Influenza B Virus Genome: Assignment of Viral Polypeptides to RNA Segments

VINCENT R. RACANIELLO AND PETER PALESE*

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

It was shown that all eight RNA segments of influenza B viruses are most likely monocistronic and code for eight virus-specific polypeptides. A genetic map of the influenza B virus genome was established, and six polypeptides (P1 protein, nucleoprotein, hemagglutinin, neuraminidase, M protein, and nonstructural protein) were unambiguously assigned to specific RNA segments. Molecular weight estimates of the eight individual genes were obtained by using the glyoxal method. These results suggest that each influenza B virus RNA segment has a greater molecular weight than the influenza A virus RNA segment which codes for the analogous gene product.

Influenza B viruses were first isolated in 1940 from patients afflicted with respiratory illness (10, 22). Since then influenza B viruses have been recognized as important human pathogens. Although influenza due to the type A virus is more frequent than influenza B, the clinical attack rate of influenza B can be very high (24), and influenza B virus infections occur in every month of the year (16). Furthermore, influenza B viruses have been associated with Reye's syndrome, an acute encephalopathy in children (9).

Extensive serological studies on different influenza B virus isolates have demonstrated a lesser degree of antigenic variation than among the influenza A viruses (5, 6, 14, 34). Other biochemical studies on influenza B viruses have been scarce. Four to six influenza B virus polypeptides have been identified previously (4, 12, 13, 15, 20, 26, 37), and very little is known about the genome of influenza B viruses (31).

As part of a study to characterize the genome of influenza B viruses and to compare it with that of influenza A viruses, we recently began work on the establishment of a genetic map for influenza B viruses. Our earlier observation (31) that the RNA of influenza B virus consists of eight segments was confirmed (30), and in a preliminary report we showed that RNA 5 of influenza B viruses codes for the hemagglutinin (HA) polypeptide (30). Our finding that RNA 5 of influenza B viruses codes for HA was confirmed by Ueda et al. (38). Furthermore, these authors succeeded in identifying RNA 6 of influenza B viruses as the gene coding for the neuraminidase (NA) polypeptide. We also presented evidence for seven virus-coded polypeptides, in-

cluding a previously unrecognized viral nonstructural (NS) protein (30).

We now report our complete results on the establishment of a genetic map of the influenza B viruses. Our analysis shows that there are most likely eight virus-specific polypeptides coded for by the eight influenza B virus RNA segments. In addition to the surface proteins HA and NA, three P polypeptides, a nucleoprotein (NP), the M protein, and one NS protein can be distinguished. (The nomenclature for these proteins is the same as that for the influenza A viruses.) The genes coding for P1 protein, NP, NA, M protein, and NS protein are assigned to specific RNA segments. Finally, molecular weight estimates of influenza B virus RNA segments and influenza B virus-specific polypeptides are presented, and the gene structure of influenza B viruses is compared with that of influenza A viruses.

(This work was carried out by V.R.R. in partial fulfillment of the requirements for a Ph.D. degree in biomedical sciences at the Mount Sinai School of Medicine of the City University of New York.)

MATERIALS AND METHODS

Cells and medium. Madin-Darby canine kidney cells (MDCK; 17) were propagated in Earle-based minimal essential medium (Grand Island Biological Co.) supplemented with 10% fetal bovine serum.

Viruses. Seed stocks of influenza B/Lee/40 (B/Lee) and B/Maryland/59 (B/Md) viruses were prepared by inoculation of the allantoic cavity of 11-day-old embryonated hen eggs as described previously (31).

Isolation of recombinant viruses. Recombinants

between B/Lee and B/Md viruses were isolated by using techniques employed for the isolation of influenza A virus recombinants (27). Briefly, MDCK monolayers on 25-cm^2 plastic dishes were mixedly infected with B/Lee and B/Md viruses. It has been noted (35) that, in such a mixed infection with influenza A viruses, the multiplicity of infection (MOI) can influence the gene composition of the resulting recombinants. Therefore, depending on which parental RNAs we wished to predominate in the recombinant, one parent was present at a MOI of 10 PFU/cell and the other parent was present at 1 PFU/cell. After a 45-min adsorption at 37°C, monolayers was washed, and maintenance medium was added. Cells were washed again after 3 h and incubated at 37°C for an additional 14 h until most cells were detached. Culture fluids were centrifuged briefly to remove cell debris and then plaqued in MDCK cells in the presence of hyperimmune rabbit antisera directed against the surface proteins of B/Lee or B/Md virus. The resulting plaques were picked and grown up in MDCK cells, and seeds were made in 11-day-old embryonated hen eggs.

Serological analysis. The derivation of the surface proteins of recombinant viruses was identified by HA inhibition and NA inhibition assays (2, 40). To avoid steric interference of the viral HA in NA inhibition assays, purified virus was used which was treated with Pronase to destroy the viral HA (6).

Labeling and extraction of viral RNA. The RNA of recombinant viruses and their parents was labeled with ^{32}P in MDCK cells and extracted from purified virus as described previously for influenza A viruses (28, 29).

Polyacrylamide gel electrophoresis of viral RNA. ^{32}P-labeled RNAs of parent and recombinant viruses were separated on 2.6% polyacrylamide gels containing 6 M urea under established conditions (32).

Labeling of viral polypeptides. Pulse-labeling of influenza B virus-infected cell polypeptides with L-[^{35}S]methionine was performed as described previously (33). Unless indicated otherwise, at 6 h postinfection cells were first incubated for 15 min in Earle salt solution containing an additional 150 mM NaCl. The use of hypertonic medium to selectively suppress host protein synthesis in RNA virus-infected cells has been reported previously (25). For pulse-labeling experiments L-[^{35}S]methionine was added directly to the medium after a 15-min preincubation in hypertonic medium. Virus continuously labeled with L-[^{35}S]methionine was purified as described previously for influenza A viruses (33).

Polyacrylamide gel electrophoresis of viral polypeptides. L-[^{35}S]methionine-labeled viral polypeptides were routinely subjected to electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels. The discontinuous gel system of Maizel (23), containing linear gradients of polyacrylamide (5 to 13%) and sucrose (0 to 20%), was employed. Running conditions and preparation of samples were as previously reported (33) except that gels were usually stained with Coomassie brilliant blue (23) before drying and autoradiography.

Electrophoresis of pulse-labeled viral polypeptides on polyacrylamide gels containing Triton X-100, urea, and acetic acid was performed by the modified method of Alfageme et al. (1). The gel dimensions were 12 cm

by 23 cm by 1.5 mm and consisted of a resolving gel with a 5 to 13% gradient of polyacrylamide and a 0 to 20% sucrose gradient. The spacer gel was 5% polyacrylamide. The gels also contained 6 M urea, 0.37% (vol/vol) Triton X-100, and 5% acetic acid. The running buffer was 5% acetic acid. Pulse-labeled infected cell pellets were dissolved in a mixture containing 6 M urea, 5% acetic acid, 4% β-mercaptoethanol, 0.02% pyronine Y, and 0.37% (vol/vol) Triton X-100. Electrophoresis was performed at room temperature at 100 V until the dye front left the resolving gel (length, 20 cm). After electrophoresis, gels were dried and exposed to X-ray film.

