Defective RNAs Inhibit the Assembly of Influenza Virus Genome Segments in a Segment-Specific Manner

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Four avian influenza viruses have been generated, each containing a single extra defective RNA segment in addition to the eight standard segments. Three of the extra RNAs were derived from segment 1 and the fourth from segment 2. Chick embryo fibroblast cells were infected with each virus, and a wild-type virus. Virus RNA was quantified in extracts of virus-infected cells and in virus released by 10 hr postinfection using reverse transcription and by Northern blot analysis. In the case of two of the viruses the presence of the defective RNA did not markedly affect the accumulation of virus RNA within the infected cell, but significantly and selectively reduced the amount of the “parent” segment in released virus. This effect was reduced in a third virus. In a fourth virus, defective RNA was found to be present at a low-input multiplicity and results were varied. Mixed infections of one of the viruses with a closely related wild-type virus resulted in reduction of the corresponding vRNA segment of the nondefective virus. We conclude that assembly of influenza virus segments is not a purely random process.

INTRODUCTION

The genome of influenza A and B viruses consists of eight segments of negative-sense RNA (reviewed by McCauley and Mahy, 1983; Lamb and Horvath, 1991). In order to be infectious, an influenza virus particle must contain a copy of all eight segments. The means by which this might be achieved have not yet been determined.

All segments of RNA are present in purified virus in equimolar amounts (McGeoch et al., 1976) but the relative quantities of virus RNA segments in the infected cell are not equimolar and are thus different from those seen in virus released from the same cells (Smith and Hay, 1982). These observations suggested that there may be some specific interaction of viral segments involved in packaging.

On the other hand, it has also been suggested that each virion might incorporate more than the required eight segments such that the whole virus population has an equimolar representation of segments, but that each virion contains a random and not necessarily complete selection of RNA segments. This hypothetical strategy of having more than eight randomly selected segments per particle would increase the probability of any one virus particle containing a complete complement of RNA segments and thus being infectious (the mathematical formulas have been derived by Lamb and Choppin, 1983; and by Pederson, Gettingby, and McCauley, unpublished results).

More recently, significant advances involving the rescue of influenza virus genes (Luytjes et al., 1989) indicated that only the first 22 and final 26 nucleotides of influenza vRNA segment 8 were required for packaging the RNA into virus. The termini of influenza virus RNAs are conserved: the terminal 5′ 13 nucleotides and 3′ 12 nucleotides are conserved in each RNA segment; these regions being followed by sequences which are specific for each segment (Skehel and Hay, 1978; Robertson, 1979). Thus, the terminal regions are potential signals for genome packaging. These regions have been shown to exist as the stems of a “pan handle” secondary structure in virus RNA (Hsu et al., 1987). Mutagenesis of these regions has shown that a mismatched adenylate residue in the “pan handle” is crucial for the packaging of RNA into virus (Luytjes et al., 1989).

Influenza viruses readily generate defective RNAs which are derived from standard influenza virus genes (Janda et al., 1979; Nakajima et al., 1979; Pons, 1980) and contain the 5′ and 3′ ends of the parent segment (Davis et al., 1980; Nayak et al., 1982; Jennings et al., 1983). These defective RNAs act as “minigenes” in that they are transcribed and replicated similarly to standard segments, but they accumulate to very high levels in the infected cell.

In this report, we examine the influence of defective RNAs on the packaging of standard influenza virus segments. The data presented indicate that the presence of a single defective RNA can specifically reduce the quantity of the segment from which it is derived in released...
DEFECTIVE RNAs IN INFLUENZA VIRUS ASSEMBLY

However, the presence of a defective RNA does not appear to affect greatly the level of vRNA of the non-defective RNAs in the cell. The data support the suggestion that packaging of influenza virus segments involves a specific mechanism.

MATERIALS AND METHODS

Cells and viruses

The influenza viruses used included A/FPV/Germany/34 (H7N1 — Rostock strain), A/FPV/Dobson/27 (H7N7 — Dobson strain), and a reassortant— SD17, containing segments 4, 6, and 7 of Rostock and 1, 2, 3, 5, and 8 of Dobson strain (Smith, 1985; McCauley and Penn, 1990). Viruses were grown in 11-day-old fertile hen eggs. Primary chick embryo fibroblasts (CEF) were maintained for infection in M199 medium with 2% calf serum.

The production of defective viruses

Reassortant virus SD17 was passaged once at a 1/10 dilution and once undiluted in eggs to produce defective virus. CEF monolayers were co-infected with standard (mRNA sense) SD17 and the progeny of low-dilution-passaged SD17. Dishes were infected with virus at a multiplicity of infection (m.o.i.) of at least 10 plaque-forming units (PFU) per cell, washed thoroughly with fresh M199 medium, and left overnight at 37°. The medium from the overnight mixed infection was diluted and applied to monolayers of CEF cells overlaid with M199 medium with agar. After incubation at 37° for 2 days, the monolayers were stained with neutral red, virus plaques were picked, and virus was amplified in eggs. Virus RNA was extracted from the purified virus and examined by end-labeling with 32P-pCp (England and Uhlenbeck, 1978). Viruses containing single defective RNAs or a restricted number of defective RNAs were selected for further study or subjected to further rounds of plaque purification.

