Proc. Natl. Acad. Sci. USA
Vol. 76, No. 7, pp. 3092–3096, July 1979
Biochemistry

Sequence relationships among defective interfering influenza viral RNAs

(defective interfering virus/influenza virus/oligonucleotide mapping/autointerference)

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Communicated by Robert M. Chanock, March 22, 1979

ABSTRACT **Each clone of ts-52 and ts$^+$ WSN influenza virus, when serially passaged at high multiplicity, gives rise to defective interfering (DI) virus with a unique set of new RNA species. The new RNAs (DI RNA) from several DI viruses were examined by the technique of RNase T1 oligonucleotide two-dimensional electrophoresis. It was found that each DI RNA arises from a specific segment of standard viral RNA. All DI RNA studied arose from the viral polymerase genes (P1, P2, and P3). DI RNAs originating from the same polymerase gene were interrelated. Certain of these DI RNAs appeared to contain completely overlapping nucleotide sequences. Others contained both overlapping and nonoverlapping nucleotide sequences. The latter DI RNAs may be formed from the progenitor viral RNA segment by a mechanism other than a common initiation (or termination) point and a simple deletion from one end.**

Animal viruses that are unable to replicate in the absence of a helper virus and are able to decrease the yield of wild-type virus have been termed defective interfering (DI) viruses (1, 2). DI influenza virus was first shown in 1951 (3) to be produced by serially undiluted passages of the virus. Recently it has been shown that DI influenza virus has new small RNA molecules (DI RNA) that are not present in the standard virus (4). Furthermore, these DI RNAs have been implicated in the interference mediated by DI virus (4). Additionally, we have shown (5) that defective particles prepared from each individual clone of infectious virus contain a unique set of DI viral RNAs. Although these DI RNAs were shown to be of viral origin (4), the specific progenitor viral RNA segment(s) was not determined. Neither was the relationship among these DI viral RNAs determined. Because influenza virus, however, has been shown by both genetic and biochemical analysis to have a segmented genome composed of eight distinct viral RNA segments (for reviews see refs. 6 and 7), these DI RNAs could arise from a single or from multiple segments. In this report we have used the technique of oligonucleotide mapping to determine the progenitor standard viral RNA segments of several influenza DI RNAs and the interrelationships among these DI RNAs. Using this technique, others have shown that each of the eight viral RNA segments is unique (8) and have demonstrated reassortment of the viral genome in nature (9). In this report we show that single DI viral RNA species originate from a discrete standard segment of viral RNA and that more than one viral RNA segment can yield DI RNA. Additionally, we have found that DI RNAs arising from the same viral RNA segment are interrelated in a novel way.

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MATERIALS AND METHODS

Viruses and Cells. Wild-type (ts$^+$) and ts-52 (a group II temperature-sensitive mutant of the WSN strain of influenza virus) have been described (10). DI virus particles were produced after four undiluted passages in MDBK (bovine kidney) cells as reported (4). These preparations reduced the yield of standard virus by 99.5% in an interference assay. Each DI virus was prepared from a single virus clone of ts-52 virus or ts$^+$ virus.

Preparation and Fractionation of Viral RNA. MDBK cells were infected with either 1 plaque-forming unit of ts-52 virus per cell or 1 plaque-forming unit of ts-52 virus and 2 defective interfering units of DI virus per cell (5). After 1 hr at 37°C, inoculum was removed and phosphate-free maintenance medium (4) containing 5.0 mCi of $^{32}P_i$ was added. Labeled viral RNA was extracted from a suspension of purified virus (11). The RNA of virus particles was fractionated on 2.2% polyacrylamide/0.6% agarose gels containing 6 M urea/36 mM Tris base/30 mM NaH_2PO_4/10 mM EDTA as described (12, 13, 5). RNA was eluted from gels by the procedure of Maxam and Gilbert (14).

