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Genetics

Biochemical evidence that "new" influenza virus strains in nature may arise by recombination (reassortment)

(influenza virus RNA/antigenic variation/avian virus strains/oligonucleotide analysis)

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ABSTRACT **Oligonucleotide analysis of two avian influenza A viruses (Hav6N2 and Hav6Nav4) isolated in nature showed identical or almost identical patterns for the corresponding *M* and *HA* genes: 24 of 25 and 13 of 13 large oligonucleotides were indistinguishable by two-dimensional gel analysis. On the other hand, remarkable differences in the oligonucleotide patterns of the remaining genes were observed. Only 2 of 11 oligonucleotide spots of the *NS* gene, 10 of 27 spots of the *NA/NP* genes, and 22 of 49 spots of the *P* genes were indistinguishable between the two strains. On the basis of this observation that at least two genes of these viruses are virtually identical whereas others show easily detectable differences, we conclude that the two avian strains are related to each other by a recombinational event. In addition, it was found that animals in nature can be doubly infected with influenza viruses. Both lines of evidence strongly suggest that recombination is at least one mechanism by which "new" influenza virus strains emerge in nature.**

Influenza viruses are unique among viruses of man because they display a seemingly endless number of genetic variants. In a single winter, epidemics can be caused by viruses that are antigenically distinguishable from each other and from other variants that circulate during the next winter. This phenomenon of small changes in the virus is referred to as antigenic drift and has been associated with mutations of the virus surface proteins. In contrast, major changes (antigenic shift) in the surface proteins of the virus have occurred every 10–20 years in this century and the emergence of these "new" influenza viruses results in pandemics.

How do these new strains arise? Theories suggesting that new pandemic strains emerge by mutation from circulating human or animal strains have not been widely accepted. Rather, human–animal virus recombinational theories have been invoked to explain the emergence of pandemic strains (1–7). This proposed mechanism involves the exchange of genes of human and animal influenza viruses by recombination (reassortment). Indirect evidence for this mechanism has been provided by peptide analysis of the surface proteins of existing strains (6, 7), by experiments demonstrating recombination of influenza viruses in laboratory animals (8, 9), and by hybridizing the RNAs of recent strains with those of earlier strains (10). In addition, many different animal influenza viruses isolated from birds have been shown by serologic analysis to share one surface protein but not the other (11, 12). All these results, although compatible with the recombinational theory, do not entirely exclude the possibility that new strains in nature arise by mutations and that recombination (reassortment) is not involved in the emergence of new influenza virus strains.

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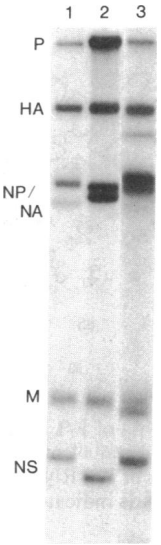

FIG. 1. Pattern of ^{32}P-labeled influenza virus RNAs separated on 2.6% polyacrylamide gels. Lanes: 1, RNA of F/2; 2, RNA of F/1; 3, RNA of isolate 3, which contains a mixture of avian influenza viruses.

Using the highly sensitive technique of oligonucleotide mapping, we now present strong evidence that recombination is one mechanism by which new influenza viruses may emerge in nature. Our results show that at least one of two avian influenza viruses recently isolated from wild ducks arose as the result of recombination in nature.

MATERIALS AND METHODS

Viruses and Cells. Several influenza virus isolates were obtained by one of us (C.H.) from cloacal swabs of wild ducks in the northern part of France. By using serologic techniques, two isolates, influenza A/duck/France/MA42/76 (isolate F/1) and influenza A/duck/France/MB42/76 (isolate F/2), were identified as members of serotypes Hav6N2 and Hav6N4, respectively (13). A third isolate from ducks obtained at the same time is referred to as isolate 3. Virus used for the RNA pattern and oligonucleotide analyses was replicated in MDCK cells in the presence of trypsin (0.5 μg/ml) and in 10- to 11-day-old embryonated chicken eggs. Purification of the virus followed published procedures (14, 15) except that, for oligonucleotide analysis, virus was further purified by a second centrifugation through a 30–60% sucrose gradient.