Virus purification

Fluid containing virus was centrifuged at 27,000 rpm for 1.5 hr on a Beckman SW28 rotor. Pellets, resuspended in phosphate-buffered saline (PBS), were loaded onto gradients of 30–60% (w/v) sucrose in PBS overlaid with 15% sucrose in PBS and centrifuged at 27,000 rpm for 1.5 hr on a Beckman SW28 rotor. Virus bands were removed and virus was pelleted by centrifugation. Pellets were resuspended in 10 mM Tris-HCl (pH 7.4)-buffered saline.

Time course of virus infection of chick embryo fibroblast cells

CEF monolayers in 15-cm dishes were infected with either a defective or a standard virus at an m.o.i. of at least 10 PFU per cell. Dishes were washed thoroughly four times in PBS or warm medium and then incubated at 37° in 10 milliliter medium per dish, the media being changed after 2 hr. Two plates for each virus were removed at infection and at subsequent time points as required for the preparation of intracellular RNA and released virus.

RNA extraction

RNA was extracted from released virus as described by Robertson (1979). Intracellular RNA was prepared as described by Hay et al. (1977). Pellets were resuspended in sterile RNase-free water and where sufficient quantities were available, the concentration of RNA was estimated on a spectrophotometer.

Oligonucleotides used

The oligonucleotide primers used corresponded to the 12 nucleotides common to the 3’ termini of all segments or the 18 mers specific for each segment of A/FPV/Rostock/34 (Robertson, 1979). Additional oligonucleotides used in reverse transcription were 1R2, AAAAAATGGAAGTTGA, Rostock segment 1, nucleotides 385 – 403 (mRNA sense); NPR, GGGAGAATGGTTAGT, Rostock segment 5, nucleotides 134 – 149. Details of oligonucleotides used for sequencing are available on request.

Plasmids

Plasmids were made by standard techniques from SD17 vRNA and cDNA was inserted into a Bluescript vector (Duhaut, 1992; J. W. McCauley and G. P. Thomas, unpublished). The plasmids used were for segment 7, a full-length cDNA; for segment 2, a cDNA corresponding to bases 9 – 1500, mRNA sense; for segment 1, three cDNAs corresponding to 8 – 600, 8 – 264, and 1541 – 2338, all mRNA sense.

Northern blotting analysis

RNA preparations containing 2 μg cell RNA, or 2 μl (10% of) virion RNA from virus purified from two 15-cm dishes, were denatured by heating to 65° for 5 min in 50% (v/v) formamide, 2 M formaldehyde, 1 × 3-[N-morpholino]propane sulfonic acid (MOPS) buffer, pH 7, and then electrophoresed on a 1.5% agarose gel containing 2 M formaldehyde and MOPS (Inglis and Darby, 1981). RNAs were blotted onto nylon membranes (Hybond N, Amersham). Run-off riboprobe transcripts (Promega transcription protocols) labeled with [α-32P]CTP of plasmids containing inserts from segments 1, 2, and 7 were used to hybridize these blots. Hybridization probes were used in excess, as determined empirically on filters with known amounts of bound vRNA. Filters were hybridized with the probe overnight at 42° as described (Thomas, 1980). Filters were washed four times, for 15 min each wash, at room temperature in 2× SSC, 0.1% SDS and then in 0.1× SSC, 0.1% SDS at 65° twice for 30 min each wash.
Reverse transcription of RNA

RNA preparations which contained 2 μg infected cell RNA or 15% of the yield of virion RNA were mixed with approximately 8 pmol of oligonucleotide in a volume of 10 μl. The samples were held in a boiling water bath for 2 min and then immediately placed on ice. The samples were then reverse transcribed using 200 units Moloney murine leukaemia virus reverse transcriptase (Bethesda Research Laboratories) and 20 units of RNase Block (Stratagene), buffer (as provided by the manufacturer), and 0.25 mM dNTPs with [α-32P]dATP (specific activity, 2 Ci/mmol) in a total volume of 20 μl for 60 min at 37°. The products were denatured and subject to electrophoresis on 4% polyacrylamide gels.

Quantification of RNA

Northern filters were exposed to X-ray film for 5–24 hr and suitably exposed autoradiographs were scanned by computer densitometry (Bio-Rad Model 620 with 1-D software and Biorad GS670 with Molecular Analyst software). Reverse transcription products were exposed to film for 3–12 days and autoradiographs were similarly quantified. Compensation for the nonlinear response of X-ray film was made by construction of a standard curve relating the optical density measured by the densitometer to measured amounts of a serial dilution of a radiolabeled sample which covered the entire range of detection by the densitometer. The densitometer readings from the Northern blots or reverse transcription analysis were thus converted to relative amounts of radiolabel.

RESULTS

Isolation and characterization of defective influenza viruses

We have carried out a series of experiments to examine the packaging of vRNAs by influenza viruses which carry single defective RNAs. To obtain suitable virus stocks a reassortant avian influenza virus, SD17 (Smith, 1985; McCauley and Penn, 1990), was serially passaged twice at high titer in eggs to generate defective virus (von Magnus, 1954). The progeny von Magnus virus from the second egg passage was then passed in primary CEF cells in a mixed infection with “nondefective” SD17 and the virus from the medium was subjected to one or several rounds of plaque purification in CEF cells. Plaques were picked, amplified in eggs, and screened for the presence of defective RNAs by polyacrylamide gel analysis of RNA from purified virus which had been labeled at its 3′ end with 32P-pCp.

