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BIOCHEMICAL AND GENETIC STUDIES OF INFLUENZA B AND C
VIRUSES

City University of New York

PH.D.

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BIOCHEMICAL AND GENETIC STUDIES
OF INFLUENZA B AND C VIRUSES

by

Vincent R. Racaniello

A dissertation submitted to the Graduate Faculty
in Biomedical Sciences in partial fulfillment of
the requirements for the degree of Doctor of
Philosophy, The City University of New York.

1979

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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I. ABSTRACT

BIOCHEMICAL AND GENETIC STUDIES
OF INFLUENZA B AND C VIRUSES

by

Vincent R. Racaniello

Advisor: Peter Palese, Ph.D.

The genes and gene products of influenza B and C viruses were studied using various biochemical and genetic techniques.

The RNAs of several different influenza B viruses were labelled with ^{32}P and analyzed by electrophoresis on denaturing polyacrylamide gels. Examination of the viral RNAs in this way indicated the presence of a divided genome containing eight segments. The RNA patterns of viruses isolated from 1940 to 1973 were different, suggesting that genetic variation occurs in all genes of influenza B viruses, not just those coding for surface glycoproteins. Electrophoresis of glyoxal-

treated RNA from several different influenza B viruses confirmed the presence of 8 RNA segments. In addition, glyoxal treatment abolished the strain-specific differences in RNA patterns; this finding indicated that mobility differences of influenza B virus RNAs on gels were most likely due to secondary structure and not to differences in molecular weight.

Eight influenza B virus-specific polypeptides were identified: P1, P2, P3, HA, NA, NP, M and NS1. Evidence was also presented for a second nonstructural protein, NS2. Analysis of viral polypeptides on SDS-polyacrylamide gels or by one-dimensional peptide mapping revealed that most of the corresponding proteins of B/Lee/40 and B/Md/59 viruses could be distinguished. This information confirmed the suggestion that genetic variation occurs in most of the genes of influenza B viruses.

A series of recombinants of B/Md/59 and B/Lee/40 viruses were isolated under conditions where the recombinants inherited most RNA segments from one parent, and one or two RNA segments from the other parent. A genetic map of influenza B viruses was established by examining the protein composition of these recombinants. Six polypeptides, P1, NP, HA, NA, M protein and NS protein, were unambiguously assigned to specific RNA segments.

Electrophoresis of glyoxalated RNA was used as a basis of a technique for rapidly mapping the genome of any influenza virus. This technique is based on the assumption that the gene order after glyoxal treatment of all influenza A and B viruses is identical. ^{32}P -labelled RNA of any influenza virus is electrophoresed, and the RNA segments are glyoxalated within individual gel slices and rerun on a second gel in the presence of whole glyoxalated RNA. Since the gene order for glyoxalated RNA is known, the identity of any band excised from the original gel can be determined by noting where the RNA migrates in relation to the pattern of whole glyoxalated RNA.

Experiments were conducted which indicate that the influenza C viruses contain seven RNA segments, as opposed to eight for influenza A and B viruses. Evidence was also presented for seven influenza C virus-specific polypeptides: P1, P2, P3, HA, NP, M and NS. A precursor to HA was identified in influenza C virus-infected cells, and a model for the influenza C virus HA was presented.

Recombinants between two different influenza C viruses were isolated. In MDCK cells, one strain, C/JJ/50, caused lytic plaques while C/JHG/66 virus did

not produce clear plaques. From a mixed infection of MDCK cells with C/JHG/66 virus and UV-irradiated 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 3' and 5' terminal nucleotides of the genes of an influenza A, B and C virus were identified by directly sequencing viral RNA using two different sequencing techniques. A high degree of conservation at the 3' ends as well as at the 5' ends was observed among the genes of each virus and among the genes of the three different virus types. A uridine-rich region was observed from positions 17 through 22 at the 5' end of the genes. The conserved 3' and 5' terminal sequences of the influenza virus genes showed partial inverted complementarity. This feature results in very similar sequences at the 3' ends of the plus and minus strand RNAs and may also enable single stranded RNAs of influenza virus to form panhandle structures. Inverted complementary repeats may play an important role in initiation of viral RNA replication.

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VI. INTRODUCTION

The influenza viruses are the sole members of the myxovirus group and include the influenza A, B and C viruses. These viruses are the causative agents for the most important of the recognized human viral respiratory tract infections, influenza. This disease has probably affected man throughout history, and today, despite intense research efforts and the availability of effective vaccines, influenza continues to be prevalent, severe and expensive.

The influenza viruses over the years have been able to produce widespread disease while other pathogens have been controlled, mainly because the antigenic character of the influenza virus is continually changing. A great deal of effort has been devoted towards understanding the basic biology of the influenza viruses in an attempt to explain this unique behavior.

This dissertation is concerned with influenza B and C viruses, which are certainly less studied than influenza A viruses but are nevertheless important human pathogens. In the following section, the myxoviruses will first be placed in perspective with other animal viruses, and then specific information on the molecular biology of influenza

viruses and the characteristics of influenza will be presented.

A. CLASSIFICATION OF VIRUSES

Viruses are conveniently classified into three broad groups depending upon whether they replicate in animals, plants or bacteria. Although this type of classification is not without ambiguities, most viruses replicate in a range of hosts which are more or less related.

Animal viruses have been discovered which infect mammals, fish, amphibia, birds and insects (reviewed in Luria et al., 1978). The large number of animal viruses which have been discovered can be further divided into two groups, depending upon whether the viral genetic material is RNA or DNA. The major DNA-containing animal virus groups are the poxviruses, herpesviruses, adenoviruses, papovaviruses, iridoviruses and parvoviruses. The first three groups contain many important human pathogens.

A list of the major groups of animal viruses which have an RNA genome is shown in Table 1. These RNA-containing viruses are further classified according to the configuration of the nucleic acid (single or double stranded, segmented or nonsegmented), the symmetry of the nucleocapsid (cubic or icosahedral) and the presence or absence of a lipid envelope. In Table 1, viruses

TABLE 1

GROUPS OF RNA-CONTAINING ANIMAL VIRUSES

<u>Group</u>	<u>RNA</u>			<u>Symmetry of capsid</u>	<u>Envelope</u>
	<u>Configuration</u>	<u>Polarity</u>	<u>MW</u>		
Picornaviridae	linear, ss	+	2.7×10^6	icosahedral	-
Togaviridae	linear, ss	+	4.0×10^6	icosahedral	+
Rhabdovirus	linear, ss	+	4.0×10^6	helical	+
Retrovirus	linear, ss	+	6.0×10^6	helical	+
Coronavirus	linear, ss	+	5.4×10^6	helical	+
Paramyxovirus	linear, ss	-	7.0×10^6	helical	+
Reoviridae	segmented, ds	±	15.0×10^6	icosahedral	-
Bunyaviridae	segmented, ss	-	$3-4.0 \times 10^6$	helical	+
Orthomyxovirus	segmented, ss	-	$4-5.0 \times 10^6$	helical	+
Arenavirus	segmented, ss	-	4.8×10^6	helical	+
Nodamura virus	segmented, ss	+	1.6×10^6	icosahedral	-

with single-stranded genomes are listed first, followed by viruses which contain segmented genomes. Segmented RNAs may be either double-stranded (reoviruses) or single-stranded, as is found in the orthomyxoviruses. Most of the segmented, single-stranded genomes are of negative (-) polarity, with the exception of Nodamura virus. This virus, which was isolated in Japan, may be the prototype for a new group of viruses which contain segmented RNA genomes of positive (+) polarity (Newman et al., 1978).

B. THE ORTHOMYXOVIRUSES

Description and classification

The orthomyxovirus group contains only the influenza viruses, a group of relatively large (100 nm diameter), enveloped RNA-containing animal viruses. The nucleocapsid of influenza viruses is of helical symmetry, and contains a segmented, single-stranded genome of negative or nonmessage polarity (reviewed in Kilbourne, 1975; see Table 1). Influenza viruses are further classified into three types, A, B and C, based on serological differences in two internal components, the nucleoprotein (NP) and the matrix (M) protein. For example, the M proteins of all influenza A viruses cross-react, but M protein of an

influenza A virus is not related to M protein of an influenza B virus.

Since the influenza virus undergoes continual antigenic change, many new strains are isolated each year, and a precise nomenclature to catalogue all of the isolates has been formulated (Bull W.H.O., 1971). Table 2 shows how different human and animal influenza viruses are classified. All influenza viruses are designated by a code which includes type (A, B or C), geographic site of isolation, isolate number, and year of isolation. In addition, influenza A viruses are subtyped according to serologic differences in the two surface glycoproteins, hemagglutinin and neuraminidase. Influenza B and C viruses have not been placed in subtypes. Several influenza virus reference strains are listed in the last column of Table 2.

As shown in Table 2, influenza A viruses can be isolated in nature from humans, horses, pigs and birds, while influenza B and C viruses are most likely restricted to humans. To date, all human influenza A virus isolates belong to one of four hemagglutinin-neuraminidase subtypes (H0N1, H1N1, H2N2, H3N2).

History

Human influenza-like illnesses have been described since 1170 AD, and a relatively complete record of

TABLE 2

CLASSIFICATION OF INFLUENZA VIRUSES

<u>Species</u>	<u>Type</u>	<u>HA subtype</u>	<u>NA subtype</u>	<u>Reference strain</u>
Human	A	HO	N1	A/PR/8/34
		H1	N1	A/FM/1/47
		H2	N2	A/Singapore/1/57
		H3	N2	A/Hong Kong/1/68
	B	—	—	B/Lee/40
	C	—	—	C/Taylor/47
Swine	A	Hsw1	N1	A/Swine/Wisconsin/15/30
Equine	A	Heq1	Neq1	A/equine/Prague/1/56
		Heq2	Neq2	A/equine/Miami/1/63
Avian	A	Hav1-Hav9	Nav1-Nav6	Viruses have been isolated from avian species possessing many com- binations of Hav and Nav antigens.

influenza in Europe is available from 1500 (reviewed in Webster and Laver, 1975). These records indicate that influenza occurred either in pandemic form, in which the disease spread over the globe, or occurred in local epidemics.

Early Florentine records (di Camugliano, 1933) indicate that in the 14th and 15th centuries an Italian family thought that epidemics of cough, cold and fever were due to the "influence" of a bad conjunction of stars and planets. In this way influenza (influenza = Ital., influence) became the name for these epidemics, and the name has remained with us since.

The first human influenza virus was isolated in 1933 (Smith et al., 1933) when influenza was successfully transmitted to ferrets by intranasal inoculation of filtered human throat washings. In 1931 Shope had already successfully transmitted swine influenza to pigs by inoculating them intranasally with filtrates of respiratory secretions (Shope, 1931). Even earlier, Centanni and Savonuzzi (cited in Stubbs, 1965) showed in 1900 that the avian disease fowl plague was due to a filterable virus, although it was not shown to be an influenza virus until 1955 (Schäfer, 1955).

A virus isolated from a human influenza case in 1940 was shown to be antigenically different from previous influenza virus isolates; it was called influenza B virus

(Francis, 1940; Magill, 1940). The first influenza C virus was isolated in 1947 (Francis et al., 1950; Taylor, 1951).

In 1955 Andrewes suggested myxoviruses (Andrewes et al., 1955) as the name for a group of viruses which have an affinity for mucoid substances (myxo = Gr., mucus). Originally included in this group were mumps virus and Newcastle disease virus, but these viruses were later placed in the paramyxovirus group due to differences in structure and replication (Waterson, 1962).

Since the influenza viruses are associated with an important human disease, they have generated a great deal of research interest. These viruses have been studied since the 1930s, and as a consequence were the subject of many pioneering studies in animal virology. The influenza virus was one of the first viruses to be studied by electron microscopy (Taylor et al., 1943) and was the first virus shown to bud from the cell membrane (Murphy and Bang, 1952). Hemagglutination (Hirst, 1941; McClelland and Hare, 1941) and genetic recombination in viruses (Burnet and Lind, 1949) were both initially demonstrated with influenza virus. The discovery of the influenza viral neuraminidase was the first time that an enzyme was shown to be a structural protein of a virus (Hirst, 1942). By leading the way in these basic

discoveries, influenza viruses have generated much of the impetus for studying other animal viruses.

Illness caused by orthomyxoviruses

Uncomplicated influenza A is an acute upper respiratory disease. Systemic symptoms abruptly appear approximately 2 days after infection and include fever, chills, headache and myalgias. These symptoms soon subside and are replaced with respiratory ailments such as cough, substernal pain and nasal discharge. Convalescence is often prolonged (2-3 weeks) and respiratory symptoms may persist. Subclinical cases of influenza A occur, and other respiratory tract syndromes may accompany influenza, such as common cold, pharyngitis and laryngitis. Other complications of influenza A, including pneumonia, myositis, myoglobinuria, and central nervous system involvement may occur (reviewed in Douglas, 1975).

In a study of symptoms in patients afflicted with influenza B, it was found that the disease was very similar to influenza A (Stansfield and Stuart-Harris, 1943). Other investigators feel that influenza B is milder than influenza A (reviewed in Douglas, 1975). However, the differences are rather small. 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

(Moffet et al., 1962), and influenza B virus infections occur in every month of the year (reviewed in Kilbourne, 1975). Influenza B viruses have been associated with Reye's syndrome, an acute encephalopathy in children (reviewed in Douglas, 1975).

Influenza C is essentially an afebrile upper respiratory illness. Serologic surveys indicate that influenza C virus infection must be quite common (Minuse et al., 1954; Taylor, 1951) although antibodies to influenza C viruses are not found in children (Hilleman et al., 1953) and the virus is responsible for only 1-2% of acute respiratory disease in children and in young adults (Hilleman et al., 1962; Mogabgab, 1963).

In general, influenza in children is more common than in adults, and the symptoms are usually milder, with fewer pulmonary complications (reviewed in Douglas, 1975). Animal influenza is quite distinct from human influenza and will not be discussed here (reviewed in Easterday, 1975).

C. THE INFLUENZA VIRION

Most of the work on the morphology, composition and structure of influenza viruses has been done with influenza A virus strains. In the following sections, the information presented will therefore pertain mainly to influenza A viruses. When similar experiments have been performed with influenza B and C viruses, those results will be discussed.

Morphology

Early electron microscope and ultrafiltration studies revealed that influenza viruses are roughly spherical particles 80-120 nm in diameter (Elford et al., 1936; Taylor et al., 1943). Later negative staining techniques were used to demonstrate that the virions were covered with closely spaced projections or "spikes" 10-12 nm in length, which were imbedded in the viral envelope. A helical nucleocapsid could be seen beneath the envelope (Horne et al., 1960; Hoyle et al., 1961). Thin sectioning of elongated particles (see below) revealed that the nucleocapsid consists of strands of electron dense material arranged parallel to the long axis of the particle.

Many of these early studies showed that the influenza virions were highly pleomorphic, and later it was found that this feature was probably an artifact of preparation for electron microscopy. If the virions were fixed before staining, this heterogeneity could be minimized (Choppin et al., 1961). In addition, when freeze drying and etching techniques were used, the virions appeared more uniformly spherical since they were not flattened by the drying process (Nermut and Frank, 1971).

Although most influenza viruses are spherical, there are some exceptions. Virus which has just budded from the cell surface is somewhat elongated or bacillary in

in shape (Compans and Dimmock, 1969; Bächli et al., 1969; Compans et al., 1970a). Preparations of freshly isolated influenza viruses often contain long, filamentous forms 80-100 nm in diameter and 4 μ m in length (Mosely and Wyckoff, 1946; Chu et al., 1949; Choppin et al., 1960; 1961). However, upon passage in culture the filamentous viruses were shown to assume the more characteristic spherical shape (Choppin et al., 1960). Production of filamentous particles is a genetic trait which can be exchanged by recombination (Kilbourne and Murphy, 1960).

No morphological differences between influenza B viruses and influenza A viruses have been reported (Waterson et al., 1963; Archetti et al., 1967; Apostolov et al., 1970). Influenza C viruses also possess a similar morphology, except that influenza C viruses are slightly larger than influenza A and B viruses and seem to contain a hexagonal lattice structure just beneath the surface (Waterson et al., 1963; Flewett and Apostolov, 1967; Archetti et al., 1967; Apostolov and Flewett, 1969; Apostolov et al., 1970; Compans et al., 1977; Meier-Ewert et al., 1978). The significance of this structure is not known.

Chemical composition

Determinations of the chemical composition of influenza viruses are not precise, due to the heterogeneity of the virions and the dependence on the host cell for the acquisition

of lipid and carbohydrates. However, several estimates have been made and the values obtained from different laboratories are in general agreement.

The approximate composition of populations of influenza virions is 0.8-1.1% RNA, 70-75% protein, 20-24% lipid and 5-8% carbohydrate (Ada and Perry, 1954; Frommhagen et al., 1959; Blough et al., 1967). In one study, the composition of an influenza B virus was found to be identical to that of an influenza A virus (Frommhagen et al., 1959).

Various estimates for the sedimentation value of the influenza virion have been reported. Values for influenza A viruses vary from 700-740S (Friedewald and Pickels, 1944; Lauffer and Stanley, 1944; Sharp et al., 1945). The influenza B virus particle has been found to have a somewhat higher sedimentation constant in the range of 800-840S (Friedewald and Pickels, 1944; Sharp et al., 1945).

RNA

The influenza viruses were one of the first groups of animal viruses shown to possess a genome of single-stranded RNA (Ada and Perry, 1954). The precise structure of the influenza virus genome was debated for twenty years after that discovery, and was finally elucidated in the late 1970s.

Early genetic studies demonstrated an unusually high frequency of recombination in influenza viruses (Burnet and Lind, 1952) suggesting that the genome may be segmented (Burnet, 1956). Later studies using the newly developed plaquing technique confirmed these findings (Simpson and Hirst, 1961). These observations led Hirst (1962) to formulate his classic hypothesis, which states, in part, that influenza RNA exists in pieces which are capable of replication and random reassortment. Other biologic evidence for a segmented genome was provided by Barry (1961) who demonstrated multiplicity reactivation of influenza viruses. The stepwise inactivation of viral functions by a chemical (Scholtissek and Rott, 1964) and by UV irradiation (Gandhi and Burke, 1970) also provided support for the concept of a divided genome. Much biochemical evidence is now available which proves that the influenza virus genome is indeed segmented.

Initially, isolated influenza virus RNA was subjected to sedimentation analysis on sucrose or glycerol gradients. The results indicated that the RNAs sedimented heterogeneously in two to three peaks (Agrawal and Bruening, 1966; Davies and Barry, 1966; Duesberg and Robinson, 1967; Nayak and Baluda, 1967; Pons, 1967). The development of polyacrylamide gel electrophoresis techniques permitted a

more precise analysis of viral RNA, and estimates of the number of RNA segments jumped to between 5 and 7 (Duesberg, 1968; Pons and Hirst, 1968a, b; Bishop et al., 1971; Lewandowski et al., 1971; Skehel, 1971). More recently, techniques for polyacrylamide gel electrophoresis of RNA on slab gels in the presence of urea (Floyd et al., 1974) have been developed. Application of this technique has permitted resolution of the influenza A virus genome into eight distinct RNA segments (Palese and Schulman, 1976a; McGeoch et al., 1976; Ritchey et al., 1976a; Pons, 1976; Scholtissek et al., 1976).

Throughout the years, there has been continuous debate about whether the recovery of RNA from influenza viruses in pieces was a valid finding or an experimental artifact. A number of experiments have been conducted which indicate that influenza viral RNA is truly segmented. Using the same conditions for extracting influenza virus RNA, the RNAs of other viruses are recovered intact (Davies and Barry, 1966). Different techniques have been employed in many laboratories for extracting the RNA from several influenza virus strains, and the results are generally in agreement. Young and Content (1971) separated 3 size classes of viral RNA by velocity sedimentation, and found that the 5' end of the molecules in each class was pppAp.

Studies of the 3' end of influenza viral RNAs isolated in a similar manner indicated that most of the molecules contained U_{OH} at the 3' end (Lewandowski et al., 1971). The latter findings indicated that the different classes of viral RNA were most likely not derived by endonucleolytic cleavage of a precursor, but are probably independently replicated.

Evidence for the existence of unique sequences in the different RNA classes was first obtained by cross-hybridizing 3 classes of vRNA with cRNA extracted from infected cells (Content and Duesberg, 1971). This study showed that sequences of RNAs in the smallest class were absent from larger RNA classes. Horst et al. (1972) separated influenza viral RNA into 3 classes, fingerprinted the RNA with RNase T1, and performed base composition analysis on the large unique oligonucleotide spots. The results indicated that oligonucleotides from different size classes had unique base compositions. Thermal transition profiles of purified double-stranded RNA segments extracted from infected cells were different, indicating that there were nucleotide sequence differences between different virion RNA segments (Bromley and Barry, 1973). Most recently, McGeoch et al. (1976) found that oligonucleotide fingerprints of the 8 RNA segments of an

influenza A virus were all different.

The influenza virus genome was originally shown to be single-stranded by demonstrating that the RNA was destroyed by RNase (Ada and Perry, 1954). This finding was subsequently confirmed by a number of investigators (Frisch-Niggemeyer and Hoyle, 1956; Agrawal and Breuning, 1966; Duesberg and Robinson, 1967). The sedimentation value of the RNA was also shown to be strongly dependent on salt concentration, suggesting a single-stranded configuration (Duesberg and Robinson, 1967). Base composition analyses from different laboratories yielded very similar results: A, 22-23%; G, 19-20%; C, 23-25%; U, 31-33.5% (reviewed in Bellett, 1967; Ritchey et al., 1976 a). These data indicated that there were probably no large double-stranded regions in the viral RNA.

The RNA of influenza viruses has clearly been shown to be of negative sense. This conclusion derives from several independent lines of investigation. The virion RNA, which is noninfectious, was first shown to be of one strand sense in self-annealing experiments (Pons, 1971; Scholtissek and Becht, 1971). Purified RNA from infected-cell polysomes completely protects virion RNA from digestion by RNase after hybridization, indicating that the virion RNA is complementary to the mRNA from polysomes (Pons, 1972; Etkind and Krug, 1975; Glass et al., 1975). This polysome-associated RNA will direct the synthesis of viral

proteins in a cell-free system, while RNA from purified virus does not (Ritchey and Palese, 1976; Etkind and Krug, 1975; Content, 1976). Influenza viruses contain an RNA-dependent RNA polymerase (Chow and Simpson, 1971; Penhoet et al., 1971) which can synthesize in vitro RNA which is complementary to vRNA. Finally, only polysome-associated RNA contains 7-methylguanosine at the 5' end (Krug et al., 1976) and poly (A) at the 3' end (Etkind and Krug, 1974). All of these data indicate that RNA contained in purified virions is of negative sense, and is complementary to mRNA found in infected cells.

Molecular weight estimates of influenza viral RNAs have varied over the years. In the earliest studies, techniques of centrifugation yielded values of $2-2.5 \times 10^6$ daltons (Ada and Perry, 1954; Frisch-Niggemeyer and Hoyle, 1956; Pons and Hirst, 1968a). With the development of polyacrylamide gel electrophoresis, values between $3.9-5 \times 10^6$ daltons were obtained (Bishop et al., 1971; Lewandowski et al., 1971; Skehel, 1971). Even higher estimates ($5.5-5.9 \times 10^6$ daltons) were found using electrophoresis of RNA in denaturing gels containing urea, or by determining the complexity of unique oligonucleotide spots derived from viral RNA (McGeoch et al., 1976; Palese and Schulman, 1976a; Ritchey et al., 1976a).

However, most of these estimates were most likely wrong. Complexity measurements of isolated oligonucleotides are made difficult because of recovery problems, and molecular weight analysis in gels is inaccurate due to the presence of secondary structure in RNA, in spite of denaturing conditions (Bailey et al., 1976; Lehrach et al., 1977). Recently it was reported that glyoxalation of RNA prevents base stacking and consequently eliminates secondary structure (McMaster and Carmichael, 1977). Glyoxalation of RNA was coupled with gel electrophoresis to obtain a very accurate MW determination (Desselberger and Palese, 1978). The segments of influenza A viruses were determined to have the following MWs ($\times 10^6$):

RNA 1, 0.89; RNA 2, 0.89; RNA 3, 0.86; RNA 4, 0.66;
RNA 5, 0.56; RNA 6, 0.48; RNA 7, 0.28; RNA 8, 0.21.

The total MW of the influenza A virus genome is 4.9×10^6 . In addition, migration differences in the RNA patterns of different influenza A viruses, which are most likely due to differences in secondary structure, were eliminated (see below).

Recently a new method for calculating MWs of influenza virus segments has been developed (Emtage et al., 1979; Sleight et al., 1979). A poly (A) tail is first added to the 3' end of the viral RNA, and then cDNA copies of the genome are made using reverse transcriptase and an oligo

d(T) primer. The cDNAs are then sized on denaturing gels with suitable DNA markers of known chain length. The authors claim that this is the most accurate technique available for obtaining MW values of RNA; however, both groups obtain different values for the chain length of influenza A virus RNAs. Most likely the cDNAs produced by this technique are not complete transcripts of the influenza virus RNA segments.

An interesting finding which resulted from the development of high-resolution gel electrophoresis techniques for RNA was the discovery that the RNA patterns of different influenza A virus isolates are different (Palese and Schulman, 1976a). Migration differences in corresponding RNA segments of two virus strains are most likely due to sequence differences in the RNAs of antigenically diverse viruses, since complete denaturation with glyoxal eliminates these migrational differences (Desselberger and Palese, 1978). Differences in RNA patterns of influenza viruses was used to construct a genetic map of influenza A viruses (see Genetics of Orthomyxoviruses).

The RNAs of influenza B and C viruses have not been extensively studied. Early experiments with influenza B viruses described the transfer of mouse lung virulence from one strain to another (Perry and Burnet, 1953; Perry et al., 1954; Ledinko, 1955) and demonstrated

multiplicity reactivation (Kilbourne, 1955, 1957; Gotlieb and Hirst, 1956; Barry, 1961). Unequivocal demonstration of high frequency genetic recombination for antigenic markers was later presented (Tobita and Kilbourne, 1974), suggesting the presence of a segmented genome in influenza B viruses similar to that of influenza A viruses.

Base composition studies of influenza B viruses yielded values which were virtually identical to those obtained for influenza A viruses (Schuster, 1960; Schaffer, 1962; Ritchey et al., 1976a). Ritchey and co-workers (1976a) showed that the genome of influenza B/Lee/40 virus consisted of eight single-stranded RNA pieces with a total molecular weight estimated on urea-polyacrylamide gels of 6.43×10^6 . The most recent estimate of the molecular weight of the influenza B virus genome, using the glyoxal technique, is 5.3×10^6 (Desselberger and Palese, 1978). There is very little evidence that influenza B virus RNA is negative-stranded; no in vitro cell free studies have been conducted. However, an RNA-dependent RNA polymerase activity has been found associated with purified influenza B virus particles (Chow and Simpson, 1971; Oxford, 1973a; 1973b).

The influenza C virus genome had been relatively ignored until recently. In the first report on the RNAs of these viruses, Ritchey et al. (1976a) showed that influenza

C virus RNA was 95% susceptible to RNase, and the base composition of the RNA ruled out the presence of large double-stranded regions. These studies also showed that the base composition of influenza C virus RNA was similar to that of influenza A and B viruses. Evidence was presented using polyacrylamide gel electrophoresis for at least 4 RNA segments with a total MW of 4.67×10^6 . A later report (Cox and Kendal, 1976) confirmed that the influenza C virus genome is single-stranded, and provided evidence for 5 RNA segments with a total MW of 4.15×10^6 . Later studies of influenza C virus RNAs indicated the presence of 6 segments with a total MW of 5.15×10^6 (Compans et al., 1977). No information is available concerning the coding sense of influenza C virus RNAs.

Recently information on the primary structure of influenza virus genes has been reported. Using the capping enzyme from vaccinia virus, Moss et al. (1978) demonstrated that the first 3 bases at the 5' end of all genes of an influenza A or B virus is pppAGU.... This finding was later confirmed by Skehel and Hay (1978b), using 5' end labelling and rapid RNA sequencing techniques. They also found that the adjacent nucleotides for all genes of several influenza A viruses and one influenza B virus were ...AGA-AAC-AAG-G(_)-UUU-UUU.... (The blanks after the guanosine indicate positions of variability from gene to gene.) The

same authors also sequenced the 5' end of ApG-primed, in vitro synthesized cRNA transcripts of the genes of fowl plague virus, and found the conserved sequence AGC-AAA-AGC-AGG.... Several investigators have suggested that the ApG-primed transcripts of influenza A virus RNAs initiate exactly at the 3' end of vRNA and therefore the 5' terminal sequences of the transcripts must be complementary to the 3' ends of the virion RNA segments (Skehel and Hay, 1978b; Plotch and Krug, 1978; Plotch et al., 1978; Smith et al., 1978). This assumption would mean that the conserved sequence at the 3' end of virion RNA is $_{OH}UCG-UUU-UCG-UCC....$ However, direct sequence data of the 3' end of vRNA is required to address this assumption.

Most recently, Both and Air (1979) polyadenylated the 3' end of the influenza A virus M gene and synthesized cDNA copies using an oligo d(T) primer and reverse transcriptase. Sequencing of the cDNA provided evidence for an initiation codon (AUG) at position 26-28 (also found by Skehel and Hay, 1978b; Desselberger, unpublished results). One tryptic peptide derived from purified M protein had a composition identical to that of an oligopeptide 9 amino acids long, which was predicted by the cDNA sequence following the initiation codon. The authors concluded that the M protein initiates at position 26-28,

a suggestion which had been made previously (Skehel and Hay, 1978b).

It is desirable to obtain nucleotide sequences of influenza virus RNAs for several reasons. Sequences at gene termini may yield valuable information on genetic signals (i.e., initiation codons, promoters, terminators, etc.) and may also suggest mechanisms for the packaging of viral RNA segments, i.e., through "sticky" ends. Complete nucleotide sequences will reveal the primary structure of known proteins and will perhaps yield information on possible second-frame proteins (see Godson et al., 1978).

Protein

The influenza virus proteins will be described here individually, and in a later section their arrangement in the virion will be considered. Influenza virus proteins were studied initially by examining purified virus preparations. Later, with the development of cell culture techniques, virus-induced polypeptides in infected cells were examined and compared to virion structural proteins.

Early studies of influenza virus proteins revolved around the fact that three proteins--HA, NA and NP--were identifiable by immunologic procedures. These three antigens were studied by disrupting viruses with various agents

and separating proteins by centrifugation or by electrophoresis on cellulose acetate strips (Laver, 1964). In this way three polypeptides were found in purified virions which corresponded to these three antigens.

Later, techniques for electrophoresis on polyacrylamide gels were developed with methods for labelling proteins in infected cells with radioactive sugars and amino acids. It soon became clear that influenza viruses contained more than three virus-specific proteins. Estimates for the number of polypeptides detected on polyacrylamide gels rose steadily from three to the now presently accepted number of eight virus-coded polypeptides: P1, P2, P3 polypeptides; hemagglutinin, neuraminidase, nucleoprotein, membrane protein and nonstructural protein (Inglis et al., 1976; Lamb and Choppin, 1976; Ritchey et al., 1977). Each of these polypeptides is coded for by one of the 8 viral RNA segments (see below). Recently, evidence has been presented for a ninth influenza viral polypeptide (Lamb et al., 1978), the information for which may be on one of the existing 8 RNA segments. This polypeptide is found only in influenza virus-infected cells and therefore has been called NS2.

The influenza B viruses have been less extensively studied and consequently all of the virus-specific poly-

peptides have not been identified. In early studies by electrophoresis on cellulose acetate, three peaks were identified which were most likely the HA, NA and NP proteins (Laver, 1964). Later, using polyacrylamide gel electrophoresis techniques, Haslam et al. (1970a, 1970b) identified the HA, NA, NP and the M protein in influenza B/Lee/40 virus. Laver and Baker (1972) also identified these four polypeptides in influenza B virus by combining techniques of electrophoresis on polyacrylamide gels and cellulose acetate. Oxford (1973b) identified two high molecular weight proteins in influenza B virions which were called P1 and P2 by analogy with influenza A viruses. Tobita and Kilbourne (1975), Allen et al. (1977) and Wrigley et al. (1973) have obtained similar results, although Choppin et al. (1975) and Kendal (1975) did not detect P proteins in influenza B viruses. When this work was begun, the number of influenza B virus proteins which had been identified was between 4 and 6. No studies of influenza B virus-infected cells have been reported.

Influenza C virus polypeptides have only recently been examined. Kendal (1975) identified three virion proteins, HA, NP and M protein, based on comparisons with influenza A and B virus proteins. Other groups have also found these three virion polypeptides (Compans et al.,

1977; Meier-Ewert et al., 1978), and recently these polypeptides have been detected in infected cells (Petri et al., 1979).

P Polypeptides The P polypeptides are the highest molecular weight components of virions and are part of the internal ribonucleoprotein (RNP) component (Compans et al., 1970b; Schulze, 1970; Klenk et al., 1972a; Bishop et al., 1972; Inglis et al., 1976). These polypeptides are most likely part of the viral transcriptase complex, since they are present in viral and cellular sub-fractions which possess RNA-dependent RNA polymerase activity (Bishop et al., 1972; Caliguiri and Compans, 1974; Inglis et al., 1976; Rochovansky, 1976). Furthermore, studies with temperature sensitive mutants indicate that these polypeptides play a role in viral RNA synthesis in infected cells (Palese et al., 1977b; Ritchey and Palese, 1977).

Influenza A viruses contain three P polypeptides (Lamb and Choppin, 1976; Inglis et al., 1976; Ritchey et al., 1977). No studies of the proteins of polymerase complexes have been performed with influenza B viruses; however, two high molecular weight P proteins have been described in purified influenza B virus preparations (Oxford, 1973 ; Wrigley et al., 1973; Allen et al., 1977).

No P polypeptides have been detected in influenza C viruses. Evidence for the existence of a third P protein in influenza B viruses and three P proteins in influenza C viruses will be presented in this dissertation.

Hemagglutinin Hirst (1941) and McClelland and Hare (1941) were the first to demonstrate hemagglutination of erythrocytes by influenza viruses. Later it was found that a subviral component released from purified virus by ether treatment could hemagglutinate red blood cells (Hoyle, 1952; Schäfer and Zillig, 1954). Throughout the 1960s, a hemagglutinating fraction of influenza viruses was isolated and studied by different techniques (Laver, 1963, 1964; Laver and Valentine, 1969; Webster and Darlington, 1969), but only with the advent of polyacrylamide gel electrophoresis was the HA equated with a polypeptide of MW 75-80,000 (see a later section for structure of the HA) (Schulze, 1970; Compans et al., 1970b; Haslam et al., 1970a, 1970b).

These studies and several others (Laver, 1971; Skehel and Schild, 1971; Stanley and Haslam, 1971) indicated that the HA isolated by a number of different methods could be dissociated by reduction into two smaller glycoproteins now called HA1 and HA2. The hemagglutinin poly-

peptide is made as a single gene product and under certain conditions may be cleaved to HA1 and HA2 (Lazarowitz et al., 1971) which are held together by disulfide bonds (Stanley and Haslam, 1971; Laver, 1971; Skehel and Schild, 1971). Cleavage of the HA most likely occurs while the polypeptide is in the plasma membrane of the infected cell. Virus particles can be obtained which contain fully cleaved HA, partly cleaved HA or completely uncleaved HA. The degree of cleavage depends on the host cell, the virus strain, and the presence or absence of proteolytic enzymes in cell culture fluids (Lazarowitz et al., 1971, 1973a, 1973b; Rifkin et al., 1972; Klenk et al., 1972b; Skehel, 1972; Stanley et al., 1973). For example, HA of one strain of WSN virus grown in the chick embryo is completely cleaved (Lazarowitz, 1973a), while the same strain grown in serum-free MDBK cells contains only uncleaved HA (Choppin et al., 1975).

Cleavage of the HA is not required for assembly of virus particles (Lazarowitz et al., 1973a; Stanley et al., 1973) since complete virions which contain uncleaved HA can be obtained. Furthermore, cleavage of the HA is not required for hemagglutination of erythrocytes (Lazarowitz et al., 1973a). Uncleaved HA can be cleaved in vitro with trypsin to yield HA1 and HA2 (Lazarowitz et al., 1973a)

and this treatment increases the infectivity of a virus preparation when compared to viruses with uncleaved HA molecules (Lazarowitz and Choppin, 1975; Klenk et al., 1975). It was therefore concluded that cleaved HA was required for full infectivity of influenza virions.

The HA protein of influenza B viruses was studied by the early techniques described above (Laver, 1963, 1964) and was later equated on polyacrylamide gels with a polypeptide of MW 78,000 (Haslam et al., 1970a, 1970b). Several investigators have identified the presumptive influenza B virus HA1 and HA2 polypeptides on gels (Laver and Baker, 1972; Oxford, 1973b; Wrigley et al., 1973; Kendal, 1975; Tobita and Kilbourne, 1975; Allen et al., 1977), and the relationship of cleaved HA to infectivity was demonstrated to be similar to that of influenza A viruses (Compans and Choppin, 1975; Choppin et al., 1975; Lazarowitz and Choppin, 1975).

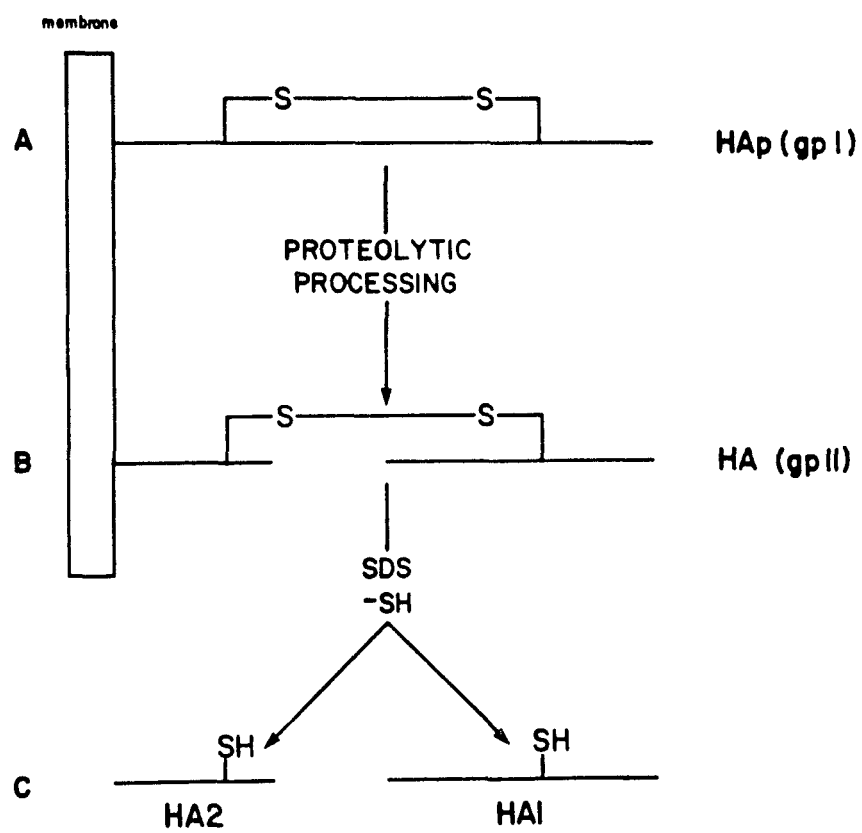
Influenza C viruses hemagglutinate erythrocytes, although the receptors for the HA are different from those of influenza A and B viruses (Hirst, 1950; Kendal, 1975; Meier-Ewert et al., 1978). Kendal (1975) first identified on polyacrylamide gels three glycoproteins (VP83, VP66, VP26) in egg-grown virus which he suggested might be related to each other in the same way that HA, HA1 and HA2 of influenza A and B viruses are related. Similar results

were obtained by Compans et al. (1977) who presented evidence for glycoproteins of MW 88,000, 65,000 and 30,000 (gp88, gp65 and gp30) in egg-grown virus. Furthermore, in virus which had been grown in chick embryo fibroblasts, only gp88 was present. When the glycoproteins of egg-grown virus were electrophoresed under non-reducing conditions, only gp88 could be detected, suggesting but not proving that gp65 and gp30 were derived from gp88 by proteolytic cleavage. Recently, evidence has been presented which indicates that a larger precursor to gp88, called gpI, may be found in virions grown in certain cell systems (Meier-Ewert et al., 1979). Virus grown in chicken embryo kidney cells contains only gpI, virus grown in chicken embryo fibroblasts contains equal amounts of gpI and gp88, and virus grown in eggs contains only gp88. Cleavage of gpI with trypsin converts gpI to gp88, which can be further reduced to gp65 and gp30. An increase in infectivity accompanies cleavage of gpI. It is thought that gpI is synthesized as a primary gene product, and may be proteolytically processed under certain conditions to gp88. gp88 consists of two smaller glycoproteins, gp65 and gp30, which are joined by disulfide bonds. A hypothetical model which summarizes these results is shown in Figure 1. Thus the

Figure 1. Model for the influenza C virus glycoprotein.

The complete form of HA, called HAp, can be detected in infected cells. HAp can also be found in virions grown in chicken embryo kidney and fibroblast cells. Step A shows HAp, which contains a disulfide bond, inserted into the viral membrane. In the presence of certain proteases, such as plasminogen, a small peptide is removed from HAp, yielding HA (Step B). HA consists of two smaller glycoproteins linked by a disulfide bond. Dissociation and reduction of HA with SDS and sulfhydryl reagents and subsequent analysis on gels demonstrates the two smaller glycoproteins (Step C) which may be analogous to HA1 and HA2 of influenza A and B viruses.

MODEL FOR THE INFLUENZA C VIRUS HEMAGGLUTININ



hemagglutinin of influenza C viruses has similarities and differences with that of influenza A and B viruses.

Neuraminidase Hirst (1942) first demonstrated the presence of a receptor destroying enzyme in influenza viruses, which has since been called neuraminidase (Gottschalk, 1957). This enzyme cleaves the glycosidic bond between N-acetylneuraminic acid and adjacent sugars. For many years it was thought that this enzyme activity was associated with the hemagglutinin molecule. Proof that HA and NA resided on unique molecules came through experiments which physically separated HA from NA (Mayron et al., 1961; Noll et al., 1962; Laver, 1963, 1964), through experiments in which the HA and NA were segregated genetically (Laver and Kilbourne, 1966), and through serologic and morphologic differentiation (Drzeniek et al., 1966, 1968; Webster et al., 1968; Laver and Valentine, 1969).

