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Homologous Interference Mediated by Defective Interfering Influenza Virus Derived from a Temperature-Sensitive Mutant of Influenza Virus

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A temperature-sensitive group II mutant of influenza virus, *ts*-52, with a presumed defect in viral RNA synthesis, readily produced von Magnus-type defective interfering virus (DI virus) when passed serially (four times) at high multiplicity in MDBK cells. The defective virus (*ts*-52 DI virus) had a high hemagglutinin and a low infectivity titer, and strongly interfered with the replication of standard infectious viruses (both *ts*-52 and wild-type ts^+) in co-infected cells. Progeny virus particles produced by co-infection of DI virus and infectious virus were also defective and also had low infectivity, high hemagglutinating activity, and a strong interfering property. Infectious viruses ts^+ and *ts*-52 were indistinguishable from *ts*-52 DI viruses by sucrose velocity or density gradient analysis. Additionally, these viruses all possessed similar morphology. However, when the RNA of DI viruses was analyzed by use of polyacrylamide gels containing 6 M urea, there was a reduction in the amount of large RNA species (V1 to V4), and a number of new smaller RNA species (D1 to D6) with molecular weights ranging from 2.9×10^5 to 1.05×10^5 appeared. Since these smaller RNA species (D1 to D6) were absent in some clones of infectious viruses, but were consistently associated with DI viruses and increased during undiluted passages and during co-infection of *ts*-52 with DI virus, they appeared to be a characteristic of DI viruses. Additionally, the UV target size of interfering activity and infectivity of DI virus indicated that interfering activity was 40 times more resistant to UV irradiation than was infectivity, further implicating small RNA molecules in interference. Our data suggest that the loss of infectivity observed among DI viruses may be due to nonspecific loss of a viral RNA segment(s), and the interfering property of DI viruses may be due to interfering RNA segments (DI RNA, D1 to D6). *ts*-52 DI virus interfered with the replication of standard virus (ts^+) at both permissive (34°C) and nonpermissive temperatures. The infectivity of the progeny virus was reduced to 0.2% for ts^+ and 0.05% for *ts*-52 virus without a reduction in hemagglutinin titer. Interference was dependent on the concentration of DI virus. A particle ratio of 1 between DI virus (0.001 PFU/cell) and infectious virus (1.0 PFU/cell) produced a maximal amount of interference. Infectious virus yield was reduced 99.9% without any reduction of the yield of DI viruses. Interference was also dependent on the time of addition of DI virus. Interference was most effective within the first 3 h of infection by infectious virus, indicating interference with an early function during viral replication.

Although defective interfering virus particles (DI virus or von Magnus virus) possessing normal or nearly normal hemagglutinating activity but decreased infectivity were first shown in 1954 to be produced by serially undiluted passages of influenza virus in a given host (31), very little is known about physical and chemical properties of DI influenza viruses. The intracellular events that lead to the formation of these DI particles remain unknown. Furthermore, the mechanism by which DI influenza viruses cause an inhibition of homologous infectious virus but not of DI virus is still obscure. DI influenza virus particles have been characterized by their marked pleomorphism and altered polypeptide composition (1, 17). However, the nature of the DI viral genome has been controversial. Earlier

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reports indicated that DI viruses lack entirely or contain only reduced amounts of the largest segment of viral RNA (4, 6, 23). However, this view that DI influenza viruses lack a specific RNA segment has been disputed by a recent observation which suggests that influenza DI viruses do not selectively lose a specific RNA segment (2). There are a number of major difficulties in studying DI influenza viruses. (i) Unlike other DI viruses, DI influenza virus particles cannot be physically separated from infectious viruses. Thus, a relatively pure population of DI viruses is difficult to obtain. (ii) A host-virus system which will readily permit the growth of DI or infectious virus in a predicted fashion under controlled conditions is not readily available. For example, WSN strain (HON1), a widely used influenza virus, preferentially produces infectious virus in MDBK cells (3), whereas in cultured chick embryo cells the same virus tends to produce defective particles. (iii) Additionally, the segmented nature of viral genome (21) and the presence of randomly incomplete virus lacking one or more RNA segments (7) have further complicated the study of DI influenza virus.

However, we have recently found that *ts*-52 (a temperature-sensitive mutant of WSN belonging to complementation-recombination group II [15, 29]), when passed serially undiluted in MDBK cells at the permissive temperature, readily produces DI virus with a high hemagglutinin (HA) titer and a low PFU/HA ratio. Contamination from infectious virus is only 0.1% or less in the DI population as measured by PFU/HA ratio. Furthermore, a relatively large amount of DI virus could be produced from the clones of this mutant (*ts*-52) by our procedure. Thus, the availability of this high-quality defective virus in large quantity permitted us to study the nature of DI influenza virus as well as the mechanism of homologous interference. In this system we found that the *ts*-52 defective virus (*ts*-52 DI virus) thus prepared markedly reduced the yield of infectious virus in MDBK cells coinfected with wild-type (ts^+) or *ts*-52 infectious virus, without any significant effect on the production of hemagglutinating particles. The progeny virus isolated from MDBK cells, co-infected with infectious virus and defective virus, possessed characteristics of von Magnus-type DI virus, namely, low infectivity and high hemagglutinating activity and ability to interfere with infectious virus replication. We also found that the interference caused by DI virus occurred early in the infectious cycle (within 3 h postinfection [p.i.]).

Finally, in this report we show that a number of new small RNA segments (DI RNA) are characteristic of *ts*-52 DI virus and propose that the interfering ability of DI virus is due to the presence of these DI RNA molecules whereas the noninfectivity of DI virus is due to a nonspecific loss of a viral RNA segment(s).

MATERIALS AND METHODS

Viruses and cells. MDBK (bovine kidney) cells were grown and maintained as described previously (30). The wild-type (ts^+) virus and the *ts*-52 mutant of the WSN strain of influenza virus have been described in detail (30). The viruses had been plaque-cloned, and each virus stock was prepared from a single plaque. Growth of the viruses and plaque assay in MDBK cells were also performed according to the methods described before (29, 30).

