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Defective segment 1 RNAs that interfere with production of infectious influenza A virus require at least 150 nucleotides of 5′ sequence: evidence from a plasmid-driven system

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The presence of at least 80–90 and more typically around 200 nucleotides (nt) at the 5′ end of the virion-sense RNA in all naturally occurring defective influenza A virus RNAs suggests that this is essential sequence, whereas the 3′-end sequence may be as short as 25 nt. The stability of defective RNA on serial passage with infectious helper virus also depends on the length of 5′-end sequence. Here, we have studied the influence of 5′-end sequences of a panel of six defective segment 1 RNAs from H3N8 and H7N7 viruses on their ability to interfere with the multiplication of plasmid-produced infectious A/WSN virus (H1N1). Four of the H3N8 defective RNAs are identical in overall length but vary in the length of 5′ sequence. Transfected defective RNAs interfered with infectious virus production in a concentration-dependent manner. The extent of interference also depended on the length of 5′-end sequence in the defective genome. This required at least 150 nt and was maximal with 220 nt of 5′ end sequence. The reduction in virus multiplication was highly significant and correlated with the presence of detectable intracellular defective RNA. Packaging of full-length segment 1 RNA by progeny virus was inversely proportional to the packaging of defective segment 1 RNA and may explain the reduction in infectivity. In summary, a critical length of 5′-end sequence is essential for the interfering properties of defective influenza virus RNAs, which indicates that this plays some vital role in the virus life cycle.

Introduction

The genome of the influenza A viruses, members of the *Orthomyxoviridae*, consists of eight single-stranded, negative-sense RNAs and encodes ten proteins. During the course of the virus life cycle, shorter defective RNAs are also generated (Choppin & Pons, 1970; Crumpton *et al.*, 1978; Davis *et al.*, 1980; Janda *et al.*, 1979; Nakajima *et al.*, 1979; Nayak *et al.*, 1978; Pons, 1980) and these arise from full-length genomic RNAs by internal deletion (Crumpton *et al.*, 1979; Davis & Nayak, 1979; Davis *et al.*, 1980; Winter *et al.*, 1981). All defective RNAs possess the 5′ and 3′ ends of the parent RNA segment and most have a large, single, usually central deletion. They are replicated and packaged alongside full-length genomic RNAs, which provide the missing genetic information *in trans*.

Defective RNAs have been isolated from all eight segments, but those from segments 1 and 2 (respectively encoding proteins PB2 and PB1) are found in the greatest abundance (Davis & Nayak, 1979; Davis *et al.*, 1980; Duhaut & Dimmock, 1998; Jennings *et al.*, 1983). Studies have shown that all the information necessary for replication, transcription and packaging is present within the terminal 1–25 nt from the 5′ and 3′ ends (Luytjes *et al.*, 1989). However, almost all defective RNAs analysed to date possess considerably more sequence than this. Jennings *et al.* (1983) isolated a number of defective RNAs, largely from segment 1, from egg-grown PR8 virus and found that all possessed at least 80–90 nt from each terminus and most had in the region of 200 nt. In a more recent study of 50 segment 1, 2 and 3 defective RNAs isolated from WSN- or EQV-infected eggs or mouse lung tissue, almost all were found to possess at least 178 nt from the 5′ end of the virion (v) RNA (Duhaut & Dimmock, 1998). The length of the 3′ end varied much more, and one segment 2 defective RNA possessed just 25 nt from the 3′ end.

At least some defective RNAs are able to interfere with the multiplication of infectious virus (Nayak *et al.*, 1978, 1985; von Magnus, 1954). Defective interfering RNAs have also been shown to protect from both homologous and heterologous

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influenza A virus subtypes *in vivo* (Dimmock, 1996; Dimmock *et al.*, 1986; McLain *et al.*, 1992; Morgan & Dimmock, 1992; Morgan *et al.*, 1993). However, to date it has not been possible to study the mechanism of this interference in detail or determine at what stage in the virus life cycle the interference occurs.

The advent of a plasmid-based transfection system (Pleschka *et al.*, 1996) has enabled more detailed study of the requirements for the propagation of defective RNAs from cell culture to cell culture in the form of defective virus particles. We isolated a defective RNA from influenza virus-infected mouse lung, cloned it into a suitable vector and constructed further clones that possessed less of the 5′ end and more of the 3′ end, such that all were of the same overall size and segment of origin. On transfection and subsequent passage, those defective RNAs that possessed at least 150 nt from the 5′ end proved stable on passage in two different cell lines and with three different subtypes of helper virus (Duhaut & Dimmock, 2000). Those with less 5′ sequence were unstable, and survived only a few passages. More recent technical developments have permitted the construction of infectious virus entirely from cloned RNAs (Fodor *et al.*, 1999; Neumann *et al.*, 1999), and these have enabled us to analyse interference by plasmid-encoded defective RNAs and hence to take these studies further.