The subunit of the NA, determined by a variety of techniques, has a MW of about 50-65,000 daltons (reviewed in Bucher and Palese, 1975; White, 1974). There was initially controversy over whether the monomer is a single glycoprotein or two species of similar size. Some workers found that NA consisted of only one polypeptide (Haslam et al., 1970b; Gregoriades, 1972; Kendal and Eckert, 1972; Wrigley et al., 1973; Kendal and Kiley, 1973) while others described two polypeptides (Webster, 1970; Skehel and Schild, 1971; Bucher and Kilbourne,

1972; Lazdins et al., 1972). The second smaller component is now believed to be proteolytically derived from the larger polypeptide and it is generally accepted that the neuraminidase subunit consists of a single polypeptide of MW 50-65,000.

The influenza B virus neuraminidase has been well studied due to its resistance to SDS and its extreme stability and many of the above studies dealt with the influenza B virus NA. Results indicate that the influenza B virus NA is composed of subunits of MW 70-80,000, which are slightly larger than the subunits of the influenza A virus NA (Lazdins et al., 1972; Wrigley et al., 1973; Oxford, 1973b; Kendal, 1975; Tobita and Kilbourne, 1975; Allen et al., 1977).

The cell receptors of influenza C viruses are not neuraminidase-labile (Hirst, 1950) although the virus does possess some type of receptor destroying enzyme. It has been conclusively shown that influenza C viruses do not possess an α -neuraminidase similar to that in influenza A and B viruses (Kendal, 1975; Nerome et al., 1976). Sialic acid, which is absent in influenza A (Klenk et al., 1970b) and is probably absent in influenza B viruses (Palese et al., 1974) has been found in the envelope of influenza C viruses (Nakamura et al., 1979).

The receptor destroying enzyme of influenza C viruses has not been well characterized (Kendal, 1975; O'Callaghan et al., 1977).

Nucleoprotein The nucleoprotein was originally called the S or "soluble" antigen of influenza viruses, since it could be found in the medium of influenza virus-infected cells. This protein, which is one of the type-specific antigens of influenza A, B and C viruses (Hoyle and Fairbrother, 1937), is the subunit of the helical viral RNP (Duesberg, 1969; Joss et al., 1969; Pons et al., 1969). On polyacrylamide gels the NP migrates as a single high molecular weight polypeptide of MW 55-65,000 (Duesberg, 1969; Joss et al., 1969; Pons et al., 1969; Content and Duesberg, 1970; Haslam et al., 1970a). An interesting property of NP, in light of its presence in RNP, is its high affinity for both + and - strands of viral RNA (Scholtissek and Becht, 1971).

A nucleoprotein has been identified in influenza B viruses based solely on its quantity in the virion and on its molecular weight (Haslam, 1970a, 1970b; Oxford, 1973b; Wrigley et al., 1973; Kendal, 1975; Choppin et al., 1975). In all of these reports, the influenza B virus NP has been found to be larger than the influenza A virus NP. An amino acid analysis of influenza A and B

virus NPs revealed extensive differences (Laver and Baker, 1972).

Kendal (1975) identified a 62,000 dalton polypeptide in influenza C viruses which he suggested was the NP. Later studies showed that purified influenza C virus RNP contained a 66,000 dalton polypeptide which was most likely the NP, by analogy with influenza A and B viruses (Compans et al., 1977).

M protein The first gel electrophoresis studies of influenza virus polypeptides revealed the presence of a small (MW 20-28,000) protein which was quite distinct from the known HA, NA and NP antigens (Haslam et al., 1970a, 1970b; Compans et al., 1970b; Schulze, 1970). This polypeptide constituted one-third of the total mass of the virion and was therefore the most plentiful virion polypeptide.

Electron microscope studies of influenza A viruses had revealed the presence of an electron-opaque zone lining the inside of the viral envelope (Apostolov and Flewett, 1969; Kendal et al., 1969; Bächli et al., 1969; Compans and Dimmock, 1969; Apostolov et al., 1970). Experiments in which viruses were subjected to degradation with proteases and detergents indicated that this layer was most likely composed of the 21-28,000 dalton polypeptide detected on gels (Schulze, 1970; Compans et al.,

1970b; Schulze, 1972). This protein was called membrane or M protein (Schulze, 1970, 1972).

Influenza B and C viruses probably also contain M protein, (Haslam et al., 1970a; Oxford, 1973b; Wrigley et al., 1973; Kendal, 1975; Compans et al., 1977) which, in addition to NP, is the other type-specific antigen of influenza viruses (Schild, 1972).

Nonstructural protein(s) Dimmock and Watson (1969) first showed that viral polypeptides could be detected in virus-infected cells which were not found in purified virions. Later the existence of a distinct nonstructural protein (NS) was conclusively demonstrated (Lazarowitz et al., 1971). In this last study, peptide maps were used to confirm the separate identity of NS protein (MW 25,000) and M protein (MW 28,000). In addition, the two proteins were further distinguished by the finding that M protein is made in limiting amounts in infected cells and is found in smooth and plasma membranes, while NS is found in great quantity in infected cells and migrates to the nucleus (Lazarowitz et al., 1971).

It has been suggested that another small nonstructural polypeptide (NS2, MW 11,000) is also present in influenza virus-infected cells (Skehel, 1972; Follet et al., 1974; Minor and Dimmock, 1975; Etkind and Krug, 1975; Lamb et al.,

1978). This NS2 differs from other virus polypeptides by its tryptic map (Lamb et al., 1978) and is translated from a unique mRNA (Stephenson et al., 1977). Recent evidence hints that the information for NS2 is contained on RNA 8, along with the information for NS1 (R.A. Lamb and S. Inglis, personal communications) and the two genes may overlap.

The function of either polypeptide is unknown, although an NS1 mutant is defective in late amplification of vRNA synthesis (Barry and Mahy, 1979).

Lipid

All of the lipid in the influenza virion is located in the envelope. This lipid is composed of phospholipid (10-13%), cholesterol (6-8%) and glycolipid (1-2%) (Fromm-hagen et al., 1959; Kates et al., 1961; Blough and Merlie, 1970; Klenk et al., 1972a).

A number of studies have now demonstrated that the lipid of enveloped viruses is derived completely from the host cell (Klenk and Choppin, 1969, 1970a, 1970b; Choppin et al., 1971; Quigley et al., 1971; McSharry and Wagner, 1971; Laine et al., 1972; Renkonen et al., 1977). The main difference between the membranes of influenza A and B viruses and cellular membranes is the absence of neuraminic acid-containing glycolipids and glycoproteins in

the viral envelope, due to the presence of the viral neuraminidase (Klenk and Choppin, 1970b; Klenk et al., 1970a; Palese et al., 1974). However, influenza C virus membranes contain sialic acid (Nakamura et al., 1979) which is not surprising since the virus lacks a neuraminidase.

Carbohydrate

Influenza virions contain 5-8% carbohydrate (Fromm-hagen et al., 1959) consisting of N-acetylglucosamine, mannose, galactose and fucose. Most of the viral carbohydrate is covalently linked to the HA and NA polypeptides. It was first observed that the electrophoretic mobility of the HA of one virus strain varied according to the cell in which the virus was grown (Haslam et al., 1970a, 1970b; Compans et al., 1970b; Schulze, 1970; Schwarz et al., 1977; Nakamura and Compans, 1978). This behavior can be explained since it is now known that the glycosylation of viral glycoproteins is achieved by cellular transferases, and therefore the glycosylation pattern of viral glycoproteins is highly host-dependent (Schwarz et al., 1977; Nakamura and Compans, 1978, 1979a, 1979b). Glycosylation occurs in the endoplasmic reticulum in a manner identical to that of cellular glycoproteins (Compans, 1973; Hay, 1974) and other viral glycoproteins (Rothman and Lodish, 1977).

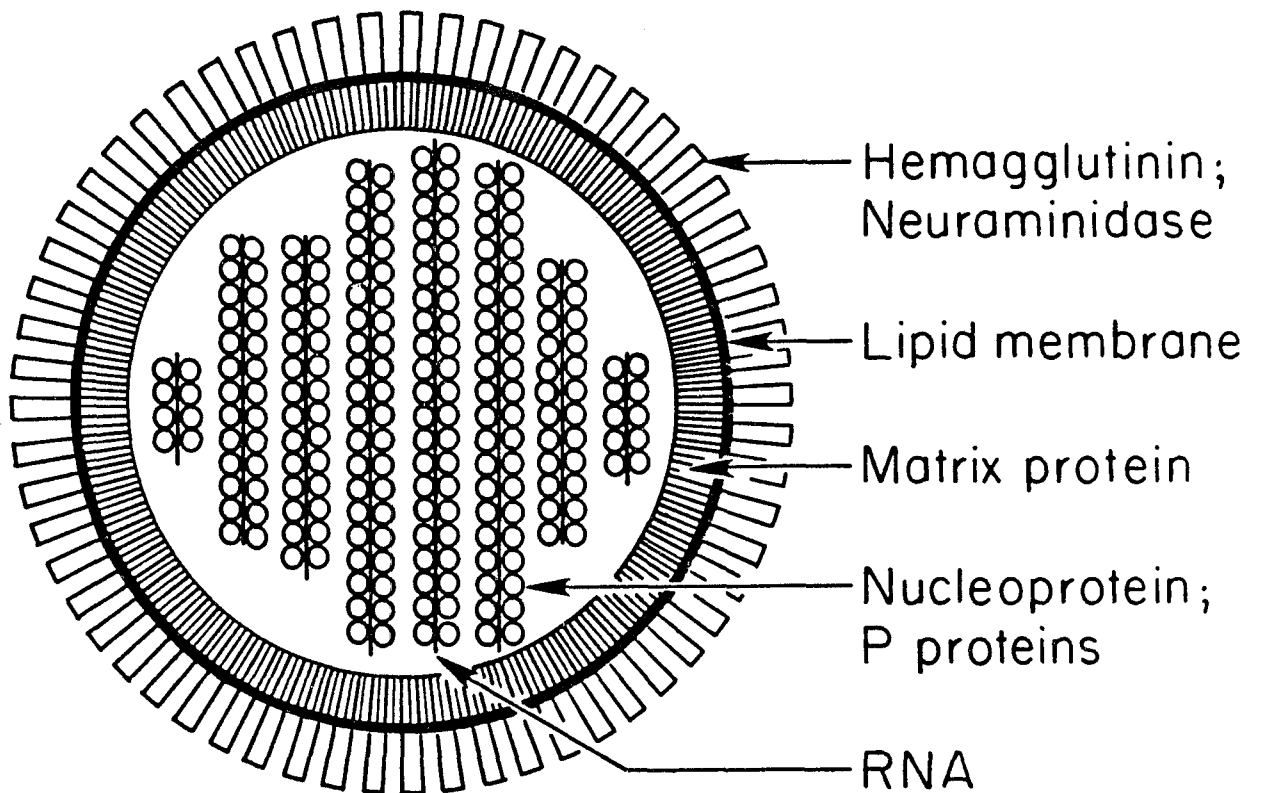


Figure 2. Schematic drawing of the influenza virus.

Structure

A schematic drawing of the influenza virion is shown in Figure 2. The virus is completely surrounded by an envelope which is a conventional lipid bilayer (Landsberger et al., 1971). The hemagglutinin and neuraminidase glycoproteins are "spikes" which are imbedded in the envelope (Laver and Valentine, 1969). When isolated from the virus, the hemagglutinin spike is shaped like a triangular rod and consists of three identical subunits which in turn are composed of disulfide-linked HA1 and HA2 moleculea (Laver and Valentine, 1969; Wiley et al., 1977). Each spike has a total MW of 215,000 daltons. The carboxyl-terminal end of the HA2 molecule is hydrophobic and is inserted into the viral membrane (Laver and Valentine, 1969; Brand and Skehel, 1972) while the HA1 portion appears to be external. The neuraminidase spike is mushroom-shaped and consists of an oblong head attached to a fiber which has a hydrophobic base and is inserted into the membrane (Laver and Valentine, 1969). It now seems likely that the neuraminidase spike is comprised of a tetramer of molecular weight 240,000, consisting of four identical subunits each 60,000 MW (Wrigley et al., 1973).

Beneath the lipid envelope is a shell of protein 6 nm thick (Apostolov and Flewett, 1969; Kendal et al., 1969; Compans and Dimmock, 1969; Bachi et al., 1969; Apostolov

et al., 1970) which is the matrix protein (Kendal et al., 1969; Compans et al., 1970a; Schulze, 1970, 1972). The M protein is of structural importance to the viral envelope, and probably also plays an important role during assembly of virions (reviewed in Compans and Choppin, 1975).

The interior core or nucleocapsid of the virions can be seen as helical, coiled filaments in negatively stained virions which are partially disrupted (Wrigley, 1979). This nucleocapsid, or RNP, can be isolated by a variety of techniques and consists of RNA in association with nucleoprotein subunits (Pons et al., 1969; Krug, 1971) and the three P proteins (Bishop et al., 1972; Inglis et al., 1976). Sedimentation and electron microscope studies indicate that the RNP is present in a discrete range of sizes (Compans et al., 1972) corresponding to the length of viral RNAs. Thus, the RNP probably exists in eight pieces containing RNA, NP, P1, P2 and P3 proteins. The RNP most likely comprises the RNA-dependent RNA polymerase of the virus particle (Bishop et al., 1972; Rochovansky, 1976). However, direct demonstration of the dependence of in vitro polymerase activity on P proteins has not been obtained, although work with ts mutants (see next section) indicates that the P proteins participate in RNA synthesis in the infected cell.

D. GENETICS OF ORTHOMYXOVIRUSES

The study of influenza virus genetics can be conveniently divided into two periods (Sugiura, 1975). In the first period, comprising the 1950s and early 1960s, experiments were performed using animal hosts. Evidence for genetic interaction of influenza viruses (Burnet and Lind, 1949), high frequency recombination (Burnet and Lind, 1951), multiplicity reactivation (Henle and Liu, 1951) and cross-reactivation (Burnet and Lind, 1954; Baron and Jensen, 1955) was obtained during this period. All of the information obtained suggested the presence of a segmented genome (see discussion in the previous section).

The second period of influenza virus genetics began with the development of plaquing techniques for influenza viruses (Simpson and Hirst, 1961). The development of this technique in Hirst's and in other laboratories led to the confirmation of earlier findings on influenza virus recombination and initiated precise biochemical studies of the genome.

The isolation of the first influenza virus temperature-sensitive (ts) mutants by Simpson and Hirst (1968) marked the beginning of the analysis of the structure and

function of the viral genome. Many ts mutants have since been isolated in a number of laboratories (Mackenzie, 1970; Mills and Chanock, 1971; Ueda, 1972; Sugiura et al., 1972; Ghendon et al., 1973; Markushin and Ghendon, 1973; Scholtissek et al., 1974; Ghendon et al., 1975; Krug et al., 1975; Scholtissek and Bowles, 1975; Spring et al., 1975; Sugiura et al., 1975; Almond et al., 1979). Many laboratories have contributed to the examination of these mutants, and several general conclusions can be drawn from these studies: (1) the ts mutants can be arranged into distinct complementation-recombination groups, (2) complementation studies support the one segment-one gene hypothesis and (3) there are a maximum of eight recombination-complementation groups, and each group contains viruses with a defect in one RNA segment.

In some cases studies have been carried out in which the phenotype of the mutant viruses was determined. However, until recently it has been very difficult to determine exactly which gene contains the lesion, with the exception of the HA and NA mutants. New techniques described below have now made it possible to determine the defective gene in any ts mutant.

The single most important advance in influenza virus genetics has been the mapping of the influenza virus genome. Techniques for separating influenza virus RNAs

have been described which led to the resolution of all eight influenza virus RNAs and their viral proteins. It is now known for several influenza virus strains exactly which RNA segments carry the information for specific polypeptides.

Basically three methods have been developed in three different laboratories for mapping the influenza virus genome. The first (reviewed in Palese, 1977) was based on the discovery that the RNAs and the proteins of two influenza virus strains, A/PR/8/34 (H0N1) and A/HK/8/68 (H3N2) migrated in unique patterns on polyacrylamide gels (Palese and Schulman, 1976a; Ritchey et al., 1977). A series of recombinants were isolated which inherited most of their RNAs from one parent and one or two RNAs from the other parent. Analysis of polypeptides of the recombinant viruses indicated which polypeptide was associated with each RNA segment. For PR/8 virus it was found that RNAs 1-8 code for P3, P1, P2, HA, NP, NA, M and NS polypeptides, respectively, while in HK/68 virus RNAs 1-8 code for P3, P1, P2, HA, NA, NP, M and NS polypeptides (Palese and Schulman, 1976b; Palese et al., 1977a; Ritchey et al., 1976b). This type of approach was also used by Almond and Barry (1979) to map the genome of two avian influenza viruses.

Another method is based on the ability of RNA from infected cells to direct the synthesis of viral proteins in an in vitro cell-free translating system. Purified RNA is annealed to different vRNA segments, and those segments which form duplexes are not translated. By noting which proteins are not made when cytoplasmic RNAs are annealed with specific vRNAs, polypeptides can be assigned to RNA segments (Inglis et al., 1977).

The third method utilizes hybridization and nucleic acid protection techniques (Scholtissek et al., 1976, 1977a, 1977b; Rohde et al., 1977, 1978). Recombinants with a wild-type phenotype are isolated from a cross between ts mutants with known defects and wild-type viruses. Different ^{32}P -labelled RNA segments of both parent viruses are annealed to an excess of complementary RNA (cRNA) extracted from cells infected with the recombinants. After calculation of the ribonuclease resistance of homologous and heterologous hybrids, the derivation of segments in the recombinant can be determined. The authors then note which RNA segment in the ts mutant is consistently "rescued" and associate that segment with a particular viral function. However, exact polypeptides cannot be assigned to specific RNA segments in cases where the function of each polypeptide is not known.

The construction of a genetic map has enabled investigators to analyze ts mutants in more detail. Palese and co-workers (Palese et al., 1977b; Ritchey and Palese, 1977) have used this new technology to identify the defective gene in several complementation-recombination groups of ts mutants. These mutants were originally isolated by Sugiura, Tobita, Ueda and Kilbourne (Ueda, 1972; Sugiura et al., 1972, 1975). Two groups contained mutants defective in complementary RNA synthesis, and two groups contained mutants defective in virion RNA synthesis (Krug et al., 1975). Two other groups contained viruses with defective HA (Ueda and Kilbourne, 1976) and NA (Palese et al., 1974) and one group consisted of viruses which did not assemble at the nonpermissive temperature (Sugiura et al., 1975). By rescuing ts mutants with wild type viruses and determining which gene was exchanged, the defective RNA in each group was determined. This analysis revealed that P1 and P3 proteins are required for cRNA synthesis (Palese et al., 1977b) and P2 protein and NP are required for vRNA synthesis (Ritchey and Palese, 1977). Mutants in the group of viruses which did not assemble at nonpermissive temperature had defects in M polypeptide (Ritchey and Palese, 1977). Recently Almond and Barry (1979) used this same method to identify the defect in 6 recombination-complementation groups of fowl plague virus.

The result of all this work is that in the last several years the protein coded for by each viral RNA segment has been identified, and precise functions of each protein have been established. This information hopefully will permit a more precise analysis of influenza virus replication.

E. REPLICATION OF ORTHOMYXOVIRUSES

The replication of influenza viruses is a complex subject which can only be dealt with briefly here. The major points of each step of replication will be discussed, with particular emphasis placed on recent developments. Nearly all of these studies have been performed with influenza A viruses.

Influenza viruses initiate infection of host cells by adsorption to cellular receptors via the hemagglutinin spike. The cellular receptors are N-acetylneuraminic acid-containing glycoproteins. The virus particle enters the cell by one of two proposed mechanisms. In viropexis (Fazekas de St. Groth, 1948), virions penetrate the cell via a pinosome which eventually fuses with a lysosome, leading to uncoating of the virus. Morgan and Rose (1968), believe that viruses enter the cytoplasm through fusion of the viral envelope with the host cell membrane. Experiments have been presented in support of both mechanisms,

and it may be that some combination of the two occurs. At any rate, after penetration the M protein is stripped from the viral core and the resulting RNP is probably not uncoated further.

Since the input RNP possesses polymerase activity it is thought that this complex is responsible for what is known as primary transcription. This term describes the RNA synthesis which occurs before viral protein synthesis begins, and is experimentally achieved by infecting cells in the presence of cycloheximide. Primary transcription results in the production of mRNAs complementary to each of the eight viral RNA segments. These molecules contain poly(A), and 7-methylguanosine in cap structures, the latter of which are added by host enzymes (Plotch et al., 1978). The mRNAs are incomplete transcripts of vRNAs since they lack sequences complementary to the 5' end of vRNA (Hay et al., 1977b). The presence of internal N⁶-methyladenosine (Krug et al., 1976) indicates that the mRNA molecules are most likely synthesized in the cell nucleus.

If cells are restricted to 1^o transcription by the presence of cycloheximide, mRNAs to all eight RNA segments are made in roughly equal amounts. Upon release of the cycloheximide block, all eight viral proteins are synthesized from these mRNAs. One or more of these

proteins is required for the generation of a second class of cRNA molecules which do not contain poly(A) and which are complete transcripts of all 8 vRNA segments (Hay et al., 1977b). The exact regulatory mechanism which allows for the synthesis of complete transcripts as opposed to virus-specific mRNA molecules is not known. The complete cRNA molecules most likely serve as templates for the synthesis of minus-strand vRNA segments. The enzyme (replicase) responsible for the synthesis of vRNA has not been isolated, but studies of ts mutants indicate that at least P2 protein and NP are required for this step (Ritchey and Palese, 1977). The site of synthesis of vRNA has not been unequivocally identified.

Newly synthesized vRNA can be used as a template for the synthesis of additional cRNA and mRNA molecules. The mRNAs for individual segments which are made as a result of secondary transcription are made in different amounts, in contrast to the mRNA products synthesized by primary transcription (Hay et al., 1977a). This differential synthesis of secondary transcripts results in a control of protein synthesis at the transcriptional level. As more vRNA molecules are made, mRNA synthesis is "amplified". Later in infection vRNAs are packaged into virions for eventual release.

Several puzzling features of influenza virus replication have become less enigmatic in light of several recent reports. It has been known that certain treatments of the host cell such as administration of mitomycin C, α -amanitin, actinomycin D, UV or removal of the cell nucleus prevents multiplication of influenza viruses (Barry et al., 1962; Rott et al., 1965; White and Cheyne, 1965; Nayak and Rasmussen, 1966; Mahy et al., 1972; Follett et al., 1974; Kelly et al., 1974). It was found that influenza viruses will replicate in the presence of α -amanitin only in cells which contain a mutant RNA polymerase II which is resistant to α -amanitin (Lamb and Choppin, 1977; Spooner and Barry, 1977). An explanation of the sensitivity to the other agents was not as clear; for example, actinomycin D has no effect on the influenza viral polymerase in vitro (Chow and Simpson, 1971; Penhoet et al., 1971).

It now seems that some of these observations might be explained by some recent discoveries. The influenza viral polymerase reaction in vitro is greatly stimulated by the addition of one of several dinucleotides (McGeoch and Kitron, 1975; Plotch and Krug, 1977) such as ApG, which may be incorporated into the 5' end of viral transcripts (Plotch et al., 1978; Plotch and Krug, 1978).

It has been postulated therefore that a cellular primer is required for influenza transcription in vivo (Plotch and Krug, 1977; Plotch and Krug, 1978). Most recently it was shown that the in vitro polymerase reaction may be primed with globin message containing 7-methylguanosine in cap structures (Bouloy et al., 1979) and that the cap and a portion of the eukaryotic message is transferred to the synthesized influenza viral cRNA (Plotch et al., 1979). Thus, the requirement for a capped cellular message as a primer for influenza virus transcription may explain why influenza virus replication depends on intact host cell nuclear functions. However, proof that this type of splicing occurs in vivo requires direct sequence data on the 5' end of viral mRNA extracted from infected cells.

Another intriguing question in influenza virus replication is the role of the NS protein(s). Only one mutant of NS1 has been isolated to date, and this mutant has a somewhat altered pattern of late RNA synthesis (Barry and Mahy, 1979). Since this polypeptide is made very early in infection, it may indeed play an important role in RNA synthesis.

Proteins in influenza virus-infected cells are synthesized starting early in infection and are made at different rates (Skehel, 1972, 1973; Meier-Ewert and Compans,

1974; Inglis et al., 1976; Lamb and Choppin, 1976; Inglis and Mahy, 1979). Glycoproteins are synthesized on rough ribosomes, migrate to smooth membranes and then to plasma membranes (Compans, 1973; Klenk et al., 1974; Hay, 1974; Meier-Ewert and Compans, 1974). During this process blocs of carbohydrate are added to glycoproteins in distinct steps by cellular transferases (Compans, 1973; Stanley et al., 1973; Klenk et al., 1977; Nakamura and Compans, 1979a, 1979b). The NS protein, NP and P proteins are synthesized in the cytoplasm, and NP and NS protein migrate rapidly to the nucleus. M protein is associated with cellular membranes (Compans, 1973; Klenk et al., 1974).

Assembly and release of influenza virions has been studied by electron microscopy (Compans and Dimmock, 1969; Bächli et al., 1969; Compans et al., 1970a) and is envisioned to occur in the following steps. The glycoproteins HA and NA are inserted into a region of the plasma membrane, at which time HA is probably cleaved (Lazarowitz et al., 1971). M protein then collects at discrete sites beneath the membrane where glycoproteins have been inserted. Assembled RNP associates with the M protein and the whole assembly pinches out and buds off from the cell as a complete virus particle. Neur-

aminidase removes N-acetylneuraminic acid from the viral glycoproteins and thus facilitates release of the virus from the cell (Palese et al., 1974).

F. EPIDEMIOLOGY OF INFLUENZA

Influenza viruses continue to cause widespread disease due to their ability to undergo antigenic change, a strategy which foils the body's immune system. Influenza A viruses cause two types of disease which are distinguished epidemiologically. Pandemic influenza involves worldwide spread of disease and epidemic influenza consists of regional outbreaks.

Influenza epidemics are associated with different variants of influenza viruses which may emerge every year. This type of change in influenza viruses has been coined antigenic drift (Burnet, 1955), and is thought to result from the selection by an immune population of variant viruses which possess a growth advantage in the presence of antiviral antibody. The precise mechanism of the antigenic drift is not known, but seems to be mediated by changes in viral proteins due to point mutations in the RNAs. Antigenic drift has been duplicated in the laboratory (reviewed in Webster and Laver, 1975) and antigenic variants selected in vitro have altered HA sequences (Laver and Webster, 1968; Laver et al.,

1979). In a recent study, a series of H1N1 variants were examined by RNA fingerprinting and sequencing techniques (Young et al., 1979). The authors showed that (1) sequential mutations occur in the genome, and (2) mutations are not restricted to the surface glycoproteins. This finding suggested that antibody pressure, as was thought previously, (Archetti and Horsfall, 1950; Hamre et al., 1958; Francis and Maassab, 1965) is not the only factor responsible for the emergence of antigenic variants.

Recently, examination of a new H1N1 variant (A/Cal/10/78) by RNA fingerprinting techniques indicated that it was a recombinant of an A/Tex/77 (H3N2)-like strain and an A/USSR/77 (H1N1)-like strain, two viruses which had co-circulated in the population for some time (Young and Palese, submitted). This finding indicates that variants of influenza viruses may arise by recombination as well as by point mutation.

Antigenic drift has also been observed with influenza B viruses (Chakraverty, 1971, 1972) and possibly with influenza C viruses (Chakraverty, 1978).

Pandemic influenza is caused by the emergence of a completely different virus to which the population is not immune. In the last 40 years new pandemic strains

have arisen every 10-15 years. Emergence of new pandemic strains involves dramatic antigenic changes in either the HA or the HA and the NA polypeptides. A widely accepted theory for the emergence of pandemic strains involves generation of new viruses by recombination of human and animal strains. Some of the evidence which has accumulated in recent years in support of this theory includes (1) isolation of human/animal virus recombinants in vitro (Kilbourne, 1968); (2) formation and transmission in animals of recombinants generated under natural conditions (Webster et al., 1973; Webster and Campbell, 1974); (3) isolation of avian virus recombinants in nature (Desselberger et al., 1978); (4) serological data (Coleman et al., 1968; Laver and Webster, 1973) and peptide mapping data (Laver and Webster, 1972) which indicates that isolated HA of H3, Hav7 and Heq2 subtypes are closely related, and peptide maps show that the NA from late Asian strains (H2N2) and early Hong Kong strains (H3N2) are markedly similar (Laver, 1978); (5) base sequence homology studies which indicate that 7 RNAs of A/HK/8/68 (H3N2) strain are closely related to 7 RNAs of H2N2 strains, and RNA 4 (the H3N2 HA gene) did not originate from the H2N2 strain and may be related to influenza A/duck/Ukraine/63 or A/equine/Miami/63 viruses

(Scholtissek et al., 1978b). Other serologic data (Pereira et al., 1967; Webster and Pereira, 1968; Schild and Newman, 1969; Meier-Ewert et al., 1970) indicate that there are certain degrees of relationship between human and animal influenza viruses.

Antigenic shift has not been observed in influenza B viruses, and it has been suggested that this absence is due to the failure of influenza B viruses to multiply in an animal reservoir (Pereira, 1969).

A discussion of an interesting recent development in influenza epidemiology concludes this section. In early 1977, H1N1 viruses were isolated from epidemics of influenza in China, and these viruses have subsequently been isolated all over the world. Analysis of the viruses by oligonucleotide mapping (Nakajima et al., 1978) and RNA-RNA hybridization (Scholtissek et al., 1978a) indicated that they were very similar to H1N1 viruses isolated in 1950 but not to H1N1 viruses isolated before or after that date. Serologic data (Kendal et al., 1978) confirmed that the surface proteins of these viruses were similar to the surface proteins of viruses isolated in 1950. The re-emergence of these viruses after 27 years in the absence of more extensive genetic changes excluded the possibility that they were generated by a recombinational

event. At the time of this writing these H1N1 viruses continue to be isolated, and many variants have already emerged. Thus, a new influenza virus pandemic has appeared in an unusual and as yet unexplained fashion.

G. GOALS OF RESEARCH

At the time this work was begun, the genetic map of influenza A viruses had just been completed. The influenza A and B viruses differ biologically and epidemiologically in many ways, and therefore it was of interest to compare these two virus types on a molecular level in an attempt to explain these differences. This project evolved from a need to more fully characterize the influenza B virus genes and gene products, and the following goals were initially established:

- (1) determine the number of RNA segments in influenza B viruses
- (2) identify all of the influenza B virus-specific polypeptides
- (3) construct a genetic map of influenza B viruses
- (4) compare the sizes of genes and gene products of influenza A and B viruses
- (5) compare the genomes of influenza B viruses by oligonucleotide mapping of RNA

While the first four projects were completed, and work was progressing on the fifth, it was realized that the sophisticated techniques which had been learned could be well applied to questions about the infrequently-studied influenza C viruses. Very little was known about the biochemistry of these viruses and therefore the following projects were proposed:

- (1) develop a plaquing system for influenza C viruses in order to study the molecular biology of these viruses
- (2) determine the number of RNA segments in the influenza C virus genome
- (3) identify all of the influenza C virus-specific polypeptides
- (4) compare the genomes of different influenza C viruses by oligonucleotide fingerprinting of RNA
- (5) investigate whether or not recombination (reassortment) occurs between different influenza C viruses

These studies elucidated the basic structure of the genomes of influenza B and C viruses. In order to compare the fine structure of RNA segments from these viruses, it was proposed to sequence the 5' and 3' ends

of genes from an influenza A, an influenza B and an influenza C virus.

All of the above aims were fully realized with the result that a great deal of basic knowledge about the genes and gene products of influenza B and C viruses has been contributed. This new information should serve as a foundation for more extensive molecular analyses on influenza B and C viruses than was previously possible.

VII. MATERIALS AND METHODS

A. CELL CULTURE AND VIRUSES

Media and buffers

Minimal essential medium (MEM) containing Earle's salts (Eagle, 1959) was purchased from Grand Island Biological Co. (GIBCO) as a 10x concentrated liquid without L-glutamine and NaHCO_3 . Reinforced MEM (REM) was specially prepared by Microbiological Associates, Inc. and contains twice the amount of amino acids, four times the amount of vitamins and four and one-half times the amount of glucose as MEM. In addition, REM contains 0.4 M $\text{Fe}(\text{NO}_3)_3$ and 1 mM sodium pyruvate (Bablanian et al., 1965). Phosphate buffered saline (PBS) is an isotonic solution buffered to pH 7.5 with $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, which also contained 0.026 M KCl (Dulbecco and Vogt, 1954). Trypsin-EDTA (disodium ethylenediamine tetraacetate) solution for dissociation of cells was prepared in PBS and contained 0.1% trypsin (w/v) (DIFCO 1:250), 0.04% (w/v) EDTA, and 0.025% (w/v) NaHCO_3 . Virus diluent is PBS containing 100 units/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate and 190 $\mu\text{g}/\text{ml}$ bovine albumin, (fraction V, Miles Biochemicals). NTE is 10 mM Tris·HCl pH 7.4, 100 mM NaCl and 1 mM EDTA.

Cell culture

Madin-Darby canine kidney cells (MDCK: Gaush et al., 1966) used in these experiments were obtained from the American Type Culture Collection, Beltsville, Md. This continuous cell line was grown in 75 cm² plastic bottles (Falcon Plastics, Oxnard, CA) in 15 ml MEM containing 10% (v/v) heat treated fetal bovine serum (Flow Laboratories, Rockville, Md.), 5% (w/v) NaHCO₃, 0.292 mg/ml L-glutamine (Flow Laboratories), 100 units/ml penicillin G and 100 µg/ml streptomycin sulfate. Monolayers were dissociated with trypsin-EDTA and cells were pelleted and seeded in new bottles at 2 x 10⁶ cells/bottle. Monolayers were usually confluent after incubation for 3-4 days at 37°C in a humidified incubator containing an atmosphere of 5% CO₂. For growing viruses, MDCK cells were seeded in 25 cm² plastic tissue culture dishes (Falcon Plastics, Oxnard, CA) at 1.0 x 10⁶ cells/dish and grown under the same conditions as described for bottles.

Viruses

Table 3 lists the different wild-type influenza viruses used in this study.

TABLE 3

VIRUSES USED IN THIS STUDY

<u>Strain</u>	<u>Source</u>	<u>Abbreviation</u>
A/PR/8/34 (H0N1)	Dept. of Microbiology Mt. Sinai School of Medicine	PR8
B/Lee/40	"	B/Lee/40
B/Great Lakes/54	"	B/GL/54
B/Maryland/59	"	B/Md/59
B/Massachusetts/66	"	B/Mass/66
B/USSR/69	M. Lobmann, Belgium	B/USSR/69
B/Hong Kong/5/72	M. Lobmann, Belgium	B/HK/72
B/Hong Kong/8/73	Dept. of Microbiology Mt. Sinai School of Medicine	B/HK/73
C/Taylor/47	"	C/Tay/47
C/JJ/50	R.J. O'Callaghan and C. Howe, New Orleans, La.	C/JJ/50
C/AA/50	H.F. Maassab, Ann Arbor, MI	C/AA/50
C/Great Lakes/54	Dept. of Microbiology Mt. Sinai School of Medicine	C/GL/54
C/Johannesburg/66	"	C/JHG/66
C/New Jersey/76	A.P. Kendal, Atlanta, GA	C/NJ/76

Propagation

In embryonated eggs Seed stocks of all viruses were prepared by inoculating 10-11 day old embryonated hen eggs with tenfold serial dilutions of virus (from 10^{-3} to 10^{-7}) in virus diluent. Influenza A and B viruses were propagated in the allantoic cavity. After 36-48 hours incubation at 35°C , eggs were chilled and the allantoic or amniotic fluid was removed and clarified by centrifugation at $1000 \times g$ for 15'. Virus was quantitated by hemagglutination (HA, see below), and those fluids containing the highest HA titre resulting from a 10^{-4} to 10^{-6} dilution of inoculum were divided into aliquots, quick frozen in dry-ice ethanol and stored at -70°C .

In tissue culture Preparation of cloned virus stocks in MDCK cells was performed by first plaquing egg-grown seed virus as described in the section on plaque assay of virus. Virus particles in individual plaques were recovered by removing the block of agar above a plaque with a plastic 1.0 ml serological pipette. The agar block was suspended in 0.5 ml of virus diluent and vortexed vigorously. Confluent MDCK monolayers in 25 cm^2 dishes were washed free of

growth medium with PBS and infected with 0.2 ml of the plaque suspension. Virus was allowed to adsorb at 37°C (influenza A and B viruses) or 34°C (influenza C viruses) for 50 minutes. After that time, 3.0 ml of maintenance medium was added per dish, which consisted of REM containing 0.21% (w/v) NaHCO₃, 0.21% (w/v) bovine albumin, 10.5 mM HEPES, Earle's balanced salt solution (Flow Laboratories), 100 units/ml penicillin G, 100 µg/ml streptomycin sulfate, 2 µg/ml trypsin (Worthington Biochemicals, Freehold, N.J.) and, for influenza C viruses, 20 µg/ml human plasminogen (Worthington Biochemicals). Dishes were incubated at 37°C (influenza A and B viruses) or 34°C (influenza C viruses) for 2 days (influenza A and B viruses) or 4 days (influenza C viruses) or until extensive cytopathic effects were observed, i.e., greater than 70% of cells were detached. Cell fluids were harvested, clarified by centrifugation at 1000 x g for 15' and either stored in aliquots or used to make additional seed in embryonated eggs.

Procedures for infecting MDCK cells for plaque assay, labelling with isotopes or formation of recombinants are described in individual sections.

Assay of virus

Hemagglutination assay (HA) The hemagglutination assay (HA) described by Salk (1944) was adapted to microtiter scale (Sever, 1967). Serial twofold dilutions of virus in PBS were made in 96 well V-bottom microtiter plates (Dynatech Labs., Inc., Alexandria, Va.). Human type "O" erythrocytes were added to each well to a final concentration of 0.25% (v/v) and the plates were mixed on a Cooke MicroMixer. For influenza A and B viruses, titrations were performed at room temperature, and titrations of influenza C viruses were done on ice. The hemagglutination titer is reported as the reciprocal of the dilution of the last well where hemagglutination is observed (the dilution of virus in the first well is 1:8).

Plaque assay Influenza viruses were routinely plaqued in MDCK cells. Conditions used for influenza A and B viruses have been described before (Gaush and Smith, 1968) and included trypsin in the agar overlay (Tobita, 1975; Tobita et al., 1975); influenza C viruses were plaqued under different conditions which were developed in this study. Ten-fold serial dilutions of virus were made, and 0.1 ml of each dilution was used to infect MDCK monolayers, in duplicate, as is described in the

section on virus propagation in tissue culture. After the adsorption period, the inoculum was left on the monolayers and 3.0 ml of overlay was added to each dish. For influenza A and B viruses, the overlay consisted of REM containing 0.12% (w/v) NaHCO_3 , 0.21% (w/v) bovine albumin, 10.5 mM HEPES, Earle's balanced salt solution (Microbiological Associates, Bethesda, Md.), 100 $\mu\text{g}/\text{ml}$ DEAE-dextran, 0.6% (w/v) agar (Oxoid Purified Agar, Oxoid, Ltd., England), 2 $\mu\text{g}/\text{ml}$ trypsin (Worthington Biochemicals, Freehold, N.J.), 100 units/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate. The overlay for influenza C viruses was similar except that it contained 0.5% agar and 20 $\mu\text{g}/\text{ml}$ human plasminogen (J.L. Schulman, personal communication). After the overlay medium solidified, dishes were inverted and incubated at 37°C (influenza A and B viruses) or 34°C (influenza C viruses) in a humidified incubator containing an atmosphere of 5% CO_2 . Plaques were visible within 3-4 days and were usually counted by illuminating the dishes from the side with a high intensity lamp.

Plaques were either isolated as described under plaque assay, or stained for permanent records and for photography, by removing the agar overlay and applying a

solution of 0.1% (w/v) crystal violet in 50% (v/v) ethanol. After 15' at room temperature, excess stain was washed off with running tap water and dishes were allowed to air dry.

Purification

Virus was purified from infected eggs by first clarifying the pooled allantoic or amniotic fluid as described in the section on growth of virus in eggs. Supernatants were then concentrated on a 1.5 ml cushion of 60% (w/v) sucrose in NTE by centrifugation in an SW27 rotor (Beckman Instruments, Palo Alto, CA) for 90 minutes at 25,000 rpm. The supernatant was aspirated off until approximately 2 ml of egg fluid remained above the sucrose cushion. The cushions were mixed and drawn up and down 15 times through a 20 ml syringe fitted with a 20-gauge needle. For influenza A and B viruses, the concentrate was layered on a continuous gradient of 30%-60% (w/v) sucrose in NTE formed in an SW27 tube, and centrifuged at 25,000 rpm for 3 hours. For influenza C viruses, the gradient was 20%-60% sucrose in NTE and centrifugation was at 25,000 rpm for 6 hours. These conditions were used for influenza C viruses to achieve better separation of the virus band from host cell

material. After centrifugation, the visible virus band was removed by puncturing the side of the tube with a syringe. The band was then diluted 1:4 (v:v) in NTE and virus was pelleted in an SW27 rotor for 90' at 25,000 rpm. Pellets were resuspended in a small volume of NTE and rebanded in sucrose gradients as described above.

The procedure for purifying virus from infected cells varied depending on the purpose of the experiment, and is therefore described in individual sections.

Isolation of recombinant viruses

Recombinants between different influenza B viruses and between different influenza C viruses were isolated in MDCK cells. Recombinants between B/Lee/40 and B/Md/59 viruses were isolated using techniques employed for the isolation of influenza A virus recombinants (Palese et al., 1977b). MDCK monolayers were mixedly infected with B/Lee and B/Md viruses. It has been noted that, in such a mixed infection with influenza A viruses, the multiplicity of infection (MOI) can influence the gene composition of the resulting recombinants (Schulman and Palese, 1976). Therefore, depending on which parental RNAs were desired to predominate in the recombinant, one parent was present at an MOI of 10 PFU/cell and the other was present at 1 PFU/cell. After a 50' adsorption at

37°C, monolayers were washed, and maintenance medium (see Growth of virus in tissue culture) was added. Cells were washed again after 3 hours and incubated at 37°C for an additional 14 hours until most cells had detached from the plastic dish. Culture fluids were centrifuged at 1000 x g for 15' to remove cell debris and then plaqued in MDCK cells in the presence of various concentrations of hyperimmune rabbit antisera (see Formation of antibodies) directed against the surface proteins of B/Lee or B/Md virus. The resulting plaques were isolated and virus within plaques was grown up in MDCK cells and seeds were made in embryonated eggs.