Oligonucleotide Fingerprinting. Labeled standard or DI RNA segments were digested for 30 min at 37°C with RNase T1 at an enzyme-to-substrate ratio (wt/wt) of 1:10. Two-dimensional slab-gel electrophoresis was performed by a modification (15) of the method of De Wachter and Fiers (16). The first dimension gel (20 × 40 cm) contained 10% acrylamide and 0.325% *N,N'*-methylene bisacrylamide and 6.0 M urea/25 mM citric acid (pH 3.5). Electrophoresis was performed at 300 V and 25°C, with a reservoir buffer of 25 mM citric acid, until bromphenol blue had migrated 21 cm. The second dimension gel (40 × 40 cm) contained 20% acrylamide, 0.65% *N,N'*-methylene bisacrylamide, and 0.5% polyacrylamide (molecular weight over 5×10^6) and 0.1 M Tris borate buffer, pH 8.3/2.5 mM EDTA. Electrophoresis in this dimension was performed at 400 V and 25°C, with a reservoir buffer of 0.1 M Tris borate buffer, pH 8.3/2.5 mM EDTA, until bromphenol blue had migrated 21 cm. The position of the two dye markers (xylene cyanol and bromphenol blue) are indicated by X.

RESULTS

Species of RNA Specified by DI Particles. DI virus preparations obtained from individual virus clones possess unique DI RNA species. DI viruses were prepared from clones B, L, and P of ts-52 virus and a clone of ts$^+$ (ts$^+$/c/c) virus. More than one DI RNA species were present in these DI virus preparations

Abbreviation: DI virus, defective interfering virus.

(Fig. 1). A number of these DI RNAs were eluted from the gel and analyzed by the technique of ribonuclease T1 oligonucleotide fingerprinting. Molecular weights of DI RNAs were determined from the linear relationship between logarithm of molecular weight and electrophoretic mobility at 25°C from the molecular weights of standard viral RNA segments (4) and 28S and 18S rRNA, 9S globin mRNA, and 4S and 5S cellular RNA as standards. From these measurements the molecular weights of DI RNAs were as follows: L1 (3.4×10^5), B1 (2.7×10^5), L2 (2.5×10^5), P1 (2.4×10^5), ts$^+$1 (2.0×10^5), P2 (1.7×10^5), ts$^+$2 (1.4×10^5), L3 (1.3×10^5), and ts$^+$3 (8.9×10^4). Because of the incomplete denaturation of RNA in gels containing 6 M urea, these molecular weights should be considered approximate (17).

Oligonucleotide Mapping of Standard Viral RNA Segments. Total viral RNA was digested with RNase T1, and its oligonucleotide pattern was analyzed (Fig. 2 *left*). Approximately 97 oligonucleotide spots were chosen for analysis of standard viral RNA and DI RNA (Fig. 2 *right*). Each standard viral RNA species (V1–V8) was examined by fingerprinting, and spots in total RNA were assigned to the appropriate viral RNA segment (Table 1). This was done by mixing approximately 1–2 $\times 10^5$ cpm of total viral RNA with each isolated segment and determining the oligonucleotide pattern of the segment against a background of the pattern of total viral RNA. Oligonucleotide spots characteristic for V1, V2, and V3 were derived as follows. RNA fingerprints of isolated V1 were made and RNA fingerprints of V2 and V3 together were made. Oligonucleotide spots characteristic of V3 were derived both by cutting only the bottom edge of V3 from polyacrylamide/agarose gels electrophoresed at 25°C (Fig. 1 *left*) and from the separated V3 segment isolated from polyacrylamide/agarose gels electrophoresed at 4°C (Fig. 1 *right*). Oligonucleotides characteristic of V2 were those present in the V2 and V3 mixture yet absent from V3.