Measurement of interference. Monolayers of MDBK cells were infected with either 1 PFU of ts^+ (or *ts*-52)/cell or an appropriate amount of defective virus (usually 0.004 PFU/cell, except where otherwise stated), or with a mixture of both. After 30 min at 34°C, maintenance medium was added without removing the inoculum, and the cells were incubated at 34°C. At 2.5 h p.i., the cultures were treated with a 1:1,000 dilution of antiserum to WSN virus for 20 min at 34°C, washed three times with phosphate-buffered saline supplemented with 0.2% bovine albumin (29, 30), and incubated in fresh maintenance medium at 34°C. At 14 h p.i., the virus yield was determined by plaque assay in MDBK cells at 34°C and HA titration with the use of 0.5% chicken erythrocytes.

UV irradiation of virus. DI virus obtained from undiluted passage 4 was exposed to a UV germicidal lamp (15 W) at a distance of 20 cm for various times (20), and samples were assayed for survival by plaque assay at 34°C. For assay of interference, samples of the UV-irradiated DI virus were co-infected with *ts*-52 as described above, and the yield after 14 h at 34°C was determined by plaque assay. The interfering ability of DI virus as a function of UV irradiation is given by 100 − percent yield, where percent yield is [(yield of *ts*-52 × DI/yield of *ts*-52 alone] × 100.

Virus purification. The culture supernatant fluid was freed from cellular debris by centrifuging at 6,000 rpm for 20 min, directly layered on a linear 30 to 60% (wt/wt) sucrose gradient in NTE buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride, 0.001 M EDTA, pH 7.4), and centrifuged at 25,000 rpm for 150 min in an SW27 rotor at 4°C. The virus band was collected, diluted, pelleted, and resuspended in NTE buffer.

To make radioactive virus for protein analysis, 2.5 μCi of a ^{14}C-labeled amino acid mixture per ml or 5 μCi of [^{3}H]fucose per ml (specific activity, 12.1 Ci/ml) was added immediately after the infection of MDBK cells with 1 PFU of ts^+/cell, 0.004 PFU of *ts*-52 DI virus/cell, or a mixture of both, and the viruses were purified as above.

Preparation of viral RNA labeled with [^{3}H]-uridine or ^{32}P. MDBK cells were infected with 1 PFU of ts^+/cell, 0.004 PFU of *ts*-52 DI virus/cell, a mixture of both, or 1 PFU of *ts*-52 infectious virus/cell. After 30 min at 34°C, the inoculum was removed and maintenance medium containing 50 μCi of [^{3}H]uridine per ml (specific activity, 29 Ci/mmol) or phosphate-free

maintenance medium containing 1 mCi of $^{32}P_i$/ml was added. Viral RNA was extracted from a suspension of purified virus by the method described by Palese and Schulman (21).

Polyacrylamide gel electrophoresis of viral proteins and RNA. The protein pattern of virus particles was examined by electrophoresis of radioactive viruses in 10 cm long, 10% polyacrylamide cylindrical gels in the Tris-glycine-sodium dodecyl sulfate (SDS) system described by Maizel (18) under the reducing condition. Electrophoresis was for 12 h at 50 V. At the end of the run, gels were cut into 1-mm slices and radioactivity was measured in a Beckman liquid scintillation counter.

The RNA of virus particles was analyzed by electrophoresis at 4°C on 2.8% polyacrylamide gels (14 by 17 by 0.15 cm) containing 6 M urea, by the method of Palese and Schulman (21). Approximately 75,000 cpm of ^{32}P-labeled RNA was applied per slot.

RESULTS

Preparation of defective virus. Plaque-cloned, infectious *ts*-52 (1.7×10^8 PFU/ml, 8,192 HAU) was inoculated into MDBK cell monolayers at 20 PFU/cell (passage 1), and the virus was harvested at 14 h p.i. Cells and culture fluid were harvested by scraping, subjected to three cycles of rapid freezing and thawing, clarified by low-speed centrifugation (800 × *g* for 10 min), and inoculated into another MDBK cell monolayer without dilution. Such an undiluted passage was repeated consecutively three more times. At each passage, culture fluid was assayed for infectivity and HA titer (Table 1). Since HA titer is a rough measure of the number of physical particles possessing HA spikes, reduction in PFU/HA ratio indicates that undiluted passages cause the production of noninfectious virus particles which still retain their HA activity. The PFU/HA ratio decreased from 2.1×10^4 (stock virus) to 2.1×10^1 (fourth passage virus), causing a 1,000-fold reduction in infectivity. This virus, after four undiluted passages, was used as defective virus (DI virus) in subsequent experiments.

TABLE 1. *Serially undiluted passages of ts-52 in MDBK cells at the permissive temperature (34°C)*[a]

Passage no.	Titer		PFU/HA ratio
	PFU/ml[a]	HAU/ml	
ts-52[b]	1.7×10^8	8,192	2.1×10^4
1[c]	7.5×10^7	8,192	9.2×10^3
2[d]	1.4×10^7	8,192	1.7×10^3
3[d]	2.1×10^6	4,096	5.1×10^2
4[d]	4.4×10^4	2,048	2.1×10^1

[a] Assay plates were incubated at 34°C and plaques were counted on day 3.

[b] Cells infected at 1 PFU/cell.

[c] Cells infected at 20 PFU/cell.

[d] Cells infected with undiluted virus (10 ml/150-cm^2 flask).

Effect of defective virus on infectious virus production. Serial fivefold dilutions of defective virus (4.4×10^4 PFU/ml, 2,048 HAU) and 1 PFU of *ts*$^+$/cell were mixed and inoculated into MDBK cell monolayers. At 2.5 h p.i., residual inoculum virus was neutralized by a 1:1,000 dilution of WSN antiserum. Incubation of the plates was continued at 34°C. The virus yield, harvested at 14 h p.i., showed a reduction in infectious virus titer with the increasing concentration of co-infecting defective virus (Fig. 1). Maximum interference was achieved when the concentration of DI virus was 0.001 PFU/cell. Since DI virus is 1,000-fold less infectious than infectious *ts*$^+$ (or *ts*-52) virus, the particle ratio between the DI virus at 0.001 PFU/cell and the infectious virus at 1 PFU/cell was approximately 1. To insure that each cell receiving an infectious virus also received at least one DI virus particle, a concentration of 0.004 PFU of DI virus/cell and 1 PFU of infectious virus/cell was employed in the present study.