Here, using an entirely plasmid-based system, we have investigated the 5′-sequence requirements for interference by a panel of defective segment 1 RNAs. We demonstrate that increasing amounts of transfected defective DNA reduced virus infectivity titres progressively and that the behaviour of the cloned defective virus was similar to that of conventionally produced, authentic defective virus. Interference required a critical length of 5′-end sequence in the defective RNA, which we estimated to be between 150 and 220 nt. A mechanism of interference was suggested by the observed reduction in the amount of full-length segment 1 RNA in released progeny virions, coupled with the appearance of defective segment 1 RNA in the same preparation. In all, these data demonstrate the importance of 5′-end internal sequences of vRNA to the interference phenomenon, and the means to manipulate it.

Methods

■ **Cells and viruses.** Vero cells (European Collection of Animal Cell Cultures, Porton Down, UK) and MDCK cells (J. McCauley, Institute of Animal Health, Compton, UK) were maintained in DMEM containing 10% heat-inactivated FCS (Gibco BRL Life Technologies) and 4 mM glutamine. Influenza virus A/WSN (H1N1) was grown in Vero cells following transfection with the plasmids that are required for infectivity (Neumann *et al.*, 1999). Progeny virus was titrated by plaque assay in MDCK cells by standard methods.

■ **Plasmids.** The 17 plasmids required for the generation of infectious WSN were kindly donated by Y. Kawaoka (Neumann *et al.*, 1999). The construction of the EQV (A/equine/Newmarket/7339/79; H3N8) defective RNAs has been described previously (Duhaut & Dimmock, 2000). Briefly, a defective 445 nt RNA, isolated from infected mouse lungs, was cloned into a POL I vector between a truncated human polymerase I promoter and a hepatitis delta virus ribozyme (Pleschka *et al.*, 1996). This comprised 225 nt of 3′ sequence and 220 nt of 5′ sequence of vRNA (POLI-220). Three further defective RNAs were derived from this with 295 nt of 3′ sequence and 150 of 5′ sequence (POLI-150), 365 nt of 3′ sequence and 80 nt of 5′ sequence (POLI-80) and 415 nt of 3′ sequence and 30 nt of 5′ sequence (POLI-30) (Fig. 1). A second EQV defective RNA of 436 nt (POLI-d136) was also isolated from infected mouse lung and cloned as before (Duhaut & Dimmock, 2000). This is identical to POLI-220 except for an additional 9 nt deletion at positions 136–144 from the 5′ end. A defective segment 1 RNA of 585 nt was isolated from cells infected with A/chicken/Dobson/27; H7N7) and cloned as before (POLI-317). This is identical to RNA 1.2 (Duhaut & McCauley, 1996).

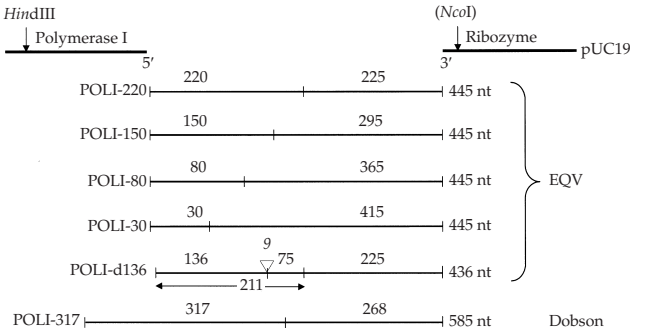

Fig. 1. Plasmids containing defective RNAs under the control of the human RNA polymerase I promoter (POLI) for transfection into Vero cells, together with WSN infectious plasmids (Neumann *et al.*, 1999). All influenza virus constructs are shown as vRNAs in 5′ to 3′ orientation. POLI-220 RNA was cloned from mouse lungs infected with A/equine/Newmarket/7339/79 (EQV; H3N8) (Duhaut & Dimmock, 1998, 2000) and POLI-30, -80 and -150 RNAs were derived from it. POLI-d136 RNA was also cloned from mouse lungs infected with EQV and POLI-317 RNA was cloned from eggs infected with A/chicken/Dobson/27 (H7N7) (Duhaut & Dimmock, 1998; Duhaut & McCauley, 1996).

■ **Transfection.** Subconfluent monolayers of Vero cells in 3 cm diameter dishes were transfected using Fugene and an adaptation of the manufacturer's instructions (Roche Diagnostics). Dishes were washed twice in PBS and then DMEM containing 10% newborn calf serum was added. Fugene was incubated with plasmid DNA (2:1) in 100 μl serum-free, antibiotic-free DMEM at room temperature for 15 min and then added dropwise to the cells. We used 0·5 μg of each of the 17 WSN genomic plasmids (Neumann *et al.*, 1999). In the negative control, we transfected all the plasmids except the PB2 expression plasmid. We used pPOLI-317 at concentrations ranging from 0·1 to 2 μg per transfection and 2 μg of each of the EQV defective plasmids. The same amount of Fugene was used for all transfections in order to exclude any differential toxic effect. Dishes were incubated for 5–6 h at 37 °C and monolayers were then rinsed twice with PBS and incubated further in serum-free DMEM containing 0·2% BSA. Aliquots of medium were removed at 24, 48 and 72 h, clarified at 1000 r.p.m. for 5 min and used for plaque assay or extraction of RNA.