One-dimensional peptide maps. Individual viral polypeptides from pulse-labeled infected cells or from continuously labeled purified virus preparations were subjected to one-dimensional peptide mapping by the procedure of Cleveland et al. (7) with minor modifications. After labeled viral polypeptides were subjected to electrophoresis on a polyacrylamide gel as described above, the gel was stained, and two lanes which contained samples of infected and uninfected cells were cut out, dried, and autoradiographed. The labeled viral polypeptides were located on the film, which was used to identify viral polypeptides in the remaining lanes of the wet, stained gel. (The HA, NP, and NS and M proteins could often be identified by staining alone.) Bands were then cut out from the wet gel and subjected to partial digestion in the stack of a second polyacrylamide gel (the resolving gel contained a 10 to 15% gradient of acrylamide). The enzymes used were *Staphylococcus aureus* V8 protease (0.5 µg/slot) and chymotrypsin (20 µg/slot). All enzyme solutions used to overlay the gel slices contained 2% β-mercaptoethanol (19). Gels were subjected to electrophoresis at 80 V until the dye front reached the resolving gel and then run at 100 V until the dye marker ran off the resolving gel (length, 15 cm). Gels were then fluorographed (3), dried onto Whatman 3MM filter paper, and exposed at −70°C to Kodak X-Omat RC film.

Estimation of molecular weights of viral RNA segments and viral polypeptides. The method of Weber and Osborn (39) was used to determine molecular weights of viral polypeptides. Pulse-labeled polypeptides of B/Lee virus-infected cells were analyzed on a SDS-polyacrylamide gel with a 3% stacking gel and a gradient-resolving gel of 5 to 13% polyacrylamide. The discontinuous buffer system used was that of Laemmli (18). Marker proteins used and their molecular weights were: phosphorylase a, 94,000; bovine serum albumin, 67,000; ovalbumin, 45,000; lactic dehydrogenase, 36,000; chymotrypsinogen, 25,000; and cytochrome c, 12,400. For unknown reasons viral proteins often split into multiple bands on polyacrylamide gels. In these cases, the slowest-migrating band was used when calculating molecular weights.

Molecular weight estimates of glyoxal-treated RNA segments of influenza B viruses were performed as described previously (8, 21). ^{32}P-labeled RNA of B/Lee virus was denatured at 56°C for 1 h in 1 M glyoxal–10 mM phosphate buffer, pH 7.0. RNA treated in this manner was subjected to electrophoresis on 2.6% polyacrylamide gels (as described above) with glyoxal-treated marker *Escherichia coli* 23S, 16S, and 4S RNAs.

Materials. ^{32}P as phosphoric acid in water was

obtained from ICN Pharmaceuticals, Irvine, Calif. L-[³⁵S]methionine was from Amersham/Searle, Arlington Heights, Ill. *S. aureus* V8 protease was from Miles Laboratories, Inc., Elkhart, Ind., and chymotrypsin was obtained from Worthington Biochemicals Corp., Freehold, N.J.

RESULTS

Analysis of recombinant viruses. The strategy for mapping the influenza B virus genome by using recombinant viruses was first developed with the influenza A viruses (28) and also proved helpful for mapping the genome of other segmented RNA viruses, such as reoviruses and bunyaviruses (11, 36). Briefly, two parental influenza B viruses are chosen whose RNA patterns on polyacrylamide gels are markedly different. Recombinants between the two viruses are then isolated which inherit one or two RNA segments from one parent virus and all other RNAs from the other parent. The parental derivation of polypeptides in the recombinant viruses is then examined by serological or electrophoretic techniques.

Recombinants between B/Md and B/Lee viruses, the parental viruses chosen for this mapping study, were isolated, and their RNAs were examined by polyacrylamide gel electrophoresis as described above. Figure 1 shows the RNAs of six different recombinants used in this mapping study. A summary of the genetic composition of these recombinants is shown in Table 1. All of the recombinants inherit most RNA segments from one parent and only one or two RNA segments from the other parent. A gene constellation of this type is useful for mapping studies because single polypeptides inherited from one parent can then be unambiguously assigned to one RNA segment. As was mentioned above, the MOI of each parent used in the recombinational mixture can influence the genotype of the recombinant. For example, recombinant R2 (Fig. 1) was isolated from a mixture in which B/Lee virus was present at an MOI of 10 PFU/cell, and B/Md virus was present at an MOI of 1 PFU/cell. This recombinant inherits only RNAs 3 and 5 from B/Md virus and all other RNAs from B/Lee virus. The other recombinants (R3 through R7) contain predominantly B/Md virus genes; these recombinants were isolated from a recombinational mixture in which B/Md virus was present at a higher MOI than B/Lee virus.

Identification of the gene coding for NA. Recombinant R6 is an example of a virus which inherits one RNA (RNA 6) from B/Lee virus and all other RNAs from B/Md virus (Fig. 1). NA inhibition tests revealed that recombinant R6 possesses a B/Lee virus NA (Table 2). From this result we conclude that RNA 6 of influenza B viruses codes for the NA. While this work was

in progress, Ueda et al. (38) also found that the information for the NA of influenza B viruses is contained on RNA 6.