Viruses in which only a single species of defective RNA were detected were further analyzed by reverse transcription of vRNA using primers corresponding to the complement of the 3′ 18 nucleotides of each vRNA segment (Robertson, 1979). Accordingly, three viruses were isolated which contained single defective RNAs derived from segment 1 (viruses 6.13.10a, 6.13.10d, and 1.2) and one virus was isolated with a single defective RNA derived from segment 2 (virus 6.13.10b). The nature of these defective viruses can be most readily seen from reverse transcription of infected cell RNA and an example is shown below in the analysis of virus assembly by reverse transcription (vide infra). The nucleotide sequences of the defective RNAs were determined by direct RNA sequencing on infected cell vRNA extracted 10 hr after infection. The sequence at the extreme 3′ terminal region was determined by sequencing infected cell cRNA. The terminal 3′ sequence of virus 6.13.10b defective RNA could not be determined, since the lower level of defective cRNA in the cell was insufficient to generate sequence data. The presence of nondefective vRNA in the cell did not interfere with the analysis of defective vRNA, since the accumulation of defective vRNA was always much greater than that of nondefective vRNA (see, for example, Figs. 2 and 3).

The results from sequencing showed that the respective lengths of the defective RNAs were 585 nucleotides (virus 1.2), 341 nucleotides (virus 6.13.10a), 448 nucleotides (6.13.10d) for the defective RNAs from segment 1, and 373 nucleotides (6.13.10b) for the defective RNA generated from segment 2. Each defective RNA had a single internal deletion and contained different proportions of the 5′ and 3′ termini of the standard RNA. No mutations of the standard RNA sequence, other than the deletion, were detected in the defective RNAs. The results are summarized in Table 1.

A time course of the release of virus was determined by plaque assay of medium, following infection of cells with standard virus. Virus was detected in the medium from 6 hr after infection and had reached a plateau by 9–12 hr postinfection (data not shown). For all subsequent experiments, medium containing virus was taken at 10 hr postinfection.

The influence of defective RNAs on virus assembly: Northern blot analysis

The accumulation of intracellular and released vRNAs was measured by Northern blot analysis of infected cell RNA and of RNA isolated from virus released from the
cells. Infected cell RNA was prepared at 4 and 10 hr postinfection and was purified from the medium, by sucrose density gradient centrifugation, at 10 hr postinfection. After phenol extraction, RNA was subject to electrophoresis through agarose gels and blotted onto nylon membranes. For each virus two membranes were prepared. One of each pair of membranes was probed in excess with approximately equimolar amounts of two 32P-labeled RNA transcripts corresponding to the cRNA of segments 1 and 7; the other membrane was probed with transcripts corresponding to the cRNA of segments 2 and 7. The inclusion of the segment 7 probe served as an internal control for any variation in the yield of RNA during sample preparation. A schematic diagram of the probes that were used in this analysis is shown in Fig. 1.

The results of Northern blot analysis of cells infected with virus clone 1.2 are shown in Fig. 2. Figure 2A shows the results obtained from probing membranes with cRNAs derived from segments 1 and 7; Fig. 2B shows the results obtained from probing replicate membranes with cRNAs of segments 2 and 7. Both the 28S and 18S ribosomal RNAs were evident in the infected cell RNA tracks of both membranes from this and all other experiments, but they were absent from the tracks of purified virus RNA. This background signal from the ribosomal RNAs served as a marker not only of virus purity, but also as an additional indication of the yield of RNA from the infected cells. As expected, full-length influenza virus RNAs corresponding to segments 1, 2, and 7 were detected, but there was an additional species revealed by the segment 1 probe in both infected cell RNA and released virus RNA, which corresponds to the single defective RNA carried by this virus. Visual inspection of the ratio of hybridization signals from vRNAs 1 and 2 relative to that of vRNA7 showed that vRNAs 1 and 2 accumulate in the cell; however, the ratio of vRNA1 to vRNA7 in purified released virus was drastically reduced compared to the ratio of vRNA1 to vRNA7 in infected cells and compared to the ratio of vRNA2 to vRNA7 seen in both infected cells and purified virus. Therefore, the principal effect of the defective RNA derived from segment 1 in virus 1.2 was that the defective segment 1 RNA inhibited the incorporation of the full-length segment 1 RNA into virions.

Figure 3 shows the results from a similar set of experiments with viruses 6.13.10a, 6.13.10b, and 6.13.10d, in which single defective RNAs from segments 1, 2, and 1, respectively, had been detected. Visual inspection of Fig. 3A, in which a probe for vRNA1 was used in conjunction with a segment 7 probe, shows that in cells infected with virus clones 6.13.10a and 6.13.10d, the defective RNAs accumulated to high levels by 4 hr postinfection. In cells infected with virus clone 6.13.10b, a virus in which only a single defective RNA derived from segment 2 had previously detected, defective RNAs derived from segment 1 were also detected by 10 hr postinfection, though at a much lower level than the defective RNAs from 1.2, 6.13.10a, and 6.13.10d. In cells infected with virus 6.13.10b, the accumulation of the additional defective RNAs, derived from segment 1, varied between experiments but in no experiment did the segment 1 defectives reach a high level. The results obtained from hybridizing a parallel membrane with probes for vRNAs derived from segments 2 and 7 are shown in Fig. 3B. The defective RNA carried by 6.13.10b, which was known to be derived from segment 2, was detectable in cells by 4 hr postinfection, but it did not accumulate to the high intracellular concentration seen with the single defectives from seg-
ryng single segment 1 defective RNAs (data not shown). Nevertheless, the parental virus was included in the analysis, so sister clones and parental viruses can be used as cross-reference for comparison with each other to examine the effects that major single species of defective RNAs have on virus genome assembly.