■ **RNA extraction.** RNA was extracted from infected cells and from clarified, virus-containing tissue culture fluids by using the hot-phenol method (Duhaut & McCauley, 1996). RNA concentrations were estimated spectrophotometrically and adjusted to 1 μg/μl.

■ **RT–PCR.** Forward primers were complementary to the first 25 nt of the 3′ end of all eight WSN RNA segments. We reverse-transcribed 0·5 μg RNA, extracted from transfected dishes at 72 h, in 20 μl reaction buffer at 37 °C for 60 min. Reaction buffer was 50 mM Tris–HCl (pH 8·3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP, 10 U ribonuclease inhibitor (Amersham Life Sciences), 13 U Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) and 200 pmol complementary primer. Second-strand synthesis used primers complementary to the first 25 nt of the 5′ end of all eight segments of WSN RNA. PCR was carried out in 20 mM Tris–HCl (pH 8·4), 50 mM KCl, 2 mM $MgCl_2$, 0·2 mM each dNTP, 2·5 U *Taq* DNA polymerase (Gibco BRL), 20 pmol primer and 20 μl first-strand synthesis reaction product. Reactions were performed in a Hybaid touchdown PCR block for one cycle of 94 °C for 120 s, 30 cycles of 94 °C for 60 s, 50 °C for 60 s and 72 °C for 120 s and a final cycle of 72 °C for 8 min. PCR products were separated on a 1·5% agarose gel.

■ **Northern blotting.** Cell RNA (2 μg) or all the RNA extracted from the medium of one 3 cm diameter dish of Vero cells was electrophoresed through agarose. Northern blotting was carried out as described previously (Duhaut & McCauley, 1996) except that run-off riboprobe transcripts (Promega Transcription Protocols) were labelled using digoxigenin–UTP. Hybridization probes were nt 1–225 complementary to the 3′ end of WSN vRNA segments 1 and 5. These were used in excess as determined empirically on filters with known amounts of bound vRNA. Filters were prehybridized for 2 h at 65 °C in 50% formamide, 1% blocking reagent (Roche Diagnostics), 5 × SSC, 45 mM sodium phosphate (pH 5) and 0·3 mg/ml calf intestinal DNA. The RNA probe was boiled with calf intestinal DNA for 5 min and cooled on ice prior to incubation overnight in hybridization buffer, constituted as above, except with 2% blocking reagent and 0·07 mg/ml boiled calf intestinal DNA. Conditions were optimized for blotting of segment 1 RNAs from H1, H3 and H7 viruses so that binding of the probe was equalized. Filters were then washed at room temperature and 68 °C (Duhaut & McCauley, 1996) and reacted according to the manufacturer's instructions (Roche DIG luminescent detection kit) except that the amount of blocking reagent was increased to 2·5% and three washes of 20 min each were used. Filters were autoradiographed with Fuji X-ray film for 30 min and bands were quantified by densitometry.

■ **Statistical analysis.** Interference data were expressed graphically and analysed to determine whether plots deviated from linearity (the runs test) and whether they deviated from the horizontal (the F-test) (Sokal & James, 1995).

Results

Carry-over of DNA or RNA from transfection does not occur

Vero cell monolayers were transfected with the 17 WSN plasmids required for infectivity or the mixture lacking just the PB2 expression plasmid as a negative control and incubated for 72 h. Nucleic acids were then extracted and subjected to RT–PCR with primers that detect virion-sense DNA or RNA, the most sensitive method of detection available. All genomic segments were present in the positive control and no bands of defective RNAs, while in the negative control no detectable plasmid DNA or newly synthesized RNA was detected. Thus, there was a positive signal only when significant amplification had taken place (data not shown).

