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Isolation of Influenza C Virus Recombinants

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Recombinants between two different influenza C viruses were isolated. In MDCK (canine kidney) cells, one strain, C/JJ/50, caused lytic plaques, whereas C/JHG/66 virus did not produce clear plaques. From a mixed infection of MDCK cells with C/JHG/66 virus and UV-inactivated C/JJ/50 virus, clones were isolated which possessed the clear-plaque phenotype. Fingerprint analyses indicated that the RNAs of parent viruses had different oligonucleotide patterns and that one of the clones derived from the mixed infection was formed by reassortment of parental genes. This recombinant clone most likely inherited RNAs 1, 2, 3, 6, and 7 from C/JHG/66 virus and RNAs 4 and 5 from C/JJ/50 virus.

The myxoviruses consist of the influenza A, B, and C viruses, which are grouped by serological differences in the nucleoprotein and M protein (16). In 1947 and 1950, hemagglutinating viruses were isolated from patients during epidemics of respiratory illness (10, 32). These viruses were serologically different from influenza A and B viruses and were named influenza C viruses. Infection with influenza C viruses usually causes only a mild or inapparent illness, and therefore these viruses are rarely isolated. For this reason, and also because influenza C viruses were not found to replicate productively in cell cultures, these viruses have been the least studied of the myxoviruses.

Early studies led several investigators to question whether the influenza C viruses were true influenza viruses (8). When viewed in the electron microscope, the influenza C viruses appeared to differ slightly from influenza A and B viruses (1-3, 6, 9, 26). It was discovered that influenza C viruses use a different receptor on erythrocytes (13, 15, 18), and the virus does not possess an α-neuraminidase which is found in influenza A and B viruses (15, 21). More recently, sialic acid, which is absent in influenza A (17) and probably absent in influenza B viruses (26), was found in the envelope of influenza C viruses (20). Differences in host range and growth temperature between influenza C and influenza A and B viruses have been discussed (16).

Despite these differences, there is evidence which indicates that influenza C viruses are closely related to influenza A and B viruses. It was shown that influenza C viruses possess a segmented, single-stranded genome (6, 7, 18, 30) and that the base composition of the genome is similar to that of influenza A and B viruses (30). The virus-specific polypeptides which have been described—M protein, nucleoprotein, and a surface glycoprotein—are also similar in general type to those found in influenza A and B viruses (6, 15, 18, 20, 28). We have found that the influenza C virus genome contains seven RNA segments which code for at least seven virus-specific polypeptides (submitted for publication), and sequencing of viral RNAs revealed that the influenza A, B, and C virus genes possess nearly identical 5′ and 3′ termini (U. Desselberger, V. R. Racaniello, J. Zazra, and P. Palese, Gene, in press). This information indicates that despite their differences, influenza C viruses are most likely true influenza viruses.

In this communication we show that influenza C viruses, like the influenza A and B viruses, may also undergo recombination (reassortment of RNA segments).

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MATERIALS AND METHODS

Cells and media. Madin-Darby canine kidney cells (MDCK [12]) were propagated in Earle-based minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum.

Viruses. Seed stocks of influenza C/Taylor/47, C/Great Lakes/54, C/Johannesburg/66 (C/JHG/66) and C/JJ/50 virus (kindly provided by R. J. O'Callaghan and C. Howe) were prepared by inoculation of the amniotic cavity of 10-day-old embryonated hen eggs as described previously (30). Amniotic fluids were harvested after incubation for 36 h at 35°C.

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Plaque assay of virus. Influenza C viruses were plaqued in MDCK cells by using a modification of the techniques used for influenza A and B viruses (34, 35). The agar overlay used contained 0.5% agar and 20 μg of human plasminogen per ml. Plates were incubated at 34°C. Under these conditions, all influenza C viruses tested, with the exception of the C/JHG/66 virus, formed clear plaques after 4 to 5 days.