Autoradiographs were analyzed by densitometry to provide a quantitative estimate of the relative abundances of various RNAs. Examples of the densitometer tracings from the autoradiographs of defective virus Northern blots are shown in Fig. 4. These demonstrate even more clearly than the photographs shown in Figs. 2 and 3 the influence of the defective RNAs on the standard RNA segments. An examination of the scans shows that there was low background radioactivity and below “film-saturating” levels of radioactivity recorded. The scans also give a qualitative estimate of the packaging efficiency of the defective RNAs. The analyses therefore reinforce the conclusions drawn from purely visual inspection. These densitometer traces shown were then used directly in quantification (see Table 2). The readings from the densitometer were standardized to allow for the nonlinear response of the film and the instrumentation by construction of a standard curve to relate the measured absorbance on the film to applied radioactivity. The standard curve related the optical density measured by the densitometer to measured amounts of a serial dilution of a radiolabeled sample and covered the entire range of detection by the densitometer. The densitometer readings from the Northern blots were thus converted to relative amounts of radiolabel. These results from four independent experiments are presented in Table 2 as the relative amounts derived from nondefective vRNA segments 1 and 2 expressed as a proportion of the amount of RNA segment 7. It should be noted that the detection of segment 2 in time course 3 was comparatively inefficient, which may have been a result of a less efficient synthesis of the segment 2 probe in that experiment.

The results showed that following infection of cells with defective viruses 6.13.10a, 6.13.10d, and 1.2, while a slight reduction in the accumulation of segment 1 vRNA may have occurred in the cell, there was a marked decrease in the amount of segment 1 RNA in virus purified from the medium. The magnitude of the relative reduction of segment 1 vRNA compared to that of segment 7 was greatest in viruses 1.2 and 6.13.10d, intermediate in virus 6.13.10a (in three of the four experiments), and least in virus 6.13.10b and the parental SD17, both of which failed to accumulate segment 1 defective RNAs to high levels. The defective RNA derived from segment 2 of virus 6.13.10b accumulated in cells to a lower level than did the segment 1 defectives of the other viruses. Despite this the virus showed, in three of the four experiments, the least relative amount of standard segment 2 vRNA in released virus when compared either with any of the viruses carrying segment 1 defective RNAs and which

FIG. 3. Time course of accumulation of vRNAs of defective influenza viruses 6.13.10a, 6.13.10b, and 6.13.10d in CEF cells, analyzed by Northern blotting. Intracellular RNA and RNA from purified virus were made and analyzed as described in Fig. 2 and probed with RNAs transcribed from plasmids derived from segments 1 and 7 (A), or segments 2 and 7 (B). Tracks marked (a) defective virus 6.13.10a, tracks (b) defective virus 6.13.10b, and (d) 6.13.10d are shown for each time point. Ribosomal RNA (28S and 18S), virus defective RNAs (def), and full-length segments 1 (seg 1), 2 (seg 2), and 7 (seg 7) are also marked.

ment 1 in viruses 6.13.10a, 6.13.10d, and 1.2. No segment 2 defective RNAs were seen in these three viruses.

The results of the analysis of the released RNAs of viruses 6.13.10a, 6.13.10b, and 6.13.10d are also seen in Fig. 3. The internal control of segment 7 shows that less RNA from purified virus was loaded in Fig. 3A than in Fig. 3B. The most striking feature discernable from this pair of Northern blots is that the accumulation of defective segment 1 RNAs to high concentration in the cell is associated in virus released from these cells with a reduction of the amount of the standard segment from which the defective was derived.

The Northern blot analysis which was described above on “sister” defective virus clones was also carried out on the parent virus that had been used to generate the defective viruses. Results from carrying out the analysis on the parent, SD17, showed that SD17 carried defective RNAs prior to its first von Magnus (high-multiplicity) passage in eggs. Despite numerous attempts to cure SD17 of these defective RNAs by plaque picking and by passaging at limit dilution in eggs, it has proved impossible. The defective RNAs that were detected were derived from segment 1 but they accumulated in cells to a much lower level than did the those of 1.2, 6.13.10a, and 6.13.10d, which had been specifically selected for carrying single segment 1 defective RNAs (data not shown). Nevertheless, the parental virus was included in the analysis, so sister clones and parental viruses can be used as cross-reference for comparison with each other to examine the effects that major single species of defective RNAs have on virus genome assembly.
FIG. 4. Time course of accumulation of vRNAs of defective influenza viruses analyzed by Northern blotting. Densitometer scans for each of the time courses of defective viruses described in Figs. 2 and 3. Scans were made of the sample prepared from virus-infected cells at 4 hr postinfection and of the RNA prepared from purified virus recovered from the medium at 10 hr following infection. The positions of 28S and 18S ribosomal RNA and vRNA segments 1 (or 2) and 7 are indicated, along with the defective RNA species. (A) Defective virus 6.13.10a, (B) defective virus 6.13.10b, (C) defective virus 6.13.10d, and (D) defective virus 1.2.

...did not accumulate segment 2 defective RNAs in the cell or when compared with the parental virus, SD17.