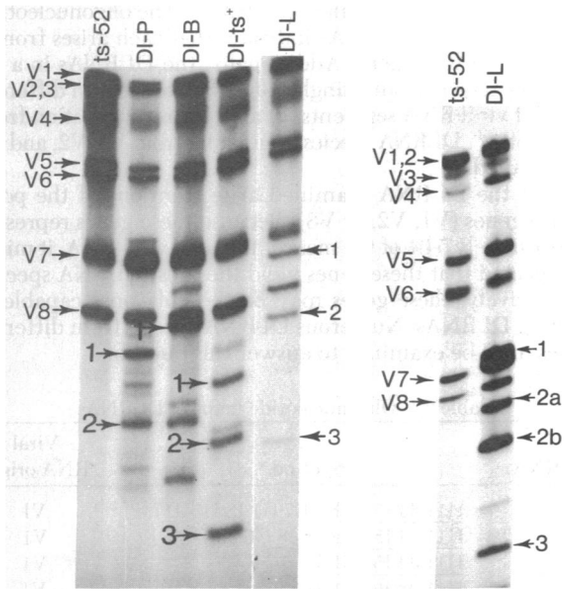

FIG. 1. Polyacrylamide/agarose gel electrophoresis of ts-52 virus and DI virus RNA. (*Left*) Cells were either infected with ts-52 virus or were coinfected with ts-52 and DI virus prepared from clones P, B, and L of ts-52 virus or a clone (ts$^+$/c/c) of wild-type virus. ^{32}P-Labeled RNA was analyzed by electrophoresis on 2.2% polyacrylamide/0.6% agarose for 19 hr at 140 V and 25°C. (*Right*) ^{32}P-Labeled viral RNA from ts-52 virus and ^{32}P-labeled viral RNA from a coinfection of DI-L and ts-52 virus were analyzed by electrophoresis for 23 hr at 180 V and 4°C.

Table 1. Oligonucleotide maps of gene segments of WSN virus

RNA segment	Protein	Spot no.
VI	P3*	D2 E1 E3 E7 E10 E11 E12 F4 F5 G1 G6 G9 G10 G12 H6 H8 H11 I5 I8 I9 I13 I15 I17 I18 J4 J5 J7 J8 J17 J19
V2	P1	B3 D1 E8 F1 F3 F4 G3 G4 G8 H2 H5 H8 H10 I6 I11 I12 I19 J12 J15 J19 J24
V3	P2	E2 E4 F4 G5 G7 H1 H6 H7 H8 H9 H14 H15 I2 I3 I5 I8 I10 I16 J2 J7 J8 J9 J10 J14 J16 J25
V4	HA	B1 C1 E2 E6 E14 G2 G11 G13 H4 H6 H7 I1 I2 I7 I9 I10 J3 J6 J9 J13 J14
V5	NP	B2 D3 G5 G7 G8 H6 H12 I6 I7 I8 I10 J7 J8 J9 J11 J21 J23 J26
V6	NA	A1 E13 F2 H6 H7 H9 H13 I7 J1 J8 J11 J13 J16 J20 J22 J26
V7	M	D4 E9 H4 H6 I10 I15
V8	NS	G6 H3 H7 I14 J3 J6 J7 J10 J18

*The gene products of RNA segments 1–8 of WSN influenza virus have been determined by Palese *et al.* (18).

Most oligonucleotides of individual segments were characteristic of that segment. However, a few spots were present in more than one segment. Similar observations have been made previously (9). However, sufficient characteristic oligonucleotides were present in viral RNA segments and DI RNAs for specific identification.

Identification of Viral RNA Segment from Which DI RNAs Originate. To determine the progenitor standard viral RNA segments giving rise to a DI RNA, oligonucleotide maps of several DI RNAs were made. DI RNA P1, P2, B1, ts$^+$1, ts$^+$2, ts$^+$3, L1, L2, and L3 were examined by fingerprinting (Fig. 3). Oligonucleotide spots in each of these DI RNAs were established (Table 2) by mixing with 2×10^5 cpm of total ts-52 viral RNA, and the standard viral RNA segment progenitor was determined. DI L2 and ts$^+$2 appeared to be mixtures of two DI RNAs. Both L2 and ts$^+$2 contained oligonucleotide spots of various intensities. Groupings were made by the light (L2a and ts$^+$2a) and dark (L2b and ts$^+$2b) spots. L2a contained oligonucleotides characteristic of V1, whereas L2b contained oligonucleotides characteristic of V2. ts$^+$2a contained oligonucleotides characteristic of V1, whereas ts$^+$2b contained oligonucleotides characteristic of V3. L2 (Fig. 1 *left*), however, could be separated into L2a and L2b when electrophoresis was at 4°C (Fig. 1 *right*) rather than at 25°C. Each was then positively identified (L2a of V1 origin and L2b of V2 origin) by fingerprinting (data not shown).