Isolation of recombinant virus. The strategy for obtaining recombinants between influenza C viruses was based on the finding that C/JJ/50 virus produced clear, well-defined plaques in MDCK cells, whereas C/JHG/66 virus yielded turbid plaques which could not be counted. A seed stock of C/JJ/50 virus (1,024 hemagglutination units per ml) was diluted 1:10, and 1.5 ml of the dilution was placed in a 25-cm^2 plastic petri dish. The virus was irradiated for 15 s with UV light from an 8-W bulb (G.8.T5; General Electric Co., Schenectady, N.Y.) placed 15 cm above the surface of the dish. The irradiated virus was then mixed 1:1 with a 1:10 dilution of C/JHG/66 virus (1,024 hemagglutination units per ml). The mixture was used to infect MDCK monolayers (29) which were incubated under serum-free liquid overlay containing 20 μg of plasminogen per ml. Control dishes were also infected with C/JHG/66 virus and UV-irradiated C/JJ/50 virus. After 3 days at 34°C, cell supernatants were harvested, clarified, and plaque assayed in MDCK cells. In dishes infected with the recombinational mixture, agar surrounding individual plaques was removed and suspended in phosphate-buffered saline. The suspension was then assayed for PFU, and after a second plaque passage, virus was propagated first in MDCK cells and then in embryonated hen eggs.

Fingerprinting viral RNA. Unlabeled RNA was obtained by growing viruses in large quantities of eggs, purifying virus, and extracting viral RNA as described before (19, 25). RNA was digested with RNase T_1, and the resulting oligonucleotides were enzymatically labeled at their 5′ ends by using [γ-^{32}P]ATP and polynucleotide kinase (11, 27), fingerprinted in two dimensions on polyacrylamide gels, and detected by autoradiography. Specific conditions for fingerprinting influenza virus RNA have been published elsewhere (19, 27). To obtain fingerprints of individual influenza C virus genes, 10 to 15 μg of unlabeled viral RNA was electrophoresed in one slot of a 2.6% polyacrylamide gel containing 7 M urea under established conditions (31). Individual RNA segments were visualized under UV illumination after the gel was immersed in Loening buffer (25) containing 4 μg of ethidium bromide per ml. RNA continuously labeled with ^{32}P was prepared as described (30) and electrophoresed in parallel with cold RNA. Areas of the gel containing RNA segments were then excised, and the RNA was electrophoresed into 0.3 ml of 0.5 M Tris-acetate buffer, pH 8.0. RNA was precipitated with 2.5 volumes of ethanol, treated with perchloric acid to remove contaminating acrylamide (14), reprecipitated with ethanol, and dissolved in T1 buffer (27) for subsequent RNase T_1 digestion.

Materials. Human plasminogen was obtained from Worthington Biochemicals Corp., Freehold, N.J.; RNase T_1 was from Calbiochem, La Jolla, Calif.; and [γ-^{32}P]ATP was purchased from New England Nuclear Corp., Cambridge, Mass.

RESULTS

Plaquing influenza C viruses. To study the molecular biology of influenza C viruses, it was necessary to develop a cell culture system in which the viruses could replicate productively. Recently, several investigators have described various cell systems for growing influenza C viruses (4–6, 18, 22, 23, 28). Here we show that most influenza C viruses will produce clear plaques in MDCK cells when plasminogen is incorporated into the agar overlay. The incorporation of trypsin into the agar overlay facilitates plaquing of influenza A and B viruses (34, 35). However, the levels of trypsin (20 to 40 μg/ml) required to plaque influenza C viruses frequently damages cell monolayers, and it was found that incorporation of plasminogen into the agar overlay (20 μg/ml) yields more consistent results (J. L. Schulman, personal communication). The absence of any protease in the agar overlay prevents plaque formation by influenza C viruses in this system. MDCK monolayers were infected with various influenza C viruses and stained with crystal violet. C/Taylor/47, C/JJ/50, and C/Great Lakes/54 viruses produce clear, easily distinguishable plaques in this cell system, whereas C/JHG/66 virus produces turbid plaques which cannot be counted (Fig. 1). The difference in plaque morphology between C/JJ/50 virus and C/JHG/66 virus was initially chosen as a genetic marker for detecting recombination.

