Vaccine 27 (2009) 6270–6275

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Characterisation of influenza A viruses with mutations in segment 5 packaging signals

Edward C. Hutchinson[a,1], Helen M. Wise[a], Katerine Kudryavtseva[a], Martin D. Curran[b], Paul Digard[a,*]

[a] Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom
[b] Health Protection Agency, Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QW, United Kingdom

ARTICLE INFO

Article history:
Received 14 April 2009
Received in revised form 12 May 2009
Accepted 21 May 2009

Keywords:
Influenza
Packaging signal
Nucleoprotein

ABSTRACT

Influenza A virus vRNA segments contain specific packaging signals at their termini that overlap the coding regions. To further characterise segment 5 packaging signals, we introduced synonymous mutations into the terminal coding regions of the vRNA and characterised the replicative fitness of the resulting viruses. Most mutations tested were well-tolerated, but a virus with alterations to NP codons 464-466, near the 5′-end of the vRNA, produced small plaques and replicated to around one-tenth of the level of wild type virus. The mutant virus supported normal levels of NP and segment 5 vRNA synthesis but packaged reduced levels of both segment 5 and segment 3 into virus particles. This suggests an interaction between segments 3 and 5 during influenza A virus assembly.

1. Introduction

The negative-sense RNA of the influenza A virus genome is divided into eight segments, which are packaged into new virions as they assemble at the plasma membrane of infected cells. As influenza virions do not typically package more than eight segments in total [1–5], packaging a random selection of segments would result in an extremely small proportion of new virions having the full complement of genes required to initiate further infections [3]. As a result, the virus has evolved a mechanism to help ensure each of its eight segments is selectively packaged [6,7]. This selective packaging mechanism utilises *cis*-acting RNA packaging signals in each of the eight segments, the location of which has been inferred from the structure of defective interfering (DI) RNAs [6,8], the packaging of recombinant virion RNA (vRNA) molecules [7,9–13] and the conservation of primary nucleotide sequences [14–16]. Additionally, reverse genetics has been used to introduce point mutations into segments 1–4, 7 and 8, identifying nucleotides that contribute to *cis*-acting RNA signals, including those required for efficient genome packaging [9,10,13–18].

Despite the identification of packaging signals in each of the eight segments of the genome, their mechanism of function remains obscure. A favoured hypothesis is that each segment interacts with a particular set of neighbouring segments, most likely through RNA–RNA interactions, to assemble a specific 'genome complex' containing all eight segments [7,19]. This is consistent with ultrastructural studies showing a complex of eight parallel and closely aligned segments at the point of viral assembly and within virions [1,4,5], as well as with studies in which mutational disruption of the packaging of particular segments affected the incorporation of various other segments in *trans* [10,15,16,18,20]. However, this hypothesis predicts a specific array of vRNAs within the virion and the order of segments in this putative genome complex is currently unknown. This uncertainty, along with the complex tertiary structure adopted by each vRNA when it is folded and encapsidated to form a ribonucleoprotein complex (RNP), has made the identification or prediction of specific interactions between the segments difficult [14,21]. One way to address this problem is to use viral reverse genetics in order to identify *trans*-interacting sequences on the various segments.

We have previously used a bioinformatics approach to identify regions of the eight vRNA coding regions under selection pressure for primary RNA sequence as well as their coding capacity [14]. Regions of low synonymous codon variation correspond to the location of *cis*-acting RNA sequences and have been successfully used to guide the mutation of packaging signals in segments 1, 6 and 7 [14,16,18]. In this paper, we extend this approach to segment 5 of influenza A virus. We found that segment 5 packaging signals were less sensitive to mutational disruption than those of segment 7. However, one cluster of mutations (in codons F464-L466) significantly affected virus fitness by disrupting packaging of segment 5 and, interestingly, also segment 3.

* Corresponding author. Tel.: +44 1223 336920; fax: +44 1223 336926.
E-mail address: pd1@mole.bio.cam.ac.uk (P. Digard).
[1] Current address: Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom.

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.05.053

2. Materials and methods

2.1. Cells, virus, plasmids and antisera

Human embryonic kidney 293T cells and Madin-Darby canine kidney (MDCK) cells were cultured as described previously [22]. Influenza A/PR/8/34 (PR8) virus was generated using an eight-plasmid reverse genetics system kindly donated by Professor R. Fouchier [23]. Site-directed mutagenesis of the reverse genetics plasmids was carried out using mismatched PCR primers and native PFU polymerase (Stratagene). Plasmids were sequenced using a combination of terminal primers and (where necessary) internal primers by the University of Cambridge Department of Biochemistry sequencing facility. Primers and PCR conditions are available on request. Plasmids pCDNA-PB2, pCDNA-PB1, and pCDNA-PA have been described previously [24]. Plasmid pHumanPolI-ffLuc was a kind gift of Dr L. Tiley [25]. Rabbit anti-NP and anti-M1 sera have been described previously [26,27]. Secondary antibodies were purchased from Molecular Probes or LiCor Biosciences (fluorescent conjugates) or DAKO (horseradish-peroxidase conjugates).

2.2. Reverse genetics and virus titrations

Recombinant viruses were produced by transfection of 293T cells with the reverse genetics plasmids, and working stocks produced by subsequent infection of MDCK cells for 48 h, as described previously [18]. Additional stocks were produced by infecting eight-day-old embryonated chicken eggs for 48 h [18]. Segment 5 from all stocks of virus was sequenced to confirm the presence of the desired mutations. RNA was extracted from infected cells using the SV Total RNA isolation system (Promega) or from virus stock using Tri Reagent LS (Sigma), reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega) and a terminal vRNA-binding primer, and amplified by PCR using terminal primers and Illustra *Taq* DNA polymerase (GE Healthcare). Primers and reaction conditions are available on request. Plaque assays were carried out on confluent MDCK cells; plaque assays and plaque size analysis were performed as described in [18].