Identification of the gene coding for NS protein. Small migrational differences on SDS-polyacrylamide gels are observed between the pulse-labeled NS proteins of B/Lee and B/Md viruses. Figure 2 shows that the NS protein of B/Md virus migrates slightly slower than the NS protein of B/Lee virus (for undetermined reasons the NS proteins of both viruses occasionally split into a doublet). We used this migrational difference as a marker to identify the parental origin of the NS protein in two recombinants, R5 and R7. Recombinant R5 inherits only RNA 1 from B/Lee virus, and recombinant R7 inherits RNAs 1 and 8 from B/Lee virus. All other RNAs in both recombinants are derived from B/Md virus. A polyacrylamide gel of the polypeptides induced in cells infected with recombinant R7 is shown in Fig. 2. The NS protein of R7 clearly comigrates with the B/Lee NS protein. By using the same technique, R5, which derives only RNA 1 from B/Lee virus, was found to possess the NS protein of B/Md virus (data not shown). We conclude, therefore, that RNA 8 of influenza B viruses codes for the NS protein.
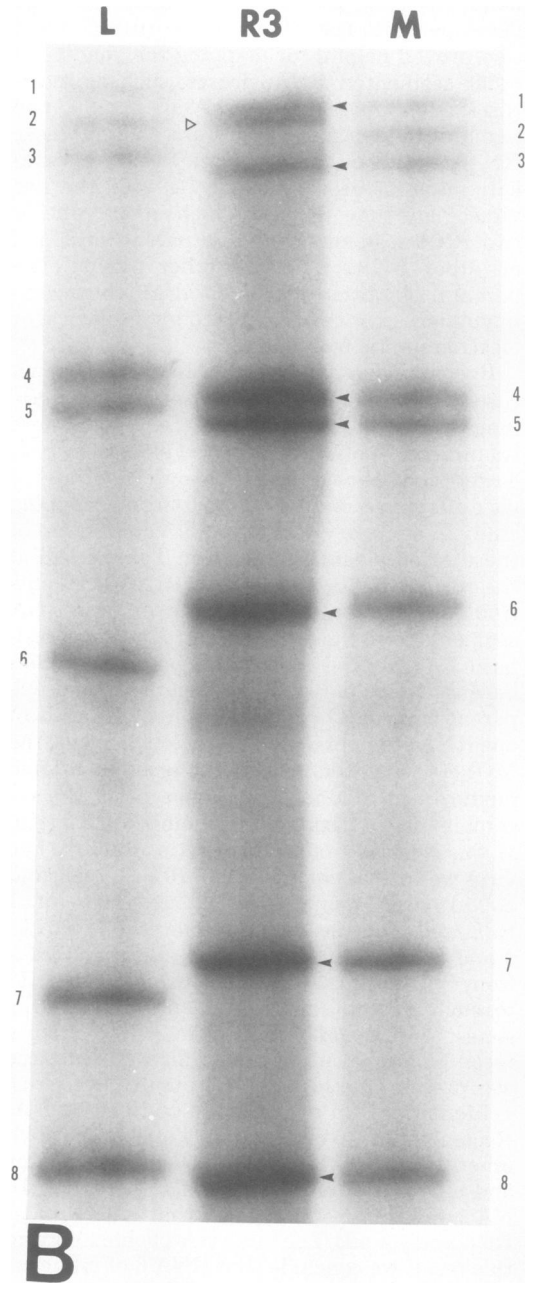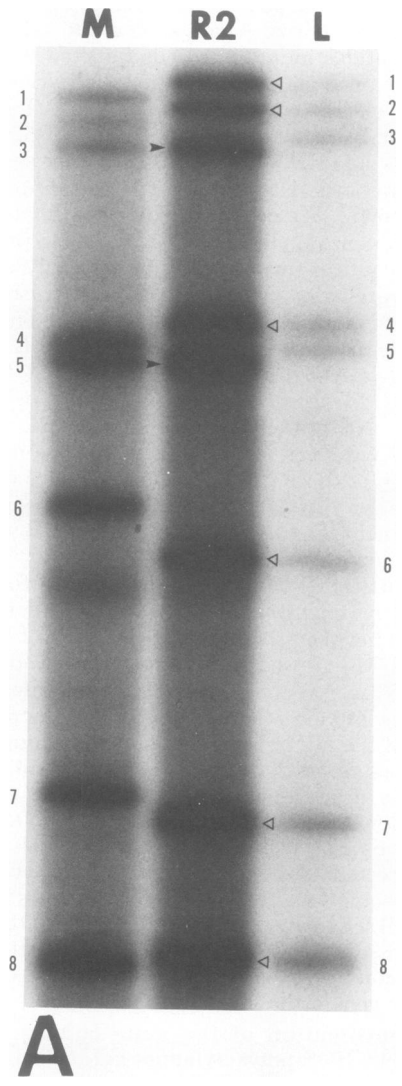
To confirm this assignment, we analyzed the NS proteins of B/Lee and B/Md viruses and recombinants R5 and R7 by one-dimensional peptide mapping in polyacrylamide gels (7). The peptide patterns of the NS proteins of B/Lee and B/Md viruses are different, as shown in Fig. 3. The NS proteins of recombinant R5 and of B/Md virus are identical, whereas the peptide pattern of the R7 NS protein resembles that of the B/Lee virus NS protein. This result confirms our conclusion that RNA 8 of influenza B viruses codes for NS protein.
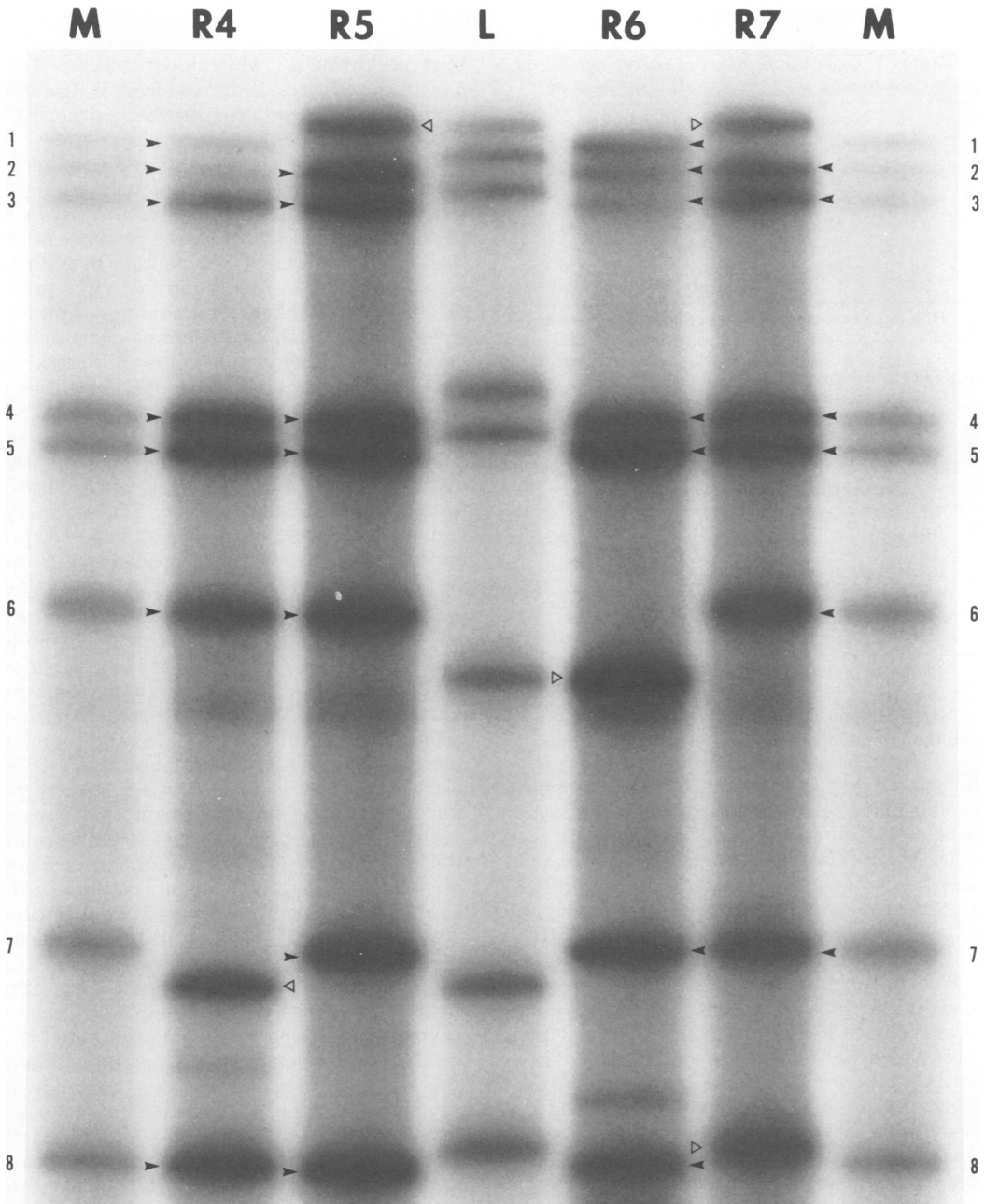
Identification of the gene coding for NP. No clear migrational differences could be detected between the pulse-labeled NPs of B/Lee and B/Md viruses on SDS-polyacrylamide gels. Therefore, the one-dimensional peptide mapping technique was used to identify the derivation of the NP in recombinants R2 through R7. Figure 4 shows that the NPs of B/Lee and B/Md viruses produce easily distinguishable one-dimensional peptide maps which differ in at least three bands. The peptide maps of the NP of each recombinant are shown in Fig. 4. According to this analysis, recombinant R2 derives its NP from B/Lee virus, and recombinants R3 through R7 possess the B/Md virus NP. This pattern is only consistent with the assignment of RNA 4 as the gene coding for NP (Table 1).