RNA Pattern Analysis. ^{32}P-Labeled RNA of influenza virus

Abbreviations: F/1, influenza A/duck/France/MA42/76 (Hav6N2) virus; F/2, influenza A/duck/France/MB42/76 (Hav6Nav4) virus; *P*, *HA*, *NA*, *NP*, *M*, and *NS* genes, genes coding for P_{1-3} polypeptides, hemagglutinin, neuraminidase, nucleoprotein, membrane protein, and nonstructural protein of influenza virus, respectively.

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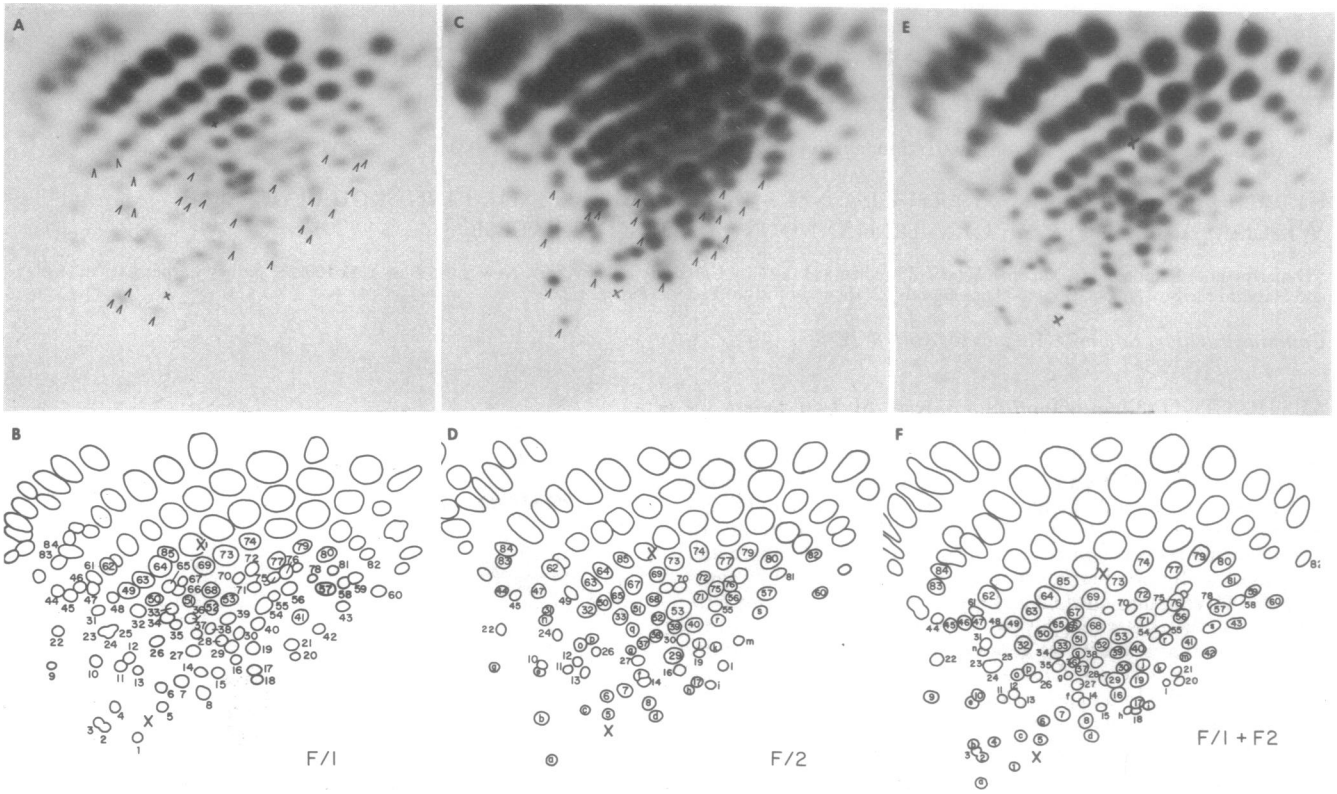

