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Selection and Identification of Influenza Virus Recombinants of Defined Genetic Composition

JEROME L. SCHULMAN* AND PETER PALESE

Department of Microbiology, Mount Sinai School of Medicine of the City University of New York, New York City, New York 10029

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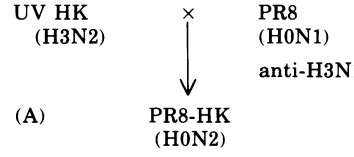
The RNAs of influenza virus recombinants were analyzed on polyacrylamide gels under conditions in which the derivation of specific RNA segments (including those coding for hemagglutinin and neuraminidase) could be determined. Analysis of the RNAs of recombinant viruses with identical hemagglutinin and neuraminidase revealed that the derivation of the remaining genes could be influenced by UV irradiation of one of the parent viruses. In five of seven such recombinants all of the remaining identifiable genes were derived from the nonirradiated parent, whereas in two others only the three largest RNA segments were derived from the nonirradiated parent. Analysis of the RNA pattern of a recombinant isolated after mixed infection in which neither parent was irradiated demonstrated a random mixture of RNA segments derived from the two parent viruses.

In previous communications we reported that RNAs extracted from different strains of influenza virus have characteristic and different migration patterns on polyacrylamide gels (5, 8). Subsequently, after analysis of RNAs from five different recombinant viruses derived from influenza A/PR/8/34 (H0N1) and A/Hong Kong/8/68 (H3N2) viruses, we were able to identify the fourth RNA segment of each (counting the slowest-moving band as no. 1) as the one coding for hemagglutinin and the fifth segment of A/Hong Kong virus and the sixth of A/PR/8 virus as the genes coding for the respective neuraminidases (6).

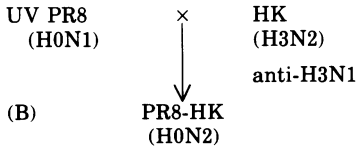
In the present report, we describe experiments in which these observations have been extended to analyze the RNA patterns of recombinant viruses obtained after the use of different selection procedures chosen to enhance the likelihood of isolating particular kinds of recombinants. As a result of these analyses, we have identified recombinant viruses with identical envelope proteins that differ from one another with respect to the derivation of genes coding for proteins other than the surface antigens.

MATERIALS AND METHODS

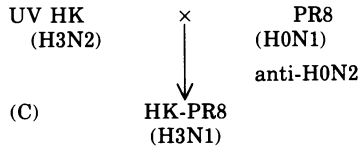
Viruses. The two parent viruses used in these experiments, influenza A/PR/8/34 (H0N1) and A/Hong Kong/8/68 (H3N2), are the MDCK cloned strains described in our previous report (6). From these two parent viruses, recombinant (H0N2 and H3N1) viruses were selected after mixed infection in embryonated eggs or after single-cycle mixed infection of MDCK (canine kidney) cells. Recombinant viruses were obtained from 10- to 11-day-old embryonated eggs as follows: allantoic fluid seed of one parent was diluted 10-fold and exposed to UV irradiation (GE G8T5 lamp, 8 W) for 30 s at a distance of 15 cm. Under these conditions of irradiation, the residual infectivity (50% egg-infective dose [EID_{50}]) of the irradiated virus was reduced by a factor of 10^5 to 10^6. Embryonated eggs were inoculated with equivalent concentrations (50 to 100 hemagglutinating units) of the UV-irradiated and nonirradiated parent viruses. After 24 h, dilutions of the mixed yield were incubated with an equal volume of a 1:10 dilution of high-titered specific rabbit antiserum and injected into eggs. To isolate one H0N2 recombinant virus, influenza A/PR/8/34 (H0)-A/Hong Kong/8/68 (N2) (PR8-HK), UV-irradiated A/Hong Kong/8/68 (H3N2), and nonirradiated A/PR/8/34 (H0N1) viruses were used, and the mixed yield was passaged in the presence of antiserum to H3N1 virus:



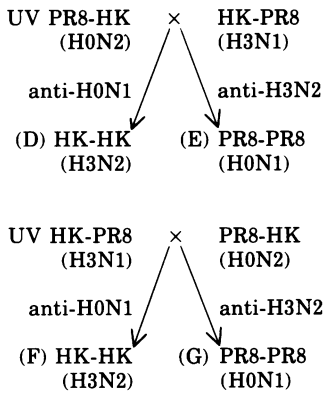
Another PR8-HK (H0N2) recombinant virus was isolated after mixed infection in which the other parent (A/PR/8/34 virus) was irradiated and after subsequent passage of the mixed yield in the presence of antiserum to H3N1 virus:



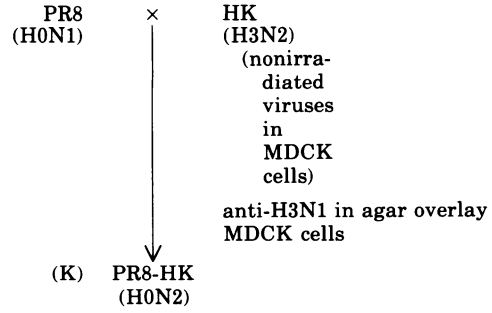
To select for a reciprocal (H3N1) recombinant virus, A/Hong Kong/8/68 (H3)-A/PR/8/34 (N1) virus (HK-PR8), the Hong Kong virus (H3N2) parent, was irradiated, and antiserum to H0N2 virus was used as the antibody screen:



Back-recombinants antigenically identical to the original H0N1 and H3N2 virus parents were isolated from two different kinds of mixed infection with PR8-HK (recombinant B) and HK-PR8 (recombinant C) viruses, as illustrated schematically below:



An eighth recombinant used in these experiments was isolated from MDCK cells after mixed infection, first with $10^{7.7}$ EID_{50} of Hong Kong virus and 1 h later with $10^{6.7}$ EID_{50} of PR8 virus. After another 9 h of incubation under liquid overlay, the mixed yield was diluted and used to inoculate MDCK cells, which then were incubated under an agar overlay containing antibody to H3N1 virus in a final concentration of 1:800. After 4 days, plaques were picked, one of which was used in the present experiments:



All viruses used were cloned by plaque-to-plaque passage in MDCK cells and then were inoculated into embryonated eggs to prepare allantoic fluid seed. The antigenic identity of each virus was confirmed by methods described previously (1, 4).

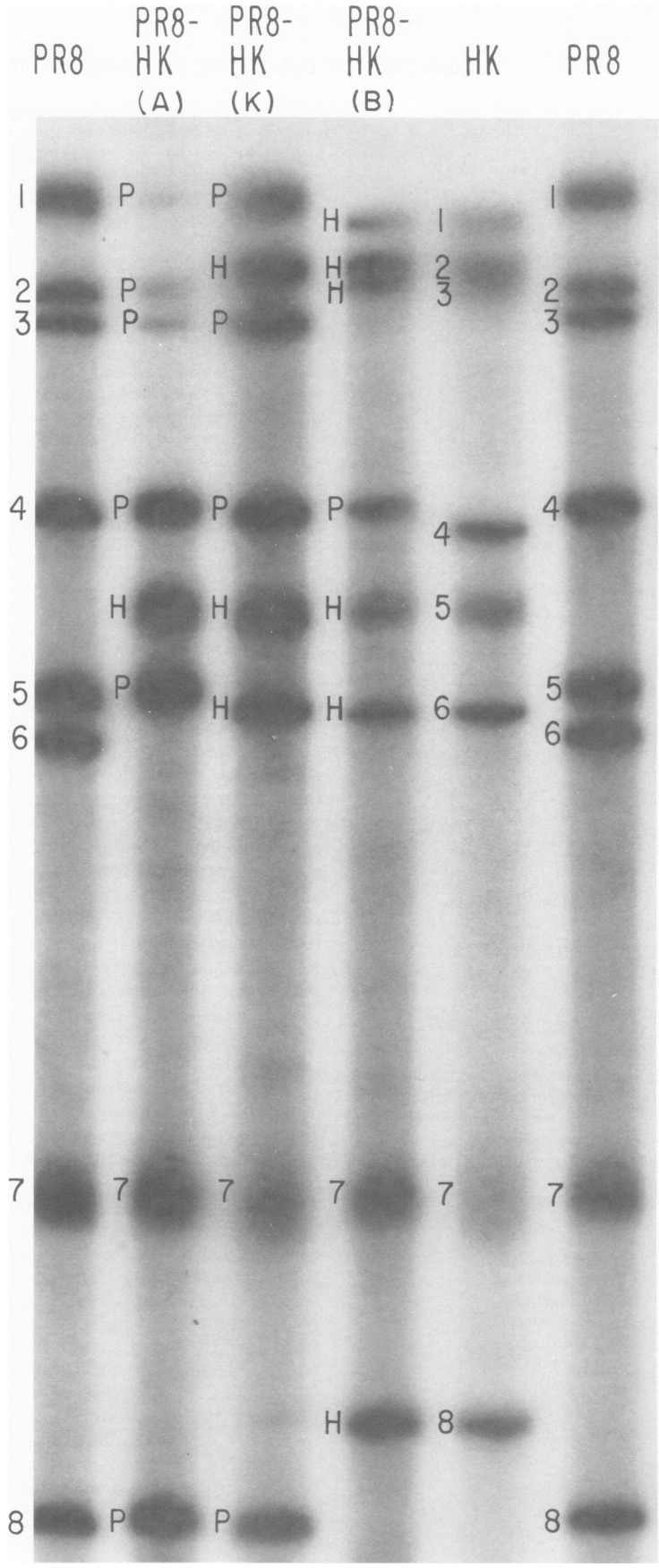
RNA. Confluent monolayers (4×10^6 cells) of MDCK cells (3) on 60-mm plastic dishes were infected at a multiplicity of 1 to 10 PFU/cell (three dishes for each virus). Two to 3 h later, approximately 3 mCi of ^{32}P (as orthophosphate, sterile in water; New England Nuclear Corp.) was added to each dish. Fluids were harvested after 12 to 18 h, and virus was purified and RNA extracted in accordance with methods described previously (5, 6). Using these viruses and these methods, 50,000 to 500,000 cpm of ^{32}P-labeled RNA was obtained from each virus pool. The extracted RNAs then were examined on polyacrylamide-urea slab gels as described originally by Floyd et al. (2) and subsequently modified in this laboratory as previously described (5). In the present experiments, all gels were run for at least 16 h at 120 V on 23-cm-long gels.

