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ANALYSIS OF VARIATION IN INFLUENZA A VIRUS GENES

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ANALYSIS OF VARIATION IN INFLUENZA A VIRUS GENES

BY

MELVYN BAEZ

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.

1981

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1.0 ABSTRACT

ANALYSIS OF VARIATION IN
INFLUENZA A VIRUS GENES

BY

MELVYN BAEZ

Advisor: Peter Palese, Ph.D.

The genes of several influenza A viruses were examined by using various biochemical techniques and molecular cloning.

The genetic compositions of eleven high yielding recombinants of influenza virus were determined by polyacrylamide gel electrophoresis of the ^{32}P-labeled RNAs obtained from the recombinants and their parents. The enhanced growth capacity of the recombinants, constructed for use in vaccine production, was associated with the presence of genes derived from the high yielding laboratory strain A/PR/8/34. In particular, all of the examined high yielding recombinants derived the M gene from the A/PR/8/34 strain.

Two dimensional electrophoresis of ribonuclease T1

resistant oligonucleotides was used to estimate the extent of genetic variation among five different influenza viruses isolated during the beginning, middle and end of the H2N2 subtype period (1957-1968). It was demonstrated that genetic variation occurred among all the corresponding RNAs of these influenza viruses and that the extent of variation was similar for all genes. It is suggested that the maximum mutational changes of 3 or 4%, that were estimated for all the genes of the H2N2 viruses, may represent the extent of genetic variation that occurred during the eleven year interval which separates the isolation of the earliest and latest H2N2 strain examined in this study.

The NS genes of the human influenza virus, A/PR/8/34, and the avian influenza virus, A/duck/Alberta/60/76, were cloned into pBR322. The complete nucleotide sequences of these two NS genes were determined and a comparison of these sequences showed a 27.3% difference. In contrast, the differences among the NS gene of the A/PR/8/34 virus and the previously sequenced NS genes of the avian virus A/FPV/Rostock/34 and the human virus A/Udorn/72 were fewer in number and ranged from 8-11%. The extensive sequence similarity among these latter three strains does not support the suggestion of species specific homology groups among the NS genes of avian and human influenza viruses. The NS genes of the A/PR/8/34, A/Udorn/72 and A/FPV/Rostock/34 strains were shown to contain open reading frames in their virion (-) RNAs which potentially encode polypeptides of 167 and 216

amino acids. Although the NS segment of the A/duck/Alberta/60/76 virus lacks an agnogene sequence in its negative strand sequence, it conserves the overlapping NS1 and NS2 gene arrangement identified in the other NS RNA segments. A comparison of the deduced amino acid sequences of the A/duck/Alberta/60/76 and A/PR/8/34 virus NS genes showed that the NS2 polypeptides are more highly conserved (18.2%) than the NS1 polypeptides (33.0% difference). Surprisingly, the NS1 polypeptides of the two strains showed this high degree of variation over their entire lengths, including the amino acids encoded by the conserved RNA domain of the NS1 and NS2 gene overlap.

2.0 ACKNOWLEDGEMENTS

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I extend a special thanks to Dr. Robert P. Aaronson who spent a great deal of time developing the computer program that was used to format this thesis.

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6.0 INTRODUCTION

6.1 DESCRIPTION AND CLASSIFICATION OF INFLUENZA VIRUSES

Influenza viruses are enveloped viruses containing segmented RNA genomes. The single-stranded RNAs are of negative polarity, are associated with an RNA-dependent RNA polymerase and are packaged within a helical nucleocapsid in the virion. The virion contains two viral coded glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which protrude from the viral envelope.

Influenza viruses exist as three distinct types, A, B, and C, which are distinguished by serological differences in two structural proteins, matrix protein (M) and nucleoprotein (NP). Influenza A viruses, for example, share antigenically cross-reacting NP and M proteins which have antigenic determinants different from those on the corresponding proteins in type B or C viruses. Influenza A viruses are further classified into subtypes on the basis of antigenic dif-

ferences among the HA and NA glycoproteins. All influenza virus isolates are designated by a code which includes type (A,B or C), geographic site of isolation, isolate number and year of isolation. Furthermore, the code for type A influenza virus isolates also includes the host species, if other than human, and the HA and NA subtypes e.g. A/duck/Alberta/60/76 (H12N5).

The influenza B and C viruses are not divided into subtypes and appear to have man as their major host whereas influenza A viruses have been isolated from other species. Recently, influenza A viruses have been reclassified on the basis of two parameters; 1. the sequence homology among the RNAs coding for the HA and NA glycoproteins and 2. the serologic cross-reactivity among the HA and NA glycoproteins. Table 1 shows the new HA and NA subtypes together with their previous designations.

6.2 INFLUENZA

Influenza is an acute tracheitis and its pathogenesis probably involves primary implantation of the virus in an alveolus. Subsequently, replication resulting in cytopathic effects occurs in the middle respiratory tract.

TABLE 1

CLASSIFICATION OF INFLUENZA A VIRUS SUBTYPES

HA SUBTYPES		NA SUBTYPES	
NEW	OLD	NEW	OLD
H1	H0, H1, Hsw1	N1	N1
H2	H2	N2	N2
H3	H3, Heq2, Hav7	N3	Nav2, Nav3
H4	Hav4	N4	Nav4
H5	Hav5	N5	Nav5
H6	Hav6	N6	Nav1
H7	Heq1, Hav1	N7	Neq1
H8	Hav8	N8	Neq2
H9	Hav9	N9	Nav6
H10	Hav2		
H11	Hav3		
H12	Hav10		

THIS CLASSIFICATION SCHEME IS TAKEN FROM CENTERS FOR DISEASE CONTROL, (1980). MMWR 29;514.

Influenza is frequently an uncomplicated viral infection. Local symptoms of influenza include cough, substernal burning pain and sore throat. Curiously, viremia has not been demonstrated in influenza virus infections although systemic manisfestations such as prostration, fever, chills, headache and myalgia are usually observed. Primary viral pneumonia is a complication of influenza virus infection that is often fatal when it occurs. It is usually limited to patients predisposed by heart or lung disease (reviewed in Kilbourne, 1978b).

Infection with influenza B virus has been associated with Reye's syndrome in children and adolescents (Corey et al., 1976). This syndrome is characterized by cerebral edema and fatty degeneration of the visceral organs, especially the liver. Other symptoms include high fever, coma and seizures. Reye's syndrome can be fatal and death is usually due to respiratory failure. The case fatality rate in Reye's syndrome can be as high as 38% (Center for Disease Control, 1978). Recently, epidemiological studies have also associated outbreaks of Reye's syndrome with influenza A virus infections (Halsey et al., 1980; Wilson et al., 1980). However, it should be noted that other agents have also been implicated in the etiology of Reye's syndrome (Corey et al., 1976).

6.3 THE INFLUENZA VIRUS

6.3.1 MORPHOLOGY AND COMPOSITION

Influenza virus particles are usually spherical with an average diameter of 100-120 nm (Wrigley et al., 1979). However, filamentous forms of influenza virions have also been described. Spikes protrude through the virion's surface and a nucleocapsid of helical symmetry is enclosed within the viral envelope. The virion envelope is composed of a lipid bilayer (Landsberger et al., 1971). Influenza A and B viruses have a similar morphology (Waterson et al., 1963; Archetti et al., 1967; Apostolov et al., 1970) which closely resembles that of the influenza C viruses. However, the influenza C viruses are slightly larger and appear to have a hexagonal lattice beneath their envelopes (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).

Several estimates of the influenza virus composition have been made and populations of influenza virions have been shown to consist of 20-40% lipid, 70-75% protein

0.8-1.1% RNA and 5-8% carbohydrate (Ada and Perry, 1954; Frommhagen et al., 1959; Blough and Merlie, 1970).

6.3.1.1 Carbohydrate

The carbohydrate of influenza viruses is found on the glycoproteins HA and NA and is derived from the host cell. Two major types of covalently linked oligosaccharide moieties have been identified on the influenza virus glycoproteins (Schwartz et al., 1977; Collins and Knight, 1978; Nakamura and Compans, 1978). Type I consists of mannose, N-acetylglucosamine, galactose and fucose whereas type II consists mostly of mannose and small amounts of glucosamine. Recently, heterogeneity in the carbohydrate side chains of the HA glycoprotein from influenza A/USSR/90/77 virus has been demonstrated (Basak et al., 1981). In particular, some oligosaccharide moieties were shown to have carbohydrate compositions that were intermediate between those of types I and II sugar side chains.

Studies on the biosynthesis of oligosaccharides of influenza virus glycoproteins suggested that the first glycosylation event is the "en bloc" transfer of a mannose rich oligosaccharide core to the viral glycoproteins (Nakamura and

Compans, 1979a). The formation of the type I oligosaccharide side chains appears to occur in several steps involving the trimming of the oligosaccharide core and subsequent modifications by host glycosyl transferases which add N-acetylglucosamine, galactose and fucose. The type II oligosaccharide side chains appear to consist of unmodified mannose rich cores.

It is believed that the glycosylation of influenza virus glycoproteins starts at the rough endoplasmic reticulum, where carbohydrate cores are added to polypeptides. At this time, trimming of the cores followed by addition of other sugars is initiated and continues during transfer of the glycoproteins to the smooth endoplasmic reticulum. Finally, the fully processed glycoproteins are inserted into the plasma membrane of the host cell. (Compans, 1973; Stanley et al., 1973; Nakamura and Compans, 1979a). The attachment of the carbohydrates to the glycoproteins of influenza viruses has been shown to be through an N-glycosidic linkage of N-acetylglucosamine to asparagine (Keil et al., 1979). The glycosylated asparagine residues always occur in the amino acid sequence asparagine-X- threonine or asparagine-X-serine (Ward and Dopheide, 1979; Waterfield et al., 1979; Nakamura et al., 1980; Wilson et al., 1981). However, it has been shown that glycosylation does not occur when X is a proline (Waterfield et al., 1980). Differences in the type and amount of carbohydrate on glycoproteins from different

viruses grown in the same cells are thought to result from the different primary structures of the glycoproteins which specify the nature of the oligosaccharide side chains to be added (Nakamura and Compans 1979b; Nakamura et al., 1980; Ward et al., 1980). In contrast, differences in the carbohydrates of the same viral glycoproteins synthesized in different host cells are known to be host determined.

6.3.1.2 Lipid

The lipid components of influenza viruses consist mostly of 10-13% phospholipids, 6-8% cholesterol and 1-2% glycolipids (Frommhagen et al., 1959; Kates et al., 1961; Blough and Merlie, 1970; Klenk et al., 1972). These lipids are in the viral membrane and are host derived (Klenk and Choppin, 1969, 1970a, b; Choppin et al., 1971; Quigley et al., 1971: McSharry and Wagner, 1971; Laine et al., 1972; Renkonen et al., 1977). One major difference between cell membranes and the membranes of the influenza A and B viruses is the lack of sialic acid residues on the glycoproteins and glycolipids in the viral envelopes. Sialic acid residues are removed from the viral envelopes by the viral neuraminidase (Klenk and Choppin, 1970b; Klenk et al., 1970; Palese

et al., 1974). This difference between host cell and virus membrane does not hold for the influenza C viruses (Nakamura et al., 1979) since they may lack an α-neuraminidase (Kendal, 1975; Nerome et al., 1976).

A recent study has reported finding fatty acid residues linked to the HA of influenza virus (Schmidt et al., 1979). It was suggested that the fatty acid may play a stabilizing role in anchoring the HA to the viral membrane. However, the significance of this finding awaits further study.

6.3.1.3 Protein

Initial studies of influenza virus proteins focused on the HA, NA and NP proteins because they could be immunologically detected. With the development of improved resolution in polyacrylamide gels and the labeling of proteins with radioactive amino acids and sugars, several other proteins were identified and studied. The genome of influenza A virus codes for at least ten polypeptides; P1, P2, P3, HA, NA, M1, and 3 nonstructural proteins, M2, NS1, and NS2 (Inglis et al., 1976; Lamb and Choppin, 1976; Ritchey et al., 1977; Lamb et al., 1978; Palese et al., 1981). The influenza B viruses code for at least eight polypeptides

(Racaniello and Palese, 1979) and eight detectable influenza C virus polypeptides have so far been reported (Petri et al., 1980). The polypeptides of influenza viruses will be individually described using the influenza A virus as an example.

P POLYPEPTIDES

The P polypeptides are the largest components (MW = 80,000-100,000 daltons) of the influenza virus and are associated with the ribonucleoprotein (RNP) complex within the virion (Schulze, 1970; Compans et al., 1972; Klenk et al., 1972; Bishop et al., 1972; Inglis et al., 1976). Influenza A viruses contain three P polypeptides (Lamb and Choppin, 1976; Inglis et al., 1976; Ritchey et al., 1977) and it is probable that these polypeptides are part of the viral transcriptase machinery because they are found in viral and cellular fractions that express RNA-dependent RNA polymerase activity (Bishop et al., 1972; Caliguiri and Compans, 1974; Inglis et al., 1976; Rochovansky, 1976). Furthermore, studies of influenza virus ts mutants with lesions in the P genes showed that these mutants were defective in RNA synthesis (Scholtissek et al., 1976; Palese et al., 1977b; Ritchey et al., 1977; Barry and Mahy, 1979).

HEMAGGLUTININ

Hemagglutination was first demonstrated with influenza virus (Hirst, 1941; McClelland and Hare, 1941). Subsequent studies showed that a fraction of ether disrupted virions had hemagglutinating activity (Hoyle, 1952; Schafer and Zillig, 1954). Using polyacrylamide gel electrophoresis, it was eventually shown that the hemagglutinin was a polypeptide with an apparent MW of 75,000-80,000 daltons (Schulze, 1970; Compans et al., 1970b; Haslam et al., 1970a, b) and that it was responsible for the receptor binding activity.

The HA molecule is synthesized as a single gene product which is subsequently glycosylated and post-translationally processed (Elder et al., 1979). An amino-terminal signal sequence is removed from the HA (McCauley et al., 1979) and under certain conditions the HA can be cleaved into two polypeptides, HA1 and HA2 (Lazarowitz et al., 1971) which are held together by disulfide bonds (Laver, 1971). This cleavage of the HA is not essential for expression of its hemagglutinating activity nor is it required for virion assembly (Lazarowitz et al., 1973a, b; Choppin et al., 1975). However, it has been demonstrated that cleavage of the HA molecule into HA1 and HA2 with trypsin increases the infectivity of a virus preparation which was propagated under conditions that yielded uncleaved HA (Lazarowitz and Choppin, 1975; Klenk et al., 1975). It is now accepted that

a cleaved HA is necessary for maximum infectivity of influenza viruses.

Sequence analysis of different HA molecules (Porter et al., 1979; Both and Sleigh, 1980; Gething et al., 1980; Min Jou et al., 1980; Sleigh et al., 1980; Verhoeyen et al., 1980; Ward and Dopheide, 1980; Hiti et al., 1981) have demonstrated that different HA polypeptides vary in length from 562-567 amino acids. All but two of the HA2 polypeptides have identical lengths of 221 residues whereas the HA1 polypeptides vary in length from 319-329 amino acids. The cleavage of the HA precursor into two polypeptides involves the excision of one connecting residue except in the FPV virus HA from which five residues are removed.

The precise three dimensional structure of the HA molecule from one influenza virus has been recently determined by X-ray diffraction of crystalline HA (Wilson et al., 1981). Direct evidence showed that the HA molecule is a trimer of identical subunits which form two structurally distinct regions. A triple-stranded coil of α-helices is thought to extend from the viral membrane and a globular "head" composed of β-sheets is believed to contain the receptor binding site.

NEURAMINIDASE

The first time an enzyme was identified as a structural component of a virus was when the influenza virus neuraminidase was discovered (Hirst, 1942). This enzyme, which was thought to be part of the viral HA, is a sialidase that hydrolyzes terminal N-acetylneuraminic acid residues in α-linkage to carbohydrate moieties. Experiments in which the HA and NA were physically separated (Mayron et al., 1961, Noll et al., 1962; Laver, 1963, 1964), genetically segregated (Laver and Kilbourne, 1966) and morphologically and serologically differentiated (Drzeniek et al., 1966, 1968; Webster et al., 1968, Laver and Valentine, 1969) conclusively demonstrated that the HA and NA were two separate molecules.

The function of the NA is the removal of sialic acid from the viral and host cell envelope and possibly from mucopolysaccharides that are not cell associated. An active NA prevents self-aggregation of the virions and facilitates their spreading to other cells. In addition, the removal of sialic acid residues from the mucopolysaccharides and host cell membrane may contribute to the rapid spread of infectious particles in the host. Evidence for these NA functions may be drawn from a study in which a 1,000-10,000 fold decrease in virus titer was reported for the ts mutant of influenza virus defective in NA (Palese et al., 1974). It

was shown that at the nonpermissive temperature large viral aggregates formed near the infected cell surface because the sialylated virions could attach to each other and the host cell membrane via their HAs.

A study using recombinants of the WSN virus showed that the NA of this strain was required for plaque formation in MDBK cells and the efficient processing of the HA into HA1 and HA2 (Schulman and Palese, 1977). It was suggested that in MDBK cells, the WSN NA has the additional function of removing the sialic acid residues which may sterically block the appropriate cleavge site on the HA molecule. Although there is no convincing evidence that the NA is needed during early stages of influenza virus replication in vivo, a recent investigation has also associated the NA with the fusion of viral and host cell membrane in vitro (Huang et al., 1980). It was demonstrated that liposomes containing NA and cleaved HA could fuse with cell membranes but that liposomes that lacked NA could not.

The sequence of the NA gene from the PR8 virus has been determined and it was shown to encode a polypeptide of 454 amino acids having a MW of 50,087 daltons without carbohydrate (Fields et al., 1981). Examination of the NA sequence revealed only one major hydrophobic region which was located near the amino terminus of the NA polypeptide. It was suggested that unlike the HA, the NA is probably anchored to

the viral membrane through its hydrophobic amino terminus (Fields et al., 1981).

NUCLEOPROTEIN

This protein has a molecular weight of approximately 60,000 daltons and is the subunit of the helical nucleocapsid in influenza virions (Duesberg, 1969; Joss et al., 1969; Pons et al., 1969). The NP is one of the type specific internal proteins of the influenza virus and its antigenic character is used to divide influenza viruses into types A, B and C. This protein is found associated with both the message and nonmessage sense (virion) RNA (Scholtissek and Becht, 1971). Studies in which the NP-RNA complexes were treated with pronase or RNAase indicated that both the RNA and NP are exposed in these structures but that each component partially protects the other from degradation (Duesberg, 1969; Pons et al., 1969).

Sequence analysis of the NP gene from the influenza PR8 virus demonstrated that the NP protein is 482 amino acids long (Van Rompuy, Min Jou, Huylebroeck and Fiers, personal communication). The deduced amino acid sequence revealed clusters of basic residues and proline, a feature that is common to other proteins which associate with nucleic acids (e.g. see Fiers et al., 1978; Van Heuverswyn et al., 1978; Pasek et al., 1979; Garoff et al., 1980; Strickland et al., 1980; Soeda et al., 1980).

POLYPEPTIDES ENCODED BY RNA 7

The matrix or membrane protein comprises one third of the influenza virus protein by weight and is therefore the most abundant protein in the virion. The M protein is the second type specific antigen used to distinguish the three types of influenza viruses (i.e. A, B and C). Electron microscopic analyses of influenza virions revealed an electron dense shell immediately beneath the inner surface of the viral envelope (Apostolov and Flewett, 1969; Kendal et al., 1969; Bachi et al., 1969; Compans and Dimmock, 1969; Apostolov et al., 1970) and this shell is believed to be a layer of M protein. This last conclusion is generally accepted since the M protein is the only virion polypeptide present in large enough quantity to form such a shell (Kendal et al., 1969; Compans et al., 1970a; Schulze, 1970, 1972). Other than providing the structural matrix of the virion, no other function has yet been associated with the M protein.

Recently, the M protein gene of the PR8 virus has been cloned and sequenced (Winter and Fields, 1980; Allen et al., 1980). The amino acid sequence deduced from the nucleotide data revealed an M protein of 252 amino acids having a MW of 27,861 daltons. In addition, a second open reading frame was observed in the M gene sequence which potentially codes

for a second polypeptide tentatively designated M2. The predicted amino acid sequence of the putative M2 is rich in isoleucine. Palese et al., (1981) have used ^{3}H-labeled isoleucine to demonstrate an isoleucine rich polypeptide (MW of approximately 15,000 daltons) in influenza virus (WSN) infected cells. When the polypeptides of WSN virus recombinants containing the M gene from a different strain were examined, the isoleucine rich polypeptide showed an altered migration pattern. This analysis demonstrated that RNA 7 of influenza A viruses most likely codes for a second polypeptide.

POLYPEPTIDES ENCODED BY RNA 8

A polypeptide present in influenza virus infected cells but not found in purified virions has been detected and designated NS (Dimmock and Watson, 1969). Subsequent studies using peptide map analysis showed that this nonstructural protein was unique (Lazarowitz et al., 1971) and not a cleavage product of the slightly larger M protein.

A second nonstructural protein (NS2) has also been detected in influenza virus infected cells (Skehel, 1972; Krug and Etkind, 1973; Follet et al., 1974; Minor and Dimmock, 1975; Lamb et al., 1978). The NS2 polypeptide is coded for by a second mRNA (Inglis et al., 1979) which partially overlaps the reading frame of the NS1 polypeptide

(Lamb and Choppin, 1979; Lamb et al., 1980). The precise functions of the NS polypeptides have not been determined. However, it is known that the NS1 polypeptide associates with the nucleus of infected cells (Dimmock, 1969; Lazarowitz et al., 1971; Krug and Soeiro, 1975) and preliminary characterization of a ts mutant in the NS gene of the A/FPV/Rostock/34 virus suggests that the gene product(s) may perform a regulatory role during late viral replication (Wolstenholme et al., 1980). In this latter study little vRNA and no NS2 polypeptide were detected at the nonpermissive temperature whereas in a more recent study, an independently isolated ts mutant of the NS gene did not show this phenomenon (Koennecke et al., 1981). Instead, this latter ts mutant expressed a decreased amount of HA and M.

The NS genes of four different viruses have been sequenced and these data will be discussed in subsequent sections.

6.3.1.4 RNA

Nucleic acid analysis of influenza A virus revealed single-stranded RNA (Ada and Perry, 1954; Frisch-Niggemeyer and Hoyle, 1956). Early studies with influenza viruses also

showed high rates of genetic recombination (Burnet and Lind, 1949; Simpson and Hirst, 1961; Hirst, 1962). A proposal evolved from these studies suggesting that influenza viruses had segmented RNA genomes and that the RNAs could freely reassort as detected by high frequency recombination. This hypothesis was subsequently confirmed by physical and chemical analysis of influenza virus RNAs.

Biochemical analyses of the influenza virus RNAs have definitively shown that these RNAs are unique genes which are physically separated and are not derived from a larger precursor RNA. Analysis of the viral RNA termini demonstrated that the three size classes of virion RNA isolated by differential centrifugation had pppAp at their 5' ends (Young and Content, 1970). It was subsequently found that each of the eight RNA segments in an influenza A virus genome had a distinct oligonucleotide fingerprint (McGeoch et al., 1976). Studies using guanylyl and methyltransferases isolated from vaccinia virus showed that the eight vRNAs of influenza A and B viruses could be "capped" and further analysis demonstrated that the 5' ends of all these vRNAs contained pppApGpUp (Moss et al., 1978). This latter study provided further confirmation that the virion RNAs are not breakdown products.

Studies on the nature of the influenza virus RNA have shown that it has a unique base composition which is rich in

uracil (i.e. 31-33% U, 23-25% C, 19-20% G, and 22-23% A; Bellet , 1967; Ritchey et al., 1976a) and that the vRNA is of nonmessage polarity. This last conclusion has been drawn from evidence provided by various techniques. Initially it was shown that the noninfectious vRNA could not self-anneal and therefore was of one sense (Pons, 1971; Scholtissek and Becht, 1971). The vRNA was subsequently shown to be complementary to viral mRNA by being protected from RNAase digestion when hybridized to RNA isolated from polysomes of virus infected cells (Pons, 1972; Etkind and Krug, 1975; Glass et al., 1975). It was also demonstrated that vRNA could not program the synthesis of viral proteins in cell-free translation systems whereas polysome associated RNA could. It should be noted however, that one study reported the in vitro translation of a protein from influenza virus vRNA but the protein could not be identified as one of the known viral proteins (Tekamp and Penhoet, 1976). The characterization of influenza virus RNAs revealed that polysome associated RNA had 7-methylguanosine at its 5' end and a poly-A tail at its 3' end (Etkind and Krug, 1974; Krug et al., 1976; Plotch et al., 1978) but that the vRNA lacked these features. Finally, in vitro translation of influenza virus specific mRNAs has been shown by several investigators to yield authentic viral polypeptides (Etkind and Krug, 1975; Ritchey and Palese, 1976; Content, 1976; Inglis et al., 1977; Etkind et al., 1977; Stephenson et al., 1977).

Molecular weight estimates of influenza virus RNAs have varied with the techniques used to determine them. The various methods used included differential centrifugation (Ada and Perry, 1954; Frisch-Niggemeyer and Hoyle, 1956; Pons and Hirst, 1968a), polyacrylamide gel electrophoresis using neutral conditions (Bishop et al., 1971; Lewandowski et al., 1971; Skehel, 1971), polyacrylamide gel electrophoresis using denaturing conditions (Palese and Schulman, 1976a; Pons, 1976; Ritchey et al., 1976a) and polyacrylamide-urea gel electrophoresis of RNA totally denatured with glyoxal (Desselberger and Palese, 1978). These studies resulted in molecular weight estimates of the influenza virus genome that ranged from 2×10^6 to 5.9×10^6 daltons. All these determinations were hampered by the secondary structure in the examined RNAs and the lack of availability of appropriate RNA size markers. The present use of DNA transcripts to determine the molecular weights of influenza virus RNAs has circumvented these problems and the reported values of 4.5 to 4.9×10^6 daltons for the influenza A virus genome are generally accepted (Emtage et al., 1979; Sleigh et al., 1979). However, it should be noted that in these latter studies different lengths of cDNA transcripts have been obtained for the the corresponding RNAs of different influenza viruses.

Within the last few years there has been a rapid accumulation of information on the primary structure of influ-

enza virus genes. Partial sequence analysis of influenza virus RNAs has demonstrated that the 5' end and the 3' end sequences are conserved in influenza A viruses (Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980). With the availability of molecular cloning and rapid sequencing techniques, the complete sequences of several influenza virus genes have been determined (Porter et al., 1979, 1980; Both and Sleigh, 1980; Both et al., 1980; Min Jou et al., 1980; Sleigh et al., 1980; Gething et al., 1980; Verhoeyen et al., 1980; Winter and Fields, 1980; Lamb and Lai, 1980; Baez et al., 1980, 1981; Allen et al., 1980; Winter et al., 1981; Fields et al., 1981; Hiti et al., 1981; Huylebroeck et al., personal communication; Van Rompuy et al., personal communication). Some observations resulting from these sequence analyses have already been presented and others will be discussed in subsequent sections.

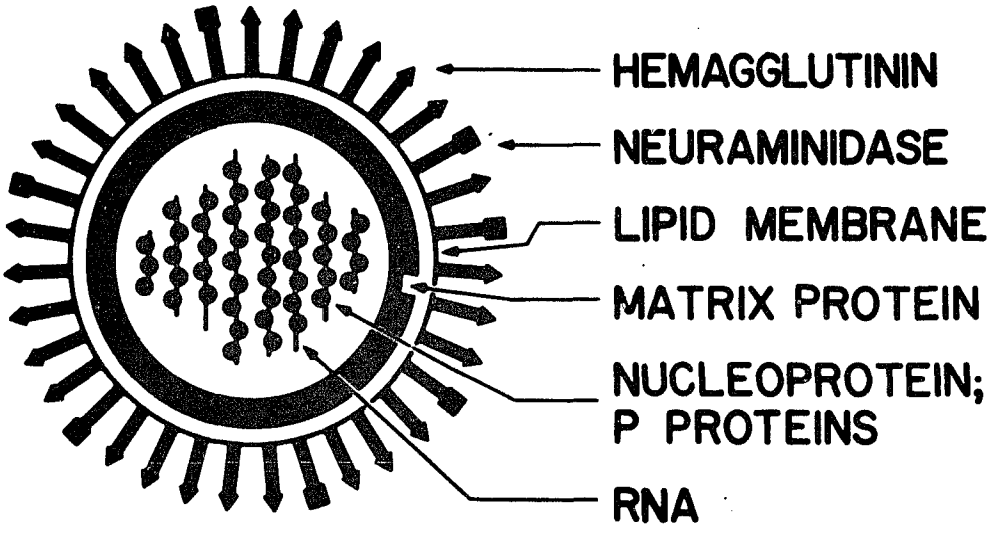
6.3.2 VIRION STRUCTURE

Figure 1 is a schematic representation of the influenza virion. Outermost, one finds the HA and NA glycoprotein spikes (Laver and Valentine, 1969) which protrude from a continuous lipid bilayer (Landsberger et al., 1971) that

FIGURE LEGEND

Figure 1. Schematic diagram of an influenza A virus.

Virions are usally spherical with hemagglutinin and neuraminidase glycoprotein spikes that protrude from a lipid membrane. Underneath the membrane is a matrix protein shell which surrounds the ribonucleoprotein complex consisting of three P proteins, NP and RNA.


HEMAGGLUTININ
NEURAMINIDASE
LIPID MEMBRANE
MATRIX PROTEIN
NUCLEOPROTEIN;
P PROTEINS
RNA

totally envelopes the virion. The isolated HA spike consists of three identical subunits which form a fibrous tail and a globular head (Wilson et al., 1981). The HA is anchored to the viral membrane by its hydrophobic carboxy-terminus (Laver and Valentine, 1969; Brand and Skehel, 1972). The NA glycoprotein consists of four identical subunits (Wrigley et al., 1973) and appears as a separate spike having a head with tetrad symmetry and a tail fiber containing a hydrophobic base which anchors the protein to the viral membrane (Laver and Valentine, 1969). The hydrophobic base of the NA is located at its amino-terminus (Fields et al., 1981).

Immediately beneath the viral envelope is a 6 nm thick layer of protein (Apostolov and Flewett, 1969; Kendal et al., 1969; Compans and Dimmock, 1969; Bachi et al., 1969; Apostolov et al., 1970) which consists of matrix protein subunits (Kendal et al., 1969; Compans et al., 1970a; Schulze, 1970, 1972). The M protein is believed to provide the skeletal support of the influenza virion. (Compans and Choppin, 1975). The M protein was found to be hydrophobic (Winter and Fields,1980) and the authors suggested that when the M protein associates with the cell plasma membrane, its basic residues line the inner face of the virion shell and interact with the negative groups of the ribonucleoproteins. Helical nucleocapsid strands can be found within the M protein shell (Wrigley, 1979).

6.3.3 INFLUENZA VIRUS GENETICS

Initial studies of genetic phenomena among influenza viruses were made possible by using infected embryonated hens' eggs and other animal hosts as model systems. These systems allowed investigators to demonstrate recombination (Burnet and Lind, 1949, 1951), multiplicity reactivation (Henle and Liu, 1951) and cross-reactivation (Burnet and Lind, 1954; Baron and Jensen, 1955). During this period of investigation, progress was limited by the inability to precisely quantitate infectious virus and to reliably isolate pure viral clones.

The advancement of influenza virus genetics began with the developement of plaquing techniques (Simpson and Hirst, 1961) followed by the isolation of the first influenza virus ts mutant (Simpson and Hirst, 1968). With these new systems began the methodical investigation of the function and structure of the influenza virus genome. A large number of ts mutants have been accumulated and studied by several investigators from which several conclusions have arisen (Mackenzie, 1970; Mills and Chanock, 1971; Ueda, 1972; Sugi-

ura 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; Koennecke et al., 1981). An important conclusion of these studies was the establishment of distinct recombination-complementation groups, each of which consisted of viruses with lesions in only one RNA.

The need to identify each of the influenza virus genes and their corresponding polypeptides resulted in the development of three methods for mapping the influenza virus genome. The physical mapping of the influenza virus genome greatly facilitated the examination of ts mutants and resulted in the identification of the defective gene in different ts mutants.

The first approach to mapping the influenza virus genome exploited the finding that the RNAs and proteins of two influenza viruses (A/PR8/34 and A/HK/8/68) exhibit unique migration patterns in polyacrylamide gels (Palese and Schulman, 1976a; Ritchey et al., 1977). By comparing recombinants of these two strains with the original strains it was possible to identify which RNA and corresponding polypeptide was transfered from each parent to the various recombinants. It was determined for the A/PR/8/34 virus that the eight genomic RNAs of increasing mobility in poly-

acrylamide gels encode the P3, P1, P2, HA, NP, NA, M and NS polypeptides respectively. In contrast, the polypeptide assignment of the A/HK/8/68 virus RNAs is reversed for RNAs 5 and 6 (i.e. RNA 5 encodes the NA polypeptide and RNA 6 encodes the NP polypeptide). This alteration in RNA mobility has proven to result from differences in the secondary structure of the RNAs. All influenza A viruses appear to have the RNA migration pattern of the A/PR/8/34 virus prototype when their RNAs are totally denatured with glyoxal prior to polyacrylamide gel electrophoresis (Desselberger and Palese, 1978).

Hybrid arrested translation was another technique used to map the influenza virus genome. Virion RNA segments were annealed to purified RNA isolated from virus infected cells. Those segments which formed RNA-RNA hybrids could not program protein synthesis in an in vitro cell-free translation system. The protein which was not made was assumed to be encoded by the input vRNA segment (Inglis et al., 1977).

The other technique used to map the influenza virus genome was based on hybridization and probe protection analysis (Scholtissek et al., 1976, 1977a, b, 1978; Rohde et al., 1977, 1978). Wild type viruses were crossed with ts mutants and recombinants having the wild type phenotype were selected. Complementary RNA isolated from cells infected with a recombinant virus was hybridized to limiting amounts

of isolated ^{32}P labeled RNA segments obtained from both parental viruses. The gene derivation of the recombinant was determined by noting which RNA segment was protected from RNAase digestion and the mutant phenotype was correlated with the RNA segment that was consistently replaced in the wildtype recombinant.