FIG. 2. Oligonucleotide fingerprints of the RNAs of F/1 (*A* and *B*) and F/2 (*C* and *D*) and of a mixture of the viruses (*E* and *F*). Large oligonucleotides of F/1 RNA are labeled 1–85. The oligonucleotides of F/2 RNA that are indistinguishable from those of F/1 RNA have the same numbers; the remaining large oligonucleotide spots of F/2 RNA are labeled a–s. The positions of the two dye markers (xylene cyanol and bromophenol blue) are indicated by X. Little arrowheads indicate spots *not* shared by the RNAs of F/1 and F/2.

was obtained by infecting MDCK (canine kidney) cells under high multiplicity, purifying the virus, and extracting the RNA according to published procedures (14, 15). The RNA was then electrophoresed on 2.6% polyacrylamide gels at 26° as described (14, 15). The assignment of the eight individual genes to specific viral proteins is based on the analysis of recombinants derived from viruses containing the Hav6 hemagglutinin (unpublished data) and follows previously established maps of influenza virus (14–16). Under the electrophoresis conditions, the top three *P* genes of both viruses and the *NP* and *NA* genes of the F/1 virus were not sufficiently separated to permit elution of single bands from gels.

Oligonucleotide Pattern Analysis. Total RNA or RNA segments (0.4–2 μg) that were eluted (17) after separation on 2.6% polyacrylamide gels were digested with RNase T1 and 5′-end labeled with [γ-^{32}P]-ATP by an unpublished procedure similar to a technique described by Frisby (18). Oligonucleotides were subjected to two-dimensional gel electrophoresis according to the method first described by deWachter and Fiers (19) and modified by Billeter *et al.* (20).

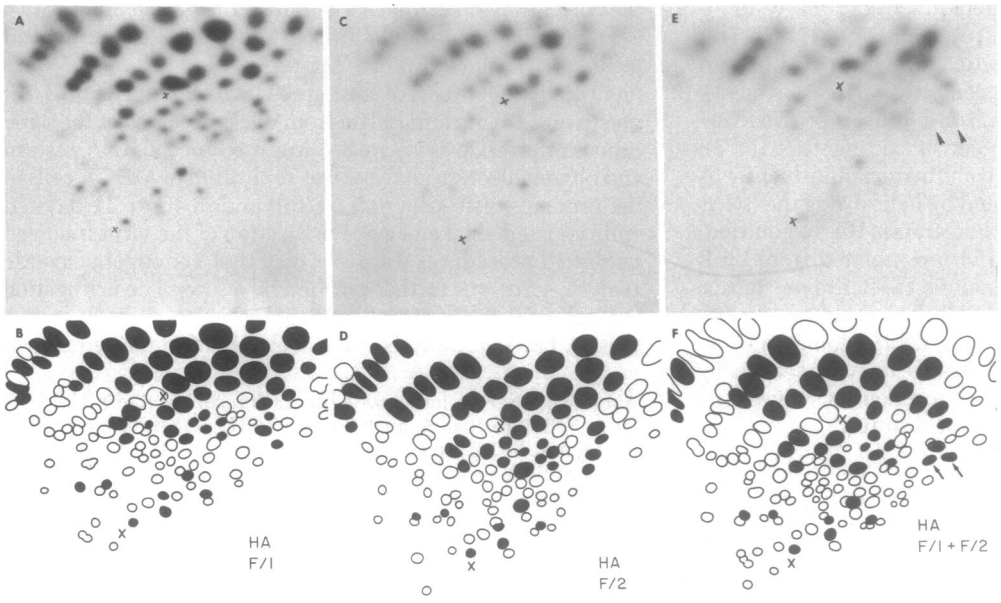

FIG. 3. Oligonucleotide fingerprints of the isolated *HA* genes of F/1 (*A* and *B*) and F/2 (*C* and *D*) and of a mixture of both genes (*E* and *F*). The diagrams show the oligonucleotides (in black) on a background of the pattern of whole F/1 RNA (taken from Fig. 2*B*), of whole F/2 RNA (taken from Fig. 2*D*), and of a mixture of both viral RNAs (taken from Fig. 2*F*). Arrows identify the only differences in the patterns of the two *HA* genes. In the oligonucleotide map of the *HA* gene of F/1, additional faint spots can be seen which are derived from contaminating breakdown products of the larger *P* genes (compare with Fig. 5*A*).