The progenitor viral RNA segment was finally confirmed for each DI RNA. Each DI RNA was mixed with its progenitor viral RNA segment and fingerprinted. In each case, the DI RNA oligonucleotides comigrated with the oligonucleotides of the progenitor viral RNA segment, with the exception of oligonucleotide a of B1, b of P1, c of L2b, and d of L1, which were new oligonucleotides not present in total viral RNA. Such new oligonucleotides may originate either by deletion or by a mechanism where the DI RNA is itself copied or a combination of both of these processes. Each DI RNA was analyzed for self-complementarity by RNase treatment. RNase resistance was less than 1.0% for all DI RNAs and approximately equivalent to the RNase resistance of each standard viral RNA segment. This suggests that extensive self-copying does not occur, although 20 nucleotides or less of 3′ and 5′ ends of DI RNA could still be complementary.

Sequence Relationships among DI RNA. DI RNAs originating from the same viral RNA segment share a common set

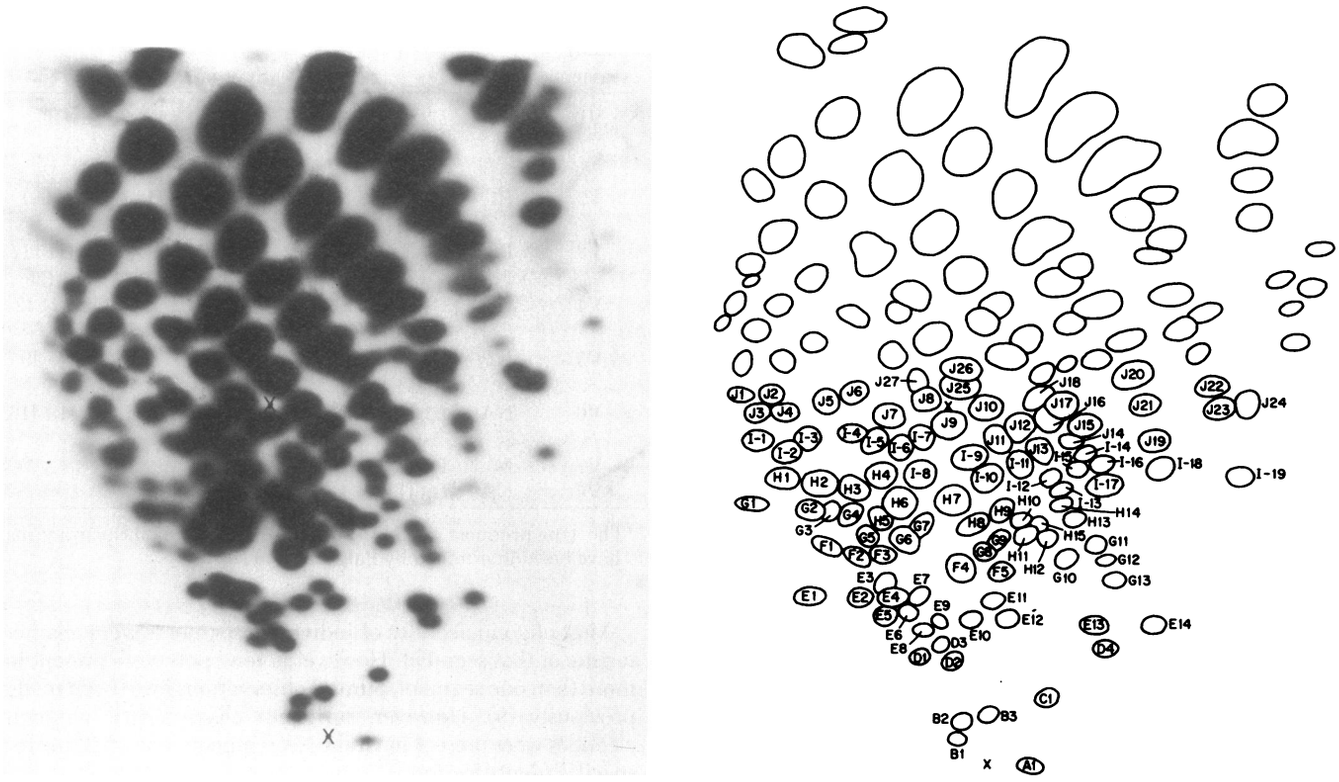

FIG. 2. Oligonucleotide fingerprinting of total ts-52 viral RNA. Total viral [^{32}P]RNA was digested with T1 RNase and fractionated by two-dimensional polyacrylamide gel electrophoresis. First dimension, right to left; second dimension, top to bottom.

