THE MOLECULAR BIOLOGY OF INFLUENZA VIRUS PATHOGENICITY

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I. INTRODUCTION

Influenza viruses are classified into types A, B, and C, of which the influenza A viruses appear to be the most important disease agents. Influenza A viruses have been isolated from humans, horses, swine, mink, seals, and whales as well as from a great variety of different avian species. There are striking differences in the type of disease resulting from influenza virus infection in the different species. Although there are variations in the severity of illness, in mammals infection is usually local and confined to the respiratory tract. The ferret has provided a suitable model to study this type of infection (Smith and Sweet, 1984). The majority of the avian influenza viruses

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also cause local infection in the respiratory tract or in the gut, which frequently remains asymptomatic. In contrast, other avian strains are highly pathogenic, inducing systemic infection that regularly leads to the death of the animal. This type of disease is designated fowl plague. The avian system is particularly useful for pathogenicity studies because a large number of naturally occurring virus strains can be analyzed in their natural host.

Disease resulting from virus infection is a complex event depending on the close interaction of viral and cellular factors. Through the application of biochemical and genetic methods, it is now possible to gain an insight into the molecular basis of these interactions. In the following discussions we will make an attempt to review current knowledge on the contribution of the individual genes of influenza virus to pathogenicity. Emphasis will thereby be put on the mechanisms underlying virus replication. Other important aspects of pathogenesis will not be covered, such as the role of immunity and of antigenic variation, which has been the topic of another contribution to this series (Air and Laver, 1986). Furthermore, rather than collecting data on cytopathogenicity we will concentrate on the ability of the virus to induce disease in the organism.

It will become clear that, although several genes may contribute to pathogenicity, certain genes definitely play a more important role than others. The most clearly defined determinant of influenza virus pathogenicity is hemagglutinin. A large part of this review will therefore be devoted to studies of this gene product.

II. Viral Components, Structure, and Function

The genome of influenza A and B viruses consists of eight segments of single-stranded RNA. The RNA of the virus has negative polarity, i.e., it is not infectious, and the mRNAs from which the proteins are translated are transcribed from the virion RNA by the virion-associated RNA polymerase (transcriptase). The eight gene segments code for the seven proteins forming the virus particle (PB1, PB2, PA, HA, NA, NP, and M1) and for at least three nonstructural proteins which are found only in the infected cell (M2, NS1, and NS2) (for review, see Lamb, 1983). An additional nonstructural protein, NB, is found only with influenza B virus. The nucleotide sequence of the complete genome has been established for several strains, and considerable information has been obtained on the structure and function of the proteins, which will be briefly reviewed in the following sections.

A. *The Polymerase-Associated Proteins PB2, PB1, and PA*

Each of the three largest RNA segments codes for one of the different polymerase molecules (PB2, PB1, and PA) that, along with the nucleocapsid protein and virion RNA, comprise the nucleocapsid of the virus. The functions of the polymerase proteins with regard to RNA transcriptase activity have been partially defined (Krug, 1983). The initial step of transcription is the binding of polymerase to capped mRNAs of the host, which are used to generate primers for viral mRNA. This step is carried out by the PB2 protein. Cross-linking experiments have also shown that the PB1 protein is associated with initiation of transcription and chain elongation (Ulmanen *et al.*, 1981, 1983; Blaas *et al.*, 1982). No function has yet been attributed to the PA protein, although during transcription a complex of the three polymerase proteins moves down the elongating mRNA (Braam *et al.*, 1983).

B. *Hemagglutinin*

The gene product of the fourth RNA segment is hemagglutinin. This glycoprotein is of considerable biological interest, because it is the major target for the protective immune response of the host and because it plays a key role in the entry of the virus into the cell. It has therefore been analyzed in great detail.

