The relative amount of an influenza A virus segment present in the viral particle is not affected by a reduction in replication of that segment

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The principles of influenza A virus replication and packaging are not fully understood. In order to investigate the signals required for these processes we have introduced mutations in the terminal non-coding region of an influenza A virus neuraminidase (NA) gene. Specifically, we have obtained two viruses, NA/X and NA/Y, which produce a reduced amount of NA-specific genomic RNA in infected cells but not in the viral particle. These data indicate that (i) specific signals which affect the amount of RNA in the viral particle are distinct from those required for viral replication and (ii) the amount of packaged RNA is not strictly dependent on the amount of RNA produced during replication. In addition, mutant NA/Y was shown to be effectively attenuated in mice. Thus, diminished replication of one viral segment might be a principle on which to base a live influenza virus vaccine.

Influenza A viruses are single-stranded negative-sense RNA viruses that possess a segmented genome. Each segment has non-coding sequences at both ends of the gene (Lamb, 1989; Lamb & Horvath, 1991). These sequences contain the signals required for transcription, replication and packaging of the gene (Luytjes et al., 1989). Extensive mutational analysis of these non-coding regions was carried out in vitro and in vivo. These data indicate that nucleotide positions 1–14 on the 3' end and 1–15 on the 5' end (nomenclature is always given as the negative sense) are important for effective transcription and replication (Parvin et al., 1989; Yamanaka et al., 1991; Li & Palese, 1992; Seong & Brownlee, 1992a; Piccone et al., 1993; Mena et al., 1994, Neumann et al., 1994). Binding studies indicate that the polymerase proteins bind to both the 3' (Seong & Brownlee, 1992b; Fodor et al., 1993) and the 5' (Fodor et al., 1994; Tiley et al., 1994) non-coding regions of the genomic viral RNA (vRNA). It was hypothesized that a fork structure formed by the two ends was important for binding (Fodor et al., 1994). Furthermore, a poly(U) tract at a certain distance from the 5' end and a panhandle structure, which is formed by the 3' and the 5' ends seem to compose the polyadenylation signal (Luo et al., 1991).

However, little information concerning the regulation of the amount of vRNA which is packaged into the progeny virus was obtained by these mutational analyses.

The packaging signal for influenza A virus RNAs is not known. In addition, there is still the question as to whether packaging of the different viral RNAs is a random or a selective process.

Viral RNAs are present in equimolar amounts in the viral particles. The quantification of the genomic RNA in the infected cell remains controversial. Smith & Hay (1982) found different amounts of the individual segments in the infected cell. In contrast, Enami et al. (1985) proposed that there are equimolar amounts of these segments in the late stage of infection. This observation could support a random packaging theory. However, the data of Smith & Hay (1982) could indicate a selective principle in the packaging process. More evidence for the selective packaging theory has been derived from experiments which describe the exclusion of certain defective RNA segments from the viral particle (Nayak et al., 1989). In contrast, a random packaging mechanism was further supported by the construction of a nine-segmented influenza A virus (Enami et al., 1991). This demonstrated that one viral particle can host at least nine different viral segments. Another mutant was generated, the NA/B-NS transfectant virus (Muster et al., 1991), which seemed to support a random packaging principle. This influenza A virus, which contained the non-coding sequences of an influenza B/Lee/40 virus on its neuraminidase (NA) segment, shows a 5- to 6-fold reduction of NA-specific RNA in the infected cell (Luo et al., 1992), when compared to the wild-type (wt) virus. Strikingly, the same reduction was found in the viral particle. It was therefore suggested that the relative...
amount of each segment packaged in the viral particle depends on the amount of this individual RNA in the cell.

We have now constructed two new mutant influenza A viruses, NA/X and NA/Y, which also contain nucleotide (nt) changes in their NA gene ends. The new mutants demonstrate that despite a downregulation of the RNA content of one segment in the infected cell, the virus particles still contain wild-type levels of all RNA segments. Furthermore, we have shown that mutant virus NA/Y is effectively attenuated in mice.