Fingerprints of RNA from C/JJ/50 and C/JHG/66 viruses. Preliminary experiments indicated that the genomes of various influenza C virus isolates possess easily distinguishable RNase T_1 fingerprint patterns. RNA fingerprints of the two viruses chosen for this study are shown in Fig. 2a and b. To the right of the autoradiograms are schematic diagrams of the fingerprints (Fig. 2d and e). In each fingerprint, only those oligonucleotides below the dotted line have been analyzed (oligonucleotides above this line are most likely found in the genome more than one time). The oligonucleotides below the dotted line represent approximately 13% of the entire influenza C virus genome. In the fingerprint of the RNA from C/JHG/66 virus (Fig. 2a and d), these oligonucleotides have been arbitrarily assigned numbers from 1 to 77. The fingerprint of the RNA of the second virus is shown in Fig. 2b and e. Differences in the patterns of the two viruses were established by fingerprinting mixtures of oligonucleotides from the two viral RNAs on the same gel (data not shown). There are 18 oligonucleotides specific for C/JHG/66 virus RNA which are absent in the pattern of C/JJ/50 virus RNA and 15 oligonu-

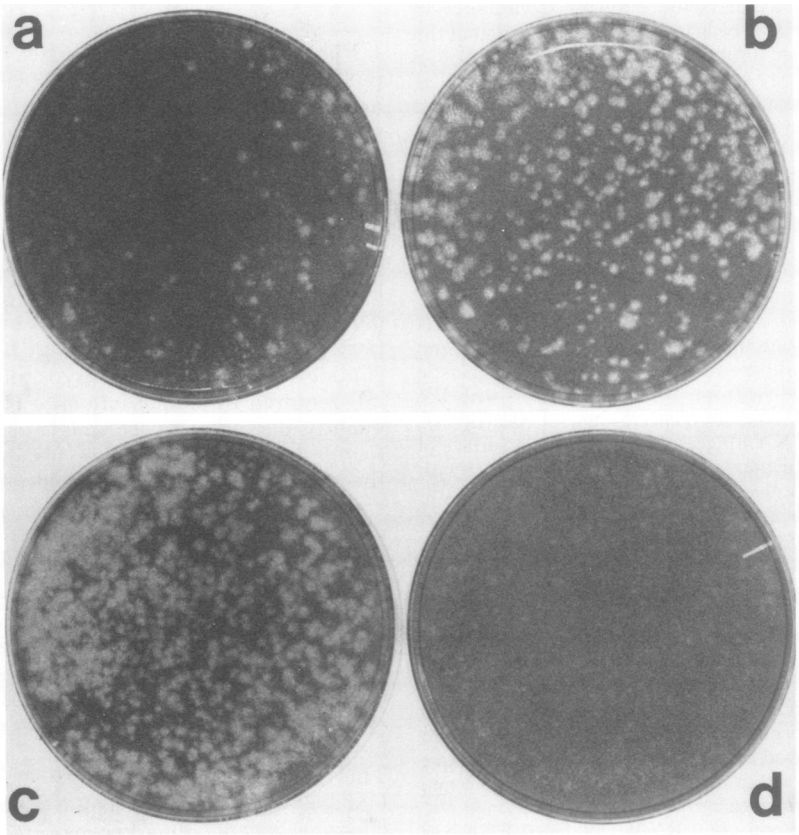


FIG. 1. *Formation of plaques by influenza C virus strains in MDCK cells. Monolayers were infected with virus and incubated under 0.5% agar overlay containing 20 μg of human plasminogen per ml as described in the text. After 4 days of incubation at 34°C, agar was removed and the monolayers were stained with crystal violet. (a, b, c) Cells infected with influenza C/Taylor/47 virus, C/JJ/50 virus, and C/Great Lakes/54 virus, respectively, which all produce clear plaques; (d) cells infected with influenza C/JHG/66 virus, which produces turbid plaques that cannot be counted.*

cleotides (numbered 100 through 114 in Fig. 2e) which are present in C/JJ/50 virus RNA and absent in C/JHG/66 virus RNA (see below).

Recombination of C/JJ/50 and C/JHG/66 viruses. Recombinants between two influenza C viruses were isolated as described above. The supernatants of MDCK cells, which had been doubly infected with C/JHG/66 virus and UV-irradiated C/JJ/50 virus, were plaqued and yielded 1.2×10^6 PFU/ml. Plaques were clear and resembled those caused by unirradiated C/JJ/50 virus. In control experiments, after one passage in MDCK cells, irradiated C/JJ/50 virus failed to produce plaques, and C/JHG/66 virus yielded turbid plaques which could not be counted (Table 1).