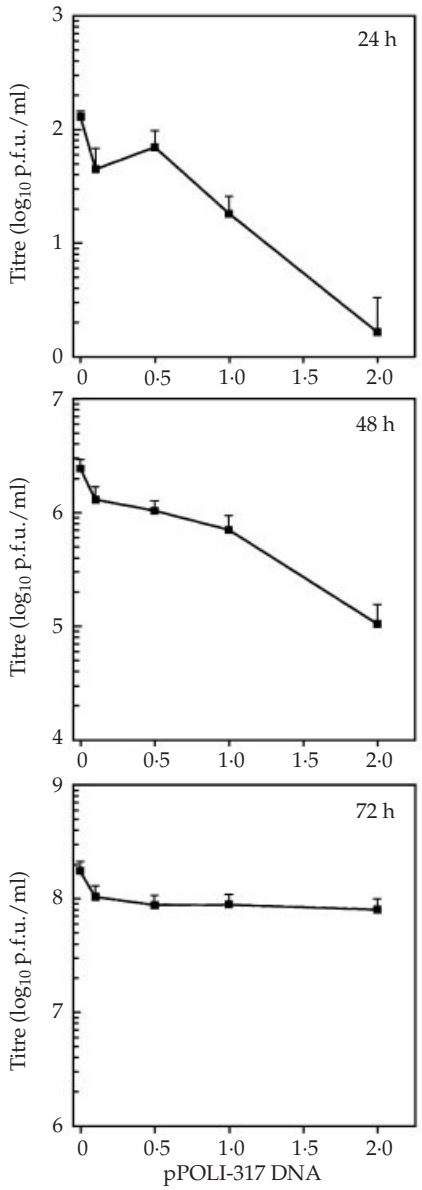

Fig. 2. Interference in the production of infectious WSN in the presence of increasing amounts of pPOLI-317 DNA. Vero cells were transfected with the 17 infectious WSN plasmids (each at 0·5 μg) and various amounts (0–2 μg) of pPOLI-317 DNA. Infectivity in clarified medium was plaque-assayed on MDCK cells at 24, 48 and 72 h post-transfection. Error bars represent the SEM of data from six separate experiments.

Defective influenza virus RNA expressed from pPOLI-317 DNA interferes with the production of infectious WSN

Vero cell monolayers were transfected with all 17 infectious WSN plasmids and increasing amounts (0·1–2 μg) of pPOLI-317 plasmid DNA. Samples of medium were removed at 24, 48 and 72 h post-transfection for plaque assay. Fig. 2 shows that 2 μg pPOLI-317 DNA caused a drop in yield of infectious virus of over 90% at 24 and 48 h post-transfection. However,

Table 1. Statistical significance of the departure from horizontal and departure from linearity of the slopes shown in Figs 2 and 4

			Departure from the horizontal (F-test)		Departure from linearity (runs test)	
Time point (h)	No. of *x* values	No. of *y* replicates	*P*	Significant?	*P*	Significant?
POLI-317 dilution series (Fig. 2)						
24	5	6	0·0001	Yes	0·9815	No
48	5	6	0·0001	Yes	0·8948	No
72	5	6	0·1017	No	0·1312	No
Possession of 5′ end: the 445 nt EQV defective RNAs (Fig. 4)*						
24	5	6	0·0001	Yes	0·3751	No
48	5	6	0·0001	Yes	0·7183	No
72	5	6	0·0076	Yes	0·9994	No
Possession of 5′ end: all defective RNAs (Fig. 4)						
24	7	6	0·0001	Yes	0·4398	No
48	7	6	0·0001	Yes	0·6787	No
72	7	6	0·0031	Yes	0·9995	No

* POLI-30, -80, -150 and -220.

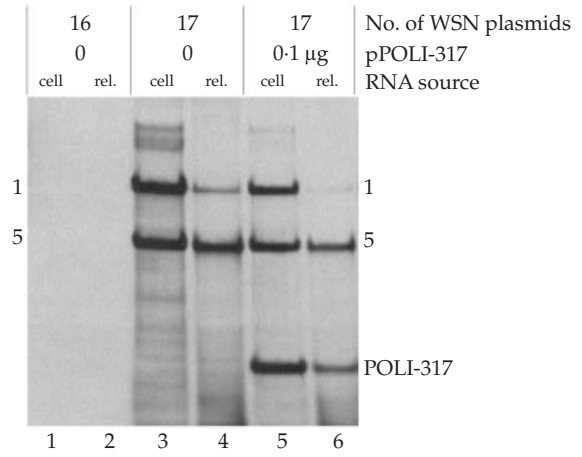

Fig. 3. Decrease in packaging of full-length segment 1 RNA when cells are transfected with infectious WSN plasmids and pPOLI-317 DNA. Vero cells were transfected with WSN plasmids and 0 (lanes 3 and 4) or 0·1 μg (lanes 5 and 6) pPOLI-317 DNA. The negative control (lanes 1 and 2) received 16 of the WSN plasmids and no PB2 expression plasmid. RNA was extracted from transfected cells (cell) and from released virus in the medium (rel.) at 72 h post-transfection and analysed on formaldehyde–agarose gels. Northern blots were probed with nt 1–225 complementary to the 3′ end of WSN vRNA segments 1 and 5, labelled with digoxigenin. The positions of vRNA segments 1 and 5 and the defective POLI-317 RNA are indicated.

little reduction in infectivity titre was seen at 72 h post-transfection. Statistical analysis using an F-test (Sokal & James, 1995) on log-transformed data showed that the departure of the slopes from zero at 24 and 48 h (i.e. the influence of the defective RNAs on virus titres, as opposed to purely random effects) was significant ($P = 0·0001$) (Table 1). A runs test (Sokal & James, 1995) on log-transformed data determined that the 24 and 48 h plots did not deviate significantly from linearity (Table 1). At 72 h, while the F-test showed that defective RNA was not having a significant effect on titres at this time-point, the runs test showed that the resulting line was linear. The combined data from six separate experiments and two statistical analyses mean that it is unlikely that the data resulted from any random transfection or toxic effects. We conclude that RNA expressed from transfected pPOLI-317 (from an H7N7 avian virus) can interfere with the production of cloned infectious human H1N1 WSN, in a concentration-dependent manner.