As an integral membrane protein hemagglutinin is translated at membrane-bound polysomes, translocated by means of an amino-terminal signal sequence into the lumen of the endoplasmic reticulum, and transported from there through the Golgi apparatus to the plasma membrane. In the course of transport hemagglutinin undergoes a series of co- and posttranslational modifications. These include, in the rough endoplasmic reticulum, the removal of the signal sequence and the attachment of N-glycosidically linked oligosaccharide chains and, in the Golgi apparatus, remodeling of the oligosaccharides and proteolytic cleavage of the precursor HA into the amino-terminal fragment HA1 and the carboxy-terminal fragment HA2 (Klenk and Rott, 1980). Another modification involves the attachment of fatty acid to HA2 (Schmidt, 1982). The problem as to whether acylation occurs in the rough endoplasmic reticulum or in the Golgi apparatus has not yet been resolved (Berger and Schmidt, 1984).

The complete nucleotide sequence of the hemagglutinin gene has been obtained for the subtypes H1, H2, H3 (for review, see Lamb, 1983), H5 (Kawaoka *et al.*, 1984), H7 (Porter *et al.*, 1979; Garten *et al.*,
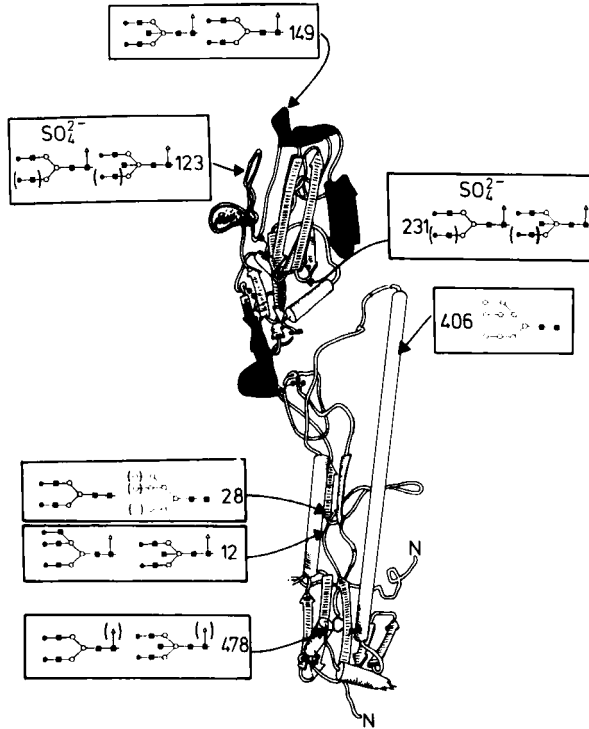
FIG. 1. The three-dimensional structure of hemagglutinin of influenza A virus. The model of Wilson *et al.* (1981) is shown. The shaded areas indicate antigenic sites. The glycosylation sites and the structure of the individual carbohydrate side chains as determined for the hemagglutinin of A/FPV/Rostock/34 are also indicated (Keil *et al.*, 1985).

1985), and H10 (Feldmann *et al.*, 1988). The primary structure of the hemagglutinin of subtypes H2 and H3 has also been elucidated by amino acid sequencing (Waterfield *et al.*, 1979; Ward, 1981).

X-Ray crystallography has revealed that the hemagglutinin spike is a trimer containing three HA1,2 subunits (Fig. 1). Each subunit comprises two structurally distinct regions, HA2 forms a triple-stranded coiled coil of α-helices extending 76 Å from the membrane, whereas HA1 forms a globular domain of antiparallel β-sheets, which is positioned on top of the stem and contains the variable antigenic determinants responsible for the characteristic recurrence of influenza infections in humans. Each subunit has a looplike topology, starting at the membrane, extending 135 Å distally, and folding back to enter the membrane (Wilson *et al.*, 1981; Wiley *et al.*, 1981). A sequence of 25–

32 hydrophobic amino acids near the C-terminus of HA2 serves to anchor the hemagglutinin spike in the virus membrane.