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, while C/JHG/66 virus yielded turbid plaques which could not be counted. An egg-grown seed stock of C/JJ/50 virus (HA 1024/ml) was diluted 1:10, and 1.5 ml of the dilution was placed in a 25 cm² plastic tissue culture dish. The virus was then irradiated for 15 seconds with UV light from an 8 watt bulb (General Electric G.8.T5) placed 15 cm above the surface of the dish. The irradiated virus was mixed 1:1 (v:v) with a 1:10 dilution of C/JHG/66 egg-grown seed virus (HA 1024/ml). The mixture was then used

to infect MDCK monolayers, allowed to adsorb 50' at 34°C, and then incubated under maintenance medium containing 20 ug/ml plasminogen. Control dishes were also infected with C/JHG/66 virus and UV-irradiated C/JJ/50 virus. After 3 days at 34°C, cell fluids were harvested, clarified, and plaque-assayed in MDCK cells. In dishes infected with the recombinational mixture, agar blocks above individual plaques were removed and assayed for plaque forming units as described under Plaque assay of virus. After a second plaque passage, virus was propagated first in MDCK cells and then in embryonated hen eggs.

B. IMMUNOLOGIC TECHNIQUES

Formation of antibodies

In order to raise antibodies against specific virus strains, influenza viruses were first grown in approximately 30 embryonated eggs and purified as described above. After the final sucrose gradient, virus was pelleted again and resuspended in 1.0 ml of NTE. An aliquot of the purified virus containing 1500 HA units was diluted to 1.5 ml with PBS and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories) by passing the mixture up and down through a 20 gauge syringe needle. One ml of the emulsion was then injected intra-

muscularly into a 6-7 lb. New Zealand white rabbit. At this time a bleeding from an ear vein was also taken. Three weeks later another ear bleeding was performed, and a booster consisting of 500 HA units of virus in 0.1 ml PBS was injected intravenously into an ear vein. Seven days after the booster a bleeding was taken, and HI assays (see Hemagglutination-inhibition) were performed. If antibody titers showed a rise after the booster injection, the rabbit was exsanguinated through the heart.

The collected blood was allowed to clot, was centrifuged (1000 x g for 15') and the serum was removed. Serum was treated with receptor destroying enzyme (RDE) as described previously (Robinson and Dowdle, 1969). V. cholerae strain 4Z RDE was obtained as a lyophilized preparation from the WHO Collaborating Center for Influenza, Center for Disease Control, Atlanta, Ga. The enzyme (10,000 units) was resuspended in water and diluted to 200 units/ml in calcium saline solution (6 mM CaCl_2 , 150 mM NaCl , 20 mM H_3BO_3 , 0.1 mM $\text{Na}_2\text{B}_4\text{O}_7$). One volume of rabbit serum was then mixed with 4 volumes of RDE in calcium saline and incubated at 37°C for 12-18 hours. The enzyme was inactivated by addition of 3 volumes

(based on serum volumes) 2.5% (w/v) sodium citrate and heating 56° for 30'. The final dilution of serum was brought to 1:10 by adding 2 volumes of PBS. Treated serum was stored at 0°C.

Hemagglutination-inhibition (HI)

The HI test of Sabin (1944) was performed with micro-titer equipment (Sever, 1967). Virus to be tested was adjusted to a titer of 16-32 HA units, and antiserum was usually RDE-treated. Twofold serial dilutions of antiserum were made in one row of a 96-well microtiter plate; the adjusted virus was added to each well (as one-fourth of the final volume) and human "O" erythrocytes were added to a final concentration of 0.25% (v/v). The plates were shaken on a Cooke MicroMixer and titers were read after 1-2 hours. The HI titer is reported as the reciprocal of the highest serum dilution at which hemagglutination is inhibited (the dilution of serum in the first well is 1:4, and the serum is already diluted 1:10 from RDE treatment, so the dilution in the first well is actually 1:40). Normal rabbit serum is also tested to assess the presence of inhibitors in the serum.

Neuraminidase-inhibition (NI)

Neuraminidase assay was performed as described by Aymard-Henry et al. (1973). Ten embryonated eggs were inoculated with each virus strain, and virus was purified as described above. Virus was pelleted again after the final sucrose gradient, and resuspended in 1.0 ml NTE. Dilutions of purified virus were made in 0.4 M sodium phosphate buffer pH 5.9, and 0.1 ml aliquots were mixed with 0.05 ml fetuin (Colorado Serum Company Laboratories, Denver, Colorado), 24 mg/ml in water, and 0.05 ml of 0.4 M sodium phosphate buffer pH 5.9. After incubation at 37°C overnight, the mixture was cooled and 0.1 ml of periodate reagent (0.2 M NaIO₄, 9 M H₃PO₄) was added. This mixture was allowed to stand 20' at room temperature and then 1 ml of arsenite reagent (0.77 M NaAsO₂, 0.5 M Na₂SO₄, 54 mM H₂SO₄) was added. The mixture was vortexed until the brown color disappeared, 2.5 ml of thiobarbituric acid reagent (0.8 M Na₂SO₄, 0.04 M thiobarbituric acid) was added, and after immersion in a boiling water bath for 15 minutes, the tubes were cooled to room temperature. The color was extracted from the reaction mixture with 4 ml of 0.55 M HCl in n-butanol, and the optical density of the organic phase was measured at 549 nanometers versus a blank which was run through the assay in the presence of saline instead of virus.

The neuraminidase-inhibition assay for influenza B viruses was performed using purified virus which was treated with pronase. This treatment was performed to avoid steric interference of the viral HA, which has been noted elsewhere (Chakraverty, 1972). Inhibition of the enzyme is carried out by mixing 0.05 ml of the virus dilution with 0.05 ml of untreated antiserum and allowing the mixture to stand 30' at room temperature. The remainder of the NA assay is then carried out as described above. Controls containing antiserum only and normal rabbit serum plus virus were also performed. The NI titer is reported as the dilution of antiserum causing 50% inhibition of NA activity.

C. BIOCHEMICAL TECHNIQUES

Labelling virus with radioactive isotopes

Viral RNA

RNA of influenza A, B and C viruses was labelled with ^{32}P according to established techniques (Ritchey et al., 1976a; Palese and Schulman, 1976a, 1976b). To label viruses in cells, MDCK monolayers in 25 cm² dishes were washed free of growth medium with phosphate-free mainte-

nance medium (maintenance medium as described in the section on growing virus in cells), which was made with phosphate-free REM specially prepared by Microbiological Associates, Bethesda, Md.). Monolayers (2 per virus) were infected with 0.2 ml of undiluted seed virus and virus was allowed to adsorb for 30' at the appropriate temperature. The inoculum was then removed and the monolayers were infected a second time. At the end of the second adsorption period, the inoculum was removed, and 3.0 ml of phosphate-free maintenance medium containing 2.5 mCi of ^{32}P (as H_3PO_4 in water, ICN Pharmaceuticals, Irvine, CA) and 2 $\mu\text{g}/\text{ml}$ trypsin were added per dish. The overlay for influenza C viruses also contained 20 $\mu\text{g}/\text{ml}$ human plasminogen. After 14-18 hours incubation at the appropriate temperatures (37°C for influenza A and B viruses, 34°C for influenza C viruses) cell fluids were harvested, clarified by brief centrifugation at 1000 x g for 15' and layered on preformed gradients of 30%-60% (w/v) sucrose in NTE (20%-60% for influenza C viruses). Unlabelled virus (2-3 ml) was also added so that the virus band would be visible after centrifugation. The gradients were centrifuged for 3 hours at 25,000 rpm in an SW27 rotor (6 hours for influenza C viruses) and the visible

virus bands were removed through the side of the centrifuge tube with a syringe. RNA was extracted from virus as described in the section on extraction of viral RNA. Influenza C virus RNA was labelled with ^{32}P in ovo by inoculating the amniotic cavity of embryonated hen eggs with 0.1 ml of a 10^{-3} dilution of virus containing 5 mCi of ^{32}P . After two days incubation at 35°C , amniotic fluid was harvested and virus was purified as described above for infected cell fluids. Usually 10 eggs per strain were inoculated in this way.

Viral proteins

Polypeptides of infected cells were pulse labelled at various times after infection with L- ^{35}S -methionine (Amersham Corp., Arlington Heights, Ill.). MDCK monolayers on 25 cm^2 dishes were infected with 0.2 ml of undiluted virus seed (MOI 1-10 PFU/cell). After adsorption, the inoculum was left on the monolayers and 3.0 ml/dish of maintenance medium containing 1 $\mu\text{g/ml}$ trypsin and, for influenza C viruses, 20 $\mu\text{g/ml}$ plasminogen was added. Unless otherwise indicated, influenza A and B virus-infected cells were pulse-labelled at 6 hours post infection, and influenza C virus-infected cells were pulse-labelled at 12-24 hours post infection. At the appropriate time, medium was removed, cells were washed with warm PBS,

and 2.0 ml of warm protein labelling medium containing 80 uCi L-[³⁵S]-methionine was added per dish. Protein labelling medium was Hanks' balanced salts solution (Ca⁺⁺ and Mg⁺⁺ free) containing 0.5% (w/v) NaHCO₃, .004% (w/v) phenol red, 100 units/ml penicillin G, 100 ug/ml streptomycin sulfate and 0.2% (w/v) glucose. Cells were incubated for an additional 15', and then the medium was removed, cells were washed with ice-cold PBS and cells were scraped into 3.0 ml of cold PBS using a rubber policeman. The cells were pelleted (1000 x g, 15'), the supernatant was discarded and the walls of the tube were dried with a paper towel. Cells were then processed as described under electrophoresis of proteins.

In some experiments, hypertonic medium was used to selectively suppress host protein synthesis in virus-infected cells (Nuss et al., 1975). At appropriate times after infection, cells were washed as described above and preincubated for 15' in protein labelling medium containing an additional 150 mM NaCl. Label was either added directly to the medium, or the cells were washed free of hypertonic medium and regular protein labelling medium containing L-[³⁵S]-methionine was added to dishes. In either case, pulse-labelling was performed as described above.

In contrast, for obtaining purified influenza A and B viruses continuously labelled with L-[³⁵S]-methionine, approximately 5 MDCK cell monolayers were infected as described above, and at 4 hours postinfection monolayers were washed with warm PBS, and 2.0 ml warm protein labelling medium containing 200 uCi L-[³⁵S]-methionine was added per dish. Incubation was continued for 4 hours, and then an additional 2.0 ml of normal maintenance medium (see the section on growing virus in cells) was added per dish. Cell fluids were harvested when CPE was more than 80% complete (usually 14-18 hours after infection). After clarification, cell fluids were layered on 30%-60% sucrose gradients in NTE, and centrifuged for 3 hours at 25,000 rpm (SW27). The virus band was removed, diluted and pelleted as described before. The final pellet was dissolved in 50 ul of H₂O, and aliquots were processed as described under electrophoresis of viral proteins.

Purified influenza C viruses continuously labelled with L-[³⁵S]-methionine were obtained either from MDCK cells or from embryonated eggs. For labelling in MDCK cells, usually 5-10 monolayers were infected with cell grown virus essentially as described above for pulse-labelling of infected cells. At 6-12 hours postinfection,

monolayers were washed and 2.0 ml/dish of Dulbecco's MEM, free of methionine (a gift of R.A. Lamb, Rockefeller University, N.Y.) was added, containing 200 uCi L-[³⁵S]-methionine. Labelling proceeded for 6-12 hours, at which time 2.0 ml of normal maintenance medium was added per dish. Cell fluids were harvested as for influenza A and B viruses, except that after clarification, virus was pelleted in an SW41 rotor at 35,000 rpm for 60', banded in the same rotor on 20%-60% sucrose gradients in NTE (35,000 rpm x 90') and repelleted. The final pellet was dissolved in approximately 50 ul of H₂O.

For labelling the proteins of purified influenza C viruses in embryonated eggs, virus stocks were diluted to 10⁻³, and 0.1 ml of this dilution containing 100 uCi L-[³⁵S]-methionine was injected into the amniotic cavity of a 10-11 day old embryonated egg. After incubation and harvesting (see section on growing virus in eggs) virus was purified from egg fluids in an SW41 rotor as described above for influenza C viruses labelled in cells.

Glycoproteins of influenza A, B and C viruses were labelled with D-[6-³H]-glucosamine hydrochloride (New England Nuclear Corp., Boston, MA) in the following way.

MDCK monolayers were infected as described above for labelling with ^{35}S , and after adsorption normal maintenance medium was added (2.0 ml/dish) containing 200 uCi ^3H -glucosamine and the appropriate proteases (see above). Normally, 5 dishes per virus were infected. Label was present throughout infection, cell fluids were harvested, and virus was purified as described above for ^{35}S -methionine labelling of virus.

Extraction of viral RNA

Purified virus (labelled or unlabelled) from sucrose gradients (see above) was placed in an ignition tube (No. 9820, 25 x 200 mm, Corning Glass Works, Corning, NY) and made 10 mM Tris-HCl pH 7.4, 10 mM KCl, 1.5 mM MgCl_2 , 0.3% sodium dodecyl sulfate (SDS) and 500 $\mu\text{g/ml}$ proteinase K (Merck Co., Darmstadt, W. Germany). The mixture was incubated in a 56° water bath for 15', then 1/10 volume of LiCl buffer (1.4 M LiCl, 5% SDS, 0.1 M sodium acetate buffer pH 4.9) was added. An equal volume of phenol (redistilled and saturated with 10 mM Tris-HCl pH 7.4, 10 mM KCl, 1.5 mM MgCl_2) was added; the tube was heated to 56°C , shaken for 5', and an equal volume of chloroform was added. The tube was then shaken for

5', and an equal volume of chloroform was added. The tube was then shaken for 20' at room temperature on a Burrell wrist action shaker (Model 76, Burrell Corp., Pittsburgh, Pa.). After centrifugation (1000 x g, 15') the aqueous phase was removed. If protein was present at the interface, the aqueous phase was re-extracted; otherwise, the aqueous phase was precipitated with 2.5 volumes of ice-cold absolute ethanol at -70°C for several hours. The RNA was pelleted (Beckman J21-C, JA21 rotor, 10,000 rpm x 20') dried and resuspended in 0.3 ml of 1 mM EDTA, 5 mM Tris-HCl, 10 mM NaCl and 0.05% SDS, transferred to a 1.5 ml conical Eppendorf centrifuge tube, and reprecipitated with ethanol. For labelled RNA, this pellet was dissolved in 25 μl of H_2O and counted for application on gels. Cold RNA was further treated to remove traces of phenol by either washing the pellet with absolute ethanol, or resuspending the pellet in 0.2 ml 20 mM Tris-HCl pH 8.0 and passing it over a Sephadex G-25 column equilibrated with 20 mM Tris-HCl pH 8.0. RNA was quantitated spectrophotometrically at 260 nm, reprecipitated with ethanol and the final pellet was made 1 $\mu\text{g}/\mu\text{l}$ in H_2O .

Electrophoresis

Viral RNA

Slab gels were cast between 16.5 cm x 26.5 cm glass plates using spacers 1.2 mm thick. The comb used had 15 teeth, each 6 mm x 1.5 cm and spaced 1.5 mm apart. The unnotched plate was sandblasted to a rough finish. Gels were polymerized, using an acrylamide: N,N'-methylene-bis-acrylamide (bis) ratio of 30:1.725, to final concentrations of 2.2% to 3.0% (w/v) and contained 6 M urea, 36 mM Tris base, 30 mM NaH₂PO₄, 1 mM EDTA, 0.1% (v/v) N,N, N',N',-tetramethylethylenediamine (TEMED) and 0.15% (w/v) (NH₄)₂S₂O₈. The running buffer consisted of 36 mM Tris base, 30 mM NaH₂PO₄, 1 mM EDTA pH7.8 (Loening's buffer). After polymerization, the comb and bottom spacer were removed and the gel was mounted on a vertical electrophoresis apparatus using rubber tubing to maintain air circulation between the plates and the apparatus. Labelled RNA was usually present in 25 µl of H₂O, and aliquots containing 20,000-30,000 cpm were made 20% (w/v) sucrose in Loening's buffer containing 0.05% (w/v) bromophenol blue and 0.01% (w/v) xylene cyanol FF. Cold RNA was dissolved in H₂O at 1 µg/µl and usually 10-15 µl were lyophilized, dissolved

in the same running buffer, and applied to one slot of a gel. Before application RNA samples were boiled for 1.5 minutes. Electrophoresis was performed at 26°C in a water jacketed incubator, with continuous buffer recirculation. Current was maintained at 120 V for various amounts of time. Several hours before the end of the run, the incubator door was opened and the chamber was allowed to equilibrate to room temperature.

After electrophoresis, gels were treated in one of several ways. Gels were removed from glass plates with Whatman 3 MM paper, covered with Saran Wrap and heat-dried under vacuum. Kodak X-Omat RC film was used to obtain autoradiograms, often in conjunction with intensifying screens (Dupont). Alternatively, wet gels containing labelled RNAs were exposed for several hours and the film was used as a template to cut out labelled segments. For visualizing cold RNAs, gels were stained for 1 hour with a solution of ethidium bromide (4 µg/ml in Loening's buffer) and viewed under ultraviolet illumination. More details on treatment of gels are found in following sections.

Viral proteins

SDS gels Proteins were electrophoresed in slab gels of similar dimensions to those used for RNA. Glass plates were not sandblasted, and the comb used to cast the sample slots had 13 teeth, 6 mm x 1.5 cm, spaced 3 mm apart. Gels containing linear gradients of polyacrylamide (5% to 13% w/v) and sucrose (0 to 20% w/v) were employed, using the discontinuous buffer system of Maizel (1971). The resolving gel (19-20 cm long) contained the appropriate gradient of polyacrylamide (acrylamide:bis ratio 30:0.8) and 0.378 M Tris-HCl pH 9.0, 0.1% (w/v) SDS, .005% (v/v) TEMED and 0.02% (w/v) $(\text{NH}_4)_2\text{S}_2\text{O}_8$. The resolving gel was poured with a two-chambered gradient maker using a Sage pump (Orion Instruments) with 1.5 mm diameter tubing. The gel solution was allowed to drip down one side of the gel mold. After polymerization of the resolving gel, a 3-4 cm stacking gel was added which consisted of 3% polyacrylamide, 60 mM Tris base, 32 mM H_3PO_4 , 0.1% (w/v) SDS, 0.005% (v/v) TEMED and 0.1% (w/v) $(\text{NH}_4)_2\text{S}_2\text{O}_8$.

After polymerization, gels were mounted in a vertical apparatus containing a running buffer of 50 mM Tris

base, 0.384 M glycine and 0.1% SDS. Buffer was not recirculated during electrophoresis, which was conducted at 10-25 mA (constant current) until the bromophenol blue dye left the bottom of the gel.

Samples for electrophoresis on SDS-polyacrylamide gels were prepared as follows. The cell pellet from a pulse-labelling experiment (see Labelling viral proteins) was resuspended in 50 μ l of H₂O and transferred to a 1.5 ml eppendorf centrifuge tube. An equal volume of 2x lysis buffer (4% (w/v) SDS, 2% (v/v) β -mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 120 mM Tris base, 64 mM H₃PO₄, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue) was added and the mixture was boiled for 5 minutes. Ten to fifteen microliters were then applied to gels. For purified virus, a volume containing 20-30,000 cpm was mixed 1:1 (v:v) with 2x lysis buffer, boiled 5' and applied to the gel.

After electrophoresis, the gels were stained for 1 hour in 1000 ml of 0.2% (w/v) Coomassie brilliant blue in 50% (v/v) methanol and 7% (v/v) acetic acid. Destaining was done in 20% methanol and 7% acetic acid using activated charcoal contained in cloth bags to remove the stain. Gels were then dried under heat and vacuum onto Whatman 3 MM filter paper and exposed to X-ray film.

For fluorography, gels were fixed in 50% methanol, 7% acetic acid and immersed in two separate dimethylsulfoxide (DMSO) baths of 500 ml for 30' each. The gel was then soaked in 100 ml of 22.2% (w/v) 2,4-diphenyloxazole (PPO) in DMSO for 3 hours, and then DMSO was removed by rinsing the gel in tap water for 1 hour (Bonner and Laskey, 1974). After drying, gels were exposed to X-ray film at -70°C .

Triton-urea gels Electrophoresis of pulse-labelled viral polypeptides on polyacrylamide gels containing Triton X-100, urea and acetic acid was performed by modifying the method of Alfageme et al. (1974). Gel dimensions were identical to those used for SDS gels 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 6M urea, 0.37% (v/v) Triton X-100, and 5% (v/v) acetic acid. The running buffer was 5% acetic acid. Pulse-labelled infected cell pellets were dissolved in a mixture containing 6M urea, 5% acetic acid, 4% β -mercaptoethanol, 0.02% (w/v) Pyronine Y, and 0.37% Triton X-100. Electrophoresis was performed at room temperature at 100 V until the dye front left the resolving gel. After electrophoresis gels were dried and exposed to X-ray film.

Molecular weight estimations

The method of Weber and Osborn (1969) was used to determine molecular weights of viral polypeptides. Labelled polypeptides were analyzed on an SDS polyacrylamide gel (see above) consisting of a 3% stacking gel and a resolving gel of 5 to 13% polyacrylamide. 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.

Molecular weight estimates of glyoxal-treated RNA segments were performed as described previously (McMaster and Carmichael, 1977; Desselberger and Palese, 1978). ³²P-labelled virion RNA (approximately 30,000 cpm per slot) was denatured at 56°C for 1 hour in 1.0 M glyoxal-10 mM phosphate buffer, pH 7.0. RNA treated in this manner was subjected to electrophoresis on urea-polyacrylamide gels (see above) with glyoxal-treated marker E. coli 23S, 16S and 4S RNAs.

One-dimensional peptide maps

Individual viral polypeptides from pulse-labelled infected cells or from continuously labelled purified virus preparations were subjected to one-dimensional

peptide mapping by the procedure of Cleveland et al. (1977) with minor modifications. After labelled viral polypeptides were subjected to electrophoresis on a polyacrylamide gel as described above, the gel was stained, and lanes containing samples of purified virus, infected and uninfected cells were cut out, dried, and autoradiographed. The labelled viral polypeptides were located on the film, which was used to identify viral polypeptides in the remaining lanes of the wet, stained gel. Bands were then cut and immersed in a solution of 0.125 M Tris-HCl pH 6.8, 0.1% SDS and 1 mM EDTA. After 10 minutes, gel slices were placed in the slots of a second polyacrylamide gel (made with a 3% acrylamide stack and a 10-15% acrylamide resolving gel) and were covered with the same solution containing enzyme, 20% glycerol, 0.005% bromophenol blue and 2% β -mercaptoethanol. The enzymes used were Staphylococcus aureus V8 protease (0.5 μ g/slot) and chymotrypsin (20 μ g/slot). Gels were subjected to electrophoresis at 80V until the dye front reached the resolving gel and then run at 100V until the dye marker ran off the resolving gel (length, 15 cm). Gels were then fluorographed, dried and exposed at -70°C (see above).

RNase T1 fingerprinting of viral RNA

Influenza viral RNA was fingerprinted according to previously published methods (Frisby, 1977; Pedersen and Haseltine, 1978; Nakajima et al., 1978) with modifications. Viruses were propagated in eggs, purified, and unlabelled viral RNA was extracted as described in previous sections. The final viral RNA pellet was dissolved in water to a concentration of 1 $\mu\text{g}/\mu\text{l}$.

All reactions were performed in 1.5 ml Eppendorf polypropylene centrifuge tubes.

Preparation of RNase T1

Ribonuclease T1 (Calbiochem 556785, from Sankyo Co., Ltd., Japan) was made 10,000 units per ml in 20 mM EDTA, 10 mM Tris-HCl pH 7.5. In order to destroy contaminating phosphatase activity, 75 μl of 1 N HCl was added per ml and the enzyme was allowed to stand 10' at room temperature. After chilling on ice, 30 μl of 1 M Tris-HCl pH 7.5 was added, and the pH was adjusted to pH 7.5 with 1N NaOH.

The treated RNase T1 was assayed as follows. The reaction mixture contained 25 μl of 0.2 M Tris-HCl pH 7.5, 8 mM EDTA, 25 μl yeast RNA (10 mg/ml), various dilutions

of RNase T1, and water to 75 μ l. The mixture was incubated 15' at 37°C, chilled on ice, the reaction stopped and its contents precipitated by addition of 25 μ l 25% (w/v) trichloroacetic acid (TCA) and 0.75% (w/v) uranyl-acetate. After precipitation, the mixtures were centrifuged (Eppendorf centrifuge) and 25 μ l of each supernatant was added to 725 μ l H₂O. The optical density of these mixtures was read at 260 nanometers. Absorbance of the mixtures was plotted versus dilution of the enzyme and the linear portion (up to an OD of approximately 0.4) of the curve was used to calculate the units of activity. An optical density at 260 nanometers of 0.1 corresponds to 12×10^{-3} units (Arima et al., 1968).

Digestion of RNA

One microliter of viral RNA (1 μ g) was mixed with an equal volume of 2x concentrated RNase T1 digestion buffer (40 mM Tris-HCl pH 7.5, 4 mM EDTA). The mixture was held for 2' in a boiling water bath, chilled on ice and spun briefly. RNase T1 was added in a volume of 2 μ l containing 2 units. Digestion was for 30' at 37°C. 100 μ Ci of γ -[³²P]-ATP (New England Nuclear Corp., Boston, MA) was lyophilized and dissolved in 46 μ l of kinasing buffer (10 mM Tris-HCl pH 8.0, 10 mM magnesium acetate,

1 mM dithiothreitol; DTT) and this mixture was added to the RNase T1 digest along with 4 units of polynucleotide kinase (New England Biolabs., Beverly, MA). After 60' at 37°C, 100 µl of phenol was added (redistilled and equilibrated with 10 mM Tris-HCl pH 8.0). The mixture was vortexed, 50 µl of stop mixture was added (2 mg/ml yeast RNA in 0.6 M ammonium acetate), mixed again, and centrifuged for 10'. The supernatant was removed and added to another tube containing 300 µl cold absolute ethanol, and chilled at -70°C for 15'. The RNA was pelleted, dried and dissolved in 25 µl of running buffer consisting of 7 M urea (Schwarz Mann ultrapure), 10% (w/v) sucrose, 0.05 M EDTA, 0.15% xylene cyanol FF, 0.15% bromophenol blue and 5 mg/ml of a mixture of digested and undigested yeast RNA. This RNA was prepared in 10 ml batches as follows. Yeast RNA (40 mg) was dissolved in 20 ml of RNase T1 digestion buffer, heat denatured and digested with 160 units RNase T1 per mg (6400 units in 0.64 ml) for 30' at 37°C. Whole RNA (10 mg in 2.0 ml), and NaCl (2.4 ml of 0.5 M) were added, and the mixture was extracted with phenol:chloroform 1:1 (v:v). RNA was precipitated from the aqueous phase with 2.5 volumes absolute ethanol at -70°C for 30'. RNA was pelleted, dried and dissolved in 10 ml of running mixture (see above).

Electrophoresis in the first dimension

The equipment for fingerprinting RNA was constructed in our own laboratory.

Glass plates for the first dimension (20 x 40 x 0.5 cm) were periodically cleansed with $K_2Cr_2O_7-H_2SO_4$ and rinsed well with H_2O . Before each use, plates were wiped with ethanol. Side spacers (42 cm x 13 mm x 2.5 mm) were lightly greased with vaseline and sandwiched between two plates using metal stationery clips. The bottom opening of the gel mold was plugged with plasticine.

Acrylamide (Eastman #5521) was prepared and purified in one-liter batches by dissolving acrylamide and bis at a ratio of 40%:1.3% (w/v) and adding 5% (w/v) charcoal (petroleum base, activated, 6-14 mesh) and 0.15% (w/v) analytical grade mixed bed resin (Bio-Rad #142-6425). After stirring 10', the mixture was filtered through 5 layers of Fisher #9-801 qualitative medium thickness filter paper.

A gel mixture (200 ml) consisting of 10% acrylamide, 0.325% bis, 7 M urea (Schwarz Mann ultrapure), 0.025 M citric acid, 0.01 mg $FeSO_4 \cdot 7 H_2O$, 0.4 mg ascorbic acid and 0.12% (w/w) H_2O_2 was poured between glass plates and a comb inserted in the top. The comb resulted in

6 slots of dimensions 5 mm x 5 mm x 2.5 mm. After polymerization of the gel, the comb, plasticine and clips were removed and the gel was immersed in a plexiglass tank (anode; 25.4 x 7.6 x 40.6 cm) filled with 4 liters of first dimension buffer 0.025 M citric acid, 7 M urea (Fisher U-15) . The top of the gel was covered with first dimension buffer, and 2 layers of Whatman 3 MM paper (33 x 20 cm) were soaked in the buffer and covered with Saran Wrap. One end of the wick was inserted into the top of the gel, and the other was placed in a second plexiglass tank (cathode) filled with 6 liters of buffer and elevated 10 cm above the level of the front tank. The gel was then prerun at 900V for approximately one hour.

After pre-running the gel, 8 ul of sample was loaded into each slot. Electrophoresis was continued at 900V until the yellow bromophenol blue front had migrated 20 cm. After each run, 0.5 liters of buffer was discarded from the front tank and replaced with 0.5 liters from the rear tank; 0.5 liters of fresh buffer was then added to the rear tank.

Under the conditions of the first dimension gel, oligonucleotides are separated mainly according to

composition. Oligonucleotides near the top of the gel are C and A rich, and those near the bottom of the gel are G and U rich (de Wachter and Fiers, 1972).

Electrophoresis in the second dimension

After electrophoresis, glass plates were separated and the gel was covered with Saran Wrap and exposed to X-ray film (Kodak X-OMAT XR-2 ready pack) for one hour. The gel was placed over a template in order to cut strips from each lane approximately 1 cm wide and 20 cm long. The top of the strip was located approximately 8 cm above the xylene cyanol dye. The upper edge of each strip was placed 4 cm from the bottom of the "second dimensional" glass plate (23.5 cm x 28.5 cm) which had been cleaned as described for "first dimensional" plates. Spacers (30.5 cm x 13 mm x 2.0 mm) greased with vaseline were placed on either side of the strip and another glass plate was placed on top of the spacers and secured with steel clips. The bottom of the plates (near the "first dimensional" gel strip) was plugged with plasticine. A gel mixture consisting of 21.9% acrylamide, 0.71% bis, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.06% TEMED and 0.06% $(\text{NH}_4)_2\text{S}_2\text{O}_8$

was poured into each mold and allowed to polymerize.

After polymerization, the clips and plasticine were removed and the gel was immersed in a plexiglass tank (30.5 cm x 28 cm x 15 cm). The tank (cathode) was filled nearly to the top with buffer (50 mM Tris-borate pH 8.3, 1 mM EDTA; Kennedy, 1976). The tops of the gels were filled with this buffer, and two layers of Whatman 3 MM paper (20 x 23 cm) were inserted in the top and draped behind into a second plexiglass tank (anode), elevated 10 cm and filled with buffer to the level of the glass plates. Current was applied at 14 mA per gel slab until the bromophenol blue dye migrated 22 cm from the bottom of the gel. When electrophoresis was complete, the gel was removed from the glass plates, sandwiched in Saran Wrap, and exposed to X-ray film at 4°C in Kodak X-ray film holders.

Under the conditions of the second dimension gel, oligonucleotides are mainly separated according to size (de Wachter and Fiers, 1972).

Fingerprinting individual RNA segments

For fingerprinting single viral RNA segments, cold viral RNA was first fractionated on polyacrylamide gels as described in the section on electrophoresis of RNA,

and eluted using a method developed by James F. Young (see Figure 3). Under ultraviolet illumination, bands were quickly excised with a sterile scalpel and placed into the small end of a cut-down pasteur pipette as shown in Figure 3. The gel slice was covered with 50 μ l of 1% agarose in Loening's buffer containing 0.01% xylene cyanol. This assembly was immersed in an Eppendorf tube containing 300 μ l of 0.5 M Tris base, 0.5 M sodium acetate pH 6.0, 0.01 M EDTA, 0.1% SDS. The modified pasteur pipette was filled with the same buffer without SDS. A platinum wire was inserted into the pasteur pipette (cathode) and a wick (Whatman 3 MM, 2 layers 0.95 x 5.7 cm) was inserted into the Eppendorf and led to a trough (anode) containing the buffer without SDS. Current was then applied at 20 mA/tube for 1 hour, which resulted in electrophoresis of RNA from the gel slice into the Eppendorf tube.

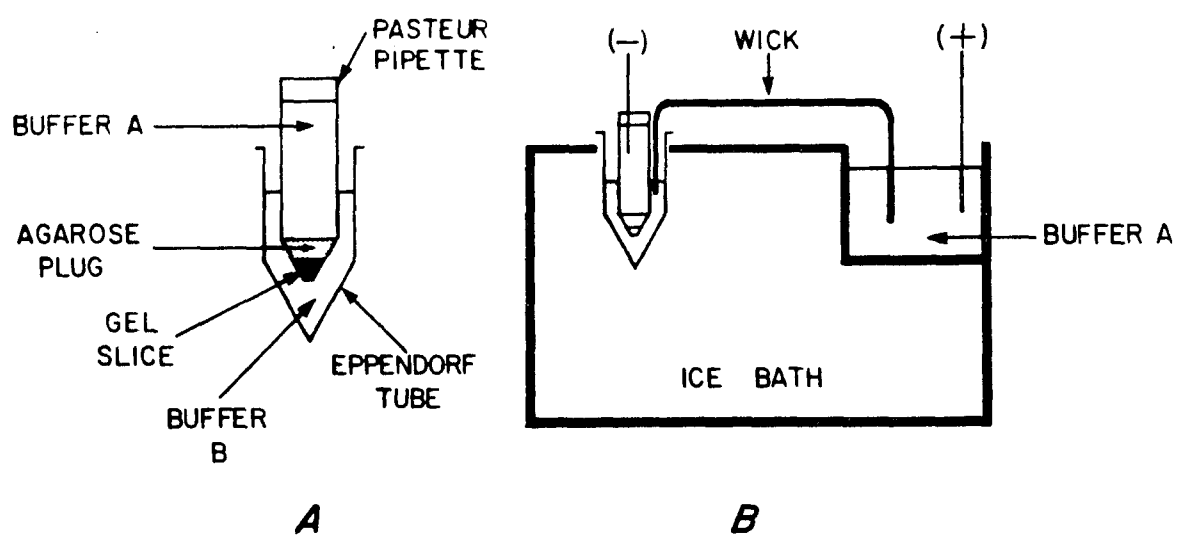
The RNA in the Eppendorf was precipitated by adding 750 μ l absolute ethanol and chilling at -70°C for 30'. After centrifuging 15' the supernatant was discarded, the pellet suspended in 50 μ l of H_2O and made 1% with HClO_4 . This treatment was used to remove acid soluble acrylamide monomers which are electrophoresed into the

Figure 3. Drawing of the apparatus used for elution of RNA from gels.

Part A: The tip is removed from a pasteur pipette which has been cut down to 2" long. The gel slice is pushed to the bottom of the pasteur pipette, covered with an agarose plug, and the pasteur pipette is covered with buffer A (0.5 M Tris base, 0.5 M sodium acetate pH 6.0, 0.01 M EDTA). The pasteur pipette is placed in a 1.5 ml Eppendorf tube containing 300 μ l of buffer B (0.5 M Tris base, 0.5 M sodium acetate pH 6.0, 0.01 M EDTA, 0.1% SDS).

Part B: The Eppendorf tube containing the pasteur pipette is suspended through a hole in a plexiglass box filled with ice water (shown here in side view). A wick leads from the Eppendorf to a trough containing buffer A (the anode) and a platinum wire (cathode) is placed in the pasteur pipette. RNA is electrophoresed from the gel slice into the Eppendorf tube.

ELUTION APPARATUS



Eppendorf tube and which are otherwise pelleted with ethanol (Jeppesen et al., 1972). The tube was chilled 10' on ice, centrifuged, and the wet pellet was suspended in 100 μ l of 5 mM Tris base, 100 mM sodium acetate pH 7.5. After precipitation at -70°C , 30' with 300 μ l ethanol, the RNA was pelleted and dried.

For RNase T1 digestion, the RNA was dissolved in 2 μ l of T1 buffer, digested and fingerprinted as described above.

Sequencing of viral RNA

3'-end labelling

The procedure for 3'-end labelling of influenza viral RNA was modified from England and Uhlenbeck (1978). Viral RNA (10-15 μ g) was lyophilized in a 1.5 ml Eppendorf tube. The pellet was resuspended in 20 μ l of 3'-end reaction mixture which consists of 50 mM HEPES pH 8.3, 10% DMSO, 5 μ M ATP, 10 mM MgCl_2 , and 3.3 mM DTT. 10 μ l of T4 RNA ligase (New England Biolabs, Beverly, MA) was added, and the mixture was transferred to a second Eppendorf tube in which 100-150 μ Ci of 5'- $[\text{}^{32}\text{P}]$ -pCp (New England Nuclear Corp.) was lyophilized. The reaction was allowed to proceed for 18 hours at 4°C ; then an additional 20 μ l

of 50 mM HEPES buffer, pH 8.3 was added. The mixture was extracted with 100 μ l of phenol (saturated with 50 mM Tris-HCl, pH 8.0) after addition of 100 μ g yeast RNA. RNA in the aqueous phase was precipitated with 3 volumes of ethanol, pelleted and resuspended in 25% sucrose in Loening's buffer containing 0.1% xylene cyanol FF and 0.1% bromophenol blue. Electrophoresis was as described above.

5'-end labelling

The procedure for 5'-end labelling of influenza virus RNA is modified from the method of Efstratiadis et al. (1977). Viral RNA (10-15 μ g) was lyophilized as for 3'-end labelling and dissolved in 10 μ l of 50 mM Tris-HCl pH 8.0. The next step, which involved removing the 5' terminal phosphates, was performed using calf alkaline phosphatase (CAP) (Boehringer-Mannheim, Indianapolis, IN) which had been purified according to the procedure of Efstratiadis et al. (1977). A suspension of the enzyme (0.4 mg = 160 units) was pelleted, dissolved in 0.1 ml H₂O and passed over a Sephadex G-75 column (0.7 x 30 cm) equilibrated with 20 mM Tris-HCl, pH 8.4, 100 mM KCl. Fractions of 0.15 ml were collected and assayed for ATPase

activity as follows. Reaction mixtures of 10 μ l were assembled which consist of 1 μ l of the column fraction or enzyme, 1 μ l γ -[^{32} P]-ATP (approximately .01 μ Ci; specific activity 3000Ci/mM) and 8 μ l of 10 mM Tris-HCl pH 8.0 containing increasing amounts of cold ATP. The mixture was incubated 30' at 37°C and then 1 μ l aliquots were spotted on a polyethyleneimine (PEI) cellulose plate (Brinkmann Instruments) which is developed in 0.75 M potassium phosphate buffer, pH 3.5. After autoradiography, the ATP spot can be easily distinguished from the inorganic phosphate spot, which are both cut out and counted by liquid scintillation. Activity of the CAP is then expressed in ATPase units (1 unit hydrolyzes 1 nmole of ATP in 30 minutes at 37°C).

Fractions containing the peak ATPase activity (usually eluted in the void volume) are pooled, mixed with an equal amount of glycerol and stored at -20°C.

One μ l (approximately 20 units) of purified CAP was added to the dissolved RNA and the mixture was incubated at 37°C for 30'. After this time, the following components were added: 1 μ l of 250 mM K_2HPO_4 , 1 μ l of 250 mM $MgCl_2$, 2 μ l 50 mM DTT, and 2 μ l T4 polynucleotide

kinase (Bethesda Research Laboratories, Rockville, Md.). This mixture was transferred to an Eppendorf tube containing 100-200 μ Ci lyophilized γ - $[^{32}\text{P}]$ -ATP (New England Nuclear Corp.; specific activity > 3000 Ci/mM) and incubated for 60' at 37°C. After this time, an additional 30 μ l of 50 mM Tris-HCl pH 8.0 was added, and the mixture was extracted with phenol and the RNA precipitated as described for 3'-end labelling. The final pellet was dissolved in sample buffer for RNA electrophoresis.

5'-or 3'-end labelled RNA was electrophoresed on polyacrylamide gels containing 6 M urea as described in the section on electrophoresis of RNA. Buffer was not recirculated, however, since trace 5'- $[^{32}\text{P}]$ -pCp or γ - $[^{32}\text{P}]$ -ATP in the sample would contaminate the entire gel; rather, fresh buffer was pumped into the top chamber, allowed to drip to the bottom chamber and then removed by another pump. After electrophoresis, the wet gel was exposed to X-ray film, viral RNA segments were located and slices of gel containing RNA bands were excised. RNA was eluted electrophoretically from the gel slices as described above, and subjected to one or more of the sequencing techniques described below.

The ladder technique

The 5'- and 3'-end labelled viral RNAs were subjected to partial digestion with RNases of different specificities (Donis-Keller et al., 1977; Simoncsits et al., 1977). Each reaction mixture consisted of at least 1500-2000 cpm of RNA in 3 μ l of H₂O, 7 μ l of the enzyme buffer, and 1 μ l of the enzyme. After the correct reaction time, mixtures were chilled, and 10 μ l of running mix (9 M urea, 0.01% xylene cyanol FF and 0.01% bromophenol blue) were added. For thin gels (see below) the reaction mixture consisted of 1 μ l of RNA and 2 μ l of enzyme in the appropriate buffer. After the reaction, 2 μ l of running mix was added.

RNase A (from bovine pancreas, Sigma #R-5000) was dissolved in 10 mM Tris-HCl pH 7.4, 1 mM EDTA to a final concentration of 100 μ g/ml. Various dilutions of this stock (usually 10^{-3} to 10^{-6}) were used. The reaction buffer consisted of 70% deionized formamide, 10 mM Tris-HCl pH 7.5, 1 mM EDTA; reaction was allowed to proceed at 37°C for 30'.

RNase Phy I was prepared from extracellular culture fluids of Physarum polycephalum (J. Zazra and W. Prenskey, manuscript in preparation) and Phy M RNase was a gift

from Helen Donis-Keller. The buffer (pH 5.2) for these two enzymes consisted of 66 mM KH_2PO_4 , 0.7 mM Na_2HPO_4 , and 10 mM EDTA.