The outcome of the mapping experiments has been the identification of the gene functions in influenza A viruses. For example, one group of investigators was able to identify the defective genes in ts mutants belonging to several recombination-complementation groups (Palese et al., 1977b; Ritchey and Palese, 1977). The ts mutants used in these analyses were isolated by Sugiura et al. (1972, 1975) and Ueda (1972) and consisted of seven recombination-complementation groups. Three groups contained defects in the HA (Ueda and Kilbourne, 1976), NA (Palese et al., 1974) and in viral assembly (Sugiura et al., 1975). The defective RNA in several ts mutants was determined by rescuing ts mutants with wild type viruses and noting which gene was replaced. These studies demonstrated that the P1 and P3 proteins were required for cRNA sythesis, that the NP and P2 proteins were necessary for vRNA synthesis and that the M protein appeared to be necessary for viral assembly (Ritchey and Palese, 1977).

6.3.4 INFLUENZA VIRUS REPLICATION

The first step in viral replication is attachment to the cell membrane. Once attached, the virus can enter the cell by fusion with the cell membrane (Morgan and Rose, 1968) which results in release of the RNP complex into the cytoplasm. It appears that cleaved HA and the presence of an NA are required for viral entry and uncoating to occur (Huang et al., 1980). An alternate mode of viral entry is viropexis (Fazekas de St. Groth, 1948). In this latter mechanism, the virion enters the cell inside a pinocytotic vesicle which then fuses with a lysosome that uncoats the virus and releases the RNP complex. However, Dourmashkin and Tyrell (1974) have reported that influenza virus directly enters susceptible cells without being engulfed in a pinocytotic vacuole and that the intact virus subsequently uncoats without the aid of a lysosome. Since this process did not require virus and cell membrane fusion it was also designated viropexis. It is not certain whether one or all of the above mechanisms for viral entry, function in vivo.

Once the RNP complex is found in the cytoplasm, primary transcription occurs. Investigators have been able to identify this stage of viral replication by treating infected cells with cycloheximide, an inhibitor of protein synthesis. The RNAs that are synthesized during primary transcription

are mRNAs that are complementary to the influenza virus vRNAs. These mRNAs have been shown to have poly-A tails at their 3' ends and 7-methylguanosine cap structures at their 5' ends (Etkind and Krug, 1974; Krug et al., 1976; Plotch and Krug, 1978). That mRNAs of influenza viruses are not complete transcripts of the vRNAs is known because when these two RNA species were hybridized, the 5' termini of the vRNAs were not protected from nuclease S1 digestion (Hay et al., 1977a). Furthermore, it has been directly shown for four vRNAs that the five to seven contiguous U residues present at approximately positions 17-22 in the 5' termini of influenza virus vRNAs are the polyadenylation sites of the transcribed mRNAs (Robertson et al., 1981).

Subsequent to the removal of protein synthesis inhibitors from influenza virus infected cells, viral proteins and complete cRNA transcripts lacking cap structures and poly-A tails are made (Hay et al., 1977b). How the viral polymerase is programed to bypass the polyadenylation site and make complete cRNA transcripts as opposed to mRNAs is not understood. It is known from "temperature shift" experiments with ts mutants that the P2 protein and the NP are essential to negative RNA synthesis (Ritchey and Palese, 1977) which is most likely programed by full length cRNAs.

Studies on influenza virus replication showed that the rate of mRNA synthesis from all eight vRNAs during primary

transcription was relatively uniform when compared to that of secondary transcription. During secondary transcription certain mRNAs are preferentially made (Hay et al., 1977b). It was shown that within the first hour of infection of chick embryo fibroblasts with fowl plague virus, mRNAs 5 and 8 are predominantly synthesized. Later in the infection mRNAs 4, 6 and 7 are produced in larger quantities. The production of mRNAs 1, 2 and 3 appears to be relatively low during the course of the infection. In contrast to this mRNA synthesis, temporal differences do not occur in the maximal production of the various full length cRNAs. (It should be noted that, using a similar system as that described above, Pons (1977) demonstrated that mRNA synthesis is not temporally controlled within the first hour of influenza virus infection.) The production of different amounts of viral proteins at different times during infection is thought to reflect fluctuations in the mRNA pool and the amplification of mRNA, during secondary transcription, results from the newly transcribed vRNA. These observations suggest that gene control in influenza virus occurs at the transcriptional level.

It is known that influenza virus RNA synthesis is dependent on a functional cell nucleus or a DNA dependent host function. For example, treatment of the host cell with agents such as actinomycin D, α-aminitin, mitomycin C and uv irradiation inhibits viral RNA synthesis (Barry et al.,

1962; Rott et al., 1965; White and Cheyne, 1965; Nayak and Rasmussen, 1966; Mahy et al., 1972). Furthermore, influenza virus fails to replicate in enucleated cells (Follet et al., 1974; Kelly et al., 1974). It has been suggested that influenza virus mRNA synthesis occurs in the infected cell nucleus because internal N^6-methyladenosine residues, which are only found in mRNAs of nuclear origin, are seen in the influenza virus mRNA (Krug et al., 1976).

One explanation for the dependence of influenza virus replication on the host cell nucleus was that it required cellular DNA transcription (Lamb and Choppin, 1977; Spooner and Barry, 1977). These studies showed that influenza virus could replicate in the presence of α-aminitin only if the host cell contained a mutant DNA-dependent RNA polymerase II that was resistant to the transcription inhibitor. In other studies it was shown that the viral polymerase could be stimulated <u>in vitro</u> by addition of certain dinucleotides (McGeoch and Kitron, 1975; Plotch and Krug, 1977) which could be incorporated into the 5' ends of the viral transcripts (Plotch et al., 1978; Plotch and Krug, 1978). From these findings it was hypothesized that <u>in vivo</u> transcription of influenza virus RNA was dependent on cellular primers. Subsequently it was shown that globin message containing a 7-methylguanosine cap structure functioned as an efficient primer for the influenza virion polymerase in vitro (Bouloy et al., 1978). In addition, the primed viral

mRNA was shown to contain the globin message cap structure (Plotch et al., 1979) and the first 12-15 5'-terminal bases of the globin mRNA (Robertson et al., 1980). A similar finding was reported for influenza virus mRNAs produced in vivo (Krug et al., 1979). Sequence analysis has confirmed that there are nonvirus coded sequences at the 5' ends of influenza virus mRNAs isolated from infected cells (Caton and Robertson, 1980; Dhar et al., 1980; Lamb and Lai, 1980). It now appears that the influenza virus dependence on a host nuclear function is due to its need for cellular mRNA from which the virus polymerase can obtain a cap structure together with several nucleotides to prime viral mRNA synthesis. Most recently, a unique cap (m^7GpppXm) dependent influenza virion endonuclease was described and it was shown that this enzyme cleaved capped mRNAs to make the primers needed for viral mRNA synthesis (Plotch et al., 1981).

Early in infection, influenza viral proteins are synthesized and are continuously produced at rates which correspond to the amount of available viral mRNAs (Skehel,1972, 1973, Meier-Ewert and Compans, 1974; Inglis et al., 1976; Lamb and Choppin, 1976; Hay et al., 1977b; Inglis and Mahy, 1979). The P, NP and NS proteins are made in the cytoplasm and the NP and NS proteins migrate to the nucleus and nucleolus respectively. The M protein has been detected on smooth membrane and plasma membrane and therefore is thought to have an affinity for membranes (Lazarowitz et al., 1971;

Compans, 1973a; Klenk et al., 1974) which may be due to its hydrophobic nature (Winter and Fields, 1980). This latter study showed that 42% of the amino acid residues in the PR8 M protein were hydrophobic. The HA and NA are synthesized on the rough endoplasmic reticulum and are glycosylated during transfer to the plasma membranes via the smooth endoplasmic reticulum (Compans, 1973b; Klenk et al., 1974, 1977; Hay, 1974; Meier-Ewert and Compans, 1974; Stanley et al., 1973; Nakamura and Compans, 1979a, b). Eventually enough viral products are made for virion assembly and budding to take place.

The first virus for which budding from a plasma membrane was demonstrated was influenza virus (Murphy and Bang, 1952). Subsequently the stages of influenza virus assembly and release were delineated by electron microscopic analysis (Compans and Dimmock, 1969; Bachi et al., 1969; Compans et al., 1970a). It is thought that the HA and NA are first inserted into the host cell membrane and that the M protein associates with the cell membrane beneath the viral glycoproteins. The RNP complex then associates with the M protein and budding occurs by a pinching off of evaginated membrane segments.

6.4 VARIATION IN INFLUENZA VIRUSES

Although influenza virus vaccines have been available for more than thirty years, disease caused by these viruses continues to plague man. One of the major reasons for the recurrence of influenza stems from the ability of influenza A viruses to undergo continuous antigenic variation. Influenza virus variants expressing novel HA and NA antigens can escape antibody neutralization and can freely reinfect the previously immune population.

Influenza viruses are capable of two types of antigenic variation. Major and sudden antigenic changes in one or both of the surface glycoproteins may give rise to new subtypes which are associated with pandemic disease. This dramatic change in antigenic character is known as antigenic shift. A second type of variation observed in influenza viruses is antigenic drift which is defined as a gradual change in the surface glycoproteins, HA and NA. Antigenic drift is associated with recurring epidemic outbreaks of influenza (for review see Kilbourne, 1975).

6.4.1 ANTIGENIC SHIFT

It has been postulated that new subtypes of influenza virus arise by recombination among human and animal viruses. The recombinant retains its infectivity and pathogenicity for man but acquires new surface antigens which possibly derive from the animal strain. The human population would then be susceptible to infection with this immunologically novel influenza virus. Several investigators using various techniques have reported evidence in support of recombination as one of the mechanisms through which new strains capable of causing disease may be generated:

(1) In vitro (Kilbourne, 1968) and in vivo (Webster et al., 1973) studies showed that recombination between human and animal influenza virus strains can occur and the latter study also showed that the recombinant strain was transmitted under conditions that simulated what may occur in nature.

(2) The use of oligonucleotide map analysis provided evidence that there were recombinant strains among avian viruses isolated in nature (Desselberger et al., 1978). Similar analysis of human influenza A viruses also isolated in nature showed that recombination contributed to the genetic variation of H1N1 strains (Young and Palese, 1979).

(3) Serologic analysis demonstrated varied extents of relatedness among the HAs and NAs of human and animal influenza virus strains (Pereira et al., 1967; Coleman et al.,

1968; Webster and Pereira, 1968; Schild and Newman, 1969; Meier-Ewert et al., 1970; Laver and Webster, 1973), suggesting that these different strains may be related through recombinational events. Similar serologic surveys surveys of the HAs and NAs of avian strains revealed antigenic hybrids (Webster et al., 1976; Shortridge et al., 1977), which implied that reassortment of the HA and NA genes may have occurred. Other surveys using peptide map analysis showed that the H3s of human, avian and equine origin were very similar (Laver and Webster, 1972) and that the NA from the H2N2 and H3N2 strains were also related (Laver, 1978). These two latter studies suggested that the H3N2 strain may be a recombinant that derived the HA gene from an avian or equine like strain but retained the NA gene from the H2N2 strain.

(4) RNA-RNA hybridization analysis was used to show that the H2N2 and H3N2 subtypes were derived from recombinational events (Scholtissek et al., 1978). It was shown that all RNAs, except for the HA gene of the H3N2 virus (A/HK/1/68) were closely related to the corresponding RNAs of the H2N2 virus (A/Sing/1/57). The H3N2 virus HA RNA was shown to be closely related to the HA RNA of the A/duck/Ukraine/1/63 influenza virus. Therefore, it was suggested that a recombinational event between an A/Sing/1/57 like virus and an A/duck/Ukraine/ 1/63 like virus gave rise to the H3N2 strains. The close homology between the HAs of the

A/duck/Ukraine/1/63 virus and that of two different H3N2 strains (A/Aichi/2/68 and A/Vic/3/75) has been confirmed by comparative nucleotide sequence analysis (D. Huylebroeck et al., personal communication).

Although there is abundant evidence supporting the recombinational origin of new pandemic strains, recombination is probably not the sole mechanism involved. In 1977, H3N2 viruses were still circulating in nature when influenza viruses of the H1N1 subtype, which was prevalent from 1946 to 1957, were isolated from epidemics in China (Kendal et al., 1978). Subsequently, similar isolates were found throughout the world. A remarkable discovery resulted from the comparative oligonucleotide map analysis of these strains (Nakajima et al., 1978). It was demonstrated that these recently isolated H1N1 strains were astonishingly similar to strains prevalent in 1950 but that they differed from other H1N1 strains isolated before or after that time. Since the RNAs of the 1977 H1N1 isolates showed few changes when compared to those of the 1950 H1N1 viruses, and since they were different from the RNAs of the H3N2 viruses, a recombinational event involving an H3N2 strain could not account for the reappearance of these H1N1 strains. RNA-RNA hybridization analysis (Scholtissek et al., 1978b) confirmed this finding.

6.4.2 ANTIGENIC DRIFT

The accepted theory for antigenic drift is that the sequential replacement of human influenza viruses by new antigenic variants results from selective antibody pressure on mutants of viral surface proteins. Early investigations showed that stable antigenic variants could be selected by antibody (Taylor, 1949; Archetti and Horsfall, 1950; Isaacs, 1950; Gerber et al., 1955). In particular, Taylor's study showed that antigenic variants could be isolated from patients who had previously been exposed to influenza virus and presented with apparent as well as inapparent infections. Consequently, he proposed that the partially immune host provided a selective environment for the emergence of antigenic variants capable of resisting neutralization by antibody. In studies that used recombination to segregate the HA and NA of several influenza viruses, it was shown that antigenic variation could independently take place in the HA and NA of naturally occurring influenza viruses (Schulman and Kilbourne, 1969). That antigenic variants are the result of mutations was shown by comparative tryptic peptide analysis of HA proteins isolated from variants selected in vitro (Laver and Webster, 1968; Laver et al., 1979). This type of analysis was also done for NA proteins isolated from naturally occurring N2 variants (Kendal and Kiley, 1975). Recently, amino acid changes in the HA1 mol-

ecule have been correlated with antigenic variation in field isolates of the H3N2 subtype (Laver et al., 1980; Sleigh et al., 1981). In the latter study the nucleotide sequence of the genes coding for the HA1 polypeptides of several H3N2 strains were determined and compared to each other in addition to two previously published HA sequences. When all the strains were analyzed with a panel of monoclonal antibodies, changes in antigenicity were correlated with particular amino acid substitutions.

A new phase in the study of genetic variation among influenza viruses has evolved from the advent of nucleotide sequence analysis. As mentioned earlier, complete nucleic acid sequences of several influenza virus genes have been determined. In addition, the complete amino acid sequence of an HA polypeptide (Ward and Dopheide, 1980) and several partial sequences of other HA polypeptides derived from peptide analysis have also been reported (Waterfield et al., 1979; Laver et al., 1980). Recently, the three dimensional structure of one HA molecule has been determined by X-ray crystallography (Wilson et al., 1981). Mapping amino acid changes detected in naturally occurring and laboratory selected antigenic variants on the three dimensional structure of the HA has tentatively established four antigenic sites. Additional nucleotide sequence analysis of other naturally occuring antigenic variants also showed changes in the same four antigenic sites (M.J. Sleigh, personal commu-

nication). Whether mutations in these four HA sites are soley responsible for the generation and selection of new antigenic variants, capable of epidemic disease, requires more extensive analysis of naturally occurring variants. For example, it should be determined if mutations in other genes can also influence the selection of variants.

6.4.3 GENETIC VARIATION IN GENES CODING FOR NONSURFACE PROTEINS

Comparison of the RNA fingerprints of H1N1 viruses isolated during a 9 month period in 1977 suggested that sequential mutations occurred throughout their genomes (Young et al., 1979). This evidence suggested that the emergence of variants is not soley dependent on antibody selection of HA and NA antigenic mutants. (Variation in the NP was independently detected by using the technique of double immunodiffusion, Schild et al., 1979; and peptide map analysis, Dimmock et al., 1980). Oligonucleotide map analysis of H1N1 strains isolated in the winter of 1978-1979 revealed that they derived from a recombinational event (Young and Palese, 1979). Specifically it was shown that the new H1N1 prototype, A/Cal/10/78, had a recombinant genotype consisting of

the HA, NA, M and NS genes from an earlier H1N1 strain and the P1, P2, P3 and NP genes which probably derived from an H3N2 virus. This finding indicated that in addition to point mutations, recombination may also be involved in generating influenza virus variants of the same subtype.

Comparative oligonucleotide map analyses that will be presented in the "Results" section, have demonstrated variation among all genes of several H2N2 viruses and have shown the extent of change among all the corresponding genes. Furthermore, nucleotide sequence analysis has also been used to directly determine the variation of two different NS genes and the sequences of these two genes were compared to those of two other NS genes.

6.5 OBJECTIVES OF THIS STUDY

The aim of this study was to analyze the genetic structure of influenza A virus genes using molecular techniques. Three goals were set during the course of my investigations:

(1) To determine the genetic compositions of several influenza virus recombinants constructed for use in vaccine production.

(2) To estimate the extent of genetic variation among all the genes of five influenza viruses, which were isolated at the beginning, middle and end of the H2N2 subtype period (1957-1968), using RNAase T1 fingerprinting.

(3) To examine the genetic structure of two different influenza virus NS genes by molecular cloning and DNA sequencing and to determine the genetic variation between these two sequences and those of two other NS gene sequences.

7.0 MATERIALS AND METHODS

7.1 VIRUS AND VIRUS PURIFICATION

Table 2 lists the influenza viruses used in this study. Virus was propagated by inoculating the allantoic cavity of 10-11 day old embryonated hens' eggs with 0.1 ml of a 1000-fold diluted virus seed. The diluent consisted of phosphate buffered saline (PBS), 136 mM NaCl, 3.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, containing 100 units/ml penicillin G, 100 ug/ml streptomycin sulfate and 190 ug/ml bovine albumin (fraction V, Miles Biochemicals, Elkhart, IN). After two days incubation at 37°C, the infected eggs were chilled to 4°C and the allantoic fluids were harvested and clarified by centrifugation at 1000 x g for 15'. Virus was quantitated by hemagglutination (Hierholzer and Sugg, 1969): Serial two-fold dilutions of virus in PBS were made in microtiter plates (Dynatech Labs Inc., Alexandria, VA) and human group "O" cells to a final concentration of 0.25% were added to each well. The contents were mixed and allowed to incubate at room temperature for one hour. The hemagglutination titer is the reciprocal of the highest dilution exhibiting hemagglutination.

Table 2

Viruses used in this study

Virus	Obtained from	Abbreviation
A/PR/8/34 (H1N1)	Dept. of Microbiology Mt. Sinai Sch. of Med.	PR8
A/Aichi/2/68 (H3N2)	"	
A/England/42/72 (H3N2)	"	
A/Port Chalmers/1/73 (H3N2)	"	
A/Scotland/840/74 (H3N2)	"	
A/Victoria/3/75 (H3N2)	"	Vic/75
A/England/864/75 (H3N2)	"	
A/New Jersey/1/76 (Hsw1N1)	"	
A/Victoria/112/76 (H3N2)	"	
A/Texas/1/77 (H3N2)	"	
A/Brazil/11/78 (H1N1)	"	
A/Ann Arbor/6/60 (H2N2)	"	A/AA/6/60
A/Nederland/84/68 (H2N2)	"	A/Ned/84/68
A/Hong Kong/8/68 (H3N2)	"	A/HK/8/68
X-31	Dr. E.D. Kilbourne	
X-37	"	
X-41	"	
X-45	"	
X-47	"	
X-49	"	
X-53	"	
X-53A	"	

TABLE 2 CONT.

VIRUS	OBTAINED FROM	ABBREVIATION
X-57	DR. E.D. KILBOURNE	
X-61	"	
X-71	"	
A/SINGAPORE/1/57 (H2N2)	DR. A.P. KENDAL	A/SING/1/57
A/TAIWAN/1/64 (H2N2)	"	A/TW/1/64
A/CALIFORNIA/1/66 (H2N2)	"	A/CAL/1/66
A/DUCK/ALBERTA/60/76 (H12N5)	DR. R.G. WEBSTER	DUCK

Aliquots of the pooled allantoic fluid were stored at -70°C for use as seed stocks and the rest of the pool was centrifuged in a 45Ti rotor at 127,000 x g for 30' to pellet the virus. Pellets were resuspended in several ml of NTE [10 mM Tris-HCl pH 7.4, 100 mM NaCl and 1 mM disodium ethylenediamine tetraacetate (EDTA)] and layered onto 30%- 60% continuous sucrose gradients formed with NTE in SW27 cellulose nitrate tubes. After centrifuging for 3 hours at 77,000 x g, the visible virus bands were harvested by puncturing the side of the tube with a syringe and aspirating the band. Concentrated virus preparations were stored at -20°C.

7.2 PLASMID AND PLASMID PROPAGATION

Three recombinant plasmids (pMEL801, pJZ101 and pAR101) constructed with pBR322 and double-stranded transcripts of influenza virus NS gene segments were used for sequence analysis. Their construction and insertion into E.coli will be detailed in subsequent sections. To propagate plasmid, E.coli transformants were grown in LB medium [1% Bacto-tryptone, 0.5% yeast extract, (Difco laboratories, Detroit, MI) and 1% NaCl] supplemented with 50 ug/ml ampicillin which

selects for cells containing the penicillinase producing plasmid. Cells were incubated at 37°C in an incubator-shaker until an absorbance at 260 nm of approximately 0.6 was reached. At this point 200 ug/ml of chloramphenicol was added to the culture to amplify the plasmid (Clewel, 1972). After 16 hours, the culture was chilled on ice and the cells harvested by centifugation at 7000 x g for 15'. Pellets were resuspended in 20 mM Tris-HCl pH 8.0 and repelleted in a 50 ml plastic screwcap tube such that each pellet contained the equivalent of 0.5 l of cells. Cells were stored at -20°C.

7.3 CELL CULTURE

Madin-Darby canine kidney (MDCK) cells were maintained in 75 cm2 plastic bottles (Falcon Plastics, Oxnard, CA) with 15 ml of growth medium [minimal essential medium (MEM) with Earles salts, (Grand Island Biological Co., Berkeley, CA) containing 10% heat treated (56°C for 60') fetal bovine serum, (Flow Laboratories, Rockville, MD) 5% $NaHCO_3$, 0.292 mg/ml L-glutamine (Flow laboratories), 100 units/ml penicillin G and 100 ug/ml streptomycin sulfate]. Using trypsin-EDTA [0.1% trypsin (Difco Laboratories) 0.04% EDTA, 0.025%

$NaHCO_3$] cells were removed from the bottles, pelleted (1000 x g for 10') and seeded into new bottles at 2 x 10^6 cells per bottle. After 3-4 days incubation at 37°C in a humidified incubator containing 5% CO_2, confluent monolayers were obtained. MDCK cells were also seeded into 25 cm2 plastic dishes (Falcon Plastics) at 1.0 x 10^6 cells per dish for use in labeling viral RNAs.

7.4 BIOCHEMICAL TECHNIQUES

7.4.1 EXTRACTION OF VIRAL RNA

Virus purified from sucrose gradients was placed into a 50 ml plastic screwcap tube and made 10 mM Tris-HCl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.3% sodium dodecyl sulphate (SDS) and 500 ug/ml proteinase K (Merck Co., Darmstadt, W. Germany). After 15' at 56°C, 1/10 volume of LiCl buffer (1.4 M LiCl, 5% SDS, 0.1 M $NaC_2H_3O_2$ buffer pH 4.9) was added and the contents mixed before adding an equal volume of phenol (which was redistilled and saturated with 10 mM Tris-HCl pH

7.4, 10 mM KCl, 1.5 M $MgCl_2$). After mixing and heating to 56°C for 5', an equal volume of $CHCl_3$ was added and the sealed tube was mixed on a wrist-action shaker for 20'. The phases were separated by centrifugation (1000 x g for 10') and the aqueous phase was reextracted if protein was present at the interface. RNA was precipitated from the aqueous phase with 2.5 volumes of cold ethanol for several hours at -70°C. RNA was pelleted (12,000 x g for 20'), washed with ethanol to remove residual phenol and lyophilized before storing at -20°C.

7.4.2 EXTRACTION OF PLASMID DNA

To isolate plasmid DNA from bacterial cells the rapid alkaline extraction method (Birnboim and Doty, 1979) was used as follows: A pellet of cells from 0.5 l of a bacterial culture (see section 7.2) was resuspended in 20 ml of solution I (25 mM Tris pH 8.3, 50 mM glucose, and 10 mM EDTA) and divided into 2 45Ti bottles. Lysozyme, 20 mg, (Sigma Chemical Co., St. Louis, MO) was added to each bottle which was gently mixed by inversion and incubated on ice for 30'. After addition of 20 ml of solution II (0.2 M NaOH and 1% SDS) each bottle was gently inverted until the solu-

tion cleared. The bottle was then chilled on ice. Five minutes after the addition of solution II, 15 ml of solution III (3 M $NaC_2H_3O_2$ adjusted to pH 4.8 with glacial acetic acid) was added to each sample which was incubated 60' on ice. Cellular DNA and debris were pelleted by centrifugation (50,000 x g for 30'). The supernatant was combined with 2.5 volumes of ethanol and chilled for 30' at -70°C to precipitate plasmid DNA and cellular RNA.

After centrifugation (12,000 x g for 20'), the drained pellets were resuspended in 10 ml of 20 mM Tris-HCl pH 7.4, 200 mM NaCl and reprecipitated with 30 ml ethanol. The resultant pellet was resuspended in 2.5 ml 10 mM Tris-HCl pH 7.4, 1 mM EDTA and treated with 2.5 ml of 180 ug/ml pancreatic RNAase A (Worthington Biochemical Corp., Freehold, NJ,). To inactivate contaminating DNAases the pancreatic RNAase A was previously heated to 100°C in 50 mM $NaC_2H_3O_2$ at pH 5.0 and then diluted to 180 ug/ml with 10 mM Tris-HCl pH 7.4, 1 mM EDTA. After digesting the RNA for 30' at 37°C, 7.5 ml of 0.3 M $NaC_2H_3O_2$ was added to the solution which was extracted several times with equal volumes of phenol:chloroform (1:1, v:v) until no protein was noted at the interface. Following ethanol precipitation, the nucleic acid was resuspended in 2 ml NTE containing 10% glycerol and layered onto a Sepharose A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) column to separate oligonucleotides from plasmid DNA. Fractions containing the purified plasmid DNA

(as determined by uv light absorbance at 260 nm) were pooled, ethanol precipitated and pelleted (12,000 x g for 20') for storage at -20°C.

7.4.3 CONSTRUCTION OF CLONES

7.4.3.1 pMEL801 plasmid construction

Double-stranded cDNA transcripts of the duck virus RNAs were prepared using modifications of the Emtage et al. (1979) technique. Virus RNA was primed with the synthetic oligonucleotide d(AGCAAAAGCAG)rG (Collaborative Research, Waltham, MA) which is complemetary to the 3' end of all influenza virus RNAs (Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980) and reverse transcriptase (provided by Dr. J.W. Beard, Division of Cancer cause and Prevention, National Cancer Institute), in a cocktail consisting of 200 ug/ml RNA, 100 ug/ml primer, 500 units/ml reverse transcriptase, 500 uM each dATP, dCTP, dGTP, and dTTP, 100 ug/ml actinomycin D (Sigma Chemical Co.) 100 uCi α-^{32}P-dCTP

(300 Ci/mmole, New England Nuclear, Boston MA) in RT buffer [50 mM Tris-HCl pH 8.5, 5 mM dithiothreitol (DTT), 0.01% triton X-100, 40 mM KCl, 10 mM $MgCl_2$]. Single-strand synthesis proceded for 2 hours at 37°C and was stopped by making the reaction 0.2% SDS, 20 mM EDTA, 300 mM $NaC_2H_3O_2$ and by extracting the mixture 3 times with equal volumes of phenol:chloroform (1:1). The nucleic acid was ethanol precipitated from the aqueous phase, washed with ethanol and lyophilized.

The DNA-RNA pellet was resuspended in 10 mM Tris-HCl pH 7.4, 1 mM EDTA and made upto 25 ug/ml of pancreatic RNAase A to digest the RNA template. After a 30' incubation at 37°C, the reaction was stopped by making it 300 mM $NaC_2H_3O_2$ and extracting it with 1 volume phenol:chloroform (1:1). The ethanol precipitated nucleic acid was resuspended in 10 mM Tris Tris-HCl pH 7.4, 10 mM NaCl, 1 mM EDTA and passed through a Sephadex G-100 (Pharmacia Fine Chemicals) column to separate the cDNA from oligonucleotides. Fractions containing the radioactive DNA were pooled and the DNA was ethanol precipitated. The yield of single-stranded DNA was calculated as the percent of available nucleotides which were incorporated into high molecular weight material.

The single-stranded DNA was resuspended in H_2O, combined with a second synthetic oligonucleotide, d(AGTAGAAACAAG) (Collaborative Research) which is complemen-

tary to the first 12 residues of the 3' end of the positive cDNA strand (Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980) and heated to 100°C for 30'' followed by quick chilling on ice. Second-strand synthesis proceded as described for first-strand synthesis except that actinomycin D was omitted, 50 uCi of α-^{32}P-dCTP were used, the DNA concentration was approximately 60 ug/ml and that of the primer was 3 ug/ml. The reaction was stopped and the DNA was purified as previously described.

The 5' ends of the double-stranded cDNAs were phosphorylated so that EcoR1 linkers (Collaborative Research) could be added (Maniatis et al., 1978). The linkers were also phosphorylated. The double-stranded DNA was resuspended in kinase-ligase buffer (66 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 15 mM DTT, 1 mM Spermidine, 200 ug/ml bovine serum albumin, 1 mM ATP) containing 240 units/ml of polynucleotide kinase. After 1 hour at 37°C the kinase activity was destroyed by heating the mixture to 68°C for 5'. The EcoR1 linkers were phosphorylated with 100 uCi of γ-^{32}P-ATP for 1 hour at 37°C in a 10 ul reaction containing kinase-ligase buffer, 100 uM linker and 1.5 units polynucleotide kinase. Three ul of kinase-ligase buffer containing 3.3 mM ATP and 1.5 units polynucleotide kinase were added to the mixture which was incubated for another 30' at 37°C. The volume of the reaction mixture was made upto 100 ul with 0.3 M $NaC_2H_3O_2$ and the mixture was extracted with an equal volume of phe-

nol:chloroform (1:1). The linkers were ethanol precipitated, washed with ethanol, dried and resuspended to a concentration of 50 uM in H_2O.

The phosphorylated DNA mixture was combined with an equal volume of kinase-ligase buffer containing 20 uM phosphorylated EcoR1 linker and 4 ul of T4 DNA ligase (a gift of Dr. K. Squires, Columbia University, NY). The optimal concentration of T4 ligase was empirically determined. The ligation reaction was incubated for 16 hours at 14^oC and heated to 68^oC for 5' to inactivate the enzyme. The DNA and linker mixture was made 100 mM Tris-HCl pH 7.2, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol and 50 mM NaCl and treated for 2 hours with 130 units of EcoR1 restriction endonuclease (Bethesda Research Laboratories, Inc., Rockville, MD) to reduce polymerized linker to monomer length. The mixture was made 0.3 M $NaC_2H_3O_2$, extracted with an equal volume of phenol:chloroform (1:1) and the DNA was precipitated with ethanol. The pellet was resuspended in 2 ml of NTE containing 10% glycerol and the solution was passed through a Sepharose CL6B column to separate DNA from unattached linker. Fractions containing the radioactive DNA were pooled and the DNA was ethanol precipitated.

INSERTION OF DNA INTO PBR322 AND TRANSFORMATION OF E.COLI

Approximately 0.5 ug of the virus specific double-stranded DNA was used for insertion into 150 ng of plasmid pBR322 (Bolivar and Bachman, 1979; the plasmid was a gift of Dr. P. Szabo, Sloan-Kettering Institute for Cancer Research, NY) in 50 ul of kinase-ligase buffer containing 1 ul T4 ligase. (The plasmid DNA was previously digested with EcoR1 restriction endonuclease and dephosphorylated, as described in a subsequent section, to prevent self-ligation.) After 16 hours at 14°C, 7.5 ul of 4 M $NaC_2H_3O_2$ were added, the mixture was extracted with phenol:chloroform and the DNA was precipitated with ethanol.

The washed and dried pellet was resuspended in 10 mM Tris-HCl ph 7.4 and E.coli C600 cells (a gift of Dr. A. Skalka , Roche Institute of Molecular Biology, Nutley NJ) were transformed with the chimeric DNA using the $CaCl_2$ technique (Dagert and Ehrlich, 1979): E.coli cells were inoculated into 100 ml of LB broth and were allowed to grow to an absorbance (at 260 nm) of 0.2 at 37°C. The cells were chilled on ice for 10', pelleted (7,000 x g for 15') and resuspended in 20 ml of 0.1 M $CaCl_2$. After 30' on ice, the cells were pelleted, resuspended in 0.5 ml of 0.1 M $CaCl_2$ and left on ice for 24 hours. Competent cells in a volume of 100 ul, were combined with 10 ul of the recombinant DNA (250 ng) and left on ice for 1 hour. The cells and DNA were

heated to 42°C for 90'', cooled to room temperature and combined with 1.5 ml LB medium which had been prewarmed to 37°C. After an hour incubation in a shaking waterbath set at 37°C, 200 ul aliquots of the cell suspension were spread onto petri plates of LB agar (LB medium + 2% agar) supplemented with 50 ug/ml ampicillin (LB + AMP plates). The plates were incubated 16 hours at 37°C.

IDENTIFICATION OF RECOMBINANT CLONES.