In infections with conventional virus in cell culture, defective influenza virus RNA 1.2 (POLI-317) interfered with the packaging of genomic segment 1 into virions of its homologous virus (A/chicken/Dobson/27; H7N7) and with those of the closely related A/chicken/Rostock/34 (H7N1) (Duhaut & McCauley, 1996). In order to determine whether this also occurred in the plasmid-based system, RNA was extracted from cells transfected with WSN plasmids alone or from cells transfected with WSN plasmids together with 0·1 μg pPOLI-317 and from released virus in the medium at 72 h post-transfection. RNAs were analysed by Northern blot (Fig. 3). The lanes from cells transfected with all the WSN plasmids except the PB2 plasmid were blank, as expected (lanes 1 and 2), showing that the blot is specific and that no virus was produced. Virion RNAs 1 and 5, but no bands of defective RNA, were seen in the extracts from cells transfected with infectious WSN plasmids and no defective RNA plasmid (lane 3), while RNA extracted from cells transfected with WSN plasmids together with pPOLI-317 DNA showed strong

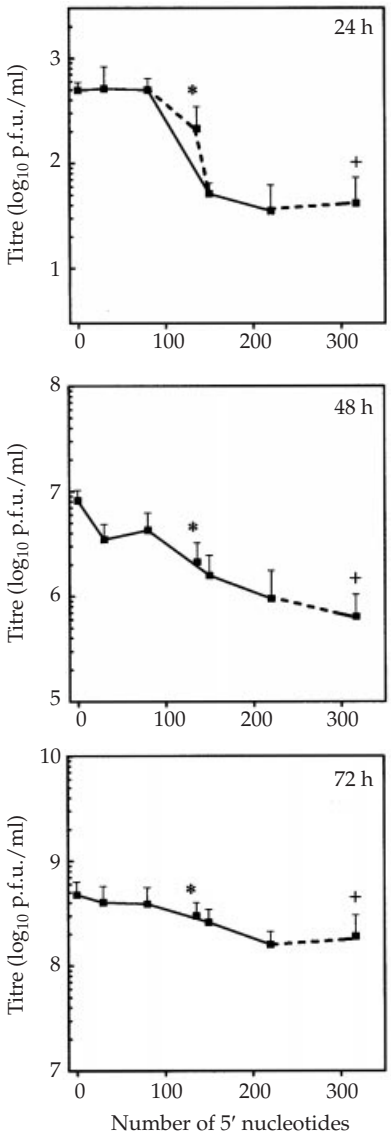

Fig. 4. Interference with the production of infectious WSN in the presence of a panel of plasmids encoding defective segment 1 EQV RNAs with increasing lengths of 5′ sequence. Vero cell monolayers were transfected with 17 infectious WSN plasmids (each 0·5 μg) and 0 or 2 μg defective plasmid. The solid lines refer to the defective plasmids pPOLI-30, pPOLI-80, pPOLI-150 and pPOLI-220, which each comprise 445 nt. Infectivity was determined in clarified medium sampled at 24, 48 and 72 h post-transfection. In addition, interference by two other defective RNAs (POLI-317 and POLI-d136) is shown for comparison. RNA expressed by pPOLI-317 (H7N7) is 585 nt in length and has 317 5′ nt; points are indicated by plus signs (+) and the curve for these is the dashed line. RNA from pPOLI-d136 (H3N8) is 436 nt in length and has 211 5′ nt and encodes the same RNA as POLI-220 apart from a unique 9 nt deletion within the 5′ region (Fig. 1). Datum points from pPOLI-d136 are plotted as if its RNA had a 5′ sequence of 136 nt (see text) and are marked by asterisks (*). All datum points represent means of six separate experiments.

bands of virion segments 1 and 5 and of defective POLI-317 RNA (lane 5). Virus released from cells transfected with infectious virus only showed bands of RNAs 1 and 5 (lane 4), whereas virus released from cultures transfected with infectious virus and defective RNA showed strong bands of RNA 5 and defective segment 1 RNA, but only a trace of full-length RNA 1 (lane 6). By densitometry, full-length segment 1 RNA was reduced, relative to segment 5, by 60% in the presence of defective pPOLI-317 RNA. We conclude that defective H7N7 segment 1 RNA expressed from transfected pPOLI-317 is replicated in cells transfected with infectious WSN (H1N1) plasmids and that defective POLI-317 RNA was incorporated into virions at the expense of full-length segment 1.