Identification of the gene coding for M protein. SDS-polyacrylamide gel analysis of the pulse-labeled polypeptides from B/Lee and

B/Md virus-infected cells failed to demonstrate mobility differences in the M protein. In addition, one-dimensional peptide maps using *S. aureus* V8 protease, chymotrypsin, tolylsulfonyl phenylalanyl chloromethyl ketone–trypsin, papain, and Pronase did not reveal differences between the M proteins of B/Lee and B/Md viruses. However, the Triton–urea polyacrylamide gel electrophoresis method of Alfageme et al. (1), as described above, demonstrated migrational differences in M proteins of B/Lee and B/Md viruses. This gel system separates proteins according to hydrophobic properties, molecular size, and charge. Figure 5 is an autoradiogram of pulse-labeled polypeptides of B/Lee and B/Md viruses and recombinant R4, sepa-

rated on a Triton–urea gel. Recombinant R4 derives all genes from B/Md virus except RNA 7, which is inherited from B/Lee virus. The influenza B virus polyptides on the Triton–urea gel were identified by cutting out the individual polypeptide bands and subjecting them to electrophoresis in a conventional SDS-polyacrylamide gradient gel along with marker influenza

FIG. 1. *Gene composition of influenza B virus recombinants.* ^{32}P-*labeled viral RNAs were run on a 2.6% polyacrylamide gel containing 6 M urea (for conditions, see text). Migration differences of the RNAs of parental viruses permit identification of gene derivation of recombinant viruses. Solid arrows next to the RNAs of recombinant viruses indicate RNA segments derived from B/Md virus (M), and open arrows indicate RNA segments derived from B/Lee virus (L). The RNAs of B/Md and B/Lee viruses are numbered in order of increasing mobility on the gel. (A) Lane 1, RNA of B/Md virus; lane 2, RNA of recombinant R2; lane 3, RNA of B/Lee virus. (B) Lane 1, RNA of B/Lee virus; lane 2, RNA of recombinant R3; lane 3, RNA of B/Md virus. (C) Lane 1, RNA of B/Md virus; lane 2, RNA of recombinant R4; lane 3, RNA of recombinant R5; lane 4, RNA of B/Lee virus; lane 5, RNA of recombinant R6; lane 6, RNA of recombinant R7; lane 7, RNA of B/Md virus. Additional faint RNA bands observed in some viral preparations are most likely due to the presence of defective particles.*

365

TABLE 1. *Gene composition of six recombinants used to map the influenza B virus genome*

Recombinant	Origin of RNA segment:							
	1[a]	2	3	4	5	6	7	8
R2[b]	L[c]	L	M[d]	L	M	L	L	L
R3	M	L	M	M	M	M	M	M
R4	M	M	M	M	M	M	L	M
R5	L	M	M	M	M	M	M	M
R6	M	M	M	M	M	L	M	M
R7	L	M	M	M	M	M	M	L

[a] RNA segments are numbered in order of increasing mobility on 2.6% polyacrylamide gels containing 6 M urea.

[b] An additional recombinant, R1, was used previously to map the HA polypeptide (30).

[c] L, RNA derived from B/Lee virus.

[d] M, RNA derived from B/Md virus.

TABLE 2. *NA inhibition titers of recombinant R6 derived from B/Lee and B/Md viruses*

Virus	Antiserum	
	B/Lee	B/Md
B/Lee	20[a]	<10
B/Md	<10	1,250
R6[b]	30	<10

[a] Dilution of antiserum causing 50% inhibition of NA activity.

[b] Recombinant R6 derives RNA 6 from B/Lee virus and the remaining seven genes from B/Md virus.

B virus polypeptides. There is a small but reproducible migration difference between the M proteins of B/Lee and B/Md viruses. The B/Lee virus M protein migrates slightly slower than does the B/Md virus M protein. Because the M protein of recombinant R4 comigrates with the B/Lee virus M protein, we conclude that RNA 7 of influenza B viruses codes for M protein.

P proteins of influenza B viruses. We previously identified, in purified virus and in infected cells, two high-molecular-weight polypeptides which were called P1 and P2 (30). These polypeptides are shown in Fig. 6. A one-dimensional peptide map of P1 protein excluded the possibility that this polypeptide is a precursor of HA (Fig. 7). The P1 protein was then assigned to a RNA segment by using the one-dimensional peptide mapping technique. In Fig. 8 it can be seen that the chymotrypsin digestion patterns for P1 protein of B/Lee and B/Md viruses are different. Recombinant R3 derives only RNA 2 from B/Lee virus, and this recombinant virus has the P1 protein of B/Lee virus. This result suggests that RNA 2 of influenza B/Lee virus codes for P1 protein. Analysis of two other recombinants is compatible with this finding. Recombinant R5 inherits only one RNA, RNA 1, from B/Lee virus and possesses a P1 protein derived by B/Md virus. Finally, the P1 protein

of recombinant R2, which inherits RNAs 3 and 5 from B/Md virus, is derived from B/Lee virus (Table 1). These data suggest that neither RNA 1 nor RNA 3 codes for the P1 polypeptide.

The polypeptide labeled P2 in Fig. 6 did not label well consistently in infected cells or in purified virus to permit extensive analysis by peptide mapping. However, the presence of this high-molecular-weight polypeptide in both infected cells and in highly purified virus preparations make it likely that this polypeptide is virus specific. Such a polypeptide has also been observed by others in influenza B viruses (15, 26). This polypeptide would map on either of the two unassigned RNA segments, RNA 1 or RNA 3.

In influenza B virus-infected cells, another distinct polypeptide band migrating slightly faster than HA can be seen in SDS-polyacrylamide gels (Fig. 6). We previously reported preliminary peptide mapping studies which revealed a similarity between this unidentified polypeptide and HA (30). However, those peptide maps were obtained by using polypeptides from infected cells, and, because the faster-moving band and HA migrate so closely, contamination with HA might have occurred. In purified virus, the HA is fully glycosylated and therefore migrates slower than pulse-labeled HA of infected cells. The distance between HA and the unidentified polypeptide is therefore larger and allows greater ease in cutting these polypeptides from a gel. Figure 7 shows one-dimensional peptide maps of the faster-moving polypeptide (labeled P3) and of HA obtained from purified virus preparations. It is clear that there are differences between these two polypeptides, so we conclude that the polypeptide migrating slightly faster than HA is a unique virus-coded polypeptide. However, it should be noted that this P3 preparation may still be contaminated with HA molecules. Because this faster-moving polypeptide is present in purified virus (30) and forms a sharp band on polyacrylamide gels, we think it is unlikely that this band represents an underglycosylated species of the HA. Therefore, we tentatively identify it as the P3 protein. However, because peptide maps of P3 protein from B/Lee and B/Md viruses failed to reveal extensive differences (data not shown), this polypeptide could not be specifically assigned to either RNA 1 or RNA 3.

The results of these mapping studies are presented in Fig. 9, which shows the genetic map for B/Lee and B/Md viruses.