Bacterial colonies containing recombinant plasmids with influenza virus gene sequences were identified by colony hybridization (Grunstein and Hogness, 1975): Each colony from the transformation experiment was transfered onto a second LB + AMP plate and onto a nitrocellulose filter which was placed onto a third LB + AMP plate. The filter containing plates were incubated at 37°C until colonies were visible (µ4 hours) whereas the replica plates were incubated for 16-24 hours and then stored at 4°C. Nitrocellulose filters were treated for indicated times with a series of solutions [0.5 N NaOH, 10'; 1 M Tris-HCl pH 8.3, 5'; 1 M Tris-HCl pH 8.3, 5'; 10 x SSC (1.5 M NaCl, 0.15 M $Na_3C_6H_5O_7$, 5'] by placing filters on 2 layers of 3MM chromatography paper (Whatman, LTD., England) saturated with these solutions. After air drying the filters, they were soaked in chloroform for 30'', air dried, soaked in 10 x SSC, air dried and baked

in a vacuum oven at 80°C for at least 2 hours to fix the DNA. Four filters per petri plate were pretreated for 1 hour with 25 ml hybridization buffer (50% formamide, 5 x SSC, 0.02% Ficoll-400, 0.02% polyvinylpyrrolidone-360, 0.5% SDS) in a slowly shaking waterbath set at 42°C. Hybridization with a cDNA probe made from duck virus RNA (see below) was for 1.5 hours using pretreatment conditions. Filters washed for several hours in hybridization buffer and for 10' in 3 x SSC at 42°C were air dried and autoradiographed using Kodak X-OMAT XR-1 film and intensifying screens.

PREPARATION OF CDNA PROBE

To make the cDNA probe, 50 ug/ml of duck virus RNA were combined with 25 ug/ml of synthetic primer, d(AGCAAAAGCAG)rG, 600 units/ml of reverse transcritase, 200 uM each dATP, dGTP and dTTP, 20 uM dCTP, 100 uCi α-^{32}P-dCTP (300Ci/mmole, New England Nuclear) and 1 ug/ml actinomycin D in RT buffer. After 1.5 hours at 37°C the DNA was purified as described above.

LENGTH DETERMINATION OF VIRAL SEQUENCES IN RECOMBINANT PLASMIDS

Several replicates of colonies containing DNA that hybridized with the probe were chosen and grown for 16 hours

in 10 ml of LB medium supplemented with 50 ug/ml ampicillin. Plasmid was isolated as described earlier, except that 1.5 ml of cells were used and the volumes of all reagents were proportionately reduced. Plasmid DNA was digested with EcoR1 endonuclease and the sizes of the viral sequences were determined by their relative electrophoretic mobility in a 1% agarose gel. One insert was tentatively identified as an NS gene based on its length of approximately 900 residues. This gene assignment was subsequently confirmed by comparative sequence analysis with the cloned NS gene derived from the A/PR/8/34 virus (see construction of pJZ101). The plasmid containing the duck virus NS gene was designated pMEL801.

7.4.3.2 pJZ101 plasmid construction

In a separate cloning experiment similar to that described above, double-stranded cDNA transcripts of A/PR/8/34 virus RNA were prepared and inserted into the HindIII site of plasmid pBR322 (by Dr. J.J. Zazra in our laboratory). The influenza virus gene sequences contained in each of these recombinants were identified by the "Northern" blot technique described by Alwine et al. (1979; see following description).

AGAROSE-UREA GEL ELECTROPHORESIS

A 1.5% agarose gel solution (Seakem, Marine Colloids Div. FMC Corp., Rockland, ME) was made with 150 ml 0.025 M $C_6H_8O_7$ pH 3.8 containing 6 M urea (Rosen et al., 1975) and poured into a flatbed gel apparatus. Sample slots were cast with a comb (2 preparative slots, 35 mm x 1.2 mm x 5 mm; each of which was flanked on either side by smaller slots, 5 mm x 1.2 mm x 5 mm) and the gel allowed to set for 16 hours at 4^oC. Running buffer (0.025 M $C_6H_8O_7$, pH 3.8, 6 M urea) sufficient to just cover the gel was added and the comb was gently removed. Unlabeled viral RNA was dissolved in running mixture (0.025 M $C_6H_8O_7$ pH 3.8, 6 M urea, 25% sucrose, 0.025% xylene cyanol-ff, 0.025% bromophenol blue) at a concentration of 600 ug/ml and loaded into each of the preparative slots. Approximately 50,000 cpm of *in vivo* labeled RNA (see section on *in vivo* RNA labeling) were dissolved in running mixture and loaded into each of the 3 smaller slots for use as gene position markers. Samples were electophoresed for 17 hours at a constant current of 40 mA. The gel was stained with 100 ml of 0.05 ug/ml ethidium bromide in H_2O and the RNA visualized by uv light illumination.

TRANSFERING RNA ONTO DIAZOBENZYLOXYMETHYL (DBM) PAPER

A large gel piece containing the sample tracks was excised and soaked in 50 mM NaOH for 30' followed by two 10' soakings in 0.2 M $NaC_2H_3O_2$ pH 4.2. A piece of DBM paper cut to fit precisely over the gel section was placed onto the gel section and 0.2 M $NaC_2H_3O_2$ pH 4.2 was allowed to diffuse through the gel and paper for 16 hours at 4°C (see figure 2 for details). After blotting, strips of DBM paper containing the radioactive RNAs were excised and autoradiographed to serve as a marker for the position of each viral RNA segment bound to the paper. The DBM paper containing the unlabeled RNA was sealed in a plastic bag together with several ml of hybridization buffer (50% formamide, 375 mM NaCl, 37.5 mM Na3C6H5O7, 25 mM Na_2HPO_4/NaH_2PO_4 pH 6.5, 0.02% ficoll-400, 0.02% polyvinylpyrolidone-360, 0.02% BSA, 1 mg/ml sonicated calf thymus DNA) containing 1% glycine. The sealed plastic bag was incubated at 42°C for at least 3 hours to saturate nonspecific binding sites and to inactivate residual diazonium groups which could react with the labeled probe. The bag containing the DBM paper was stored at 4°C.

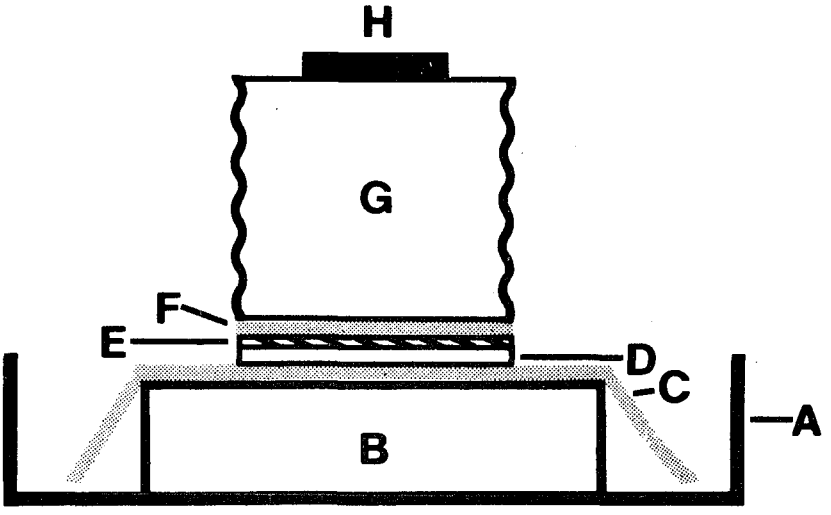
ACTIVATION OF DBM PAPER

Nitrobenzyloxymethyl paper (Schleicher and Schuell Inc., Keene, NH) was converted to aminobenzyloxymethyl paper

FIGURE LEGEND

Figure 2. Transfer of RNA to activated DBM paper.

Trough A contains 200 mM $NaC_2H_3O_2$, pH 4.2 and a platform (B) on which a wick (C), consisting of 2 layers of Whatmann 3MM paper saturated with the above buffer is placed. The gel (D) containing the RNA is placed on the wick and the area surrounding the gel is covered with Saran wrap. The activated DBM paper (E) is placed on the gel and covered with 2 layers of Whatman 3MM paper (F) followed by a stack of paper towels (G) upon which a weight (H) is placed. RNA transfer proceeds for approximately 16 hours (overnight) at $4^{o}C$.


H
G
F
E
D
C
A
B

by incubating 30' at 60°C with a 20% (w/v) solution of sodium dithionite (0.4 ml per cm2 of paper). The paper was washed with large volumes of H_2O for 3' and for 5' in 30% $C_2H_4O_2$. Then the paper was repeatedly washed with H_2O until no odor of H2S remained. The amino form of the paper is placed in cold 1.2 M HCl containing 3 mg/ml $NaNO_2$ (0.3 ml of solution per cm2 of paper) and incubated for 30' on ice to convert the amino groups to diazonium groups. When ready to use, the paper was washed twice with cold H_2O and twice with cold 200 mM $NaC_2H_3O_2$ pH 4.2.

PREPARATION OF NICK TRANSLATED DNA PROBE

Nick translated (Maniatis et al., 1975a) plasmid DNA was hybridized to the RNA containing DBM paper to determine the gene specificity of its viral sequences. One ug of plasmid DNA was treated for 2 hours at 14°C in a cocktail containing 50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 ug/ml BSA, 2 uM each dCTP, dGTP and dTTP and 2 uM α-^{32}P-dATP (300 Ci/mmole, New England Nuclear), 45 units/ml DNA polymerase I (New England Biolabs) and 1 ng/ml DNAase I (DN-EP, Sigma Chemical Co.). The mixture was extracted with an equal volume of phenol:chloroform (1:1) and the aqueous phase was loaded onto a Sephadex G-100 (Pharmacia Fine Chemicals) column equilibrated with NTE.

Fractions containing the radioactive peak were pooled and the DNA was ethanol precipitated.

DNA-RNA HYBRIDIZATION

The labeled DNA was resuspended in hybridization buffer, heated to 100°C and quick-chilled on ice. A 3 mm wide strip of DBM paper containing viral RNA was combined with the hybridization mixture in a plastic bag which was sealed and incubated in a shaking water bath set at 42°C for 1 hour. Following several washes with 50% formamide, 375 mM NaCl, 37.5 mM $Na_3C_6H_5O_7$, 25 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 at 42°C, the DBM paper strip was blotted dry with paper towels and autoradiographed using Kodak X-OMAT X-R1 film and an intensifyinng screen (Cronex, Dupont, Wilmington DE). The gene specificity of the viral sequences in the hybridizing DNA was determined by analysis of the labeled hybrid bands on DBM-RNA paper.

7.4.3.3 pAR109 plasmid construction

Plasmid pAR109 containing a PR8 virus NS gene specific sequence was constructed by Drs. A.R. Reisfeld and

A.M. Skalka (Roche Institute of Molecular Biology, Nutley, NJ) and its preparation will only be briefly described. DNA complementary to PR8 virus RNA was made using conditions described above except that the second strand was primed by the small double-stranded "hairpin" formed by reverse transcritase at the 3' end of the first strand cDNA transcript (Efstratiadis et al., 1975). After digestion with S1 nuclease, the blunt ended dsDNAs were methylated with EcoR1 methylase and EcoR1 linkers were added (Maniatis et al., 1978). Following digestion with EcoR1 nuclease, the cDNAs were ligated to the left and right arms of λgtWESλB and recombinant phages were produced by in vitro packaging of the chimeric DNAs (Sternberg et al., 1977). E.coli DP50/supF cells were infected with phage and the resulting plaques were examined for influenza virus gene sequences by in situ hybridization (Benton and Davis, 1979) using as probe viral RNA which was partially digested with alkali and 5' end labeled with γ-^{32}P-ATP using polynucleotide kinase (Chaconas and van de Sande, 1980; see next section). The influenza virus gene derivation of each recombinant phage was identified by the "Northern" blot technique described above. One PR8 virus NS gene specific insert was subcloned into pBR322 and designated pAR109.

7.4.4 PREPARATION OF LABELED DNA FRAGMENTS FOR SEQUENCE ANALYSIS

7.4.4.1 5' end labeling of DNA fragments

Restriction endonuclease fragments of cloned DNA were prepared using digestion conditions specified for the different enzymes in the New England Biolabs Inc. catalogue (1980-1981). DNA fragments (3-5 ug) were treated with several units (10-80 units) of calf intestine alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IN) in 15-25 ul of 50 mM Tris- HCl pH 8.3 (Efstratiadis et al., 1977). After 30' incubation at 37°C, the reaction was made upto 100 ul with 0.3 M $NaC_2H_3O_2$ and extracted twice with phenol:chloroform (1:1). DNA was ethanol precipitated, washed with ethanol and dried. DNA resuspended in 8.5 ul H_2O was combined with 12.5 ul of 20 mM K_2HPO_4 pH 9.2, 20 mM $Mg(C_2H_3O_2)_2$ and 10 mM DTT. The mixture was transfered to a 1.5 ml microcentrifuge tube containing 300 uCi of lyophilized γ-^{32}P-ATP (1000-3000 Ci/mmole, New England Nuclear) and incubated for 1 hour with 4 ul of polynucleotide kinase (6 units, New England Biolabs, Beverly, MA) at 37°C. (This

labeling technique is a modification of the method of Chaconas and van de Sande, 1980.) The reaction mixture was made upto 100 ul with 0.3 M $NaC_2H_3O_2$ and extracted with an equal volume of phenol:chloroform (1:1). The DNA was precipitated with 3 volumes of ethanol, pelleted (12,000 x g for 15'), dried and resuspended in 30 ul TBE (90 mM Tris-Base, 90 mM H3BO3, 2.5 mM EDTA) containing 0.05% SDS, 10% glycerol, 0.025% xylene cyanol-FF and 0.025% bromophenol blue. Samples were electrophoresed in low-melting-point agarose (Bethesda Research Laboratories) gels to separate individual fragments and unincorporated label. The concentration of the agarose gels varied from 0.8%-1.5% depending on the expected sizes of the DNA fragments.

7.4.4.2 Electrophoretic separation of DNA on agarose gels

Agarose gels were prepared by dissolving appropriate amounts of agarose (1.6-3.0 g) in 200 ml of TBE, containing 0.5 ug/ml ethidium bromide (Sigma Chemical Co.), on a heated magnetic stirrer until the solution boiled. After the gel solution was cooled to 37^{o}C, it was poured into a flatbed electrophoresis apparatus (Biorad Laboratories, Richmond, CA) and different combs were used to cast sample wells.

Once the gel set, enough TBE (containing 0.5 ug/ml ethidium bromide) to cover its surface was added and the comb was gently removed. DNA fragment mixtures were loaded into the wells and electophoresed at $4^{o}C$ with a constant current of 70 mA until separation of all expected fragments was effected. The DNA fragments were detected by uv light illumination.

Gel pieces containing individual DNA fragments were excised, melted at $65^{o}C$ and diluted to 0.3% agarose with 20 mM Tris-HCl pH 7.4, 200 mM $NaC_2H_3O_2$. Agarose was removed from the solution by three successive extractions with 0.75 volumes of phenol (redistilled and saturated with 10 mM Tris-HCl pH 7.4 containing 10 mM KCl and 1.5 mM $MgCl_2$). DNA was precipitated with 3 volumes of ethanol and the resulting pellets were washed with ethanol and dried. Further digestion of labeled DNA fragments with a second restriction endonuclease proceded as described above and resulted in fragments labeled at one end. These fragments were separated in 4% polyacrylamide gels (Maniatis et al., 1975).

7.4.4.3 Electrophoretic separation on polyacrylamide gels

Solutions of 3.87% acrylamide, 0.13% N,N'-methylene-bis acrylamide (bis), 1% $(NH_4)_2S_2O_8$ and 0.1% N,N,N',N',-tetramethylethylenediamine (TEMED) in TBE (Maniatis et al., 1975b) were prepared for casting gels between glass plates (265 mm x 165 mm x 4.5 mm) separated by 1.2 mm thick spacers. An appropriate comb was used to mold the sample wells. After the gel polymerized, the comb and bottom spacer were removed and the gel was mounted onto a vertical electrophoresis apparatus. TBE was used as the running buffer and gels were prerun for 0.5 hours at a constant voltage of 200 V. DNA resuspended as for agarose gels was loaded into the wells and electrophoresed until the fragments separated (the duration of electrophoresis was empirically determined). Labeled DNA bands were detected by autoradiography using Kodak X-OMAT XR-1 film together with intensifying screens.

An autoradiogram of the labeled DNAs was used as a template to excise gel pieces containing individual DNA fragments which were electroeluted into 0.3 ml of electroelution buffer (0.5 M Tris-C2H3O2 pH 6.0, 0.5 M $NaC_2H_3O_2$): The gel piece was inserted into the tip of a medicine dropper and overlayed with a 1% agarose plug made with electroelution buffer and 0.05% xylene cyanol-FF. The dropper tip was placed into a 1.5 ml microcentifuge tube containing 0.3 ml of electroelution buffer and the dropper was filled with the same buffer. The tube was placed in an electrophoresis

apparatus and a wick (consisting of 2 layers of Whatman 3MM paper measuring 5 cm x 0.3 cm) was used to make contact between the tube buffer and the buffer in the anode chamber. A platinum cathode was placed into the open end of the dropper and a constant current of 15 mA was applied until the marker dye electrophoresed out of the end of the dropper. The DNA was recovered by ethanol precipitation and the pellet was resuspended in 300 ul 50 mM $NaC_2H_3O_2$. After a second ethanol precipitation, the pellet was washed with ethanol and dried for storage at -20°C.

7.4.5 DNA SEQUENCING

Three sequencing techniques were used to obtain the NS gene sequences reported in these studies; the chemical method of Maxam and Gilbert, (1980), the Forward-Backward method of Seif et al., (1980), and the chain termination method of Sanger et al., (1977) in which restriction fragments of cloned DNA served as primers for DNA synthesis using reverse transcriptase programed by viral RNA (Both et al., 1980).

7.4.5.1 Chemical sequencing

The Maxam and Gilbert protocol was used to sequence 5' end labeled restriction fragments (see previous section for preparation). This technique involves the chemical modification of a specific base (A,G,C or T) followed by removal of that base with subsequent cleavage of the DNA strand at the point of modification. DNA subjected to 4 partial cleavage reactions results in fragments with known 3' end residues and these fragments are sized on a polyacrylamide gel. An autoradiogram is made and the sequence of the starting DNA is determined by noting which base specific treatment cleaved at each successive residue along the DNA.

LIMITED CLEAVAGE AT GUANINES

Five ul of end labeled DNA in H_2O (containing a minimum of 3,000 cpm) were combined with 200 ul DMS buffer [50 mM Na(CH3)2AsO2 pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA] in a 1.5 ml microcentrifuge tube, mixed and chilled on ice. One ul of dimethylsulfate (Aldrich Chemical Co., Milwaukee, WI) was added, the tube was capped, mixed and incubated at 20°C for 10'. The reaction was stopped by addition of 50 ul cold DMS stop mix (1.5 M $NaC_2H_3O_2$ pH 7.0, 1 M 2-mercaptoethanol, 40 ug/ml sonicated and deproteinized herring sperm DNA) and 750 ul cold ethanol. After mixing, the tube was chilled in dry

ice-ethanol for 15' and centrifuged (12,000 x g) for 15'. The supernatant was discarded (into 5 M NaOH to neutralize the DMS), the pellet was resuspended in 250 ul cold 0.3 M $NaC_2H_3O_2$ and the DNA precipitated as before. The final pellet was washed with 1 ml ethanol and dried by lyophilization for 5'. (The steps that follow are identical for the four base-specific reactions and will not be repeated in the description of the other 3 reactions).

The dried pellet was resuspended in 100 ul 1 M piperidine (Fisher Scientific Co. Pittsburgh, PA), the tube opening was covered with 2 layers of teflon tape, capped, placed in an aluminum rack and covered with an aluminum plate that was bolted into place to prevent the cap from opening. The tube was heated to 90°C for 30', removed from the rack, unsealed and the contents lyophilized . The residue was resuspended in 10 ul H_2O, lyophilized , resuspended in 10 ul H_2O and dried again. The final pellet was taken up in 10 ul running mix (80% formamide, TBE and 0.05% each of xylene cyanol-FF and bromophenol blue). The solution was heated to 100°C for 2' and quick chilled in ice.

LIMITED CLEAVAGE AT GUANINES AND ADENINES

Ten ul of end labeled DNA (containing at least 6,000 cpm) and 10 ul H_2O were combined in a 1.5 ml microcentrifuge tube and chilled on ice. Two ul of 1 M piperidinium formate

pH 2.0 (10 ml 4% formic acid + 15 ul piperidine) were added to the tube which was heated to 30°C for 2 hours. The reaction was stopped by rapidly freezing the contents in a dry ice-ethanol bath and lyophilization. DNA was dissolved in 20 ul H_2O, dried and subjected to cleavage as described for the guanine cleavage reaction.

LIMITED CLEAVAGE AT CYTOSINES AND THYMIDINES

Ten ul of end labeled DNA (containing at least 6,000 cpm) were combined with 10 ul H_2O and chilled in an ice bath. Following addition of 30 ul 95% hydrazine (Eastman Organic Chemicals, supplied by Fisher Scientific Co.) the tube was capped, mixed and heated to 30°C for 10'. The reaction was stopped by adding 200 ul of hydrazine stop mix (0.3 M $NaC_2H_3O_2$, 0.1 mM EDTA, 10 ug/ml sonicated and deproteinized herring sperm DNA) and 750 ul ethanol. After mixing, the tube was placed in dry ice-ethanol for 15' and centrifuged (12,000 x g) for 15'. The supernatant was discarded (into 3 M FeCl3 to neutralize the hydrazine), the DNA was resuspended with 250 ul cold 0.3 M sodium acetate and it was ethanol precipitated as before. Subsequent treatments followed those described for the guanine reaction.

LIMITED CLEAVAGE AT CYTOSINES

Five ul of end labeled DNA (containing at least 3,000 cpm) were combined with 15 ul of 5.0 M NaCl in a 1.5 ml microcentrifuge tube and chilled in an ice bath. Subsequent treatment was exactly as that described for cleavage at cytosines and thymidines.

ELECTROPHORESIS

The fragments resulting from the base specific cleavages were fractionated in an 8% polyacrylamide gel. Gel solutions were prepared to contain 7.6% acrylamide, 0.4% bis, 7 M urea, 0.03% $(NH_4)_2S_2O_8$ and 0.1% TEMED in TBE. The gel was cast between 2 glass plates (one plate measured 910 mm x 200 mm x 4.5 mm, the second plate was 25.4 mm shorter) separated by vaseline coated teflon spacers which were 0.38 mm thick. Sample wells were cast with a teflon comb containing 18 teeth (10 mm x 5 mm) interspaced by 2 mm. The gel was allowed to polymerize for at least 1 hour. The comb and bottom spacer were removed, the sample slots were flushed and filled with TBE as was the space at the bottom of the gel plates. The gel was mounted onto an upright electrophoresis apparatus. Two glass spacers (25.4 mm x 12.5 mm x 4.5 mm) were coated with vaseline and placed onto the top edge of shorter plate to creat a notch that coincided with that of the buffer chamber. The buffer chambers

were filled with TBE and a constant voltage of 1,000 V was applied to the gel for 0.5 hours. Three ul of each cleavage reaction were loaded into adjacent wells of the gel and electrophoresed until the xylene cyanol-FF dye reached the bottom of the gel. At this point the gel was either reloaded and run as before to allow for the reading of sequences past those of the first loading or the electrophoresis was stopped and the gel cut into 30 cm sections which were autoradiographed using x-ray film and intensifying screens. To obtain the sequence of the 20 residues proximal to the 5' end label, the samples were loaded onto a shorter gel (400mm x 200mm) containing 19% acrylamide and 1% bis (all other components were the same as for the 8% acrylamide gel) and electrophoresed until the bromophenol blue migrated 12 cm into the gel.

7.4.5.2 The Forward-Backward technique

This method is a modification of the nick-translation method of Maat and Smith (1978) and the "plus-minus" method of Sanger and Coulson (1975). In the forward reactions, 5' end labeled DNA is treated with DNAase to create random single-stranded nicks. This is done in the presence of E.coli

DNA polymerase I (pol I) which is provided with deoxynucleoside triphosphates (dA, dC, dG, dT) and one dideoxynucleoside triphosphate chain terminator (i.e. ddA, ddC, ddG, or ddT) in four separate reactions. The pol I binds to sites where the DNA was cleaved. Using its 5'-3' exonuclease activity, pol I creates single-stranded gaps which are then repaired using the provided nucleotides. Random termination occurs at guanine residues, for example, when ddG is incorporated. The fragments resulting from the four reactions are ordered by size in a polyacrylamide gel identical to that used in the chemical sequencing technique. After autoradiography the sequence of the starting DNA is determined by noting which terminator was incorporated at each successive position along the DNA.

In the backward reaction only one dideoxynucleotide is provided so that the pol I degrades the DNA at the DNAase cleavage site until the provided residue can be added. The four backward reactions are loaded adjacent to the forward reactions on the gel and both sets of reactions serve to confirm the nucleotide sequence.

In four separate 1.5 ml microcentrifuge tubes, 0.4 pmoles of 5' end labeled DNA (containing at least 9,000 cpm) were resuspended with a 5 ul cocktail containing 6.6 mM Tris-HCl pH 7.5, 6.6 mM $MgCl_2$, 2 mM DTT, 2 mM NaCl, 1 mM ddX, 1 mM each of dA, dC, dG, dT and 2.5 units of pol I

(Boerhinger-Mannheim). The backward reaction conditions were similar to those above except that dA, dC, dG and dT were omitted and the concentration of the one dideoxynucleotide was 5 mM. After heating to 37°C for 30', 5 ul of stop mix (99% formamide, 10 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue) were added to each tube and samples were heated to 100°C for 3'. Samples were electrophoresed and analyzed as described in the electrophoresis section of the chemical sequencing technique.

7.4.5.3 Sequencing RNA by reverse transcription and chain termination

In this technique restriction fragments obtained from cloned DNA were used to prime DNA synthesis from viral RNA in the presence of chain terminators and reverse transcriptase. Viral RNA (4.5 pmoles) in 5 ul H_2O was combined with approximately 0.5 pmoles of restriction fragment in 5 ul of 20 mM Tris-HCl pH 8.0, 13 mM KCl and 0.2 mM EDTA. The mixture was sealed in a capillary tube and heated first to 100°C for 30'' and then kept at 68°C for 30'. The contents of the capillary tube were combined with 5 ul of H_2O and 2.5 ul aliquots were combined with 7.5 ul of the ddA, ddC, ddG

and ddT sequencing cocktails in separate 1.5 ml microcentrifuge tubes. All sequencing cocktails contained 66.6 mM Tris-HCl pH 8.3, 80 mM NaCl, 26.6 mM DTT, 16 mM $MgCl_2$, 80 uM dG and 80 uM dT. In addition, the ddA cocktail contained 133 uM ddA, 80 um dA, 25 uCi α-32-P-dC (2,000-3,000 Ci/mmole, Amersham Corp., Arlington heights, IL); the ddC cocktail contained 6.6 uM ddC, 80 uM dC, 25 uCi α-^{32}P-dA (2,000-3,000 Ci/mmole, Amersham Corp.), the ddG cocktail contained 18.6 uM ddG, 80 um dC, 25 uCi α-^{32}P-dA; and the ddT cocktail contained 66.6 uM ddT, 80 uM dC, 25 uCi α-^{32}P-dA. A second ddA cocktail in which the ddA concentration was lowered to 33.3 uM was also used. This was necessary because in some instances the DNA chain elongation was prematurely terminated by the higher concentration of ddA in the first cocktail.

The 10 ul reaction mixtures (7.5 ul cocktail + 2.5 ul RNA and primer) were combined with 0.5 ul of reverse transcriptase (7.5 units) and heated to 37^{o}C for 20'. The α-^{32}P-dC containing reactions were "chased" for 10' at 25^{o}C with 1 ul of unlabeled 500 uM dC. The α-^{32}P-dA containing reactions were similarly treated with 1 ul of 500 uM dA. Each reaction cocktail was combined with 8 ul of running mixture (formamide containing 0.1% xylene cyanol-FF, 0.1% bromophenol blue and 5 mM EDTA) and heated to 100^{o}C for 2' followed by quick chilling on ice. Three to five ul of each reaction were loaded onto 8% polyacrylamide gels which were

made, electrophoresed and autoradiographed as described in the electrophoresis section of chemical sequencing.

7.4.6 GENOTYPING INFLUENZA VIRUS RECOMBINANTS

Labeled RNAs of parental and recombinant viruses were separated for analysis in polyacrylamide gels (Ritchey et al., 1976b) The genetic derivation of each recombinant was determined by comparing the migration patterns of its RNAs to those of its parental strains.

7.4.6.1 Isolation of recombinant viruses

The recombinant strains genotyped in this study were isolated by Dr. Kilbourne and their construction will be briefly described. Recombinants of PR8 virus and other influenza virus strains were prepared in an effort to transfer the high yield phenotype of the PR8 virus to field isolates which were needed for vaccine production. Recombinants of PR8 virus and other influenza A viruses were

obtained by dual infection of 11-day-old embryonated hens' eggs and subsequent passage of the recombinants in the presence of antiserum to the HA and NA antigens of the PR8 virus (Kilbourne et al., 1971). The serotypes of the recombinants (i.e. HA and NA derivation) were determined by established hemagglutination inhibition and neuraminidase inhibition assays (Wong and Kilbourne, 1961; Aymard-Henry et al., 1973) while HA titers were determined by the tube-dilution method (Wong and Kilbourne, 1961).

7.4.6.2 Labeling RNA in vivo

Virus specific RNA was labeled *in vivo* with ^{32}P in virus infected MDCK cells (Gaush and Smith, 1968) as described by Palese and Schulman (1976b): Two monolayers of MDCK cells in 25 cm2 dishes were used per virus. Monolayers were washed free of growth medium with phosphate-free reinforced MEM (REM, Grand Island Biological Corp.). Each dish was inoculated with 0.2 ml of undiluted seed virus which was allowed to adsorb for 30' at 37°C. Three ml of phosphate-free growth medium containing 2.5 mCi ^{32}P (as orthophosphoric acid in H_2O, New England Nuclear) and 2 ug/ml trypsin were added to each dish.

After 18 hours at 37°C, the cell supernatants were harvested and clarified by centrifugation (1,000 x g for 10'). Each virus containing fluid was layered onto a 30%-60% linear sucrose gradient together with 3 ml of unlabeled virus fluid (the unlabeled virus facilitates visualization of the virus in the gradient and also provides carrier RNA needed for precipitation of extracted RNA) and viral RNA was extracted as described in the section on viruses. Viral RNA was resuspended in Loening's buffer (36 mM Tris-Base, 30 mM NaH_2PO_4, 1 mM EDTA; pH 7.8) containing 20% sucrose, 0.05% xylene cyanol-FF, and 0.05% bromophenol blue. Ten ul of viral RNA mixture contained approximately 20,000-30,000 cpm.

7.4.6.3 Electrophoresis

A gel solution (50ml) was prepared to contain 2.4%, 2.6% or 3% acrylamide (from a stock solution containing 30% acrylamide, 1.725% bis) 6 M urea, 0.15% $(NH_4)_2S_2O_8$ and 0.1% TEMED in Loening's buffer. Gels were cast between glass plates measuring 265 mm x 165 mm x 4.5 mm. (One plate was sandblasted to a rough finish and the other was notched). The plates were separated by 1.2 mm thick spacers and sample

wells were formed by a comb containing 15 teeth (measuring 15 mm x 6 mm) interspaced by 1.5 mm. After polymerization, the comb and bottom spacer were removed and the gel was mounted on a vertical electrophoresis apparatus. The chambers were filled with Loening's buffer and 10 ul of each sample were loaded into adjacent wells. Electrophoresis proceded at a constant voltage of 120 V for 18-20 hours in a water jacketed incubator set at 26^{o}C. After electrophoresis, the gel was removed from the roughened glass plate with a piece of Whatman 3MM paper, covered with saran wrap, and heat dried under vacuum for 1 hour. The dried gel was autoradiographed using Kodak X-OMAT XR-1 film and an intensifying screen.

7.4.7 COMPARATIVE T1 MAPPING ANALYSIS OF INFLUENZA VIRUS RNAS

Ribonuclease T1 digests of total RNA and of isolated RNA segments were subjected to fingerprint analysis (Pedersen and Haseltine, 1980). The genes coding the NS proteins, the M protein(s) and the HA were individually analyzed. However, two gene mixtures were also analyzed because adequate separation between particular genes was not possible.

One mixture consisted of the NP and NA genes and the other of the three P genes.

7.4.7.1 Isolation of RNA segments

Viral RNA used for analysis was isolated as previously described. To isolate individual RNA segments, whole RNA was fractionated on polyacrylamide gels as described in the electrophoresis section of *in vivo* labeled RNAs. Gels were stained by overlaying them with several ml of 5 ug/ml ethidium bromide in Loenings's buffer and the RNA segments were seen by uv light illumination. Gel pieces containing RNA segments were excised and the RNAs were electroeluted using the same method as described for the isolation of 5' end labeled DNA fragments.

7.4.7.2 Preparation of RNAase T1

Ribonuclease T1 (Calbiochem, San Diego, CA) was dissolved to 10,000 units/ml with 20 mM EDTA, 10 mM Tris-HCl pH

7.5. Seventy-five ul of 1 N HCl per ml of enzyme was added and the mixture was kept at room temperature for 10' to inactivate contaminating phosphatase activity. After chilling on ice, 30 ul of 1 M Tris-HCl pH 7.5 was added, and the pH of the mixture was adjusted to pH 7.5 with 1 N NaOH. The above preparation was assayed in 75 ul reactions containing 66.6 mM Tris-HCl pH 7.5, 2.66 mM EDTA, 250 ug yeast RNA and several dilutions of the enzyme. After heating to 37^{o}C for 15', each reaction was chilled on ice and the undigested RNA was precipitated with 25 ul 25% trichloroacetic acid containing 0.75% uranyl acetate. Following centrifugation (12,000 x g) for 10', 25 ul of the supernatants were each combined with 750 ul H_2O and the solutions were read in a spectrophotometer (at 260 nm) to determine the units of activity in each enzyme dilution. An absorbance of 0.1 is equated to 12 x 10^{-3} enzyme units (Arima et al., 1968).