Overall the conclusions drawn from the four independent experiments carried out and examined by Northern blot analysis were (1) that in all cases for viruses 6.13.10d and 1.2 a marked reduction in the assembly of segment 1 into released virus was observed; (2) similarly, virus 6.13.10a showed a reduction in standard segment 1, albeit less marked, in three of the four time courses; (3) of the four Northern blot experiments carried out with virus 6.13.10b three experiments showed a significant reduction in the assembly of standard segment 2 compared to the that of other viruses.

Thus defective RNAs can result in a radical reduction...
Table 2
Relative Quantities of Influenza Virus Segments 1 and 2 as a Proportion of Segment 7 Analyzed over Four 10-hr Time Courses by Northern Blotting

<table>
<thead>
<tr>
<th>Time course 1</th>
<th>Defective Viruses</th>
<th>Parent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Def 1.2</td>
<td>6.13.10a</td>
</tr>
<tr>
<td>4 hr s1/s7</td>
<td>0.14</td>
<td>0.29</td>
</tr>
<tr>
<td>4 hr s2/s7</td>
<td>0.15</td>
<td>0.10</td>
</tr>
<tr>
<td>Rel s1/s7</td>
<td>0.06</td>
<td>0.16</td>
</tr>
<tr>
<td>Rel s2/s7</td>
<td>0.59</td>
<td>0.42</td>
</tr>
<tr>
<td>Time course 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr s1/s7</td>
<td>0.30</td>
<td>0.70</td>
</tr>
<tr>
<td>4 hr s2/s7</td>
<td>0.33</td>
<td>0.53</td>
</tr>
<tr>
<td>Rel s1/s7</td>
<td>0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>Rel s2/s7</td>
<td>0.57</td>
<td>0.92</td>
</tr>
<tr>
<td>Time course 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr s1/s7</td>
<td>0.38</td>
<td>0.34</td>
</tr>
<tr>
<td>4 hr s2/s7</td>
<td>nd</td>
<td>0.19</td>
</tr>
<tr>
<td>Rel s1/s7</td>
<td>0.09</td>
<td>0.41</td>
</tr>
<tr>
<td>Rel s2/s7</td>
<td>nd</td>
<td>0.14</td>
</tr>
<tr>
<td>Time course 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr s1/s7</td>
<td>0.29</td>
<td>0.34</td>
</tr>
<tr>
<td>4 hr s2/s7</td>
<td>0.26</td>
<td>0.28</td>
</tr>
<tr>
<td>Rel s1/s7</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>Rel s2/s7</td>
<td>0.12</td>
<td>0.58</td>
</tr>
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</table>

The influence of defective RNA accumulation on the replication and assembly of standard RNAs: Analysis by reverse transcription

The results described above indicate that there seems to be a segment-selective competition in assembly between a defective RNA and the segment from which it was derived. However, no obvious competition was seen between the defective and the other two segments examined by Northern blot analysis. To examine any possible selective effects on other virus RNA segments we have also analyzed virus assembly by using reverse transcription to estimate the levels of vRNA in cells at 4 and 10 hr and in released virus at 10 hr.

Figure 5 shows the products of reverse transcription using an oligonucleotide primer corresponding to the complement of the conserved 3' terminal 12 nucleotides, present on all influenza virus vRNAs, to prime reverse transcription on RNA isolated from cells at 4 hr, 10 hr, and from released virus in medium at 10 hr. The high level of the accumulation of defective RNAs in cells can be clearly seen and the packaging of the defective RNAs into virus is also evident. cDNAs corresponding to copies of RNA segments 1 and 2 cannot be separated on the gels used, since they are identical in size; nonetheless, all other segments are separated from each other. We have consistently noted that segments 4 and 6 appear not to be efficiently packaged compared to the efficiency of packaging of segments 5, 7, and 8 when analyzed by reverse transcription. No selective relative reduction in the assembly of segments 3, 4, 5, 6, 7, and 8 from this analysis was evident when comparing the four individual defective viruses with each other.

The data from reverse transcription failed to detect any further selective inhibition of the packaging of segments other than segments 1 and 2 mediated by the accumulation of defective RNAs into virus. Taken in conjunction with the data from Northern blot analysis, therefore, defective RNAs can inhibit the assembly into virus of nondefective RNAs of the same segment selectively.

Assembly of a nondefective virus is influenced by defective virus in a mixed infection