Interference of defective influenza virus RNAs with different lengths of 5′-end sequence with the production of infectious WSN

Vero cell monolayers were transfected with the 17 infectious WSN plasmids and 2 μg of one of the EQV (H3N8) 445 nt defective segment 1 plasmids containing 220, 150, 80 or 30 nt from the 5′ end and correspondingly greater lengths of 3′-end sequence. Infectivity in medium removed at 24, 48 and 72 h was plotted against the length of the 5′ end that each RNA possessed (Fig. 4). This shows that infectivity was reduced by $\geqslant$ 90% at 24 h post-transfection and $\geqslant$ 80% at 48 h post-transfection in the presence of RNAs with 150 or 220 nt of 5′ sequence, but was little affected by RNAs having 30 or 80 nt of 5′ sequence. Thus, interference in production of virus infectivity required a defective RNA with at least 150 nt of 5′-end sequence. Statistical analysis with the F-test showed that all plots deviated significantly from the horizontal (P = 0·0001). Reductions were much less, but still statistically significant, by 72 h post-transfection (Table 1). All the plots were significantly linear by the runs test (Table 1). The six repetitions of this experiment, taken together with the highly significant statistical analysis, makes any contribution due to random transfection or toxic effects unlikely.

RNA was extracted from transfected cells to determine whether the various defective RNAs were detectable at 72 h post-transfection. Fig. 5(a) shows clearly the presence of defective RNAs of the size expected in cells transfected with pPOLI-150 and WSN plasmids or pPOLI-220 and WSN plasmids. However, no defective RNAs were detected in cells transfected with pPOLI-30 or pPOLI-80 or in cells receiving WSN plasmids alone. This agrees with previous data that suggest that defective RNAs that have less than 150 nt from the 5′ end are unstable on passage (Duhaut & Dimmock, 2000) when all other factors (size, segment and virus of origin, cell type) are identical.

Influence of the 5′ sequence of defective influenza virus RNAs on interference with production of WSN infectivity: (i) subtype and host species of origin and (ii) possible secondary structure

The effect on production of virus infectivity of two segment 1 defective RNA constructs that differ in length of 5′ end

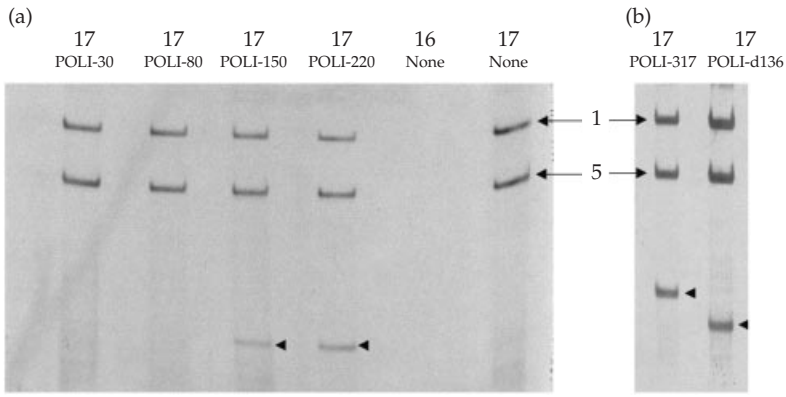

Fig. 5. Stability of defective segment 1 EQV RNAs possessing different lengths of the 5′-end sequence in WSN-transfected cells as shown by Northern blotting. (a) Vero cell monolayers were transfected with 17 infectious WSN plasmids (each 0·5 μg) as shown and either 0 or 2 μg plasmid encoding defective RNA (pPOLI-30, pPOLI-80, pPOLI-150 and pPOLI-220, as shown). Cells were also transfected with 16 of the 17 WSN plasmids (no PB2 expression plasmid). None, lanes from cells that received no defective RNA-encoding plasmid. RNAs were extracted from cells at 72 h post-transfection and analysed on formaldehyde–agarose gels. Blots were probed with nt 1–225 complementary to the 3′ end of WSN vRNA segments 1 and 5 that had been labelled with digoxigenin. The positions of segments 1 and 5 are marked and defective RNAs are indicated by arrowheads. (b) A similar Northern blot of RNA extracted from cells transfected with infectious WSN plasmids and pPOLI-317 or POLI-d136. All data are representative of three separate experiments.

sequence were compared with the defective RNAs examined in the previous section (POLI-30, -80, -150 and -220). pPOLI-317 encodes an avian H7N7 defective RNA of 585 nt and 317 nt of 5′ end sequence. As before, pPOLI-317 was transfected into Vero cells along with infectious WSN plasmids and infectivity of released virus was plotted against the length of 5′-end sequence possessed by each RNA (Fig. 4; datum points indicated by plus signs). This figure shows that pPOLI-317 and pPOLI-220 reduced infectivity titres by a similar amount at all times tested.