7.4.7.3 RNAase T1 digestion and 5' end labeling of RNA

Viral RNA or RNA segments (1 ug) were resuspended in 2 ul of digestion buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA) in 1.5 ml microcentrifuges tube and heated to 100^{o}C for 2' followed by quick chilling on ice. RNAase T1 (2 units in 2 ul

of digestion buffer) was added to each tube and the mixtures were heated to 37°C for 30'. The reaction mixtures were combined with 43 ul kinase buffer [10 mM Tris-HCl pH 8.0, 10 mM $Mg(C_2H_3O_2)_2$, 1 mM DTT], containing 100 uCi γ-^{32}P-ATP (1,000- 3,000 Ci/mmole, New England Nuclear) and 3 ul polynucleotide kinase (4 units, New England Biolabs). After heating to 37°C for 30', the reactions were terminated by adding 100 ul phenol, mixing, ad adding 50 ul stop mixture (2 mg/ml yeast RNA in 0.6 M $NH_4C_2H_3O_2$). Following centrifugation (12,000 x g for 10'), the supernatants were collected and the RNA precipitated with 300 ul ethanol. Dried pellets were resuspended in 25 ul running mixture (7M urea, 10% sucrose, 0.05 M EDTA, 0.15% xylene cyanol-FF, 0.15% bromophenol blue, 5 mg/ml of T1 digested and undigested yeast RNA). The RNA in the running mixture was prepared by digesting 40 mg of yeast RNA in 20 ml RNAase T1 digestion buffer with 160 units of RNAase T1 per mg of RNA for 30' at 37°C. After adding 10 mg of whole yeast RNA in 2 ml of H_2O and 2.4 ml of 0.5 M NaCl, the mixture was extracted with an equal volume of phenol:chloroform (1:1). The RNA was ethanol precipitated and resuspended in 10 ml of running mixture (described above).

7.4.7.4 Two dimensional electrophoresis

Gel solutions (200 ml) were prepared to contain 10% acrylamide, 0.325% bis, 7 M urea, 0.025 M $C_6H_8O_7$, 0.01 mg, $FeSO_4$-$7H_2O$, 0.4 mg ascorbic acid and 0.012% H_2O_2 and poured between glass plates (measuring 40 cm x 20 cm x 0.5 cm) separated by vaseline coated teflon side spacers (which were 2.5 mm thick). The bottom space between the plates had been previously sealed with plasticine. A comb containing 6 teeth was used to cast sample wells measuring 5 mm x 5 mm x 2.5 mm. Following polymerization the comb and plasticine were removed and the gel was placed upright into an anode chamber containing 4 l of 1D running buffer (0.025 M $C_6H_8O_7$, 7 M urea). The top of the gel was overlayed with the same buffer and a wick, consisting of 2 layers of buffer saturated Whatman 3MM paper covered with Saran wrap, was inserted into the top of the gel such that the wick was flush with the side spacers. The other end of the wick was placed in the cathode chamber which contained 6 l of 1D buffer and was elevated such that its buffer level was 10 cm above that of the anode chamber. The gel was prerun for 1 hour at a constant voltage of 900 V and 8 ul of each sample were loaded and electrophoresed until the bromophenol blue migrated 18 cm. Following electrophoresis, one plate was removed and the gel was covered with Saran wrap for autoradiography using Kodak X-OMAT XR-2 film.

Gel strips (1 cm wide and covering from 10 cm to 30 cm from the origin) containing the oligonucleotides were excised with a razor blade. Each gel strip was placed on a glass plate (measuring 28.5 cm x 23.5 cm x 0.45 cm) 4 cm above the bottom edge and 2 vaseline coated teflon spacers that were 0.2 cm thick were placed on either side of each gel strip. A second glass plate was placed over the gel strip and the bottom space between the plates was sealed with plasticine. A gel solution consisting of 21.9% acrylamide, 0.71% bis, 50 mM Tris-H_3BO_3 pH 8.3, 1 mM EDTA, 0.06% TEMED and 0.06% $(NH_4)_2S_2O_8$ was poured between the plates containing each gel strip to a level 2 cm from the top of the plates. After polymerization, the plasticine was removed and the gels were placed in a cathode chamber containing enough 2D buffer (50 mM Tris-H_3BO_3 pH 8.3, 1 mM EDTA) to reach the level of the polyacrylamide. Each gel was overlayed with the 2D buffer and a buffer saturated wick (as described above) was inserted into the top of each gel. The other end of the wick was placed into the anode chamber and a constant current of 14 mA per gel was applied until the bromophenol blue migrated 18 cm from the first dimension gel strip. Following electrophoresis, one glass plate was removed from each gel, the gels were covered with Saran wrap and autoradiographed.

Using the above electrophoretic conditions, the separation of oligonucleotides in the first dimension is mostly

dependent on their base composition. The C or A rich oligonucleotides migrate slower than the G or U rich oligonucleotides . In second dimension electrophoresis, the oligonucleotides are separated mostly by size differences (DeWachter and Fiers, 1972).

7.4.7.5 Oligonucleotide size determination

To determine the size of the oligonucleotides used for analysis, representative oligonucleotides were eluted from gel pieces (Maxam and Gilbert, 1980) which were excised from an RNA fingerprint gel of the A/Sing/1/57 virus. Gel pieces were placed in a 1.5 ml microcentrifuge tube and crushed with a glass rod. One ml of elution buffer [0.5 M $NH_4C_2H_3O_2$, 0.01 M $Mg(C_2H_3O_2)_2$, 0.1% SDS, 0.1 mM EDTA] was added to each tube which was then capped and heated to 37°C for 16 hours. The suspension was passed through a sterilized 1 ml plastic pipette tip plugged with siliconized glass wool to remove gel pieces from the eluate which was then combined with 25 ul stop mixture (see section 7.4.7.3). The RNA was precipitated with 2.5 volumes of ethanol and pelleted by centrifugation (12,000 x g for 20'). The dried pellet was resuspended in 1 ml H_2O, combined with 0.1 ml 10%

perchloric acid, and chilled on ice for 10'. This treatment selectively precipitates the RNA and leaves the soluble acrylamide in solution (Jeppesen et al., 1972). After centrifugation (12,000 x g for 15') the pellet was combined with 0.3 ml of 100 mM Tris-HCl pH 8.0, 20 mM KCl and the dissolved RNA was precipitated with 2 volumes of ethanol. The pellet was washed with ethanol and dried.

7.4.7.6 Partial alkali digestion of oligonucleotides

Each isolated oligonucleotide was resuspended in 5 ul of 50 mM $NaHCO_3$ pH 9.5, 1 mM EDTA and heated to 93°C for 30'. Digestions were terminated by addition of 5 ul stop mixture (10 M urea, 0.05% xylene cyanol-FF, 0.05% bromophenol blue). A 50 ml gel solution was prepared to contain 20% acrylamide 0.15% bis), 7 M urea, 50 mM Tris-H_3BO_3 pH 8.3, 1 mM EDTA, 0.06% $(NH_4)_2S_2O_8$ and 0.06% TEMED. This was poured between 2 plates (measuring 400 mm x 330 mm x 4.5 mm; one of which was notched) separated by 0.5 mm thick vaseline coated teflon spacers. Sample wells were cast with a teflon comb containing 18 teeth (measuring 10 mm x 14 mm) interspaced by 3 mm. Following polymerization, the comb and bottom spacer were removed and the gel was mounted on an electrophoresis

apparatus. The buffer chambers were filled with running buffer (50 mM Tris-borate pH 8.3, 1 mM EDTA) and the gel was prerun several hours at a constant voltage of 2,000 V. Five ul of each sample were loaded into adjacent slots on the gel and electrophoresed until the xylene cyanol-FF migrated 9.5 cm. Electrophoresis was stopped, one glass plate was removed, the gel was covered with Saran wrap and autoradiographed using Kodak X-OMAT XR-5 film and an intensifying screen. The number of residues in each oligonucleotide was determined by counting the number of bands the partially digested products yielded in the autoradiogram. This procedure allowed for the construction of a "reference map" on which the lengths of all oligonucleotides were identified. Superimposing the reference map on the oligonucletide fingerprint of any viral RNA permitted the size estimation of its oligonucleotides.

8.0 RESULTS

Some of the data presented in this section have either been published or are in press:

(1) Baez, M., Palese, P. and Kilbourne, E.D. 1980. Gene composition of high-yielding influenza vaccine strains obtained by recombination. J. Inf. Dis. 141:362-365

(2) Baez, M., Taussig, R., Zazra, J.J., Young, J.F. and Palese, P. 1980. Complete nucleotide sequence of the influenza A/PR/8/34 virus NS gene and comparison with the NS genes of the A/Udorn/72 and A/FPV/Rostock/34 strains. Nucl. Acids Res. 8:5845-5858.

(3) Palese, P., Brand, C., Young, J.F., Baez, M., Six, H.R., and Kasel, J.A. 1981. Molecular epidemiology of influenza viruses. In: "Perspectives in virology" vol. 11, pp. 115-127 (M. Pollard, ed.) Raven Press, New York.

(4) Palese, P., Elliott, R.M., Baez, M., Zazra, J.J., and Young, J.F. 1981. Genome diversity among influenza A, B and C viruses and genetic structure of RNA 7 and 8 of influenza A viruses. In: "Genetic variation among influenza A viruses" ICN-UCLA symposia on molecular and cellular biology, vol. XXII (D. Nayak and C.F. Fox eds.) Academic Press, New York. (in press)

(5) Baez, M., Zazra, J.J., Elliot, R.M., Young,J.F. and Palese, P. 1980. Nucleotide sequence of the influenza A/duck/Alberta/60/76 virus NS RNA: Conservation of the NS1/NS2 overlapping gene structure in a divergent influenza virus RNA segment. Virology. (in press)

8.1 GENOTYPE DETERMINATION OF INFLUENZA VIRUS RECOMBINANTS CONSTRUCTED FOR USE IN VACCINE PRODUCTION

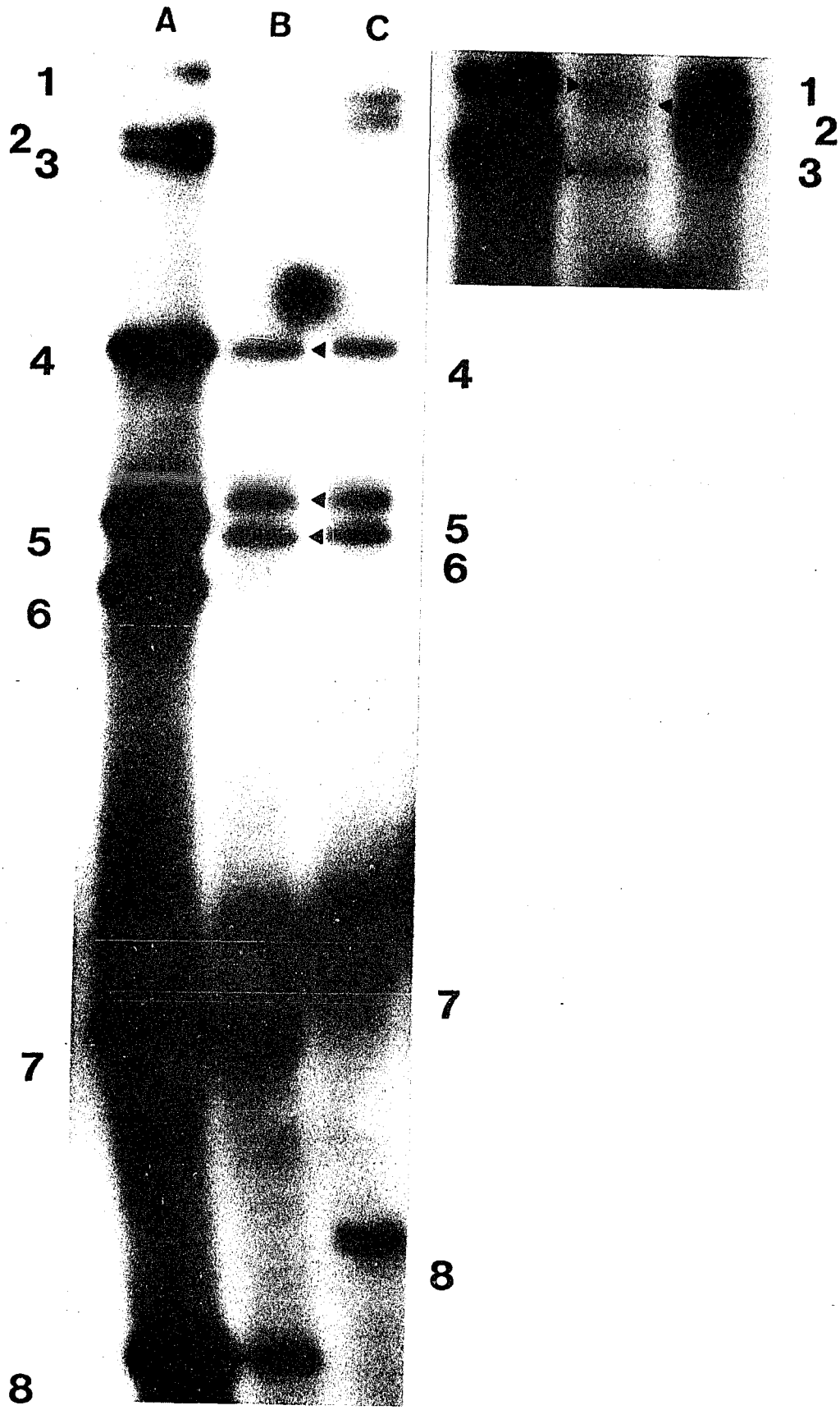
The undertaking of the first project occurred at a time when a breakthrough in electrophoretic techniques permitted the physical mapping of the influenza virus genome (see section 6.3.3 of the introduction). Influenza virus recombinants expressing an enhanced growth phenotype were constructed from PR8 virus and several field isolates using established procedures (Kilbourne et al., 1971). Since these recombinants were constructed for use or potential use in vaccine production, it was of interest to examine their genetic composition with the recently developed electrophoretic techniques and determine if any particular genes could be correlated with the high yield phenomenon.

The genetic derivation of each recombinant was determined by comparison of the polyacrylamide gel migration patterns of the ^{32}P-labeled RNAs isolated from the recombinants and parental strains. If an RNA segment of a recombinant strain can be shown to comigrate with the corresponding RNA of one of its parental strains, it is assumed to be derived from that parental strain. Several labeling experiments and different polyacrylamide gel concentrations (2.4%, 2.6% and 2.8%) were used to identify the gene composition of each recombinant. Figure 3 shows an autoradiogram of a 2.6%

FIGURE LEGEND

Figure 3. Polyacrylamide gel electrophoresis of influenza PR8, X-47 and Vic/75 virus RNAs.

Viral RNAs were labeled *in vivo* with ^{32}P and separated on a 2.4% polyacrylamide gel containing 6 M urea (see "Materials and Methods"). Electrophoresis was from top to bottom and the RNAs are numbered according to increasing mobility. Differences in the RNA migration patterns of the parental strains PR8 (lane A) and Vic/75 (lane C) were used to identify the gene derivation of recombinant X-47 (see arrowheads in lane B). RNA 4 of X-47, which codes for the HA, is known to derive from Vic/75 (see "Materials and Methods" and text). A longer exposure of RNAs 1-3 is also shown.


A
B
C
1
2
3
4
5
6
7
8
1
2
3
4
5
6
7
8

polyacrylamide gel containing the ^{32}P-labeled RNAs of the PR8, X-47 and Vic/75 viruses in lanes A, B and C respectively. The arrowheads on either side of lane B indicate which of the recombinant's RNAs comigrate with the parental strain RNAs. It is clear that RNAs 1, 3, 7 and 8 of the recombinant strain derive from the PR8 parental virus. These four RNAs respectively code for the P3, P2, M and NS genes of the X-47 strain. RNAs 2, 5 and 6 of the recombinant strain derive from the Vic/75 parental virus and respectively encode the P1, NA and NP genes. In this particular example, differences in the migrational patterns of the HA genes (band 4) were not distinguishable but serological analysis of the surface antigens of the recombinant showed that it derives the HA gene from the Vic/75 parental virus (see preparation of recombinants in Materials and Methods).

Table 3 presents the genetic composition of all the recombinant strains examined. Except for X-45, X-49 and X-57, all the recombinants analyzed have been used in commercial vaccine production. The serotype of the HA and NA of each recombinant is the same as its low-yielding unadapted parent, but the recombinants grow to 2-32 fold higher HA titers (resulting in HA titers of 512-4096) than the corresponding low yielding parents. Recombinant X-47 has also been analyzed by oligonucleotide mapping and its genotype

Table 3. Genetic composition of high yielding influenza virus recombinants derived from A/PR/8/34 virus and different field strains.

The genetic composition of each recombinant was determined by comparing the mobility of its ^{32}P-labeled RNAs with those of the parental strains in polyacrylamide gels (see "Materials and Methods" and text).

GENETIC COMPOSITION OF HIGH-YIELDING INFLUENZA VIRUS RECOMBINANTS DERIVED FROM A/PR/8/34 VIRUS AND DIFFERENT FIELD ISOLATES

		GENE COMPOSITION OF RECOMBINANT							
RECOMBINANT	FIELD ISOLATE	P1	P2	P3	HA	NA	NP	M	NS
X-31	A/AICHI/2/68	P	P	P	+	+	P	P	P
X-37	A/ENGLAND/42/72	+	P	+	+	+	P	P	P
X-41	A/PORT CHALMERS/1/73	+	P	+	+	+	+	P	+
X-45	A/SCOTLAND/840/74	+	P	+	+	+	+	P	P
X-47	A/VICTORIA/3/75	+	P	P	+	+	+	P	P
X-49	A/ENGLAND/864/75	P	P	ND	+	+	P	P	P
X-53	A/NEW JERSEY/11/76	P	P	P	+	+	P	P	P
X-53A	A/NEW JERSEY/11/76	P	P	P	+	+	P	P	P
X-57	A/VICTORIA/112/76	+	P	+	+	+	P	P	P
X-61	A/TEXAS/1/77	+	P	P	+	+	P	P	P
X-71	A/BRAZIL/11/78	+	+	ND	+	+	+	P	P

P = GENE DERIVED FROM A/PR/8/34 VIRUS; + = GENE DERIVED FROM FIELD ISOLATE; ND = NOT DONE

has been confirmed (J.Young, personal communication). In addition, a study of NPs of different influenza viruses has independently confirmed the presence of the PR8 NP gene in X-31 and X-49 and the NP gene from the low-yielding parent in X-47 (Schild et al., 1979).

It is clear that a recombinant strain was selected during each isolation procedure since all isolates have genes derived from both parents. All recombinant strains contained the M gene of the PR8 virus and many recombinants had derived additional genes from this high-yielding laboratory strain. For example, 9 of 11 recombinants contain both the P2 and the NS genes from the PR8 virus. However, as will be discussed later, it was not possible to definitively correlate a characteristic pattern of gene distribution with high yield.

8.2 GENETIC VARIATION AMONG H2N2 INFLUENZA A VIRUS GENES

Having completed the first project, I became interested in examining influenza A viruses for genetic variation. At that time, a quantitation of the extent of variation among the genes of influenza viruses belonging to one subtype had

not been accomplished. Such an analysis was thought to be helpful in better understanding genetic variation in influenza A viruses. In particular, I was interested in determining whether genes coding for the surface glycoproteins, which were under selective antibody pressure, showed greater variation than did the genes coding for nonsurface proteins. Viruses for this study were chosen from isolates of the H2N2 subtype period (1957-1968). This period was the most recent time span in which viruses of only one subtype circulated. Strains were selected to represent the beginning, middle and end of this period so that the frequency of mutations in the genes of a group of viruses that were passaged in nature for a defined amount of time could be estimated. The technique of RNAase T1 oligonucleotide mapping was used to accomplish these aims.

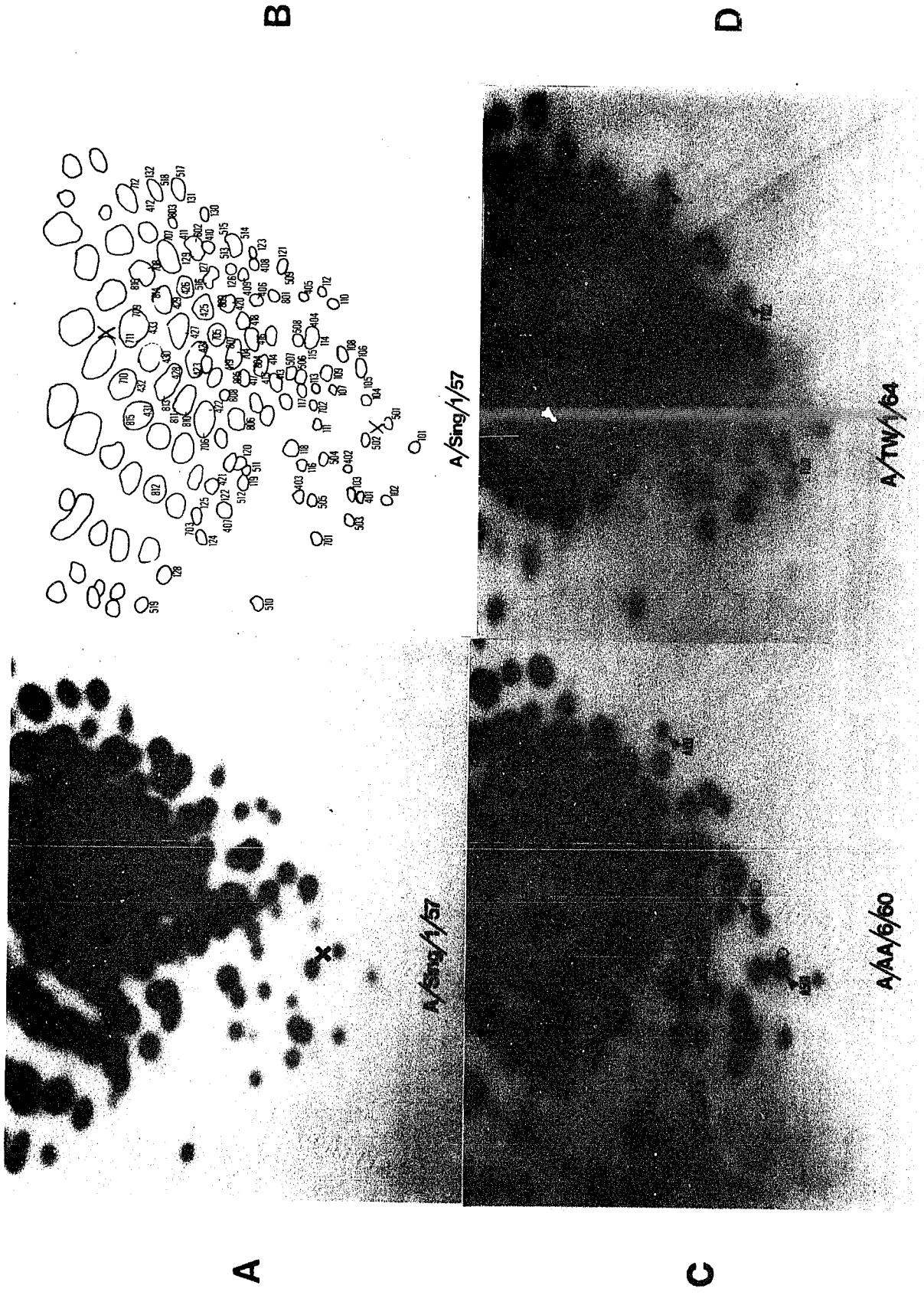
8.2.1 OLIGONUCLEOTIDE MAP ANALYSIS OF INFLUENZA VIRUS GENOMES

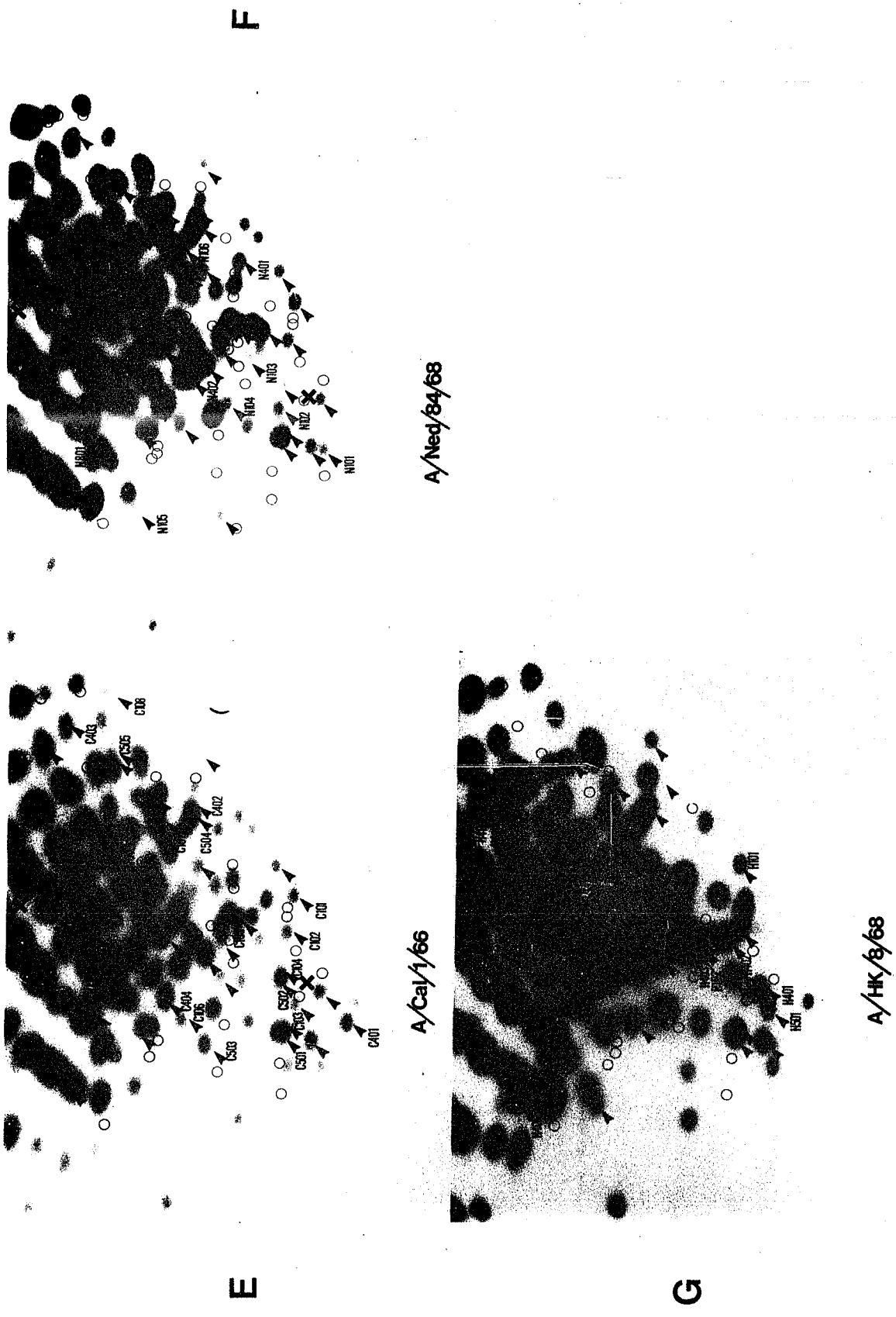
The RNAs of five H2N2 influenza viruses, A/Sing/1/57, A/AA/6/60, A/TW/1/64, A/Cal/1/66, A/Ned/84/68) and one H3N2 strain (A/HK/8/68) were extracted and examined by RNAase T1 oligonucleotide map analysis. The RNA fingerprints of these

FIGURE LEGEND

Figure 4. Oligonucleotide fingerprints of influenza virus genomes.

The oligonucleotide fingerprint of the RNA from each strain was compared to that of the earliest H2N2 isolate A/Sing/1/57 (see "Materials and Methods"). Differences in the fingerprints of the later strains are designated by arrowheads and open circles, which represent "new" spots and "missing" spots respectively. Oligonucleotides obtained from the RNA of A/Sing/1/57 virus are numbered according to gene derivation (see panel B). The gene assignment of a spot was done by fingerprint analysis of isolated RNAs or RNA mixtures. Spot numbers with a 100, 400, 500, 700 and 800 series were assigned to oligonucleotides in the P gene mixture, HA gene, NP/NA gene mixture, M gene and NS gene respectively. All "new" oligonucleotides not present in the fingerprint of the A/Sing/1/57 virus RNA are noted by an arrowhead. The letter A, T, C, N or H next to an arrowhead indicates that a particular oligonucleotide was first observed in the fingerprint of the A/AA/6/60, A/TW/1/64, A/Cal/1/66, A/Ned/84/68 or A/HK/8/68 virus respectively. To maintain clarity in these RNA fingerprints, letters and numbers next to arrowheads are shown only in the fingerprints of the strain in which the oligonucleotide was first observed. X's indicate the positions of the dye markers bromophenol blue and xylene cyanol FF.


A
B
C
D
A/Sing/1/57
A/Sing/1/57
A/AA/6/60
A/TW/1/64


F
A/Ned/84/68
E
A/Cal/1/66
G
A/HK/8/68

strains are presented in figure 4 and a diagram of the RNA fingerprint of the earliest strain, A/Sing/1/57, is shown in figure 4B. The oligonucleotides used in the analysis of this RNA are numbered according to gene derivation (see below and legend to figure 4). Differences between the RNA fingerprints of the earliest isolate (A/Sing/1/57) and that of each of the other strains are identified by arrowheads which indicate "new" oligonucleotides, and by open circles which indicate "missing" oligonucleotides. Mixtures of digested RNAs from different viruses were coelectrophoresed to confirm differences in the RNA fingerprints. The oligonucleotide patterns of the H2N2 viruses demonstated that their RNAs were related because they had several common oligonucleotides which could be identified by their identical map positions. Although the oligonucleotide maps of these strains are similar, an increasing number of differences was noted when late H2N2 isolates were compared to the A/Sing/1/57 strain. For example, the RNA fingerprint of the 1960 isolate (A/AA/6/60) revealed 27 changes while the latest isolate, A/Ned/84/68, showed 94 changes.

The A/HK/8/68 virus is of the H3N2 subtype but its oligonucleotide pattern (figure 4G) was similar to those of the H2N2 viruses in accordance with earlier RNA-RNA hybridization analyses (Scholtissek et al., 1978). This latter study suggested that the H3N2 viruses originated from the recombination of an H2N2 virus with an unknown strain.

8.2.2 OLIGONUCLEOTIDE MAP ANALYSIS OF ISOLATED RNA SEGMENTS

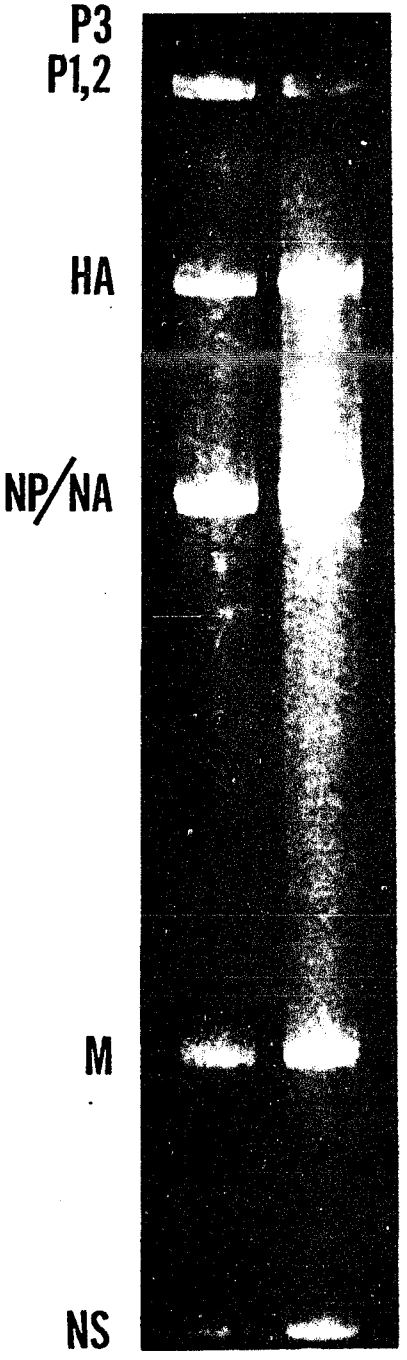
Since oligonucleotide map differences were observed among all the genomes of the examined viruses, individual genes were isolated to determine the number of differences among the corresponding genes of each virus. Figure 5 shows a 2.6% polyacrylamide gel containing the fractionated and ethidium bromide stained RNAs of the A/AA/6/60 virus. It can be seen in this example that the three P gene RNAs separate into two bands (a single and a double band) and that the NP and NA gene RNAs comigrate. This separation pattern was typical for the other H2N2 strains and allowed for the individual analysis of the HA, M and NS genes from each virus. Mixtures of the three P genes and of the NP/NA genes were also examined. Gel pieces containing RNA segments were excised and the RNA was electroeluted for analysis by RNAase T1 fingerprinting.

The oligonucleotide fingerprints of the P gene RNAs isolated from the different viruses were compared to that of the A/Sing/1/57 virus (see figure 6 and table 4). Only the large oligonucleotides below the dotted lines in the figure

FIGURE LEGEND

Figure 5. Polyacrylamide gel electrophoresis of the A/AA/6/60 virus RNAs.

Each lane represents approximately 10 ug of A/AA/6/60 virus RNA which was fractionated in a 2.6% polyacrylamide gel containing 6 M urea (see "Materials and Methods"). Electrophoresis was from top to bottom. RNA was stained with ethidium bromide and visualized by uv light illumination. The gene product of each RNA is indicated.


