 mutation creates an alternative AUG translation initiation codon (complement of UAC; Fig. 1B) in the corresponding mRNA. Whilst we cannot exclude the possibility that this will affect translation of NS1 and NEP in NS D2 virus-infected cells, and PA in PA D2 virus-infected cells, previous work on the NA D2 virus suggests that this would be unlikely since the NA D1/2 virus, which lacked this AUG codon yet incorporated the D2 mutation, was shown to have very similar properties to the NA D2 virus (Fodor et al., 1998). As in the case of the NA D2 virus-infected cells (Fodor et al., 1998), if translation is initiated from the D2 AUG codon in NS D2 or the D2 AUG codon in PA D2 virus-infected cells, a stop codon is reached soon after (5 and 2 aa residues, respectively) translation initiation and therefore, only a short peptide would be produced. Therefore, we have not isolated NS D1/2 or PA D1/2 viruses as the results in Fodor et al. (1998) suggest this to be unnecessary.

Interestingly, the PA D2 virus is able to achieve virus titres similar to wild-type but the maximum titre of the NS D2 virus is 3 log lower (Fig. 2). Both viruses have been shown to have similar phenotypes, i.e. decreased vRNA, mRNA and protein levels. We propose that, under these conditions, influenza virus growth can better tolerate a reduction in PA protein levels than NS1 and/or NEP. Western blot analysis of cells infected with the PA D2 virus illustrated that the reduced PA protein levels did not result in a decrease of NS1 protein detected in virus-infected cells (Fig. 7A, lane 3). These results suggest that, under these conditions, the PA protein may be in excess. This is the first time that an influenza virus has been reported that is able to replicate with reduced levels of one of its polymerase proteins.

Another reason why influenza virus may not be able to tolerate a reduction in NS1 protein levels in MDBK cells is that NS1 is needed to protect the virus against the IFN-I antiviral response, as NS1 is known to act as an IFN-I antagonist (reviewed by García-Sastre, 2001). It has been demonstrated that NS1-deficient influenza viruses replicate efficiently in cells that have deficiencies in the IFN-I response, such as Vero cells, but are attenuated in cell lines with normal IFN-I systems (Egorov et al., 1998; García-Sastre et al., 1998). Our preliminary results (data not shown), which suggest the NS D2 virus is less attenuated in Vero cells than MDBK cells, support this hypothesis. These results also suggest that the attenuation of the NS D2 virus is mainly due to the reduction in NS1 protein and not NEP. Further support for this hypothesis is that an influenza virus mutant that produces very little NEP replicates normally (Elton et al., 2001; Smith & Inglis, 1985).

In conclusion, we have shown that the base pair mutations in the vRNA promoter cause a similar but probably not identical effect in the different genes tested. Therefore, our results are consistent with the hypothesis that the control of transcription and replication is not solely influenced by the minimal vRNA promoter (residues 1–13 of the 5′ end and 1–12 of the 3′ end). This is the first time the effects of such vRNA promoter mutations have been compared in different genes. Specifically, we have demonstrated that the ability of the D2 promoter mutation to attenuate virus growth is not specific to the NA gene but attenuates influenza virus when present in any of the genes tested and therefore, may be considered to be a general response. Therefore, we propose that the introduction of alternative base pairs into the duplex region of the conserved influenza virus vRNA promoter of any of the eight influenza gene segments could be used in the production of live-attenuated vaccine strains.

**ACKNOWLEDGEMENTS**

We thank P. Palese and A. Portela for antibodies and A. Taylor and D. Peiris for DNA sequencing. This work was supported by an MRC programme grant, G9523972, to G. G. B.

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