of characteristic oligonucleotides (Table 2). For example, DI RNAs originating from V2 all contain D1, G4, H5, I6, I11, and J15 spots. DI RNAs originating from V1 similarly share H11 and J4 spots. Additionally, P2 and ts$^+$2a, found in defective virus preparations from two different clones (one temperature sensitive and the other wild type), contain identical characteristic oligonucleotides. Certain DI RNAs appear to be complete subsets of others originating from the same viral RNA segment. For example, B1 contains all the oligonucleotides present in L3. This result suggests that all the nucleotide sequences of L3 are present in B1. Thus, if L3 and B1 share a common initiation (or termination) point, L3 may be a simple deletion of B1 from one end.

The sequence relationship among other DI RNAs arising from the same segment cannot, however, be explained by a common initiation (or termination) point and a simple deletion from one end. Several examples are present in Table 2. With the exception of the new spot b, B1 contains all the oligonucleotides present in P1. B1 also contains all the oligonucleotides present in ts$^+$1. Yet P1 and ts$^+$1 each contains an oligonucleotide not present in the other. Spot J12 is not present in ts$^+$1 and spot H10 is not present in P1 (Table 2 and Fig. 3). This indicates that P1 and ts$^+$1 contain nonoverlapping nucleotide sequences in addition to common sequences and that both cannot be related to B1 or to each other by a common initiation or termination point and simple deletion from one end.

L1 is related to P2 and ts$^+$2a in a similar fashion. Each contains oligonucleotides H11, J4, and I5. Yet L1 contains many oligonucleotides (J7, E1, E7, G1, I8, and J17) not present in either P2 or ts$^+$2a. Additionally, P2 and ts$^+$2a contain E11 and J8, which are not found in L1. ts$^+$3 (9.9×10^4 daltons) comprising approximately 9% of the sequences of VI (1×10^6 daltons) also contains both common and nonoverlapping nucleotide sequences with the other DI RNA originating from V1.

DISCUSSION

When influenza virus is passed serially at high multiplicity, a virus is produced that strongly interferes with the replication of standard virus and is also defective. These defective viruses contain multiple new RNAs (4, 5). Recently, Crumpton *et al.* (19) have also made a similar observation. The oligonucleotide analysis of these new RNAs indicates that each arises from a specific viral RNA gene. Additionally, the DI RNAs in a DI virus preparation from a single clone may arise from different standard viral RNA segments. In a DI virus preparation from a clone of ts$^+$, DI RNA species originating from V1, V2, and V3 are observed.

All of the DI RNA examined arise from one of the polymerase genes (V1, V2, or V3). Because these genes represent approximately 54% of the mass of the total viral RNA, it might be expected that these genes yield the most DI RNA species. Alternatively, these genes may be the only ones capable of yielding DI RNAs. Numerous DI RNA species from different clones must be examined to answer this question.

Table 2. Oligonucleotide maps of DI RNAs

DI RNA	Spot no.*	Viral RNA origin
L1	H11 J4 I5 J7 E1 E7 G1 I8 I15 J17 d	VI
L2a	H11 J4 I5 J7 E11 J8 G6 J5	V1
P2	H11 J4 I5 E11 J8	V1
ts$^+$2a	H11 J4 I5 E11 J8	V1
ts$^+$3	H11 J4 H8	V1
B1	D1 G4 H5 I6 I11 J15 H10 J12 a	V2
P1	D1 G4 H5 I6 I11 J15 J12 b	V2
L2b	D1 G4 H5 I6 I11 J15 J12 c	V2
ts$^+$1	D1 G4 H5 I6 I11 J15 H10	V2
L3	D1 G4 H5 I6 I11 J15	V2
ts$^+$2b	E4 H7 H14 J10 J16 J25	V3

*Spots present in more than one DI RNA are underlined.