P3
P1,2
HA
NP/NA
M
NS

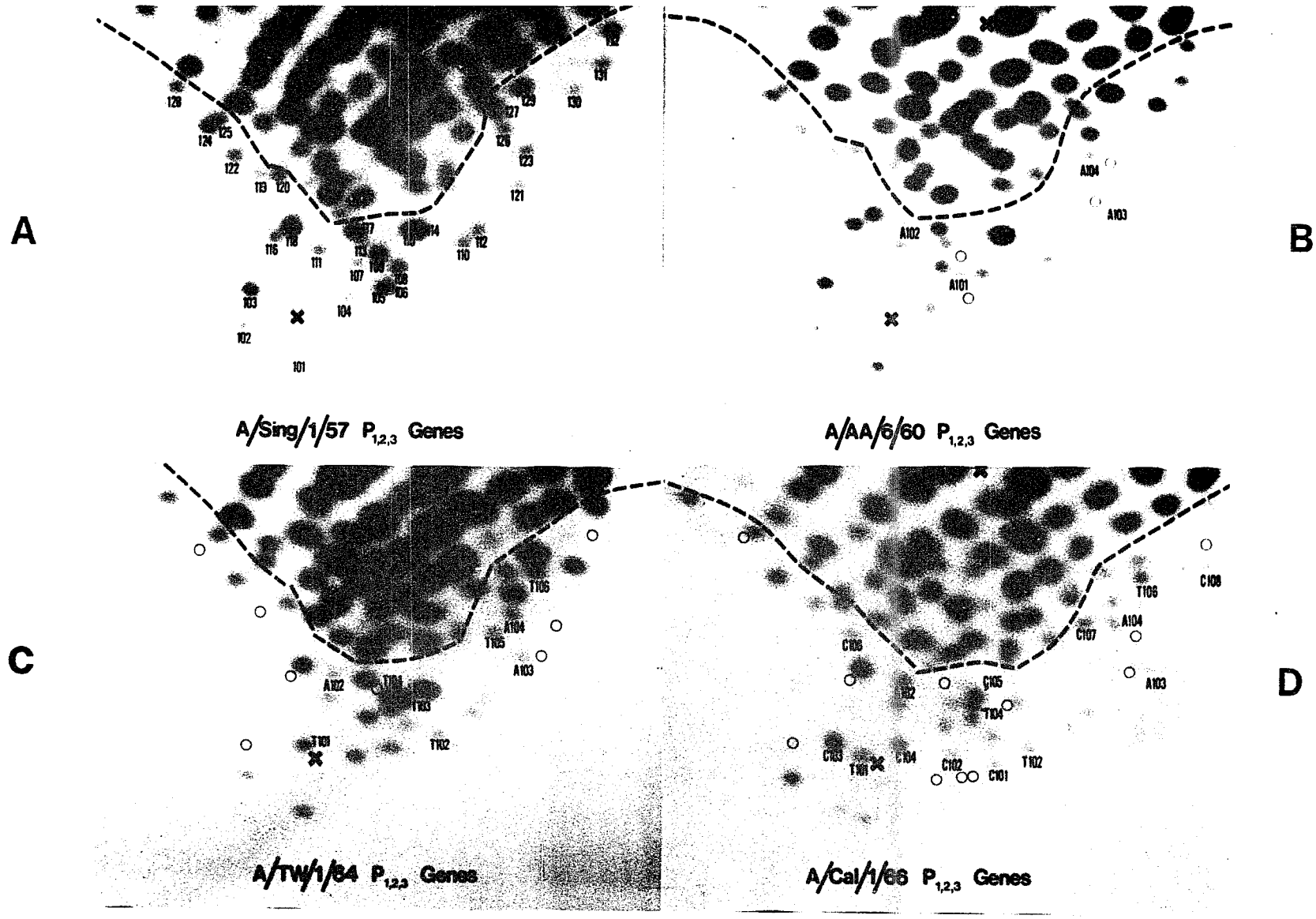
were considered in the analysis and each of the 32 oligonucleotides in the RNA fingerprint of the A/Sing/1/57 virus P genes were assigned a 100 series number. Open circles in panels B-F indicate "missing" spots that are present in the P gene RNA fingerprint of the A/Sing/1/57 virus and the letters and numbers designate "new" spots. The letters A,T,C,N or H next to a number indicate that a particular oligonucleotide was first observed in the fingerprint of the A/AA/6/60, A/TW/1/64, A/Cal/1/66, A/Ned/84/68 or A/HK/8/68 virus respectively.

It is apparent that several oligonucleotides are common to the P genes of all six viruses. Twelve of the 32 large oligonucleotides seen in the RNA fingerprint of the A/Sing/1/57 virus P genes can also be identified in the P gene RNA fingerprints of the later isolates by their identical map positions (e.g. see spots 101 102 and 107). In addition, changes in the P gene RNA fingerprints of earlier isolates can also be found in the P gene RNA fingerprints of later isolates. For example, the "new" oligonucleotide A103 in the RNA fingerprint of the A/AA/6/60 virus P genes is also seen in the P gene RNA fingerprints of all the later isolates. Similarly, the "missing" oligonucleotide 123 (see figure 6A for map location; and table 4 for a summary of all changes) in the P gene RNA fingerprint of the A/AA/6/60 virus is also "missing" in those of the later isolates. The

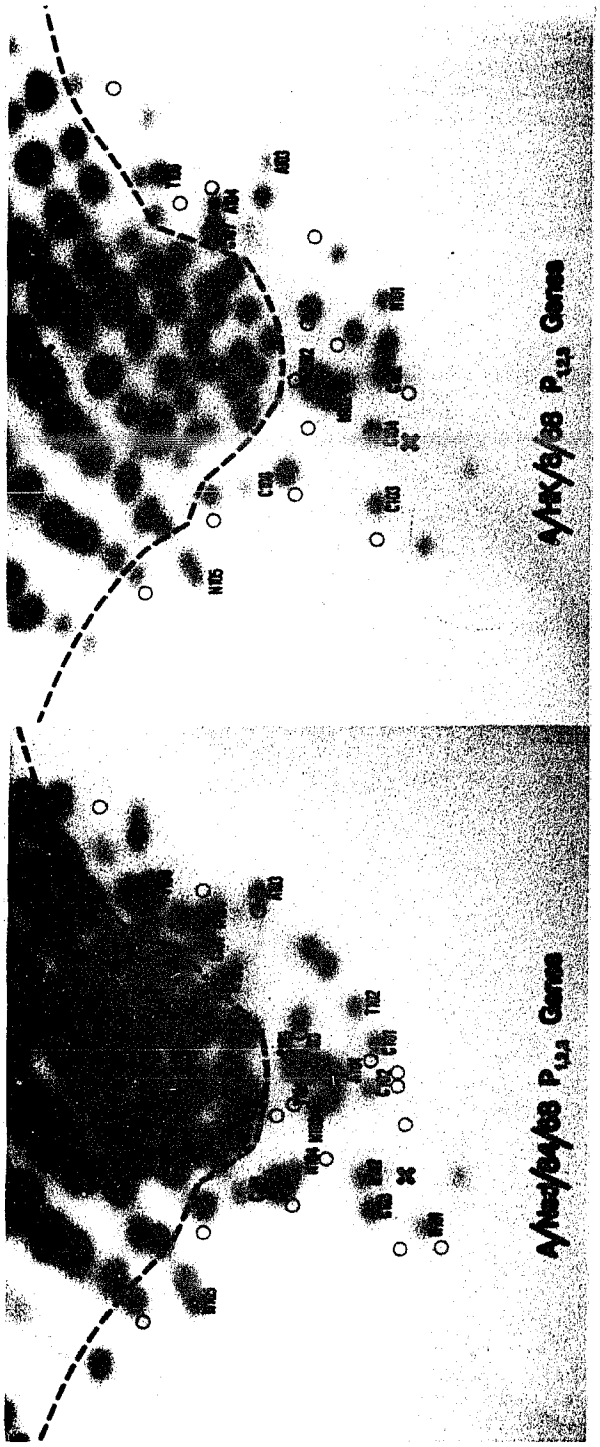
FIGURE LEGEND

Figure 6. Oligonucleotide fingerprints of isolated P gene mixtures.

Oligonucleotide fingerprints were obtained from the isolated P gene mixtures of each virus representd in panels A-F. Each of the 32 oligonucleotides in the fingerprint of the A/Sing/1/57 virus was assigned a 100 series number. Open circles in panels B-F indicate spots in the P gene mixture fingerprint of the A/Sing/1/57 virus that are missing while letters and numbers designate "new" spots. (see legend to figure 4 and the text for details.) Only large oligonucleotides (10 residues or longer) lying below the dotted line were considered in the analysis.


A
B
C
D
A/Sing/1/57 P1,2,3 Genes
A/AA/6/60 P1,2,3 Genes
A/TW/1/64 P1,2,3 Genes
A/Cal/1/66 P1,2,3 Genes

F



E

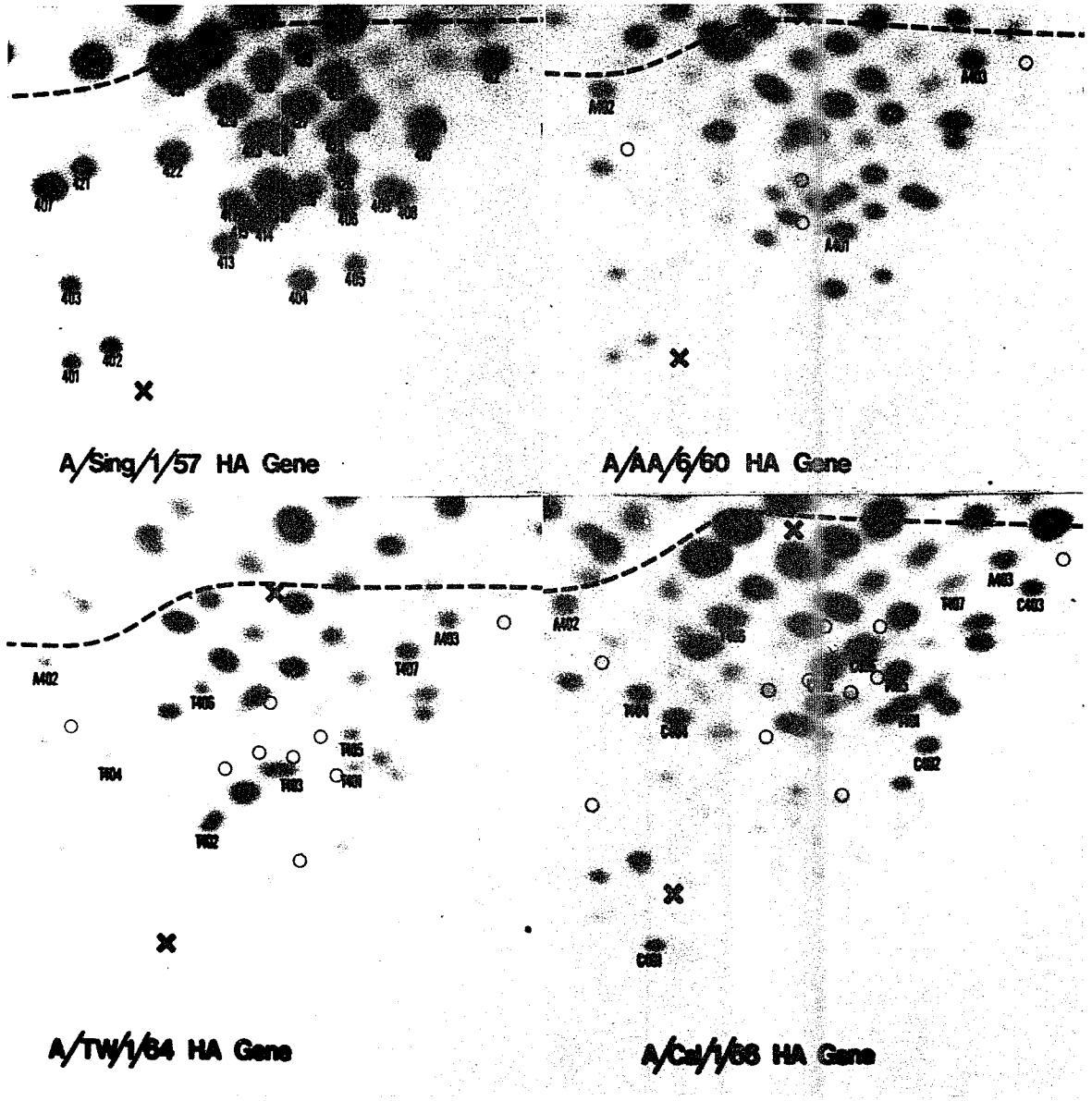
common oligonucleotides and common changes found among the P gene RNA fingerprints indicate that the P genes of the six viruses examined are most likely related through a series of sequential mutations. However, some changes were unique to a particular strain and indicate that divergent mutations also occur among the P genes of these six viruses. For example, the presence of spot number N101 and the absence of spot number 108 (see figure 6A for map location) are changes unique to the oligonucleotide map of the A/Ned/84/68 virus P genes.

The oligonucleotide fingerprints of the HA genes were also examined by a comparative analysis (see figure 7 and table 4) similar to that performed on the P gene maps. The HA gene patterns of the H2N2 viruses (figure 7A-E) show that these genes are related. In contrast to what was found for the P genes, the HA gene patterns of the H2N2 viruses are strikingly different from that of the H3N2 virus (figure 7F). Of the 33 oligonucleotides seen in the RNA fingerprint of the A/Sing/1/57 virus HA gene, 26 are missing from that of the A/HK/8/68 virus HA gene. In addition, there are fewer large oligonucleotides in the RNA fingerprint of the A/HK/8/68 virus HA gene than in that of the A/Sing/1/57 virus. This suggests that the distribution (or content) of guanosine residues in the H3 gene differs from that in the H2 genes. In any case, the HA RNA of the A/HK/8/68 virus does not appear to be closely related to the HA genes of the

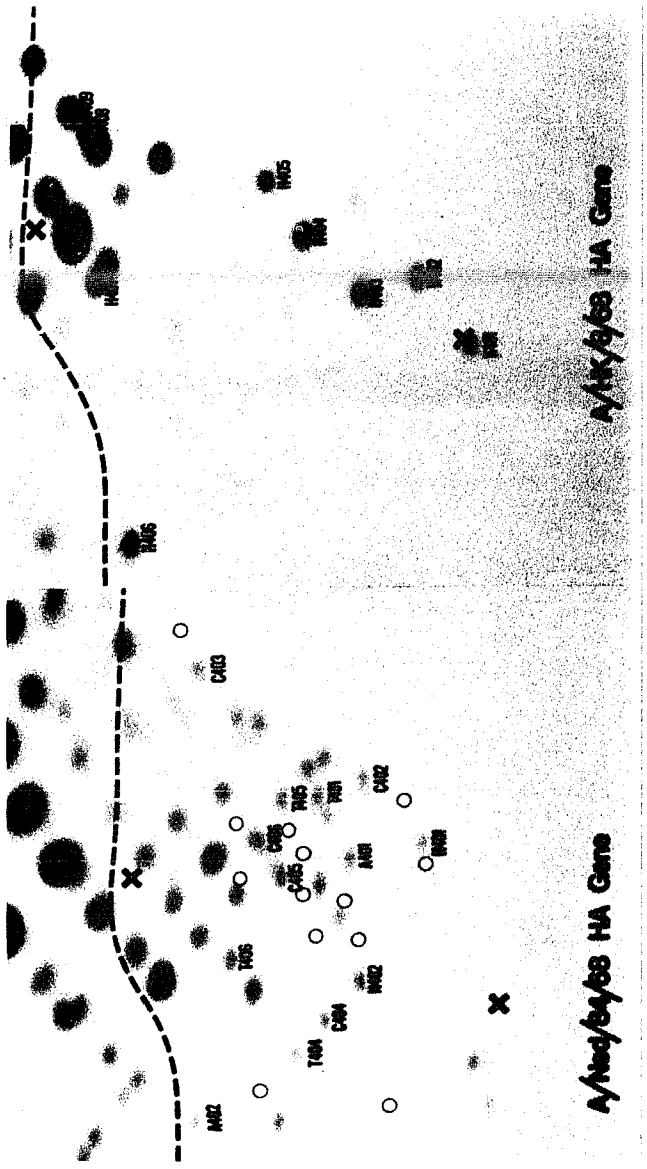
FIGURE LEGEND

Figure 7. Oligonucleotide fingerprints of isolated HA genes.

Oligonucleotide fingerprints were obtained from the isolated HA genes of each virus represented in panels A-F. Each of the 33 oligonucleotides in the fingerprint of the A/Sing/1/57 virus was assigned a 400 series number. Open circles in panels B-F indicate spots in the HA fingerprint of the A/Sing/1/57 virus that are missing while letters and numbers designate "new" spots. (see legend to figure 4 and the text for details.) Only large oligonucleotides (10 residues or longer) lying below the dotted line were considered in the analysis.


A
B
C
D
A/Sing/1/57 HA Gene
A/AA/6/60 HA Gene
A/TW/1/64 HA Gene
A/Cal/1/68 HA Gene

F



E

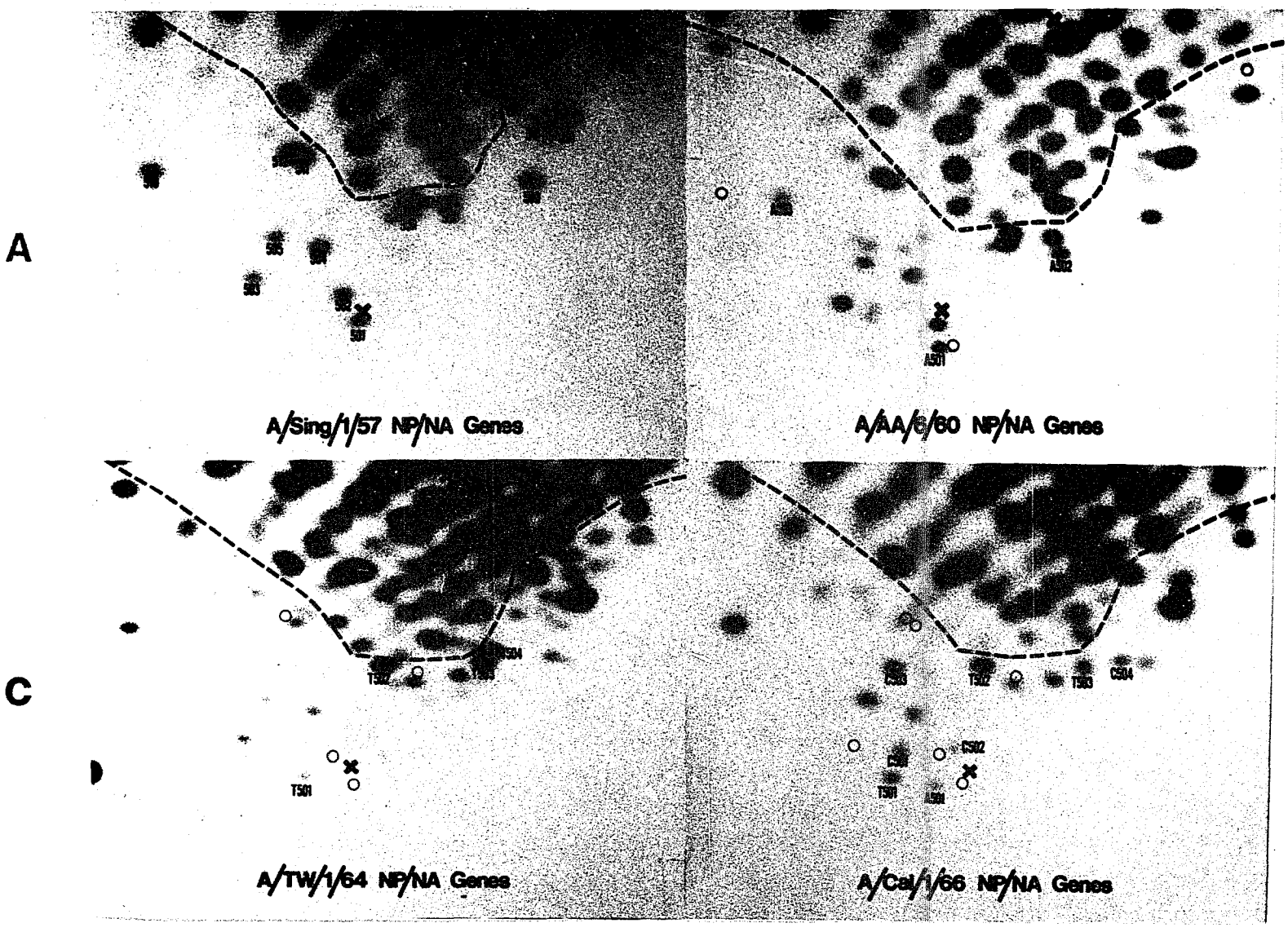
H2N2 viruses. The results obtained by DNA sequencing (Min Jou et al., 1980; Gething et al., 1980; Verhoeyen et al., 1980) serologic (Coleman et al., 1968), peptide mapping (Laver and Webster, 1972), RNA-RNA hybridization (Scholtissek et al., 1978a) and partial amino acid sequencing (Waterfield et al., 1979) techniques are in accord with this observation.

The variation among the oligonucleotide fingerprints of the NP/NA, M and NS genes was also examined (see figures 8, 9 and 10 respectively). The results of the comparative analysis of isolated genes and gene mixtures are summarized in table 4. It is clear that mutations have occurred in all the represented genes or gene mixtures. Nevertheless, the common oligonucleotides and common changes observed among corresponding RNAs of the H2N2 viruses demonstrated that their corresponding genes or gene mixtures were closely related. In addition, all the genes or gene mixtures except for the HA RNA of the H3N2 virus appeared to have sequences similar to those of the H2N2 viruses.

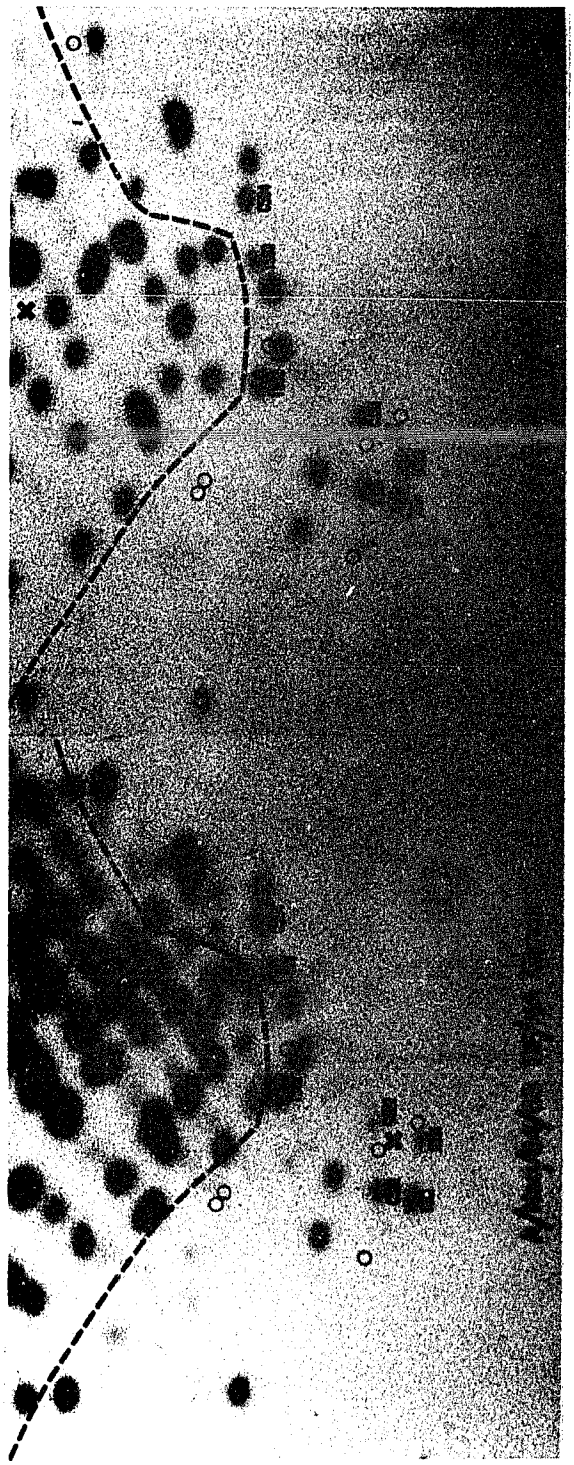
FIGURE LEGEND

Figure 8. Oligonucleotide fingerprints of isolated NP/NA gene mixtures.

Oligonucleotide fingerprints were obtained from the isolated NP/NA gene mixtures of each virus represented in panels A-F. Each of the 19 oligonucleotides in the fingerprint of the A/Sing/1/57 virus was assigned a 500 series number. Open circles in panels B-F indicate spots in the fingerprint of the NP/NA gene mixture of the A/Sing/1/57 virus that are missing while letters and numbers designate "new" spots. (see legend to figure 4 and the text for details.) Only large oligonucleotides (10 residues or longer) lying below the dotted line were considered in the analysis.


A
B
C
D
A/Sing/1/57 NP/NA Genes
A/AA/6/60 NP/NA Genes
A/TW/1/64 NP/NA Genes
A/Cal/1/66 NP/NA Genes

F

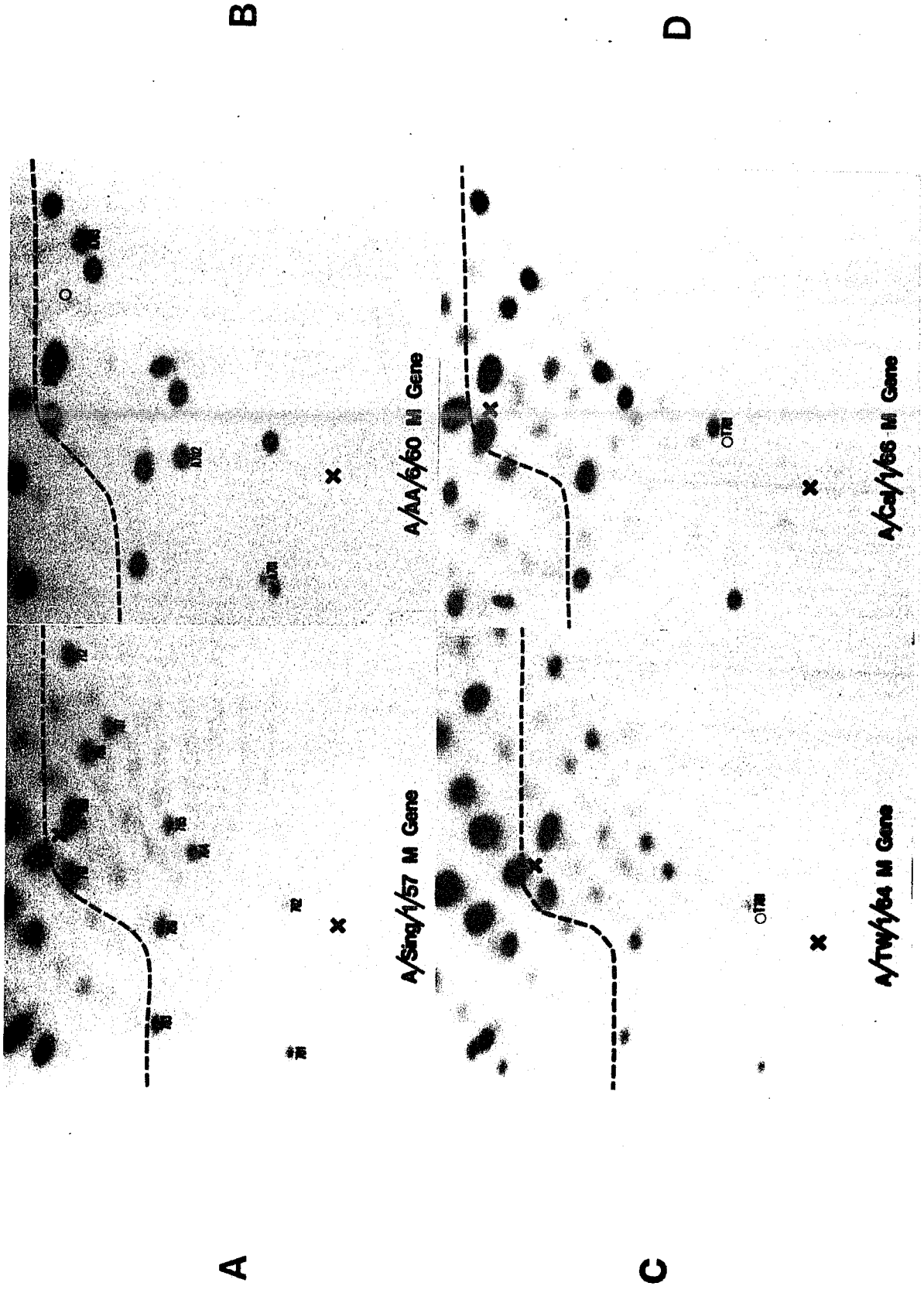


E

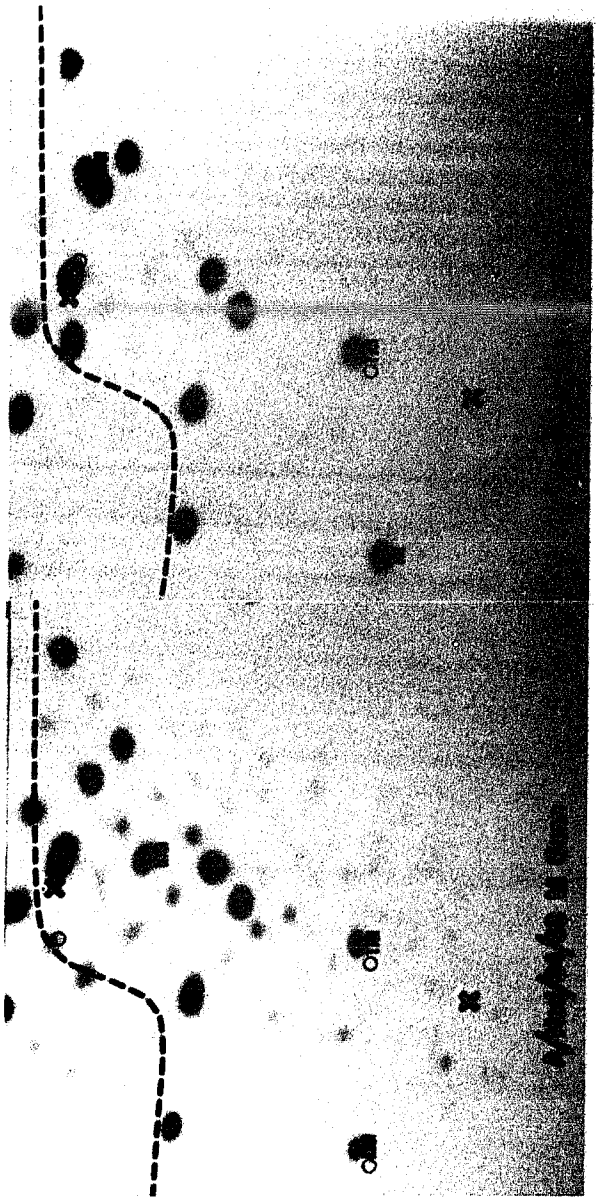
FIGURE LEGEND

Figure 9. Oligonucleotide fingerprints of isolated M genes.

Oligonucleotide fingerprints were obtained from the isolated M genes of each virus represented in panels A-F. Each of the 12 oligonucleotides in the fingerprint of the A/Sing/1/57 virus was assigned a 700 series number. Open circles in panels B-F indicate spots in the M gene fingerprint of the A/Sing/1/57 virus that are missing while letters and numbers designate "new" spots. (see legend to figure 4 and the text for details.) Only large oligonucleotides (10 residues or longer) lying below the dotted line were considered in the analysis.


A
B
C
D
A/Sing/1/57 M Gene
A/AA/6/60 M Gene
A/Tw/1/64 M Gene
A/Cal/1/65 M Gene

F

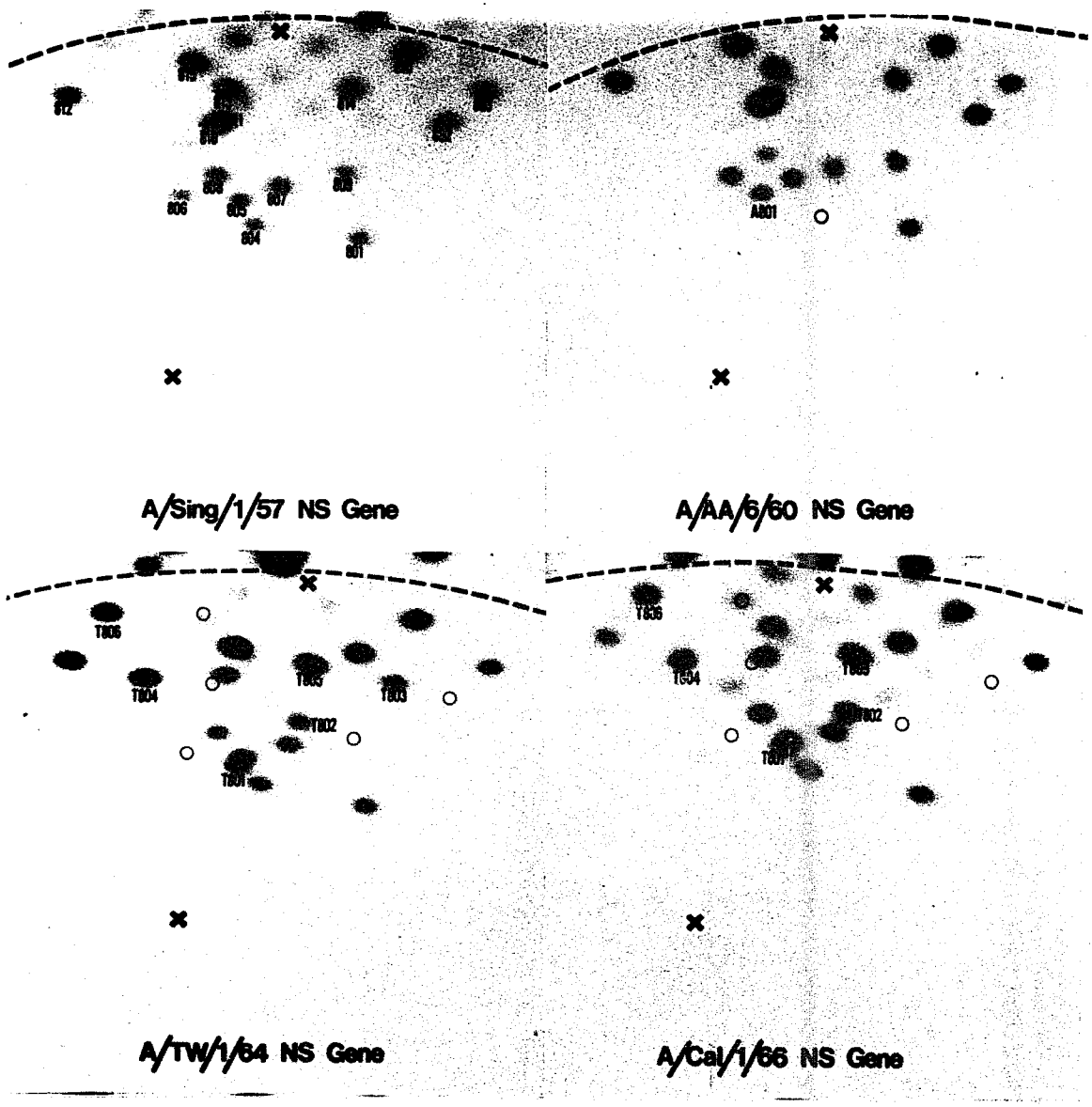


E

FIGURE LEGEND

Figure 10. Oligonucleotide fingerprints of isolated NS genes.