Mutant viruses NA/X and NA/Y contain mutations in the non-coding region of the NA segment of the influenza A/WSN/33 virus. The introduced mutations are also present in the NA/B-NS virus. However, in contrast to the NA/B-NS virus we have now introduced parts of the influenza B virus non-coding region in these influenza A virus mutants. The sequences of the non-coding region of the new mutants are shown in Fig. 1. In virus NA/X the uridine residue in position 5 at the 3’ end is replaced by a cytidine. Virus NA/Y has a computer-predicted stem-loop stem structure of 6 nt exchanged for the corresponding 8 nt of the influenza B virus promoter. Mutant viruses were generated by a ribonucleoprotein (RNP) transfection method (Enami et al., 1990; Enami & Palese, 1991), which was developed to construct novel influenza A viruses (García-Sastre & Palese, 1993). First, the mutations were introduced into plasmid pT3NAv1 (Enami et al., 1990), which codes for the influenza A/WSN/33 virus NA gene under the control of a T3 promoter. Plasmid pT3NA/X codes for the NA segment of mutant NA/X. Plasmid pT7NA/Y codes for the NA segment of mutant NA/Y. Both plasmids were constructed by exchanging the 3’ and the 3’ and 5’ termini, respectively, of the viral gene with corresponding polymerase chain reaction (PCR) products which contained the corresponding mutations. Briefly, an EcoRI–NarI fragment was replaced to obtain pT3NA/X and in the case of pT3NA/Y the EcoRI–NarI and a SpeI–HindIII fragment were exchanged. Plasmid-derived synthetic WSN-NA-like RNA was then incubated with purified influenza A virus proteins and transfected in influenza A/WSN-HK virus-infected cells. The influenza A/WSN-HK virus is a WSN-like virus, which contains the NA segment of the influenza A/Hong Kong/68 virus and does not grow on Madin-Darby bovine kidney (MDBK) cells in the absence of a protease. Selection for a virus which contained the synthetic NA segment, but had lost the HK-NA gene was done on MDBK cells. The viruses were plaque purified three times on MDBK cells. Introduced mutations were sequenced at the 5’ end by direct RNA sequencing. To confirm the mutations at the 3’ end, 100 ng of viral RNA was polyadenylated using poly A polymerase (Gibco-BRL) according to the manufacturer’s protocol. This RNA was then amplified.
Fig. 3. Analysis of NA- and NS-specific genomic RNA in the infected cell extracts. RNA preparations were obtained as described in the text and subjected to Northern blot analysis. Viruses are indicated at the top, time points are indicated on the bottom. NA- and NS-specific bands are indicated on the right. (a) Comparison of viruses WSN-wt and mutant NA/X. (b) Comparison of viruses WSN-wt and mutant NA/Y. Key: WSN, WSN wild-type virus; NA, neuraminidase gene; NS, non-structural protein gene. The bands were quantified by a phosphor-imaging scanner. The intensity of the bands was in the linear range for quantification only for the last three time points. The calculated NA/NS ratios for the different viruses are: (a) WSN 6 h p.i., 0.729; 7.5 h p.i., 0.765; 9 h p.i., 0.952. NA/X 6 h p.i., 0.269; 7.5 h p.i., 0.273; 9 h p.i., 0.335. The percentage of the amount of the NA/NS ratio of virus NA/X was compared to the NA/NS ratio of wt virus: 6 h p.i., 37%; 7.5 h p.i., 35.6%; 9 h p.i., 35.2%. (b) WSN 6 h p.i., 0.428; 7.5 h p.i., 0.536; 9 h p.i., 0.631; NA/Y 6 h p.i., 0.075; 7.5 h p.i., 0.122; 9 h p.i., 0.096. The percentage of the amount of the NA/NS ratio of virus NA/Y was not as compared to the NA/NS ratio of wt virus: 6 h p.i., 17.5%; 7.5 h p.i., 23%; 9 h p.i., 15%. Similar ratios were obtained in a second experiment.

by reverse transcription–PCR (RT–PCR) using a poly(T) primer and a reverse primer which annealed to positions 223–243 of the NA segment. The resulting product was sequenced. Both viruses were found to contain the mutated sequences.

In order to investigate the effect of the introduced mutations on the regulation of genomic RNA we quantified the vRNA of the mutants in the progeny virus and in the infected cell. We first determined the amount of NA-specific RNA present in the virus particles. Viruses were grown on MDBK cells and purified on a 30–60% sucrose gradient. Viral RNA was extracted with phenol–chloroform (Enami et al., 1990), separated on a 2.8% polyacrylamide gel and visualized by silver staining. The two mutants and the wt virus appeared to contain equimolar amounts of all viral segments (data not shown). A more precise quantification was then obtained by a Northern blot analysis. In this assay the amount of NA-specific RNA was compared to that of the non-structural protein (NS) -specific RNA (Fig. 2). The NS segment was chosen at random as an internal control. We used NA- and NS-specific riboprobes of positive-sense polarity to detect the genomic RNA. Briefly, viral RNA was run on a 1–5% agarose gel, transferred to a Nytran membrane (Schleicher & Schuell), blotted and hybridized with [α-32P]UTP-labelled riboprobes, which were complementary to the negative-sense NA- and NS-specific RNAs. The NA-specific riboprobe, which spans nt 1–240 of the NA segment in positive polarity was obtained by T7 transcription of FokI-digested pIBI30-NA (Luo et al., 1992). The NS-specific riboprobe, which spans nt 1–300 of the NS segment in positive polarity was obtained by T3 transcription of TaqI-digested pIBI30-NS (Luo et al.,
Quantification of the bands was done using a phosphor-imaging system (Molecular Dynamics). The ratio of NA-specific RNA to NS-specific RNA was the same for the mutant viruses as for the wt virus. Thus, the Northern blot data confirmed the results of the silver staining.