Molecular weight estimates of influenza B virus RNA segments and polypeptides. Molecular weights of B/Lee virus polypeptides
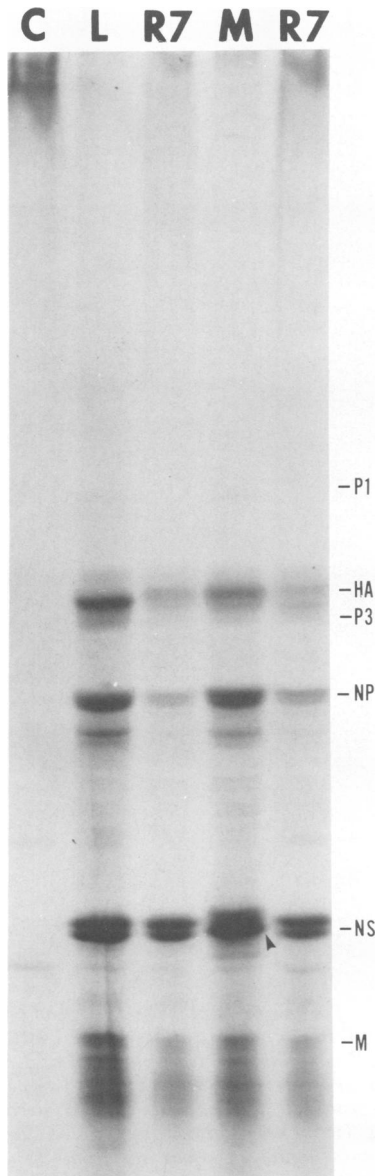
FIG. 2. *Derivation of NS protein of recombinant R7 using SDS-polyacrylamide gel electrophoresis. MDCK cells infected with parental and recombinant viruses were preincubated in hypertonic medium and pulse-labeled with L-[³⁵S]methionine as described in the text. Infected cell polypeptides were subjected to electrophoresis on a 5 to 13% gradient polyacrylamide-SDS gel (see text) and visualized by autoradiography. Lane 1, Uninfected cell polypeptides (C); lane 2, polypeptides of B/Lee virus (L)-infected cells; lane 3, polypeptides of recombinant R7-infected cells; lane 4, polypeptides of B/Md virus (M)-infected cells; lane 5, polypeptides of recombinant R7-infected cells. Viral proteins P1, HA, P3, NP, NS, and M are labeled to the right (30); the arrow indicates the slower-mov-*
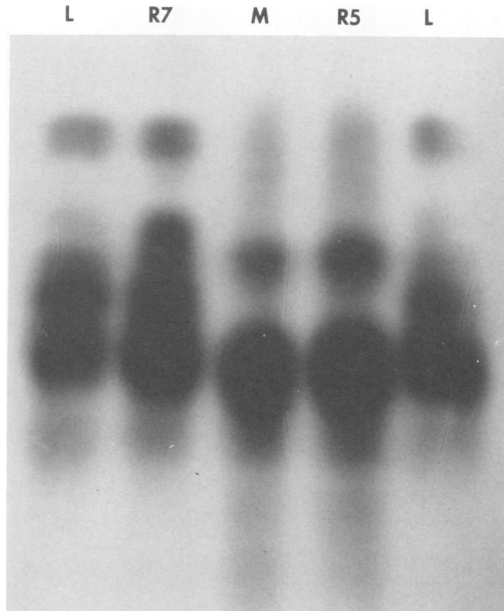

FIG. 3. *Identification of the NS protein in recombinants R5 and R7 by using one-dimensional peptide maps. After separation of parent and recombinant virus-infected cell polypeptides on 5 to 13% gradient polyacrylamide-SDS gels, the NS proteins were cut out from the gel and subjected to partial digestion in the stack of a second polyacrylamide gel in the presence of 20 μg of chymotrypsin per slot. Separation of the oligopeptides was performed in a 10 to 15% gradient polyacrylamide-SDS gel (see text). Lane 1, Digestion pattern of B/Lee virus (L) NS protein; lane 2, digestion pattern of recombinant R7 NS protein; lane 3, digestion pattern of B/Md virus (M) NS protein; lane 4, digestion pattern of recombinant R5 NS protein; lane 5, digestion pattern of B/Lee virus (L) NS protein. This analysis shows that recombinant R7 possesses B/Lee virus NS protein, whereas recombinant R5 derives its NS protein from B/Md virus.*

were determined as described above. The results are presented in Table 3. The calculated molecular weights are compared with molecular weight estimates of the individual influenza B virus RNA segments. The latter values were obtained by using the glyoxalation technique, which permits measurement of the absolute molecular weights of nucleic acids on polyacrylamide gels (21). The molecular weights for the individual influenza B virus genes were determined by eluting the RNA segments from a 2.6% polyacrylamide gel, denaturing them with glyoxal, and rerunning the segments on a second

ing NS protein of B/Md virus. (Note that the NS proteins of all viruses are split into doublets). This analysis indicates that recombinant R7 inherits the B/Lee virus NS protein.
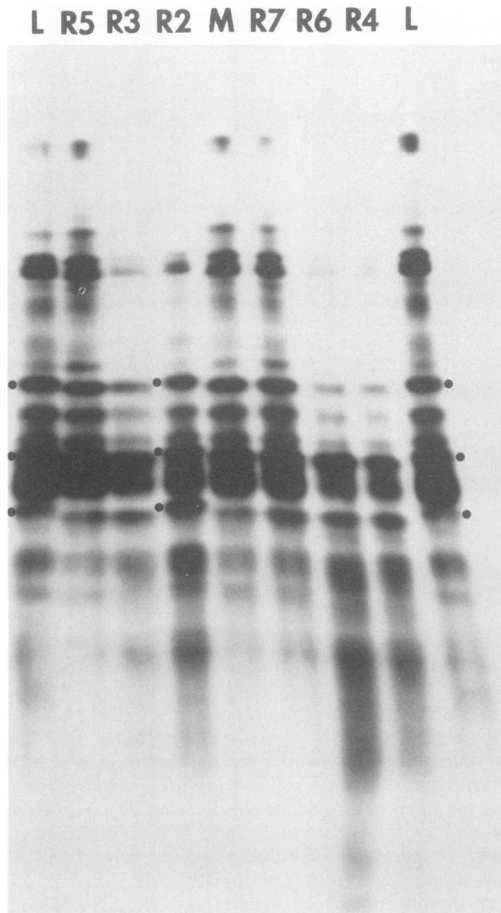
FIG. 4. *Identification of the NP in recombinants R2 through R7 by using one-dimensional peptide maps. Experimental conditions were as described in the legend to Fig. 3. The NPs of parent and recombinant viruses were partially digested with S. aureus V8 protease (0.5 μg/slot) in the stack of an SDS-polyacrylamide gel, and the digestion products were separated on a 10 to 15% gradient gel. Dots indicate B/Lee virus (L)-specific bands. Lane 1, Digestion pattern of B/Lee virus (L) NP; lane 2, digestion pattern of recombinant R5 NP; lane 3, digestion pattern of recombinant R3 NP; lane 4, digestion pattern of recombinant R2 NP; lane 5, digestion pattern of B/Md virus (M) NP; lane 6, digestion pattern of recombinant R7 NP; lane 7, digestion pattern of recombinant R6 NP; lane 8, digestion pattern of recombinant R4 NP; lane 9, digestion pattern of B/Lee virus (L) NP. This analysis shows that recombinant R2 possesses B/Lee virus NP and recombinants R3 through R7 possess B/Md virus NP.*

FIG. 5. *Derivation of M protein of recombinant R4 by using Triton–urea gels. Pulse-labeled polypeptides of MDCK cells infected with parental and recombinant viruses were separated on a 5 to 13% polyacrylamide gradient gel containing 6 M urea, 0.37% (vol/vol) Triton X-100, and 5% acetic acid. Individual polypeptide bands were cut out from the gel and identified by reelectrophoresis on a conventional SDS gel in the presence of viral marker proteins. Lane 1, Polypeptides of B/Lee virus (L)-infected cells; lane 2, polypeptides of recombinant R4-infected cells; lane 3, polypeptides of B/Md virus (M)-infected cells; lane 4, polypeptides of B/Lee virus-infected cells. In this gel system the P proteins are not resolved, and the HA splits into four bands. The M protein of B/Md virus migrates faster than the B/Lee virus M protein. By this analysis recombinant R4 is shown to possess a B/Lee virus M protein.*