Oligonucleotide fingerprints were obtained from the isolated NS genes of each virus represented in panels A-F. Each of the 16 oligonucleotides in the fingerprint of the A/Sing/1/57 virus was assigned an 800 series number. Open circles in panels B-F indicate spots in the NS gene fingerprint of the A/Sing/1/57 virus that are missing while letters and numbers designate "new" spots. (see legend to figure 4 and the text for details.) Only large oligonucleotides (10 residues or longer) lying below the dotted line were considered in the analysis.


A
B
C
D
A/Sing/1/57 NS Gene
A/AA/6/60 NS Gene
A/TW/1/64 NS Gene
A/Cal/1/66 NS Gene

F

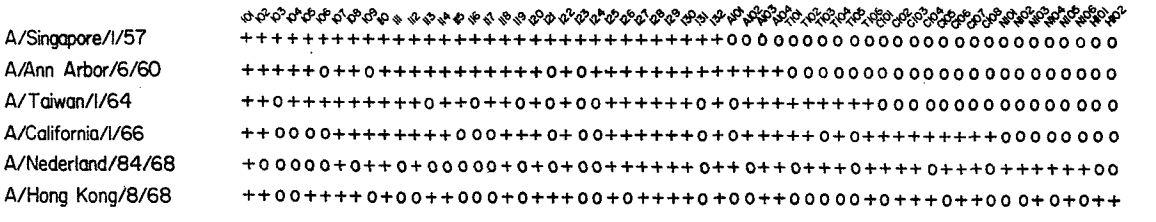
E

Table 4. Comparison of RNAase T1 resistant oligonucleotides derived from the RNAs of different influenza A viruses.

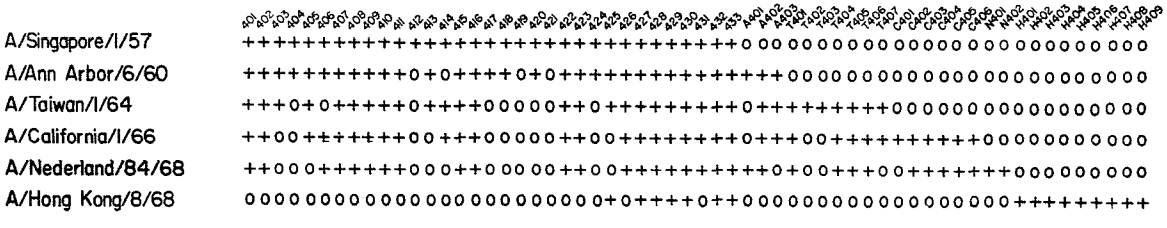
Data are derived from oligonucleotide fingerprint analysis of isolated genes or gene mixtures (see figures 6-10). The presence or absence of an oligonucleotide in an RNA fingerprint of a particular strain is indicated by (+) or (o) respectively. Blocks of oligonucleotides are arranged and numbered according to gene derivation as indicated by P1, P2, P3 for polymerase genes; HA for hemagglutinin genes; NP/NA for nucleoprotein and neuraminidase genes; M for membrane protein genes and NS for nonstructural protein genes.

Comparison of RNAase T1 resistant oligonucleotides derived from the RNAs of different influenza A viruses

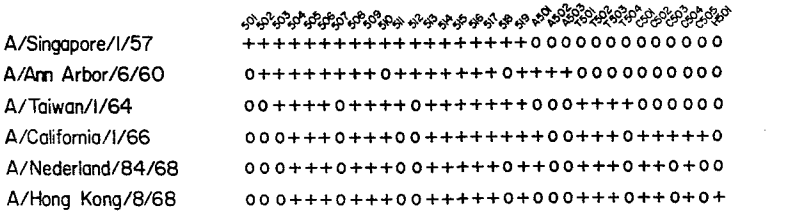
PI, P2, P3



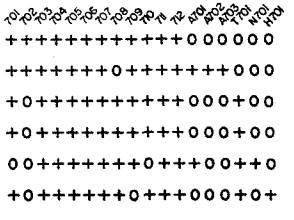
HA



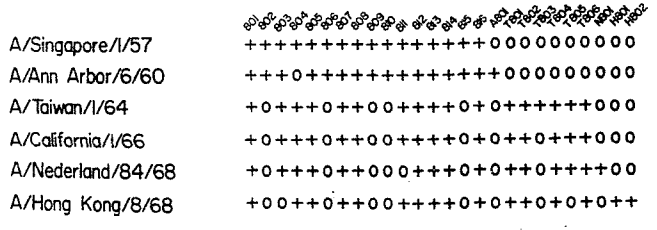
NP, NA



M



NS



8.2.3 EXTENT OF VARIATION AMONG GENES OR GENE MIXTURES

The differences in the oligonucleotide fingerprints of corresponding genes or gene mixtures were used to estimate base sequence differences among the genes: For example, the RNA fingerprint of the A/HK/8/68 virus M gene has two "missing" spots and two "new" spots when compared to the RNA fingerprint of the A/Sing/1/57 virus M gene (see figure 9A and F). These four changes may be the result of four independent mutations (i.e., the loss of the two "missing" spots in the M gene map of the A/HK/8/68 virus RNA results from mutations to G residues within those sequences. The two "new" spots result from mutations that replaced G residues in areas of the RNA which are not overlapping with the sequences of the "missing" spots). Alternatively, these four changes may result from two mutations (i.e., the loss of each of the two "missing" spots in the M gene fingerprint of the A/HK/8/68 virus RNA is due to a mutation which altered the moblity of each spot in the gene map). By taking the average of the minimum (two) and the maximum (four) number of changes, an estimate of the actual number of mutations (three) can be obtained (Nakajima et al., 1978). The estimated percent change between the two M genes used in this example is 1.56% and is calculated by dividing the average number of mutations by the average number of resi-

dues (192) represented in their oligonucleotide maps (3/192 x 100%; see Materials and Methods for determination of oligonucleotide size). Although the estimated mutational change between two RNAs is reported to two decimal places, the percent error of this value has not been determined.

The estimated extent of variation determined for the isolated P genes of the six viruses examined is shown in table 5. The P genes from the A/AA/6/60, A/TW/1/64, A/Cal/1/66, A/Ned/84/68 and A/HK/8/68 viruses differ from that of the A/Sing/1/57 virus by 0.85%, 1.83%, 2.89%, 3.81% and 2.68% respectively. When all possible comparisons of the P genes are made, a maximum change of 3.81% is noted between the A/Sing/1/57 and A/Ned/84/68 strains.

The variation among the HA genes of the H2N2 viruses was also determined (table 6). The HA genes from the A/AA/6/60, A/TW/1/64, A/Cal/1/66 and A/Ned/84/68 viruses differed from that of the A/Sing/1/57 virus by 0.96%, 2.36%, 3.28% and 3.40% respectively. A comparison of all the H2 HAs showed that the maximum change of 3.40% occured between the HAs of the 1957 and 1968 strains. Since most of the oligonucleotides in the H3 HA fingerprint are different from those of the H2 HA fingerprints, an estimation of nucleotide sequence homology based on a comparison of oligonucleotide maps was not possible.

Table 5. Percent changes in the P genes.

The RNA fingerprints of the isolated P genes were compared and the differences were used to estimate the extent of variation among the corresponding RNAs (see text for derivation of percent change values).

PERCENT CHANGES IN THE P1,2,3 GENES
(BASED ON DIFFERENCES IN OLIGONUCLEOTIDE MAPS)

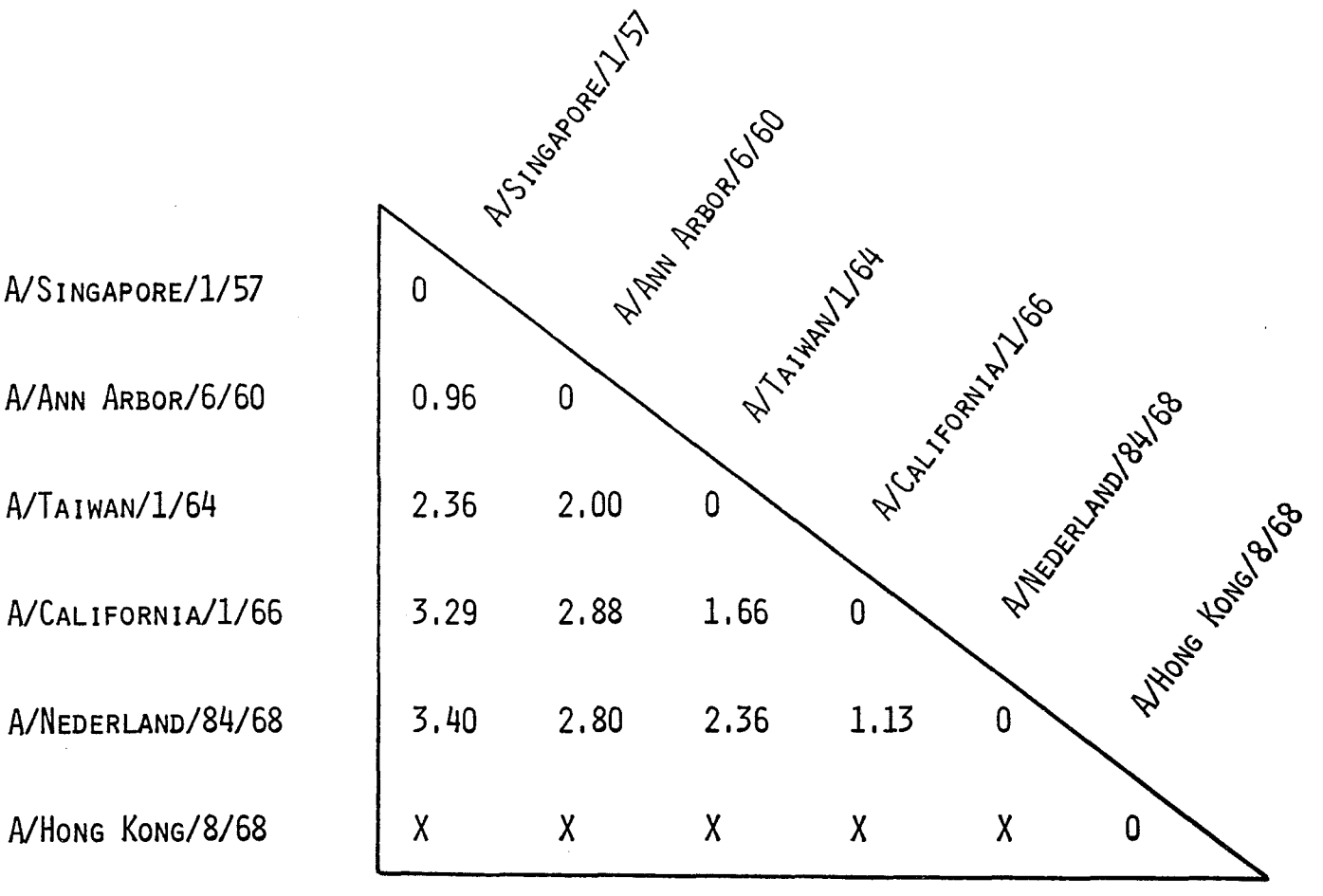
	A/SINGAPORE/1/57	A/ANN ARBOR/6/60	A/TAIWAN/1/64	A/CALIFRONIA/1/66	A/NEDERLAND/84/68	A/HONG KONG/8/68
A/SINGAPORE/1/57	0					
A/ANN ARBOR/6/60	0.85	0				
A/TAIWAN/1/64	1.83	1.62	0			
A/CALIFORNIA/1/66	2.89	2.47	1.90	0		
A/NEDERLAND/84/68	3.81	2.96	2.61	1.83	0	
A/HONG KONG/8/68	2.68	2.68	2.61	2.19	2.47	0

Table 6. Percent changes in the HA genes.

The RNA fingerprints of the isolated HA genes were compared and the differences were used to estimate the extent of variation among the corresponding RNAs (see text for derivation of percent change values).

PERCENT CHANGES IN THE HA GENES

(BASED ON DIFFERENCES IN OLIGONUCLEOTIDE MAPS)



Tables 7-9. Percent changes among the corresponding NP/NA gene mixtures; M genes; and NS genes.

Differences among the RNA fingerprints of the NP/NA gene mixtures; the M genes; and the NS genes were used to estimate the extent of variation among the corresponding RNAs of these different groups of genes respectively (see text for details).

PERCENT CHANGES IN THE NP,NA GENES

(BASED ON DIFFERENCES IN OLIGONUCLEOTIDE MAPS)

	A/SINGAPORE/1/57	A/ANN ARBOR/6/60	A/TAIWAN/1/64	A/CALIFORNIA/1/66	A/NEDERLAND/84/68	A/HONG KONG/8/68
A/SINGAPORE/1/57	0					
A/ANN ARBOR/6/60	1.13	0				
A/TAIWAN/1/64	1.51	2.27	0			
A/CALIFORNIA/1/66	3.02	2.14	1.89	0		
A/NEDERLAND/84/68	2.64	2.64	1.51	0.88	0	
A/HONG KONG/8/68	2.64	3.02	1.51	1.13	0.38	0

PERCENT CHANGES IN THE M GENES

(BASED ON DIFFERENCES IN OLIGONUCLEOTIDE MAPS)

	A/SINGAPORE/1/57	A/ANN ARBOR/6/60	A/TAIWAN/1/64	A/CALIFORNIA/1/66	A/NEDERLAND/84/68	A/HONG KONG/8/68
A/SINGAPORE/1/57	0					
A/ANN ARBOR/6/60	1.82	0				
A/TAIWAN/1/64	0.78	2.60	0			
A/CALIFORNIA/1/66	0.78	2.60	0	0		
A/NEDERLAND/84/68	2.34	3.39	1.56	1.56	0	
A/HONG KONG/8/68	1.56	3.13	0.52	0.52	1.82	0

PERCENT CHANGES IN THE NS GENES

(BASED ON DIFFERENCES IN OLIGONUCLEOTIDE MAPS)

	A/SINGAPORE/1/57	A/ANN ARBOR/6/60	A/TAIWAN/1/64	A/CALIFORNIA/1/66	A/NEDERLAND/84/68	A/HONG KONG/8/68
A/SINGAPORE/1/57	0					
A/ANN ARBOR/6/60	0.58	0				
A/TAIWAN/1/64	3.29	3.88	0			
A/CALIFORNIA/1/66	2.90	3.49	0.39	0		
A/NEDERLAND/84/68	3.49	4.07	0.97	0.58	0	
A/HONG KONG/8/68	3.49	4.07	1.55	1.16	1.74	0

Tables 7, 8 and 9 present the variation among the NP/NA, M and NS genes respectively. Estimated maximum changes of 3.02% among the NP/NA genes, 3.39% among the M genes and 4.07% among the NS genes were found. This analysis demonstrated that all the examined genes, with the exception of the H3 HA gene, have a similar extent of variation.

8.3 CLONING AND SEQUENCE ANALYSIS OF TWO DIFFERENT INFLUENZA VIRUS NS GENES

The last part of my study was aimed at obtaining direct evidence for genetic variation of influenza virus NS genes. Upon completing the cloning and sequencing of the PR8 virus NS gene, the sequences of two different influenza A virus NS genes were reported (Porter et al., 1980; Lamb and Lai, 1980). I then took the opportunity to compare these NS gene sequences with the one I had obtained and determined the extent of genetic variation among three NS genes. Other studies in our laboratory, using cDNA-RNA hybridization analysis to examine a wide variety of influenza viruses, revealed that the NS gene of the A/duck/Alberta/60/76 virus

differed strikingly from that of the PR8 virus (R.M. Elliott, unpublished). It was found that this duck virus RNA protected only 12% of a cDNA probe specific for the PR8 virus NS gene in a nuclease S1 resistance assay. Since the duck virus NS gene appeared to share little homology with the PR8 virus NS gene, I decided to clone and sequence this gene to compare its primary structure to that of the PR8 virus NS gene. I was especially interested in determining if the (-) RNA sense of the duck virus NS gene also contained an agnogene sequence (Seif et al., 1979) similar to the ones I had observed in the nonmessage sense of the PR8, FPV and Udorn strain NS genes (see below).

8.3.1 CLONING

Double-stranded DNA transcripts of the influenza duck and PR8 virus RNAs were prepared using reverse transcriptase. Usually a 3-6% conversion of RNA to DNA was achieved. The novel approach of priming the synthesis of the first and second strand DNA with two different synthetic oligonucleotides was used as a selection method for obtaining full length dsDNA transcripts from viral RNA. Restriction enzyme linkers were ligated to the virus specific dsDNA transcripts

which were then inserted into the appropriate sites of the plasmid cloning vector pBR322. Recombinant pBR322 plasmids (see "Materials and Methods" for their construction) were used to transform E.coli C600 cells. Colonies containing recombinant plasmids were identified by in situ hybridization using a ^{32}P-labeled cDNA probe made from viral RNA with reverse transcriptase and a synthetic oligonucleotide in the presence of α-^{32}P-dCTP. Figure 11 shows an autoradiogram of a nitrocelluslose filter with several colonies containing DNA that hybridized to the probe. Replicates of several of these colonies were used to isolate recombinant plasmids which were digested with a restriction endonuclease. The sizes of the virus specific sequences in the digested DNAs were estimated by agarose gel electrophoresis. One plasmid containing a 900 residue insert derived from the duck virus cDNA was designated pMEL801. Plasmids pAR109 and pJZ101 were shown to contain PR8 viral cDNAs of approximately 500 and 850 residues respectively.

The gene designation of the PR8 virus specific inserts in the phage and plasmid vectors were identified by the "Northern" blot technique. Figure 12 shows an agarose-urea gel containing fractionated and ethidium bromide stained PR8 virus RNA which was subsequently transfered to activated DBM paper. PR8 virus RNA labeled in vivo with ^{32}P was also fractionated in the gel to mark gene positions (see figure

FIGURE LEGEND

Figure 11. Colony hybridization

E.coli C600 colonies containing recombinant plasmids with sequences of influenza virus genes were identified by colony hybridization. The DNAs from several transformed colonies were were bound to a nitrocellulose filter (see "Materials and Methods" for details) and then hybridized with a ^{32}P-labeled cDNA probe. The probe was made from PR8 virus RNA by reverse transcription primed with a synthetic oligonucleotide in the presence of α-^{32}P-dCTP. A colony transformed with the pBR322 cloning vector alone was used as a negative hybridization control (see arrow for location).

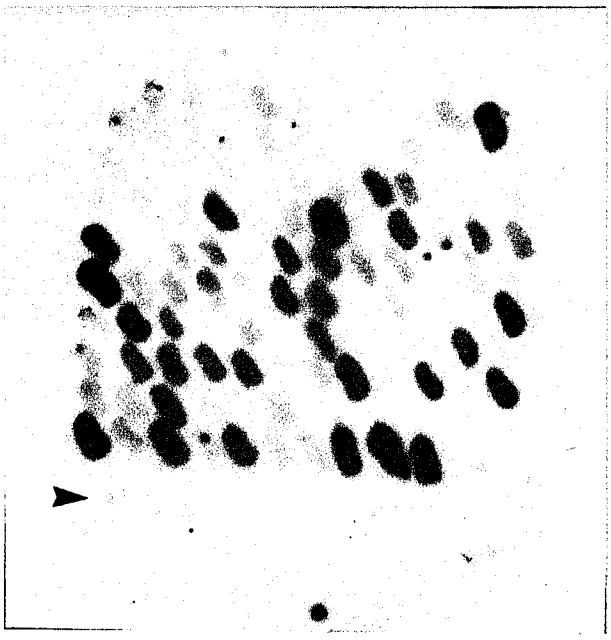


FIGURE LEGEND

Figure 12. Preparative fractionation of PR8 virus RNA by agarose-urea gel electrophoresis.

PR8 virus RNA was preparatively fractionated in a 1.5% agarose gel containing 6 M urea and the separated RNAs were transfered to activated DBM paper (see "Materials and Methods"). Approximately 50,000 cpm of PR8 virus RNA labeled in vivo with ^{32}P was also fractionated in each of 3 slots which flanked the 2 preparative slots. The ^{32}P-labeled RNA served as gene position markers.

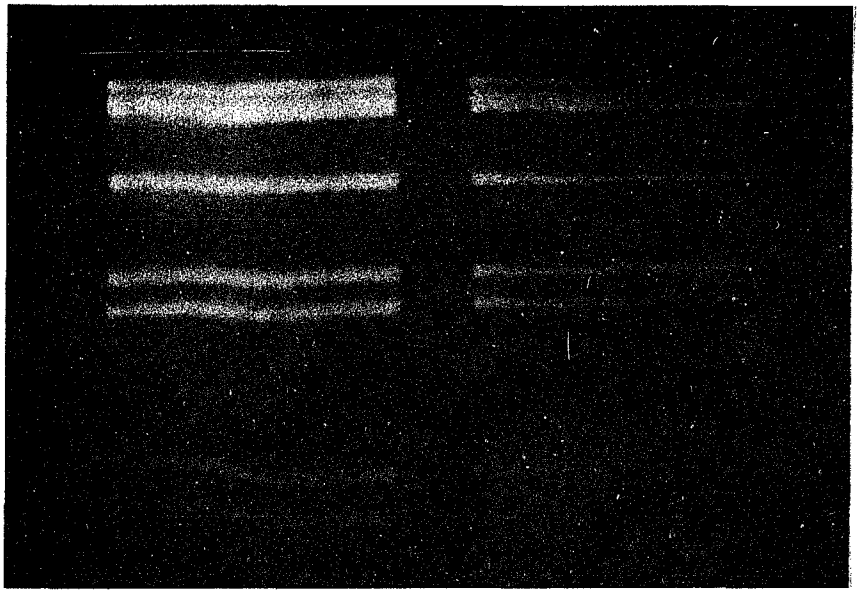
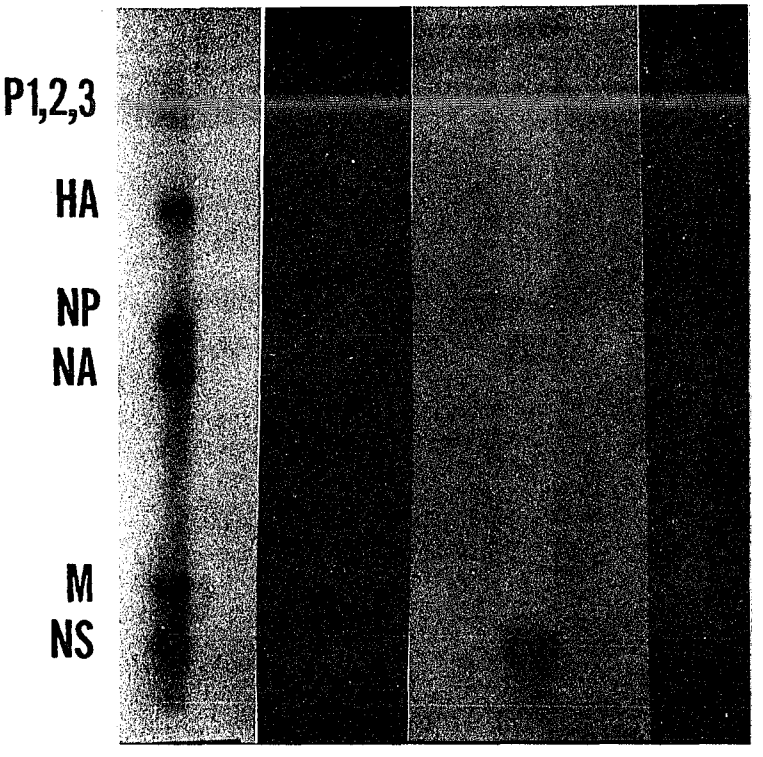


FIGURE LEGEND

Figure 13. Hybridization of recombinant DNA to PR8 virus RNAs bound to DBM paper.

PR8 virus RNA was fractionated in agarose-urea gels and transfered to activated DBM paper (see legend to figure 12). The recombinant DNAs from 1 and pBR322 vectors were "nick-translated" and hybridized to strips of the DBM-RNA paper to determine the gene specificity of their PR8 virus cDNA sequences (see "Materials and Methods"). Lane 1 shows the ^{32}P-labeled RNAs of PR8 virus bound to DBM paper. Lanes 2, 3 and 4 show strips of DBM-RNA paper with the hybridized DNAs of a recombinant λ phage, plasmid pAR109 and plasmid pJZ101 respectively.


P1,2,3
HA
NP
NA
M
NS

legend). Strips of the RNA containing DBM paper were used in the hybridization analysis of nick-translated DNA prepared from the recombinant phage and plasmids. A composite autoradiogram of three hybridization experiments is shown in figure 13. Lane 1 shows the PR8 gene markers while lanes 2, 3 and 4 show to which PR8 virus RNAs the labeled DNAs of the recombinant phage and the recombinant plasmids, pAR109 and pJZ101 respectively hybridized. It is clear from this analysis that the three examined DNAs contain PR8 NS gene specific sequences. The plasmids that contained DNA which hybridized to the NS RNA in lanes 3 and 4 (i.e. pAR109 and pJZ101) were used to determine the sequence of the PR8 NS gene.

8.3.2 DNA SEQUENCING

The NS gene specific DNAs from the plasmids pAR101 and pJZ101 were isolated and subjected to restiction endonuclease analysis with several enzymes. The strategy used to determine the nucleic acid sequence of the PR8 virus NS gene is shown in figure 14. Three sequencing techniques were used to determine the sequence of the PR8 virus NS gene:

FIGURE LEGEND

Figure 14. Sequencing strategy and restriction endonuclease maps of the inserts of the PR8 virus NS gene in pAR109 and pJZ101

The DNA inserts from clones pAR101 and pJZ101 are shown with their restriction endonuclease sites and are aligned with respect to the PR8 virus NS gene. The large rectangles represent the synthetic HindIII and EcoRI linkers at the ends of the cloned DNAs. Arrows indicate the direction of sequencing and the length of the sequence determined by a particular technique; (O) the Forward-Backward technique of Seif et al. (1980), (■) the chemical method of Maxam and Gilbert (1980), (□) the chain termination technique of Sanger et al. (1977) as modified by Both et al. (1980).

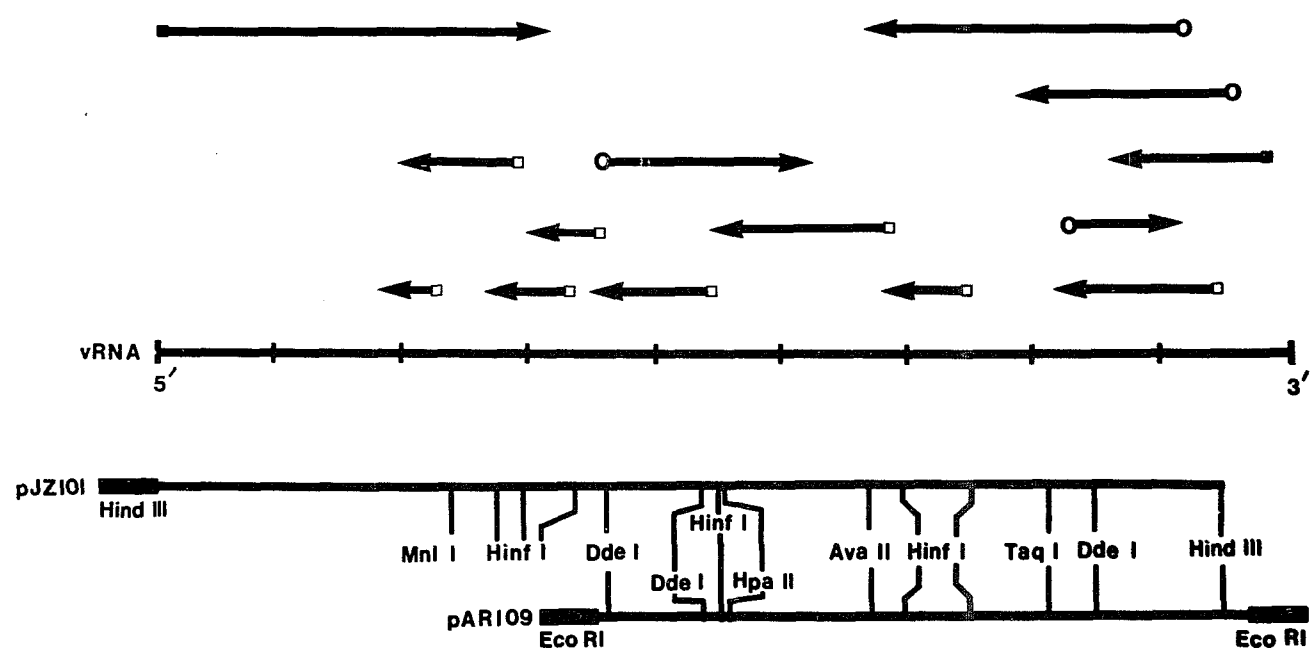
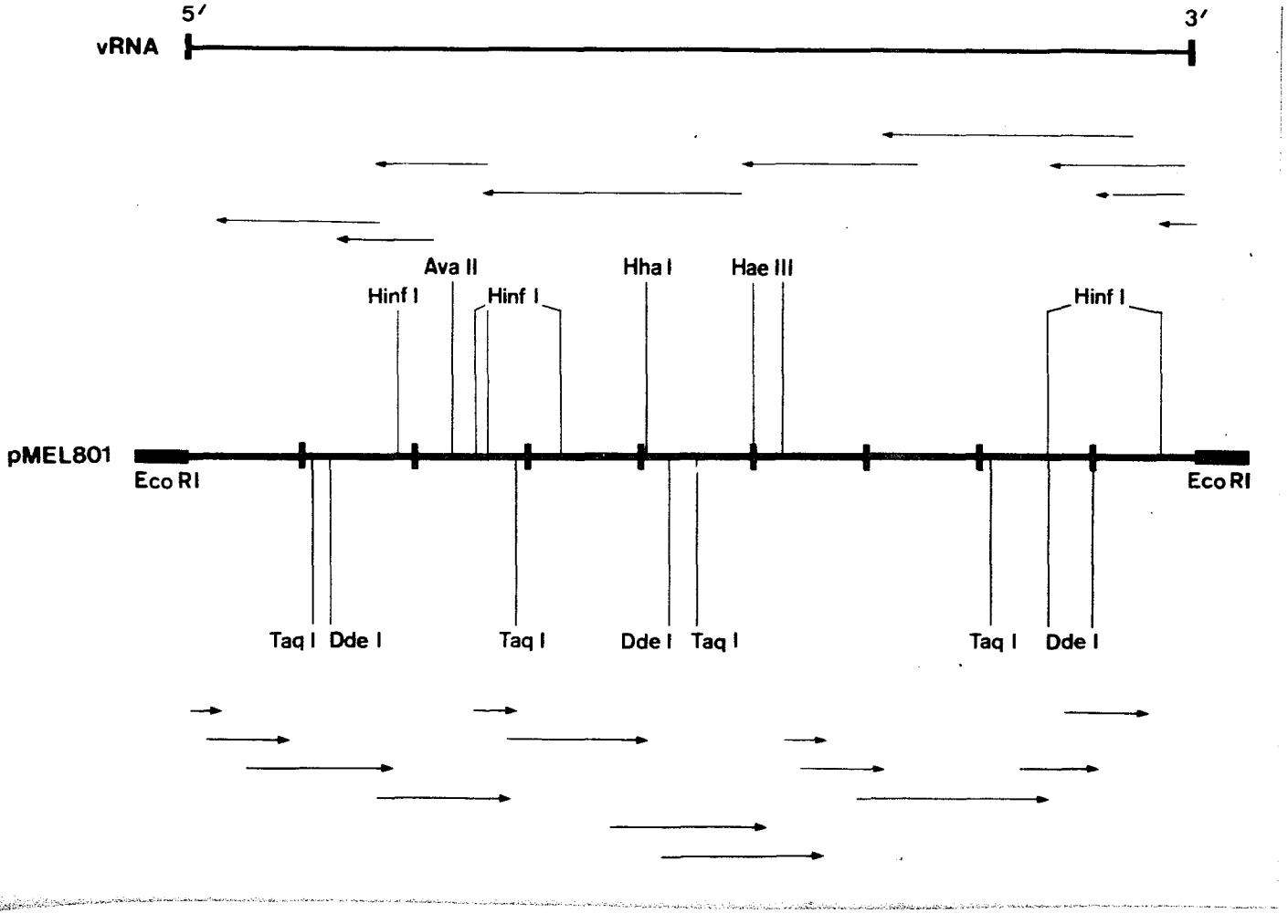

vRNA
5′
3′
pJZ101
Hind III
Mnl I
Hinf I
Dde I
Hinf I
Dde I
Hpa II
Ava II
Hinf I
Taq I
Dde I
Hind III
pAR109
Eco RI
Eco RI

FIGURE LEGEND

Figure 15. Sequencing strategy and restriction endonuclease map of the insert of the duck virus NS gene in pMEL801.

The viral cDNA insert from recombinant plasmid pMEL801 is aligned with the vRNA of the duck virus NS gene and several restriction endonuclease sites are shown in the cloned gene. The large rectangles represent synthetic EcoRI linkers. Arrows indicate the direction of sequencing and the length of a sequence determined by a particular experiment. The chemical technique of Maxam and Gilbert, (1980) was used to determine the complete sequence of the duck virus NS gene.