The second defective RNA, encoded by POLI-d136, was isolated from EQV-infected mice (Duhaut & Dimmock, 1998). It is identical in sequence to the RNA of POLI-220 and has the same central deletion but differs by virtue of a second, 9 nt deletion (residues 136–144) in the 5′ sequence and hence has a total of 211 nt of 5′ end sequence (Fig. 1). Cells were transfected with pPOLI-d136 and infectious WSN plasmids. The result presented in Fig. 4 (datum points indicated by asterisks) shows that, at 24 h, POLI-d136 RNA clearly inhibited production of infectious virus, but it did so by an amount equivalent to an RNA with slightly less than 136 nt 5′ sequence, rather than an RNA with 211 nt 5′ sequence. At 48 and 72 h post-transfection, POLI-d136 RNA interfered as if it had a 5′ sequence of 136 nt, and hence was plotted in that position on the *x*-axis in Fig. 4. It therefore appears that the 9 nt deletion disrupts and reduces the interfering activity of POLI-d136 RNA, and it may be that interference requires an RNA with a particular 5′ region secondary structure rather than a certain length of 5′ sequence. All three plots were significant by the F-test and all the plots were linear by the runs test (Table 1). Northern blot analysis showed that POLI-d136 and POLI-317 defective RNAs were both clearly present in WSN-transfected Vero cells at 72 h post-transfection (Fig. 5b).

Discussion

The ability of defective influenza virus RNAs to interfere with virus multiplication has been noted both *in vivo* and *in vitro* (Dimmock, 1996; Dimmock *et al.*, 1986; McLain *et al.*, 1992; Morgan & Dimmock, 1992; Morgan *et al.*, 1993; Nayak *et al.*, 1978, 1985; von Magnus, 1954). However, these studies used mixed populations of many different-sized defective RNAs and infectious virus that was probably contaminated with defective RNAs (Janda *et al.*, 1979). Only now is it possible, through the use of cloned defective RNA and cloned infectious virus, to analyse interference by a single defective RNA, and this is the first such study.

In this report, we have shown that transfected POLI-317 DNA gave rise to a defective RNA that was replicated stably in the presence of transfected virus (Fig. 3) and strongly inhibited (by nearly 99%) the production of infectious virus (Fig. 2). It also inhibited the incorporation of full-length segment 1 into virions (Fig. 3). This repeats earlier work using conventionally produced infectious virus (Duhaut, 1992; Duhaut & McCauley, 1996) and demonstrates that defective RNA derived from the plasmid system behaved authentically.

We investigated the importance of the length of 5′-end sequence to interfering activity by using constructs that encoded a panel of four defective EQV RNAs, all segment 1 and all the same length, but varying in the length of their 5′-end sequence. Data showed that RNAs with 150 or 220 nt of 5′ sequence (POLI-150 and POLI-220) interfered with infectious virus production, while those with 30 or 80 nt of 5′ sequence (POLI-30 and POLI-80) did not (Fig. 4). Thus, interference required at least 150 nt of 5′-end sequence. Northern blotting of RNA extracted from cultures at 72 h post-transfection detected POLI-150 and POLI-220 RNAs but

not POLI-30 and POLI-80 RNAs (Fig. 5a). Interference is thus consistent with the physical presence of defective RNA, and both properties are apparently dependent on the possession of a critical length of internal 5′ sequence. Defective POLI-317 RNA is longer than POLI-220 RNA and has more 5′-end sequence, but it did not interfere significantly more with the production of infectious virus (Fig. 4). This may be because POLI-220 carries sufficient 5′ sequence for interference, and interference is not improved by the presence of additional 5′ sequence. These and other data presented above also demonstrate cross-subtype interference, with defective RNAs from an avian H7N7 virus (POLI-317) and from an equine H3N8 virus (POLI-d136, POLI-150 and POLI–220) interfering well with a human H1N1 virus.

It is self-evident that a defective RNA that is unstable in infected cells will not interfere significantly. However, detection of a less than optimally stable defective RNA depends on the sensitivity of the technique used. Thus, RT–PCR with specific primers detected POLI-80 RNA on passage, albeit faintly and intermittently (Duhaut & Dimmock, 2000), while Northern blotting failed to detect it at all (Fig. 5). Interference with infectious virus production is therefore probably the best measure of the length of the 5′ sequence required for the stable expression of defective RNAs.