RNase U2 was obtained from Calbiochem., San Diego, CA. Ampoules containing 100 units were resuspended to 5 units/ml in 50 mM sodium acetate buffer pH 4.5, 2 mM EDTA, 100 $\mu\text{g}/\text{ml}$ bovine serum albumin. The reaction took place in 20 mM citrate buffer pH 5.0 for 15' at 37°C. This enzyme was used undiluted or diluted 1:2 to 1:10.

RNase T1 was prepared as described in the section on fingerprinting of viral RNA. The enzyme was stored in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and the reaction was performed in a 20 mM citrate buffer pH 5.0, 1 mM EDTA, 7 M urea. Digestion was performed at 50°C for 15 min.

Alkali digestion of RNA was performed by dissolving lyophilized RNA in 10 μl of 50 mM NaHCO_3 pH 9.5, 1 mM EDTA and heating in capillary tubes at 95°C for 30-40 min. Alternatively, RNA was boiled for 3 hours in 70% deionized formamide. Both procedures generate a series of fragments which, when separated on a gel, yields a gel pattern containing bands representing every possible chain length from 1 base on up ("ladder").

Partial digests of end-labelled RNA were separated on gels as described by Maxam and Gilbert (1977). Gels consisting of 20% acrylamide, 0.67% bis, 7 M urea, 50 mM Tris-borate pH 8.3, 1 mM EDTA, 0.07% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and 0.01% TEMED were cast between glass plates 33 cm x 40 cm, using spacers 1 cm wide and either 0.38 mm, 0.5 mm or 1.5 mm thick. One glass plate had a notch at the top 2 cm x 30 cm in order to allow contact between buffer in the upper chamber and the gel. Thick (1.5 mm) gels were sealed by greasing the spacers and inserting a plasticine plug at the bottom; the thin gels were sealed without grease, using spacers on the side and the bottom, and taping the sides securely with teflon tape. Combs were used which yielded 18 slots with dimensions 1 cm x 1 cm.

The running buffer for sequencing gels was 50 mM Tris-borate pH 8.3, 1 mM EDTA. Gels were prerun for at least 2 hours before the samples were loaded. Electrophoresis was done at 1000-2000 V, and the bromophenol blue dye was run approximately 20 cm down the gel.

After electrophoresis, the gel was removed from the plates, covered with Saran Wrap, and exposed at -70°C to Kodak X-Omat XR-2 film using an intensifying screen (Dupont).

"Wandering spot" technique

End-labelled RNAs were sequenced by mobility shift as described previously (Semler et al., 1978). In this procedure, end-labelled RNAs are digested under conditions which result in a series of fragments each of which differs in length by one base from the preceding fragment. These fragments are then separated in the two-dimensional fingerprinting system described above. When the autoradiogram of the second dimension is viewed, the fastest moving spot at the top of the gel should be one base long, and the next one down, two bases, etc. Each spot shifts to the left or to the right upon addition of the next base, depending on the identity of the new base. Thus, addition of a C or A shifts the spot to the left, and addition of a G or U shifts the spot to the right. The method derives its name from the series of "wandering spots" (see Figures 40 and 42 for examples).

RNA (minimum 5,000 cpm) was digested with alkali or formamide as described under the ladder technique, and then fractionated in two dimensions as described for fingerprinting RNA. In this case, the bromophenol blue dye was run 11 cm in the second dimension. After electrophoresis, the gel was removed from the glass plates, covered with Saran Wrap, and exposed to X-ray film at 4°C with an intensifying screen.

VIII. RESULTS

Portions of the results reported in this section have either been published, are in press or are submitted:

- 1.) Racaniello, V.R. and P. Palese. 1978. The genes of influenza virus: analysis of influenza B virus strains. In: Negative Strand Viruses and the Host Cell (R.D. Barry and B.W.J. Mahy, eds.) pp. 27-36. Academic Press, London.
- 2.) Racaniello, V.R. and P. Palese. 1979. Influenza B virus genome: assignment of viral polypeptides to RNA segments. *J. Virol.* 29:361-373.
- 3.) Palese, P. V.R. Racaniello, U. Desselberger, J.F. Young and M. Baez. 1979. Genetic structure and genetic variation of influenza viruses. *Proceedings of the Royal Society, London.*
(in press)
- 4.) Racaniello, V.R. and P. Palese. 1979. Isolation of influenza C virus recombinants. *J. Virology*, in press.
- 5.) Desselberger, U., V.R. Racaniello, J. Zazra and P. Palese. 1979. The 5'- and 3'-terminal sequences of influenza A, B and C virus genes are

highly conserved and show partial inverted complementarity. Gene, in press.

- 6.) Racaniello, V.R. and P. Palese. 1979. Influenza C virus RNAs and proteins. Submitted, Virology.

and one abstract:

Racaniello, V.R. and P. Palese. 1979. Biochemical analyses of influenza C virus RNA. Abstr. Ann. Meeting Amer. Soc. Microbiol., Los Angeles, S207.

A. STUDIES ON INFLUENZA B VIRUSES

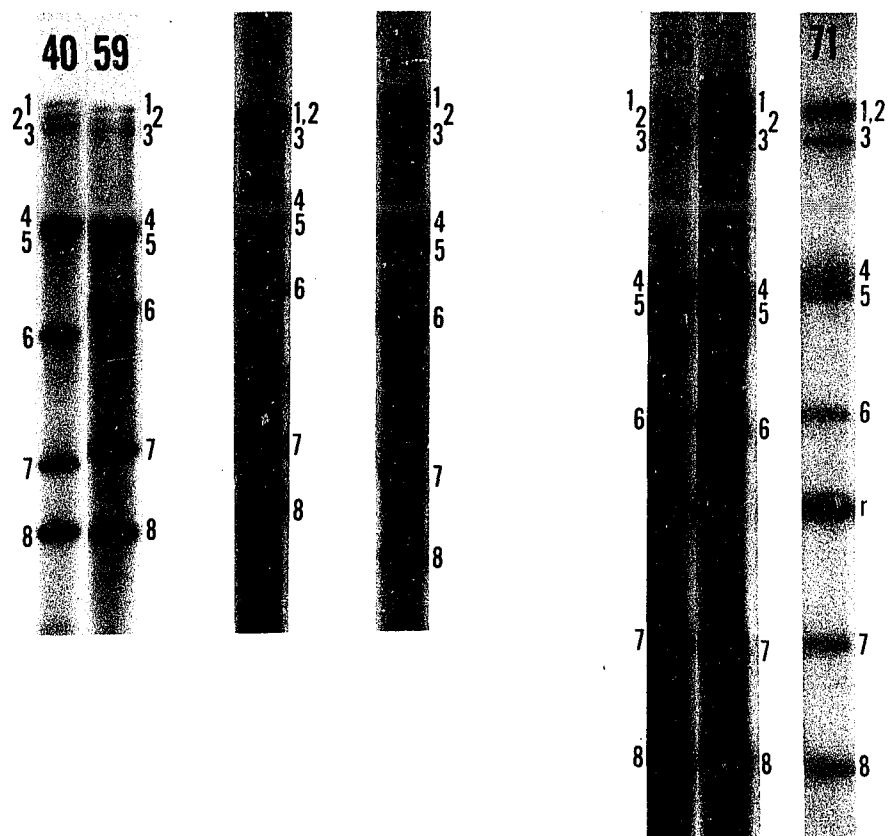
Influenza B viruses contain eight RNA segments

In a previous report, Ritchey et al. (1976a) found that the genome of B/Lee/40 virus consisted of eight RNA segments. In order to confirm this finding and to determine whether the genome of all influenza B viruses contains 8 RNA segments, different influenza B virus isolates were grown in MDCK cells in the presence of ^{32}P . Virus was then purified and the RNA extracted and electrophoresed on denaturing polyacrylamide gels.

Figure 4 is a composite of autoradiograms from several polyacrylamide gels of the ^{32}P -labelled RNA of 7 different influenza B viruses isolated from 1940 to 1973. The RNA of each isolate, which is numbered so that the slowest moving RNA is number 1, separates into eight bands. It is interesting to note that, for RNAs which have been analyzed on the same gel, different migration patterns can be observed. For example, in the patterns of B/Lee and B/Md viruses (Figure 4) each RNA segment migrates to a different position. From these gels it cannot be determined whether the various RNA patterns result from differences in molecular weight

Figure 4. RNA patterns of different influenza B virus strains.

Viral RNA was labelled with ^{32}P in MDCK cells and electrophoresed on 3.0% (lanes 1-4) or 2.6% (lanes 5-7) polyacrylamide gels containing 6 M urea. This figure is a composite of autoradiograms from several gels; lanes 1-2 are from one gel, lanes 3 and 4 are from separate gels, and lanes 5-7 are from the same gel. The RNAs are numbered 1-8 in order of increasing mobility on the gel. Lane 1, RNA pattern of influenza B/Lee/40 virus; lane 2, RNA pattern of influenza B/Md/59 virus; lane 3, RNA pattern of influenza B/USSR/69 virus; lane 4, RNA pattern of influenza B/HK/5/72 virus; lane 5, RNA pattern of influenza B/Mass/66 virus; lane 6, RNA pattern of influenza B/HK/8/73 virus; lane 7, RNA pattern of influenza B/Michigan/71 virus. Additional faint RNA bands observed in some viral preparations are most likely due to the presence of 18S ribosomal RNA (r, lane 7) or defective particles.

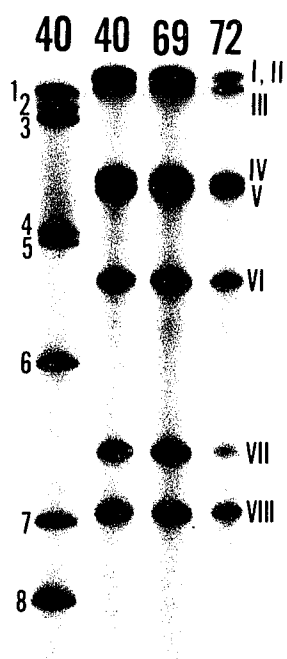


or nucleotide sequence. The relationship of these observed differences to genetic drift of influenza B viruses is also not clear at this time.

Figure 5 shows the RNA pattern of the glyoxal-treated RNA of 3 different influenza B viruses. Glyoxal treatment of RNA prevents base stacking and therefore should eliminate secondary structure (McMaster and Carmichael, 1977), which influences migration of RNAs in gels. It has been found that the patterns of glyoxal treated RNAs from different influenza A viruses are nearly identical (Desselberger and Palese, 1978) and 8 RNA segments can be identified. Glyoxalation of influenza B virus RNA also abolished strain-specific migration differences as shown in Figure 5. The untreated RNA of these strains migrates in different patterns on gels; however, after glyoxal treatment, all three strains possess an identical RNA pattern (Figure 5). RNAs 1 and 2 comigrate after glyoxal treatment, as shown in the section which describes a rapid technique for mapping the genome of any influenza virus. The analysis in Figure 5 shows that the genomes of all three influenza B viruses examined contain, when fully denatured, eight RNA segments. These results also indicate

Figure 5. RNA pattern of glyoxal treated RNA from three influenza B virus strains.

Viral RNA was labelled with ^{32}P in MDCK cells and electrophoresed on 2.6% polyacrylamide gels containing 6 M urea; RNA was either untreated or treated with glyoxal. Untreated viral RNAs are numbered 1-8 in order of increasing mobility on the gel; glyoxalated RNAs are numbered I - VIII in order of decreasing molecular weight (see the section which describes a rapid technique for mapping the genome of any influenza virus). Lane 1, pattern of untreated influenza B/Lee/40 virus RNA; lane 2, pattern of glyoxalated influenza B/Lee/40 virus RNA; lane 3, pattern of glyoxalated influenza B/USSR/69 virus RNA; lane 4, pattern of glyoxalated influenza B/HK/5/72 virus RNA.



that differences in RNA patterns of influenza B viruses are probably due to secondary structure and not molecular weight.

In summary, like influenza A viruses, influenza B viruses possess a segmented RNA genome consisting of eight genes.

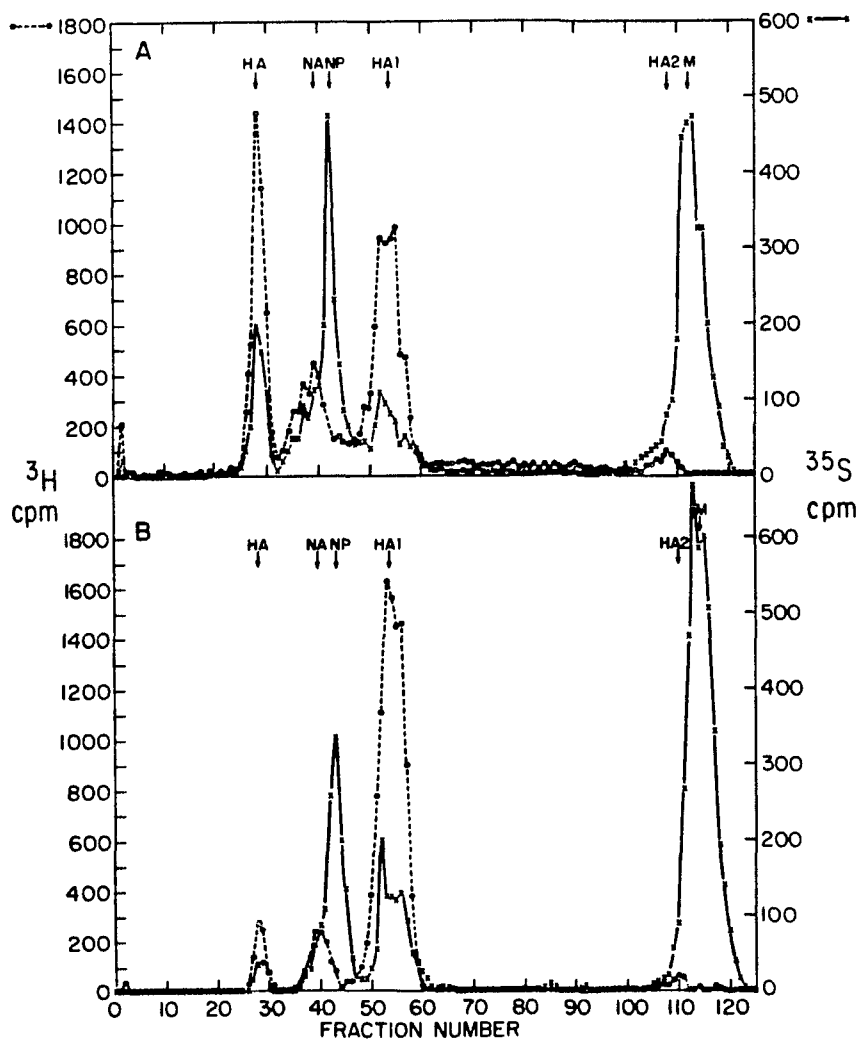
Influenza B virus-specific polypeptides

As discussed in the Introduction, when this thesis work was begun, only six influenza B virus-specific polypeptides had been described in the literature. In an attempt to identify all of the influenza B virus-specific polypeptides, viral proteins were examined both in infected cells and in purified virus.

An analysis of the polypeptides of purified B/Lee/40 virus is shown in Figure 6. Virus was labelled with ^{35}S -methionine and ^3H -glucosamine, disrupted, reduced, and electrophoresed on a 13% SDS-polyacrylamide gel, which was then sliced and counted. In panel A, six protein peaks are labelled, four of which are glycosylated. The nonglycosylated peaks are identified as NP and M, since their molecular weights (66,000 and 25,000; determined as described in the section on estimation of molecular weights) correspond with previously

Figure 6. Electrophoresis of purified influenza
B/Lee/40 virus.

Virus was doubly labelled with ^{35}S -methionine and ^3H -glucosamine in MDCK cells in the absence of serum, purified from cell fluids and processed for electrophoresis as described in Materials and Methods. Samples were electrophoresed on a 13% polyacrylamide gel containing SDS. After drying the gel was sliced, and gel slices were dissolved in 30% H_2O_2 and counted by liquid scintillation. A: untreated virus. B: virus treated with TPCK-trypsin, 1 $\mu\text{g}/\text{ml}$, for 20 minutes at 37°C . Equal amounts of protein were added to each slot.



determined values (Oxford, 1973b; Kendal, 1975; Choppin et al., 1975). The hemagglutinin polypeptide (HA) was identified by treating the double-labelled B/Lee virions with TPCK-trypsin. In panel B of figure 6 it can be seen that after trypsin treatment, the first glycosylated peak (HA) has nearly disappeared while the third glycosylated peak (HA1) increases in size. The HA2 peak does not change upon trypsin treatment; the reason for this unexpected finding is not known, but it is possible that HA2 is further degraded into smaller products. The presence of HA1 and HA2 in untreated virus suggests that B/Lee virus HA is partially cleaved in MDCK cells in the absence of serum. Under these conditions the second glycoprotein, most likely NA, migrates slightly slower than NP.

Analysis on gradient polyacrylamide gels of ^{35}S - methionine-labelled polypeptides in infected cells revealed, in addition to the HA, NP and M proteins, four additional polypeptides (Figure 7). One of these polypeptides migrates between the NP and the M protein. This protein is absent from the pattern of purified virus shown in Figure 8, and is also absent in the gel pattern of purified virus shown in Figure 6. Therefore this

Figure 7. Electrophoresis of influenza B virus-infected cell proteins on a 5-13% gradient polyacrylamide-SDS gel. MDCK cells were infected with influenza B/Lee or B/MD viruses at an MOI of 10 PFU/cell. At 6 hr. p.i., cell proteins were pulse-labelled with ^{35}S -methionine for 15', and then cells were processed for electrophoresis as described in Materials and Methods. After electrophoresis, the gel was fixed, dried and exposed to X-ray film. Lane 1, polypeptides of uninfected (U) MDCK cells; lane 2, polypeptides of influenza B/Lee/40 virus-infected cells (L); lane 3, polypeptides of influenza B/Md/59 virus-infected cells (M). Electrophoresis is from top to bottom, and influenza B virus-specific polypeptides are labelled (in this gel system the NA polypeptide comigrates with the NP).

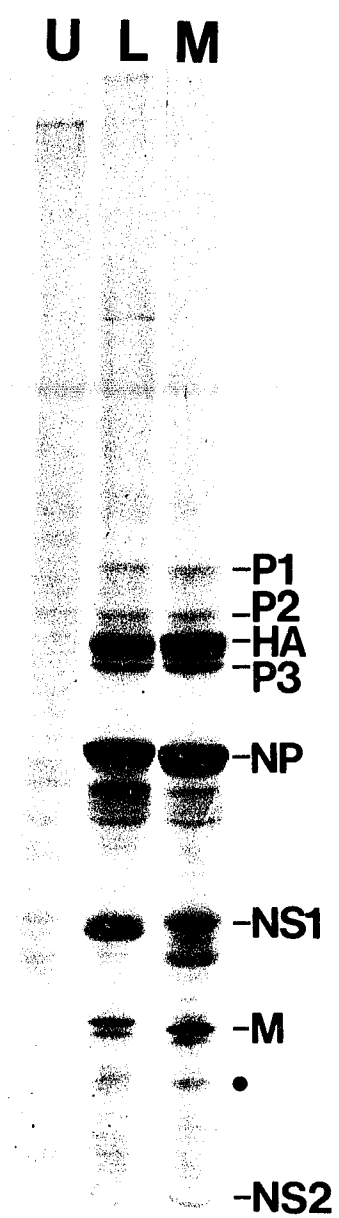
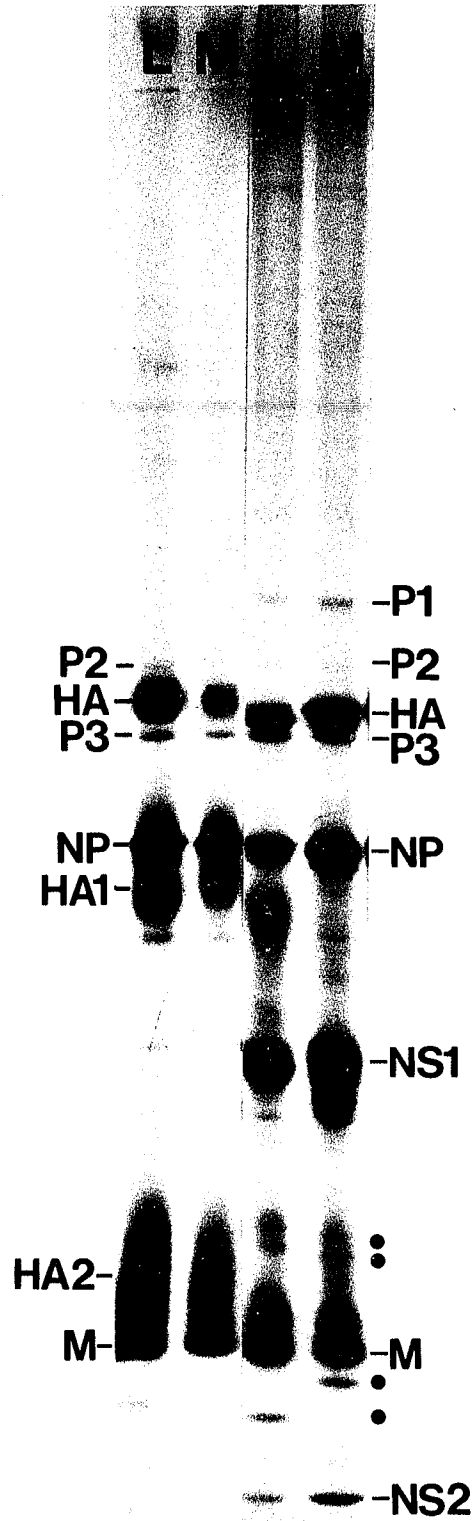


Figure 8. Electrophoresis of influenza B virus-specific proteins on a 5-13% gradient polyacrylamide gel. Purified influenza B/Lee/40 and B/Md/59 viruses were continuously labelled in MDCK cells with ^{35}S -methionine. MDCK cells infected with influenza B/Lee/40 and B/Md/59 viruses were preincubated in hypertonic medium and polypeptides were pulse-labelled with ^{35}S -methionine as described in Materials and Methods. Polypeptides were subjected to electrophoresis on a 5 to 13% gradient polyacrylamide-SDS gel and visualized by autoradiography. Lane 1, polypeptides of purified influenza B/Lee/40 virus (L); lane 2, polypeptides of influenza B/Md/59 virus (M); lane 3, polypeptides of influenza B/Lee/40 virus-infected cells (L); lane 4, polypeptides of influenza B/Md/59 virus-infected cells (M). The polypeptide in purified virus (lanes 1, 2) which migrates lightly slower than NS1 in infected cells (lanes 3,4) is most likely actin, which may be incorporated into virus particles.



band is an influenza B virus nonstructural protein (NS1), which was called NS in a previous publication (Racaniello and Palese, 1978).

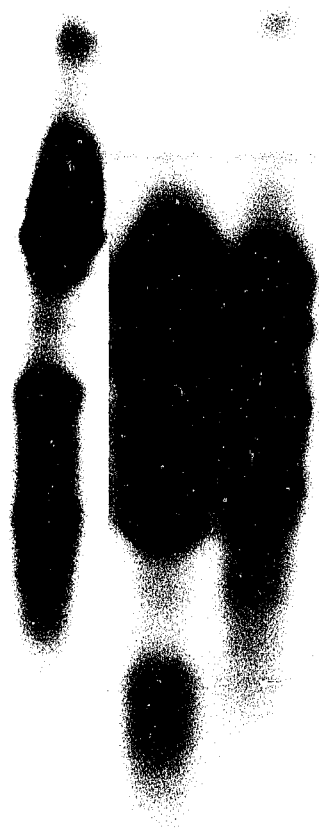
The other three polypeptides are labelled P1, P2 and P3 (Figures 7 and 8). These high molecular weight polypeptides can be detected in infected cells (Figure 7), and Figure 8 shows that P2 and P3 proteins can also be found in purified virus. P1 protein is not visible in this exposure, but has been observed in other viral preparations.

A one-dimensional map of P1 protein excluded the possibility that this polypeptide is a precursor of HA (Figure 9). The polypeptide P2 did not consistently label well enough in infected cells or in purified virus to permit extensive analysis by peptide mapping. However, the presence of this high molecular weight polypeptide both in 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 (Oxford, 1973b; Kendal, 1973; Wrigley et al., 1973; Allen et al., 1977).

In influenza B virus-infected cells, another distinct polypeptide band migrating slightly faster than HA can

Figure 9. One-dimensional peptide maps of HA and P1 and P3 proteins of influenza B/Lee/40 virus. Pulse-labelled 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.

P1 HA P3



be seen in SDS-polyacrylamide gels (Figure 7). Figure 8 shows that this polypeptide, labelled P3, is also present in purified virus. To rule out that this polypeptide represented unglycosylated HA, peptide maps of HA and P3 obtained from infected cells were analyzed (Figure 9). From this figure it is clear that there are differences between these two polypeptides, so it is concluded that the polypeptide P3 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 and forms a sharp band on polyacrylamide gels, it is most likely not an underglycosylated species of HA. Therefore, it is tentatively identified as the P3 protein.

Recently evidence has been presented, in influenza A viruses, for a ninth unique virus-coded polypeptide which is called NS2 (see Introduction). A second small nonstructural polypeptide can be found in cells infected with different influenza B viruses (Figures 7 and 8). This polypeptide may be NS2; however, other small proteins are also present which have not been identified (marked by dots in Figures 7 and 8).

In conclusion, at least eight influenza B virus-specific polypeptides have been identified: P1, P2, P3, HA, NA, NP, NS1 and M.

Strategy for mapping the influenza B virus genome

The discovery of eight RNA segments and at least eight virus-specific polypeptides initiated work on establishment of a complete genetic map of the influenza B virus genome. Such a map identifies the protein product of each RNA segment.

The strategy for mapping the influenza B virus genome by using recombinant viruses was first developed with the influenza A viruses (Palese and Schulman, 1976b) and was later used for mapping the genome of other segmented RNA viruses, such as reoviruses (Sharpe et al., 1978) and bunyaviruses (Gentsch et al., 1977). Figure 10 summarizes the mapping technique. This figure shows a schematic diagram of a polyacrylamide gel on which the RNAs of two parent viruses, X and Y, have been separated. Parent viruses are chosen which have readily distinguishable RNA patterns. Recombinants of the two parents are then isolated under conditions such

STRATEGY FOR MAPPING THE INFLUENZA B VIRUS GENOME

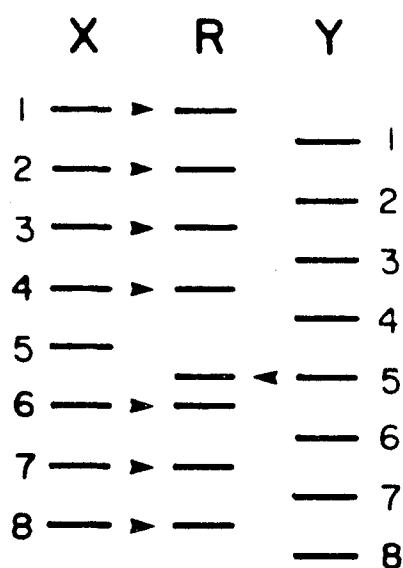


Figure 10. Schematic diagram of a polyacrylamide gel on which the 8 RNAs of two parent viruses, X and Y, have been separated. The middle lane shows the RNA pattern of recombinant R which inherits RNAs 1, 2, 3, 4, 6, 7 and 8 from parent X and RNA 5 from parent Y. A genetic map is constructed by examining the RNA and protein composition of a number of such recombinants which possess different gene constellations.

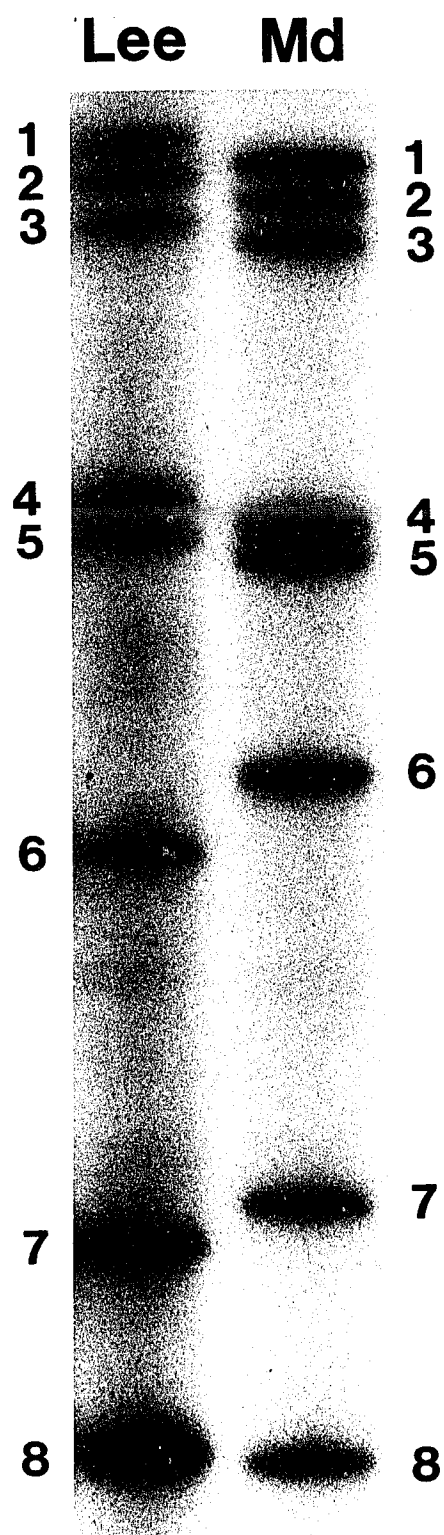
that most of the RNA segments are inherited from one parent virus and one or two RNA segments are inherited from the other parent. In Figure 10, the middle lane shows the RNA of a recombinant which derives RNAs 1, 2, 3, 4, 6, 7 and 8 from parent X and RNA 5 from parent Y. A series of such recombinants are isolated which have different gene constellations, and the polypeptide composition of each recombinant is examined. If the parental polypeptides are distinguishable, then single polypeptides may be correlated with specific RNA segments and a genetic map can be obtained.

Isolation of influenza B virus recombinants

The RNA patterns of the two parent viruses chosen for the mapping study are shown in Figure 11. From this gel it can be seen that each RNA segment of B/Lee/40 virus and B/Md/59 virus migrates to a different position, and therefore the derivation of RNA segments in recombinants of these two viruses can be easily established.

To obtain recombinants of B/Lee and B/Md viruses, yields from MDCK cells doubly infected with these two viruses were plaqued in the presence of antiserum to

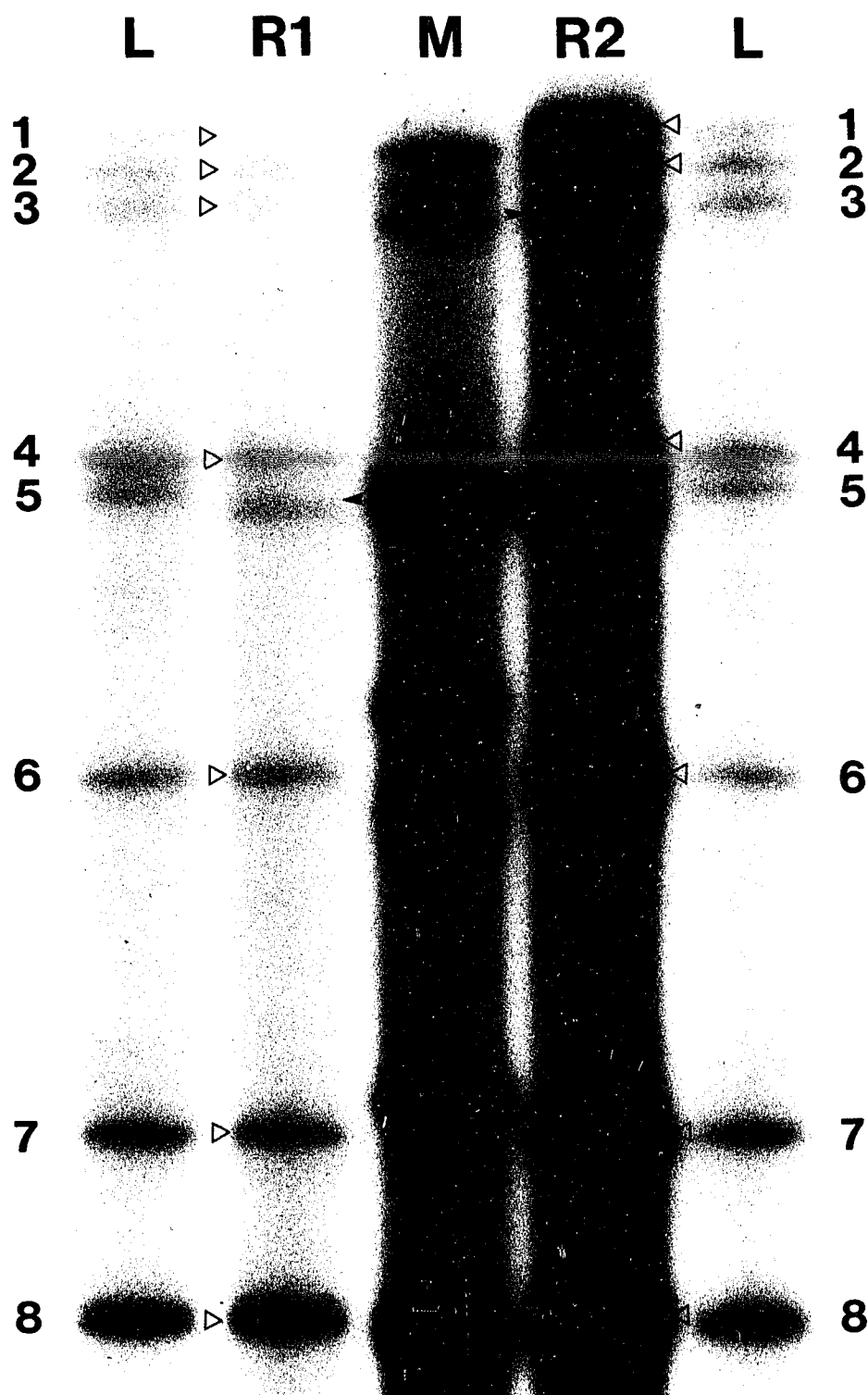
Figure 11. RNA patterns of influenza B/Lee/40 and B/Md/59 viruses. RNAs are numbered from 1-8 in order of increasing mobility on the gel. Lane 1, RNA pattern of influenza B/Lee/40 virus; lane 2, RNA pattern of influenza B/Md/50 virus.

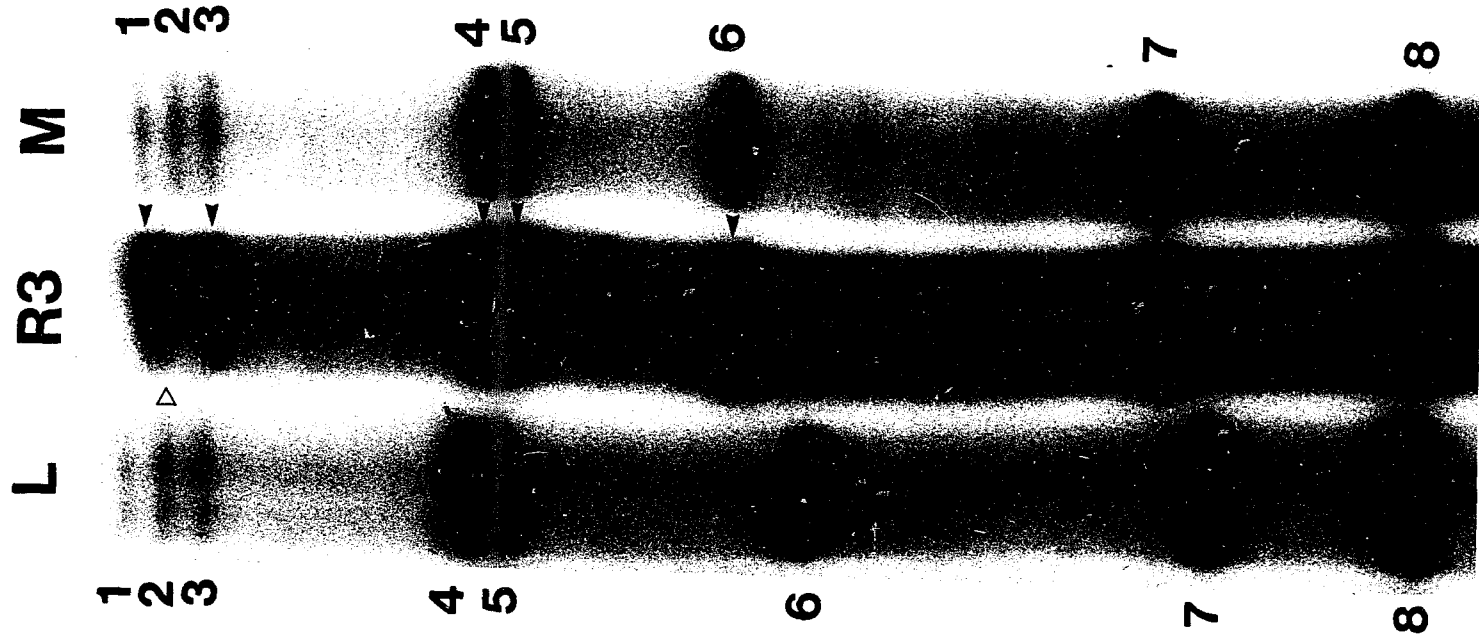


the surface glycoproteins of one virus. The RNAs of the recombinants were analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. Figure 12 shows the RNAs of seven different recombinants used in this mapping study. For example, it can be seen that recombinant R1 inherits RNAs 1, 2, 3, 4, 6, 7 and 8 from B/Lee virus and RNA 5 from B/Md virus. A summary of the genetic composition of these recombinants is shown in Table 4. All of these recombinants inherit most RNA segments from one parent and only one or two RNA segments from the other parent

In the Materials and Methods section it was discussed how the MOI of each parent used in the recombinational mixture can influence the genotype of the recombinant (see also Schulman and Palese, 1976). The effect of this phenomenon can be noted by examining the gene composition of the recombinants in Table 4. Recombinants R1 and R2 were 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. Recombinant R1 inherits only RNA 5 from B/Md virus, and recombinant R2 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

Figure 12. Gene composition of influenza B virus recombinants. ^{32}P -labelled viral RNAs were run on a 2.6% polyacrylamide gel containing 6 M urea. Migration differences of parental virus RNAs permitted 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/Lee virus; lane 2, RNA of recombinant R1; lane 3, RNA of B/Md virus; lane 4, RNA of recombinant R2; lane 5, 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 are most likely due to the presence of defective particles.

**A**



B

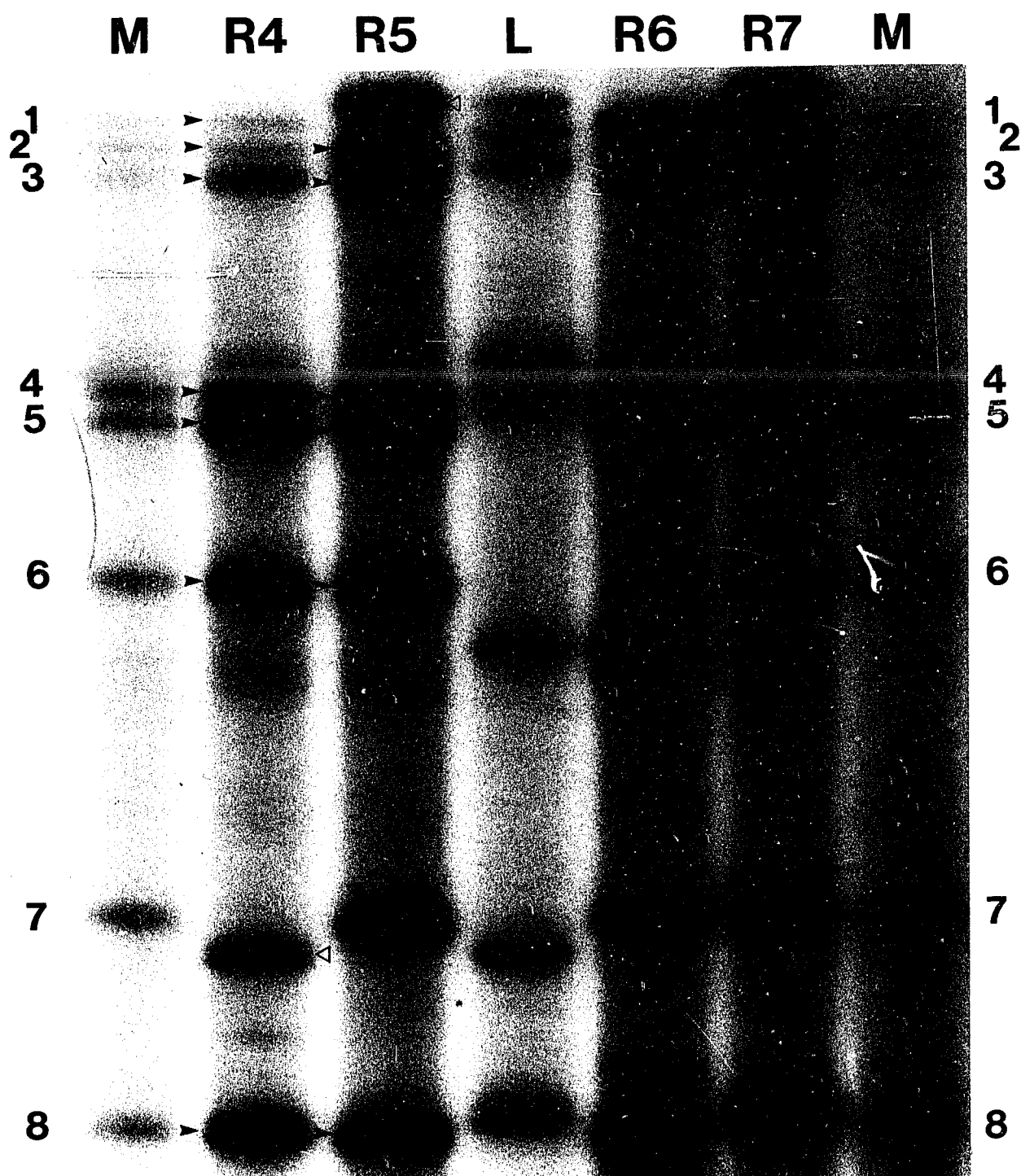


TABLE 4

GENE COMPOSITION OF SEVEN RECOMBINANTS USED
TO MAP THE INFLUENZA B VIRUS GENOME

Recombinant	RNA Segment							
	1 ^a	2	3	4	5	6	7	8
R1	L ^b	L	L	L	M ^c	L	L	L
R2	L	L	M	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 numbered in order of increasing mobility
on polyacrylamide gels

^b RNA derived from B/Lee virus

^c RNA derived from 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 HA

Recombinant R1 (Table 4) inherits RNAs 1, 2, 3, 4, 6, 7 and 8 from B/Lee virus and one RNA, RNA 5, from B/Md virus. The result of hemagglutination inhibition tests for these viruses is shown in Table 5. The hemagglutinins of the two parent viruses cross-react to some extent, but the recombinant clearly resembles B/Md virus in its reactivity of the HA. This serologic information, considered with the RNA composition of the recombinant (Table 4) suggests that RNA 5 of influenza B viruses codes for HA. Derivation of the HA polypeptide was confirmed by analysis on SDS-polyacrylamide gels under conditions where HA of B/Lee and B/Md viruses can be separated.