5′
3′
vRNA
Ava II
Hha I
Hae III
Hinf I
Hinf I
Hinf I
pMEL801
Eco RI
Eco RI
Taq I
Dde I
Taq I
Dde I
Taq I
Taq I
Dde I

the chemical method of Maxam and Gilbert, (1980); the Forward-Backward method of Seif et al., (1980); and the chain termination method of Sanger et al., (1977). The arrows indicate the direction of sequencing and the length of the sequence obtained by a particular technique (see figure legend). When the end sequences of the cloned DNAs were compared to the known 5' and 3' terminal sequences of the PR8 virus NS RNA (Desselberger, unpublished), it was observed that the pAR109 insert (530 residues long) lacked 23 nucleotides found at the 3' end of the NS gene vRNA. The pJZ101 insert was found to be identical to the 5' end of the NS gene vRNA and to extend 843 residues to a position 47 nucleotides from the 3' end of the NS gene vRNA.

A similar strategy was used to clone and sequence the duck virus NS gene (see figure 15). The complete nucleotide sequence of the viral cDNA in this plasmid was determined using the Maxam and Gilbert technique (1980) and figure 16 shows an example of A DNA fragment sequenced by this method. End sequence analysis and comparison to the PR8 virus NS gene sequence demonstrated that the viral cDNA in plasmid pMEL801 was a complete copy of the duck virus NS gene. The complete sequences of the duck virus and PR8 virus NS genes and their deduced amino acid sequences are presented in figure 17. The first initiation codon occurs in the message sense (+) RNA at positions 27-29 and an open reading frame

FIGURE LEGEND

Figure 16. Nucleotide sequence of a 5' end ^{32}P-labeled DNA fragment.

The Maxam and Gilbert chemical sequencing technique (1980) was used to determine the complete nucleotide sequence of the duck virus NS gene and portions of the PR8 virus NS gene (see "Materials and Methods"). As an example, the products of a 5' end, ^{32}P-labeled DNA fragment resulting from four base specific modification and cleavage reactions (G, G+A, T+C and C) were fractionated in an 8% polyacrylamide gel containing 7 M urea. Electrophoresis was from top to bottom and the sequence of this representative DNA fragment is read in the 5'-3' direction by reading from bottom to top.

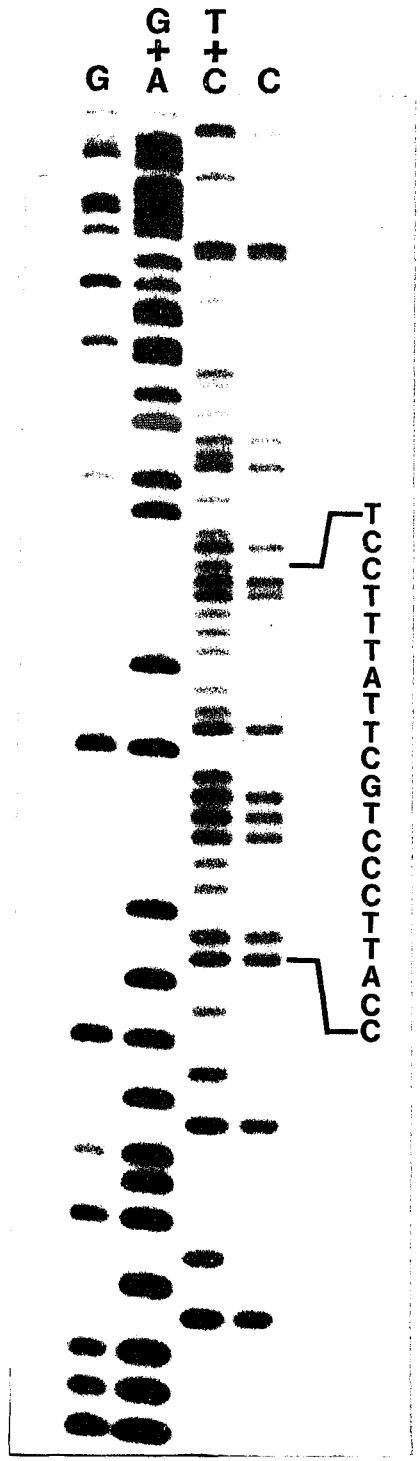
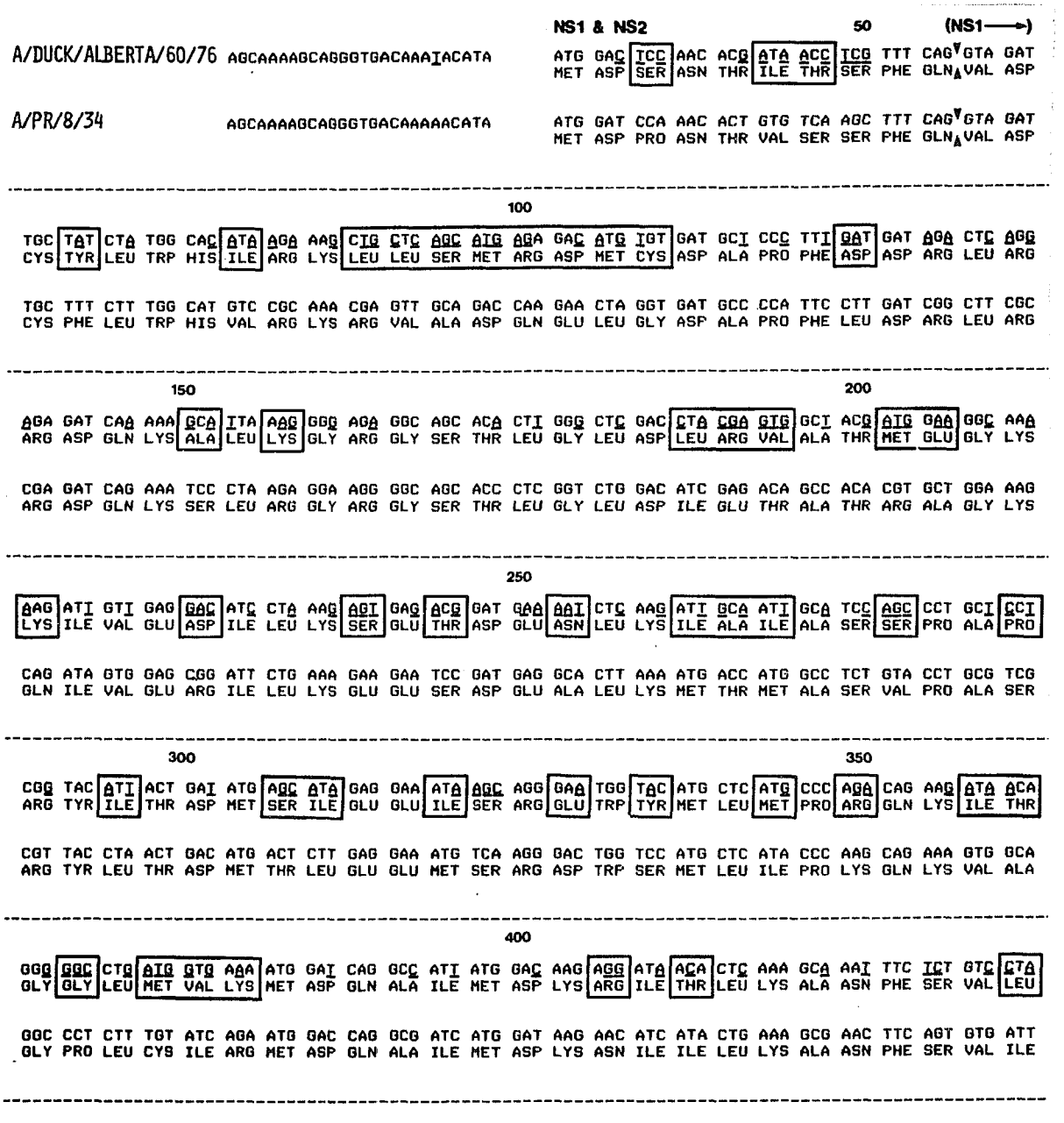
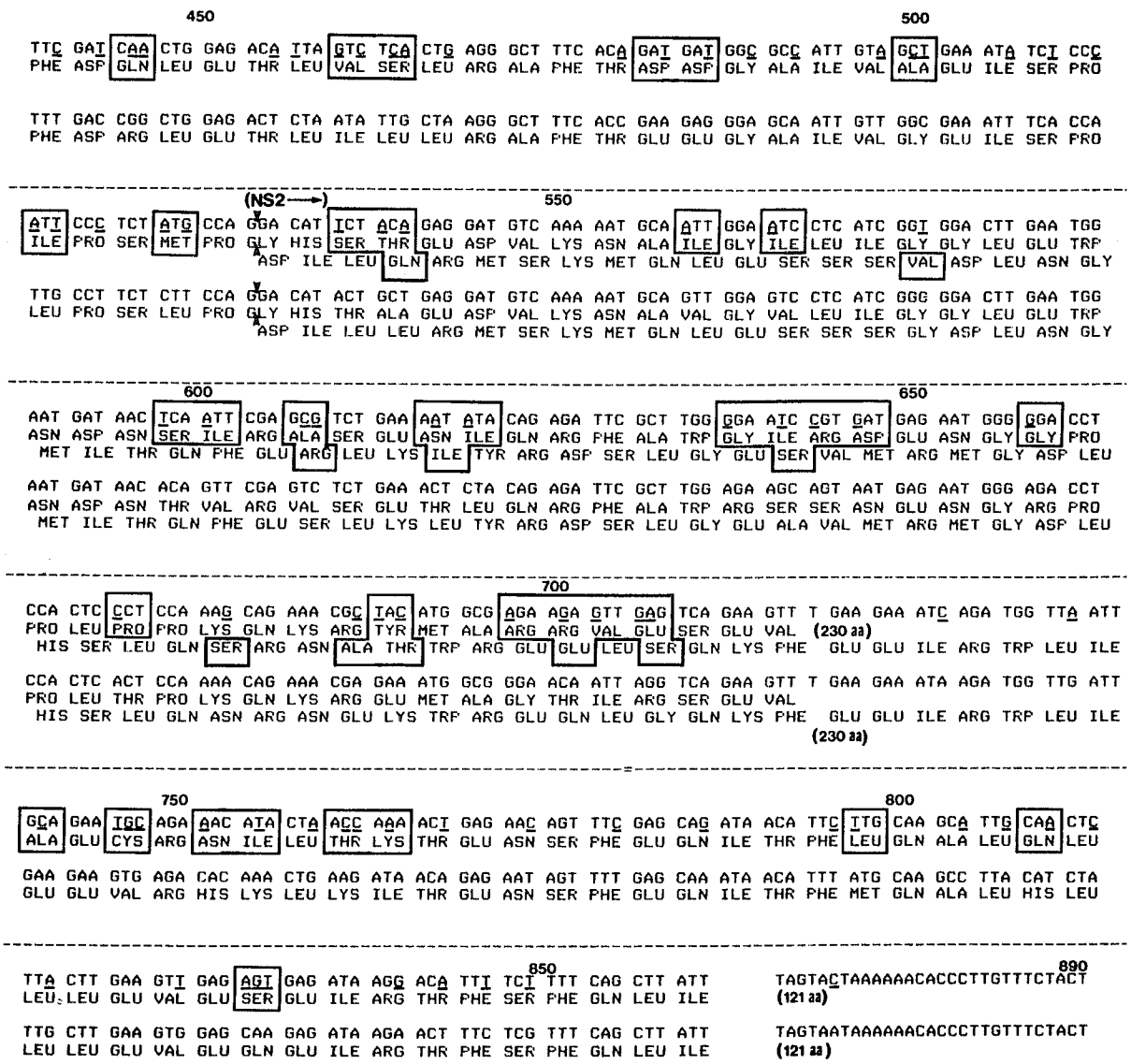

G
G+A
T+C
C
TCCTTTTATTCGTCCCTTACC

FIGURE LEGEND

Figure 17. Comparison of the PR8 and duck virus NS gene sequences and their deduced amino acid sequences.

The complete nucleotide and predicted amino acid sequences of the A/PR/8/34 virus NS gene and their comparison to those of the A/duck/Alberta/60/76 virus NS gene are presented. Nucleotide differences among the sequences are underlined and amino acid changes are boxed. The arrowheads indicate the probable splicing sites for the NS2 mRNA.


NS1 & NS2
50
(NS1——→)
A/DUCK/ALBERTA/60/76
AGCAAAAGCAGGGTGACAAAIACATA
ATG GAC TCC AAC ACG ATA ACC TCG TTT CAG GTA GAT
MET ASP SER ASN THR ILE THR SER PHE GLN VAL ASP
A/PR/8/34
AGCAAAAGCAGGGTGACAAAAACATA
ATG GAT CCA AAC ACT GTG TCA AGC TTT CAG GTA GAT
MET ASP PRO ASN THR VAL SER SER PHE GLN VAL ASP
100
TGC TAT CTA TGG CAC ATA AGA AAG CIG CTC AGC ATG AGA GAC ATG IGT GAT GCI CCC TTI GAT GAT AGA CTC AGG
CYS TYR LEU TRP HIS ILE ARG LYS LEU LEU SER MET ARG ASP MET CYS ASP ALA PRO PHE ASP ASP ARG LEU ARG
TGC TTT CTT TGG CAT GTC CGC AAA CGA GTT GCA GAC CAA GAA CTA GGT GAT GCC CCA TTC CTT GAT CGG CTT CGC
CYS PHE LEU TRP HIS VAL ARG LYS ARG VAL ALA ASP GLN GLU LEU GLY ASP ALA PRO PHE LEU ASP ARG LEU ARG
150
200
AGA GAT CAA AAA GCA ITA AAG GGG AGA GGC AGC ACA CTI GGG CTC GAC CTA CGA GTG GCI ACG ATG GAA GGC AAA
ARG ASP GLN LYS ALA LEU LYS GLY ARG GLY SER THR LEU GLY LEU ASP LEU ARG VAL ALA THR MET GLU GLY LYS
CGA GAT CAG AAA TCC CTA AGA GGA AGG GGC AGC ACC CTC GGT CTG GAC ATC GAG ACA GCC ACA CGT GCT GGA AAG
ARG ASP GLN LYS SER LEU ARG GLY ARG GLY SER THR LEU GLY LEU ASP ILE GLU THR ALA THR ARG ALA GLY LYS
250
AAG ATI GTI GAG GAC ATC CTA AAG AGI GAG ACG GAT GAA AAI CTC AAG ATI GCA ATI GCA TCC AGC CCT GCI CCI
LYS ILE VAL GLU ASP ILE LEU LYS SER GLU THR ASP GLU ASN LEU LYS ILE ALA ILE ALA SER SER PRO ALA PRO
CAG ATA GTG GAG CGG ATT CTG AAA GAA GAA TCC GAT GAG GCA CTT AAA ATG ACC ATG GCC TCT GTA CCT GCG TCG
GLN ILE VAL GLU ARG ILE LEU LYS GLU GLU SER ASP GLU ALA LEU LYS MET THR MET ALA SER VAL PRO ALA SER
300
350
CGG TAC ATI ACT GAI ATG AGC ATA GAG GAA ATA AGC AGG GAA TGG TAC ATG CTC ATG CCC AGA CAG AAG ATA ACA
ARG TYR ILE THR ASP MET SER ILE GLU GLU ILE SER ARG GLU TRP TYR MET LEU MET PRO ARG GLN LYS ILE THR
CGT TAC CTA ACT GAC ATG ACT CTT GAG GAA ATG TCA AGG GAC TGG TCC ATG CTC ATA CCC AAG CAG AAA GTG GCA
ARG TYR LEU THR ASP MET THR LEU GLU GLU MET SER ARG ASP TRP SER MET LEU ILE PRO LYS GLN LYS VAL ALA
400
GGG GGC CTG ATG GTG AAA ATG GAI CAG GCC ATI ATG GAC AAG AGG ATA ACA CTC AAA GCA AAI TTC ICT GTC CTA
GLY GLY LEU MET VAL LYS MET ASP GLN ALA ILE MET ASP LYS ARG ILE THR LEU LYS ALA ASN PHE SER VAL LEU
GGC CCT CTT TGT ATC AGA ATG GAC CAG GCG ATC ATG GAT AAG AAC ATC ATA CTG AAA GCG AAC TTC AGT GTG ATT
GLY PRO LEU CYS ILE ARG MET ASP GLN ALA ILE MET ASP LYS ASN ILE ILE LEU LYS ALA ASN PHE SER VAL ILE


450
500
TTC GAT CAA CTG GAG ACA ITA GTC TCA CTG AGG GCT TTC ACA GAT GAT GGC GCC ATT GTA GCI GAA ATA TCI CCC
PHE ASP GLN LEU GLU THR LEU VAL SER LEU ARG ALA PHE THR ASP ASP GLY ALA ILE VAL ALA GLU ILE SER PRO
TTT GAC CGG CTG GAG ACT CTA ATA TTG CTA AGG GCT TTC ACC GAA GAG GGA GCA ATT GTT GGC GAA ATT TCA CCA
PHE ASP ARG LEU GLU THR LEU ILE LEU LEU ARG ALA PHE THR GLU GLU GLY ALA ILE VAL GLY GLU ILE SER PRO
(NS2→)
550
ATI CCC TCT ATG CCA GGA CAT ICT ACA GAG GAT GTC AAA AAT GCA ATT GGA ATC CTC ATC GGI GGA CTT GAA TGG
ILE PRO SER MET PRO GLY HIS SER THR GLU ASP VAL LYS ASN ALA ILE GLY ILE LEU ILE GLY GLY LEU GLU TRP
ASP ILE LEU GLN ARG MET SER LYS MET GLN LEU GLU SER SER SER VAL ASP LEU ASN GLY
TTG CCT TCT CTT CCA GGA CAT ACT GCT GAG GAT GTC AAA AAT GCA GTT GGA GTC CTC ATC GGG GGA CTT GAA TGG
LEU PRO SER LEU PRO GLY HIS THR ALA GLU ASP VAL LYS ASN ALA VAL GLY VAL LEU ILE GLY GLY LEU GLU TRP
ASP ILE LEU LEU ARG MET SER LYS MET GLN LEU GLU SER SER SER GLY ASP LEU ASN GLY
600
650
AAT GAT AAC ICA ATT CGA GCG TCT GAA AAT ATA CAG AGA TTC GCT TGG GGA ATC CGT GAT GAG AAT GGG GGA CCT
ASN ASP ASN SER ILE ARG ALA SER GLU ASN ILE GLN ARG PHE ALA TRP GLY ILE ARG ASP GLU ASN GLY GLY PRO
MET ILE THR GLN PHE GLU ARG LEU LYS ILE TYR ARG ASP SER LEU GLY GLU SER VAL MET ARG MET GLY ASP LEU
AAT GAT AAC ACA GTT CGA GTC TCT GAA ACT CTA CAG AGA TTC GCT TGG AGA AGC AGT AAT GAG AAT GGG AGA CCT
ASN ASP ASN THR VAL ARG VAL SER GLU THR LEU GLN ARG PHE ALA TRP ARG SER SER ASN GLU ASN GLY ARG PRO
MET ILE THR GLN PHE GLU SER LEU LYS LEU TYR ARG ASP SER LEU GLY GLU ALA VAL MET ARG MET GLY ASP LEU
700
CCA CTC CCT CCA AAG CAG AAA CGC IAC ATG GCG AGA AGA GTT GAG TCA GAA GTT T GAA GAA ATC AGA TGG TTA ATT
PRO LEU PRO PRO LYS GLN LYS ARG TYR MET ALA ARG ARG VAL GLU SER GLU VAL (230 aa)
HIS SER LEU GLN SER ARG ASN ALA THR TRP ARG GLU GLU LEU SER GLN LYS PHE GLU GLU ILE ARG TRP LEU ILE
CCA CTC ACT CCA AAA CAG AAA CGA GAA ATG GCG GGA ACA ATT AGG TCA GAA GTT T GAA GAA ATA AGA TGG TTG ATT
PRO LEU THR PRO LYS GLN LYS ARG GLU MET ALA GLY THR ILE ARG SER GLU VAL
HIS SER LEU GLN ASN ARG ASN GLU LYS TRP ARG GLU GLN LEU GLY GLN LYS PHE GLU GLU ILE ARG TRP LEU ILE
(230 aa)
750
800
GCA GAA IGC AGA AAC ATA CTA ACC AAA ACI GAG AAC AGT TTC GAG CAG ATA ACA TTC ITG CAA GCA TTG CAA CTC
ALA GLU CYS ARG ASN ILE LEU THR LYS THR GLU ASN SER PHE GLU GLN ILE THR PHE LEU GLN ALA LEU GLN LEU
GAA GAA GTG AGA CAC AAA CTG AAG ATA ACA GAG AAT AGT TTT GAG CAA ATA ACA TTT ATG CAA GCC TTA CAT CTA
GLU GLU VAL ARG HIS LYS LEU LYS ILE THR GLU ASN SER PHE GLU GLN ILE THR PHE MET GLN ALA LEU HIS LEU
850
890
TTA CTT GAA GTI GAG AGI GAG ATA AGG ACA TTI TCT TTT CAG CTT ATT
LEU LEU GLU VAL GLU SER GLU ILE ARG THR PHE SER PHE GLN LEU ILE
TAGTACTAAAAAACACCCTTGTTTCTACT
(121 aa)
TTG CTT GAA GTG GAG CAA GAG ATA AGA ACT TTC TCG TTT CAG CTT ATT
LEU LEU GLU VAL GLU GLN GLU ILE ARG THR PHE SER PHE GLN LEU ILE
TAGTAATAAAAAACACCCTTGTTTCTACT
(121 aa)

extends to a termination codon at positions 717-719 in both sequences. These regions potentially encode polypeptides of 230 amino acid acids in length (NS1). Another open reading frame begins in both sequences with positions 469- 471 and ends at positions 859-861. Consensus RNA splicing signals (Lerner et al., 1980) are found at positions 54-57 and at positions 526-529 in the NS genes of the duck and PR8 viruses. Splicing of the RNAs at these points could result in a second mRNA coding for a polypeptide of 121 amino acids (NS2) in both NS genes, which are in accordance with the model of the NS gene structure presented by Lamb and Lai (1980).

It is clear from the sequences of the duck and PR8 virus NS genes that these genes have identical termini and total lengths of 890 residues. (Conserved termini are common to all genes of all influenza viruses; Skehel and Hay, 1978; Robertson, 1979; Desselberger et al., 1980). Other common features of these two RNAs include the length of the sequences coding for the NS1 polypeptides (690 residues), the conservation of putative RNA splicing sites (see arrowheads in figure 17) and the length of the sequences coding for the NS2 polypeptides (363 residues). In addition, six contiguous A residues at positions 869-874 have also been conserved in both RNA segments. These regions have been recently identified as the polyadenylation sites for influ-

enza virus mRNAs (Robertson et al., 1981). The structural organization of the duck and PR8 virus NS genes are therefore similar to each other and also to those reported for the other two NS gene sequences (Porter et al., 1980, Lamb and Lai, 1980; recently Winter et al., 1981, have independently determined the PR8 virus NS gene sequence). In contrast, there are many differences between the nucleic acid sequences of the duck and PR8 virus NS genes as indicated by the underlined residues in figure 16. The deduced amino acid differences are shown by boxes in the duck virus gene sequence.

Table 10 lists the percentage of single base differences among the nucleotide sequences of the duck, PR8, FPV and Udorn virus NS genes. In comparing the total nucleotide sequences of the duck virus NS gene to any of the other three NS genes, a difference of approximately 27% is noted. In contrast, when the PR8, FPV and Udorn virus NS gene sequences are compared to each other, the total nucleotide differences range from 8-11%. It is also apparent that the portions of the RNAs encoding the NS1 polypeptides vary to a greater extent than do the regions coding for the NS2 polypeptides (also, see below).

Table 11 presents the differences among the amino acid sequences encoded by the four RNA segments. The NS1 and NS2 polypeptides encoded by the duck virus NS gene differ from

Table 10. Differences in nucleotide sequences among the NS genes of four influenza viruses.

The lengths of the NS genes, NS1 coding regions and NS2 coding regions are 890, 690 and 363 nucleotides respectively. The differences reported for the NS1 coding regions were calculated for the "common" 690 nucleotides. It should be noted that the Udorn virus NS1 is 21 nucleotides longer than those of the other viruses.

DIFFERENCES IN NUCLEOTIDE SEQUENCES AMONG THE NS GENES OF FOUR INFLUENZA VIRUSES

NS GENE DERIVATION	TOTAL (%)	NS1 (%)	NS2 (%)
A/DUCK/ALBERTA/60/76 - A/PR/8/34	243/890 (27.3)	210/690 (30.4)	69/363 (19.0)
A/DUCK/ALBERTA/60/76 - A/FPV/ROSTOCK/34	236/890 (26.5)	198/690 (28.7)	65/363 (17.9)
A/DUCK/ALBERTA/60/76 - A/UDORN/72	245/890 (27.5)	206/690 (29.9)	72/363 (19.8)
A/PR/8/34 - A/FPV/ROSTOCK/34	71/890 (8.0)	61/690 (8.8)	24/363 (6.6)
A/PR/8/34 - A/UDORN/72	78/890 (8.8)	63/690 (9.1)	29/363 (8.0)
A/UDORN/72 - A/FPV/ROSTOCK/34	96/890 (10.8)	80/690 (11.6)	31/363 (8.5)

Table 11. Differences in amino acid sequences among the NS polypeptides of four influenza viruses.

The lengths of the NS1 and NS2 polypeptides are 230 and 121 amino acids respectively. The differences for the NS1 polypeptides were calculated with respect to the "common" 230 amino acid regions. It should be noted that the Udorn virus NS1 polypeptide is seven residues longer than those of the other viruses.

DIFFERENCES IN AMINO ACID SEQUENCES AMONG THE NS POLYPEPTIDES OF FOUR DIFFERENT INFLUENZA VIRUSES

NS GENE DERIVATION	NS1 (%)	NS2 (%)
A/DUCK/ALBERTA/60/76 - A/PR/8/34	76/230 (33.0)	22/121 (18.2)
A/DUCK/ALBERTA/60/76 - A/FPV/ROSTOCK/34	69/230 (30.0)	20/121 (16.5)
A/DUCK/ALBERTA/60/76 - A/UDORN/72	73/230 (31.7)	23/121 (19.0)
A/PR/8/34 - A/FPV/ROSTOCK/34	24/230 (10.4)	10/121 (8.3)
A/PR/8/34 - A/UDORN/72	26/230 (11.3)	9/121 (7.4)
A/UDORN/72 - A/FPV/ROSTOCK/34	35/230 (15.2)	7/121 (5.8)

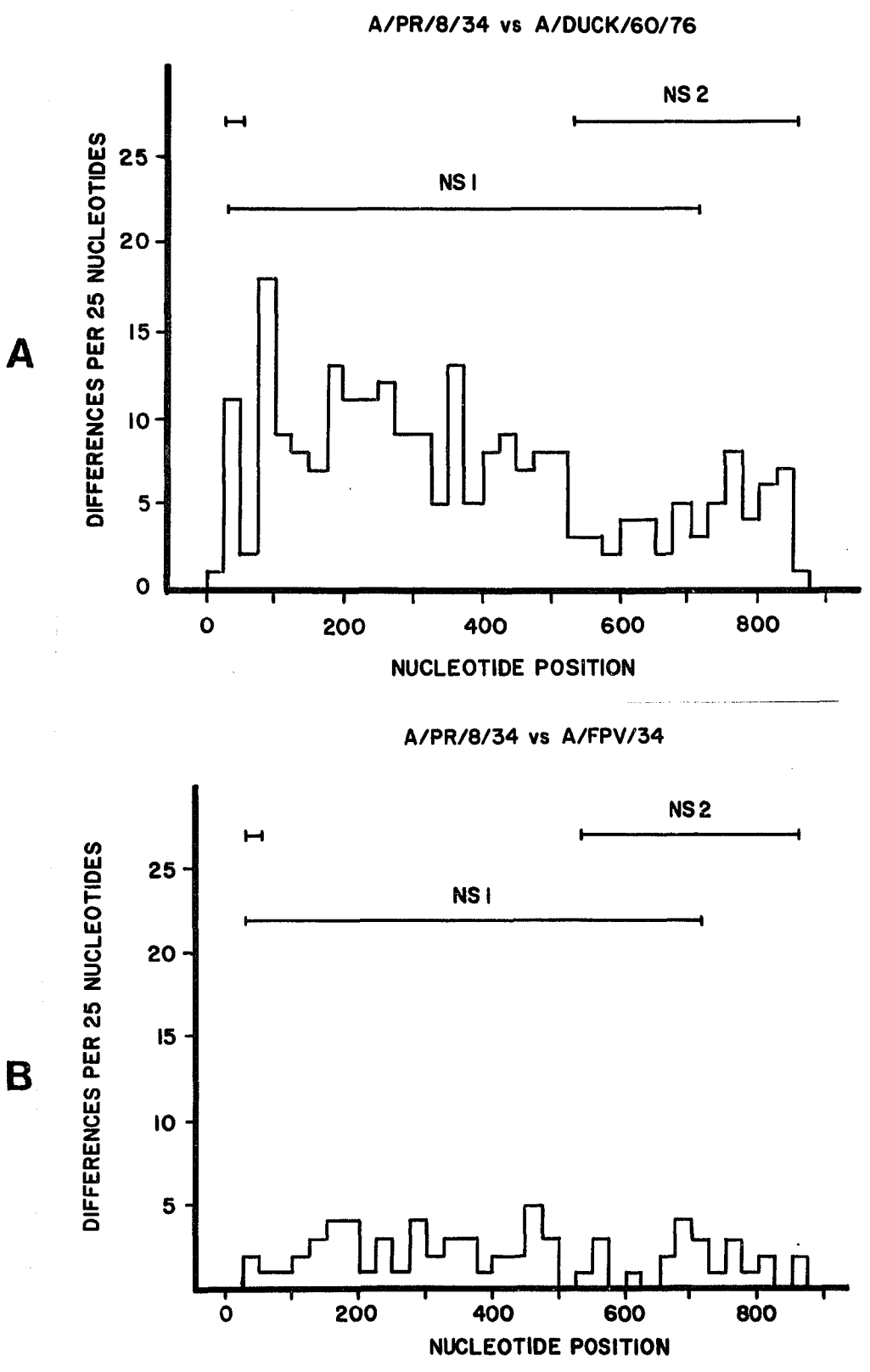
those of the other viruses by an average of 32% and 18% respectively. When the PR8, FPV and Udorn viruses were compared, average differences of only 12% and 7% were observed in their NS1 and NS2 polypeptides, respectively.

The positions of nucleotide differences between the duck and PR8 virus NS genes (+ RNA sense) are represented by the histogram in figure 18A. Differences can be seen throughout the entire length of these two genes although a greater number of mutations is apparent at their 5' proximal regions. One section of 25 residues in particular (positions 75 through 100) has sustained 18 mutations which represents a 72% change for this small region. Other sections of the genome also show substantial variation (i.e. 52% differences among positions 175 through 200 and among positions 350 through 375, 48% difference among positions 250 through 275 and 44% difference among positions 25 through 50). It is not known if these apparently hypervariable regions result in functional differences of the two genes. In contrast, a comparison of the PR8 virus and FPV virus NS gene sequences yields a histogram which does not clearly distinguish areas of conservation or hypervariability (see figure 18B). Obviously, the extent of genetic variation between these two RNAs is lower than that between the duck virus and PR8 virus NS genes.

FIGURE LEGEND

Figure 18. Positions of nucleotide differences among three NS genes.

Histograms of the positions of nucleotide differences, per 25 residues, between the NS genes of the (A) A/duck/Alberta/60/76 and A/PR/8/34 viruses, and (B) A/PR/8/34 and A/FPV/Rostock/34 viruses are presented. The regions of the RNA encoding the NS1 and NS2 polypeptides are indicated above the histograms. The numbering of nucleotide positions proceeds from the 5' end to the 3' end of the message-sense RNA. The FPV NS gene sequence is taken from Porter et al., (1980).