gel in the presence of glyoxalated marker RNAs. This analysis showed that the order of the B/Lee and B/Md virus RNA segments in polyacrylamide gels changes after glyoxal treatment.
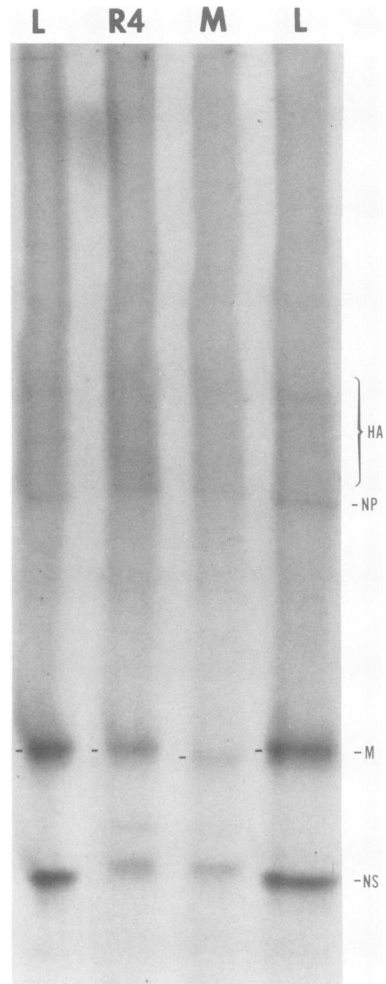
When untreated influenza B virus RNA is run on a 2.6% polyacrylamide gel containing 6 M urea, the NP gene migrates as the fourth-fastest segment, and the HA gene migrates as the fifth-
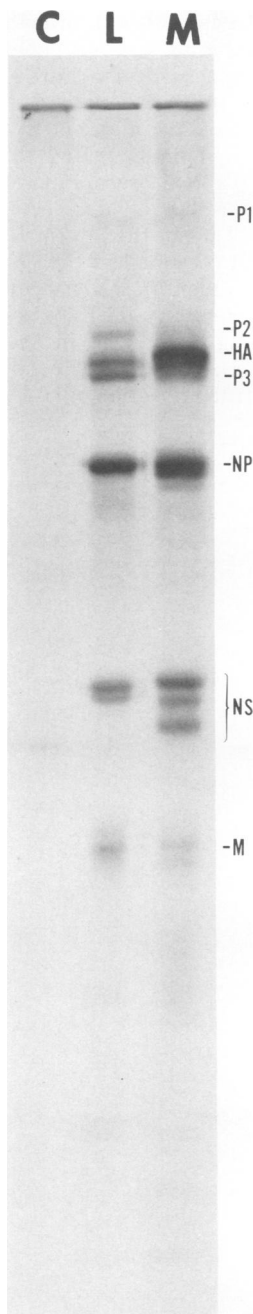
FIG. 6. Separation of P proteins of influenza B virus-infected MDCK cells by SDS-polyacrylamide gel electrophoresis. Pulse-labeled infected cell polypeptides were separated on a 10 to 15% gradient polyacrylamide-SDS gel. Lane 1, Uninfected cell polypeptides (C); lane 2, polypeptides of B/Lee virus (L)-infected cells; lane 3, polypeptides of B/Md virus (M)-infected cells.
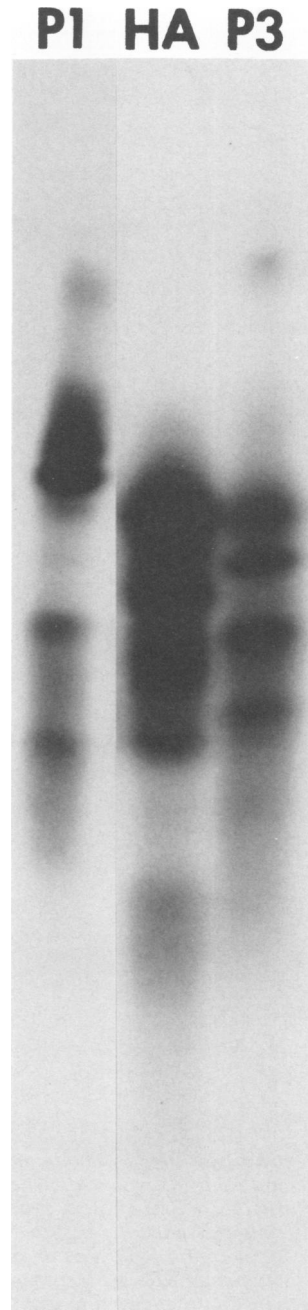
FIG. 7. One-dimensional peptide maps of HA and P1 and P3 proteins of B/Lee virus. Pulse-labeled polypeptides from virus-infected MDCK cells were partially digested with S. aureus V8 protease (0.5 µg/slot) and separated on a 10 to 15% gradient polyacrylamide-SDS gel. Lane 1, Digestion pattern of P1 protein; lane 2, digestion pattern of HA; lane 3, digestion pattern of P3 protein. This analysis shows that HA and P1 and P3 proteins have different one-dimensional peptide maps.
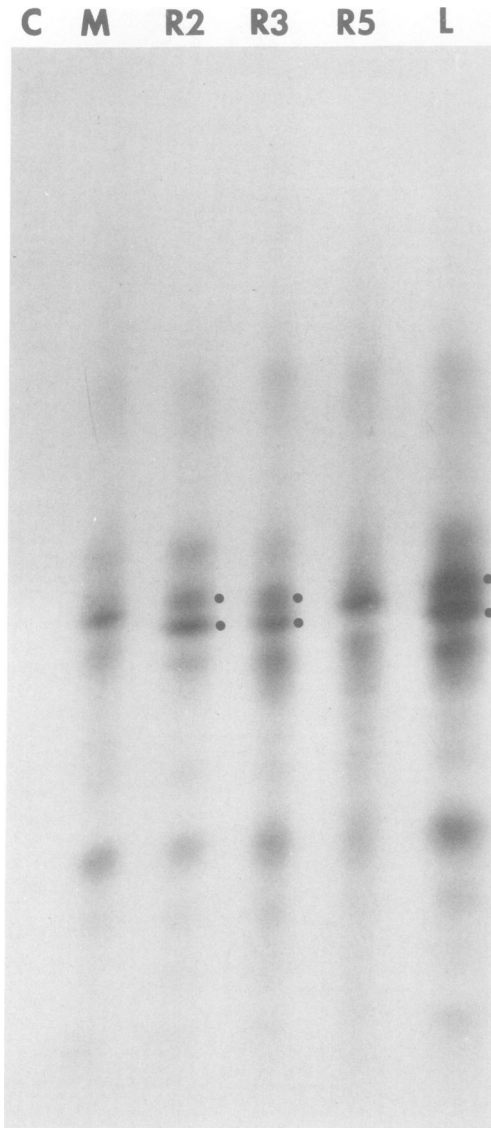
FIG. 8. *Identification of P1 protein in recombinants R2, R3, and R5 by using one-dimensional peptide maps. Pulse-labeled P1 protein from virus-infected MDCK cells was partially digested with chymotrypsin (20 µg/slot) and separated on a 10 to 15% gradient polyacrylamide-SDS gel (experimental conditions as described in the legend to Fig. 3). Lane 1, Digestion pattern of a mock P1 band obtained from uninfected cells; lane 2, digestion pattern of B/Md virus (M) P1 protein; lane 3, digestion pattern of recombinant R2 P1 protein; lane 4, digestion pattern of recombinant R3 P1 protein; lane 5, digestion pattern of recombinant R5 P1 protein; lane 6, digestion pattern of B/Lee (L) virus P1 protein. Dots indicate specific bands characteristic of B/Lee virus P1 protein. This analysis indicates that recombinants R2 and R3 possess B/Lee virus P1 protein and that recombinant R5 inherits a B/Md virus P1 protein.*