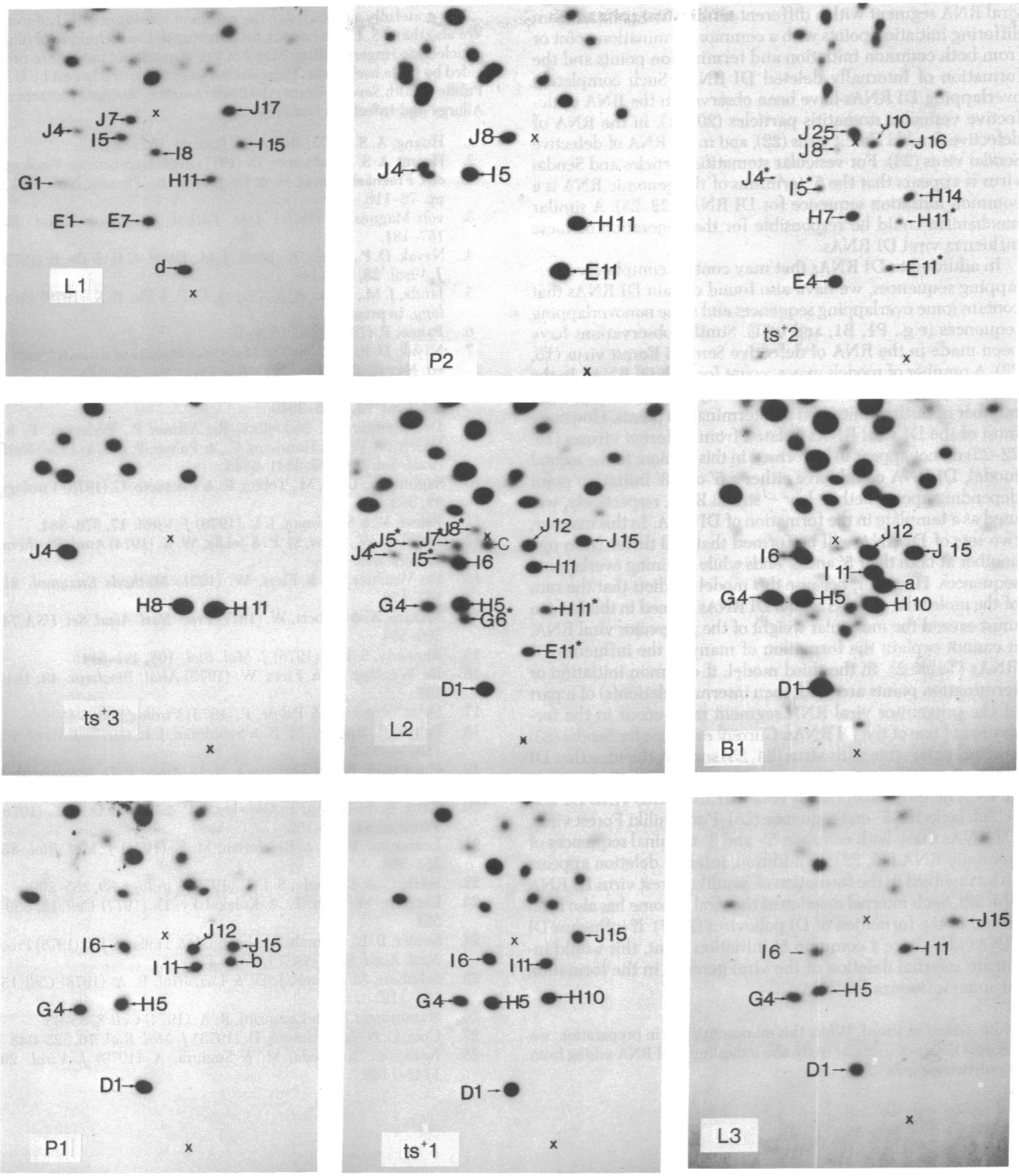

FIG. 3. Oligonucleotide fingerprinting of DI RNAs (L1, P2, ts+2, ts+3, L2, B1, P1, ts+1, and L3). The DI RNAs (Fig. 1 *left*) were eluted from polyacrylamide/agarose gels and fingerprinted. B1 is a mixture of B1 and V2 and V3. Asterisks show light oligonucleotide spots belonging to one DI RNA, whereas dark spots belong to another different DI RNA. First dimension, right to left; second dimension, top to bottom.