The oligosaccharides attached to individual glycosylation sites are either complex or oligomannosidic, with the latter type being specifically localized in niches at interfaces between different domains (Keil *et al.*, 1984, 1985). None of these carbohydrate structures is unique to the hemagglutinin of influenza virus. They are also present in a large variety of other membrane and secretory glycoproteins. This observation supports the concept that it is not so much the specific structure of the oligosaccharides that contributes to the antigenic properties of hemagglutinin, but their position on the polypeptide. Evidence has been obtained that immune recognition of an antigenic site of hemagglutinin is modulated by the presence or absence of an oligosaccharide (Skehel *et al.*, 1984). It is therefore tempting to speculate that the acquisition of oligosaccharides antigenically indifferent to the host is one of the mechanisms underlying antigenic drift and, thus, helps the virus to escape the immune defense of the host organism (Keil *et al.*, 1985). In contrast to these variable oligosaccharides, there are other side chains that are highly conserved. They are located predominantly at the base of the spike, and it is conceivable that they are important in maintaining the hemagglutinin structure.

Hemagglutinin plays an essential role in the initiation of infection, and it is now clear that it has two different functions in this process. It has long been known that it is responsible for the attachment of the virus to neuraminic acid-containing receptors at the cell surface. The receptor binding site is located in the globular region of the hemagglutinin spike. More recently it has become apparent that hemagglutinin is also involved in penetration by triggering fusion of the viral envelope with cellular membranes. The first indication that hemagglutinin possesses a function in addition to adsorption came from the observation that cleavage of the precursor HA, while irrelevant for adsorption, is necessary for infectivity (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975). Evidence has since been obtained that cleavage is necessary for the fusion capacity (Nicolau *et al.*, 1978; Kurrle *et al.*, 1979; Huang *et al.*, 1980a). The amino terminus of HA2 that is created in the cleavage reaction has attracted special attention as the crucial site for this activity. It is hydrophobic, highly conserved among the hemagglutinin subtypes, and shows structural similarity with the amino terminus of another fusion factor, the F1 polypeptide of paramyxoviruses (Gething *et al.*, 1978; Scheid *et al.*, 1978). Furthermore, there is evidence that, in addition to hydrophobicity, a specific amino acid sequence in this region is also required for fusion (Richard-

son *et al.*, 1980; Garten *et al.*, 1981). The fusion capacity of hemagglutinin is expressed only at low pH (Maeda and Ohnishi, 1980; Huang *et al.*, 1981a; White *et al.*, 1981), and it has been shown that under these conditions the molecule undergoes a conformational change (Skehel *et al.*, 1982). It is reasonable to assume that the amino terminus of HA2 is exposed in such a way that it can insert into the target membrane, thereby forming a bridge between the two membranes. Alternatively, exposure of the hydrophobic amino termini of HA2 may result in an aggregation of hemagglutinin in the plane of the membrane and in the concomitant formation of areas of the viral envelope devoid of protein. Both mechanisms could result in the close apposition of the lipid layers of the cellular and viral membranes and thus facilitate their fusion. The dependency on low pH has led to the concept that the fusion of the influenza virus envelope takes place in endosomes (White *et al.*, 1981). However, there is also evidence that fusion may occur at the plasma membrane (Kurrle *et al.*, 1979; Huang *et al.*, 1981b).

C. The Nucleocapsid Protein

RNA segment 5 encodes the nucleocapsid protein (NP). It is the major constituent of the nucleocapsid, and, according to electron microscopic studies, has a helical left-handed configuration (Compans *et al.*, 1972). Nucleotide sequence analysis has revealed that the protein is rich in arginine and has a net positive charge at pH 6.5 (Winter *et al.*, 1981; van Rompuy *et al.*, 1981; Huddleston and Brownlee, 1982). Since there are no clusters of basic residues, it appears that the RNA is associated with many regions of the NP molecule to neutralize the charges. Based on the total length of the influenza virus genome and the number of NP molecules associated with one virus particle (Compans and Choppin, 1975), it can be estimated that approximately 20 nucleotides interact with a single protein subunit. It is assumed that the RNA is exposed at the outside of the ribonucleoprotein (RNP) structure, since it can be displaced by polyvinyl sulfate (Pons *et al.*, 1969) and is susceptible to digestion with ribonuclease without disrupting the RNP structure (Schäfer and Wecker, 1958; Duesberg, 1969). *In vitro* studies have shown that NP forms complexes equally well with viral and cellular RNAs (vRNA and cRNA) (Scholtissek and Becht, 1971). This might explain why after disrupting infected cells, vRNA and cRNA were found in nucleocapsid structures (Pons, 1971; Krug, 1972).