fastest segment (Fig. 9). When glyoxalated, however, the HA genes of both influenza B viruses migrate slower than the NP genes. Therefore, the HA gene is actually the fourth-largest RNA segment, and the NP gene is the fifth-largest RNA segment of the influenza B virus genome (Table 3). It should be noted that the RNA segments in Table 3 are numbered by Roman numerals in order of decreasing molecular weight, RNA I being the largest RNA segment and RNA VIII the smallest. The Arabic numerals are used to identify RNA segments in order of increasing mobility on 2.6% polyacrylamide gels containing 6 M urea in the absence of glyoxal (Fig. 1). Under these conditions migration is not a true indication of molecular weight.
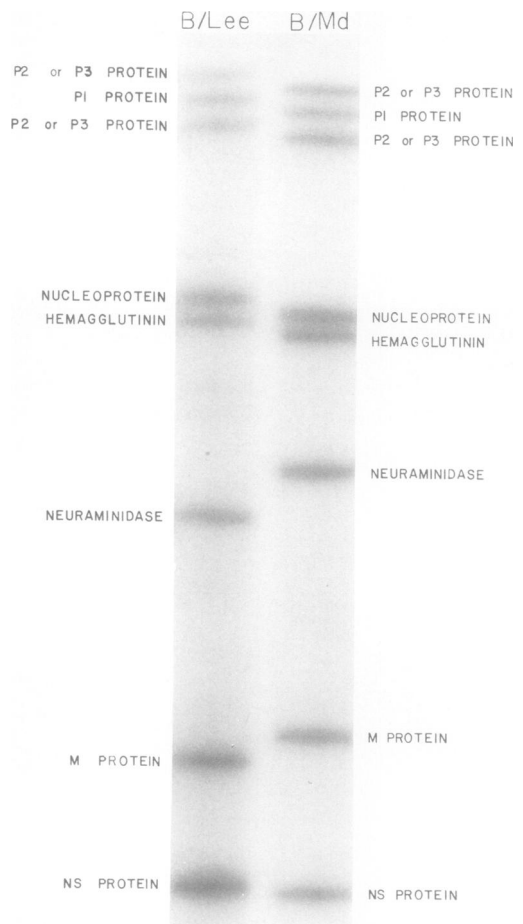

FIG. 9. *Influenza B virus genome. RNAs of B/Lee and B/Md viruses were labeled with ^{32}P, subjected to electrophoresis on 2.6% polyacrylamide gels, and detected by autoradiography. Each RNA segment contains information for the polypeptide indicated. It was not possible to specifically assign P2 or P3 proteins to RNA segments 1 or 3.*

TABLE 3. *Molecular weights of polypeptides and RNA segments of influenza A and B viruses*

Influenza B viruses				Influenza A viruses			
RNA	Mol wt[a] ($\times 10^6$)	Protein	Mol wt[b] ($\times 10^3$)	RNA	Mol wt[a] ($\times 10^6$)	Protein	Mol wt[b] ($\times 10^3$)
I	0.91	P2 or P3	93 or 80	I	0.89	P3	80
II	0.91	P1	102	II	0.89	P1	97
III	0.88	P2 or P3	93 or 80	III	0.86	P2	86
IV	0.70	HA	84	IV	0.66	HA	72
V	0.68	NP	66	V	0.56	NP	55
VI	0.55	NA	66	VI	0.48	NA	ND[c]
VII	0.38	M	25	VII	0.28	M	35
VIII	0.33	NS	40	VIII	0.21	NS	25

[a] Molecular weights of RNA segments were determined by using the glyoxalation technique (8, 21). Values for influenza A virus RNA segments are taken from reference 8.

[b] Molecular weights of polypeptides were determined as described in the text.

[c] ND, Not determined.

A careful comparison of the molecular weights of influenza B virus polypeptides and RNA segments indicates that RNA segments I, II, III, V, and VII appear to be the right size to encode the corresponding polypeptides. The apparent molecular weights of the HA and NA are greater than the values which are suggested by the coding capacity of RNAs IV and VI, respectively. This difference is probably due to the glycosylated nature of these two polypeptides. Because RNA VIII has been shown unambiguously to code for the NS protein, it is therefore likely that the apparent molecular weight of the NS protein, estimated in SDS-polyacrylamide gradient gels, is incorrect. A similar discrepancy in molecular weight estimation, when SDS-polyacrylamide gradient gels are used, is noted in the case of the M and NS proteins of influenza A viruses.

For comparison, the molecular weights of influenza A virus RNA segments and proteins are also presented in Table 3. Note that the molecular weight of each of the influenza B virus RNAs is greater than the molecular weight of the corresponding influenza A virus RNA. In accord with this finding, most of the influenza B virus proteins appear to be larger than the corresponding influenza A virus proteins. The exceptions are the M and P3 proteins. Choppin et al. (4) and Oxford (26) have obtained similar data for HA, NP, NA, and M proteins.

DISCUSSION

Based on epidemiological, biological, and biochemical similarities, the influenza A and B viruses are both placed in the orthomyxovirus group. This classification is in accord with our finding that, like the influenza A viruses, the influenza B viruses also contain eight monocistronic RNA segments which code for eight polypeptides. Similar to influenza A viruses, the influenza B viruses also code for two surface gly-coproteins, HA and NA, a NS protein whose function is not known, and two internal proteins, NP and M protein. The influenza B viruses also contain three P proteins which, like those of influenza A viruses, may be involved in the synthesis of virus-specific RNA.

Table 3 lists the genes of influenza A and B viruses according to their molecular weight. This comparison indicates that the gene order of both viruses is probably identical. The smallest RNAs of both influenza A and B viruses, RNAs VII and VIII, code for M and NS proteins, respectively. Among the medium-sized segments of both influenza A and B viruses, RNAs IV, V, and VI code for HA, NP, and NA, respectively. One of the largest RNA segments (RNA II) has been found to code for P1 protein in both influenza A and B viruses. Although we were not able to assign P2 and P3 proteins to specific influenza B virus RNA segments, we would expect that RNA I codes for P3 protein and RNA II codes for P2 protein. This suggestion is based on the identity of the gene order for the other six RNA segments of influenza A and B viruses. Because the genetic map of influenza B viruses was established by using two strains isolated 19 years apart, we feel that the gene order of other influenza B virus isolates may not differ from that of B/Lee and B/Md viruses.

Table 3 lists absolute molecular weight estimates of influenza A and B virus RNAs based on glyoxalation and analysis on polyacrylamide gels. The molecular weight of the total influenza B virus RNA is approximately 10% larger than that of the total influenza A virus RNA. It is clear that each influenza B virus RNA segment has a higher molecular weight than the corresponding influenza A virus RNA segment. Most likely, this molecular weight difference cannot be accounted for by the presence of spacer or untranslated regions in the influenza B virus RNA segments, because most of the influenza B

virus proteins appear to be larger than the corresponding influenza A virus proteins. The exception is the influenza B virus M protein which, at a molecular weight of 26,000, appears to be smaller than the influenza A virus M protein (molecular weight, 35,000). As noted previously, there may be uncertainties in the determination of molecular weights of proteins in the SDS-polyacrylamide gradient gel system. A careful examination of the molecular weights of RNAs presented in Table 3 also reveals that the molecular weight differences between the influenza B and A virus genes generally increase with decreasing size of the RNA segments. The significance of this observation is not clear at present. Future analysis may clarify the evolutionary relationship between individual influenza A and B virus genes.