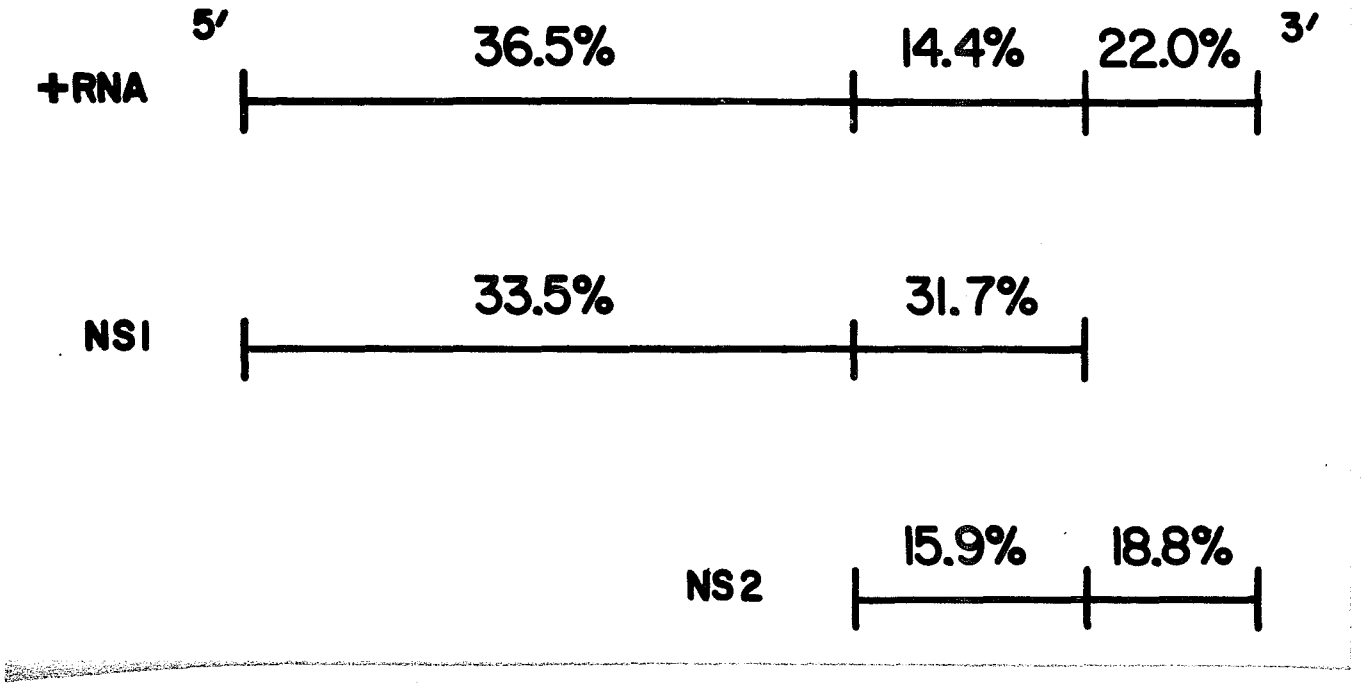

A
A/PR/8/34 vs A/DUCK/60/76
NS 2
NS I
DIFFERENCES PER 25 NUCLEOTIDES
0
5
10
15
20
25
0
200
400
600
800
NUCLEOTIDE POSITION
B
A/PR/8/34 vs A/FPV/34
NS 2
NS I
DIFFERENCES PER 25 NUCLEOTIDES
5
10
15
20
25
0
200
400
600
800
NUCLEOTIDE POSITION

A close examination of the "peaks" and "troughs" in figure 18A suggests that the NS genes of the duck and PR8 viruses possess regions of distinct homology. A division of the NS gene into three distinguishable domains based on sequence differences is representd in figure 19. The top line in the diagram shows the nucleotide variation between the duck and PR8 virus NS genes for the regions of the (+) RNA that correspond to positions 27 through 528 (first domain), 529 through 716 (second domain) and 720 through 861 (third domain) respectively. The first domain encodes over 70% of the NS polypeptide and shows a 36.5% difference while the third domain which encodes 40% of the NS2 polypeptide shows a difference of only 18.8%. The first and third domains code for polypeptides in only one reading frame. The second domain is the most conserved and shows the lowest difference of 14.4% in nucleotide sequence. This region encodes the carboxy-terminal portion of the NS1 polypeptides and a 52% portion of the NS2 polypeptides in different reading frames. Examination of the amino acid substitutions among the first 70% of the NS1 polypeptides reveals a 33.5% difference which is comparable to the number of changes detected between the corresponding regions of the RNAs. Although the nucleotide sequences encoding the carboxy-terminal portions of the NS1 polypeptides are highly conserved (14.4%) between the duck and PR8 virus NS genes, surpris-

FIGURE LEGEND

Figure 19. Division of the A/duck/Alberta/60/76 and A/PR/8/34 virus NS genes into three domains of distinct homology.

The comparison of the duck and PR8 virus NS genes suggested that they could be divided into three domains of distinct sequence homology. The message sense RNA (top line) shows the percent of nucleotide differences within these three regions: (a) unique for NS1 gene, (b) overlap of NS1 and NS2 genes and (c) unique for NS2 genes. The second and third lines show the NS1 and NS2 polypeptides and the percent amino acid differences within the regions defined by the RNA domains. The ten amino terminal residues of the NS2 polypeptides are most likely identical to those of the NS1 polypeptides and are not shown.


5′
36.5%
14.4%
22.0%
3′
+RNA
33.5%
31.7%
NS1
15.9%
18.8%
NS2

ingly the corresponding domains of the NS1 polypeptides are not conserved (a 31.7% difference is noted). Thus the NS1 polypeptides of these two viruses show an amino acid sequence difference of greater than 30% over their entire lengths. This is in contrast to the NS2 polypeptides which show only a 15.9% and 18.8% change in the regions encoded by the second and third domains of the NS genes.

An interesting finding of this analysis was the observation of open reading frames in the nonmessage sense of the PR8, FPV and Udorn virus NS genes. Surprisingly, the duck virus NS gene did not have a similar agnogene sequence in its RNA sense (see discussion for details).

9.0 DISCUSSION

9.1 GENOTYPE DETERMINATION OF INFLUENZA VIRUS RECOMBINANTS CONSTRUCTED FOR USE IN VACCINE PRODUCTION

The gene constellations of eleven influenza virus recombinants, which were constructed for use in vaccine production, were determined by comparing their RNA migration patterns to those of the parental strains in polyacrylamide gels. This project was aimed at determining whether a particular genotype could be correlated with the high yield phenotype that was used to select each recombinant vaccine strain. It was found that all the recombinants analyzed derive the gene for the M protein from the PR8 virus. This would suggest that this gene is associated with high yield in embryonated hens' eggs.

Although the PR8 virus M gene was consistently found in the eleven recombinants, it may not be the only factor responsible for the enhanced growth capacity. To confirm its role as the high yield determinant, one should be able to demonstrate that recombinants containing only the M gene from PR8 virus also show an increased yield. Furthermore,

the absence of the PR8 M gene in a recombinant should correlate with that strain's expression of poor growth in embryonated hens' eggs. However, in this study, such strains were not available for analysis because only one high yielding recombinant was selected from each crossing of the PR8 virus with a field isolate. Consequently, a systematic correlation of viral genotype with the high yield phenotype was not possible. In addition, a precise correlation between titer and genotype was hampered by the different passage histories of the recombinants and field isolates used in this analysis.

Previously, it was shown for recombinants of PR8, an H1N1 virus, and A/HK/8/68, an H3N2 virus, that the PR8 virus M and NP genes were associated with the high yield property (Schulman and Palese, 1978). Crossing PR8 virus with another H3N2 virus, A/England/69, Oxford et al., (1978) suggested that the transfer of the PR8 virus P2 and NP genes into recombinants resulted in increased yields in eggs. However, in that analysis only seven of the eight influenza virus genes were considered because the M genes of the two parents were not distinguishable by the techniques used. Since different genes have been associated with enhanced growth in embryonated hens' eggs, it is possible that high yield is a polygenic trait. In addition, different genes or combinations of genes may be responsible for the increased growth capacity of recombinants derived from different parental viruses.

The fact that all but one of the highest yield recombinants in this study contain the greatest number of PR8 genes suggests that several PR8 genes may function in an additive manner in the enhancement of viral replication in the chicken embryo. For example, it is possible that the addition of the P1 and P3 genes of PR8 virus to the X-57 recombinant in which these genes are not present (table 4) would result in a higher yielding virus. However, without a study of all possible gene combinations for each parental pair, the optimal gene combination for high yield cannot be definitively established.

Although it seems probable that mutations have occurred in all of the PR8 virus genes in the course of adapting it for maximal replication in the chicken embryo, selection of such mutations most likely has been influenced by the interdependence of these genes as they function together. For example, the combination of genes optimally functional in the PR8 parental virus may be less efficient in a recombinant which contains different HA and NA genes. Furthermore, it is apparent that the nature of the viral HA can be restrictive with respect to yield because the X-53 and X-53a recombinants contain the same six PR8 genes (table 4) but grow to different titers (Kilbourne et al., 1978a).

Since the precise gene constellation responsible for the high yield phenotype of each recombinant is not known, the practical goal of recombination for the production of high-yield vaccine strains may be to combine the HA and NA genes of the currently circulating virus with the 6 nonsurface-protein genes of PR8 virus. Similar approaches have been used to transfer the attenuated virulence of ts- and cold adapted-mutants of influenza viruses into recombinants constructed for live virus vaccine production (e.g. see Markoff et al., 1979; Lazar et al., 1980).

9.2 GENETIC VARIATION AMONG H2N2 INFLUENZA A VIRUS GENES

In this second project, oligonucleotide mapping was used to estimate the genetic variation of five influenza A viruses isolated at the beginning, middle and end of the H2N2 pandemic period. It was demonstrated that mutations occurred in all the genes (gene mixtures) of these viruses. Furthermore, the extent of genetic variation was shown to be similar for all the influenza virus genes. Since changes did not preferentially occur among genes coding for surface glycoproteins, it is likely that mechanisms in addition to selective antibody pressure may result in genetic variants.

A similar observation was made by Young et al. (1979), when it was found that mutations were not limited to the HA and NA genes of naturally occuring H1N1 influenza A viruses. Recently, the genetic variability of influenza A viruses from the H3N2 pandemic period was also examined by oligonucletide fingerprint analysis (Ortin et al., 1980). These investigators examined the genomes as well as the individual HA, NA and NP RNAs of viruses isolated from 1968 to 1977 and also estimated that similar differences occurred among all the corresponding RNAs studied.

As the time between the isolation of different influenza virus strains increases, the extent of variation among their corresponding genes should also increase if it is assumed that the mutation rate of all influenza virus genes is the same. The data presented in this study do not, in every case, support this premise (see tables 6-10). For example, the percent difference between the M genes of the A/Sing/1/57 and A/AA/6/60 viruses is 1.82 whereas that between the A/Sing/1/57 virus and the later isolate A/TW/1/64 is 0.78. This phenomenon may reflect the limitations of RNA fingerprint analysis (to be discussed later) in estimating genetic variation or it may result from the small sampling of a heterogeneous population of viruses for analysis.

It is possible that genetically divergent strains of the same subtype are cocirculating in nature and that limited sampling of these strains over a period of time would select viruses derived from different clonal lines. The comparison of these viruses, which may have different passage histories, could result in apparently spurious values when their extent of genetic variation is estimated and correlated with their time of isolation. Furthermore, it is also possible that these cocirculating genetic variants may reassort their genes and thereby perturb a linear increase in their observable genetic "drift". That recombination does occur among different viruses cocirculating during an interpandemic period has been demonstrated (Young and Palese, 1979). These investigators showed that several genes of an H1N1 isolate derived from a cocirculating H3N2 virus. Finally, in addition to reassortment, one cannot preclude the possibility that other mechanisms, such as intragenic recombination, also contribute to the nonlinear increase in variation that was observed among the genes of the H2N2 viruses.

The oligonucleotide map of the H3N2 (A/HK/8/68) virus HA gene lacks 26 of the 33 oligonucleotides seen in that of the H2N2 (A/Sing/1/57) virus HA gene. Furthermore, the number of oligonucleotides in the map of the H3N2 virus HA gene is approximately half of what is observed in the HA gene maps of the five H2N2 strains. These findings indicate that

the difference between the sequences of the H3 and H2 HAs is too great to be determined by comparative oligonucleotide map analysis (see below). Recently, sequence analysis has permitted the direct comparison of an H2 HA with two different H3 HA genes (Gething et al., 1980; Min Jou et al., 1980; Verhoeyen et al., 1980). These studies revealed that there was only a 27% sequence homology between the H2 HA and either of the H3 HAs, whereas the sequences of the two H3 HAs shared 96% homology. The sequence data also showed that the H3 HAs had a 20% G content but that of the H2 HA was only 18%. Although a 2% increase in G residues would be expected to reduce the number of large (10 residues or greater), T1 resistant oligonucleotides in an RNA, it can not by itself account for the 50% reduction in oligonucleotides found in the RNA fingerprint of the H3 HA when it was compared to that of an H2 HA. It is therefore likely that the distribution of G residues in the RNA of the H3 HA contributes to its relatively low number of large T1 resistant oligonucleotides.

Oligonucleotide map analysis of the RNAs from five H2N2 viruses and one H3N2 virus has demonstrated that all genes but the HA RNA of the A/HK/8/68 (H3N2) virus are closely related to those of the H2N2 strains. This finding confirms the proposal that the H3N2 strains derived from a recombinational event that replaced the HA gene of an H2N2 strain with the novel H3 HA gene (Scholtissek et al., 1978a).

As mentioned above, RNA fingerprint analysis has limitations when used to quantitate differences among viral RNAs. Only a fraction of the RNA can be directly observed in its fingerprint map and only the large oligonuceotides in the map provide reasonable assurance that each "spot" represents a unique sequence. It should be noted that occasionally, two large oligonucleotides with different sequences may comigrate in a fingerprint if they happen to have the same size and base composition. However, if one compares closely related RNAs, the frequency of such instances should be low. As described in the "Results" section, it is also possible for one mutation in an RNA to result in two changes of its fingerprint if the mutation is one that alters the position of an oligonucleotide within the map. One other limitation to RNA fingerprint analysis is that all oligonucleotides may not be equally labeled and consequently those with less radioactivity may not be included in a comparative analysis. The above mentioned factors indicate that oligonucleotide map analysis is limited to providing estimates rather than absolute determinations of variation among closely related RNAs.

Despite its limitation, oligonucleotide map analysis provides a rapid and efficient means of comparing the sequences of a large number of related RNAs. This technique has been shown to be reliable in determining the variation among RNAs that share 90% or greater sequence homology

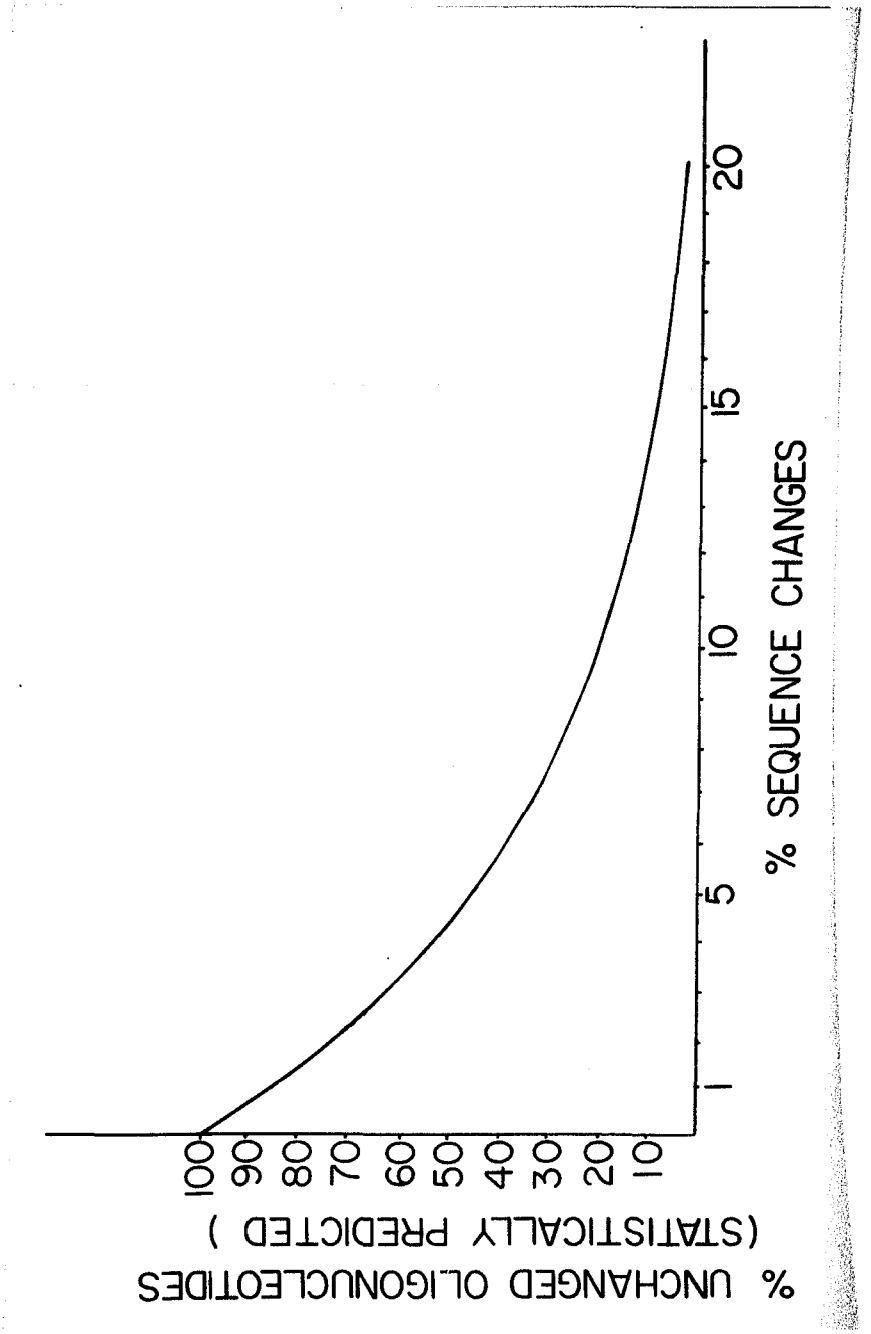
(Young et al., 1981). Based on these authors' computer analysis of random mutations in a randomly generated nucleotide sequence, RNAs having less than 90% of their sequences in common would be expected to yield completely different fingerprints (see figure 20). In the present study, the corresponding genes and gene mixtures of the earliest (A/Sing/1/57) and latest (A/Ned/84/68) H2N2 isolates yielded RNA fingerprints that had a minimum of 47% of their oligonucleotides in common. Clearly the corresponding RNA sequences of these viruses are sufficiently similar to be within the range of sensitivity required for a reliable oligonucleotide map analysis of their differences. In the analysis of the HA genes, approximately 570 of 1760 residues were examined (29% of the HA gene) and in the analysis of the P genes, approximately 700 of 7070 residues were used (10% of the P genes). In addition, all gene fractions analyzed by RNA fingerprinting consisted of large oligonucleotides (10 residues or longer) that, in most instances, should have represented unique sequences.

It is possible that a disproportionate number of large oligonucleotides in a fingerprint may derive from a hypervariable or conserved region of the influenza virus genome. However, since large gene fractions were examined in this study, it is unlikely that they derive from a continuous segment in the viral genome. Therefore it is assumed that

FIGURE LEGEND

Figure 20. Percent of unchanged oligonucleotides in a random RNA sequence as a function of increasing base changes.

The statistical number of unchanged oligonucleotides 16 bases in length was calculated for a given (random) nucleotide sequence following the introduction of random sequence changes. (This figure is taken from Young et al., 1981.)


% UNCHANGED OLIGONUCLEOTIDES
(STATISTICALLY PREDICTED)
100
90
80
70
60
50
40
30
20
10
1
5
10
15
20
% SEQUENCE CHANGES

the viral RNAs contain RNAase T1 resistant oligonucleotides throughout their nucleotide sequences. Finally, the average of the minimum and maximum possible changes between two RNA fingerprints was used to express their estimated variation. This was done in an attempt to correct for the possibility that two oligonucleotide map changes may result from one mutation. It should be noted that the percent error for the estimates of genetic varition reported in this study were not determined. However, an indication of the sensitivity of RNA fingerprint analysis in determining sequence differences among RNAs was obtained when the RNAs of the PR8 and Udorn virus NS genes were compared by this technique (Young et al., 1981). An estimated nucleotide difference of 5-6% was predicted for the PR8 and Udorn virus NS RNAs which by direct sequence analysis were shown to have an actual sequence difference of 8.9%.

Variation was demonstrated among all the genes of several H2N2 viruses. Furthermore, a maximum variation of three or four changes per hundred nucleotides has been estimated for all the genes of these influenza viruses. It is believed that the mutational changes estimated for all the H2N2 virus genes may represent the extent of genetic variation which occurred during the eleven year interval separating the isolation of the earliest and latest H2N2 strains analyzed in this study.

9.3 CLONING AND SEQUENCING OF TWO DIFFERENT INFLUENZA VIRUS NS GENES

9.3.1 STRUCTURE AND VARIATION OF NS GENES

The complete nucleotide sequences of the cloned duck and PR8 virus NS genes have been determined. A comparison of these NS gene sequences with those of the FPV (Porter et al., 1980; and Udorn (Lamb and Lai, 1980) viruses revealed that the duck virus NS gene differed by approximately 27% from the other NS genes. Despite the large number of changes detected in the duck virus NS gene, this RNA has precisely retained the NS1 and NS2 gene arrangement in the PR8 NS RNA. The duck and PR8 virus NS genes contained the same structural elements described in the Udorn virus NS RNA (Lamb and Lai, 1980). The conserved features of the duck virus as well as of the PR8 virus NS gene structures include a gene length of 890 nucleotides, overlapping reading frames which encode the NS1 and NS2 polypeptides, the putative RNA splicing signals and the presence of a polyadenylation site. By analogy to the NS gene structure of the Udorn and FPV viruses, the NS genes of the duck and PR8 viruses most likely code

for the NS1 and NS2 polypeptides. Although the duck virus NS gene has many features in common with the other NS genes, the large number of differences detected in both nucleotide and amino acid sequences indicate that this gene has diverged significantly from the evolutionary pathway that the other NS genes appear to have in common.

A study using RNA-RNA hybridization analysis to determine sequence differences among influenza virus RNAs suggested extensive genetic variation among different NS genes and a division of NS genes into one human-strain specific and two avian-strain specific homology groups (Scholtissek et al., 1976; Scholtissek and von Hoyningen-Huene, 1980). For example, this analysis showed that the NS gene of the avian strain, FPV, was in a distinct homology group from that of the human strain, PR8. In contrast, the sequence data presented here demonstrated an 8% difference between the PR8 and FPV virus NS genes and a slightly greater difference of 8.9% between the NS genes of PR8 virus and another human strain, Udorn. Consequently, the available sequence data do not permit the classification of the FPV and PR8 virus NS genes into distinguishable homology groups. However, the sequence data presented here did show a large degree of variation between the duck virus NS gene and each of the NS genes of the other viruses (approximately 27% differences were noted).

The duck virus NS gene was selected for analysis because a cDNA-RNA hybridization study showed that it shared only 12% homology with the PR8 virus NS gene (R.M. Elliott, unpublished observations) and therefore provided the opportunity to study a very different NS gene. However, the direct nucleotide sequence comparison of these two genes showed a 72.8% homology. An analogous situation has been described for the nucleotide sequence comparison of the BK and SV40 viruses (Yang and Wu, 1979). It was noted in this latter study that hybridization analysis can result in highly variable estimates of homology between two genes if different hybridization conditions and assay methods are used to detect sequence differences.

A detailed analysis of the positions of nucleotide differences between the PR8 and duck virus NS genes revealed three domains of different homology (figure 19). The first region, nucleotides 27-529, encodes the amino terminal portion (73%) of the NS1 polypeptide and is the least conserved domain. Another region, nucleotides 717-861, encoding the carboxy-terminal of the NS2 polypeptides is much more conserved. The remaining region of the NS gene, nucleotides 529-716, which directs synthesis of portions of both the NS1 and NS2 polypeptides in overlapping reading frames, separates the other two regions and is the most conserved. The conservation in this last region is reflected by a low number of amino acid changes (10/63) detected between corre-

sponding portions of the duck and PR8 virus NS2 polypeptides (figure 19). Surprisingly, this same RNA region codes for twice as many amino acid changes (20/63) in the reading frame used to synthesize the carboxy terminal portions of the NS1 polypeptides. First position changes in the codons of the reading frames used for the synthesis of the NS1 polypeptides result in third position mutations in the triplets of the reading frames coding for the NS2 polypeptides. Since the variation in all regions of the NS1 polypeptides is greater than that found between the entire NS2 polypeptides, it is probable that selective mechanisms are acting to more strictly conserve the NS2 polypeptides.

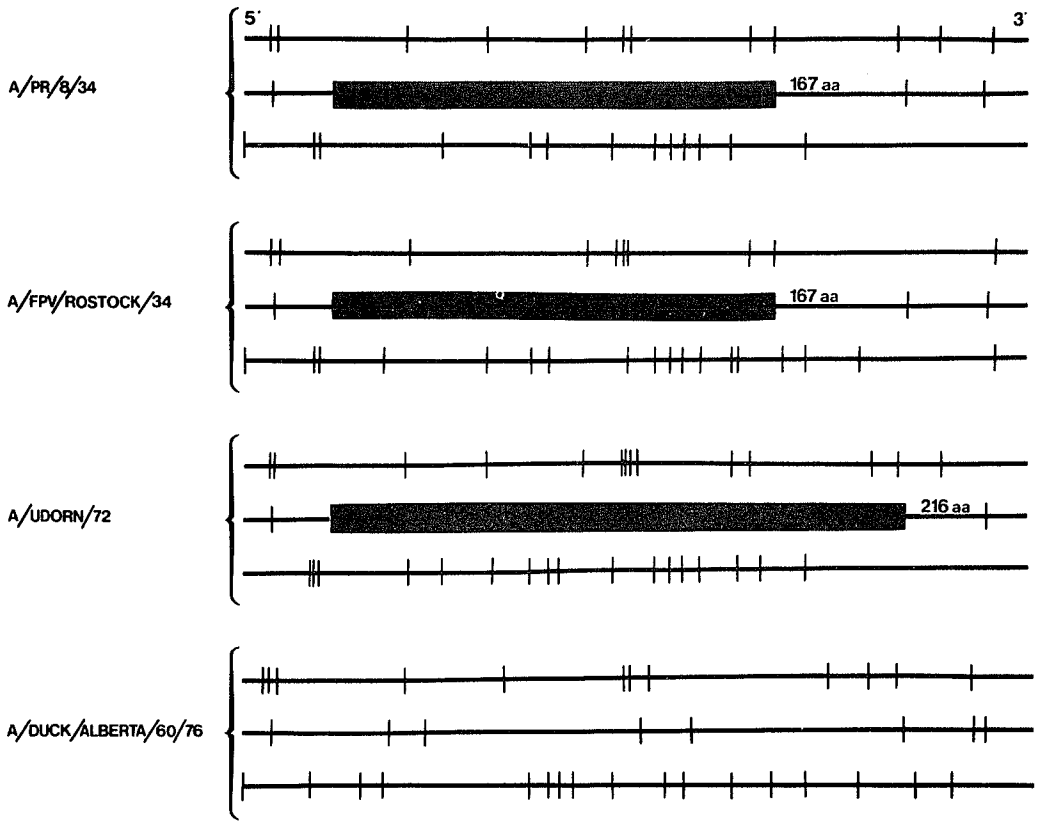
Figure 21 shows an examination of the three reading frames in the (-) RNA of four influenza virus NS genes. The PR8, FPV and Udorn viruses have the coding potential for a third NS polypeptide in the virion RNA. It seems unlikely that open reading frames spanning 501 nucleotides in the NS genes of PR8 and FPV viruses and 648 residues in that of the Udorn virus would occur by chance. The random probability of finding open reading frames of any length can be determined by assuming the random occurrence of the 64 codons, three of which are termination codons. An open reading frame of 216 codons, which was observed in the (-) RNA sense of the Udorn virus NS genes has a probability of $(61/64)^{216}$ or 3.13×10^{-5} of occurring by chance. If one restricts the selection of such open reading frames to those that start

FIGURE LEGEND

Figure 21. Examination of open reading frames in the virion (-) RNA of four influenza virus NS genes.

The virion (-) RNA of four influenza virus NS genes were examined for the presence of open reading frames starting with an AUG. Verticle lines indicate the positions of termination codons and the long dark rectangles represent open reading frames of 501 and 648 nucleotides, respectively.

READING FRAMES IN NS SEGMENTS (−RNA SENSE)



with an AUG (1 of 64 codons) and end with a termination codon (3 of 64 codons), the probability that one occurs by chance becomes $1/64 \times (61/64)^{215} \times 3/64$ or 2.40×10^{-8}. Finally, since there are 81 possible starting positions for 216 contiguous codons in the NS gene, the random probability of finding the one described above increases to 1.59×10^{-6}. A similar computation for the random occurrence of the open reading frame (167 codons long) in the (-) RNA of the PR8 or FPV virus NS gene yields a value of 3.29×10^{-5}. In contrast, the probability of randomly finding an open reading frame of 100 codons in an NS gene is only 1.24×10^{-3}. Using random probability one would also predict the occurrence of 8 and 11 termination codons in regions containing 167 and 216 triplets respectively. This prediction is confirmed by the observation of 6-14 termination codons within the other reading frames that span the presumptive NS3 genes.

Subsequent to these studies another group of investigators independently cloned and sequenced the NS gene of PR8 virus with a different passage history (Winter et al., 1981). Their sequence contained ten nucleotide differences and four amino acid changes when compared to the PR8 virus NS gene sequence determined by the present study. Nevertheless, the DNA sequence reported by this group also showed an agnogene sequence in the (-) RNA sense of the NS gene. The

putative polypeptides encoded by these open reading frames have not been identified in influenza virus infected cells or in purified viruses. It should also be noted that translation of viral proteins from the virion strand RNA would require a unique mechanism which has not yet been identified in negative strand viruses. Influenza virion RNA has been shown to lack cap structures and poly-A tails. Only the viral mRNAs (which contain these structural features) have been shown to yield authentic viral polypeptides in cell-free translation systems (Etkind and Krug, 1974, 1975; Krug et al., 1976; Content, 1976; Ritchey and Palese, 1976; Etkind et al., 1977; Inglis et al., 1977; Stepehenson et a ., 1977; Plotch et al., 1978). One possible exception was reported by Tekamp and Penhoet (1976). These investigators found that a novel protein, similar in size to the NP, appeared to be translated from influenza virion RNA in a cell-free, wheat germ, translation system. However, they could not rule out that the synthesized polypeptide might have derived from contaminating host cell mRNA. (That an NP-like polypeptide could be translated from influenza virus vRNA was earlier shown with an E.coli, cell-free, translation system; Siegert et al., 1973.)

An examination of the (-) RNA sequence of the duck virus NS gene did not reveal an open reading frame similar to those previously identified in the NS genes of the other three (four) strains. Instead there were four termination

codons within the region of the reading frame expected to contain the agnogene sequence. Unless a specific splicing mechanism to circumvent the stop signals is invoked, it appears that the duck virus does not have the coding potential for a third NS polypeptide. This may indicate that the presence of the open reading frames in the (-) RNAs of the three other NS genes is fortuitous and has no biological significance, despite the low statistical probability that the presence of the NS agnogene is a chance event. However, at the present time it has not been determined whether the a sence of the open reading frame in the duck virus NS gene is specific for the DNA clone analyzed or if its absence is specific for the viral isolate used in this study. Should subsequent analyses using cells infected with PR8, Udorn and other influenza viruses establish the existence of an NS3 polypeptide, it would be apparent that the NS3 polypeptide is not essential for the replication of the duck virus.

On the basis of its extensive genetic difference from the NS genes of the PR8, FPV and Udorn viruses the evolution of the duck virus NS gene appears to have diverged from that of the other three NS genes. It is possible that during its evolution the duck virus NS gene may have lost the coding capacity for a third polypeptide and its agnogene has become a pseudogene analogous to those described in the mouse and human globin gene families (e.g. see Leder et al., 1980; Proudfoot et al., 1980). Conversely, it may be that the

other three NS genes evolved an open reading frame encoding a third polypeptide that provided a selective advantage to the viruses. If the putative NS3 gene is expressed in some but not all viruses, its role maybe associated with the host specificity of influenza viruses.

Subsequent to this study, Casino et al., (1981) have also identified agnogene sequences in the nonmessage strand of the human β- and ϵ-globin genes. In particular it was shown that the agnogene sequence in the human ϵ-globin gene contained all the appropriate signals necessary for the transcription and translation of a spliced eukaryotic gene. Futhermore, the authors noted that the ϵ-globin gene sequence could not be distinguished from the agnogene sequence in the absence of amino acid sequence data.

9.4 SIGNIFICANCE OF THIS WORK.

The genotyping study has augmented earlier evidence that introduction of PR8 virus genes by recombination into low yielding field isolates is a rapid and efficient means of obtaining high yielding vaccine strains of desired serotype (Kilbourne et al., 1971; Palese et al., 1976). The precise gene composition of eleven vaccine strains was bio-

chemically determined and the presence of PR8 genes was associated with enchanced growth in embryonated hens' eggs. In particular, the presence of the PR8 virus M gene was consistently noted in the strains selected for high yield. This study indicates that it is feasable to use the available biochemical techniques to establish the content of PR8 genes in recombinant strains. The routine use of such procedures should greatly facilitate the selection of vaccine strains that have a desired serotype and a precisely defined genotype.

In the second study, oligonucleotide map analysis was used to estimate the variation among influenza A viruses isolated over the 11 year duration of the H2N2 subtype period. Additonal evidence was provided (Young and Palese, 1979) that variation occurs among all the genes in influenza viruses. Furthermore, it was for the first time demonstrated that the maximum extent of variation was similar for all the genes of several H2N2 viruses. This finding provides some understanding as to the degree of change that influenza viruses can undergo during a subtype period. Since it was shown that the genes coding for nonsurface proteins of influenza virus appeared to change to the same extent as the HA and NA genes, this study further supports the proposal that mechanisms in addition to antibody selection may be responsible for the emergence of genetic variants.

In the final study molecular cloning and DNA sequence analysis was used to examine the NS genes of an avian (duck) and a human (PR8) influenza virus. In addition, the sequences of these genes were compared with those of two other NS genes (Porter et al., 1980; Lamb and Lai, 1980) to directly determine the extent of genetic variation among four different NS genes. The duck and PR8 virus NS gene sequences confirmed that the NS1 and NS2 polypeptides are coded for by overlapping reading frames in the smallest RNA segment of the influenza virus genome (Lamb and Lai, 1980). A surprising discovery resulting from this study was that the (-) RNA sense of the PR8 virus NS gene has the coding potential for a third polypeptide and that the Udorn and FPV virus NS gene sequences also have this coding potential in the (-) RNA sense. The occurence of an open reading frame starting with an AUG and extending for 167-216 codons in the nonmessage sense of a gene appears to be a unique feature. However, the significance of the agnogenes in the NS genes is not yet known. If a functional role can be ascribed to these open reading frames, another mechanism for the extension of the coding capacity of a gene will have been described.

The analysis also showed that the duck virus NS gene is a divergent RNA segment because its nucleotide sequence contained a large number of differences when it was compared to those of the PR8, FPV and Udorn virus NS genes. An addi-

tional difference between the duck and PR8 virus NS genes is that the duck virus NS gene does not have an open reading frame in its (-)RNA sense. It is hoped that the present sequence characterization of two very different NS genes will provide the impetus for further studies on the putative NS3 polypeptide. For example, the deduced amino acid sequence of the predicted NS3 polypeptide is serine rich and therefore, the predicted NS3 polypeptide may be detected by polyacrylamide gel electrophoretic analysis of influenza virus polypeptides labeled with ^{14}C-serine *in vivo*. Alternatively, one might synthesize an oligopeptide of the deduced NS3 polypeptide to raise specific antibody which can be used to precipitate the NS3 polypeptide from a lysate of influenza virus infected cells. (Sutcliffe et al., 1980, have used this latter strategy to identify the polypeptide produced from an agnogene which they identified in a retrovirus.) These suggested approaches may help to establish whether an NS3 polypeptide exists and whether the duck virus NS gene analyzed in this investigation is unique or representative of a second class of NS genes.

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