RESULTS

The RNA patterns of the two parent viruses, A/PR/8/34 (H0N1) (lanes 1 and 6) and A/Hong Kong/8/68 (H3N2) (lane 5), are shown in Fig. 1. The RNA patterns of three PR8-HK (H0N2) viruses are shown in the center (lanes 2 through 4). Lane 2 demonstrates the RNA pattern of an H0N2 recombinant virus obtained after mixed infection of eggs with UV-irradiated HK virus and nonirradiated PR8 virus and after passage of the mixed yield in the presence of antibody to H3N1 virus (recombinant A, Materials and Methods). The RNA pattern of this recombinant virus is identical to that of its PR8 virus parent except for its fifth segment, which was derived from Hong Kong virus, the parent with which it shares a common neuraminidase. An RNA band migrating in the same position as the sixth band of PR8 virus is not present in this recombinant or in any of the other H0N2 recombinants demonstrated in Fig. 1, which confirms our earlier evidence that it is the sixth and not the fifth segment of PR8 virus that is the gene for neuraminidase (6).

Lane 4 of Fig. 1 demonstrates the RNA pattern of an H0N2 recombinant obtained after mixed infection in eggs in which the PR8 virus parent and not Hong Kong virus was irradiated (recombinant B, Materials and Methods). This recombinant derived all its RNA segments from its HK virus parent except for band 4, which was derived from PR8 virus. (The origin of segment 7 in all of these recombinants is unknown because band 7 migrates identically in all five viruses under these conditions.)

The RNA in lane 3 was extracted from the recombinant isolated after single-cycle mixed


PR8
PR8-HK (A)
PR8-HK (K)
PR8-HK (B)
HK
PR8
1
2
3
4
5
6
7
8
P
H

infection of MDCK cells in which neither parent was irradiated (recombinant K). Like the other two H0N2 recombinants, it derived its fourth segment (hemagglutinin gene) from PR8 virus and its fifth (neuraminidase gene) from HK virus. However, the remaining RNA segments are of mixed derivation. Segments 2 and 6 were derived from HK virus, whereas segments 1, 3, and 8 are of PR8 virus origin. (Again, the origin of band 7 cannot be distinguished.) Thus, of the seven RNA segments that could be distinguished, the recombinant in lane 2 derived six from PR8 and one from HK, the recombinant in lane 3 derived three from HK virus and four from PR8, and the recombinant in lane 4 derived six from HK virus and only one from PR8 virus.