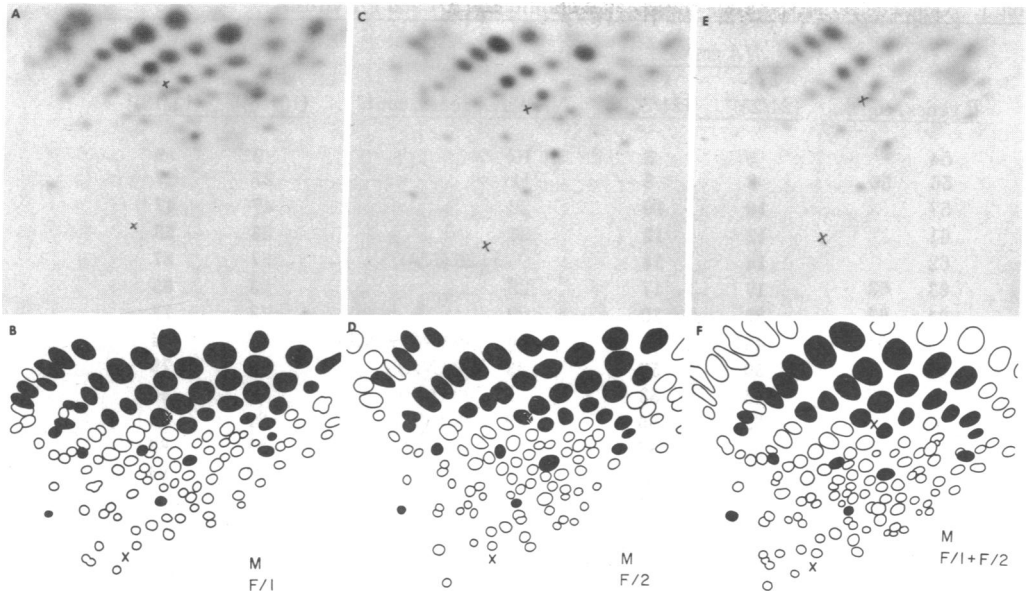

FIG. 4. Oligonucleotide fingerprints of the isolated *M* genes of F/1 RNA (*A* and *B*) and F/2 RNA (*C* and *D*) and of a mixture of the two genes (*E* and *F*). The diagrams show the oligonucleotides (in black) on a background of the patterns of whole F/1 RNA (taken from Fig. 2*B*), of whole F/2 virus RNA (taken from Fig. 2*D*), and of a mixture of both viral RNAs (taken from Fig. 2*F*).

RESULTS

RNA Patterns. Two influenza A viruses (F/1 and F/2) isolated from wild ducks were shown to share a serologically indistinguishable hemagglutinin (Hav6) (13). In contrast, their neuraminidases were classified as an N2 and an Nav4 neuraminidase type, respectively (13). Analysis of the RNAs of these two influenza viruses on polyacrylamide gels (Fig. 1, lanes 1