Interference mediated by DI virus. One PFU of plaque-purified *ts*$^+$ or *ts*-52/cell was inoculated into MDBK cells singly or mixedly with 0.004 PFU of DI virus/cell. Progeny viruses were harvested at 14 h p.i., and yield was determined by plaque assay and HA titration.

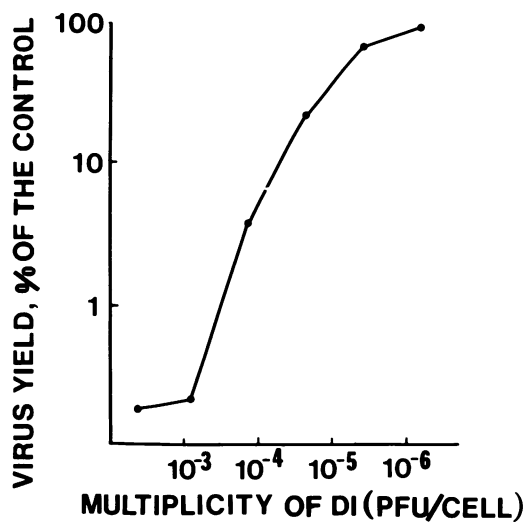

FIG. 1. *Dose response relationship between concentration of ts-52 DI virus and degree of interference. Serial fivefold dilutions of DI virus and 1 PFU of ts*$^+$ *virus/cell were mixed and inoculated into MDBK cells. At 2.5 h p.i., residual inoculum virus was neutralized by antiserum to strain WSN. After 14 h at 34°C, the cultures were harvested and assayed for infectivity in MDBK cells. Since the DI virus preparation is approximately 1,000-fold less infectious, the particle ratio of DI virus at 0.001 PFU/cell to ts-52 infectious virus at 1 PFU/cell will be one.*

Co-infection of DI and infectious virus reduced the final yield of infectious virus from cells infected with ts^+ to 0.2% (i.e., 99.8% reduction in infectivity) of the control at both permissive (34°C) and nonpermissive (39.5°C) temperatures. Also, a defective virus reduced the yield of infectious virus from *ts*-52-infected cells to 0.05% of the control at the permissive temperature (Table 2).

In both cases, the HA activity of virus was not affected. Defective virus reduced the degree of cytopathic effect caused by ts^+ or *ts*-52, but still a significant degree of cell degeneration was observed.

To test whether HA activity observed in the culture fluid from mixed infection of cells with ts^+ and defective virus was free or was associated with particle structure, cells mixedly infected with ts^+ and defective virus were labeled with 7.5 μCi of [^{3}H]uridine/ml. Culture fluid was harvested at 14 h p.i. and analyzed by velocity sedimentation in a linear 10 to 40% sucrose gradient for 45 min at 25,000 rpm (Fig. 2). The peak of HA activity coincided with the peak of radioactivity in both DI and ts^+ virus. Both ts^+ and DI virus preparations also had the same density of 1.18 g/ml when they were analyzed by equilibrium centrifugation at 49,000 rpm for 3 h in a 20 to 65% linear sucrose gradient (data not shown).

The hemagglutinating, noninfectious particles which emerged from cells mixedly infected with ts^+ and DI viruses also strongly interfered with the replication of ts^+ infectious virus: 0.004 PFU of such a virus preparation/cell reduced the infectious virus yield to less than 0.1% of the control (data not shown). Morphological properties of progeny virus from ts^+, ts^+ × DI virus-, and DI virus-infected cells were examined by negative stain with phosphotungstic acid. The visible bands isolated from sucrose gradients were used for electron microscopy. DI viruses did not have any characteristic morphological feature markedly different from that of

TABLE 2. *Homologous interference mediated by defective virus derived from ts-52*

Infected with	Incubated at:	
	34°C	39.5°C
ts^+ alone	5.5×10^7[a] (2,048)[b]	6.0×10^7 (2,048)
ts^+ × DI	1.0×10^5 (2,048)	1.2×10^5 (1,024)
ts-52 alone	5.6×10^7 (2,048)	NT[c]
ts-52 × DI	2.4×10^4 (2,048)	NT
DI alone	2.0×10^2 (256)	10 (<8)

[a] Infectivity (PFU/ml). Plaques were counted on day 3, after incubation at 34°C.
[b] HA titer.
[c] Not tested.

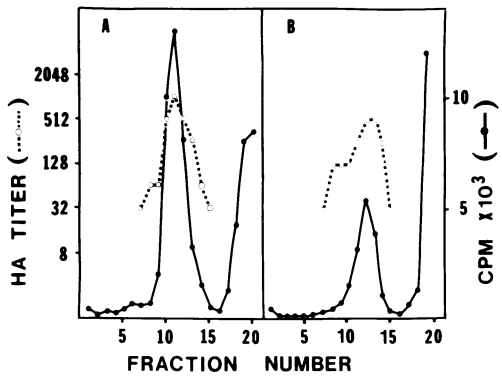

FIG. 2. *Velocity sedimentation patterns of DI virus labeled with [^{3}H]uridine. MDBK cells were infected with 1 PFU of ts^+/cell or with a mixture of 1 PFU of ts^+/cell and 0.004 PFU of DI virus/cell, and the viruses were grown in the presence of 7.5 μCi of [^{3}H]uridine/ml. After 14 h at 34°C, culture fluid was harvested, clarified by centrifugation for 20 min at 6,000 rpm, and sedimented through a linear 10 to 40% sucrose gradient in NTE buffer for 45 min at 25,000 rpm in a Beckman SW27 rotor. Fractions of 1.5 ml were collected from the bottom of the tube, and samples were measured for HA activity and acid-precipitable radioactivity. (A) ts-52 infectious virus, (B) DI virus.*

standard viruses (Fig. 3), but, as reported by Lenard and Compans (17), there was considerable heterogeneity in the shape and size of DI viruses from preparation to preparation. Some DI viruses also contained irregular forms (Fig. 3D) reported by Lenard and Compans (17).