Figure 2 compares the RNA patterns of two HK-HK (H3N2) back-recombinants (center lanes) with those of the two recombinants from which they were derived, recombinant B (PR8-HK) in lane 1 and recombinant C (HK-PR8) in lane 4. Recombinant B is the same PR8-HK virus that was shown in Fig. 1 (lane 4) to derive only its fourth RNA segment from PR8 virus. Recombinant C was shown previously (6) to derive all of its RNA segments from PR8 virus with the exception of RNA 4 (and possibly band 7). The two HK-HK (H3N2) back-recombinants in lanes 2 and 3 of Fig. 2 were obtained after passage in eggs of two different recombinational mixtures of recombinants B and C in the presence of antibody to PR8 (H0N1) virus. Recombinant D (lane 2) was obtained after mixed infection in eggs with recombinant C (HK-PR8) and UV-irradiated recombinant B (PR8-HK), and recombinant F (lane 3) was obtained after mixed infection in which the other parent, recombinant C (HK-PR8), was exposed to UV irradiation. Once again, the fourth and fifth RNA segments of the two back-recombinants have similar migration rates, reflecting their possession of antigenically identical hemagglutinins and neuraminidases. The fourth band is identical to that of HK-PR8 virus (lane 4), with which both back-recombinants share a common hemagglutinin, and the fifth segment of each is identical to that of PR8-HK virus (lane 1), with which both back-recombinants share a common neuraminidase. Except for the fifth segment, all RNA segments of back-recombinant D in lane 2 coincide with those of HK-PR8 virus (lane 4). Therefore this virus derives both its surface antigens from HK virus but derives all of its other genes (except possibly segment 7) from PR8 virus. In contrast, the three largest RNAs of HK-HK back-recombinant F in lane 3 are identical to those of PR8-HK virus (lane 1, Fig. 2), which as shown in Fig. 1 were derived from Hong Kong virus. Thus, of the seven RNA segments whose origin could be determined in the two back-recombinants, four are identical, but in each case at least the three largest RNA segments were derived from the nonirradiated parent.

Figure 3 depicts the RNA patterns of two PR8-PR8 (H0N1) back-recombinants, comparing them this time with the RNA patterns of the 2 "grandparents," PR8 virus in lane 1 and HK virus in lane 4. These two back-recombinants were obtained after mixed infection of eggs with one UV-irradiated parent and one nonirradiated parent and subsequent passage of the mixed yield with antibody to H3N2 virus. The RNA pattern in lane 2 is that of a PR8-PR8 back-recombinant (E) obtained after mixed infection with UV-irradiated recombinant B (PR8-HK) and nonirradiated recombinant C (HK-PR8), whereas the RNA in lane 4 was obtained from a back-recombinant (G) isolated from mixed infection in which the HK-PR8 parent was irradiated. The RNA pattern of the PR8-PR8 back-recombinant E in lane 2 is identical to that of PR8 virus (lane 1), whereas back-recombinant G in lane 3 derived its three largest RNA segments from Hong Kong virus (lane 4). Thus, as was the case with the HK-HK back-recombinants in Fig. 2, each PR8-PR8 back-recombinant derived its three largest RNA segments from the nonirradiated virus parent.

DISCUSSION

Three factors have made it possible to obtain much more precise definition of the genetic composition of influenza virus recombinants than heretofore had been possible. The first is the development of the polyacrylamide-urea slab gel system (2), which produces a much clearer separation of the eight RNA segments

FIG. 1. *Analysis of ^{32}P-labeled RNAs of influenza A/PR/8/34 (HON1) (lanes 1 and 6) and influenza A/Hong Kong/8/68 (H3N2) (lane 5). The RNAs of three PR8-HK (HON2) recombinants (A, K, and B) are shown in lanes 2, 3, and 4, respectively. (See Materials and Methods for a description of the derivation of each of these recombinants.) The numbers 1 through 8 adjacent to the RNA patterns of the parent viruses indicate the RNA segments, starting with the largest segment as 1. The P or H designation adjacent to the RNA patterns of the recombinant viruses indicates whether the segment was derived from PR8 virus (P) or HK virus (H).*