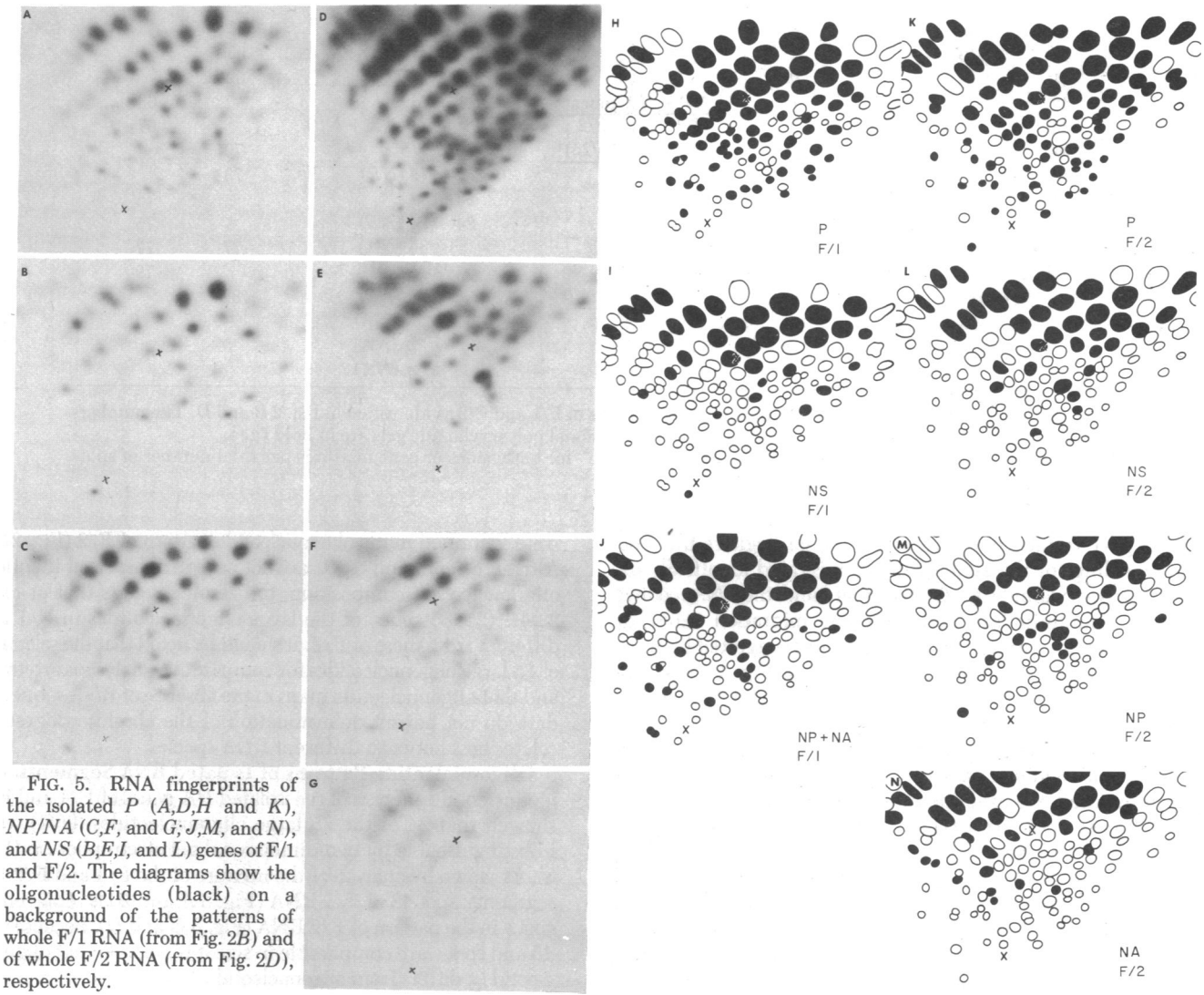

FIG. 5. RNA fingerprints of the isolated *P* (*A,D,H* and *K*), *NP/NA* (*C,F,* and *G; J,M,* and *N*), and *NS* (*B,E,I,* and *L*) genes of F/1 and F/2. The diagrams show the oligonucleotides (black) on a background of the patterns of whole F/1 RNA (from Fig. 2*B*) and of whole F/2 RNA (from Fig. 2*D*), respectively.

Table 1. Oligonucleotide spots of isolated genes of influenza F/1 and F/2 viruses