Since UV-irradiated C/JJ/50 virus did not form plaques, whereas C/JHG/66 virus produced turbid plaques, the presence of clear plaques in the recombinational mixture suggested that recombination had occurred. To obtain further evidence that recombination between parental viruses had taken place, 14 clones isolated from cells infected with the recombinational mixture were plaque purified two times as described above. These cloned viruses were then subjected to the biochemical analysis described below.

Fingerprint of RNA of influenza C virus recombinant. To confirm that the cloned viruses were recombinants, RNA from four isolates was prepared and fingerprinted as described above. The fingerprint of one clone, virus P1, is shown in Fig. 2c. To the right of the autoradiogram is a drawing of the oligonucleotide spots (Fig. 2f). The identification of parental spots in virus P1 RNA was accomplished by fingerprinting mixtures of oligonucleotides derived from RNAs of the recombinant and its parents (data not shown). It is clear from Fig. 2

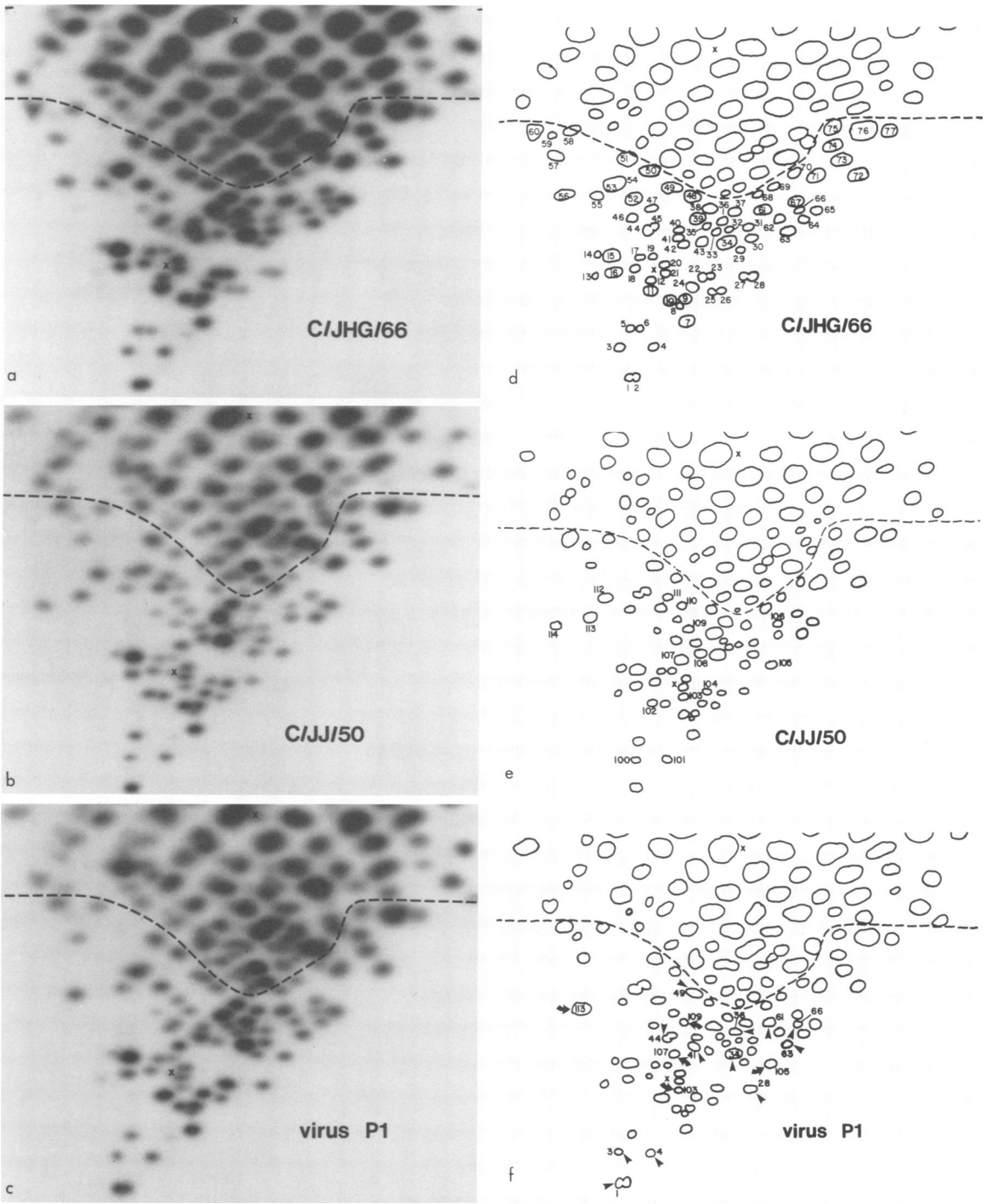


FIG. 2. *Fingerprints of RNA from C/JHG/66 virus, C/JJ/50 virus, and recombinant P1. RNA was extracted from purified virus and digested with RNase T_1, and the resulting oligonucleotides were labeled at their 5' ends with ^{32}P and fingerprinted in two dimensions on polyacrylamide gels, as described in the text. (a) Autoradiogram of fingerprint of influenza C/JHG/66 virus RNA. (d) Drawing of autoradiogram in a; oligonucleotides used for the analysis (below the dotted line) are numbered 1 to 77. (b) Autoradiogram of fingerprint of influenza C/JJ/50 virus RNA. (e) Drawing of autoradiogram in b; oligonucleotide spots present in the fingerprint of C/JJ/50 virus RNA and not present in the fingerprint of C/JHG/66 virus RNA are numbered 100 to 114. Unlabeled spots below the dotted line are common to both viruses. The numbers of these common spots are the same as shown in d. (c) Autoradiogram of fingerprint of virus P1 RNA. (f) Drawing of autoradiogram in c; unique oligonucleotide spots inherited from C/JHG/66 virus are indicated by an arrowhead and the appropriate number, and unique oligonucleotide spots inherited from C/JJ/50 virus are indicated by an arrow and the appropriate number. Lower ×, Xylene cyanol FF dye; upper ×, bromophenol blue dye. In a, b, and c, faint oligonucleotide spots below the dotted line which are not numbered were not observed consistently.*