The observations outlined above suggest that defective RNAs are capable of eliciting a selective reduction of the assembly into virus particles of the corresponding standard segment. Two likely explanations for these results have been considered. The first suggestion is that during the assembly of the virus genome there is simply a competition between the defective RNA and the standard segment from which it was derived based on some segment-specific signal; thus, the relative overproduction of the defective RNA during replication allows it to compete effectively with its standard segment but apparently not, or much less so, with other segments. The second suggestion is that the standard segment of the viruses we
pared to the assembly of RNA segment 7 (data not shown). The accumulation of vRNA in cells and in released virus was analyzed as before, in parallel infections of cells with defective virus 1.2 alone, nondefective virus S3 alone, and these two viruses together. Figure 6 shows the results of Northern blot analysis of the assembly of RNA segment 1 and RNA segment 7. The defective RNA can be seen to accumulate in cells infected with the defective virus and in the dual infection: No defective RNAs can be seen in the S3-infected cells. The assembly of RNA segment 1 can clearly be seen to be reduced in cells infected with virus 1.2, though the overall yield of virus from cells infected with the defective virus was apparently lower than the yield from cells infected with S3 virus in this experiment. However, the assembly of RNA segment 7 serves as an internal control and a comparison of the relative levels of segment 1 assembly in each set of infected cells can be made. From Fig. 6, the relative efficiency of the assembly into released virus of RNA segment 1 can be seen to be reduced qualitatively in the mixed infection, vis-à-vis cells infected with S3 alone, albeit the reduction was less extensive than in cells infected with defective 1.2 alone. There is also a slightly lower level of accumulation of RNA segment 1 in the dual-infected cell. The results from densitometry and quantification of this blot and a blot from another time course are shown in Table 3. The probes used in each set of experiments were not synthesized at the same time, so the relative intensities of the signal of segment 1 and segment 7 between the two experiments varies. In both experiments the same observations were made: (1) defective 1.2 showed the lowest level of incorporation of segment 1 into progeny virus compared with that of segment 7 assembly, (2) the virus from mixed infection of cells showed an intermediate inhibition in segment 1 packaging, but (3) the nondefective virus, S3, showed an efficient packaging of the segment 1 compared to segment 7. These results suggest therefore that the defective virus inhibits the assembly of segment 1 of a nondefective virus.

We have analyzed the assembly of a virus in which we could detect few defective RNAs (and none which accumulate to high levels in the cell)—S3, a plaque-purified virus derived from A/FPV/Rostock/34 (Almond et al., 1979). Northern blot analysis followed by densitometry showed that this nondefective virus packaged its segment 1 and segment 2 RNAs with similar efficiency, compared to the assembly of RNA segment 7 (data not shown).
Relative Quantities of Influenza Virus Segment 1 as a Proportion of Segment 7 in Single or Mixed Infections over Two 10-hr Time Courses Analyzed by Northern Blot Analysis (Viruses 1.2 and S3)

<table>
<thead>
<tr>
<th>Time point</th>
<th>Virus</th>
<th>Ratio s1/s7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time course 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr</td>
<td>1.2</td>
<td>0.53</td>
</tr>
<tr>
<td>4 hr</td>
<td>S3</td>
<td>0.48</td>
</tr>
<tr>
<td>4 hr</td>
<td>Mixed</td>
<td>0.63</td>
</tr>
<tr>
<td>Released</td>
<td>1.2</td>
<td>0.24</td>
</tr>
<tr>
<td>Released</td>
<td>S3</td>
<td>1.45</td>
</tr>
<tr>
<td>Released</td>
<td>Mixed</td>
<td>0.45</td>
</tr>
<tr>
<td>Time course 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr</td>
<td>1.2</td>
<td>0.15</td>
</tr>
<tr>
<td>4 hr</td>
<td>S3</td>
<td>0.33</td>
</tr>
<tr>
<td>4 hr</td>
<td>Mixed</td>
<td>0.30</td>
</tr>
<tr>
<td>Released</td>
<td>1.2</td>
<td>&lt;</td>
</tr>
<tr>
<td>Released</td>
<td>S3</td>
<td>0.37</td>
</tr>
<tr>
<td>Released</td>
<td>Mixed</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Note. <, value too low to quantify.

However, these results do not conclusively show that in the dual-infected cell the reduction in the assembly of standard segment 1 into virus resulted from reductions in the assembly of standard segment 1 from both defective and nondefective virus, but shows an overall reduction in the relative amount of segment 1 compared to that of segment 7.

To assess independently the assembly of segment 1 from the nondefective and the defective viruses, another set of experiments was carried out in which the standard RNA segment 1 from each virus in the dual infection could be differentiated. Since the defective virus was a reassortant between a Rostock-derived clone and a Dobson-derived clone, whereas the nondefective virus S3 was entirely Rostock derived, the origin of segment 1 was different and therefore segment 1 of each virus was distinguishable. Experiments were set up in which oligonucleotides specific for segment 1 of each virus were used to prime reverse transcription of RNA extracted from infected cell or from virus purified from medium and the products subjected to electrophoresis on polyacrylamide gels. The following oligonucleotides were used: (1) 7, 1–18, a segment 7-specific oligonucleotide which detects the accumulation of segment 7 of both viruses in the dual infection since they both have the same segment 7; (2) NPR, a segment 5 Rostock-specific oligonucleotide to detect the replication of the nondefective virus and (3) 1R2, a segment 1-specific oligonucleotide which detects only the Rostock-derived RNA segment 1. (The Rostock-specific oligonucleotides primed reverse transcription internally and so give rise to smaller cDNA products than oligonucleotides corresponding to terminal sequences of vRNA.) In Fig. 7, an autoradiograph of the cDNAs produced in such an experiment is shown. The essential results are in the tracks which show the analysis of Rostock RNA segments 1 and 5, and both Rostock and defective virus RNA segment 7 in single and dual infections. The amount of the product of reverse transcription of RNA segment 5 serves as a quantitative measure of the level of Rostock RNA. It is evident that a similar level of accumulation in the cell of Rostock–RNA segment 1 relative to segment 5 (or segment 7) has occurred in both the single and dual infection, but the amount of Rostock–RNA segment 1 was reduced relative to that of segment 5 (or segment 7) in the released virus purified from the medium from the dual infection, compared to virus in the equivalent medium from the single, Rostock-alone, infection. Other parallel results showed that the oligonucleotides used were indeed specific for their respective segments as designed. As expected, the single infection of cells with virus 1.2 led to a profound reduction in the assembly of RNA segment 1 into the progeny virus (data not shown). The autoradiographs were scanned by a densitometer. Although the amounts of the reverse transcripts of RNA segment 1 at 4 hr and in the released virus in the dual