After its synthesis in the cytoplasm the NP protein migrates to the cell nucleus, where it can be identified by fluorescent antibodies. Later

in the infection cycle it accumulates also in the cytoplasm (Breitenfeld and Schäfer, 1957). In abortively infected cells (Franklin and Breitenfeld, 1959) or under von Magnus conditions NP is seen only in the nucleus (Rott and Scholtissek, 1963). Since the NP protein interacts in the nucleocapsid not only with itself and the RNA but also with the three P proteins, it is reasonable to assume that it is part of the transcriptive and replicative complex. However, it is not clear whether it has a catalytic or only a structural function in this complex. NP is a phosphoprotein (Privalsky and Penhoet, 1977). The phosphorylation pattern varies depending on the host and on the virus strain. Although the role of the phosphate groups is not clear, it has been speculated that these variations may account for differences in host range and pathogenicity (Kistner et al., 1985). NP is the type-specific antigen by which influenza A, B, and C viruses can be distinguished from one another. However, minor differences in antigenicity have also been observed among the NP proteins of different influenza A viruses (Davenport et al., 1960; Schild et al., 1979). The nucleocapsid protein also appears to play a major role in cell-mediated immunity, since it has been shown that the NP gene controls the induction of both subtype-specific and cross-reactive T cells (Towsend and Skehel, 1984; Fleischer et al., 1985). This observation implies that the nucleocapsid protein is exposed at the surface of the infected cell. The mechanism by which this is accomplished is obscure.

D. Neuraminidase

The second glycoprotein of the influenza virus envelope is neuraminidase (NA), which is encoded by the sixth RNA segment of the viral genome. The nucleotide sequence of the NA gene has been elucidated with the N1 and the N2 subtypes of influenza A virus (Fields et al., 1981; Hiti and Nayak, 1982; Markoff and Lai, 1982; Bentley and Brownlee, 1982; Steuler et al., 1984) and with influenza B virus (Shaw et al., 1982). These studies have shown that the protein contains close to its amino terminus a single hydrophobic region that is long enough to span the lipid bilayer of the virus or the cell. That this region is inserted in the lipid membrane has been confirmed by protein sequencing studies of the intact molecule and of its ectodomain liberated by protease treatment (Blok et al., 1982). Neuraminidase therefore differs from hemagglutinin not only by its opposite membrane orientation, but also by a signal that is not removed proteolytically and serves as a membrane anchor. Posttranslational proteolytic cleavage, necessary for the biological function of hemagglutinin, does not occur with neuraminidase. The intracellular transport of neuraminidase has

not been analyzed as extensively as that of hemagglutinin, but it is believed that both glycoproteins follow the same pathway from the rough endoplasmic reticulum via the Golgi apparatus to the plasma membrane (Compans, 1973; Klenk *et al.*, 1974).