Polypeptides of defective viruses. The polypeptides of defective and ts^+ standard virus particles were compared by electrophoresis of the virus on a 10% polyacrylamide cylindrical gel under the reducing condition (Fig. 4). The overall polypeptide patterns were essentially the same (Fig. 4, panels B and C), with an increase in the amount of M polypeptide in DI virus. If the amount of each polypeptide was normalized to the amount of M, the content of NP as well as glycopeptides was reduced 30% in DI virus when compared to that present in infectious virus. Although these results are somewhat different from those reported by Lenard and Compans (17), who found an increased glycoprotein content of incomplete virus, the heterogeneity among the DI viruses may explain these minor differences in protein patterns.

RNA pattern of *ts*-52 infectious and DI viruses. RNA patterns of the virus particles were analyzed by electrophoresis of viral RNA in 2.8% polyacrylamide slab gels containing 6 M urea (21). Infectious *ts*-52 virus contained eight segments, as has been reported previously (21). Under our conditions, V1 and V2 segments mi-

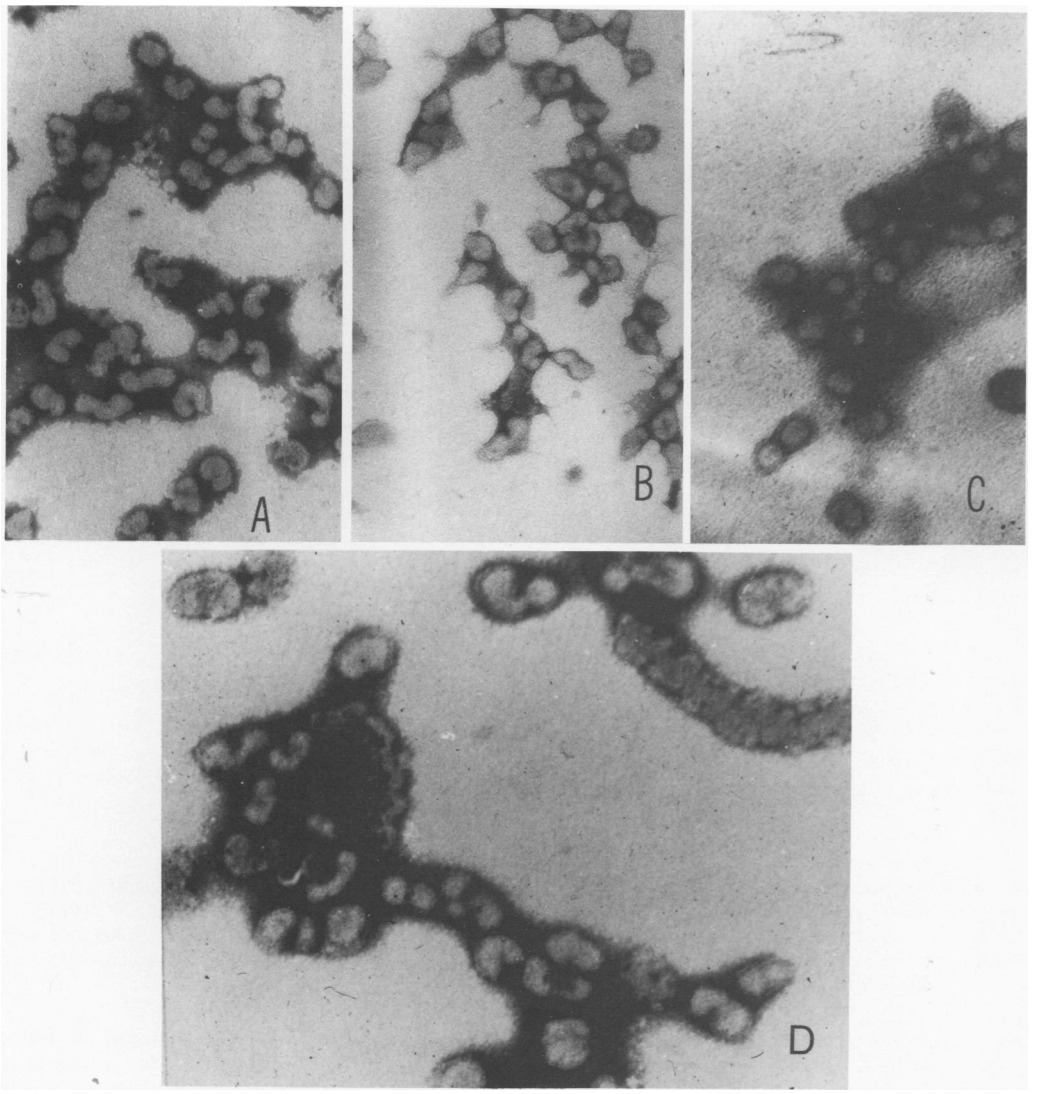

FIG. 3. *Morphology of ts-52 infectious and DI virus by negative staining with phosphotungstic acid: (a) ts-52 standard, ×96,000; (b) ts-52 DI preparation 1, ×96,000; (c) ts-52 DI preparation 2, ×115,000; and (d) ts-52 DI preparation 3, ×150,000.*

grated together, although V3 became separated when electrophoresis was run at a higher voltage (Fig. 5). The RNA pattern of ts^+ RNA was identical to that of *ts*-52 RNA (data not shown). RNA patterns of *ts*-52 (clone c) viral RNA at either 1 PFU/cell or 20 PFU/cell (passage 1) were indistinguishable. However, upon subsequent undiluted passages, smaller RNA segments began to appear (passage 2), which became more prominent with undiluted passages (passages 3 and 4). These RNA segments (D1 to D6) were also present in virus particles produced from cells co-infected with *ts*-52 and *ts*-52 DI virus. The molar ratios of viral RNA segments (V1 to V8) and DI RNA segments (D1 to D6) were determined from the counts per minute in each band (Table 3). Relative to segment V6, the larger RNA segments (V1, V2, V3, and V4) were present in reduced amount even in a three times cloned *ts*-52 virus. Although no visible band was present in the DI region, there was some radioactivity present in the region corresponding to the DI bands. From the undiluted passage 2 on, D4 was the predominant DI segment and was present in a molar ratio of 2 compared to V6 in undiluted passages 2, 3, and