![Fig. 7. Time course of assembly of RNAs of mixed infections of defective and nondefective influenza viruses in CEF cells.](image-url)
DEFECTIVE RNAs IN INFLUENZA VIRUS ASSEMBLY

Infection were too low to be quantified by densitometry, it was nonetheless clear that the packaging of segment 1 from S3 was reduced in the dual infection: The amounts of segments 5 and 7 in released virus were higher than in the samples from the cells taken at either 4 or 10 hr, yet the amount of S3 segment 1 was lower than it was in the 10-hr time point. Moreover, the amounts of segments 5 and 7 in the released virus from the mixed infection were higher than the amounts of these segments in the virus released from cells infected with S3 alone but the amount of segment 1 was lower in the virus from the mixed infection compared to that of virus from cells infected with S3 alone.

Essentially similar results by Northern blot analysis and reverse transcription from a mixed infection of Rosstock S3 nondefective virus and defective virus 6.13.10d have been obtained, though with a slightly less marked reduction of segment 1 in released vRNA of both single and mixed infections, as was seen using defective 1.2.

These results show that a defective RNA can inhibit the assembly of a nondefective RNA segment from another virus, in addition to the inhibition of assembly of standard RNA from the defective virus itself, in a segment-specific manner and are consistent with a model in which the assembly of the RNA segments into virus is controlled in a segment-specific way.

DISCUSSION

In this paper we present observations carried out in tissue culture on the effect of the accumulation of single defective RNA segments on the assembly of standard vRNA segments in progeny virus. Four viruses, which carry single defective RNAs, were generated and used to show that a correlation could be drawn between the accumulation of large amounts of single defective RNAs intracellularly by 4 hr postinfection, and a marked reduction of the amount detected in progeny virus of the standard vRNA segment from which the defective was derived. This effect on vRNA assembly was not equally paralleled by the synthesis of the standard segment in the infected cell. On the basis of these observations, we submit that there is segment-specific competition between the defective vRNA and the standard vRNA for assembly into virus. We therefore infer that the assembly of the influenza virus genome into progeny virus is a specific process and that segment-specific packaging signals are present within the genome.

The results can be summarized as follows. Consistently, two out of three viruses which carried a defective RNA derived from RNA segment 1 also showed an impaired packaging of the standard RNA segment 1, irrespective of whether the standard RNA was from the "helper" virus or a "nondefective" virus stock. However, we also analyzed two other virus stocks that carried single species of defective RNA molecules, one from segment 1 and one from segment 2: the results from these two viruses were less striking or less consistent.

The defective RNA in virus 6.13.10a showed a lower level of packaging than the defective RNAs of 6.13.10d or 1.2, but in three of four experiments a reduction in the assembly of the standard segment 1 of this defective virus was seen, albeit this reduction in the assembly of the standard segment 1 was less striking than it was in cells infected with 6.13.10d or 1.2. In virus 6.13.10a, the level of the defective RNA in the inoculum was about the same as for the other two viruses which carried defects from RNA segment 1, viruses 1.2 and 6.13.10d. We reiterate that no mutations were found in any of the defective RNAs compared to the undeleted standard segments, so we presume that the defective RNA carried in 6.13.10a is inherently hampered in assembly in a manner, as yet, undefined. The size of defective RNAs of Sendai virus has been proposed to be a factor in the budding efficiency of defective RNAs (Re and Kingsbury, 1988; Mottet and Roux, 1989). If the size of the defective RNA of influenza viruses may also play a role in assembly, it is noteworthy that the defective RNA carried by 6.13.10a is the smallest RNA that we have examined (Table 1). Thus the defective carried by 6.13.10a may be the least efficient in the assembly of a segment 1-derived defective RNA because of its small size and hence also least efficient in competition with the standard segment.

On the other hand in the only defective virus which we isolated carrying a single defective RNA derived from segment 2, virus 6.13.10b, the amount of defective vRNA that accumulated in the infected cells was much lower than that in the others we analyzed (and late in infection defective RNAs from segment 1 were detected). Northern blot analysis showed that the segment 2-derived defective RNA was present in the virus inoculum at about one-tenth the input multiplicity of the segment 1-derived defective RNAs of the other defective viruses (data not shown). Therefore, failure to establish replication of the defective RNA in a large proportion of cells is likely to have resulted in failure to detect a reduction in the assembly of the corresponding standard vRNA segment (due to an overshadowing of the selective effects of the defective RNA by virus produced from cells in which the defective RNA did not replicate). We suggest that the lower and less consistent competition in assembly seen in virus 6.13.10b could have arisen from a variable and lower multiplicity of infection by defective RNA.

Notwithstanding our reservations concerning the results of two of the defective viruses, the others consistently showed a very marked reduction in the assembly of RNA segment 1 compared either to any effect on its synthesis or to any inhibition of the assembly of other standard segments. We propose that this effect is caused by a segment-specific competition with the standard segment during assembly and from this we infer the existence of segment-specific packaging signals.