Identification of the gene coding for NA

Recombinant R6 inherits RNA 6 from B/Lee virus and all other RNAs from B/Md virus (Table 4). NA-inhibition tests revealed that recombinant R6 possesses

TABLE 5

HEMAGGLUTINATION - INHIBITION TITERS

<u>Virus</u>	<u>Antiserum</u>		
	B/Lee/40	B/Md/59	NRS ^b
B/Lee/40	1,280 ^a	160	80
B/Md/50	320	1,280	80
R1 ^c	320	1,280	80

^a reciprocal of highest serum dilution which causes inhibition of hemagglutination

^b normal rabbit serum

^c Recombinant R1 derives RNA 5 from B/Md/59 virus and the remaining seven RNAs from B/Lee/40 virus.

a B/Lee virus NA (Table 6). This result indicates that RNA 6 of influenza B/Lee and B/Md viruses codes for the NA. While this work was in progress, Ueda et al. (1978) also found that the information for the NA of influenza B/Lee/40 and B/Massachusetts/66 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-labelled NS proteins of B/Lee and B/Md viruses. Figure 13 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). This migrational difference was used 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 proteins induced in cells infected with recombinant R7 is shown in Figure 13. The NS protein of R7 clearly comigrates with the B/Lee NS protein. Using similar techniques, R5, which

TABLE 6

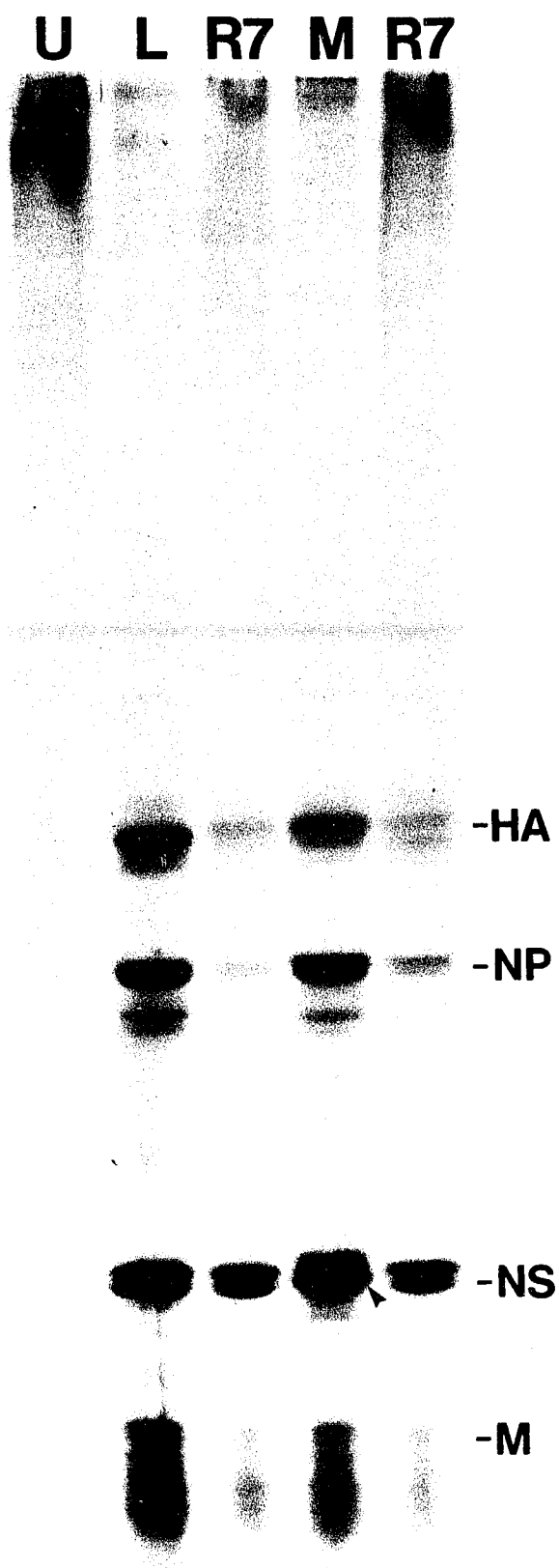
NEURAMINIDASE - INHIBITION TITERS

<u>Virus</u>	<u>Antiserum</u>	
	B/Lee/40	B/Md/59
B/Lee/40	20 ^a	< 10
B/Md/59	< 10	1,250
R6 ^b	30	< 10

^a dilution of antiserum causing 50% inhibition of neuraminidase activity

^b Recombinant R6 derives RNA 6 from B/Lee/40 virus and the remaining seven genes from B/Md/59 virus (Table 4).

Figure 13. Derivation of NS protein of recombinant R7 using SDS-polyacrylamide gel electrophoresis. Infected MDCK cells were preincubated in hypertonic medium and polypeptides were pulse-labelled with ^{35}S -methionine and analyzed on a 5 to 13% gradient polyacrylamide SDS gel. Lane 1, uninfected cell polypeptides (U); 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. The arrow indicates the slower-moving B/Md virus NS1 protein. This analysis indicates that recombinant R7 inherits the B/Lee virus NS protein.



derives only RNA 1 from B/Lee virus, was found to possess the NS protein of B/Md virus. Therefore RNA 8 of influenza B/Lee and B/Md viruses codes for the NS protein.

To confirm this assignment, the NS proteins of B/Lee and B/Md viruses and recombinants R5 and R7 were analyzed by one dimensional peptide mapping in polyacrylamide gels (Cleveland et al., 1977). The peptide patterns of the NS proteins of B/Lee and B/Md viruses reveal several differences, as seen in Figure 14. The NS proteins of recombinant R5 and of B/Md virus are identical, while the peptide pattern of the R7 NS protein resembles that of the B/Lee virus NS protein. This result confirms the conclusion that RNA 8 of influenza B/Lee and B/Md viruses codes for NS protein.

Identification of the gene coding for NP

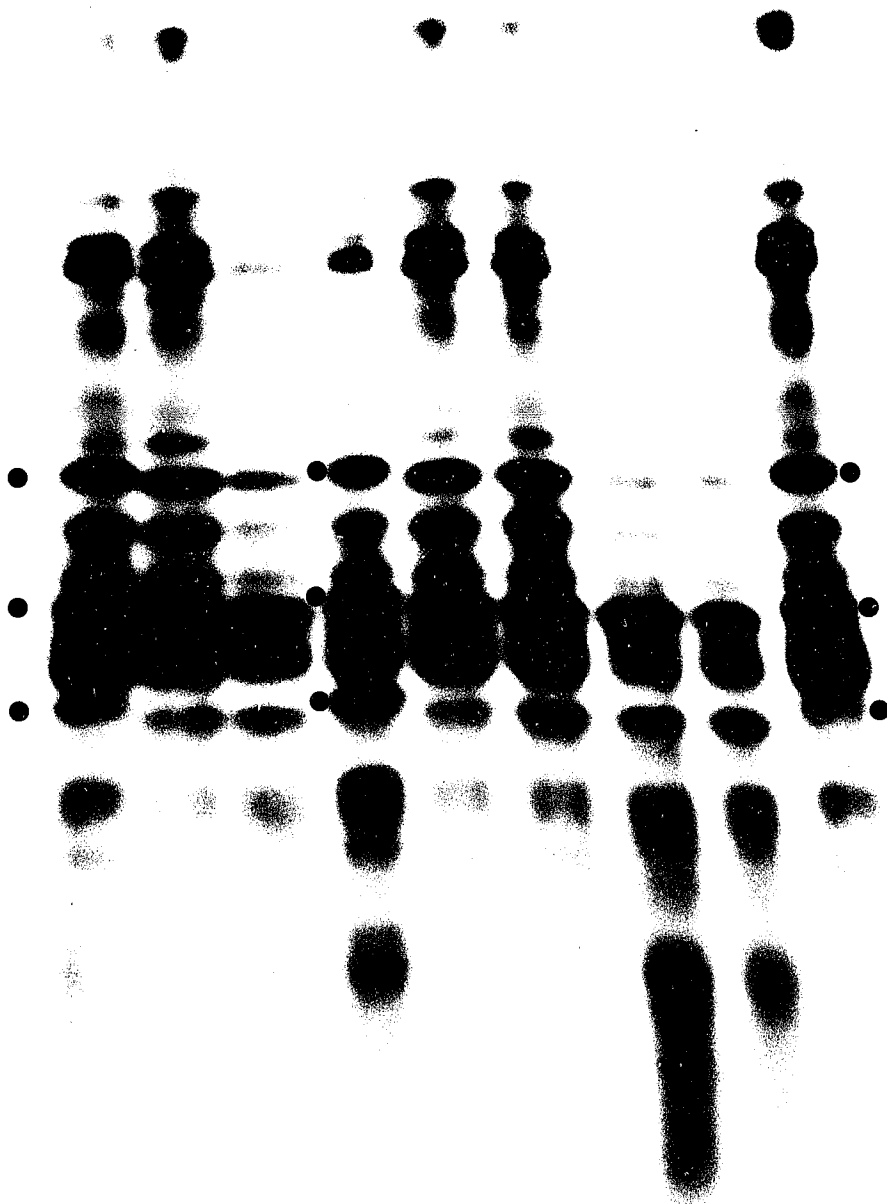
No clear migrational differences could be detected between the pulse-labelled nucleoproteins of B/Lee and B/Md viruses on SDS-polyacrylamide gels. Therefore, the one-dimensional peptide mapping technique was used to identify the derivation of the nucleoprotein in recombinants R2, R3, R4, R5, R6 and R7. Figure 15 shows that the nucleoproteins of B/Lee and B/Md viruses produce easily distinguishable one-dimensional peptide maps

Figure 14. Identification of the NS protein in recombinants R5 and R7 by using one-dimensional peptide maps. NS proteins were separated on a 5 to 13% gradient polyacrylamide-SDS gel, cut out and partially digested in the stack of a 10 to 15% gradient gel with 20 μ g chymotrypsin per slot. Lane 1, pattern of B/Lee virus (L) NS protein; lane 2, pattern of recombinant R7 NS protein; lane 3, pattern of B/Md virus (M) NS protein; lane 4, pattern of recombinant R5 NS protein; lane 5, pattern of B/Lee virus (L) NS protein. This analysis shows that recombinant R7 possesses B/Lee virus NS protein, while recombinant R5 derives its NS protein from B/Md virus.

L**R7****M****R5****L**

Figure 15. Identification of the NP in recombinants R2 through R7 by using one-dimensional peptide maps. Experimental conditions were as described in the legend to Figure 14. The NP was partially digested with S. aureus V8 protease (0.5 µg/slot) and the oligopeptides were separated on a 10 to 15% gradient polyacrylamide-SDS gel. Dots indicate B/Lee virus (L)-specific bands. Lane 1, pattern of B/Lee virus (L) NP; lane 2, pattern of recombinant R5 NP; lane 3, pattern of recombinant R3 NP; lane 4, pattern of recombinant R2 NP; lane 5, pattern of B/Md virus (M) NP; lane 6, pattern of recombinant R7 NP; lane 7, pattern of recombinant R6 NP; lane 8, pattern of recombinant R4 NP; lane 9, pattern of B/Lee virus (L) NP. This analysis shows that recombinant R2 possesses B/Lee virus NP and recombinants R3, R4, R5, R6, and R7 B/Md virus NP.

L R5 R3 R2 M R7 R6 R4 L

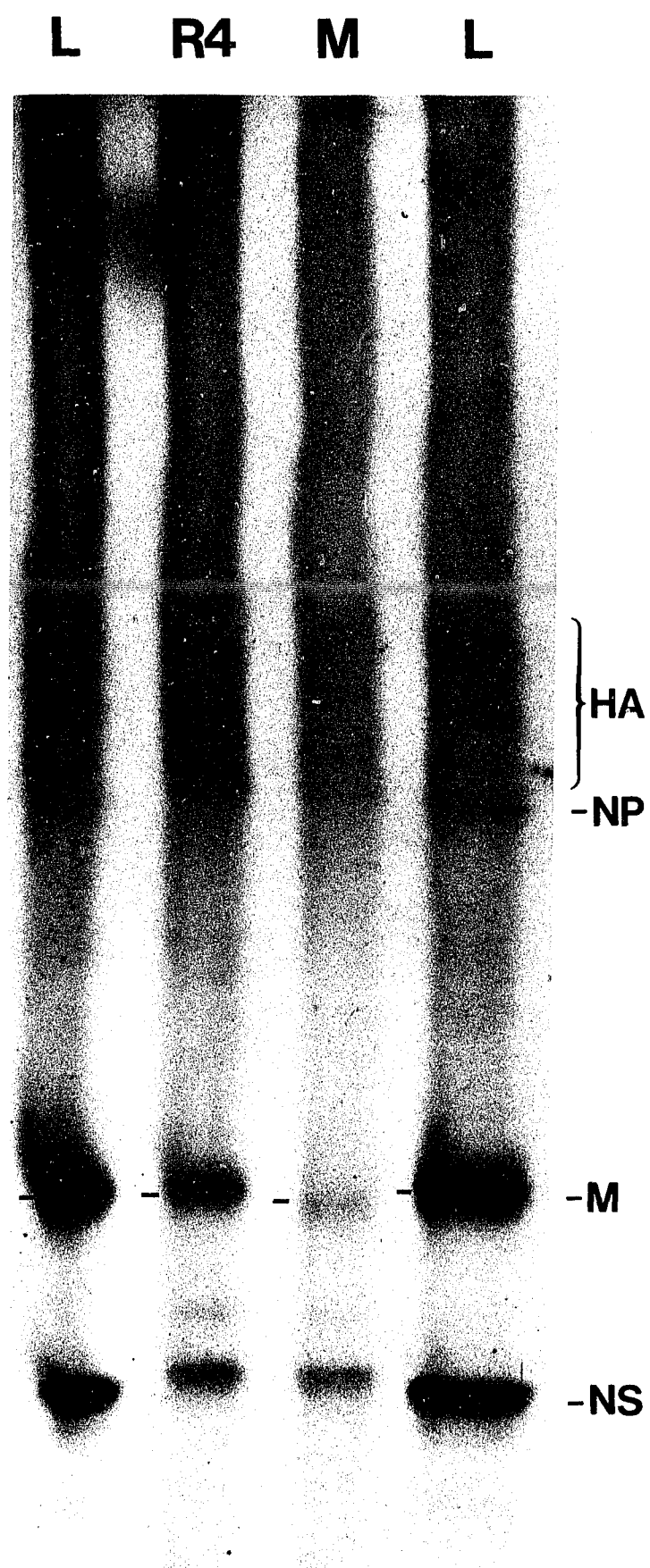


which differ in at least three bands. The peptide maps of the nucleoprotein of each recombinant is shown in Figure 15. According to this analysis, recombinant R2 derives its nucleoprotein from B/Lee virus and recombinants R3, R4, R5, R6 and R7 possess the B/Md nucleoprotein. This pattern is only consistent with the assignment of RNA 4 as the gene coding for nucleoprotein (see also Table 4).

Identification of the gene coding for M protein

SDS-polyacrylamide gel analysis of the pulse-labelled proteins 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, TPCK-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. (1974) as described in the Methods section, 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 16 shows

Figure 16. Derivation of M protein of recombinant R4 by using Triton-urea gels. ³⁵S-methionine-labelled infected cell polypeptides were analyzed on a 5 to 13% gradient polyacrylamide gel containing 6 M urea, 0.37% (V/V) Triton X-100, and 5% acetic acid. 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.



the autoradiogram of a Triton-urea gel of pulse-labelled proteins from B/Lee and B/Md viruses and recombinant R4. Recombinant R4 derives all genes from B/Md virus except RNA 7, which is inherited from B/Lee virus. The influenza B virus proteins on a Triton-urea gel were identified by cutting out the individual polypeptide bands and electrophoresing them in a conventional SDS-polyacrylamide gradient gel along with marker influenza B virus proteins. There is a small but reproducible migration difference between the M proteins of B/Lee and B/Md virus. The B/Lee virus M protein migrates slightly slower than the B/Md virus M protein. Since the M protein of recombinant R4 comigrates with the B/Lee virus M protein, RNA 7 of influenza B/Lee and B/Md viruses codes for M protein. Later analysis of the M protein using improved techniques of SDS-polyacrylamide gel electrophoresis demonstrated migrational differences between the M proteins of B/Lee and B/Md viruses (Figure 7). It was therefore possible to confirm the gene assignment made with Triton-urea gels (data not shown).

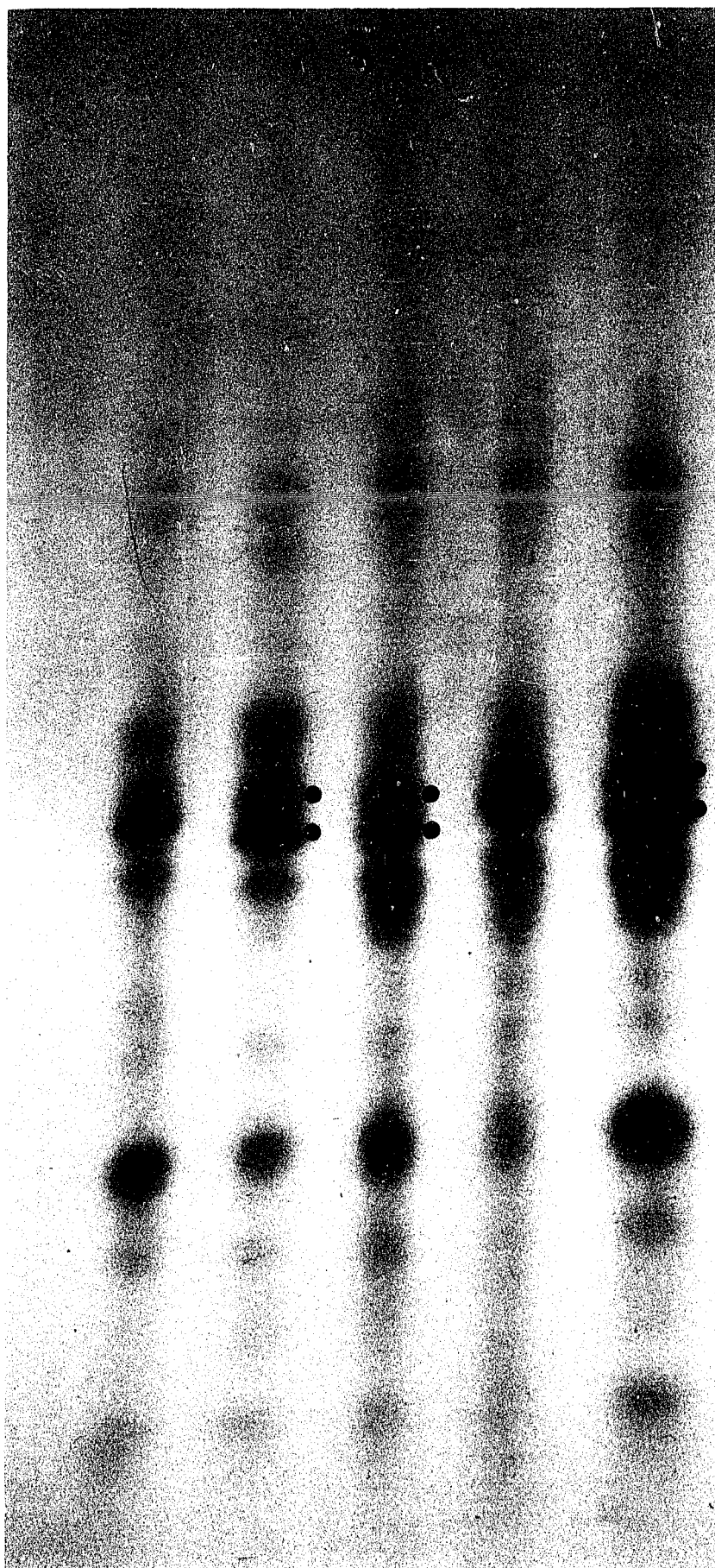
Identification of the genes coding for P proteins

The P1 protein was assigned to an RNA segment by using the one-dimensional peptide mapping technique. In Figure 17 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 and B/Md viruses 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 from 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 4). These data suggest that neither RNA 1 nor RNA 3 codes for the P1 polypeptide.

P2 protein did not label well enough consistently to permit analysis by peptide mapping, and therefore this protein could not be assigned to a specific RNA segment. Based on a comparison of the sizes of RNAs and proteins discussed later, P2 protein would map on either

Figure 17. Identification of P1 protein in recombinants R2, R3 and R5 by using one-dimensional peptide maps. Pulse-labelled P1 protein from virus-infected MDCK cells was partially digested with chymotrypsin (20 μ g/slot) and the oligopeptides were separated on a 10 to 15% gradient polyacrylamide-SDS gel. Lane 1, pattern of a mock P1 band obtained from uninfected cells; lane 2, pattern of B/Md virus (M) P1 protein; lane 3, pattern of recombinant R2 P1 protein; lane 4, pattern of recombinant R3 P1 protein; lane 5, pattern of recombinant R5 P1 protein; lane 6, pattern of B/Lee virus (L) 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.

C M R2 R3 R5 L



of the two unassigned RNA segments, RNA 1 or RNA 3.

Since peptide maps of P3 protein from B/Lee and B/Md viruses failed to reveal extensive differences, this polypeptide could not be assigned to a specific RNA. However, it is likely, based on the coding capacity of the RNAs, that RNA 1 or RNA 3 codes for P3 polypeptide.

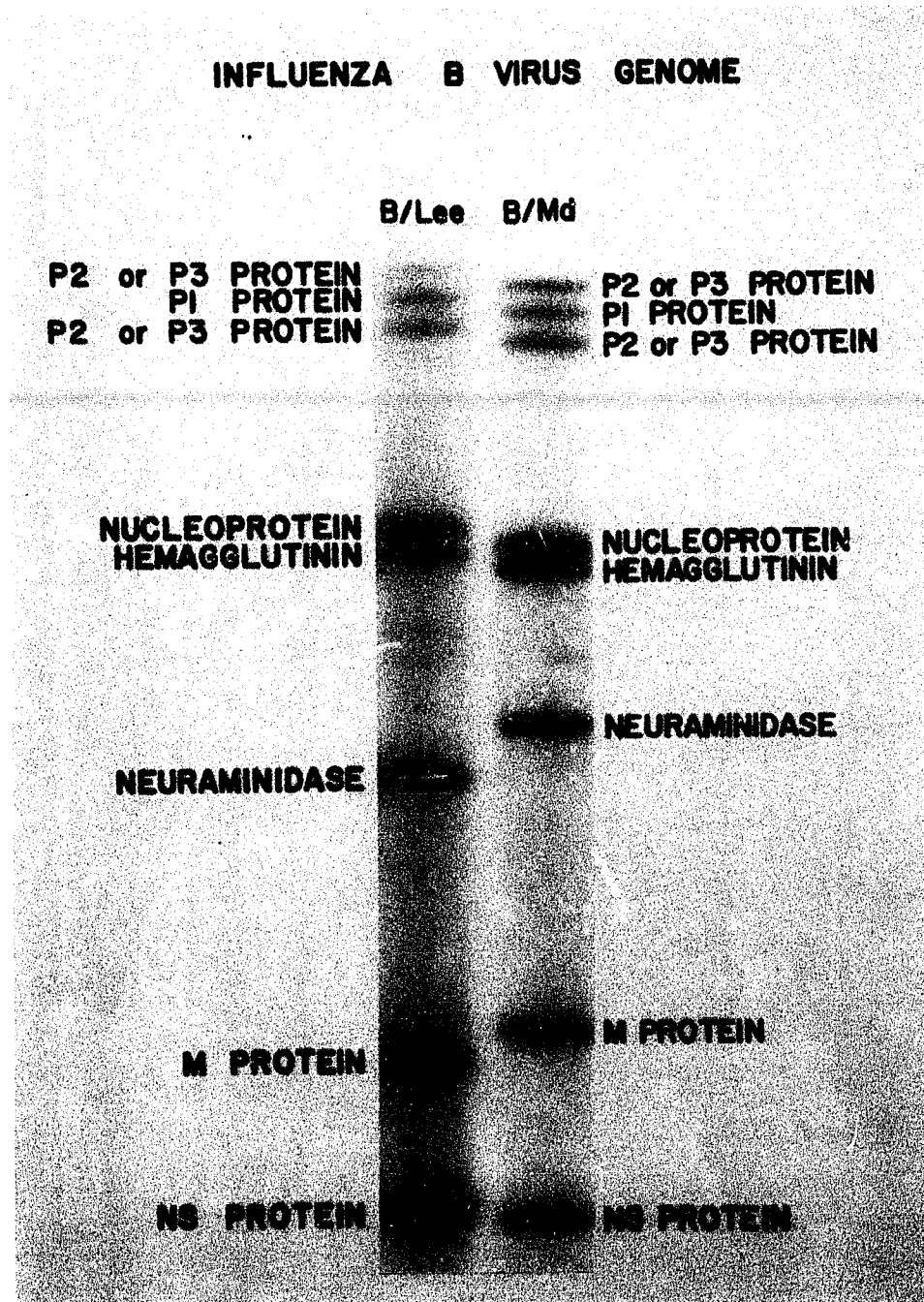
The results of these mapping studies are presented in Figure 18, which shows the genetic map for B/Lee and B/Md viruses.

A rapid technique for mapping the genome of any influenza virus

The establishment of genetic maps for influenza viruses is not a routine task. It would be helpful to have a method for rapidly mapping genomes of, for example, new virus isolates. Such a technique was developed in the course of studies on the molecular weight of RNAs reported in the next section.

In order to determine MWs of individual RNA segments, it was necessary to determine the order of migration of glyoxal-treated RNAs. For example, it was important to know whether or not RNA 4 migrated as the fourth largest

Figure 18. Map of the influenza B virus genome. RNAs of B/Lee and B/Md viruses were labelled 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.



RNA after glyoxal treatment. The method described below was developed to answer this question, and the results can be seen in Table 7. This analysis shows that the order of migration of the B/Lee and B/Md virus RNA segments in polyacrylamide gels changes after glyoxal treatment. When untreated influenza B/Lee/40 or B/Md/59 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 fastest segment (Figure 18). 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 in these two influenza B viruses (Table 7). It should be noted that the RNA segments in Table 7 are numbered by Roman numerals in order of decreasing molecular weight; RNA I is the largest RNA segment and RNA VIII is 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 (i.e., Figure 18). Under these conditions migration is not a true indication of molecular weight.

TABLE 7

MOLECULAR WEIGHTS OF PROTEINS AND RNA SEGMENTS OF INFLUENZA VIRUSES

Influenza A Viruses				Influenza B Viruses				Influenza C Viruses	
RNA ^a : MW x 10 ⁶	PROTEIN ^b : MW x 10 ³			RNA ^b : MW x 10 ⁶	PROTEIN ^b : MW x 10 ³			RNA ^b : MW x 10 ⁶	
I	0.89	P3	80	I	0.91	P2 or P3	93 or 80	I	0.92
II	0.89	P1	97	II	0.91	P1	102	II	0.92
III	0.86	P2	86	III	0.88	P2 or P3	93 or 80	III	0.85
IV	0.66	HA	72	IV	0.70	NP	66	IV	0.82
V	0.56	NP	55	V	0.68	HA	84	V	0.69
VI	0.48	NA	ND ^c	VI	0.55	NA	66	VI	0.36
VII	0.28	M	35	VII	0.38	M	25	VII	0.23
VIII	0.21	NS	25	VIII	0.33	NS	40		
Total: 4.83				Total: 5.34				Total: 4.79	

^aMolecular weights of RNA segments of influenza A/PR/8/34 virus are from Desselberger and Palese (1978).

^bMolecular weights of B/Lee/40 virus and C/GL/54 virus determined as described in Materials and Methods.

^cNot determined.

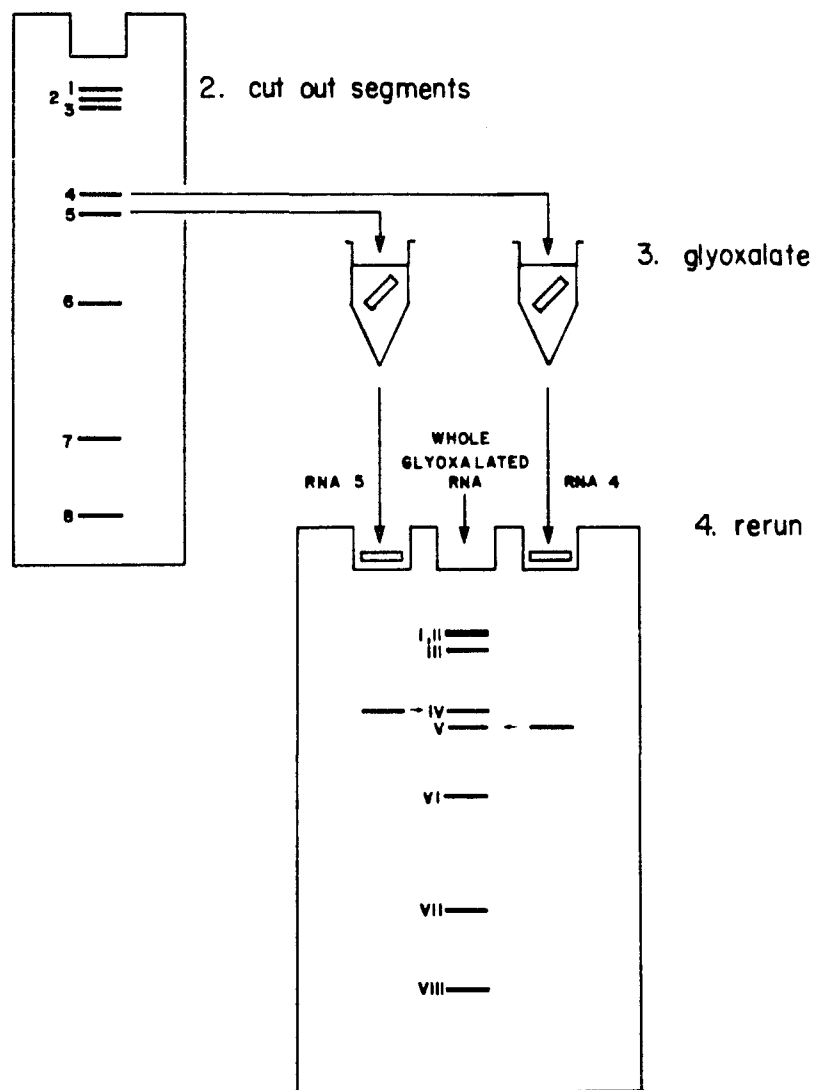
Table 7 compares the gene order of influenza A/PR/8/34 and B/Lee/40 viruses. The term "gene order" specifically means the ordering of genes solely according to molecular weight. Glyoxalation results in migration of RNA according to true molecular weight, and therefore electrophoresis under these conditions results in a pattern which represents the true gene order for any influenza virus. In our laboratory it has been found that the gene order for a large number of different influenza A and B viruses is identical, and therefore the gene order presented in Table 7 is most likely the same for all influenza A and B viruses.

The technique for rapidly mapping influenza virus genomes is based on the assumption that the gene order (the order of migration of genes after glyoxalation) is identical for all influenza viruses. This method is summarized in Figure 19. ^{32}P -RNA of any influenza virus is first separated on a polyacrylamide gel. Individual bands are located by autoradiography, cut out, and the whole gel slice containing an RNA segment is glyoxal treated. The gel slice, which now contains glyoxalated RNA, is placed in the slot of a second gel and run along with whole, glyoxal-treated ^{32}P -RNA. Since the gene

Figure 19. A technique for rapid mapping of the influenza virus genome. Step 1, ^{32}P -labelled RNA is electrophoresed, and segments are detected by autoradiography. Step 2, RNA segments are cut out of the gel. Step 3, whole gel slices containing RNA segments are glyoxalated. Step 4, gel slices containing glyoxalated RNA are placed in slots of a second polyacrylamide gel and electrophoresed with whole glyoxalated ^{32}P -labelled RNA. After autoradiography, segments cut from the original gel can be identified by comparison with the pattern of whole glyoxalated RNA. Example illustrated here is for segments 4 and 5 of B/Md/59 virus.

RAPID MAPPING OF THE INFLUENZA VIRUS GENOME

1. electrophorese untreated RNA



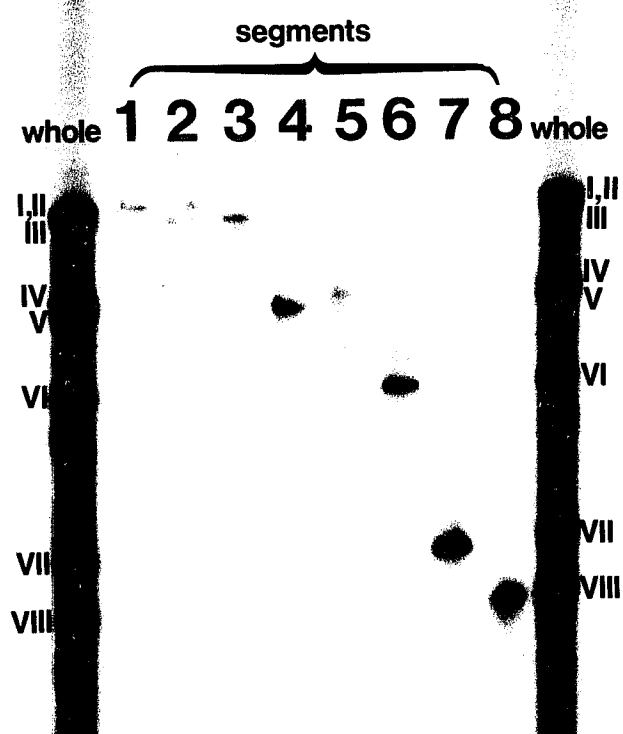
order is known, then the identity of any band excised from the original gel can be determined by noting where the isolated gene migrates in relation to whole glyoxalated RNA. The diagram in Figure 19 demonstrates how RNA 4 of untreated influenza B/Md/59 virus RNA migrates as the fifth largest RNA, gene V, after glyoxalation, and how RNA 5 migrates as the fourth largest RNA, gene IV, after glyoxalation. An example of the technique in use is shown in Figure 20. In this figure the gene order for B/Md/59 virus has been determined.

This technique is useful not only for mapping the genomes of new influenza virus isolates, but also for identifying genes separated in different electrophoresis systems, since I have found that the migration order of RNAs varies with different buffer systems. Such an identification is necessary when individual genes are studied in terms of their biologic function, when the fine structure of genes is analyzed or when genes are subjected to sequence analysis.

Molecular weight estimates of genes and gene products of influenza B viruses

Molecular weights of B/Lee virus polypeptides were determined as described in Materials and Methods. The

Figure 20. Determination of the gene order for influenza B/Md/59 virus. Gene order of B/Md/59 virus RNAs after glyoxal treatment was determined using the method shown in Figure 19. Lanes 1, 7 contain whole glyoxal treated B/Md/59 virus RNA which can be resolved into 8 segments. The glyoxal treated RNAs are identified by Roman numerals to indicate that they are separated according to molecular weight. Lanes 2-9 contain, respectively, glyoxalated individual RNA segments 1-8 of B/Md/59 virus. By this analysis, RNA 4 of B/Md virus, the NP gene, is the fifth largest RNA after glyoxal treatment, and RNA 5, the HA gene, is the fourth largest RNA after glyoxal treatment. A bubble under slot number 3 resulted in aberrant migration of RNA 2, which comigrates with RNA 1 after glyoxal treatment.



results are presented in Table 7. 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 measurements of the absolute molecular weights of nucleic acids on polyacrylamide gels (McMaster and Carmichael, 1977). These values are shown in Table 7, which also shows, for comparison, molecular weight estimations of the RNAs and proteins of an influenza A virus, and the RNAs of an influenza C virus.

Studies on the gene composition of candidate influenza B virus live vaccines

Considerable interest has been generated in recent years toward developing live attenuated influenza virus vaccines. Several attenuated influenza A virus strains have been developed and tested, and recently Recherche et Industrie Therapeutiques (RIT) has developed several candidate influenza B virus vaccines. Using the technology which had been developed in the course of this thesis work, these candidate vaccines were genotyped.

The theory behind the development of the vaccine strains is first to obtain a suitably attenuated parent

virus. Recombinants of this attenuated virus and recent circulating viruses are isolated, such that the recombinants retain the surface antigens of the current strain yet are still attenuated. (The attenuation character and the immunogenicity of the recombinant virus is then tested in clinical trials.)

Three candidate vaccine strains were obtained from RIT: R5, R22 and R75. In all of these viruses the attenuated parent was called "Brigit", which was derived from influenza B/USSR/69 virus. Virus R5 is a recombinant of Brigit and B/HK/8/73 virus and viruses R22 and R75 are recombinants of Brigit and B/HK/5/72 virus.

The RNAs of the parent and recombinant viruses were analyzed on polyacrylamide gels as described in Materials and Methods. The RNA patterns of the parent viruses were very similar, and therefore it was necessary to use gel concentrations of 2.4%, 2.6% or 3.0% to distinguish different RNAs.

A summary of the genotype of the three recombinants is shown in Table 8. From this analysis it can be seen that recombinants R22 and R75 inherited all RNAs from Brigit virus except RNA 5, the HA gene in these viruses, which was derived from B/HK/5/72 virus. Recombinant R5

TABLE 8

GENOTYPES OF CANDIDATE LIVE INFLUENZA
B VIRUS VACCINE STRAINS

Virus	RNA Segment							
	1 ^a	2	3	4 (NP)	5 (HA)	6 (NA)	7 (M)	8 (NS)
R22	B ^b	B	B	B	H ₇₂ ^c	B	B	B
R75	B	B	B	B	H ₇₂	B	B	B
R5	H ₇₃ ^d	B	B	H ₇₃	H ₇₃	?	?	H ₇₃

^a RNA segments numbered in order of increasing mobility on polyacrylamide gels

^b RNA derived from Brigit virus

^c RNA derived from B/HK/5/72 virus

^d RNA derived from B/HK/8/73 virus

inherited RNAs 1, 4 (NP), 5 (HA) and 8 (NS) from B/HK/8/73 virus and RNAs 2 and 3 from Brigit virus. The derivation of RNAs 6 and 7 in R5 could not be determined.

In a recent report (Miller et al., 1977) recombinant R75 was tested in human volunteers and was found to be attenuated. Interestingly, in this report it was stated that R75 virus possessed the surface glycoproteins of B/HK/5/72 virus. From the results of my analyses it is clear that R75 does not possess a B/HK neuraminidase, since R75 inherits the NA gene (RNA 6) from B/USSR virus. Because influenza B virus neuraminidases are antigenically related (Chakraverty, 1972), it is possible to make this type of mistake when serologic tests are being used to determine the genealogy of influenza B virus surface proteins. This error was revealed by the RNA genotyping system, which distinguishes parental genes according to base sequence and can be more sensitive than serologic tests.

Oligonucleotide mapping of RNA from different influenza B virus strains

It was thought that the completion of a genetic map for influenza B viruses would make it possible to compare

the fine structure of all eight genes coding for specific polypeptides, using oligonucleotide fingerprinting techniques. This method has been very useful in our laboratory for detecting differences in the genomes of different influenza A virus strains (Desselberger et al., 1978; Nakajima et al., 1978; Young et al., 1979; Young and Palese, submitted). In this procedure, viral RNA is first digested with RNase T1, and the resulting oligonucleotides are labelled at the 5' end using γ -[^{32}P]-ATP and polynucleotide kinase. The labelled oligonucleotides are separated in two dimensions on polyacrylamide gels and detected by autoradiography. Comparison of the genomes of different influenza B viruses with this technique might provide information on the amount of variation in influenza B virus genes compared with that in influenza A virus genes.

Figure 21 shows the RNA fingerprint of B/Lee/40 virus. Oligonucleotides are separated from bottom to top according to size, and the largest spot is about 40 bases long, while the smallest spot is two to three bases in length. Oligonucleotides to the left are C and A rich, and oligonucleotides to the right are G and U rich. The map represents approximately 10-12% of the total genome, presumably a random sampling over each RNA. A fingerprint of another



Figure 21. Fingerprint of RNA from B/Lee/40 virus. RNA was extracted from purified virus, digested with RNase T1, and the resulting oligonucleotides were labelled at their 5' ends using γ - ^{32}P -ATP and polynucleotide kinase as described in Materials and Methods. Lower X, xylene cyanol FF dye; upper X, bromophenol blue dye.

influenza B virus isolated in 1972 is shown in Figure 22. The fingerprint of B/HK/5/72 virus RNA has 50 out of 100 of large oligonucleotides in common with the fingerprint of B/Lee/40 virus RNA. This analysis is performed by co-electrophoresing mixtures of oligonucleotides from the two viruses on the same gel. In contrast, the fingerprints of RNA from two viruses isolated only one year apart, influenza B/HK/5/72 and B/HK/8/73 viruses, share 91 out of 100 large oligonucleotides and are therefore much more similar (Figures 22, 23).

This type of information simply indicates that viruses isolated many years apart are more different than viruses isolated one year apart. It should be noted that this statement is based on data obtained from a limited number of samples. It has been shown with influenza A viruses that mutations may occur sequentially in the genomes of different strains (Young et al., 1979). This result was obtained by comparing oligonucleotide maps of many influenza H1N1 viruses isolated within several months' time. However, it was not possible to investigate whether sequential mutation also occurs in influenza B viruses, since only a small number of viruses were available, most of which had been isolated years



Figure 22. Fingerprint of RNA from influenza B/HK/5/72 virus. Experimental conditions are as described in the legend to Figure 21.