Specific reduction of the polymerase genes is also observed in the DI virus preparations studied. In DI ts+, all three polymerase genes appear reduced relative to other genes. This suggests that interference may be specific, and it might be speculated that a DI RNA would interfere specifically with the segment it comes from.

Multiple sets of DI RNA arising from the same gene have been found. These DI RNAs represent deleted forms. Two types of such deleted forms have been found. The first, where one DI RNA most likely contains all the sequences of another larger DI RNA, can be explained by a common initiation or termination point and simple deletion from one end (e.g., L3 and B1). Some possible mechanisms for formation of these DI RNAs are synthesis either from a common initiation point on the standard

viral RNA segment with a different termination point or from differing initiation points with a common termination point or from both common initiation and termination points and the formation of internally deleted DI RNAs. Such completely overlapping DI RNAs have been observed in the RNA of defective vesicular stomatitis particles (20, 21), in the RNA of defective Semliki Forest virus (22), and in the RNA of defective Sendai virus (23). For vesicular stomatitis particles and Sendai virus it appears that the 5′ terminus of the genomic RNA is a common initiation sequence for DI RNAs (22–25). A similar mechanism could be responsible for the generation of these influenza viral DI RNAs.

In addition to DI RNAs that may contain completely overlapping sequences, we have also found certain DI RNAs that contain some overlapping sequences and some nonoverlapping sequences (e.g., P1, B1, and ts^+1). Similar observations have been made in the RNA of defective Semliki Forest virus (15, 22). A number of models may account for such DI RNAs. In the first model, such DI RNAs could be synthesized by using a number of distinct initiation and termination points. However, most of the DI viral RNAs isolated from different viruses (15, 22–25) do not appear to be formed in this fashion. In the second model, DI RNA could have either a 5′ or a 3′ initiation point depending upon whether + or − strand RNA, respectively, was used as a template in the formation of DI RNA. In this manner, two sets of DI RNA will be formed that will differ from one another at both their 5′ and 3′ ends while retaining overlapping sequences. However, because this model predicts that the sum of the molecular weights of two DI RNAs formed in this fashion must exceed the molecular weight of the progenitor viral RNA, it cannot explain the formation of many of the influenza DI RNAs (Table 2). In the third model, if common initiation or termination points are used, then internal deletion(s) of a part of the progenitor viral RNA segment must occur in the formation of one of the DI RNAs. Current evidence for Sendai (23) and vesicular stomatitis virus (24, 25) support the idea that DI RNAs share a common 5′-terminal sequence with the genomic RNA. The only exception is vesicular stomatitis virus DI LT, which lacks the 5′-end sequence (26). For Semliki Forest virus, DI RNAs share both common 5′- and 3′-terminal sequences of genomic RNA (15, 22). In addition, internal deletion appears to be involved in the formation of Semliki Forest virus DI RNA (14, 22). Such internal deletion of the viral genome has also been found in the formation of DI poliovirus (2, 27). If influenza DI RNAs also share a common 5′-initiation point, this would indicate internal deletion of the viral genome in the formation of some influenza DI RNAs.

Note Added in Proof. While this manuscript was in preparation, we became aware of a similar study also indicating a DI RNA arising from a polymerase gene (28).

We gratefully acknowledge the excellent assistance of J. Lofgren. We also thank S. I. T. Kennedy for teaching us the technique of oligonucleotide fingerprinting. Many of the virus clones used were provided by J. Michael Janda. These studies were supported in part by U.S. Public Health Service Grant AI-12749 from the National Institute of Allergy and Infectious Diseases.

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