Our inference that there are segment-specific packag-
ing signals in influenza viruses is consistent with some other reports. Smith and Hay (1982) examined the synthesis and packaging of [3H]uridine-labeled vRNA and showed that vRNA segments 1, 2, and 3 were underrepresented in progeny virus compared to the levels in the cell. They proposed that the reduction of the assembly of segments 1, 2, and 3 which they observed may have been associated with defective RNAs in their virus stocks.

A nonrandom loss of polymerase genes of defective virus was also noted by Ueda et al. (1980), but although these authors did not compare the amounts of vRNA in the cell in relation to the levels of vRNA seen in the virus, they concluded that there was a segment-specific inhibition of the synthesis of vRNAs influenced by the defective RNA. Ueda et al. used T1 fingerprints to identify the genetic origin of the defective RNAs that they studied and concluded that the defective RNA did not interfere with the RNA of the segment from which it was derived, but interfered with a different segment. Our results and conclusion are at variance in two crucial respects with those of Ueda et al. (1980), however. The competition we observed was not primarily during synthesis but in assembly, and we did not see a defective RNA exerting a segment-specific effect on a standard segment from which it was not derived.

Odagiri and Tobita (1990) examined a defective virus with defective RNAs from only the PA gene and noted that segment 3 was present at a low level in virus, but the level of vRNA 3 in the cell was not examined, but subsequently Odagiri et al. (1994) examined the synthesis of vRNA and cRNA in the cell showed that the replication of the PA gene was primarily suppressed at the level of cRNA synthesis, but in addition assembly was also affected. In the light of our results, we might propose that the selective inhibition of nondefective virus segments seen in the above studies was initially likely to be due to a defect in the assembly of an individual segment.

The results obtained by Luytjes et al. (1989), Muster et al. (1991), and Enami et al. (1991) from experiments in which virus was produced by the rescue of RNAs from cloned DNA have been taken to indicate that the packaging of influenza virus RNA segments is a random process. Three types of experiment have been carried out. In the first, reported by Luytjes et al. (1989), the chloramphenicol acetyl transferase gene was flanked by influenza virus-specific terminal sequences from segment 8. This artificial RNA could be assembled into virus, but the chimeric RNA behaved in some ways like a defective RNA: it varied in titer between passages and a low titer of CAT could be reactivated by superinfection with nondefective virus. Hence, these experiments do not rule out the possibility that a selective packaging mechanism could have occurred, although the selective packaging would have had to have been effected by a relatively small number of influenza virus-specific nucleotides. In another similar set of experiments, an influenza virus type A neuraminidase gene was constructed with influenza virus type B termini. The resultant chimera was still capable of packaging into influenza virus type A progeny. Thus, although there is a low level of sequence homology between the termini of type A and type B viruses, the type B virus termini were recognized by type A virus (Muster et al., 1991). The efficiency of the assembly of these heterotypic constructs remains to be established. A later study using transcription of a RNA polymerase 1-based transcription system also concluded from their work on the packaging of CAT vRNA-like constructs that assembly was a neutral process (Neumann and Hobom, 1995).

In another series of experiments, Enami et al. (1991) managed to produce, under a double selection protocol, a virus which was reckoned to carry two copies of RNA segment 8. The virus was only stable in the presence of double selection. If the model is proposed that the packaging of each segment of vRNA is random, then packaging 8 segments of RNA from eight species of RNA leads to a very low level of virus with a full genome complement. The level of virus with a full genome increases as more segments of RNA are packaged and comes close to the typical particle:PFU ratio of influenza viruses when 10 or 11 RNA segments are packaged into each virion (Enami et al., 1991; also mathematically calculated by Pedersen, Gettinby, and McCauley, unpublished observations). Thus the packaging of greater than 8 vRNA segments is thought to be a requirement for a random packaging model and a lower particle to PFU ratio will ensue. One caveat to this is that the particle:PFU ratio observed for influenza viruses is not widely different from that observed in other RNA viruses that have a nonsegmented genome. Particle:PFU ratios of 140 to 300:1 (Joklik and Darnell, 1961) for poliovirus and 400 to 2000:1 for rhinovirus (Korant et al., 1972) are not obviously significantly lower than figures for influenza virus of 10:1 to 100:1 (Donald and Isaacs, 1954). It is not clear, therefore, that the PFU:particle ratio seen in influenza virus reflects a proportion of particles which do not contain a full genetic complement.

The packaging of the genome RNAs of bacteriophage φ6 offers some interesting parallels with the packaging of influenza virus genes. The genome of φ6 is made up of three dsRNA segments (S, M, and L) which are packaged as single-stranded positive-sense transcripts. The conclusions drawn from two independent studies (Qiao et al., 1995; Frilander and Bamford, 1995) on the packaging of the bacteriophage genome were that (1) packaging is strongly ordered: the packaging of S, precedes alone, the packaging of M requires S, and the packaging of L requires M and S; and (2) the signals for packaging are located about 200 nucleotides from the 5' end of the transcript. From the results discussed above from the rescue of artificial RNAs it is clear that at least some (if not all) of the packaging signals for influenza virus RNAs are located at the termini but is feasible that the efficiency
of packaging may be influenced by sequences elsewhere in the RNA.

The results presented in this paper on the influence of "natural" defective RNAs on packaging strongly suggest that packaging of influenza virus segments is not purely random and we infer the presence of a segment-specific selective packaging signal. However, taking our results together with those from other laboratories, the problem is not a simple one and many questions remain unanswered.

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REFERENCES