We next analysed the viral genomic RNA (vRNA) in infected cell extracts. MDBK cells were infected with the wt and the mutant viruses at an m.o.i. of 4. Infected cells were harvested at 3-0, 4-5, 6-0, 7-5 and 9-0 h after infection and RNA was extracted from the cells by the RNAzol protocol (Biotex Laboratories). Extracted total RNA (3 μg) was then used for quantification in a Northern blot. Again, the amount of NA-specific RNA of the mutant viruses was compared to that of the wild-type virus. The NS gene was used as an internal control. The assay was done as described above. We did separate experiments for the viruses NA/X and NA/Y in comparison to the wt virus (Fig. 3). For both viruses NA/X and NA/Y we observed a reduction of the NA-specific vRNA in a time-dependent manner. At the endpoint of the experiment the amount of the reduction of the NA segment of virus NA/Y was about 5-7-fold and that of virus NA/X was approximately 3-fold, when compared to the wt virus.

We then investigated the stability of the mutant viruses. We passaged each virus twice undiluted (approximately 10⁷ p.f.u./ml) in 10-day-old embryonated chicken eggs. Viruses were then plaque purified on MDBK cells and virus from two plaques was sequenced. Virus NA/X, which contained the U5C change had reverted to the original uridine residue. Mutant NA/Y was shown to be stable in this experiment.

One of the practical applications of mutations in the viral promoter is the generation of stable attenuated viruses suitable for a live virus vaccine. The maximum titre of virus NA/Y in tissue culture was only 3 x 10⁷ p.f.u./ml, which is 10-fold less than that of WSN-wt virus. We then evaluated the LD₅₀ of mutant virus NA/Y in BALB/c mice. Eight mice per group were inoculated intranasally with 50 μl of different dilutions of the viruses NA/Y and WSN-wt as indicated in Table 1. The immunizations were performed under slight ether anaesthesia. Deaths of mice were recorded daily during an observation period of 16 days. The LD₅₀ value was calculated according to the method of Reed & Muench (1938). Mutant virus NA/Y was attenuated by at least 3 logs in this assay. Thus, the growth disadvantage of mutant NA/Y was augmented in mice as compared to tissue culture.

The influenza A virus non-coding region contains the signals necessary for transcription, replication and packaging of the viral genes. Mutational analysis might be one way of dissecting this region in order to investigate these different processes separately. We have succeeded in generating viral mutants, NA/X and NA/Y, respectively, which contain a reduced amount of the vRNA of one segment in the infected cell but normal RNA levels in the viral particle. Genetic signals which regulate viral replication might be distinct from those which regulate the amount of the same gene in the progeny virus.

In previous experiments we have also obtained viruses like the NA/B-NS transfectant virus and other transfectant viruses with multiple mutations in the terminal non-coding region (Muster et al., 1991; M. Bergmann & A. García-Sastre, unpublished results), which contain a reduced amount of NA-segment specific replication and a diminished amount of the NA-RNA in the progeny virus. Thus, these viruses might contain two defects; one which reduces the amount of replication and a second which leads to a downregulation of segment-specific RNA in the progeny virus.

To the authors’ knowledge it is not known what percentage of the absolute amount of vRNA, which is made intracellularly gets packaged in an influenza A virus infection. In the case of viruses NA/X and NA/Y the reduction of the NA segment in the cell is not reflected in an increased amount of defective particles, which lack the NA gene. This suggests that the relative excess of the non-mutated segments present in the cell is not packaged into the particles.

We cannot rule out that the mutated NA segments contain a superpackaging signal, which would mean that these segments get packaged more efficiently than the other segments. Given the fact that the progeny virus of viruses NA/X and NA/Y contain exactly wt amounts of the NA genes we do not think that this is a likely explanation.

Introduction of mutations in the non-coding sequences of the viral genes might be a reasonable strategy for the attenuation of the virus in order to construct a live virus vaccine. In respect to the previously constructed NA/B-NS virus it seemed that the reduction of one segment in the viral particle was the relevant feature of the attenuation characteristics. However, virus NA/Y indicates that the efficient reduction of one viral segment in...
the cell alone can also correspond to an attenuated phenotype in mice.

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