TABLE 1. *Virus yield after infection of MDCK cells with UV-irradiated C/JJ/50 virus, C/JHG/66 virus, and virus mixture*

Virus	Yield (PFU/ml)[a]
UV-C/JJ/50[b]	<10
C/JHG/66[b]	<10[c]
Recombinational mixture[b]	1.2×10^6

[a] Determined in MDCK cells.

[b] MDCK cells were infected with 0.1 ml of UV-irradiated C/JJ/50 virus (100 hemagglutination units per ml), C/JHG/66 virus (100 hemagglutination units per ml), and a 1:1 mixture of the two virus preparations. Yields were assayed after 3 days of incubation at 34°C as described in the text.

[c] Plaques were turbid and could not be counted.

that virus P1 derives specific oligonucleotides from both parents and lacks several oligonucleotides which are specific for either C/JJ/50 or C/JHG/66 virus, indicating that this clone is not merely a mixture.

Fingerprints of isolated C/JHG/66 virus genes. In influenza A and B viruses, recombination between two strains occurs by reassortment of RNA segments. We assume that influenza C viruses also exchange genetic information in this manner, although the data presented above do not exclude the possibility that virus P1 arose by intragenic recombination. Examination of the RNA patterns of C/JHG/66, C/JJ/50, and virus P1 did not permit analysis of derivation of genes in the recombinant virus. To obtain evidence that recombination of influenza C viruses occurs by reassortment, oligonucleotide maps of individual genes were analyzed. If recombination involves the exchange of entire RNA segments, then all of the oligonucleotide spots assigned to a particular gene would be transferred in a recombinational event.

For the assignment of oligonucleotides to individual segments, RNA from C/JHG/66 virus was used, since it could be obtained in higher yields than C/JJ/50 virus RNA. Unlabeled C/JHG/66 viral RNA was electrophoresed on 2.6% polyacrylamide gels containing 7 M urea (see text). Under these conditions, influenza C virus RNA can be separated into five bands containing RNA segments 1 and 2, segment 3, segment 4, segment 5, and segments 6 and 7. (RNAs 1 and 2 migrate too closely to be eluted separately, and RNAs 6 and 7 comigrate as a dense band [Fig. 3].) The characterization of the seven RNAs of influenza C viruses will be discussed in a separate paper (manuscript in preparation). Five gel slices containing these seven RNA segments were excised, and RNA was eluted from the gel, digested with RNase T_1, and fingerprinted as described above.