Figure 23. Fingerprint of RNA from influenza B/HK/8/73 virus. Experimental conditions are as described in the legend to Figure 21.

apart. Since oligonucleotide maps of RNA from influenza B virus strains isolated several years apart were very different, it was not possible to demonstrate sequential mutations in the genome of these isolates.

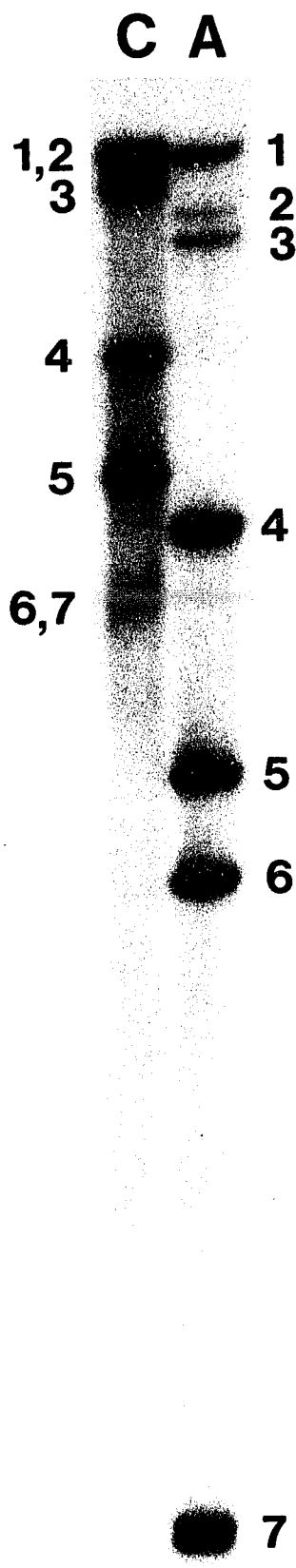
B. STUDIES ON INFLUENZA C VIRUSES

Influenza C viruses contain 7 RNA segments

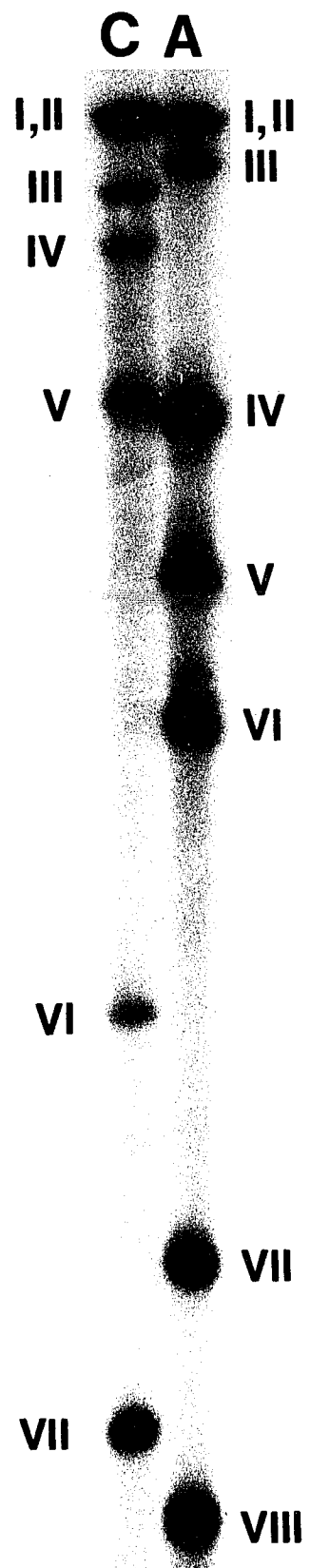
The influenza C virus genome has been reported to consist of from 4 to 6 RNA species (see Introduction). Using the techniques which I had developed for the analysis of influenza B viruses, the genome of influenza C viruses was investigated.

When this work was begun, a productive cell culture system for influenza C viruses was not available, and it was necessary to use the embryonated egg as the host. Viral RNA was labelled with ^{32}P in ovo by including the isotope in the inoculum. Virus was purified from amniotic fluids, and RNA extracted and electrophoresed on polyacrylamide gels containing 6 M urea. An autoradiogram of the separated RNAs of influenza C/GL/54 virus is shown in Figure 24a. For comparison the RNA of influenza A/PR/8/34 virus has been electrophoresed in the next lane. The RNAs of the influenza A virus

Figure 24. RNA patterns of influenza C/GL/54 and A/PR/8/34 viruses. Influenza C virus RNA was labelled with ^{32}P in ovo and influenza A virus RNA was labelled with ^{32}P in MDCK cells. Untreated and glyoxal-treated RNA was separated on 2.6% polyacrylamide gels and detected by autoradiography. Untreated RNAs are numbered in order of increasing mobility on the gel, and glyoxalated RNAs are given Roman numerals in order of decreasing molecular weight. A. Lane 1, untreated RNA of C/GL/54 virus; lane 2, untreated RNA of A/PR/8/34 virus (RNA 8 has been run off the gel). B. Lane 1, glyoxalated RNA of C/GL/54 virus; lane 2, glyoxalated RNA of A/PR/8/34 virus.



A

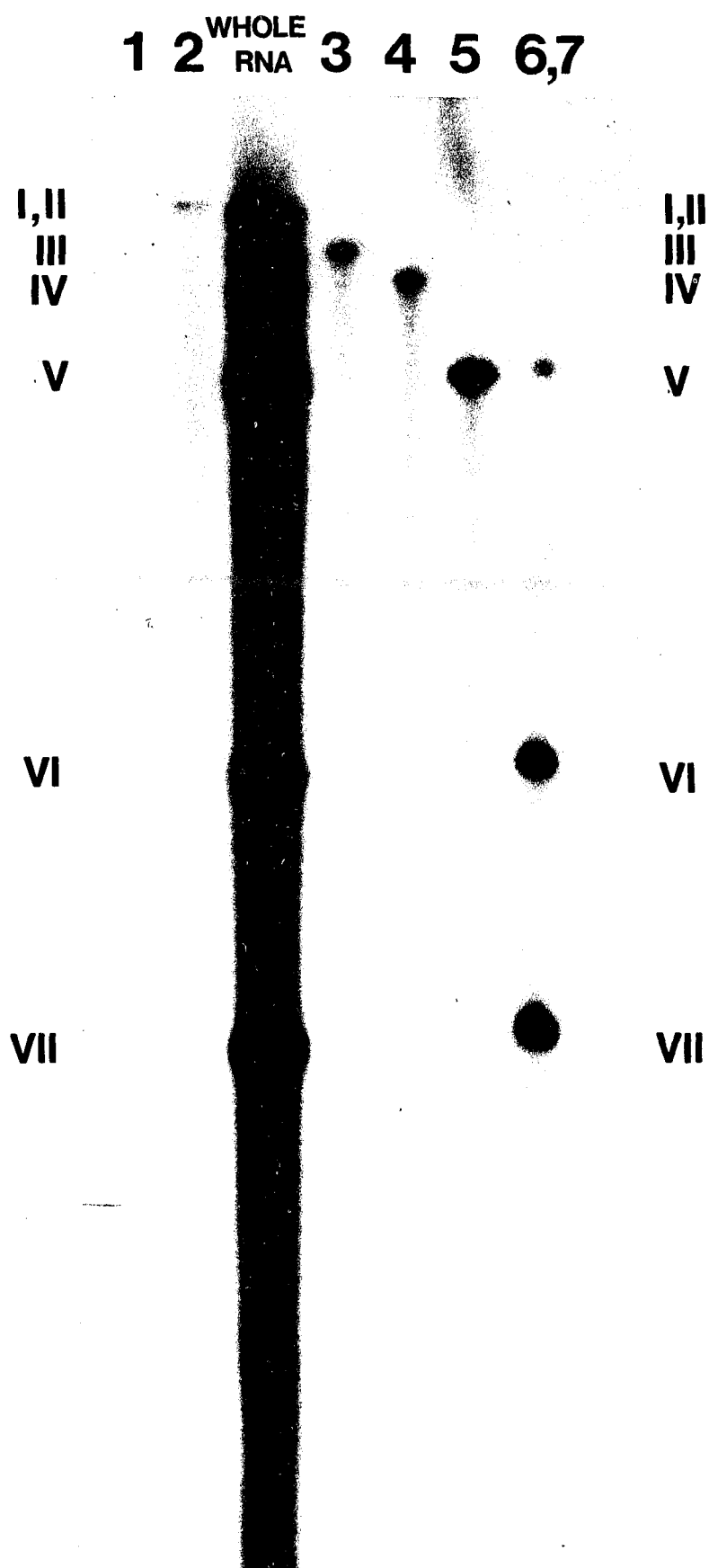


B

are numbered 1-8 (RNA 8 has run off the bottom of the gel). Seven influenza C virus RNAs are labelled: 3 RNAs which migrate near RNA 1 of A/PR/8 virus, two distinct middle sized RNAs and two smaller RNAs which migrate as a very diffuse band. No other smaller RNAs were observed when the gel was run for a very short period of time.

In Figure 24b, the same influenza A and C virus RNAs have been electrophoresed after glyoxal treatment. The order of migration of the influenza A virus RNAs after glyoxal treatment was determined previously (Desselberger and Palese, 1978). The migration order of influenza C virus genes after glyoxal treatment was identified by using the previously described glyoxal technique. Briefly, untreated influenza C virus RNA is separated and then gel slices containing individual RNAs are glyoxalated and rerun on another gel in the presence of whole glyoxalated RNA. The analysis of influenza C/GL/54 virus RNA is shown in Figure 25. Whole glyoxalated RNA has been run in lane 3, and lanes 1, 2, 4, 5, 6 and 7 contain individual glyoxalated segments. RNAs 1 and 2 migrate in this gel (Figure 25) as a single band after

Figure 25. Determination of the gene order for influenza C/GL/54 virus. Gene order of C/GL/54 virus RNAs after glyoxal treatment was determined using the method shown in Figure 19. Lanes 1, 2, 4, 5, 6 and 7 contain, respectively, glyoxalated individual RNA segments 1, 2, 3, 4, 5 and 6 plus 7 of C/GL/54 virus. Lane 3 contains whole glyoxalated C/GL/54 virus RNA. Glyoxalated segments are identified by Roman numerals in order of decreasing molecular weight.



glyoxal treatment, which can be resolved by a longer electrophoretic run. RNA 3 migrates faster and RNAs 4 and 5 migrate slower after glyoxal treatment, and glyoxalation of the diffuse RNA band labelled 6, 7 in Figure 24a generates two discrete faster moving RNAs (Figure 25). From these data it appears that the genome of these viruses contains seven RNA segments.

The RNAs of two other influenza C virus isolates, C/Tay/47 and C/JJ/50, were also analyzed in the same way as described above, and the same migration patterns were obtained (Figure 26). In addition, glyoxal treatment of the RNA abolishes the slight, strain-specific migration differences observed among RNAs 3, 4 and 5 of the three viruses when the RNA is run without glyoxal treatment (Figure 26, lanes 4, 5, 6).

All of this information led to the conclusion that the influenza C virus genome consists of seven RNA segments. This result contrasts with the finding of eight RNA segments for influenza A and B viruses.

Figure 27 shows the glyoxalated RNA patterns of an influenza A, an influenza B and an influenza C virus. The eight RNA segments of the influenza A and B viruses are identified by their protein product, and the seven

Figure 26. RNA patterns of different influenza C virus strains. RNA of influenza C viruses was labelled with ^{32}P in ovo, electrophoresed on 2.6% polyacrylamide gels, and detected by autoradiography. Lanes 1-3 contain glyoxal treated RNA; lanes 4-6 contain untreated RNA. Numbering of untreated and glyoxalated RNA segments is as described in Figure 25. Lanes 1 and 4, RNA of C/Tay/47 virus; lanes 2 and 5, RNA of C/GL/54 virus; lanes 3 and 6, RNA of C/JJ/50 virus.

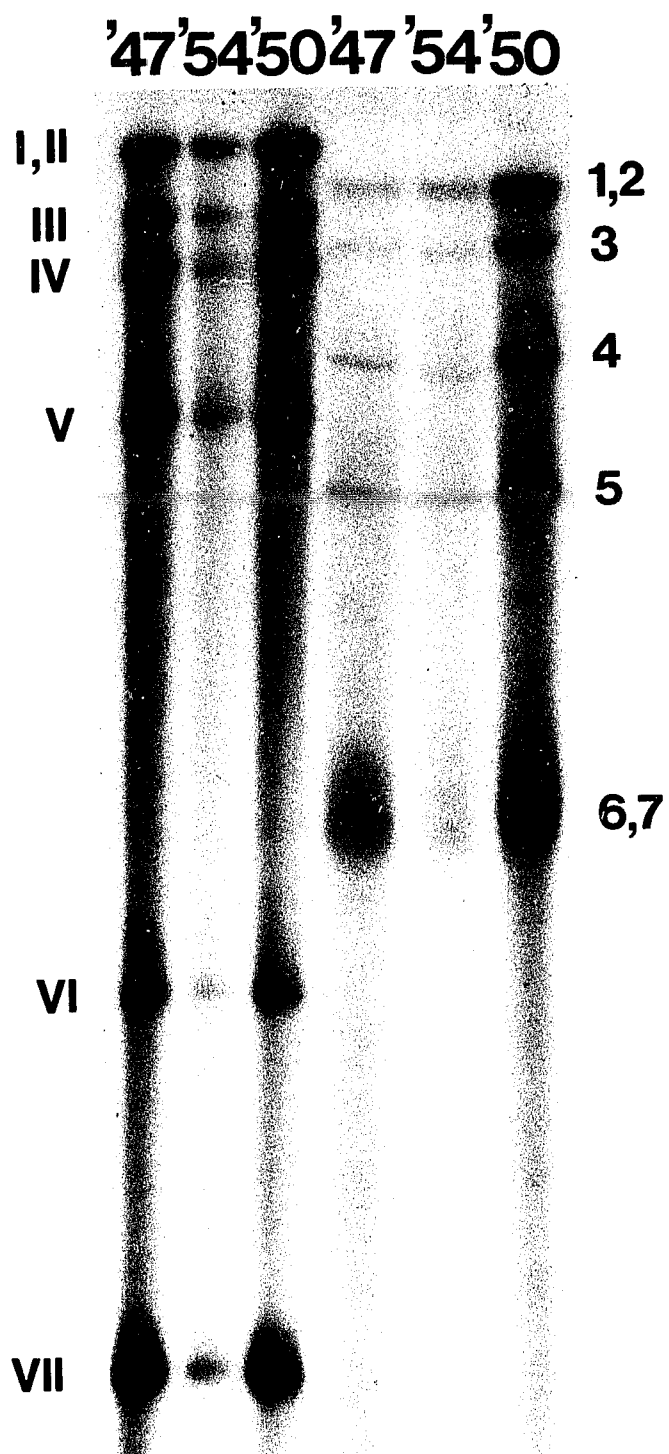
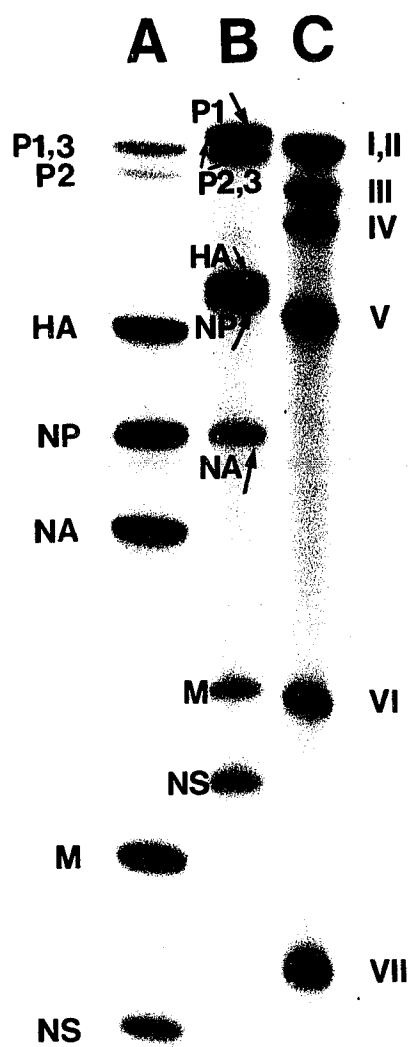


Figure 27. Patterns of glyoxal treated RNA from an influenza A, B and C virus. RNAs of influenza A/PR/8/34 and B/Lee/40 viruses were labelled with ^{32}P in MDCK cells, and RNA of C/GL/54 virus was labelled with ^{32}P in ovo. After glyoxal treatment, RNA was electrophoresed on a 2.6% polyacrylamide gel and detected by autoradiography. RNA segments of the influenza A and B virus are identified by their protein product. RNAs of the influenza C virus are given Roman numerals in order of decreasing molecular weight. Lane 1, RNA of A/PR/8/34 virus; lane 2, RNA of B/Lee/40 virus; lane 3, RNA of C/GL/54 virus.



influenza C virus RNAs are given Roman numerals which indicates that the RNAs are separated according to molecular weight. The molecular weights of glyoxalated RNA segments of influenza C/GL/54 virus were determined using marker RNAs (see Table 7). The total molecular weight of the influenza B virus genome (5.34×10^6) is the highest, followed by the influenza A virus genome (4.83×10^6) and the influenza C virus genome (4.79×10^6).

Analysis of the influenza C virus genome by oligonucleotide mapping

Investigators have found, using various serologic techniques, that antigenic variation in influenza C viruses is minor, and that all influenza C virus strains are related (Pereira, 1969; Czekalowski and Prasad, 1973; Chakraverty, 1978; reviewed in Kilbourne, 1975). In these studies, the serologic techniques were used primarily to compare the surface glycoprotein of different strains.

Examination of the RNAs of different influenza C virus isolates can yield information on genetic variation

of the entire genome. In Figure 26 it can be seen that RNAs 3, 4 and 5 of three viruses isolated in 1947, 1950 and 1954 have different migration rates. This finding suggested that there are genetic differences among several genes of different influenza C virus isolates. These differences were further investigated using the oligonucleotide fingerprinting technique. Careful comparison of the fingerprint patterns of influenza C viruses isolated in 1947, 1950, 1954, 1966 and 1976 indicates that, although the genomes of the viruses are very similar, there are readily distinguishable differences (Figures 28-32). For example, the patterns of influenza C/JJ/50 and C/JHG/66 viruses appear to be very similar, but detailed analysis by co-electrophoresis of the oligonucleotides from both viral RNAs reveals that the patterns of the two viruses differ by 33 spots out of 77 spots which were used for the analysis (Figures 31, 32; see Isolation of influenza C virus recombinants). These results indicate that the RNA fingerprinting techniques can be used to readily distinguish different influenza C virus isolates.



Figure 28. Fingerprint of RNA from influenza C/Taylor/47 virus. Experimental conditions are as described in the legend to Figure 21.

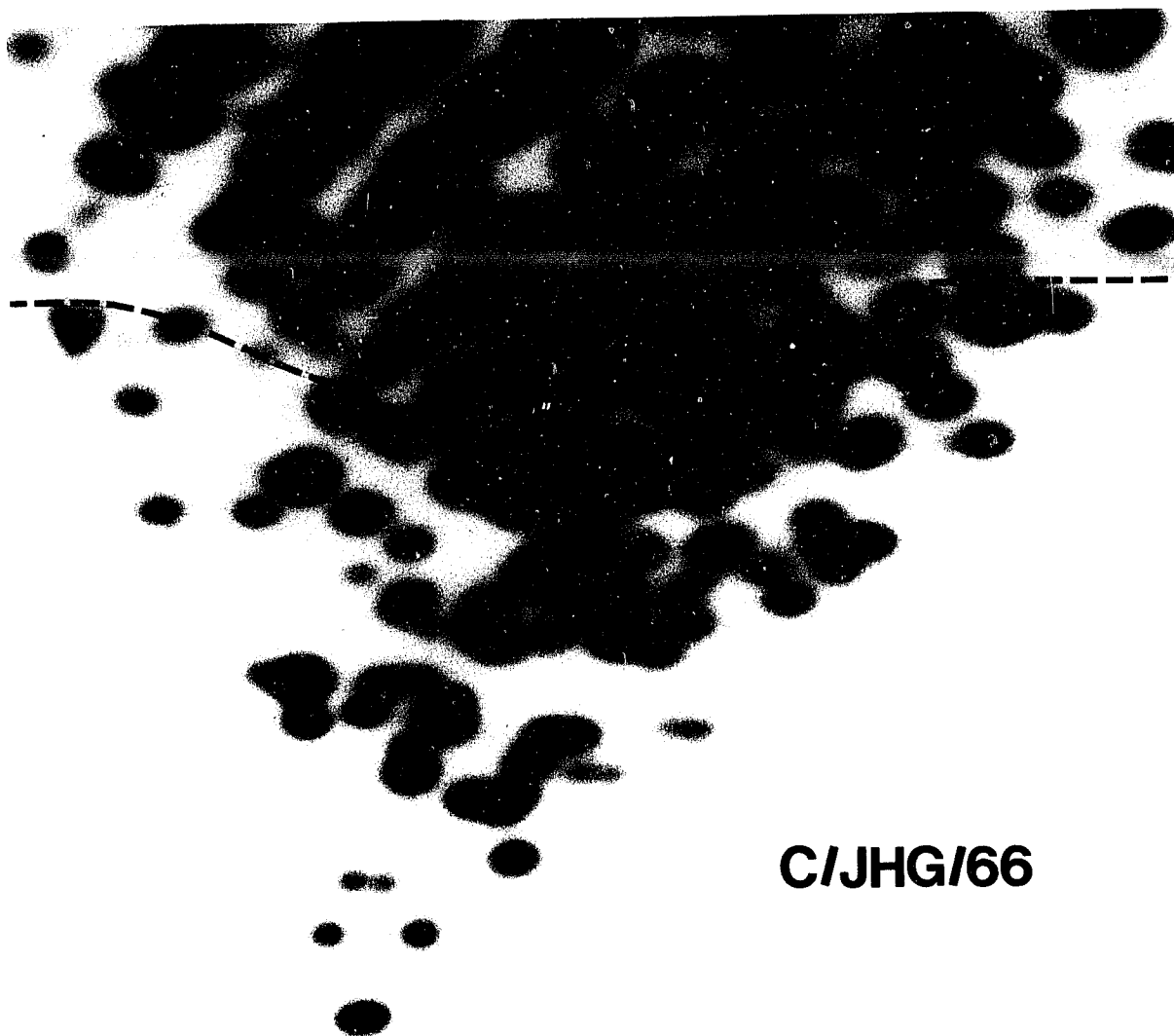


Figure 29. Fingerprint of RNA from influenza C/GL/54 virus. Experimental conditions are as described in the legend to Figure 21.



Figure 30. Fingerprint of RNA from influenza C/NJ/76 virus. Experimental conditions are as described in the legend to Figure 21.

Figure 31. Fingerprint of RNA from influenza C/JHG/66 virus. Experimental conditions are as described in the legend to Figure 21. A, autoradiogram of the fingerprint of C/JHG/66 virus RNA; B, drawing of autoradiogram in A; oligonucleotides used for analysis (below the dotted line) are numbered 1-77. Faint oligonucleotide spots below the dotted line which are not numbered were not observed consistently.



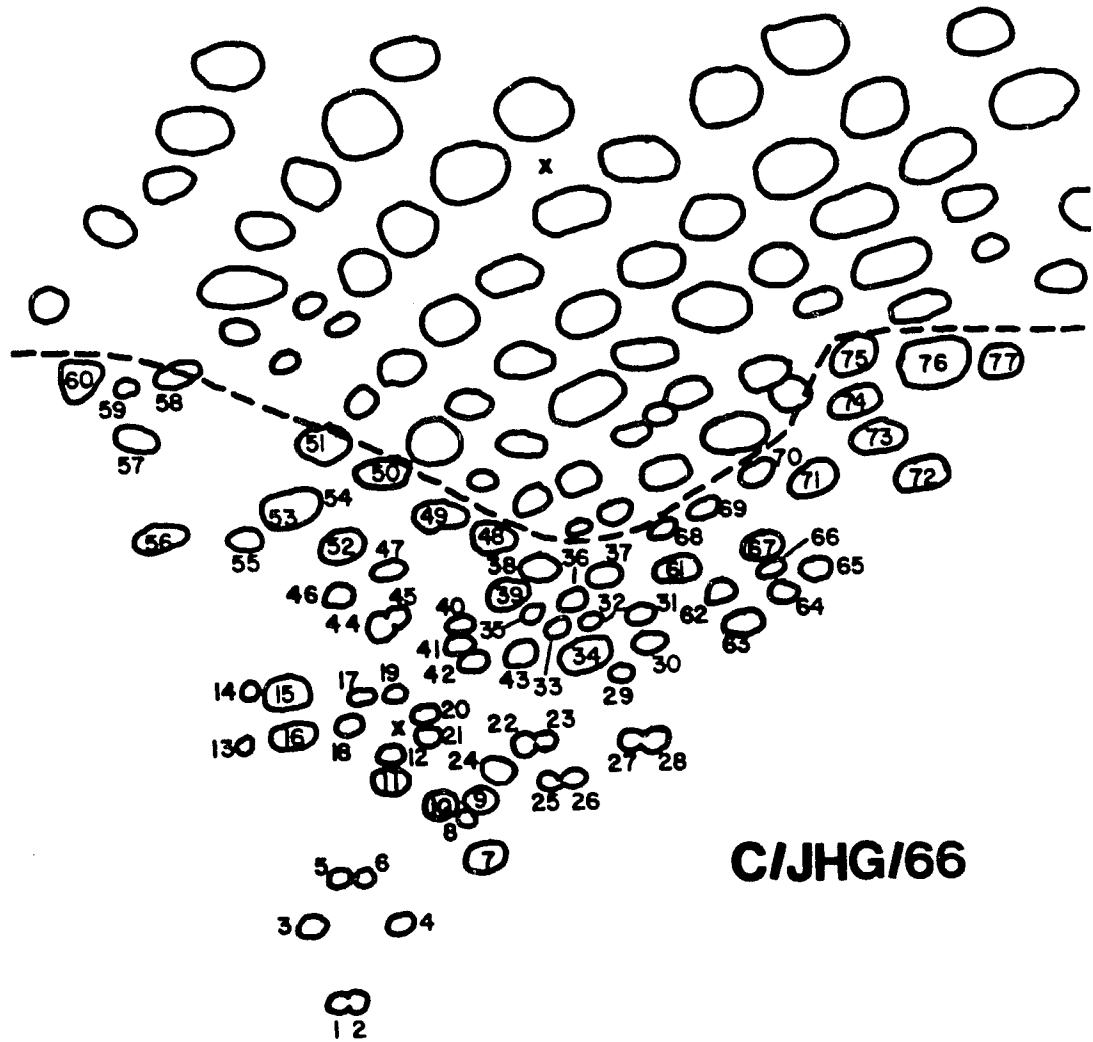
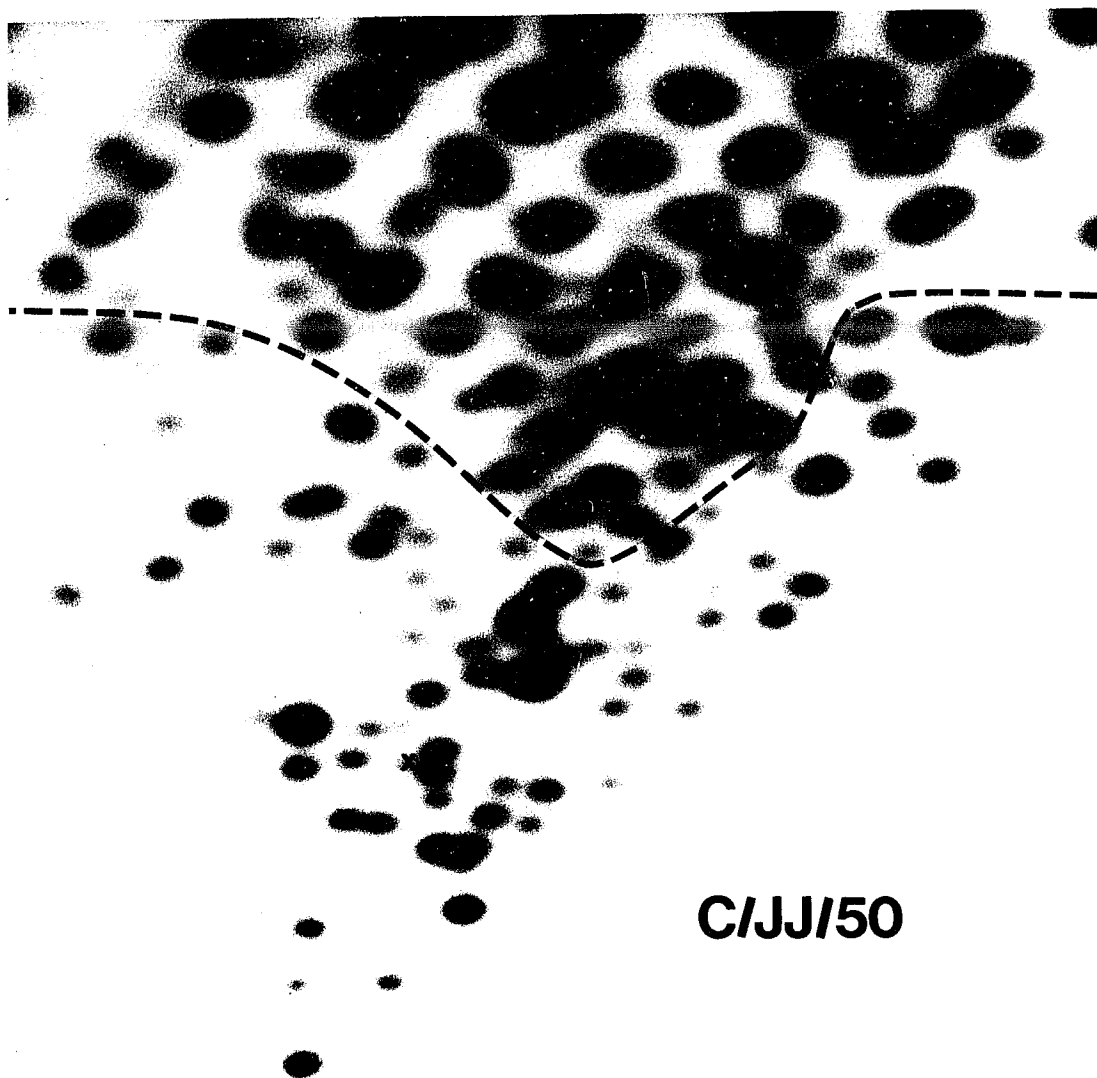
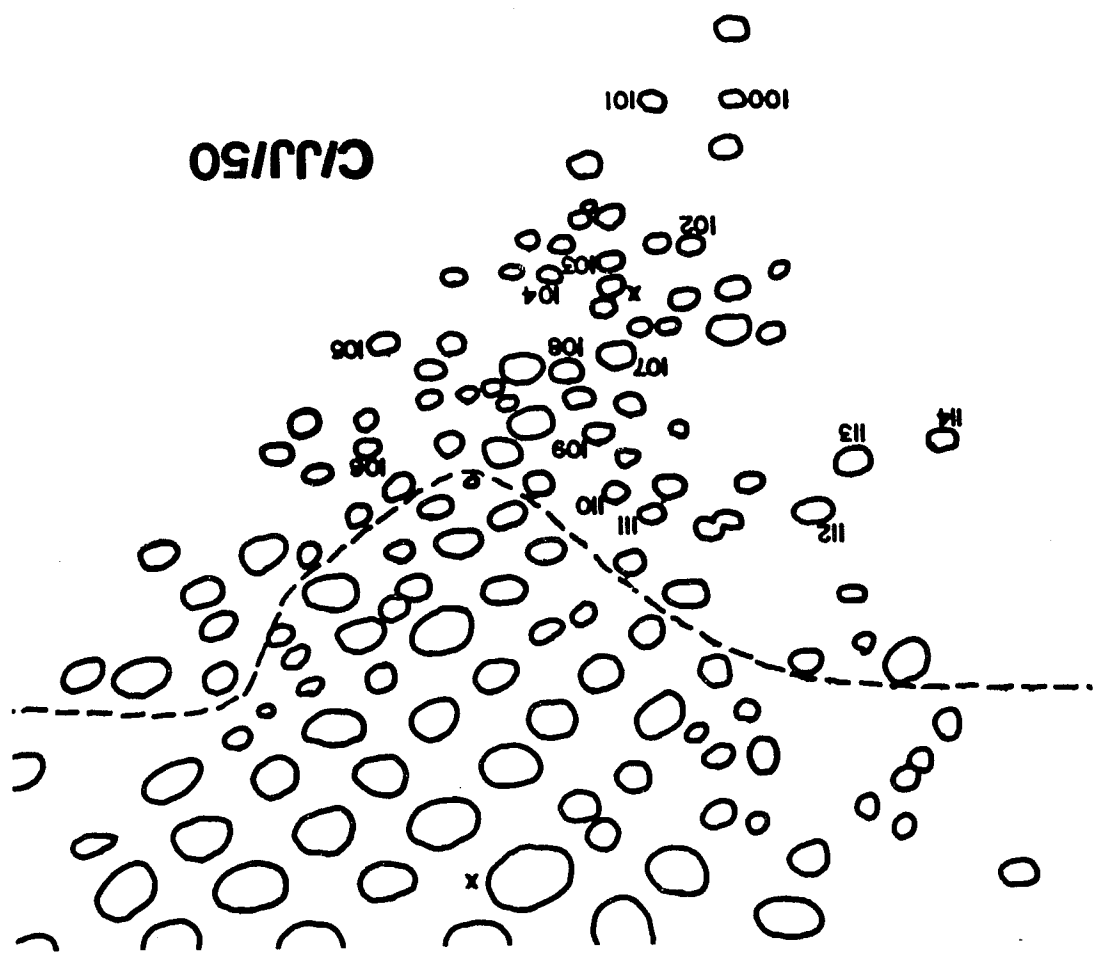


Figure 32. Fingerprint of RNA from influenza C/JJ/50 virus. Experimental conditions are as described in the legend to Figure 21. A, autoradiogram of fingerprint of C/JJ/50 virus RNA; B, drawing of autoradiogram in A; oligonucleotide spots not present in the fingerprint of C/JHG/66 virus RNA (see Figure 31B) are numbered 100-114. Unlabelled spots below the dotted line are common to both C/JJ/50 and C/JHG/66 viruses. The numbers of these common spots are the same as shown in Figure 31B.





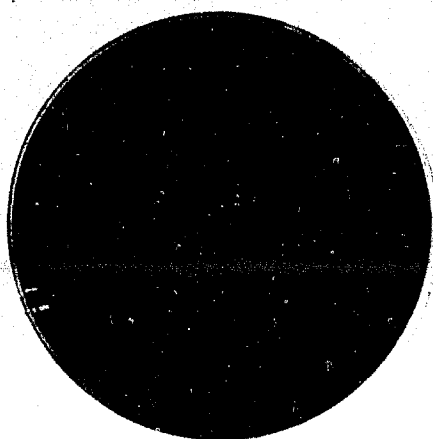
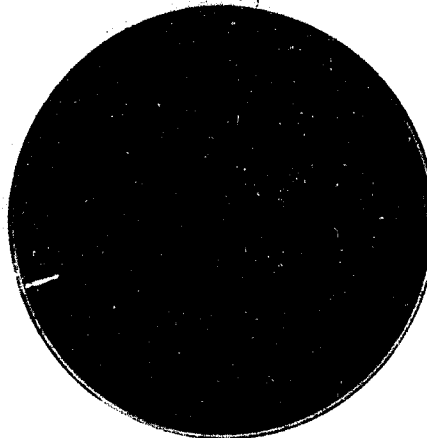
Development of a plaque assay system for influenza C
viruses

In order 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 (Chakraverty, 1974; Compans et al., 1977; O'Callaghan et al., 1977; Austin et al., 1978; Meier-Ewert et al., 1978; Nerome and Ishida, 1978; Petri et al., 1979; Nerome et al., 1979). In one report (Nerome and Ishida, 1978) it was shown that influenza C/JJ/50 virus forms plaques in MDCK cells in the presence of trypsin (20-40 $\mu\text{g/ml}$). The incorporation of trypsin into the agar overlay facilitates plaquing of influenza A and B viruses (Tobita et al., 1975; Tobita, 1975) which is most likely due to cleavage of the HA by the protease (Lazarowitz and Choppin, 1975). However, this high level of trypsin (20-40 $\mu\text{g/ml}$) required to plaque influenza C viruses frequently damages cell monolayers. Lazarowitz et al. (1973b) have found that the serum zymogen plasminogen is converted to plasmin by an activator present in MDBK cells, and that plasmin is most likely responsible for the cleavage

of HA of newly formed virus. Later it was shown that incorporation of plasminogen into the agar overlay of MDCK cells facilitated plaquing of influenza A viruses (Schulman, unpublished results). In addition, MDCK monolayers can tolerate high (20-40 $\mu\text{g/ml}$) levels of plasminogen (Schulman, personal communication).

It was found that most influenza C viruses will produce clear plaques in MDCK cells when human plasminogen is incorporated into the agar overlay. MDCK monolayers were infected with different influenza C viruses and stained with crystal violet as described in Materials and Methods. From Figure 33 it can be seen that influenza C/Taylor/47, C/JJ/50 and C/GL/54 viruses produce clear, easily distinguishable plaques in this cell system while influenza C/JHG/66 virus produces turbid plaques which cannot be counted. In addition, it was found that C/JJ/50 virus will form clear plaques when 10% fetal bovine serum is incorporated into the agar overlay. The absence of any protease in the agar overlay prevents plaque formation by influenza C viruses in this system.

Figure 33. 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/ml human plasminogen. After 4 days incubation at 34°C, agar was removed and the monolayers were stained with crystal violet. A, B, C: cells infected with influenza C/Tay/47, C/JJ/50 and C/GL/54 viruses, respectively, which all produce clear plaques; D, cells infected with influenza C/JHG/66 virus, which produces turbid plaques that cannot be counted.

A**B****C****D**

Isolation of influenza C virus recombinants

Influenza A and B viruses both possess segmented genomes, and recombination occurs by reassortment of RNA segments (Tobita and Kilbourne, 1974; reviewed in Kilbourne, 1975). It has been shown here, as well as by others, that influenza C viruses possess a segmented genome. However, it has not been possible to demonstrate recombination of influenza C viruses, since genetic and biochemical markers were not available. The work reported in previous sections made it possible to demonstrate recombination of influenza C viruses by using two markers:

(1) differences in plaque morphology between C/JJ/50 virus and C/JHG/66 virus, and (2) differences in RNase T1 fingerprint patterns of the two viral RNAs.

RNA fingerprints of the two viruses chosen for the recombination study are shown in Figures 31a and 32a; Figures 31b and 32b are schematic diagrams of the fingerprints. 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 (Figure 31), these oligonucleotides have been arbitrarily assigned numbers from 1 to 77. The fingerprint of the RNA of the second virus is shown in Figure 32. Differences in the patterns of the two viruses were established by fingerprinting mixtures of oligonucleotides from the two viral RNAs on the same gel. There are 18 oligonucleotides specific for C/JHG/66 virus which are absent in the pattern of C/JJ/50 virus RNA, and 15 oligonucleotides (numbered 100-114, Figure 32b) which are present in C/JJ/50 virus RNA and absent in C/JHG/66 virus RNA.

In order to isolate recombinants of influenza C viruses, supernatants of MDCK cells, which had been doubly infected with C/JHG/66 virus and UV-irradiated C/JJ/50 virus, were plaqued in MDCK cells. 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. The experiment is summarized in Table 9.

Since UV-irradiated C/JJ/50 virus did not form plaques, while C/JHG/66 virus produced turbid plaques,

TABLE 9

VIRUS YIELD AFTER INFECTION OF MDCK CELLS WITH UV-IRRADIATED
C/JJ/50 VIRUS, C/JHG/66 VIRUS AND VIRUS MIXTURE

<u>Virus</u>	<u>PFU/ml</u> ^a
UV-C/JJ/50 ^b	<10
C/JHG/66 ^b	<10 ^c
Recombinational mixture ^b	1.2 x 10 ⁶

^a determined in MDCK cells

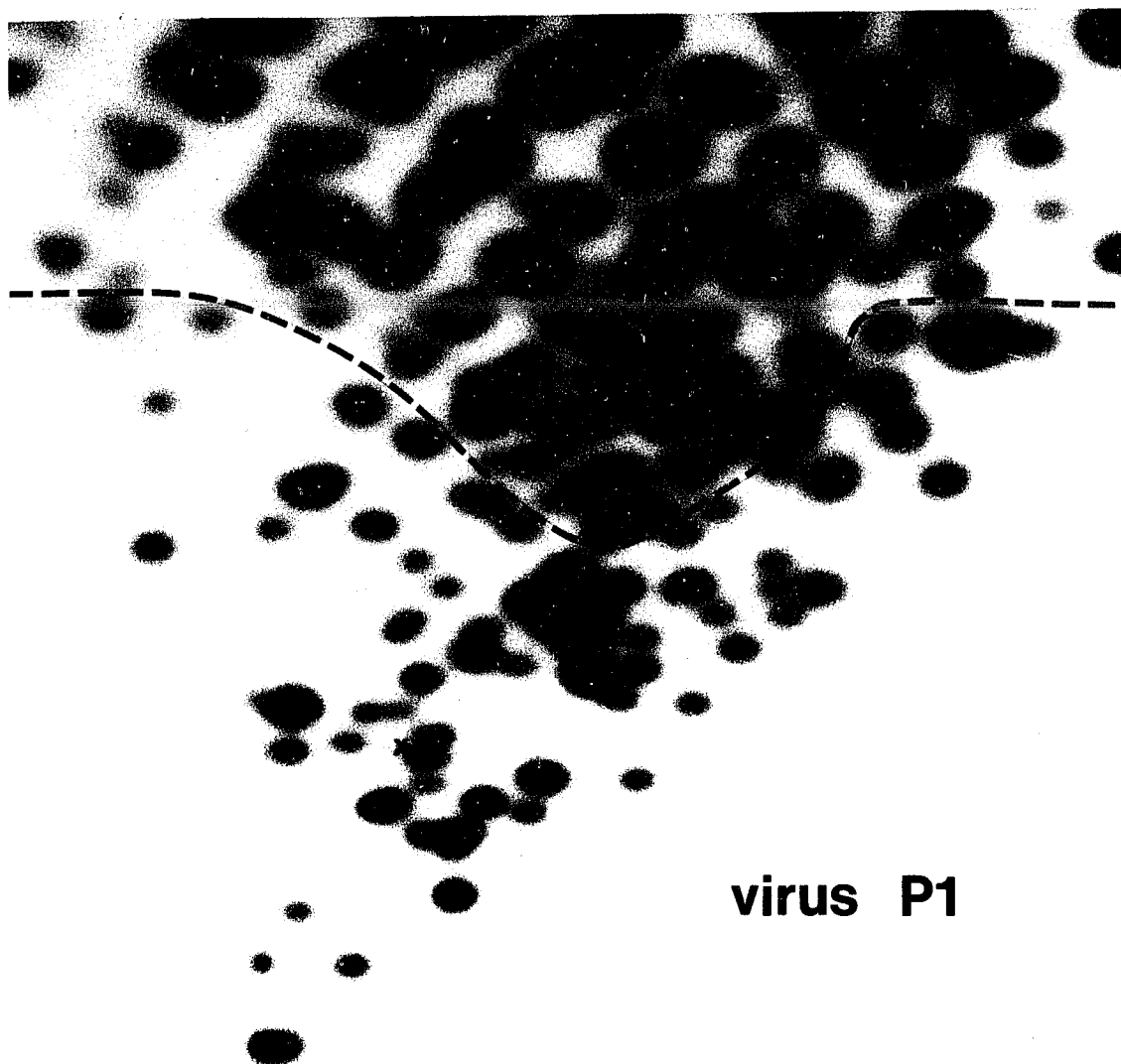
^b MDCK cells were infected with 0.1 ml of UV-irradiated C/JJ/50 virus (100 HAU/ml), C/JHG/66 virus (100 HAU/ml) and a 1:1 mixture of the two virus preparations (200 HAU/ml). Yields were assayed after 3 days incubation at 34°C as described in Materials and Methods.