2.3. Protein and RNA analyses

Infected cell lysates were analysed by SDS-PAGE and western blotting according to standard procedures. Blots were imaged by chemiluminescence using horseradish-peroxidase conjugated secondary antibodies and X-ray film, or by fluorescence using IRDye 800 conjugated secondary antibodies on a Li-Cor Biosciences Odyssey near-infrared imaging platform. To examine the protein content of virus particles, virus stocks were cleared of debris by low-speed centrifugation and then pelleted through a cushion of 33% sucrose in PBS at 91,000 × *g* for 45 min at 4 °C, as described elsewhere [18]; pellets were resuspended in 20 μl SDS-PAGE sample buffer. Reverse transcription to detect segment 5 or 7 vRNA in 500 ng of total RNA extracted from infected cells 8 h p.i. was carried out using avian myeloblastosis virus reverse transcriptase (Promega) and a terminal vRNA-binding primer (5′-AGC GAA AGC AGG AGT TTA AAA TG). Aliquots of these reactions were used in 25 cycles of PCR with Illustra *Taq* DNA polymerase (GE Healthcare) and terminal vRNA- and cRNA-binding primers (as above, and 5′- AGT AGA AAC AAG GAG TTT TTT GAA CAG, respectively); reaction conditions are available on request. The total vRNA content of virus particles was analysed by 6% urea-PAGE and silver staining as previously described [18]. Densitometry was carried out using the program ImageJ [28]. Quantitative RT-PCR was performed essentially as previously described [18] using the SuperScript III Platinum one-step qRT-PCR system (Invitrogen) and a Rotor-Gene 3000 real-time thermal cycler (Corbett Research Limited), using protocols based on UK National Standard method VSOP 25 (www.hpa-standardmethods.org.uk). Reaction conditions, primers, and TaqMan probe sequences are available upon request.

2.4. RNP reconstitution assay

1×10^6 293 T cells per 35 mm well were transfected in suspension using Lipofectin (Invitrogen). To reconstitute RNPs, 250 ng each of pCDNA-PB2, pCDNA-PB1, and pCDNA-PA were transfected along with 250 ng of an NP-expressing reverse genetics plasmid and 100 ng of pHumanPolI-ffLuc. Following incubation at 37 °C for 48 h, cells were lysed and luciferase levels determined with an AutoLumat LB953 luminometer (EG&G Berthold), using 0.6 mM beetle luciferin (Promega).

3. Results

3.1. Mutation of conserved codons in the terminal regions of segment 5

Previously, we showed that the introduction of synonymous mutations into normally highly conserved codons in the terminal regions of segments 1, 6 and 7 caused defects in vRNA incorporation into virus particles, consistent with the presence of *cis*-acting packaging signals [14,18]. Here, we applied the same techniques to study *cis*-acting RNA signals in segment 5 of the virus. The structure of a DI RNA derived from segment 5 [29], our previous bioinformatics analysis [14], as well as the minimal segment 5-derived sequences required to package a reporter gene [12] indicated that, similarly to the other segments of the influenza A genome, the terminal regions of segment 5 vRNA contained packaging signals (Fig. 1A). However, to the best of our knowledge, the structure/function of these packaging signals had not been previously investigated experimentally at the nucleotide level.

Accordingly, guided by our previous bioinformatics analysis [14], clusters of three adjacent, highly conserved codons were identified in the terminal coding regions of segment 5 and the

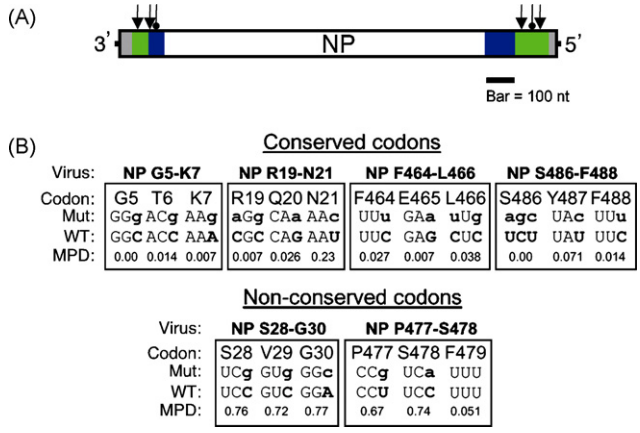

Fig. 1. Mutation of putative *cis*-acting signals in segment 5. (A) Scale diagram of segment 5 vRNA, showing non-coding regions in grey, and the coding region in white. Areas of the coding region defined as the minimal regions required for efficient packaging of a reporter construct are shown in green [12] while additional coding regions present in the shortest reported DI RNA are shown in blue [29]. Pointed arrows indicate mutations introduced to conserved codons and round-headed arrows indicate mutations in non-conserved codons. (B) Details of mutations. For each altered codon the mutant (mut) and wild type (WT) nucleotide sequences are shown in coding sense, with mutations indicated by lowercase bold letters. Also shown is the mean pairwise distance (MPD) score of each codon, scaled from 0 (absolutely conserved) to 1 (no conservation beyond that expected from amino acid constraint) [14](For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).