In Fig. 4 the fingerprints of C/JHG/66 virus RNA segments are shown. The oligonucleotide spots in each gene are numbered by their location in the fingerprint of the total RNA of C/

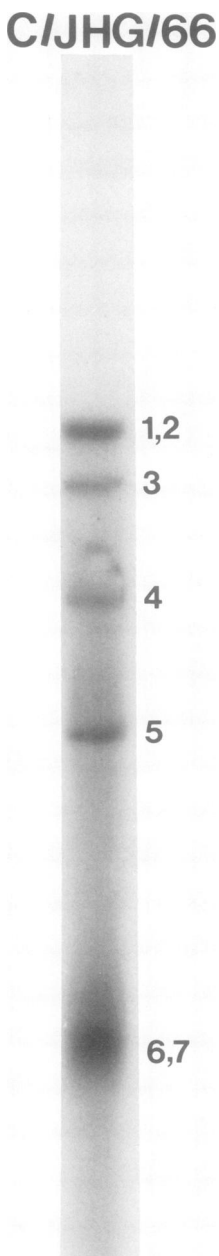


FIG. 3. *Separation of ^{32}P-labeled influenza C/JHG/66 virus RNA segments. RNA was continuously labeled with ^{32}P in ovo, extracted from purified virus, and run on 2.6% polyacrylamide gels containing 7 M urea, as described in the text. Migration is from top to bottom, and RNA segments are numbered according to results obtained in this laboratory (submitted for publication).*

JHG/66 virus (Fig. 2a and d). Of the 77 large, unique oligonucleotides, 72 can be assigned to a gene or group of genes, whereas 5 oligonucleotides could not be unambiguously assigned to RNA segments.

Figure 5 summarizes the oligonucleotide data presented in Fig. 2 and 4. The upper left-hand panel lists the 18 oligonucleotide spots present in C/JHG/66 virus RNA which are absent in C/JJ/50 virus RNA. These spots unique to C/JJ/50 are listed by the RNA segment to which they were assigned (see Fig. 3). The upper right-hand panel lists the 15 oligonucleotide spots (numbered 100 through 114) which are present in C/JJ/50 virus RNA and are absent in C/JHG/66 virus RNA. These oligonucleotides were not assigned to specific RNA segments, since it was difficult to obtain C/JJ/50 viral RNA in large quantities. The lower panel of Fig. 5 indicates the derivation of oligonucleotide spots in recombinant virus P1. It can be seen that recombinant P1 derives all of the C/JHG/66-specific oligonucleotide spots assigned to RNA segments 1 and 2, segment 3, and segments 6 and 7. None of the C/JHG/66-specific spots from RNAs 4 and 5 are present in recombinant P1. In addition, recombinant P1 inherits five oligonucleotide spots (103, 105, 107, 109, and 113) which are unique to C/JJ/50 virus.

From this information we conclude that virus P1 is a recombinant between C/JHG/66 and C/JJ/50 viruses. This recombinant most likely inherits RNAs 1, 2, 3, 6, and 7 from C/JHG/66 virus and RNAs 4 and 5 from C/JJ/50 virus. Examination of the fingerprints of RNA from three other clones indicated that they appear to have the same genotype (data not shown).