The genetic characterization of the influenza B virus genome which we present in this communication now permits the association of biological properties with certain viral genes. It may be possible to establish which gene or combinations of genes enable influenza B viruses to grow in some cells and not in others. Similarly, genes controlling drug resistance and virulence may be identified in the future. It may also be possible to identify which genes correlate with attenuation in live virus vaccine strains. With influenza A and B viruses, efforts are currently under way to transfer specific genes from prototype vaccine strains to isolates of the latest serotype for use as live virus vaccines. Identification of the gene(s) responsible for this biological property may be of practical importance in preparing commercial influenza virus vaccines.

Now that a genetic map for influenza B viruses is available, the fine structure of all eight genes coding for specific polypeptides can be examined by using oligonucleotide fingerprinting and RNA sequencing techniques. The extent of changes between genes in different influenza B virus strains can be compared with genetic variation in the genome of influenza A viruses. Such studies may help elucidate the molecular basis for the biological differences between influenza A and B viruses.

LITERATURE CITED

1. Alfageme, C. R., A. Zweidler, A. Mahowald, and L. H. Cohen. 1974. Histones of *Drosophila* embryos. J. Biol. Chem. 249:3729-3736.

2. Aymard-Henry, M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster. 1973. Influenzavirus neuraminidase and neuraminidase-inhibition test procedures. Bull. W. H. O. 48:199-202.

3. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.

4. Choppin, P. W., S. G. Lazarowitz, and A. R. Goldberg. 1975. Studies on proteolytic cleavage and glycosylation of the hemagglutinin of influenza A and B viruses, p. 105-119. *In* R. D. Barry and B. W. J. Mahy (ed.), Negative strand viruses, vol. 1. Academic Press Inc., New York.

5. Chakraverty, P. 1971. Antigenic relationship between influenza B viruses. Bull W. H. O. 45:755-766.

6. Chakraverty, P. 1972. Antigenic relationships between the neuraminidases of influenza B virus. Bull. W. H. O. 46:473-476.

7. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252:1102-1106.

8. Desselberger, U., and P. Palese. 1978. Molecular weights of RNA segments of influenza A and B viruses. Virology 88:394-399.

9. Douglas, R. G., Jr. 1975. Influenza in man, p. 395-447. *In* E. D. Kilbourne (ed.), The influenza viruses and influenza. Academic Press, Inc., New York.

10. Francis, T., Jr. 1940. A new type of virus from epidemic influenza. Science 92:405-408.

11. Gentsch, J., L. R. Wynne, J. P. Clewley, R. E. Shope, and D. H. L. Bishop. 1977. Formation of recombinants between snowshoe hare and La Crosse bunyaviruses. J. Virol. 24:893-902.

12. Haslam, E. A., A. W. Hampson, J. A. Egan, and D. O. White. 1970. The polypeptides of influenza virus. II. Interpretation of polyacrylamide gel electrophoresis patterns. Virology 42:555-565.

13. Haslam, E. A., A. W. Hampson, I. Radiskevics, and D. O. White. 1970. The polypeptides of influenza virus. III. Identification of the hemagglutinin, neuraminidase and nucleocapsid proteins. Virology 42:566-575.

14. Hennessy, A. V., E. Minuse, and F. M. Davenport. 1965. A twenty-one year experience with antigenic variation among influenza B viruses. J. Immunol. 94:301-306.

15. Kendal. A. P. 1975. A comparison of "influenza C" with prototype myxoviruses: receptor-destroying activity (neuraminidase) and structural polypeptides. Virology 65:87-99.

16. Kilbourne, E. D. 1975. Epidemiology of influenza, p. 483-538. *In* E. D. Kilbourne (ed.), The influenza viruses and influenza. Academic Press Inc., New York.

17. Krug, R. M. 1972. Cytoplasmic and nucleoplasmic viral RNPs in influenza virus-infected MDCK cells. Virology 50:103-113.

18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

19. Lamb, R. A., and P. W. Choppin. 1977. The synthesis of Sendai virus polypeptides in infected cells. III. Phosphorylation of polypeptides. Virology 81:382-397.

20. Lazdins, I., E. A. Haslam, and D. O. White. 1972. The polypeptides of influenza virus. VI. Composition of the neuraminidase. Virology 49:758-765.

21. McMaster, G. K., and G. G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. 74:4835-4838.

22. Magill, T. P. 1940. A virus from cases of influenza-like upper-respiratory infection. Proc. Soc. Exp. Biol. Med. 45:162-164.

23. **Maizel, J. V.** 1971. Polyacrylamide gel electrophoresis of viral proteins, p. 179–246. *In* K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 5. Academic Press Inc., New York.

24. **Moffet, H. L., H. G. Cramblett, G. K. Middleton, J. P. Black, H. K. Schulenberger, and A. M. Yongue.** 1962. Outbreak of influenza B in a children's home. J. Am. Med. Assoc. **182:**834–838.

25. **Nuss, D. L., H. Oppermann, and G. Koch.** 1975. Selective blockage of initiation of host protein synthesis in RNA-virus-infected cells. Proc. Natl. Acad. Sci. U.S.A. **72:**1258–1262.

26. **Oxford, J. S.** 1973. Polypeptide composition of influenza B viruses and enzymes associated with the purified virus particles. J. Virol. **12:**827–835.

27. **Palese, P., M. B. Ritchey, and J. L. Schulman.** 1977. P1 and P3 proteins of influenza virus are required for complementary RNA synthesis. J. Virol. **21:**1187–1195.

28. **Palese, P., and J. L. Schulman.** 1976. Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. Proc. Natl. Acad. Sci. U.S.A. **73:**2142–2146.

29. **Palese, P., and J. L. Schulman.** 1976. Differences in RNA patterns of influenza A viruses. J. Virol. **17:**876–884.

30. **Racaniello, V. R., and P. Palese.** 1978. The genes of influenza virus: analysis of influenza B virus strains, p. 27–36. *In* R. D. Barry and B. W. J. Mahy (ed.), Negative strand viruses and the host cell. Academic Press Inc., London.

31. **Ritchey, M. B., P. Palese, and E. D. Kilbourne.** 1976. RNAs of influenza A, B, and C viruses. J. Virol. **18:**738–744.

32. **Ritchey, M. B., P. Palese, and J. L. Schulman.** 1976. Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. J. Virol. **20:**307–313.

33. **Ritchey, M. B., P. Palese, and J. L. Schulman.** 1977. Differences in protein patterns of influenza A viruses. Virology **76:**122–128.

34. **Schild, G. C., M. S. Pereira, P. Chakraverty, M. T. Coleman, W. R. Dowdle, and W. K. Chang.** 1973. Antigenic variants of influenza B virus. Br. Med. J. **4:**127–131.

35. **Schulman, J. L., and P. Palese** 1976. Selection and identification of influenza virus recombinants of defined genetic composition. J. Virol. **20:**248–254.

36. **Sharpe, A. H., R. F. Ramig, T. A. Mustoe, and B. N. Fields.** 1978. Genetic map of reovirus. I. Correlation of genome RNAs between serotypes 1, 2 and 3. Virology **84:**63–74.

37. **Tobita, K., and E. D. Kilbourne.** 1975. Structural polypeptides of antigenically distinct strains of influenza B virus. Arch. Virol. **47:**367–374.

38. **Ueda, M., K. Tobita, A. Sugiura, and C. Enomoto.** 1978. Identification of hemagglutinin and neuraminidase genes of influenza B virus. J. Virol. **25:**685–686.

39. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244:**4406–4412.

40. **Wong, S. C., and E. D. Kilbourne.** 1961. Changing viral susceptibility of a human cell line in continuous cultivation. I. Production of infective virus in a variant of the Chang conjunctival cell following infection with swine or N-WS influenza viruses. J. Exp. Med. **113:**95–110.