P genes*				*HA* genes*				*M* gene*	
F/1 (22/49)†	F/2 (22/47)†	*P* genes, cont.		F/1 (24/25)†	F/2 (24/25)†	*NP* + *NA* genes, cont.		F/1 (13/13)†	F/2 (13/13)†
4		54		**5**	**5**	10		**9**	**9**
7	**7**	**56**	**56**	8	8	11		**27**	**27**
10		57		**10**	**10**	22		**47**	**47**
11	**11**	61		**12**	**12**	24		**53**	**53**
13	**13**	62		**14**	**14**		26 (*NA*)	**57**	**57**
14		**63**	**63**	**17**	**17**	28		**65**	**65**
15		**64**	**64**	**29**	**29**	29		**73**	**73**
16			65	43		**30**	**30** (*NP* + *NA*)	**74**	**74**
18		66		**50**	**50**	**32**	**32** (*NA*)	**79**	**79**
19	**19**		67	**53**	**53**		33 (*NP*)	**80**	**80**
20		68		**55**	**55**	**39**	**39** (*NP*)	**81**	**81**
21		**69**	**69**	**57**	**57**		40 (*NP*)	**83**	**83**
	22		71	**63**	**63**		44 (*NA*)	**84**	**84**
23		**72**	**72**	**64**	**64**		45 (*NA*)		
	24	73		**67**	**67**	46		*NS* gene*	
25		**74**	**74**	**68**	**68**		51 (*NP*)	F/1 (2/11)†	F/2 (2/11)†
26		**76**	**76**	**69**	**69**	52			
	29		77	**70**	**70**	**53**	**53** (*NP*)		
31	**31**	**79**	**79**	**71**	**71**	54		1	
32		**80**	**80**	**72**	**72**	**56**	**56** (*NP*)		32
33	**33**		81	**73**	**73**	58		35	
34			83	**74**	**74**	59			40
35			84	**75**	**75**	**60**	**60** (*NP*)	41	
36		**85**	**85**	**80**	**80**		63 (*NA*)	45	
38	**38**		*a*	**81**	**81**		64 (*NP*)	**53**	**53**
40	**40**		*c*		*s*	**68**	**68** (*NP*)	57	
41			*d*			69		62	
42			*e*	*NP* + *NA* genes*		**73**	**73** (*NP*)		63
44			*f*	F/1	F/2		74 (*NA* + *NP*)		67
48			*g*	(10/27)†	(10/25)†	78		70	
49	**49**		*h*			**79**	**79** (*NP*)	73	
50	**50**		*i*	2		83			74
51	**51**		*j*	3		**84**	**84** (*NP*)		75
52	**52**		*k*	7			85 (*NA*)	**77**	**77**
53			*l*				b (*NP*)		79
			m				*o* (*NP*)	82	
			o				*n* (*NA*)		*f*
			r				*p* (*NA*)		*j*
			s				*q* (*NA*)		

* Numbers and letters indicate specific oligonucleotide spots of F/1 and F/2 as identified in Fig. 2 *B* and *D*. The numbers of those spots that show identical migration in two-dimensional polyacrylamide gels are in **bold face**.

† Numbers in parentheses give number of spots in "common" for both genes or gene mixtures per total number of spots evaluated per gene or gene mixture.

and 2) gave results compatible with the serologic result. The RNAs coding for the hemagglutinin migrated identically on a 2.6% polyacrylamide gel. On the other hand, the RNAs coding for the neuraminidase had different migration rates, in agreement with differences in the proteins suggested by serologic analysis. In addition, lanes 1 and 2 in Fig. 1 show that several other segments, such as the genes coding for *NP* and *NS*, migrated differently in the two strains.

Oligonucleotide Patterns of Total RNAs. In order to obtain biochemical evidence that F/1 and F/2 viruses emerged by recombination, we examined the oligonucleotide patterns of the RNAs of both viruses (Fig. 2). Eighty-five large oligonucleotides of the RNA of F/1 are labeled in Fig. 2*B*; 60 of these are indistinguishable from spots identified in the pattern of F/2 (Fig. 2*D*) as demonstrated by coelectrophoresis of both viral RNAs (Fig. 2*F*). Indistinguishable spots in both patterns are labeled with the same number in the diagrams. In addition to the 60 "common" spots, 19 unique oligonucleotides lettered *a*–*s* were identified in the oligonucleotide pattern of F/2 (Fig. 2*D*). Although "common" spots are not necessarily spots of identical oligonucleotide composition, the result suggests that at least 29.4% (25/85 spots) of the large oligonucleotides in F/1 are different from those of F/2. It should be noted that the selection of 85 large oligonucleotides for comparative analysis is arbitrary and that oligonucleotide maps in the absence of RNA sequence data do not permit determination of the absolute degree of relatedness between different RNA species.