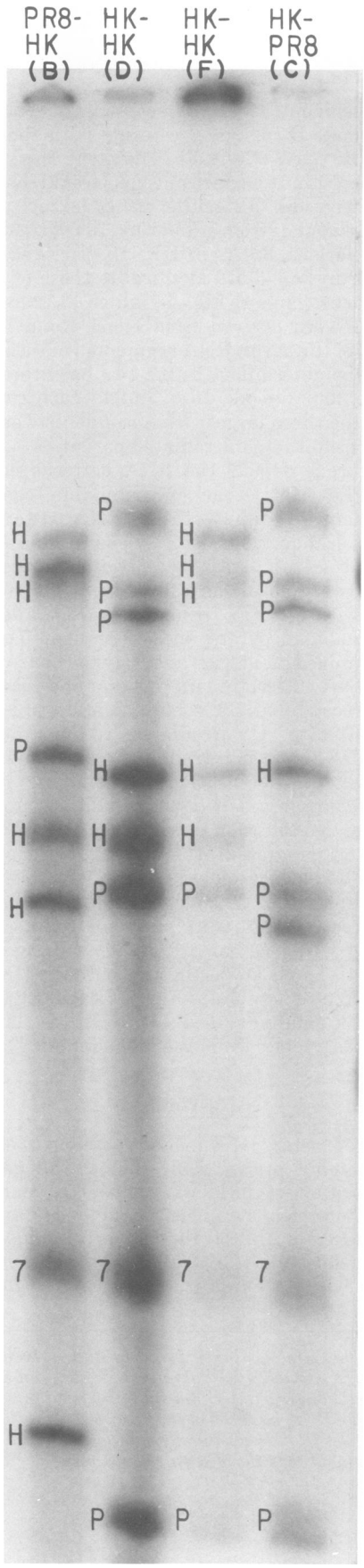


FIG. 2. *Polyacrylamide gel analysis of ^{32}P-labeled RNA of recombinant viruses. (Lane 1) Recombinant B, PR8-HK; (lane 2) recombinant D, HK-HK; (lane 3) recombinant F, HK-HK; (lane 4) recombinant C, HK-PR8. The conditions under which these recombinants were obtained are described in Materials and Methods. The P or H designation adjacent to the RNA patterns indicates whether the segment was derived from PR8 virus (P) or Hong Kong virus (H).*