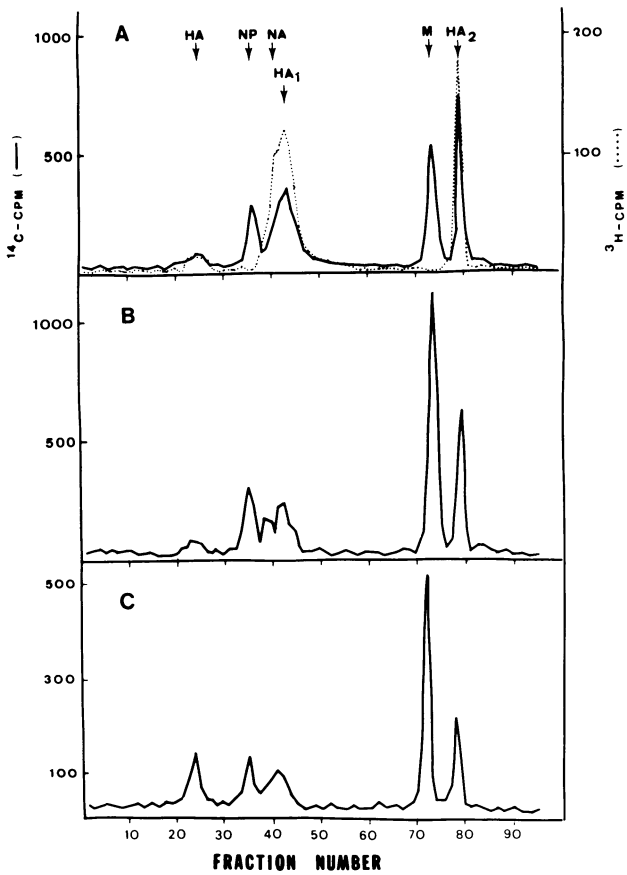

FIG. 4. *Polypeptide analysis of virus particles. MDBK cells were infected with 1 PFU of ts$^+$/cell, 0.004 PFU of defective virus/cell, or mixture of both, and the viruses were grown in the presence of 2.5 μCi of ^{14}C-labeled amino acids/ml and 5 μCi of [^{3}H]fucose/ml, added at the time of infection. Viruses were purified and viral proteins were analyzed on a polyacrylamide gel as described in Materials and Methods. Virus was obtained from cells infected with (A) ts$^+$ alone, (B) ts$^+$ and DI, and (C) DI alone.*

4 as well as in DI virus produced by co-infection with *ts*-52 and DI virus. Essentially similar results were observed in the densitometer tracing of the autoradiographs (Fig. 6); i.e., no DI RNA segment was apparent in *ts*-52 virus produced from cells infected with 1 or 20 PFU/cell. However, DI segments appeared at passage 2 and became prominent with further undiluted passages. Again, D4 was the predominant DI segment.

In an attempt to determine the presence of DI RNA segments among the virus population, a number of clones of *ts*$^+$ and *ts*-52 and DI virus preparations obtained from undiluted passage 4 of different *ts*-52 clones were examined (Fig. 7). All DI viruses invariably contained smaller DI RNA segments. Some of *ts*$^+$ and *ts*-52 clones also contained one or more DI RNA segments. In many cases, further cloning eliminated DI RNA segments from infectious virus preparations, but they reappeared after serial undiluted passages (Fig. 5). These results, taken together, suggest that DI RNA segments (D1 to D6) are associated with DI particles. To determine whether the predominant D4 segment was of viral origin, it was eluted and hybridized to viral complementary DNA. The results (Table 4) showed that D4 RNA did not self-anneal but hybridized to complementary DNA, indicating viral origin of the D4 segment.

During undiluted passages, an overall decrease in the larger segments, particularly V1, V2, V3, and V4, was noticed (Fig. 5 and 6, Table 3). The specific loss of one viral RNA segment was not observed even though there was a drastic reduction of infectivity. Thus, in agreement with Bean and Simpson (2), we conclude that the loss of infectivity among DI viruses cannot be accounted for by the loss of a specific RNA segment. However, our data suggest that the