Oligonucleotide Patterns of Isolated RNA Segments. Oligonucleotide analysis of the isolated *HA* genes of F/1 and F/2 showed that 24 of the 25 large oligonucleotides (96%) were indistinguishable by two-dimensional gel electrophoresis (Fig. 3). As shown by analysis of the mixture of the F/1 and F/2 HA segments, spot 43 of F/1 RNA (Fig. 3 *A* and *B*) is replaced by spot *s* in the pattern of F/2 RNA (Fig. 3 *C* and *D*). Because spot 43 and spot *s* are comparable in size it is possible that oligonucleotide *s* differs from oligonucleotide 43 by only one (or a few)

nucleotides. Similar analysis of the *M* genes on two-dimensional gels demonstrated identity of both oligonucleotide patterns (13 of 13 large oligonucleotides) (Fig. 4). However, the *P* genes, the *NP/NA* genes, and the *NS* genes have only 22 of 49 (44.9%), 10 of 27 (37.0%), and 2 of 11 (18.2%) spots in "common" (Table 1) (Fig. 5). Some of the isolated RNA segments eluted from acrylamide gels were still contaminated with sequences from larger genes. Repeated analysis of individual segments and the difference in intensities of contaminating and "real" spots permitted us to identify the characteristic oligonucleotide pattern of each gene. Although more than 50% of the nucleotides of the combined *P* genes were different, we cannot exclude the possibility that, in addition to the *M* and *HA* genes, one or two of the *P* genes of F/1 and F/2 have identical nucleotide patterns. (It should be mentioned that some spots of medium-sized oligonucleotides were not unique for any particular segment and that three spots of F/1 and five spots of F/2 could not be assigned with certainty to a specific gene.)

Identification of a Viral Mixture in an Isolate from Nature. Analysis of the RNA pattern of virus isolate 3 showed several "double" bands (Fig. 1, lane 3). From this viral mixture we were able to plaque-isolate six viruses which possessed different RNA patterns (not shown), suggesting that they are recombinants. Three of them possessed the Hav6 hemagglutinin and three had an Hav1 hemagglutinin. This finding suggested that infection of a single animal with two different influenza viruses may occur in nature.

DISCUSSION

Oligonucleotide analysis of the RNAs of two influenza A viruses isolated in nature showed that they share identical or almost identical *HA* and *M* genes whereas the other genes—such as the *NS*, *NP*, and *NA* genes and most likely all three *P* genes—are different. From these results we conclude that F/1 and F/2 viruses are related to each other by a recombinational event which occurred in nature. (It should be noted that one of the two viruses theoretically could have been a parent of the other strain.)

Previous evidence obtained by peptide mapping of different hemagglutinins and analysis of RNA hybridization supports the idea that some strains such as the H3N2 subtype were derived in nature by recombination. However, the techniques used could not exclude the possibility that some genes are capable of extensive mutation whereas other genes might be conserved by structural constraints. Extensive silent mutations in the latter group would not be detectable by analysis of gene products and might not be discernible by hybridization.

In the present study the exquisitely sensitive technique of oligonucleotide analysis demonstrated virtually identical patterns for the *HA* and for the *M* genes of the two strains and extensive differences in the patterns of the other genes. If evolution of all genes had occurred independently, many more mutations, including silent ones in the *HA* and *M* genes of the two viruses, would be expected. In this context we have found that the oligonucleotide patterns of influenza viruses of the H1N1 period isolated between 1946 and 1956 change considerably as a result of mutations and in this instance we have obtained no evidence of invariability of particular genes (21). Although our results on the variability of influenza virus genes are limited at this time, we think that it is unlikely that two genes, the *HA* and the *M* genes, of the avian strains should have been conserved while other genes "freely" mutated. In the light of the isolation of many avian influenza viruses with serologically different hemagglutinins (11, 12), it appears that nature permits the expression of many different avian hemagglutinin molecules. The virtual identity of the nucleotide patterns of the *HA* genes and of the *M* genes of F/1 and F/2 viruses and the differences in the other genes, therefore, can best be explained by recombination in the sense of genetic reassortment. The fortuitous finding that a single isolate obtained from one wild duck contained a mixture of viruses also suggests that such recombinational events occur in nature. In this instance, however, it cannot be excluded that actual recombination between the viruses may have taken place subsequent to isolation of the sample. The isolation of two influenza virus strains from a single animal was also recently reported by Shortridge *et al.* (12).

Finally, recombination may not be the only mechanism by which new strains emerge and further studies are required to establish whether or not recombination is the universal principle for the emergence of new pandemic influenza virus strains.

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