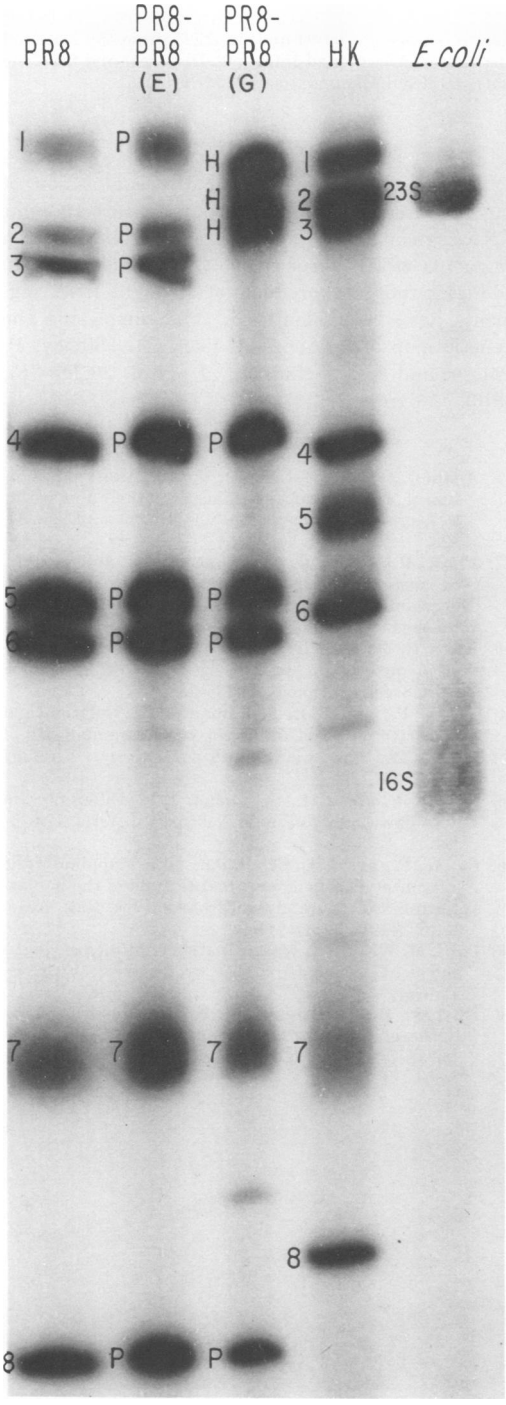


FIG. 3. *Polyacrylamide gel analysis of RNAs from PR8 (H0N1) virus (lane 1) and HK (H3N2) virus (lane 4). Lanes 2 and 3 contain the RNAs of PR8-PR8 (H0N1) back-recombinants (E and G). The numbering and lettering system of the RNA segments is the same as used in Fig. 1. Lane 5 shows 23S (molecular weight, 1.07 × 10⁶) and 16S (molecular weight, 0.55 × 10⁶) Escherichia coli rRNA. The derivation of these two recombinant viruses from recombinants B (PR8-HK) and C (HK-PR8) is described in Materials and Methods.*

that comprise the influenza A virus genome (5–8). The second is the fortuitous circumstance that RNAs extracted from different strains of virus produce different migration patterns in this gel system (5). At present it is not possible to differentiate the extent to which small differences in migration rates of comparable RNA segments reflect differences in molecular weights of the RNAs from effects produced by differences in secondary structure. Nevertheless, because these migration patterns are reproducible, they are useful in defining the derivation of specific genes in influenza virus recombinants. The third factor is the capacity of a wide variety of influenza viruses to replicate in a one-step cycle in MDCK cells to titers that permit analysis of radioactively labeled RNA.

In the present experiments as well as in our previous report (6), we used this system to identify the derivation of individual RNA segments in influenza virus recombinants. In our initial experiments we used recombinational mixtures in which one of the parent viruses was exposed to UV irradiation, subsequently selecting antigenic hybrids by passage through appropriate antiserum screens. Analysis of these recombinants facilitated the identification of the genes coding for hemagglutinin and neuraminidase (6). In the present experiments we have demonstrated that by selecting recombinants after mixed infection in which neither parent virus was irradiated or after mixed infection in which one or the other parent virus was irradiated, we could isolate recombinants with identical glycoprotein antigens with selected differences in the derivation of genes coding for the other viral proteins. Thus all of the three PR8-HK (H0N2) recombinants in Fig. 1 derive their fourth RNA segments from PR8 virus and their fifth RNA bands from HK virus. Only recombinant K in the center (lane 3) of Fig. 1, obtained after mixed infection without irradiation, contains a random mixture of its other genes, deriving its second and sixth RNA segments from Hong Kong virus and its first, third, and eighth RNA segments from PR8 virus. Each of the other two recombinants isolated from mixed yields obtained after UV irradiation of one or the other parent virus derives all of its other RNA segments (except possibly band 7) from its nonirradiated virus parent. As a result, each of the latter two recombinants may be isogenic

with respect to one of the parent viruses. Recombinant A in lane 2 is different from PR8 virus only with respect to its fifth segment, whereas recombinant B in lane 5 appears to be identical to Hong Kong virus except for its fourth segment.

Because of the greater likelihood of UV irradiation damaging the longer RNA segments, irradiation proved to be especially effective as a selection mechanism for the three largest RNA segments, as illustrated by the two HK-HK (H3N2) back-recombinants in Fig. 2 and the two PR8-PR8 (H0N1) back-recombinants in Fig. 3. In each case at least the three largest RNA segments were derived from the nonirradiated virus parent.

Thus, by a combination of specific antibody and UV irradiation of one parent, it is possible to facilitate the isolation of recombinant viruses of selected genetic composition. Comparison of similar pairs of viruses may provide a unique opportunity to relate differences in specific genes or groups of genes to strain-related differences in host range and/or virulence.

To make meaningful comparisons of recombinants requires that we isolate a wider range of recombinant viruses and identify their genetic composition. Such recombinants are currently being selected without UV irradiation, under conditions that provide a greater opportunity for random reassortment of genes and as far as possible exclude the introduction of random mutations. Experiments designed for this purpose have already permitted the identification of a more diverse assortment of recombinants, which will be described in forthcoming reports.

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ADDENDUM

Techniques developed after the submission of this manuscript now permit us to separate all eight RNA segments of PR8 virus from equivalent RNA segments of HK virus on polyacrylamide gels. In addition, the gene products of all eight RNA segments have been identified, thus completing the genetic map of influenza A virus (M. Ritchey, P. Palese, and J. L. Schulman, J. Virol. 20:307-313, 1976).

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