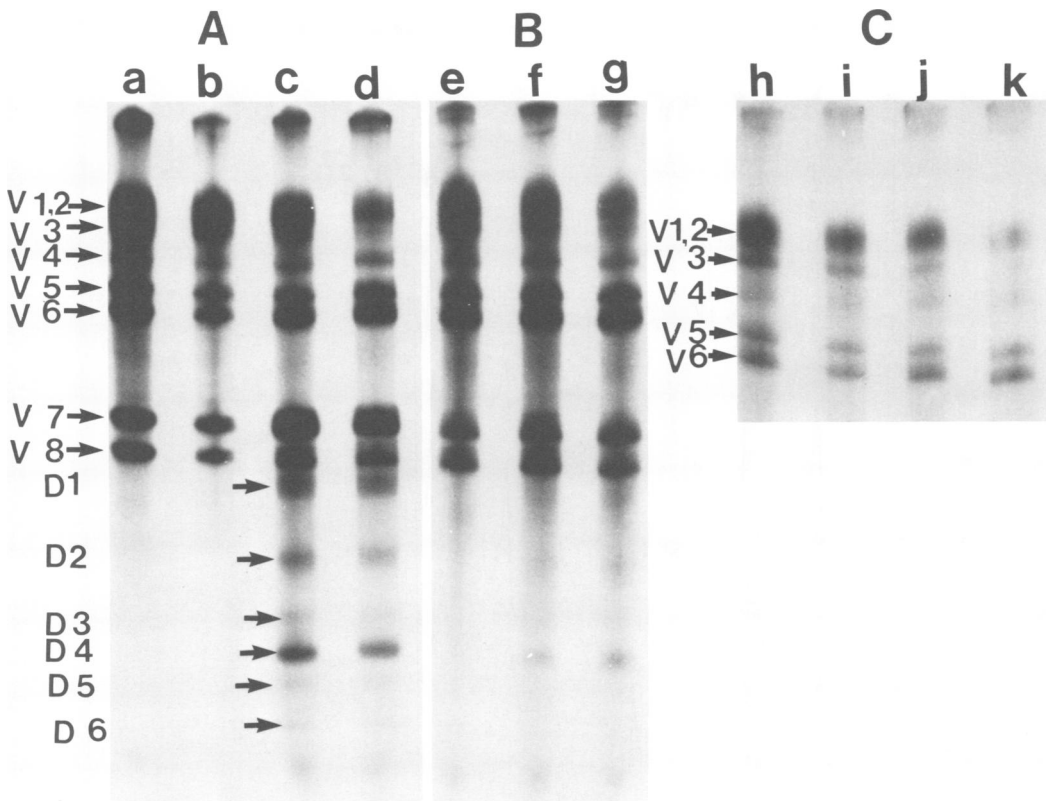

FIG. 5. *Analysis of [^{32}P]RNAs of ts-52 clone c after serial undiluted passage in MDBK cells. The ts-52 viral RNA segments are numbered V1 to V8 and DI segments, D1 to D6. (A) Columns a, b, and c show cells infected with ts-52 virus at 1 PFU/cell (a), ts-52 virus at 20 PFU/cell (b), and ts-52 × DI passage 4 (c); column d shows the virion RNA patterns after four undiluted passages. (B) Column e shows cells infected with ts-52 virus at 1 PFU/cell; f and g represent the virion RNA patterns after two and three undiluted passages, respectively. (C) Columns h, i, and j show cells infected with ts-52 virus at 1 PFU/cell (h), ts-52 virus at 20 PFU/cell (i), and ts-52 × DI (j); k shows the virion RNA of ts-52 virus after four serial undiluted passages. Conditions of electrophoresis were (A and B) 19 h at 50 V and (C) 24 h at 60 V.*

TABLE 3. *Molecular weight and molar ratio of RNA bands in infectious and DI virus preparations*

RNA Bands	Mol wt × 10^{-5}	Molar ratio of RNA band[a]					
		ts-52 PFU 1	DI P-1	DI P-2	DI P-3	DI P-4	*ts*-52 × DI P-5
V1, V2, V3	10[b]	0.7	0.8	0.6	0.5	0.3	0.5
V4	8.2	0.7	0.8	0.4	0.4	0.4	0.5
V5	6.2	0.9	1.0	0.7	1.8	0.8	0.7
V6	5.8	1.0	1.0	1.0	1.0	1.0	1.0
V7	4.0	1.6	1.1	1.2	2.5	2.6	3.4
V8	3.5	1.8	2.2	1.0	1.9	1.4	1.7
D1	2.9	0.4	0.6	0.6	0.7	1.4	2.2
D2	2.1	0.4	0.3	0.8	1.0	1.2	1.3
D3	1.6	0.3	0.2	0.5	0.4	1.0	1.0
D4	1.4	0.4	0.2	1.0	2.2	2.4	2.6
D5	1.25	0.3	0.5	0.4	0.3	0.7	0.7
D6	1.05	<0.1	<0.1	0.2	0.1	0.4	0.3

[a] Average molar ratio relative to band V6 was calculated from the counts present in the specific band. Each ratio represents an average of three RNA preparations.

[b] Molecular weight of RNA bands was calculated from the molecular weight of markers of: rRNA, 28S and 18S; globin mRNA, 9S; and 5S and 4S cellular RNAs that were run in parallel slots.

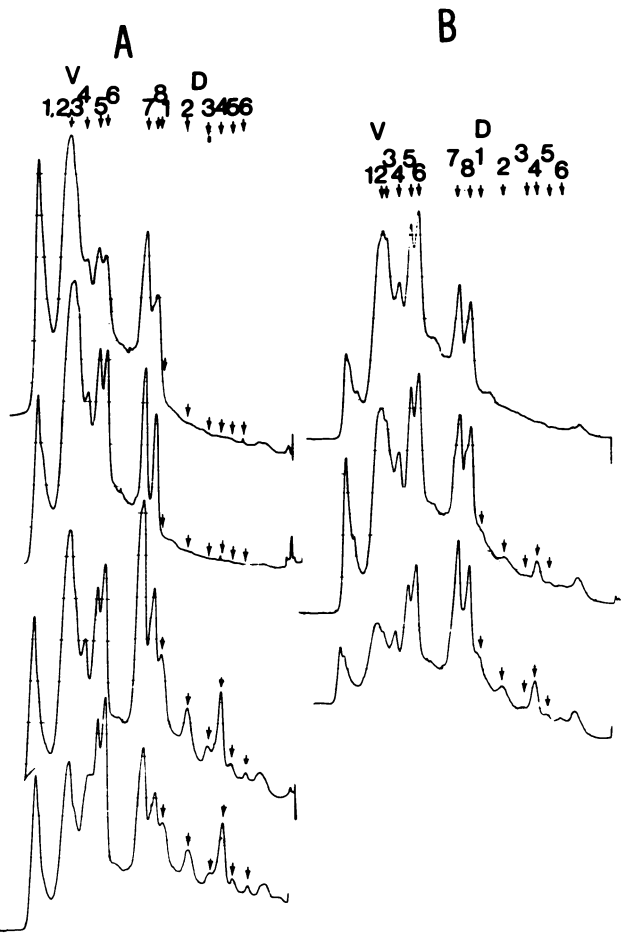

FIG. 6. *Densitometer tracing of autoradiographs of RNA gel. Left side, from top: ts-52, 1 PFU/cell and 20 PFU/cell; ts-52 × DI (passage 4); DI passage 4. Right side from top: ts-52 (1 PFU/cell); DI passage 2; DI passage 3.*

loss of infectivity could be accounted for by a nonspecific loss of one or more RNA segments (possibly larger segments) among DI virus particles.

UV target size of infectivity and interfering ability of DI virus. DI virus (passage 4) was irradiated with UV. The loss of infectivity of DI virus was determined as a function of UV dose (Fig. 8). The *ts*-52 standard virus yielded similar UV inactivation kinetics (data not shown). To measure the inactivation kinetics of interfering ability, DI virus that had been treated with UV for various periods of time was co-infected with *ts*-52 standard virus, and the yield of infectious virus was measured. The results show that the UV target size of interfering ability is approximately 40 times smaller than that of infectivity.