^c Plaques were turbid and could not be counted.

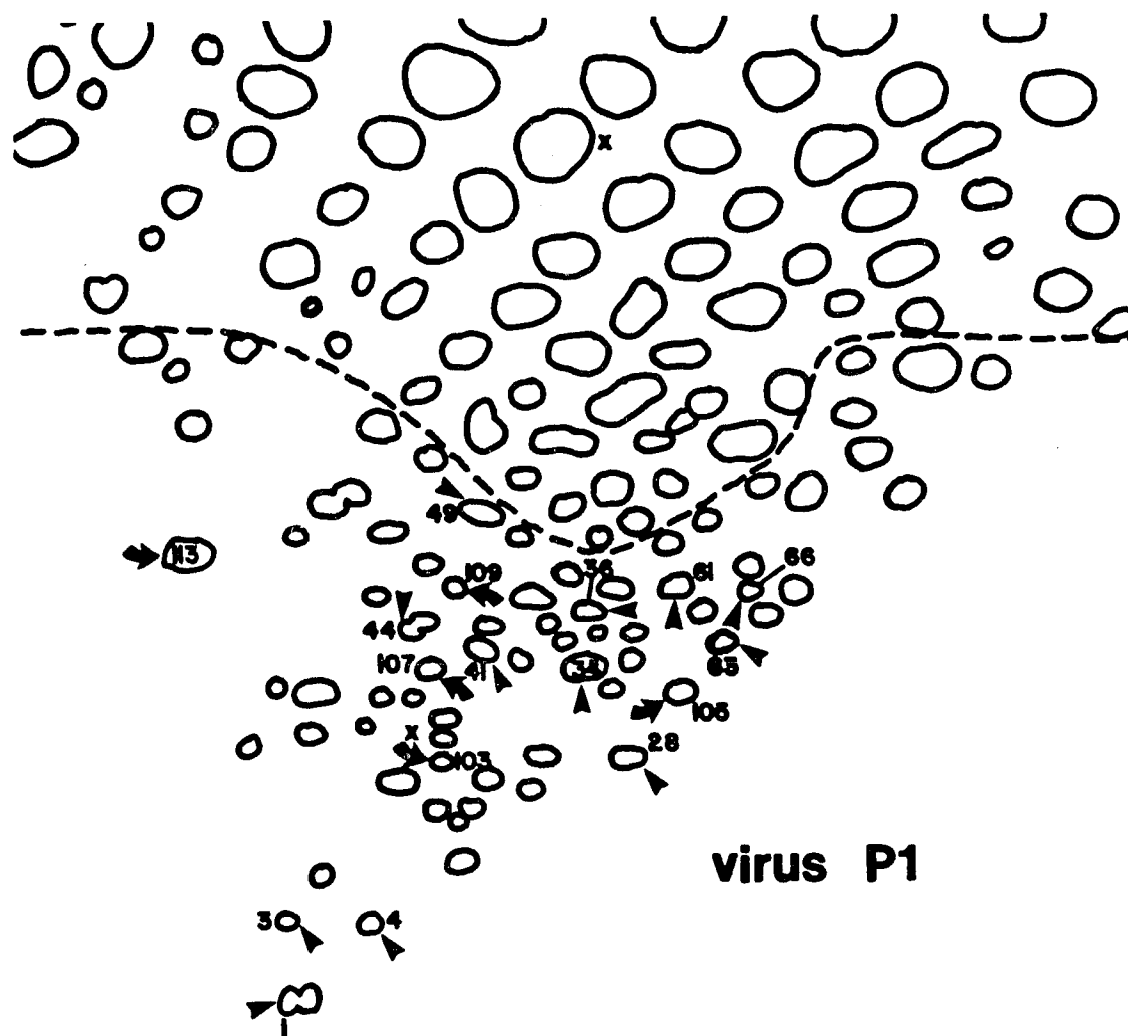
the presence of lytic plaques in the recombinational mixture suggested that recombination had occurred. In order to obtain further evidence that recombination between parental viruses had taken place, fourteen clones isolated from cells infected with the recombinational mixture were plaque-purified two times as described in Materials and Methods. These cloned viruses were then subjected to the biochemical analysis described below.

In order to confirm that the cloned viruses were recombinants, RNA from four isolates was prepared and fingerprinted as described in Materials and Methods. The fingerprint of one clone, virus P1, is shown in Figure 34a; Figure 34b is a drawing of the oligonucleotide spots. On this drawing, oligonucleotides derived from C/JHG/66 virus are indicated by an arrowhead and the appropriate number, and oligonucleotides derived from C/JJ/50 virus are labelled with an arrow and the appropriate number. 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. It is clear from Figure 34b that virus P1 derives specific oligonucleotides from both parents and lacks several oligonucleotides which are specific for either C/JJ/50

Figure 34. Fingerprint of RNA from virus P1. Experimental conditions are as described in the legend to Figure 21. A, autoradiogram of the fingerprint of virus P1 RNA; B, drawing of autoradiogram in A; unique oligonucleotide spots inherited from C/JHG/66 virus (see Figure 31B) are indicated by an arrowhead and the appropriate number, and oligonucleotide spots inherited from C/JJ/50 virus (see Figure 32B) are indicated by an arrow and the appropriate number.



virus P1



or C/JHG/66 virus, indicating that this clone is not merely a mixture.

In influenza A and B viruses, recombination between two strains occurs by reassortment of RNA segments. I assume that influenza C viruses also exchange genetic information in this manner, although the data presented above does not exclude the possibility that virus P1 arose by intragenic recombination. Examination of the RNA patterns of C/JHG/66 and C/JJ/50 viruses and virus P1 did not permit analysis of derivation of genes in the recombinant virus. In order to obtain evidence that recombination of influenza C viruses occurs by reassortment, oligonucleotide maps of individual genes were analyzed. If recombination involves 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. Unlabelled C/JHG/66 viral RNA was electrophoresed on 2.6% polyacrylamide gels containing 7 M urea (see Materials

and Methods). Under these conditions, influenza C virus RNA can be separated into 5 bands containing RNA segments 1 and 2, 3, 4, 5, and 6 and 7. (RNAs 1 and 2 migrate too closely to be eluted separately, and RNAs 6 and 7 comigrate as a dense band, Figure 24a). Five gel slices containing these seven RNA segments were excised, and RNA was eluted from the gel, digested with RNase T1 and fingerprinted as described in Materials and Methods.

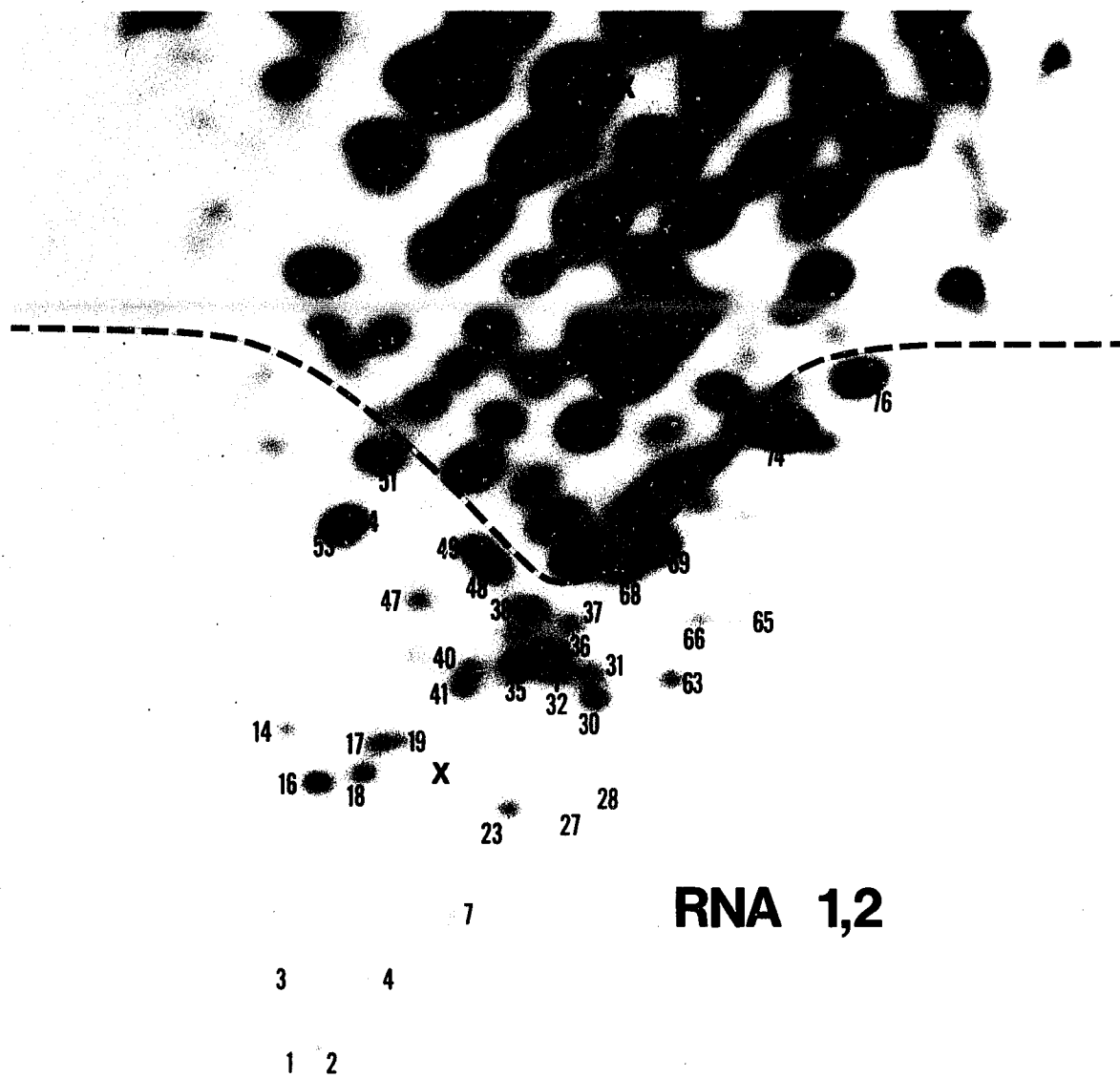
In Figure 35 the fingerprints of C/JHG/66 virus RNA segments are shown. The oligonucleotide spots in each gene are numbered according to their location in the fingerprint of the total RNA of C/JHG/66 virus (Figure 31b). Seventy-two of the seventy-seven large, unique oligonucleotides can be assigned to a gene or group of genes, while five oligonucleotides could not be unambiguously assigned to RNA segments.

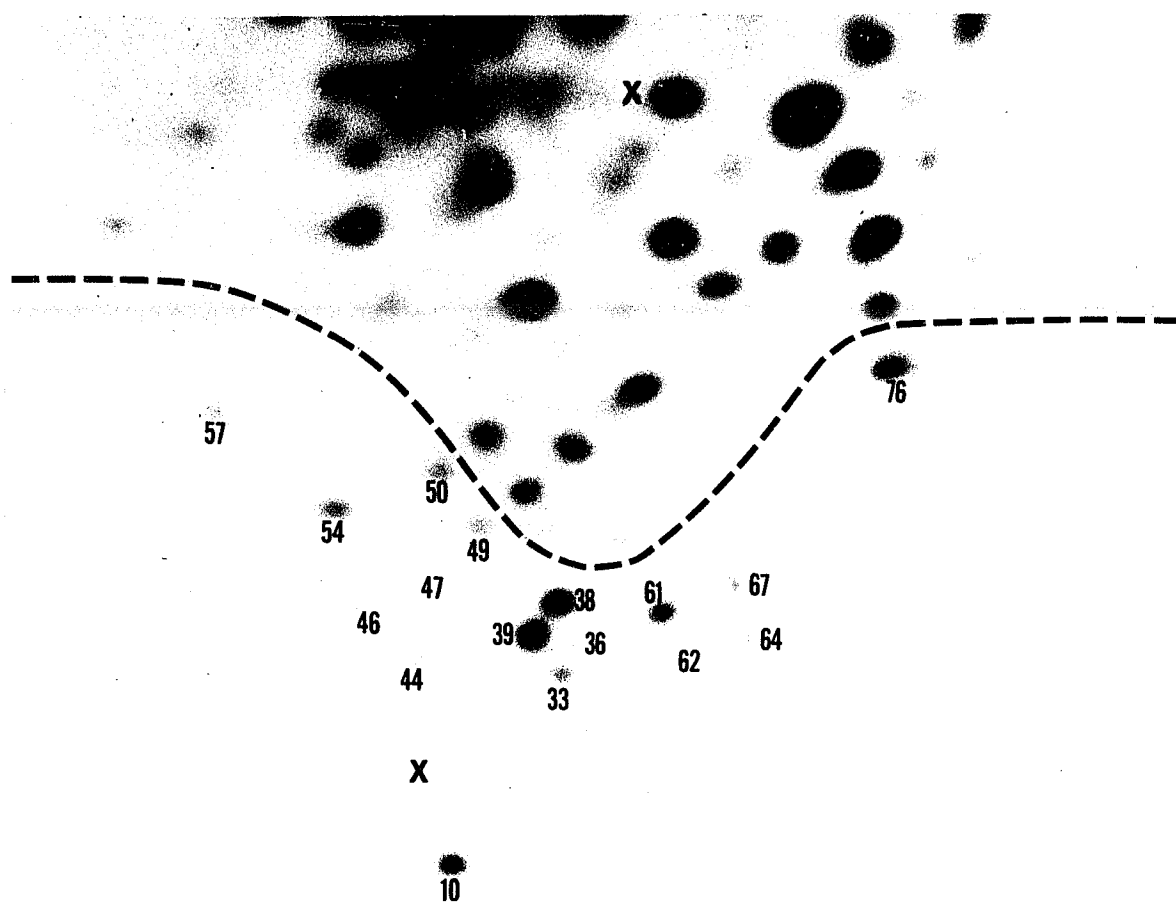
Table 10 is a summary of the oligonucleotide data presented in Figures 31-35. The upper left-hand panel lists the 18 oligonucleotide spots present in C/JHG/66 virus RNA which are absent in C/JJ/50 virus RNA. These unique spots are listed according to the RNA segment to which they were assigned (Figure 35). The upper right-

Figure 35. Fingerprints of influenza C/JHG/66 virus RNA segments. RNA from egg-grown virus was separated on polyacrylamide gels, eluted and digested with RNase T1, and the resulting oligonucleotides were labelled at their 5' ends and fingerprinted as described in the legend to Figure 21. Gene-specific oligonucleotide spots are numbered according to their location in the fingerprint of whole C/JHG/66 virus RNA (Figures 31A, 31B). Some spots are present in more than one gene and most likely represent non-unique sequences. 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 labelling efficiency of the 5' ends of different oligonucleotides.

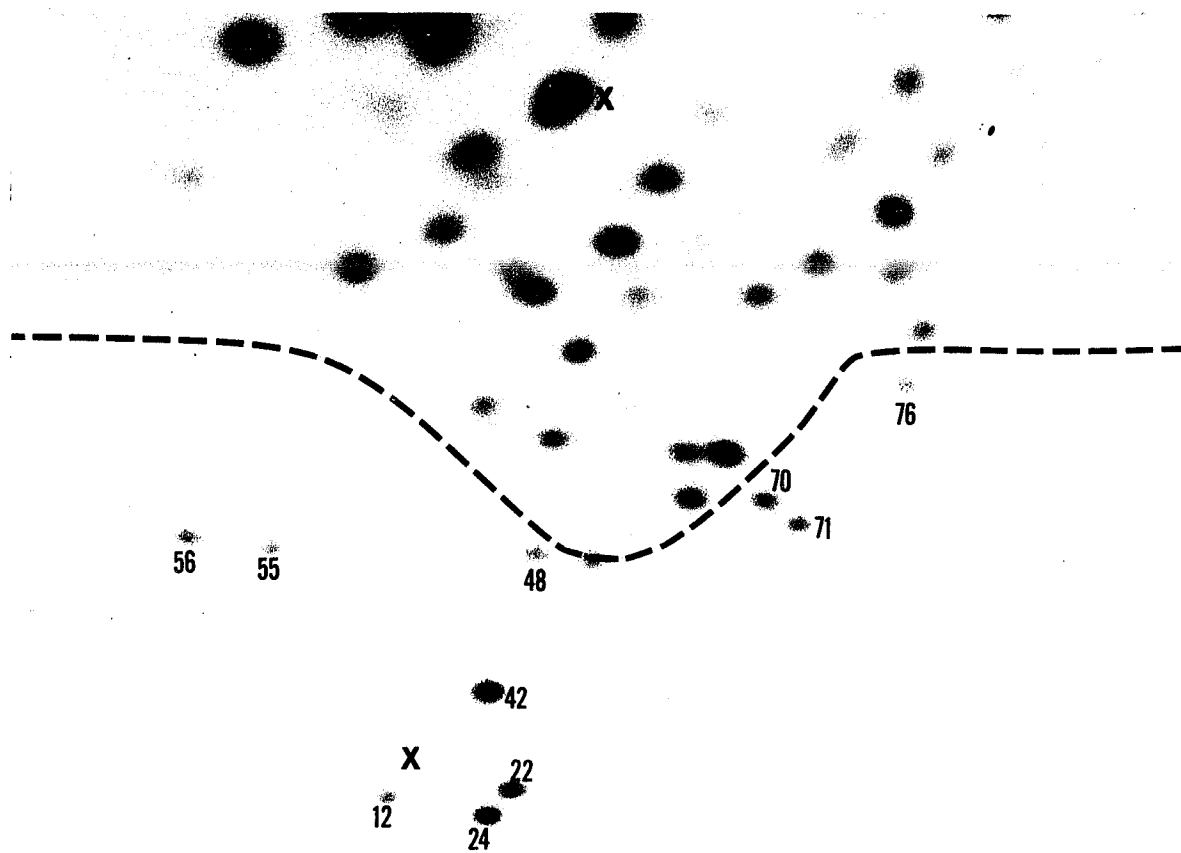
A, autoradiogram of fingerprint of RNA segments 1 and 2;
B, autoradiogram of fingerprint of RNA segment 3;
C, autoradiogram of fingerprint of RNA segment 4;
D, autoradiogram of fingerprint of RNA segment 5;
E, autoradiogram of fingerprint of RNA segments 6 and 7.

Lower X, xylene cyanol FF dye; upper X, bromophenol blue dye.

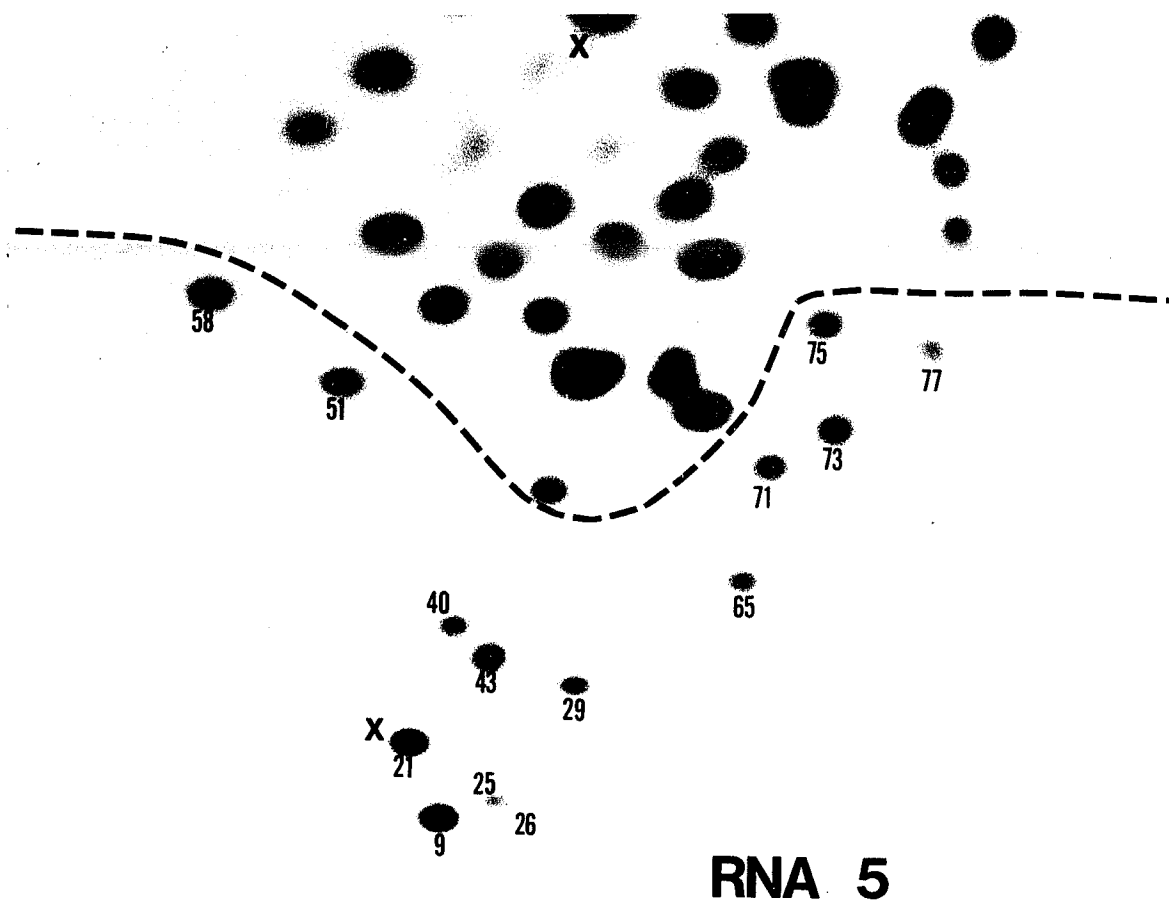




RNA 3



RNA 4



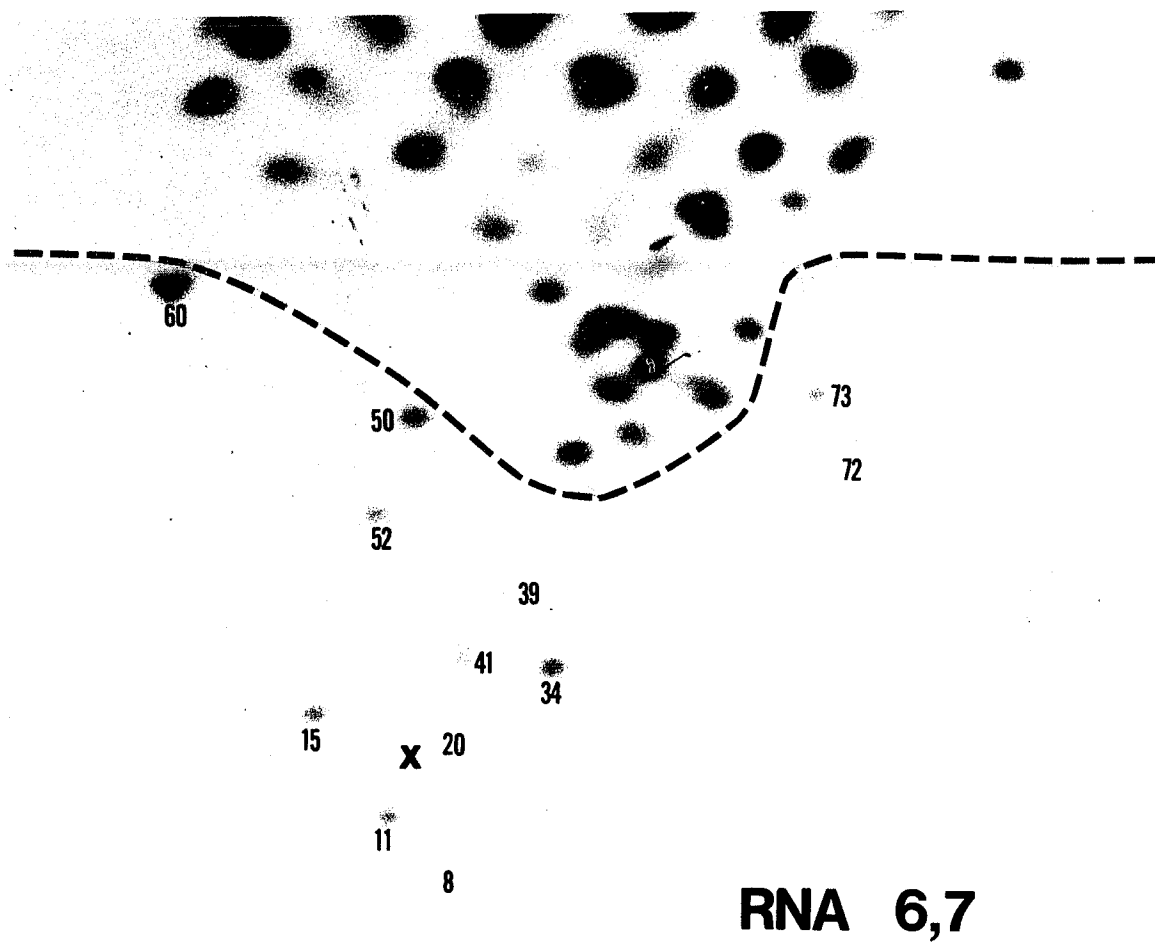


TABLE 10

UNIQUE OLIGONUCLEOTIDES PRESENT IN C/JJ/50 VIRUS,
C/JHG/66 VIRUS AND RECOMBINANT VIRUS P1

<u>Oligonucleotides unique to C/JJ/50 virus</u>	<u>Oligonucleotides unique to C/JHG/66 virus</u>
100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114	RNA segment: 1, 2: 1, 3, 4, 28, 36, 41, 49, 63, 66 3: 36, 44, 49, 61 4: 12, 22, 42, 56 5: 26 6, 7: 34, 41

Derivation of unique oligonucleotides in recombinant virus P1

from C/JHG/66:	RNA 1, 2: 1, 3, 4, 28, 36, 41, 49, 63, 66 3: 36, 44, 49, 61 4: none 5: none 6, 7: 34, 41
from C/JJ/50:	103, 105, 107, 109, 113

hand panel lists the 15 oligonucleotide spots (numbered 100-114) 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 Table 10 indicates the derivation of oligonucleotide spots in recombinant virus P1. From this table it can be seen that recombinant P1 derives all of the C/JHG/66-specific oligonucleotide spots assigned to RNAs 1 and 2, 3, and 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, 113) which are unique to C/JJ/50 virus.

This information indicates 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.

Identification of influenza C virus-specific polypeptides

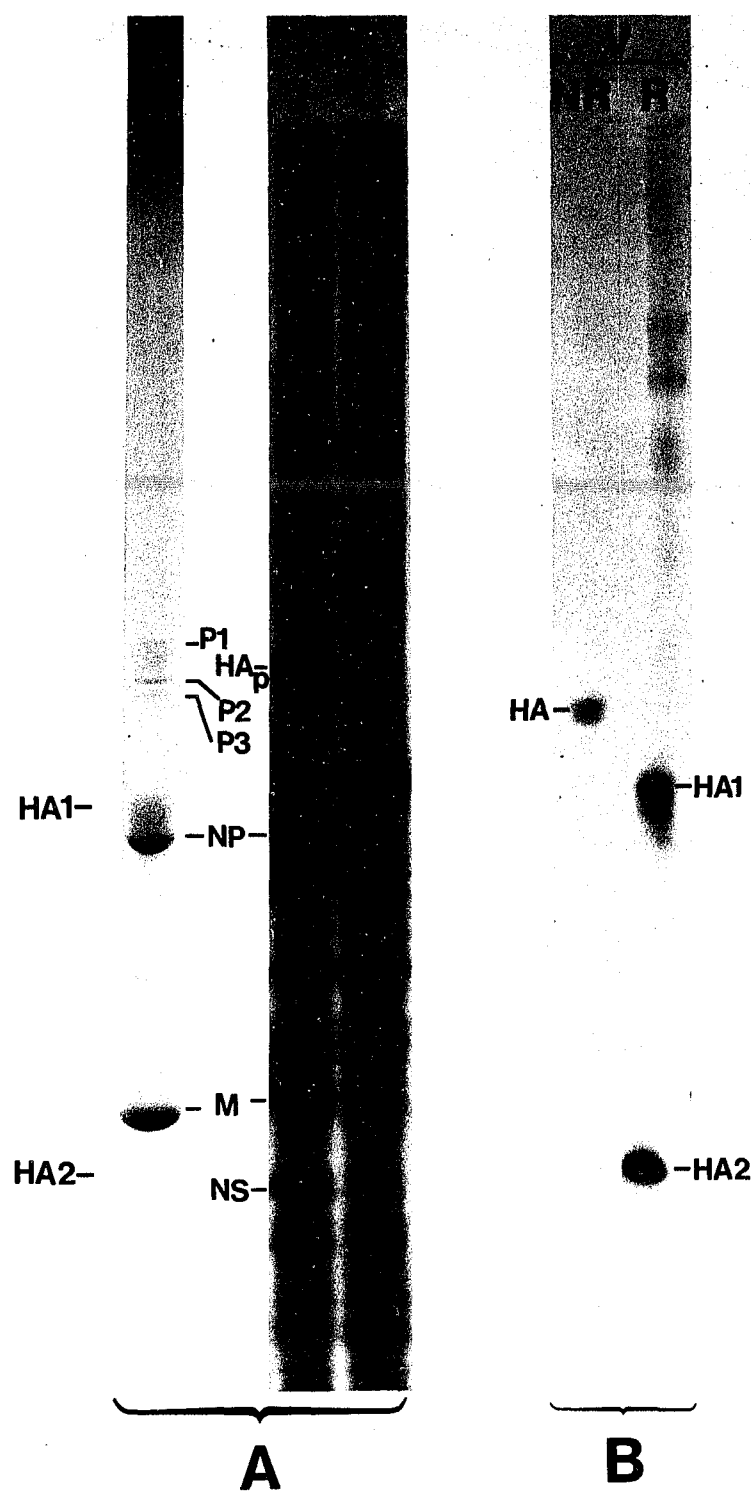
Studies on the influenza C virus polypeptides have been reviewed in the Introduction. Briefly, evidence has been presented for three virus-specific polypeptides, which include the nucleoprotein (NP) and the matrix (M) protein, and one surface glycoprotein. This glycoprotein (gpI; Meier-Ewert et al., 1979) is most likely synthesized as a primary product in infected cells (Compans, personal communication) and may then be processed to a smaller size (gpII/gp88; Meier-Ewert et al., 1979). Unprocessed gpI may be found in virions grown in chicken embryo kidney or fibroblast cells (Meier-Ewert et al., 1979). The smaller glycoprotein, gpII, yields upon reduction two smaller components which may be analogous to HA1 and HA2 of influenza A and B viruses. A hypothetical model of the influenza C virus glycoprotein is shown in Figure 1.

Since it is shown here that influenza C viruses contain seven RNA segments, an effort was made to identify other viral gene products in addition to the NP, M and HA polypeptides. Therefore, polypeptides of purified influenza C virus and of influenza C virus-infected cells were examined by SDS-polyacrylamide gel electrophoresis.

Figure 36 is a composite of several autoradiograms which summarize the findings on influenza C virus polypeptides. Lane 1 of panel A shows the pattern obtained from ^{35}S -methionine-labelled purified egg-grown virus. The polypeptides NP and M were identified according to previously published molecular weights (Compans et al., 1977). To identify glycoproteins, virus was grown in MDCK cells in the presence of ^3H -glucosamine. Panel B of Figure 36 shows the autoradiogram of polypeptides of purified virus labelled in this way. Under non-reducing conditions (Figure 36b, lane 1) most of the glucosamine label migrates as a band which most likely corresponds to the previously reported gp88 (Compans et al., 1977). This glycoprotein, which is called HA in this dissertation, can be further dissociated into two smaller glycoproteins under reducing conditions (Figure 36b, lane 2). These smaller glycoproteins (gp65 and gp30, Meier-Ewert et al., 1978) most likely correspond to HA1 and HA2 of influenza A and B viruses. By comigrating ^3H -glucosamine and ^{35}S -methionine labelled viruses on the same gel, it can be seen that HA1 migrates slower than NP, and HA2 migrates faster than M protein.

Lanes 2 and 3 of Figure 36a show the pattern of ^{35}S -methionine-labelled polypeptides of MDCK cells infected with influenza C virus and uninfected MDCK cells.

Figure 36. Electrophoresis of influenza C virus-specific proteins on 5-13% gradient polyacrylamide-SDS gels. Purified influenza C/JJ/50 virus was continuously labelled with ^{35}S -methionine in ovo or was labelled with ^3H -glucosamine in MDCK cells. MDCK cells infected with C/JJ/50 virus were pulse-labelled with ^{35}S -methionine at 18 hours p.i. Polypeptides were electrophoresed on 5-13% gradient polyacrylamide-SDS gels and detected by autoradiography. Lanes in panel A were from one gel, and lanes in panel B were from a separate gel. A: lane 1, polypeptides of ^{35}S -methionine-labelled, purified C/JJ/50 virus (V) electrophoresed under reducing conditions; lane 2, polypeptides of C/JJ/50 virus-infected cells (I) electrophoresed under reducing conditions; lane 3, polypeptides of uninfected MDCK cells (U) run under reducing conditions. B: lane 1, polypeptides of ^3H -glucosamine labelled, purified C/JJ/50 virus electrophoresed under nonreducing conditions (NR); lane 2, polypeptides of ^3H -glucosamine labelled, purified C/JJ/50 virus electrophoresed under reducing conditions (R). The gel in panel B was fluorographed. Note that HA (B, lane 1) for unknown reasons often runs as a doublet.



The NP and M polypeptides can be seen by comparing the pattern of purified virus (Figure 36a, lane 1). In addition, a low molecular weight nonstructural protein (NS) can be identified which is present only in infected cells (compare polypeptides of purified virus, lane 1). Such a virus-specific polypeptide has not been described before in influenza C virus-infected cells. In addition, a large virus-specific polypeptide can be distinguished in infected cells which is labelled HAp. The existence of a precursor molecule to HA has been reported (gpI; Meier-Ewert et al., 1979; Compans, personal communication). The polypeptide found here in infected cells is most likely analogous to gpI, since it migrates slightly slower than HA (Figure 36a and 36b) and gpI is reported to be synthesized in infected cells as a primary product (Compans, personal communication). Therefore this polypeptide band is tentatively labelled HAp. However, it was not possible to demonstrate whether or not HAp is glycosylated, and no peptide mapping studies were done.

The top three influenza C virus RNAs (Figure 24a) migrate in a pattern similar to that of RNAs 1-3 of influenza A and B viruses (Figures 11 and 24a). Since these three high molecular weight RNAs in influenza A and B viruses code for P polypeptides, a search was made for

corresponding proteins in influenza C viruses. Three high molecular weight polypeptides can be seen in ^{35}S -methionine labelled purified virus which has been run under reducing conditions (Figure 36a, lane 1). Other high molecular weight proteins observed in purified virus are most likely cellular contaminants, since they co-migrate with cellular proteins and are not found consistently from one virus preparation to another.

Three P polypeptides of influenza A viruses can be found in association with the viral RNP (Inglis et al., 1976). Therefore, attempts were made to isolate RNP from influenza C viruses. ^{35}S -methionine-labelled purified virus preparations were disrupted with lysolecithin (Rochovansky, 1976). Disrupted virus was centrifuged on discontinuous (33%, 40%, 50%, 70% w/w) glycerol gradients which were then fractionated and counted. Usually 85% of the total counts were found at the top of the gradient, and 5% of the counts were located in the 70% glycerol layer, which most likely represented undisrupted virus. A peak comprising 1-2% of the total counts was found in the 50% glycerol layer, which was probably the viral RNP. Analysis of this fraction on polyacrylamide gels indicated that it contained M, NP and P1, P2 and P3 proteins, and did not contain the

surface glycoprotein. However, since very small amounts of radioactivity were obtained, it was not possible to positively identify P1, P2 and P3 proteins with this method.

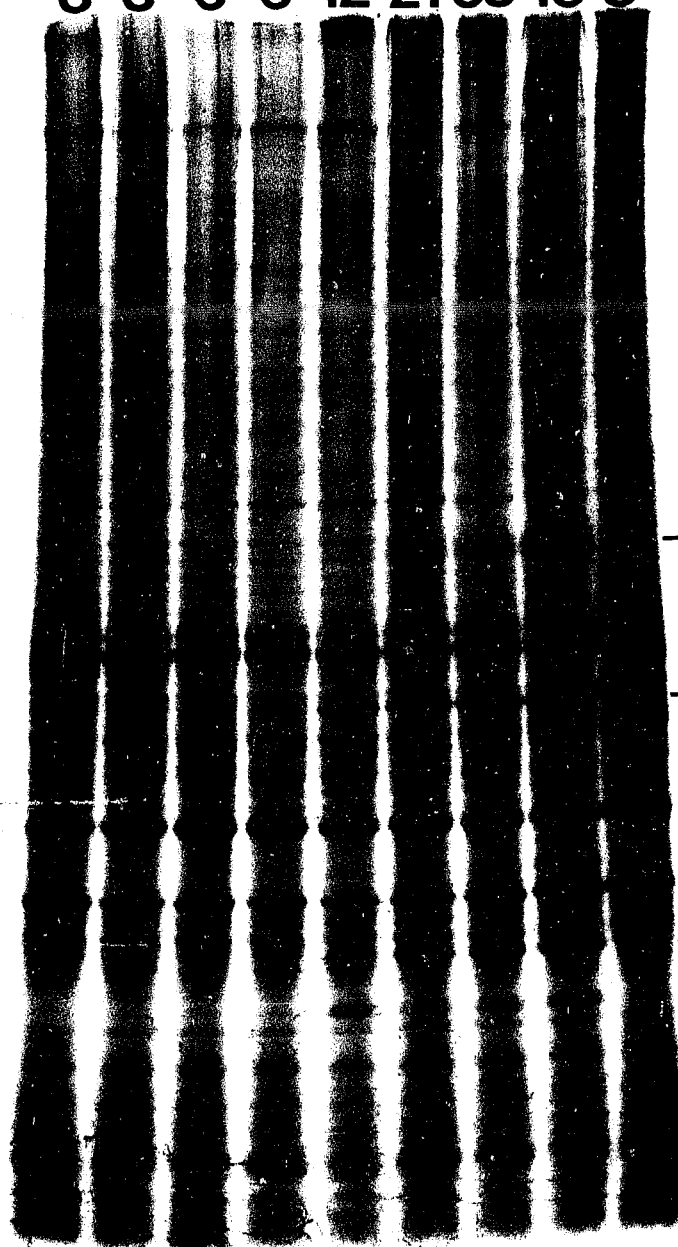
The tentative identification of P1, P2, P3 proteins and demonstration of an NS protein here brings the total number of influenza C virus-specific polypeptides to seven. Although this number matches the number of influenza C virus RNA segments which were observed, it may not represent the maximum number of virus-induced polypeptides.

The kinetics of the induction of influenza C virus polypeptides was studied in the MDCK cell system. MDCK cells were infected with influenza C/JJ/50 virus at an MOI of 1-3 PFU/cell in the presence of plasminogen, and proteins were pulse-labelled with ^{35}S -methionine at various times after infection. Figure 37 shows the analysis of the polypeptides of infected cells pulse-labelled at 0, 3, 6, 9, 12, 21, 33 and 48 hours post-infection. This analysis indicates that at 12 hours postinfection, the NP, M and NS polypeptides are detectable, while the HAp polypeptide can be seen at 21 hours postinfection. In addition a virus induced polypeptide which migrates between M and NP can be detected later

Figure 37. Kinetics of induction of influenza C virus polypeptides in MDCK cells. MDCK cells were infected with C/JJ/50 virus at an MOI of 1-3 PFU/cell and pulse-labelled with ^{35}S -methionine at various times postinfection. Polypeptides were analyzed on a 5-13% gradient polyacrylamide-SDS gel and detected by autoradiography. Lanes 1 and 9, polypeptides of uninfected MDCK cells; lanes 2-8, polypeptides of C/JJ/50 virus-infected MDCK cells pulse-labelled at 3, 6, 9, 12, 21, 33 and 48 hours, respectively. Influenza C virus-specific polypeptides are identified to the right.

hours p.i.:

U 3 6 9 12 21 33 48 U



-HA_p

-NP

-M

-NS

in infection. No further attempts were made to identify this polypeptide.

The time course of synthesis of influenza C virus proteins differs from that of influenza A viruses, where virus-specific polypeptide synthesis of A/WSN virus can be detected in MDCK cells as little as 1 hour after infection (Lamb and Choppin, 1978). It is also clear that infection with an influenza C virus does not depress host protein synthesis as is observed with influenza A viruses.

C. THE 5' AND 3' TERMINAL SEQUENCES OF INFLUENZA A, B AND C VIRUS GENES

The information which has been presented on the genomes of influenza B and C viruses has made it possible to compare the fine structure of the individual RNAs of all three types of influenza viruses. One of the methods available for such a comparison is oligonucleotide fingerprinting, which has been discussed in a previous section. Another technique which yields a great deal of information on gene structure is RNA sequencing, which in recent years has rapidly developed into a relatively fast and convenient method. The new enzymatic and chemical RNA sequencing techniques are best suited for

investigating short stretches of nucleotides at the 5' or 3' ends of nucleic acids.