It is an absolute requirement for interference by defective RNA firstly that it is replicated in the transfected cell and secondly that it is packaged into progeny virions. It begins to look as if the $\geqslant$ 150 nt of 5′ sequence, which is needed for interference and RNA passage stability (Duhaut & Dimmock, 2000), is also implicated in the packaging of segment 1 defective RNA (Fig. 3) and by implication in the packaging of full-length segment 1 RNA. However, earlier studies, using RNA that proved unstable on passage, concluded that RNA-packaging signals reside in the first 25 nt of each segment (Luytjes *et al*., 1989; Odagiri & Tashiro, 1997). We also have found that packaging of unstable defective RNAs does occur (Duhaut & Dimmock, 1998) and we conclude that the earlier data are consistent with the findings reported here. The work of Odagiri & Tashiro (1997) was limited by the difficulty of studying the role of internal sequences with the technology available at the time. This was also true of other studies, which suggested that the amounts of genomic segments could be reduced by the presence of defective RNAs (Akkina *et al*., 1984; Duhaut & McCauley, 1996; Ueda *et al*., 1980). Further work, using plasmid-based systems and more quantification, will establish the relationship between 5′-end sequence and its function(s).

Defective POLI-d136 RNA has the same central deletion as POLI-220 RNA and is identical in sequence except for a second deletion, of 9 nt, situated 136 nt from the 5′ end, and was replicated in cells (Fig. 5) and interfered with production of infectious virus (Fig. 4). However, the level of interference was less than that given by POLI-150 RNA (150 nt of 5′-end sequence) and was consistent with that expected from an RNA with a 5′-end sequence of approximately 136 nt rather than the 211 nt that it possesses. This, together with the stepped nature of the interference response at 24 h derived with the POLI-30, -80, -150 and -220 defective RNAs (Fig. 4), suggests that the interference may not be determined simply by the total length of 5′ sequence present, but rather may involve other properties of the RNA such as secondary structure. However, the 24 h plots of the POLI-30, -80, -150 and -220 defective RNA data or data from all defective RNAs (Fig. 4) were not significantly non-linear (Table 1). Thus, there remains some doubt as to their interpretation and more defective RNAs need to be analysed to determine whether secondary structure is indeed important for their interference activity.

Statistical analysis by the F-test of interference data from Figs 2 and 4 indicated that the probability that the defective RNAs had no effect on virus infectivity titres at 24 and 48 h or that effect was random was 0·0001% (Table 1). This is highly significant and provides strong evidence of the involvement of internal 5′ sequences during the virus life cycle. However, there was less interference at 72 h post-transfection, and in the data shown in Fig. 2 this was not significantly different from the virus control that received no transfected defective DNA. We believe this arises because of the successive rounds of infection by infectious and defective progeny virions that take place over 72 h. Initially, a defective RNA is replicated and interferes with infectious virus production if it enters the same cell as infectious virus. As there was strongest interference at early times post-transfection, we conclude that, at a relatively high initial input of defective RNA, the majority of virus was replicating in cells that also contained defective RNA. Thus, little infectious virus was made and released for subsequent rounds of infection. As a result, the number of p.f.u. per cell dropped below 1 and propagation of the defective RNA inevitably followed suit (Palma & Huang, 1974). This allows infectivity to escape from interference, so that yields of infectivity at 72 h are near maximal across the board.

The runs test, used to determine whether the plots were significantly non-linear, showed that all plots in Figs 2 and 4 were significantly linear with, in some cases, a 98–99% chance that they were linear (Table 1). More data are needed to determine the point at which the plot deviates from linearity, i.e. where no more interference is taking place. This will define the minimum length of 5′ sequence required. Our studies have concentrated on the 5′ end of vRNA, because no defective RNAs lacking internal sequences from the 5′ end have been found. Sequences at the 3′ end are more diverse, and can be short – one apparently stable segment 2 defective RNA possesses only 25 nt from the 3′ end (Duhaut & Dimmock, 1998). Work to address the significance of the 3′-end sequence is currently ongoing.

Previous studies have identified influenza virus promoter sequences within nt 1 to 15/16 of each segment (Flick & Hobom, 1999; Flick *et al*., 1996; Fodor *et al*., 1998; Kim *et al*., 1997). In addition, replication and polyadenylation signals

have been identified within nt 1 to 25–30 (Luo *et al.*, 1991; Poon *et al.*, 1998; Pritlove *et al.*, 1999; Zheng *et al.*, 1996). The data presented here take our knowledge further and suggest that $\geqslant$ 150 nt from the 5′ end of defective segment 1 RNAs are essential for interference as well as passage stability (Duhaut & Dimmock, 2000) and possibly packaging. It remains to be determined how much 5′ sequence of virion segments 2–8 is needed. Our work has implications, too, for understanding reassortment of RNA segments between different virus subtypes, since incompatibilities between 5′ sequences may affect the ability of segments to be propagated and hence the formation of recombinants. It is also likely that influenza virus expression systems function most efficiently when RNAs possess enough 5′ sequence. In all, the appreciation that $\geqslant$ 150 nt from the 5′ end of segment 1 RNA are essential for various aspects of the virus life cycle other than protein coding represents an important advance in our understanding and provides new insights into the biology of influenza A viruses.

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