Effect of time of addition of defective virus on infectious virus production. Defective virus was added at various times before or after infection of MDBK cells with 1 PFU of ts^+/cell. Reduction in infectious virus yield was significant when defective virus was added within the first 3 h of ts^+ infection (Fig. 9). When defective virus was added later, reduction was less marked, suggesting that interference occurs in an early phase of viral replication. This experiment also excluded the possibility that interference occurred at the level of adsorption and penetration of the virus to the host cells. Our preliminary data (D. P. Nayak and K. Tobita, unpublished data) suggest that, as in other DI virus systems (8, 10, 11, 13, 22, 29), interference involves the replicative steps rather than the transcriptive steps of viral RNA synthesis.

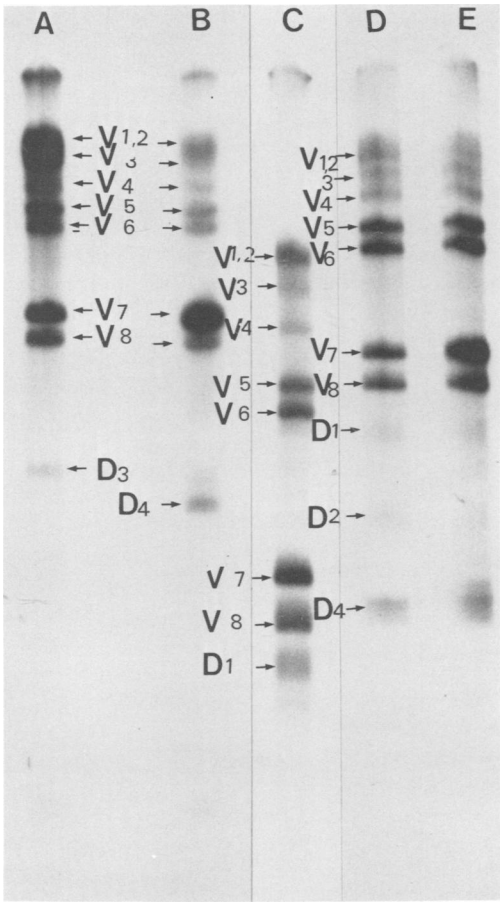

FIG. 7. *Analysis of [^{32}P]RNAs of different ts$^+$, ts-52, and DI virus clones. (A) Cells were infected with a clone of ts-52 at 1 PFU/cell. (B) The ts-52 clone depicted in A was co-infected with DI passage 4 derived from this clone. Electrophoresis for A and B was 15 h at 50 V. (C) Cells were infected with a clone of ts$^+$ virus at 1 PFU/cell; electrophoresis was for 23 h at 65 V. (D and E) Cells were infected with ts$^+$ × two other DI passage 4; electrophoresis was for 19 h at 50 V.*

DISCUSSION

Four consecutive undiluted passages of *ts*-52, a temperature-sensitive mutant of the WSN strain of influenza A virus, in MDBK cells at the permissive temperature produced von Magnus-type DI virus with a high HA titer and low infectivity. In fact, DI virus derived from *ts*-52 still maintained high hemagglutinating activity but with approximately a 4,000-fold drop in infectivity. Thus, *ts*-52 provided us with the means to prepare DI virus very efficiently. Whether this unique feature of *ts*-52 was due to a mutation in the P2 gene remains undetermined. Other members of this *ts* group did not show a similar property (Tobita and Nayak, unpublished data). The *ts*-52-derived DI virus replicated in the presence of infectious virus and interfered strongly with the replication of *ts*$^+$ or *ts*-52 infectious virus. A progeny DI virus with a high HA titer, low infectivity, and high interfering activity was produced.

Interference by defective virus occurred only when the defective virus was added within the first 3 h after infection with infectious virus, indicating that interference occurred early after infection and not at the stage of viral assembly or maturation. On the other hand, since simultaneous infection with infectious and defective viruses was not a prerequisite for interference, the possibility that interference was occurring at a stage of viral adsorption and penetration was also excluded. Our preliminary data (Nayak and Tobita, unpublished data) suggest that, as with other DI virus systems (8, 10, 11, 13, 22, 29), complementary RNA synthesis was not affected, at least during the first 6 h of infection, but that viral RNA synthesis was significantly suppressed (50 to 60%) during interference.

TABLE 4. *Hybridization of D4 RNA to complementary DNA (cDNA)*[a]

Labeled RNA	cDNA (ng)[b]	Annealing	Input cpm	Hybridized cpm	% Input hybridized[c]
D4	0	−	770	4.0	—
D4	0	+	770	6.0	0.3
D4	24	+	770	693	89.5
D4	48	+	770	732	94.5
D4	96	+	770	726	93.8
V7	0	−	1,021	10	—
V7	0	+	1,021	16	0.6
V7	6	+	1,021	852	82.5
V7	6	+	1,021	834	80.7

[a] ^{32}P-labeled segments D4 or V7 were eluted from polyacrylamide gels by the method of Maxam and Gilbert (19). Samples (50 μl) were hybridized to cDNA in 5 × SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) for 18 h at 68°C. Hybridization was assayed by treatment with RNase A (20 μg/ml) and RNase T-1 (10 U/ml) for 30 min at 37°C in 2 × SSC. Values represent the average of duplicate samples.

[b] cDNA was prepared by use of *ts*-52 viral RNA (vRNA) and the DNase-digestion end product of calf thymus DNA as primer as previously described (5). This cDNA protects 71% of ^{32}P-labeled *ts*-52 vRNA at a cDNA-to-vRNA ratio of 1.4 and 62% of ^{32}P-labeled *ts*-52 vRNA at a cDNA-to-vRNA ratio of 0.71. Additionally, the cDNA hybridized 86% to *ts*-52 vRNA at a vRNA-to-cDNA ratio of 1.6 and hybridized less than 5% to uninfected cell RNA.

[c] Corrected for RNase resistance of unannealed samples (0.5%, D4; 0.98%, V7).