DISCUSSION

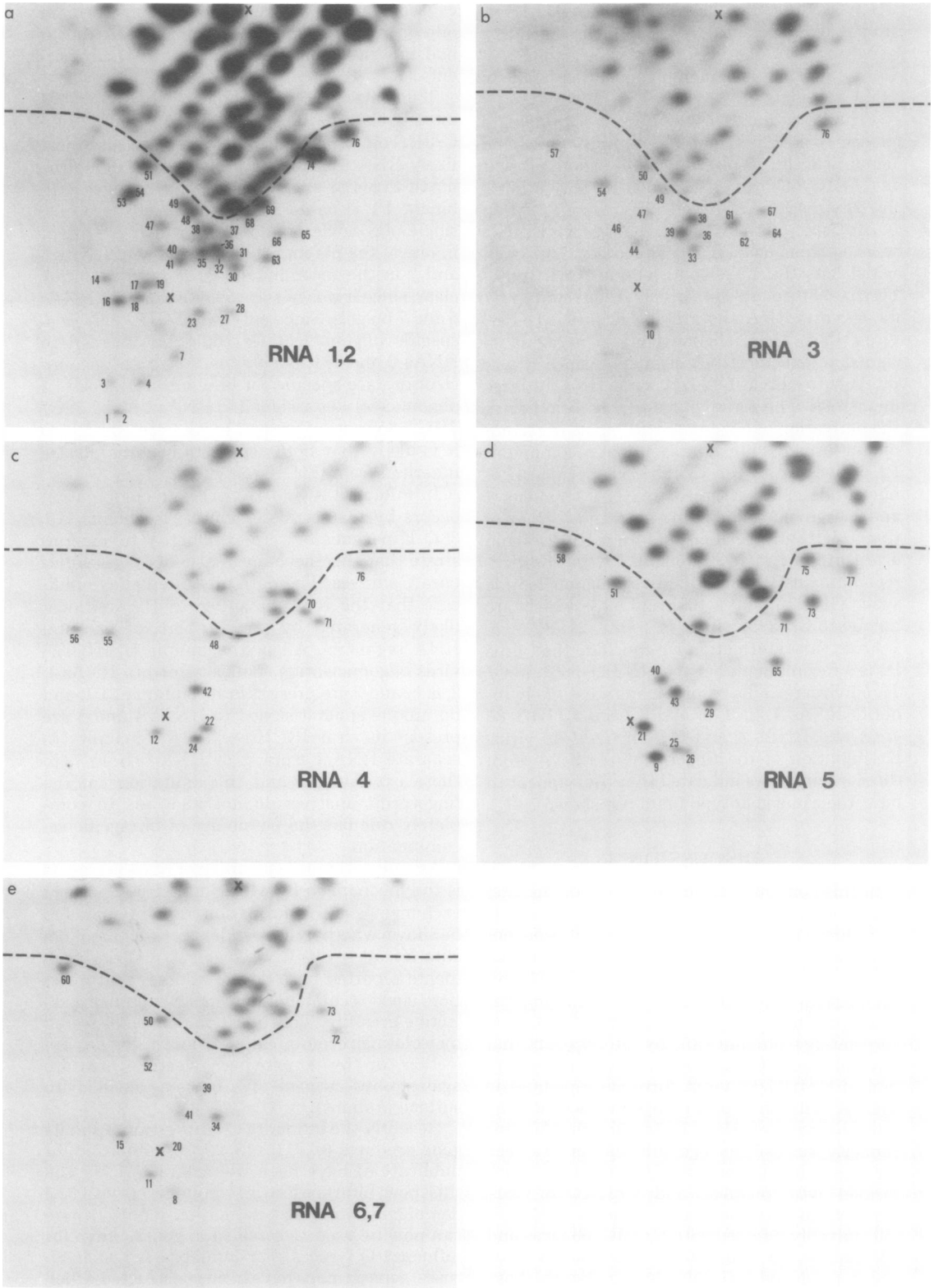
In this communication we report for the first time evidence for recombination (reassortment) in influenza C viruses. Previously it was not possible to demonstrate recombination between influenza C viruses, since genetic and biochemical markers were not available. Our finding was made possible by the development of RNA fingerprinting techniques and by the discovery that most influenza C virus isolates, with the exception of C/JHG/66 virus, form clear plaques in MDCK cells when plasminogen is incorporated into the agar overlay. Difference in plaquing capacity was used as a genetic marker to screen for recombinants between C/JJ/50 virus, which formed lytic plaques, and C/JHG/66 virus, which formed turbid plaques. Yields of MDCK cells mixedly infected with C/JHG/66 virus and UV-inactivated C/JJ/50 virus contained virus which produced lytic plaques similar to those caused by C/JJ/50 virus. An RNA fingerprint of one of the isolated clones demonstrated that it had inherited oligonucleotides from both parents.

The data presented indicate that virus P1 most likely inherits RNAs 1, 2, 3, 6, and 7 from C/JHG/66 virus and inherits RNA 4 and most likely RNA 5 from C/JJ/50 virus. It should be noted that the assignment of RNA 5 in recombinant P1 is based solely on the absence of a single oligonucleotide (no. 26) which is present in one of the parents. The genealogy of RNAs 6 and 7 in the recombinant P1 is not absolutely clear, since it was not possible to separate RNAs 6 and 7 on gels. Since only two C/JHG/66 virus-specific oligonucleotides could be assigned to RNAs 6 and 7, it is possible that both oligonucleotides are specific for only one RNA. All 34 oligonucleotides assigned to RNAs 1 and 2 of C/JHG/66 virus are present in recombinant P1, so it is likely that both segments in virus P1 are inherited from C/JHG/66 virus.