Sequences at the ends of influenza virus genes may have special features which enable them to participate in viral replication and packaging of RNAs. In addition, it is interesting to determine whether or not the conservation of terminal sequences of individual genes, which has been reported for two influenza A viruses and one influenza B virus (Skehel and Hay, 1978b), extends to other influenza viruses. Also, direct sequencing of the 3' ends of influenza viral RNA had not been reported when this work was begun.

With these objectives in mind, the 5' and 3' ends of the genes of an influenza A, an influenza B and an influenza C virus were sequenced in collaboration with Dr. Ulrich Desselberger in this laboratory.

3' terminal sequences

Purified RNAs from an influenza A, an influenza B and an influenza C virus were 3' end-labelled using 5'-[³²P]-pCp and T4 RNA ligase (England and Uhlenbeck, 1978). After labelling, the RNAs were separated on a denaturing polyacrylamide gel as shown in Figure 38.

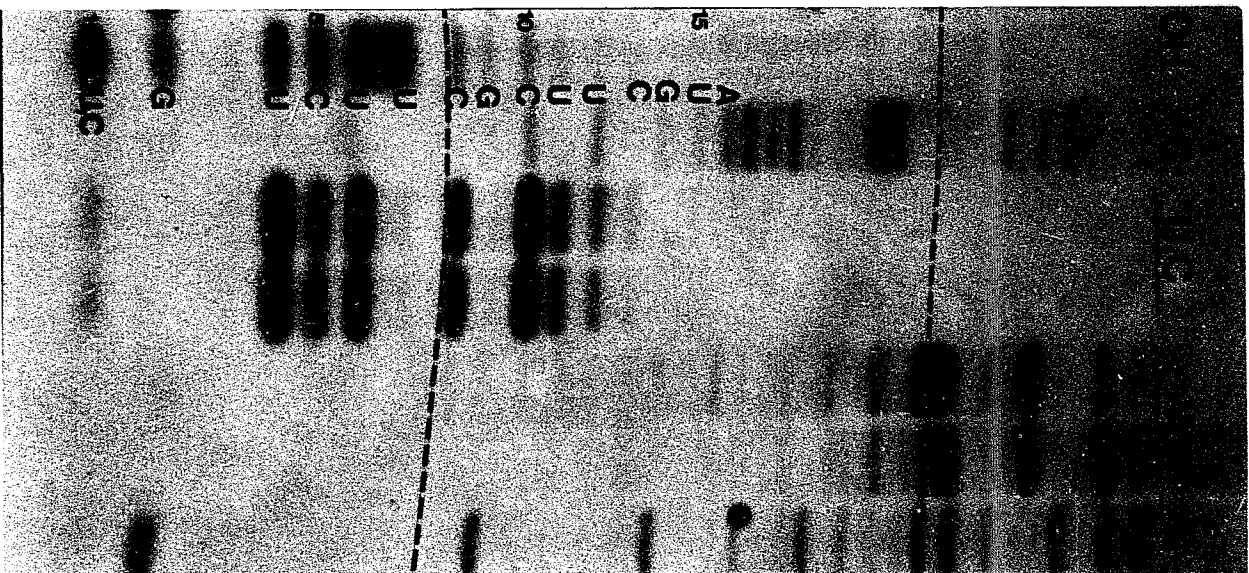


Figure 38. Separation of 3' end labelled influenza A, B and C virus RNAs. The RNAs of influenza A/PR/8/34 (A), B/HK/8/73 (B) and C/JHG/66 (C) viruses were labelled at the 3' end using 5'- ^{32}P -pCp and RNA ligase. After electrophoresis on a 2.6% polyacrylamide gel, the wet gel was exposed to X-ray film to obtain the autoradiogram. The influenza A and B virus segments (lanes 1, 2 and 3, 4) are labelled according to their protein product, and the influenza C virus RNAs (lanes 5, 6) are numbered in order of increasing mobility on the gel.

For this study, certain genes were not resolved; for example, genes 1-3 and genes 6 plus 7 of influenza C/JHG/66 virus were not separated. After electrophoresis, genes were eluted from gel slices and subjected to partial digestion with ribonucleases A, T1, U2, Phy I or Phy M. These partial digests were then separated on polyacrylamide "ladder" gels and the sequence was read from the autoradiograms. Figure 39 shows a ladder gel of the 3' end-labelled NA gene of influenza B/HK/8/73 virus.

This technique was useful for identifying the first 17-30 3'-terminal nucleotides of each RNA preparation. When ladder gels presented ambiguities in sequence, other methods were used. Often it was difficult to determine the first and second nucleotides in an end-labelled preparation. However, the use of end group analysis by Dr. Desselberger in our laboratory indicated that uridine is the 3' terminal nucleoside for all the genes of influenza A/PR/8/34, B/HK/8/73 and C/JHG/66 viruses (Volkaert and Fiers, 1977). In other cases, it was difficult to distinguish between U and C on ladder gels, and the "wandering spot" technique was used to circumvent this problem. In this method, the end-labelled RNAs are partially digested with either

Figure 39. Ladder gel of the 3' end labelled influenza B/HK/8/73 virus NA gene. 3' end labelled NA gene was eluted from a gel such as that shown in Figure 38, and subjected to partial digestion with base-specific ribonucleases. The digests were then separated on 20% polyacrylamide ladder gels and detected by autoradiography. The lanes are labelled according to the specificity of enzymes used: -C, U+C, A, G. End labelled RNA was also digested with alkali and separated on the gel (OH) to yield a series of fragments differing in length by one base. Upper dotted line, xylene cyanol FF dye; lower dotted line, bromophenol blue dye. The sequence marked on this gel was determined from a number of ladder gels, wandering spot gels (see Figure 40) and end group analyses.



alkali or formamide and separated in two dimensions on polyacrylamide gels (Semler et al., 1978). Figure 40 shows the wandering spot gel of the 3' end-labelled NA gene of influenza B/HK/8/73 virus.

Table 11 presents the 3' terminal nucleotide sequences for an influenza A, B and C virus. This information is compiled from a large number of ladder gels, wandering spot gels and end group analyses.

A comparison of the 3' terminal sequences of influenza A/PR/8/34 virus genes reveals a high degree of conservation (shaded area, Table 11). The sequence UCG-UUU-UCG-UCC for the first twelve positions is invariant except for position 4, which is C in the P1, P2, P3, NA and M genes. Beyond position 12 regions of homology cannot be detected.

The 3' terminal nucleotide sequence of the influenza B virus is conserved for the first 16 nucleotides, and the sequence UCG-UCU-UCG-CUU-CGU-A is found in most of the genes. The exception is position 11 of the P1-3 and NP genes, which is G, and position 14 of the NS gene, which is C. For the M gene, only the sequence of the first 12 nucleotides was positively identified. A comparison of the A and B virus genomes reveals very similar sequences for the first 9 nucleotides, with variations

Figure 40. Wandering spot gel of the 3' end labelled influenza B/HK/8/73 virus NA gene. 3' end labelled NA gene (obtained as discussed in the legend to figure 39) was digested with alkali and the resulting fragments were fingerprinted in two dimensions on polyacrylamide gels and detected by autoradiography. X, bromophenol blue dye. The sequence shown here was obtained by comparing the results of ladder gels (Figure 39), wandering spot gels and end group analyses.

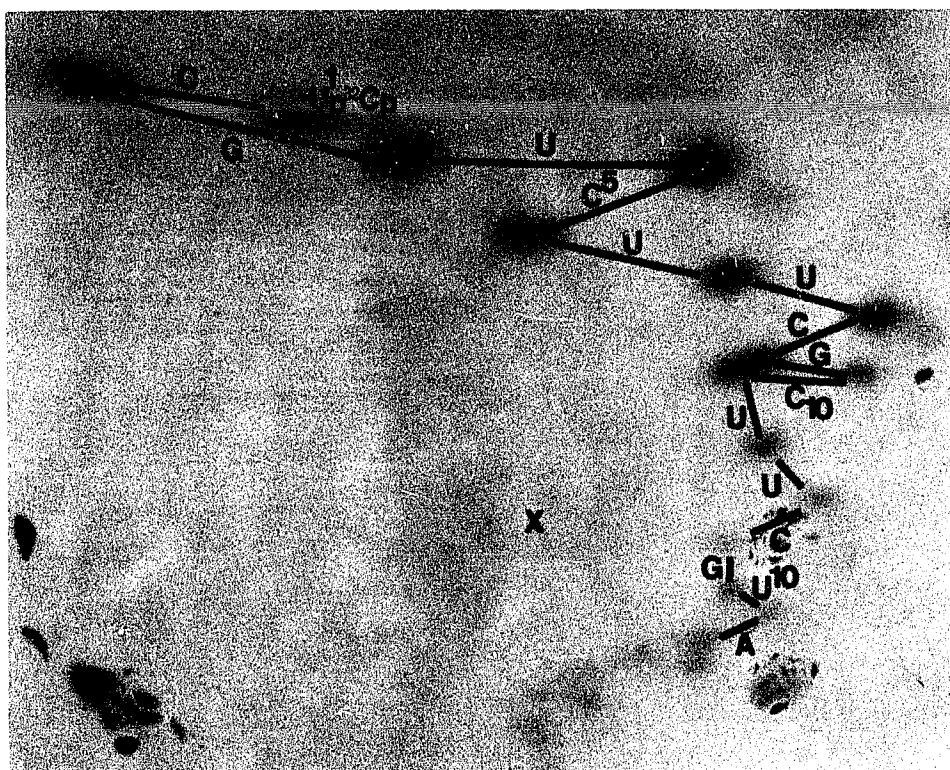


TABLE 11

3' TERMINAL NUCLEOTIDE SEQUENCES OF THE RNA SEGMENTS
OF INFLUENZA A/PR/8/34, B/HK/8/73 AND C/JHG/66 VIRUSES

A/PR/8/34 Virus

<u>Gene</u>	3	6	9	12	15	18	21	24	27
P1	U C G C U U U C G U C C A U G A - G - G G								
P2	U C G C U U U C G U C C A U G A C A A G U G					U U			
P3	U C G C U U U C G U C C A G U U A A U A								
HA	U C G U U U U C G U C C C C U U A U A U U U U G U U G G					U			
NP	U C G U U U U C G U C C C A U U A U U A G					U			
NA	U C G C U U U C G U C C C C A A U U U U A C U U A G G				C A				
M	U C G C U U U C G U C C A U C U A U A A C U U C U A C U						U		
NS	U C G U U U U C G U C C C A C U G U A U U A G U A U U A C C					U	U		

B/HK/8/73 Virus

P1+P2+	3	6	9	12	15	18	21
P3	U C G U C U U C G C U C G U A A A - G			U G			
HA	U C G U C U U C G C U U C G U A A A A G						
NP	U C G U C U U C G C G U C G U A A A A G						
NA	U C G U C U U C G C U U C G U A G A A G						
M	U C G U C U U C G C U U						
NS	U C G U C U U C G C U U C C U A A U U A					C	

C/JHG/66 Virus

1+2+	3	6	9	12	15	18	21	24	27
3	U C G U U U U C G U C C C U U A A					G A			
4	U C G U U U U C G U C C C U A A					U A			
5	U C G U C U U C G U C C U C U A A A - U A A A A G U A								
6+7	U C G U C U U C G U C C C C A U G A A A A A G								

in positions 4 and 5 only.

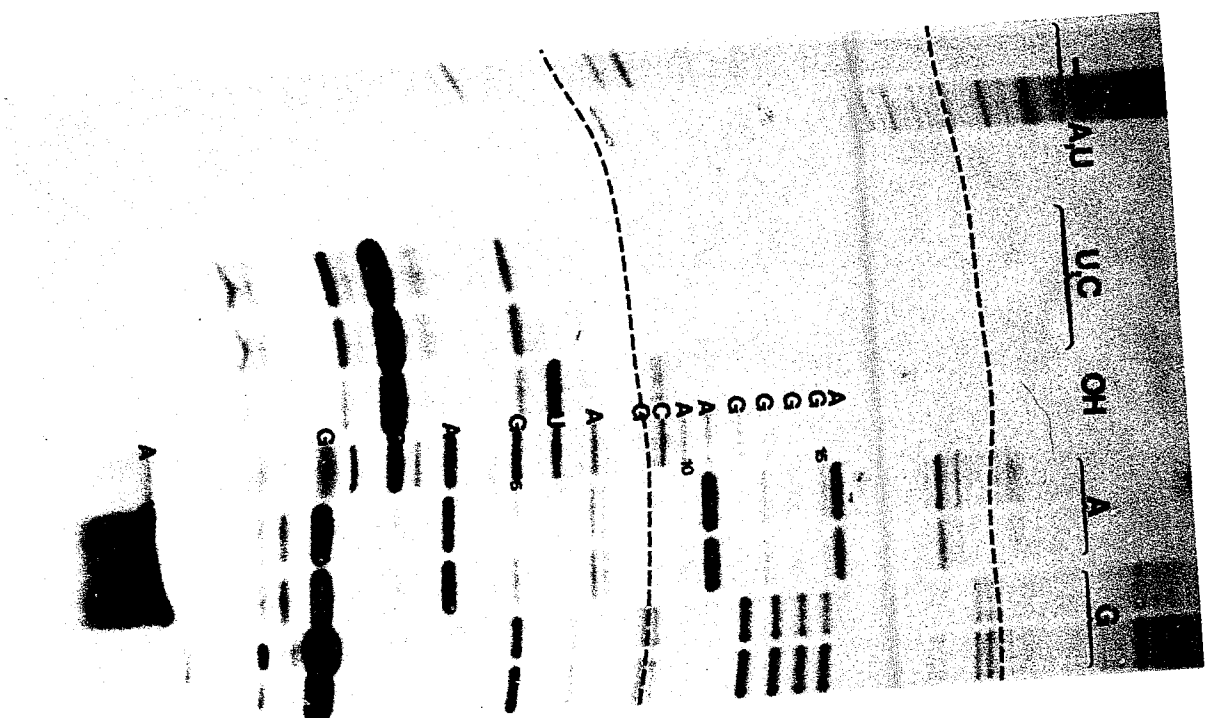
The sequence for the first 12 bases of the 3' end of influenza C virus genes, UCG-UUU-UCG-UCC is conserved for all genes except for position 5 of genes 5, 6 and 7, which is C. This sequence differs only in positions 4 and 5 from the corresponding 3' terminal sequences of influenza A virus genes.

5' terminal sequences

RNA was purified from an influenza A, B and C virus and radioactively labelled at the 5' end with γ - ^{32}P -ATP and polynucleotide kinase (Frisby, 1977). Labelled RNAs were separated on denaturing polyacrylamide gels, eluted and digested as described above for 3' end labelled RNAs. Sequence analysis was performed using ladder gels and wandering spot gels. The 5' terminal base of all genes was found to be adenosine by Dr. U. Desselberger, using methods of end group analysis (Lockard and RajBhandary, 1976; Wurst et al., 1978). Examples of a ladder gel (Figure 41) and a wandering spot gel (Figure 42) of the 5' end labelled RNAs 6 and 7 of influenza C/JHG/66 virus are shown.

Table 12 summarizes all of the 5' terminal sequences obtained by these methods.

Figure 41. Ladder gel of the 5'-end labelled RNAs 6 and 7 of influenza C/JHG/66 virus. RNA of C/JHG/66 virus was 5'-end labelled using γ - ^{32}P -ATP and polynucleotide kinase, and segments 6 and 7 were obtained as described for 3'-end labelling (Figure 39) of influenza C virus RNA. After partial digestion of the RNAs, the products were separated in different lanes on a 20% polyacrylamide ladder gel. The lanes indicate the specificity of the RNases used: -C, A+U, U+C, A and G. An alkali digestion (OH) was also performed. The final sequence indicated here was compiled from several ladder gels, wandering spot gels (see Figure 42) and end group analyses.



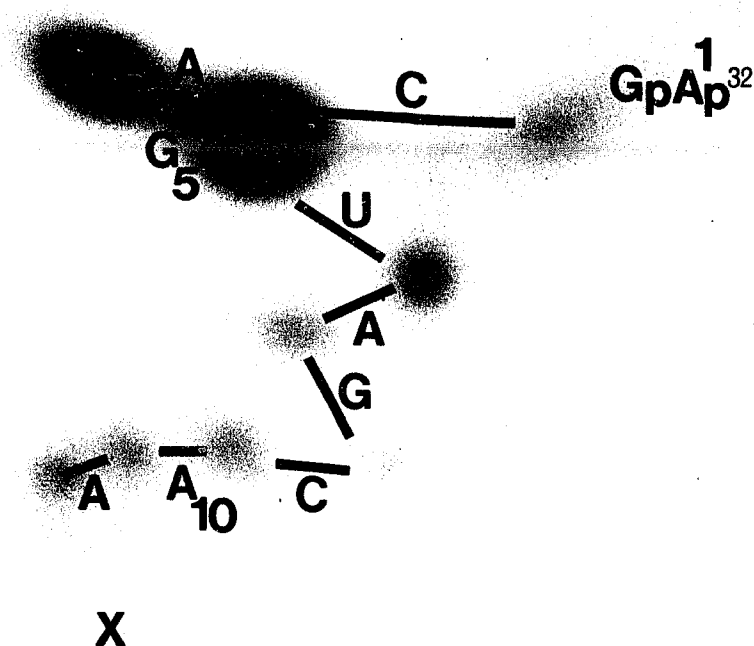


Figure 42. Wandering spot gel of 5' end labelled RNAs 6 and 7 of influenza C/JHG/66 virus. 5' end labelled RNAs 6 and 7 of C/JHG/66 virus were obtained as described in the legend to Figure 41. The RNAs were digested with alkali and fingerprinted as described in the legend to Figure 40. The sequence shown here is compiled from a number of ladder gels (see Figure 41), wandering spot gels and end group analyses.

TABLE 12

5' TERMINAL NUCLEOTIDE SEQUENCES OF THE RNA SEGMENTS OF
INFLUENZA A/PR/8/34, B/HK/8/73 AND C/JHG/66 VIRUSES

A/PR/8/34 Virus

<u>Gene</u>	3	6	9	12	15	18	21
P1 + P2	A G U A G A A A C A A G G U A U U U U U U U						
P3	A G U A - A A A C A A - -				U U C C	- - - -	U - C -
HA	A G U A G A A A C A A G G G U G U U U - - -						
NP	A G U A G A A A C A A G G G U A U U U U U U U						
NA	A G U A G A A A C A A G G A G				A U	U U U U U G	
M	A G U A G A A A C A A G G U A G U U U U U U						
NS	A G U A G A A A C A A G G G U G U U U U U U U						

B/HK/8/73 Virus

	3	6	9	12	15	18	21
P1+P2+P3	A G U A G A A A C A A G A G				U C	A U U U U U U	
HA	A G U A G U A A C A A G A G C A U U U U U U U						
NP	A G U A G A A A C A A C A G C A U U U U U U U						
NA	A G U A G U A A C A A G A G				U C	A U U U U	
M	A G U A G A A A C A A C G C A				C A	U U U U U U U	
NS	A G U A G U A A C A A G A G A A U U U U U U U						

C/JHG/66 Virus

	3	6	9	12	15	18	21
1+2+3	A G C A G U A G C A A - A - - A						
4	A G C A G U A G C A A G A - - A U U U U U U						
5	A G C A G U A G C A A - - A - A						
6 + 7	A G C A G U A G C A A G G G G A U U U U U U A						

The sequence of the first 13 nucleotides at the 5' end of influenza A virus genes, AGU-AGA-AAC-AAG-G is present in all of the genes. The influenza B virus 5' sequence, AGU-AGA-AAC-AA is conserved in all genes for the first 11 nucleotides, except for position 6 of HA, NA and NS genes, which is U. The B virus sequence is identical to that of the A virus for positions 1 through 11, with the exception of the sixth nucleotide. The sequence at the 5' end of influenza C virus genes, AGC-AGU-AGC-AA, is conserved for the first 11 nucleotides in all genes, and differs from the A virus sequence in positions 3, 6 and 8.

In all three viruses, a uridine-rich region can be identified some distance from the 5' end. The influenza A virus genes possess a string of U from position 17 through 22; the same observation can be made for the influenza B virus genes. This phenomenon was previously observed for influenza A and B virus genes (Barry and Mahy, 1979; Skehel and Hay, 1978b). Here it can be seen that influenza C virus genes most likely have a uridine rich region in the same area (positions 17-22).

IX. DISCUSSION

A. THE INFLUENZA B VIRUSES

This report presents the genetic map of the influenza B virus genome. Such a genetic map, which associates viral polypeptides with specific RNA segments, has been established for other segmented RNA viruses, and has led to many new findings on the biology and the biochemistry of these viruses. It is hoped that the results presented here will stimulate further study on the influenza B viruses.

In order to establish a genetic map for the influenza B viruses, it was first necessary to characterize the viral genes and gene products. Examination of the RNAs from several different influenza B viruses indicated the presence of a divided genome containing eight segments. It was also found that RNA patterns of viruses isolated from 1940 to 1973 were different, suggesting that genetic variation might occur in all genes of influenza B viruses, not just the two genes coding for the surface glycoproteins. Oligonucleotide maps of the RNA from different influenza B virus strains were also distinguishable and

it was found that this method is useful for examining genetic drift among viruses isolated within a short period of time. This technique is also useful for examining changes among individual genes of viruses isolated at different times.

A study of influenza B virus gene products revealed the presence of eight virus-specific proteins. In addition to the previously described P1, P2, HA, NA, NP and M polypeptides, evidence was presented, on SDS-polyacrylamide gels, for a P3 polypeptide and one or two NS polypeptides. Analysis of viral polypeptides by migration on SDS gels or by one-dimensional peptide mapping showed that most of the corresponding proteins of B/Lee/40 and B/Md/59 viruses could be distinguished. This information confirmed the suggestion that genetic variation occurs in most of the genes of influenza B viruses. In addition, differences in proteins were used to identify the protein composition of a series of recombinants of B/Lee and B/Md viruses. These recombinants were isolated under conditions where most RNA segments were inherited from one parent, and 1 or 2 RNA segments were inherited from the other parent. By noting which RNA segments segregated with a specific protein, the genetic map of influenza B viruses was established.

A careful comparison of the molecular weights of influenza B virus polypeptides and RNA segments (Table 7) indicates that RNA segments I, II, III, V, and VII appear to be the right size to encode the corresponding polypeptide. 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 (Table 7).

The comparison of molecular weights presented in Table 7 shows that the size of each of the influenza B virus RNAs is greater than the size 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 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.

Table 7 presents the gene order for influenza A/PR/8/34 and B/Lee/40 viruses. The comparison here shows 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-size 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 it was not possible to assign P2 and P3 proteins to specific RNA segments of the influenza B virus, it is expected that RNA I codes for P3 protein and RNA II codes for P2 protein, in analogy to influenza A viruses. This suggestion is based on the identity of the gene order for the other six RNA segments of influenza A and B viruses.

In our laboratory it has been observed that the gene order for a number of influenza A and B viruses is identical. In other words, in all influenza A and B viruses

tested, corresponding genes had identical molecular weights. The only exception was the finding that the size of the HA and NA genes of different influenza A viruses varied slightly (Desselberger and Palese, 1978). This small variation in size did not change the gene order of all the human, avian, equine and swine influenza A viruses tested. This information indicates that all influenza A and B viruses most likely have the same gene order.

Electrophoresis of glyoxalated RNA was used as a basis of a technique for rapidly identifying influenza virus genes separated in different gel systems. ^{32}P -labelled RNA of any influenza virus is first separated, and then gel slices containing RNA segments are excised and glyoxalated. The slices, containing glyoxalated RNA, are placed in the slot of a second gel and electrophoresed with whole glyoxalated RNA. Since the gene order for glyoxalated RNA is known, the identity of any band excised from the original gel can be determined by noting where the RNA migrates in relation to the pattern of whole glyoxalated RNA. This technique can be employed to identify genes separated in different gel systems

(i.e., using various temperature conditions, buffers, or gel media) and is also useful for mapping the genomes of new influenza virus isolates and for comparing genetic maps of different viruses which have been isolated in different laboratories. Identifying individual genes is necessary when genes are studied in terms of their biologic function, when the fine structure of genes is analyzed or when genes are subjected to sequence analysis.

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 the finding that, like influenza A viruses, the influenza B viruses also contain eight RNA segments which code for at least eight polypeptides. Similar to influenza A viruses, the influenza B viruses also contain two surface glycoproteins, HA and NA, one or two NS proteins whose functions are 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.

The genetic characterization of the influenza B virus genome presented here now permits the association

of biological properties with certain viral genes.

It may be possible to establish which gene or combination 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 vaccines. With influenza A and B viruses, efforts are currently underway 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 special importance in preparing commercial influenza virus vaccines. An analysis of several such candidate live vaccine strains has been presented here.

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. Some of the techniques which can be used for such an analysis have been employed in this study. Oligonucleotide fingerprinting of RNA and RNA sequencing techniques can be used to determine the extent of changes between genes in different influenza B virus strains, and 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, and may clarify the evolutionary relationship between these two types of viruses.

B. THE INFLUENZA C VIRUSES

The influenza C viruses have only in recent years begun to receive the attention of molecular virologists. Development of biochemical techniques suitable for analysis of very small amounts of material has improved our knowledge of these viruses. The data obtained in this study has provided answers to many questions. It was established that influenza C viruses contain seven RNA segments, as opposed to eight for influenza A and B viruses. Electrophoresis of untreated and glyoxalated RNA from several different strains has repeatedly confirmed this number. It is tempting to speculate that influenza C viruses contain one less gene than the influenza A and B viruses because they lack a neuraminidase. However, it should be noted that influenza C viruses possess a receptor destroying enzyme. If this enzyme is virus-coded, it will be necessary to identify

the gene or genes which code for this activity. The information for the enzyme might reside on a separate RNA, or it might lie next to another gene on one of the larger RNA segments. Alternatively, the enzyme may be translated from a gene in a second reading frame, or the enzyme might be part of a multifunctional protein, such as the HN polypeptide of paramyxoviruses. Clearly, more work is required to determine which of these alternatives is correct.

Experiments have also been performed which indicate that influenza C viruses code for at least seven polypeptides. Two previously discovered internal components, M and NP, could be detected in purified virus and in virus-infected cells. A previously unrecognized viral nonstructural (NS) protein was detected in virus-infected cells, which migrated faster than M protein on SDS gels. Three high molecular weight proteins were tentatively identified as P1, P2 and P3 by analogy with influenza A and B viruses. These polypeptides were consistently found in purified virus preparations, and were also detected in an RNP fraction obtained by lysolecithin treatment of virions. However, it was not possible to unequivocally identify the P polypeptides using this method.

Finally, the previously described HA was identified, bringing the total number of influenza C virus gene products to seven.

In the last year, Compans and co-workers have obtained interesting information on the nature of the influenza C virus HA (Meier-Ewert et al., 1979; Compans, personal communication). It is believed that the HA molecule is synthesized in cells as a precursor called gpI. This form of the HA may be detected in virions grown in chicken embryo fibroblast or kidney cells. In virions grown in eggs, however, a slightly smaller glycoprotein is present (gpII) which actually consists of two disulfide-linked polypeptides. These findings can be explained by the model shown in Figure 1. gpI may be a polypeptide containing a disulfide bond. Removal of a small peptide from gpI generates a smaller glycoprotein (gpII) which actually consists of two chains linked by a disulfide bond. Presumably, this form of the glycoprotein is required for infectivity, since cleavage of gpI in vitro with proteases increases the infectivity of the virus preparation. Reduction of gpII and analysis on gels separates the two small glycoproteins which may be analogous to HA1 and HA2 of influenza A and B viruses.

In this dissertation, two glycoproteins (HA1 and HA2) were identified in cell grown influenza C viruses which migrate as a single band under nonreducing conditions (HA/gpII). On the other hand, in infected MDCK cells a prominent virus-induced band can be detected which migrates slightly slower than unreduced HA of purified virus (gpII). This band in infected cells is most likely the uncleaved form of HA, and here is called HAP instead of gpI. Since all the virus used in this study was grown either in embryonated eggs or in MDCK cells in the presence of proteases, it is not surprising that HAP was not detected in purified virus. In order to confirm that the band in infected cells is indeed uncleaved HA (gpI), it will be necessary to compare peptide maps of this protein with HA from purified virus.

Previously, it was not possible to demonstrate recombination between influenza C viruses, since genetic and biochemical markers were not available. The demonstration of influenza C virus recombination reported here 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.

Recombination in influenza A and B viruses occurs by reassortment of RNA segments (Tobita and Kilbourne, 1974; reviewed in Kilbourne, 1975). Experiments were conducted to demonstrate that this mechanism also occurs in influenza C viruses. The data presented 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 in recombinant P1, and no unique spots assigned to RNAs 4 and 5 are present in virus P1. By this analysis virus P1 most likely inherits RNAs 1, 2, 3, 6 and 7 from C/JHG/66 virus, and 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 (#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.

It still remains to be shown whether independent reassortment occurs for all seven influenza C virus 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).

The influenza C virus genes and gene products have been characterized here, and this new information, in conjunction with the demonstration of recombination reported in this dissertation, can now be used to establish a genetic map for influenza C viruses. For influenza A viruses, the construction of a genetic map has led to new information concerning the function of influenza virus RNAs and proteins, and hopefully the influenza B virus genetic map reported here will have the same impact. Establishment 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 permit comparison of genetic drift of individual influenza C virus genes with those of influenza A and B viruses.

The techniques which can be used for such analyses have been discussed. These types of studies might clarify the relationships between influenza A, B and C viruses.

C. FEATURES OF THE 5' AND 3' TERMINAL NUCLEOTIDE
SEQUENCES OF INFLUENZA A, B AND C VIRUS GENES

The information on the genomes of influenza B and C viruses reported here now permits a sensible comparison of the fine structure of genetic material from all three virus types. With this idea in mind, recently developed rapid RNA sequencing techniques were employed to investigate the nucleotide sequences at the ends of genes of an influenza A, B and C virus. Specifically, since it had been reported that sequences at the 5' ends of influenza A and B viruses are conserved (Skehel and Hay, 1978b) it was of interest to see if this conservation also extended to influenza C virus RNAs. It was also desirable to directly sequence the 3' end of influenza vRNA, which had not been done previously. Finally, sequencing of the ends of influenza virus genes might reveal the presence of unique structures which might participate in replication of viral RNA or in the packaging of RNA segments.

Table 11 summarizes the nucleotide sequences at the 3' end of RNA segments from one influenza A, one influenza

B and one influenza C virus. This data directly demonstrates that the 3' terminal nucleotide sequences of genes or groups of genes of an influenza A, an influenza B and an influenza C virus are highly conserved. In influenza A and C viruses, the conservation extends for the first 12 bases, and in influenza B viruses the first 16 bases are conserved (see shaded areas, Table 11). In addition, conservation of the sequences can be observed among the three virus types.

Several groups have suggested that the in vitro ApG-primed RNA transcripts initiate exactly at the 3' end of influenza A virus genes (Skehel and Hay, 1978b; Smith et al., 1978; Plotch et al., 1978; Plotch and Krug, 1978). However, when this work was begun, no direct sequences of the 3' end of vRNA had been reported which might be compared to the published 5' sequences of in vitro transcripts (Skehel and Hay, 1978b). The data reported here directly confirm this hypothesis. The 3' terminal influenza A virus sequence UCG- $\overset{\text{U}}{\underset{\text{C}}{\text{UU}}}$ -UCG-UCC is complementary to the sequence at the 5' end of in vitro ApG primed transcripts reported by Skehel and Hay (1978b): AGC-AAA-AGC-AGG-. While this work was in progress, Both and Air (1979) obtained evidence that in vitro ApG primed transcripts of the M gene initiate precisely at the 3' end of vRNA.

Analysis of the data in Table 11 reveals three possible triplets which may serve as initiation codons (Kozak, 1979) for protein synthesis. Positions 21 to 23 of the NA gene, positions 26 to 28 of the M gene, and positions 27-29 of the NS gene of the influenza A virus (underlined in Table 11) would be transcribed to AUG (see Skehel and Hay, 1978b; Both and Air, 1979).

Table 12 shows the sequences at the 5' end of RNA segments from one influenza A, one influenza B and one influenza C virus. The data reported here confirm and extend the earlier finding that the 5' ends of influenza A and B virus genes are conserved (Skehel and Hay, 1978b; Barry and Mahy, 1979). In addition, it is shown that this conservation of sequence also applies to the influenza C virus genes. A direct comparison of the influenza A/PR/8/34 virus sequences with those of fowl plague virus (Skehel and Hay, 1978b; Barry and Mahy, 1979) reveals several differences, which may be due to genetic variation from strain to strain.

In all three viruses, a uridine-rich region can be identified some distance from the 5' end. The significance of this region is not clear, although Skehel and Hay (1978a) have suggested that this sequence may serve as a transcription-termination signal.

Several of the sequences reported here were derived from mixtures of RNA segments. Although electrophoresis on gels of varying pore size indicated that all segments of the three virus types could be end-labelled, it was not possible to show that each segment of a mixture was labelled enough to be visible on the sequencing gel. Therefore, the sequence reported for a mixture of genes may not be the correct sequence for all of the genes in the mixture.

The differences in biology, biochemistry and epidemiology between influenza C and influenza A and B viruses have been discussed. Some investigators have even proposed that influenza C viruses should be reclassified (reviewed in Davenport, 1976). However, the 3' and 5' terminal nucleotide sequences of influenza C virus genes reported here show a high degree of homology with those of influenza A and B viruses. Since sequences at the 5' and 3' ends of RNAs of other animal viruses differ markedly from the influenza virus sequences (Haseltine, 1977; Schwartz et al., 1977; Keene et al., 1977, 1978; Porter et al., 1978; Semler et al., 1978; Zimmern and Kaesberg, 1978), this sequence information indicates that influenza C viruses are probably true influenza viruses.

The sequences at the 5' and 3' ends of the influenza virus RNAs show inverted partial complementarity. This feature may permit formation of a panhandle structure as shown in Figure 43a. Such a double-stranded structure may represent a polymerase binding site. The stability of such a structure is not known, but the addition of an RNA binding protein such as a polymerase might confer greater stability to the panhandle. Fiers (1975) has discussed the significance of complementary nucleotide sequences at the ends of molecules in terms of another mechanism of replication. If the polymerase recognizes a sequence at the 3' end of vRNA, after completing synthesis of the (+) strand, the polymerase will then find a similar sequence at the 3' end of the new strand (see Figure 43b). This sequence may then serve as another initiation point for the polymerase in the synthesis of a minus (-) strand. In this way the inverted complementary repeats of influenza A, B and C virus genes may play a role in viral replication.

Similar conservation of terminal inverted sequences has been found for VSV (Colonno and Banerjee, 1978; Keene et al., 1977, 1978; Semler et al., 1978). Such a feature may be common in RNA-containing viruses and may serve some important role in the life of a virus.

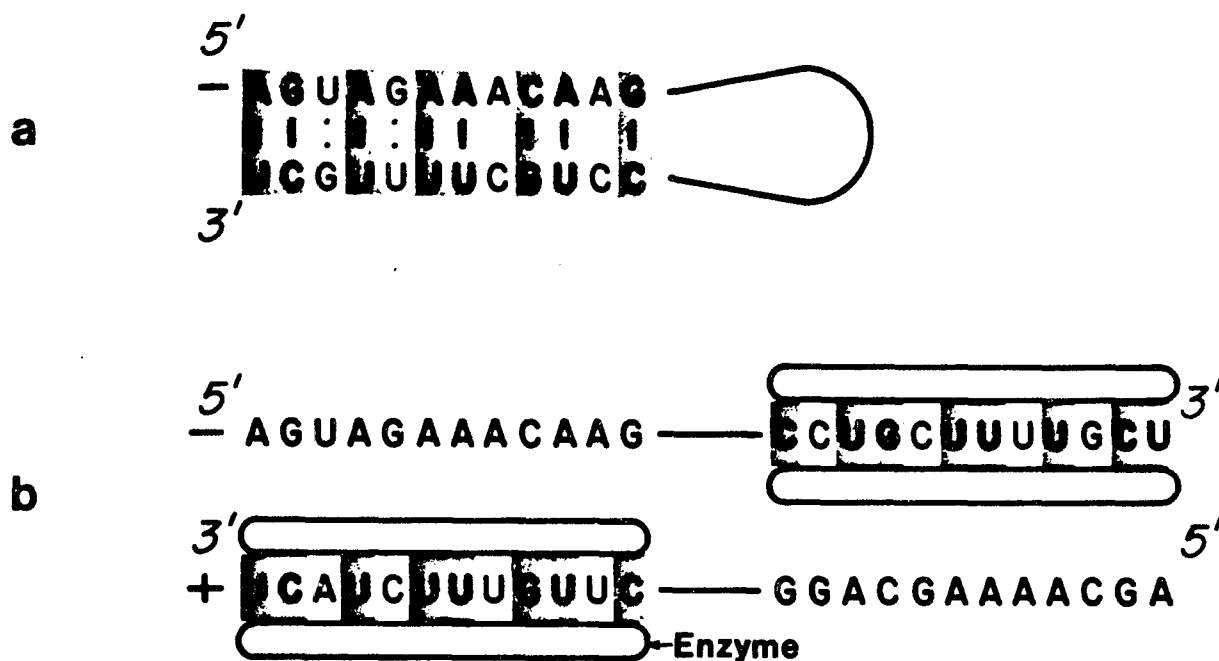


Figure 43. Hypothetical models for the role of inverted complementary termini in viral RNA synthesis. Partial inverted complementarity at the 5' and 3' ends of influenza virus genes can lead to formation of a panhandle, which may be a recognition site for polymerases (A). Alternatively, the terminal sequences may be preserved because inverted complementary termini can result in similar polymerase initiation sites at the 3' ends of minus and of plus strand RNAs; shaded nucleotides indicate preserved sequences at the 3' ends of minus and plus strand RNAs (B). As an example the HA gene of A/PR/8/34 virus is shown.

D. CONCLUDING REMARKS

A large amount of basic information on influenza B and C viruses has been presented in this thesis. This new knowledge should provide the impetus for future experimentation designed to reveal the reasons why influenza A, B and C viruses differ biologically and epidemiologically. If this project were to continue, some of the following problems might be addressed.

Techniques are now available for synthesizing full-length cDNA copies of RNA, and for cloning these DNAs and amplifying them in suitable vectors. It is now possible, for example, to obtain large amounts of ds DNAs representing any influenza virus gene. These DNAs may be cut with restriction enzymes, and the resulting fragments can be sequenced by rapid DNA sequencing techniques. Alternatively, restriction fragments may be used as primers for sequencing DNA by the chain-termination technique. The result is that now entire genes may be sequenced. A comparison of such sequences within the genes of a virus and between genes of different virus types will indicate whether or not internal variable and conserved regions exist; signal sequences and genetic control regions, such as ribosome binding sites, promoters,

or initiation codons, may also be identified. A comparison of sequences may supply information on the evolutionary relationship between influenza A, B and C viruses. It is now very important to obtain the sequence of in vivo mRNA, for several reasons. First, it must be determined whether splicing of a cap structure plus additional nucleotides occurs in vivo as has been observed in vitro (see Genetics of orthomyxoviruses). Sequencing of the 5' end of in vivo mRNA would answer this question and would indicate perhaps how mRNA is made in the cell. A comparison of the complete vRNA and in vivo mRNA sequences would also indicate whether or not splicing occurs elsewhere in the genome of influenza viruses. Second, sequencing the 3' end of in vivo mRNA would indicate exactly where transcription ends and would perhaps reveal the mechanisms for transcription termination. Finally, by identifying viral RNA sequences which participate in viral replication, it may be possible to employ specific oligonucleotides as antiviral compounds, as was done with RNA tumor viruses (Zamecnik and Stephenson, 1978).

The recent development of techniques for the production of monoclonal antibody now opens a new era for the

use of immunology in the study of viruses. Monoclonal antibody can be made toward the HA of all three virus types, and these can be used to investigate the presence of shared or unique determinants, the extent of antigenic variation and the antigenic structure of the molecule. Variants of each type of virus selected in the presence of monoclonal antibody may be analyzed by RNA fingerprinting or sequencing techniques, and the extent and types of changes which occur can be compared and related to the epidemiological behavior of the virus.

Intragenic recombination may be another mechanism by which influenza viruses evolve in nature, in addition to reassortment and mutation. The question of whether or not intragenic recombination occurs in influenza viruses may also be addressed using these recently developed techniques. For example, a monoclonal antibody is chosen which is directed toward the determinant at the N-terminus of the HA from one strain, and another monoclonal antibody is chosen which is directed toward a determinant at the C-terminus of HA from another strain. Each antibody must be specific for the one virus strain. The yield of a mixed infection with the

two viruses is then passed in the presence of both monoclonal antibodies. This procedure will enrich for viruses which contain recombinant HA genes that have lost both the N- and C-terminal determinants.

Influenza viruses are unique not only because of their continual genetic variation, but also because they exist as three distinct types, with many biological and epidemiological differences. Advantage should be taken of these new techniques to relate biochemical characteristics with the biological properties of the viruses in an effort to understand the unusual behavior of the orthomyxoviruses.

X. APPENDIX: ABBREVIATIONS

BIS	N, N'-methylene-bis-acrylamide
CAP	calf alkaline phosphatase
cDNA	complementary DNA
cRNA	complementary RNA
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	disodium ethylenediamine tetraacetate
gp	glycoprotein
HA	hemagglutinin
HEPES	hydroxyethylpiperazine ethylsulfonate
M protein	matrix or membrane protein
MW	molecular weight
MEM	minimal essential medium
MOI	multiplicity of infection
MDCK	Madin-Darby canine kidney
NA	neuraminidase
NP	nucleoprotein
NS	nonstructural
NTE	NaCl-Tris-HCl-EDTA
OD	optical density
PBS	phosphate buffered saline

PEI	polyethyleneimine
PFU	plaque forming units
PPO	diphenyloxazole
PMSF	phenylmethanesulfonyl fluoride
RDE	receptor destroying enzyme
REM	reinforced MEM
RNP	ribonucleoprotein
SDS	sodium dodecyl sulfate
ts	temperature sensitive
TCA	trichloroacetic acid
TPCK	tosylsulfonyl phenylalanyl chloromethyl ketone
TEMED	N,N,N',N',-tetramethylethylenediamine
UV	ultraviolet
vRNA	virion RNA

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