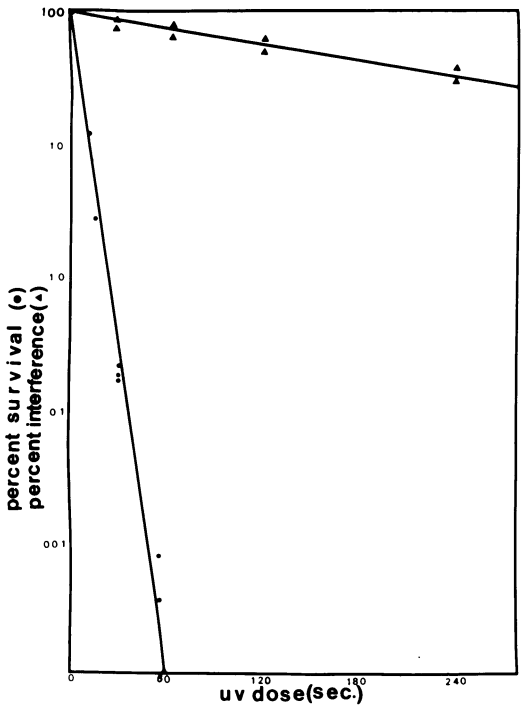

FIG. 8. *Effect of UV irradiation on the infectivity of DI virus and on the interference mediated by DI particles. DI virus (undiluted passage 4) was exposed to UV irradiation for the indicated time and assayed for survival and interfering ability as described in Materials and Methods. Untreated DI virus had 4 × 10⁴ PFU/ml and 512 HAU/ml, and it reduced the yield of ts-52 virus by 93%.*

Our data indicate that there was a significant reduction in the amount of the larger RNA segments (V1 to V4) in the DI virus. However, unlike earlier reports (4, 6, 23), we did not observe loss of any specific viral RNA segment in the DI virus. On the other hand, we found that DI viruses contained a number of smaller species of RNA (D1 to D6) which migrated faster than the smallest segment (V8) of ts^+ (or *ts*-52) virus RNA. They were absent in many cloned viruses but became apparent in undiluted passage 2 and became more prominent with subsequent undiluted passages. Although some DI RNA segments were occasionally present in significant amounts in some infectious virus preparations, they were always eliminated by recloning. Similar smaller RNA segments were reported by other investigators in both infectious and defective virus preparations (4, 21). The absence of any visible small RNA segments in many cloned *ts*-52 virus indicated that these smaller RNA segments are not required for infectivity of virus particles. These segments were also not present in equimolar amounts in infectious virus preparations.

It is possible that each DI segment represents a deletion from a separate viral RNA segment (V1 to V8) and that the presence of differing DI segments indicates the heterogeneity among DI viruses. However, it is also possible that some of these minor segments may be the result rather than the cause of interference with RNA replication by a DI virus. At present, it is not possible to distinguish between these two alternatives. Furthermore, the origin of these DI RNA segments and the nature of deletion is unknown at present.

Our data show that, as with other DI viruses (8, 10), DI influenza viruses are also noninfectious and interfere with homologous infectious virus replication. We propose that the interfering ability of DI influenza virus is due to the presence of DI RNA molecules in DI virus.

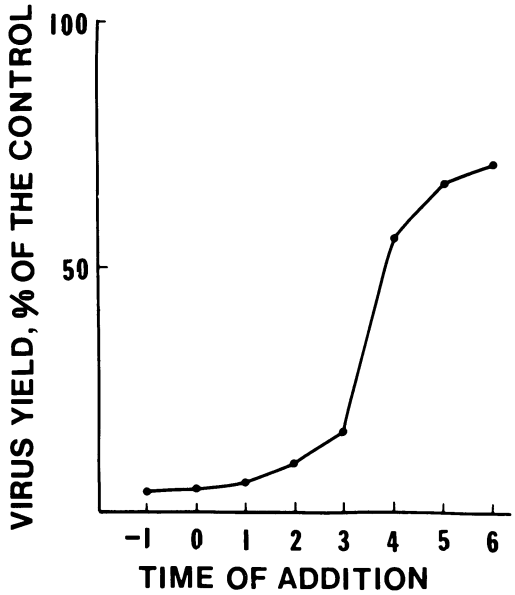

FIG. 9. *Effect of time of addition of defective virus on the yield of infectious virus from ts^+-infected MDBK cells. MDBK cell monolayers were infected with 1 PFU of ts^+/cell. After absorption for 30 min at 34°C, the inoculum was removed, and monolayers were overlaid with maintenance medium and incubated at 34°C. At various times after infection, 0.004 PFU of DI virus/cell was added. After 30 min at 34°C, inoculum was removed, and monolayers were washed twice with phosphate-buffered saline supplemented with 0.2% bovine albumin and overlaid with fresh maintenance medium. One culture received defective virus 1 h before infection with ts^+ virus. Virus was harvested after 14 h at 34°C. Virus yield from the control culture which had not received defective virus was taken as 100%.*

Consistent with this is the invariable appearance of small DI RNA segments, not present in infectious virus, in DI virus particles. Additionally, the UV target size of the interfering RNA molecule was 40 times smaller than that of infectivity and was therefore smaller than any of the viral RNA segments. Thus, DI influenza viruses have properties similar to those observed for other DI viruses; i.e., DI viruses contain a deleted RNA segment not present in infectious virus (7-10, 12-14, 16, 24, 27). However, unlike other DI viruses, DI influenza virus also contains, in addition to the DI RNA molecules, viral RNA segments present in infectious virus.

Our data, in agreement with the results of Bean and Simpson (2) but unlike earlier reports (4, 6, 23), do not show the loss of a specific viral RNA segment among DI viruses. Our results, on the other hand, show a relative reduction of the larger viral RNA segments (V1 to V4) among the DI virus population. We would therefore suggest that the drastic loss of infectivity (99.9%) observed among the DI virus population is due to a nonspecific rather than a specific loss of one or more viral RNA segments. Models proposed for the interference with viral RNA replication by a DI RNA segment(s) would support a preferential reduction of larger viral RNA segments (10). Among influenza virus populations, randomly incomplete particles have been previously observed (7). However, the presence of DI RNA segments in such incomplete particles would prevent their expected multiplicity-dependent reactivation and would explain why such reactivation has not been observed in DI influenza viruses.

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ADDENDUM IN PROOF

Our recent results show that each clone of *ts*-52 and *ts*$^+$ WSN virus produces DI virus with a characteristic set of DI RNA segments.

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