In influenza A and B viruses, recombination occurs by reassortment of RNA segments (16, 33). Experiments were conducted to demonstrate that this mechanism also occurs in influenza C viruses. The data presented above indicate that the recombinant virus P1 was most likely generated by reassortment of parental RNA segments. All of the unique C/JHG/66 virus oligonucleotide spots assigned to RNAs 1, 2, 3, 6, and 7 are present in recombinant P1, and no unique spots assigned to RNAs 4 and 5 are present in virus P1. However, the inability to separate RNA 1 from 2, or RNA 6 from 7, in these experiments and the limitations of the fingerprint analysis do not allow us to completely rule out the possibility of intragenic recombination.

Assuming that reassortment of RNA segments is the mechanism by which influenza C viruses exchange genetic information, it still remains to be shown whether independent reassortment occurs for all seven RNA segments. The experiments reported here do not address that question, since recombinants were isolated using selective pressure. This question can be answered by isolating recombinants without using any selection or by employing selective pressure against only one gene (e.g., by using antibody to surface proteins).

Recently the influenza C virus genome and its gene products (6, 7, 15, 18, 20, 28; submitted for publication) have been well characterized, and this new information in conjunction with the demonstration of recombination reported here can now be used to establish a genetic map for influenza C viruses. For influenza A and B viruses, genetic maps have been constructed which


a
RNA 1,2
b
RNA 3
c
RNA 4
d
RNA 5
e
RNA 6,7

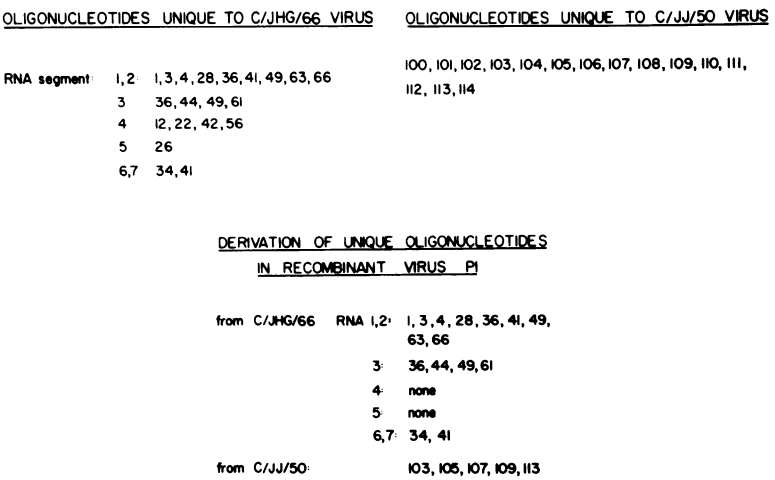


FIG. 5. *Unique oligonucleotides present in C/JHG/66 virus, C/JJ/50 virus and recombinant virus P1.*

associate one RNA segment with one polypeptide. Those studies have led to new information concerning the function of influenza virus RNAs and proteins. Construction of an influenza C virus genetic map would lead to studies which elucidate the function of influenza C virus genes and gene products and would perhaps clarify the relationship of these viruses to influenza A and B viruses.

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FIG. 4. *Fingerprints of influenza C/JHG/66 virus RNA segments. RNA from egg-grown virus was separated on polyacrylamide gels and eluted and digested with RNase T_1, and the resulting oligonucleotides were labeled at their 5′ ends and fingerprinted as described in the text. Gene-specific oligonucleotide spots are numbered by their location in the fingerprint of whole C/JHG/66 virus RNA (Fig. 2a and d). Some spots are present in more than one gene and most likely represent sequences that are not unique. Faint spots below the dotted line are not numbered and are probably caused by contaminating RNAs. Variability in the intensities of spots may be due to a difference in the labeling efficiency of the 5′ ends of different oligonucleotides. Autoradiograms of fingerprints are as follows: (a) RNA segments 1 and 2; (b) RNA segment 3; (c) RNA segment 4; (d) RNA segment 5; and (e) RNA segments 6 and 7. Lower ×, Xylene cyanol FF dye; upper ×, bromophenol blue dye.*

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