The mature neuraminidase spike is a tetramer with a box-shaped head, $100 \times 100 \times 60$ Å, made out of four coplanar and roughly spherical subunits, and a centrically attached stalk containing the membrane anchor. The heads can be removed by protease treatment (Drzeniek *et al.*, 1966; Wrighley *et al.*, 1977) and the three-dimensional structure of the heads of the N2 neuraminidase has been determined by X-ray crystallography at 2.9 Å resolution (Varghese *et al.*, 1983). Each monomer is composed of six topologically identical β-sheets arranged in a propeller formation. The tetrameric enzyme has circular fourfold symmetry stabilized in part by metal ions bound on the symmetry axis. The biologically important domains, i.e., the catalytic sites and the antigenic determinants, are located in the head (Drzeniek *et al.*, 1966). The catalytic sites are located in deep clefts which occur on the upper corners of the box-shaped tetramer. Sugar residues, which are all of the N-glycosidic type, are attached to four of the five potential glycosylation sites.

Neuraminidase was discovered more then 40 years ago as an enzyme that destroys the receptors for the virus on erythrocytes (Hirst, 1942). Later it was recognized that it hydrolyzes the glycosidic bond between the keto group of neuraminic acid (sialic acid) and adjacent sugars, mostly D-galactose or D-galactosamine, in glycoproteins and glycolipids (Klenk *et al.*, 1955; Gottschalk, 1957). The biological role of neuraminidase is still unclear, although a number of different concepts have been proposed for its function in virus replication. It has been assumed that neuraminidase permits transport of the virus through mucin in the respiratory tract, thus allowing the virus access to the target epithelial cell. Similarly, in systemic infection the enzyme may prevent the virus from being trapped by serum inhibitors. Since the enzyme is responsible for the absence of neuraminic acid on the surface of virus particles (Klenk *et al.*, 1970), it may prevent self-aggregation of the virions and, by the same mechanisms, promote release of budding virus from the host cell membrane (Seto and Rott, 1966; Palese *et al.*, 1974). Finally, evidence has been obtained with reconstituted viral envelopes that neuraminidase may also be involved in the fusion process, permitting virus penetration (Huang *et al.*, 1980b). The neuraminidase of influenza A viruses has been found to differ in substrate specificity from the enzymes of paramyxoviruses and bacteria (Drzeniek, 1972). It should be most interesting to analyze whether such differences exist also among the neuraminidases of different influ-

enza A and B viruses and whether they contribute to cell tropism, host range, and spread of infection.

RNA segment 6 of influenza B virus codes for two distinct gene products, using overlapping reading frames. Thus, in addition to NA a second glycoprotein, NB, is created (Shaw *et al.*, 1982). NB contains four potential glycosylation sites and has been found to be glycosylated in infected cells. It has not been observed in virions but is present on the surface of infected cells (R.A. Lamb, personal communication). A protein analogous to NB has not been found in influenza A virus.

E. The Membrane Protein M1 and the Nonstructural Protein M2

Gene segment 7 generates three separate mRNA species. The first one is a colinear transcript and codes for the membrane protein M1. The second one is generated by splicing and codes for the nonstructural protein M2. The third one is also a spliced mRNA for which no translation product has yet been identified (Lamb *et al.*, 1981).

The membrane or matrix protein M1 is the most abundant protein in the virion and is present in large enough amounts to form a shell beneath the lipid bilayer (Compans *et al.*, 1972; Schulze, 1972). The elucidation of the nucleotide sequence of RNA segment 7 has revealed that a region of hydrophobic amino acids exists in the middle of the molecule which could be involved in hydrophobic interactions with either protein or lipid (Winter and Fields, 1980; Lamb and Lai, 1982; McCauley *et al.*, 1982). In addition to providing structural stability to the virion envelope, M1 may recognize the viral glycoproteins and form a domain on the inner surface of the plasma membrane, which subsequently provides a binding site for the ribonucleoprotein segments during virus assembly (Choppin *et al.*, 1972). This concept was supported by genetic studies analyzing reassortants of different influenza A strains, in which it was demonstrated that the genes for hemagglutinin and the M1 protein in general did not segregate (Scholtissek *et al.*, 1976; Rott *et al.*, 1979). M1 has also been observed in the nucleus, and it will be interesting to see whether it is also therein associated with the NP protein (Giesendorf *et al.*, 1986).

M1 is synthesized relatively late in the infectious cycle, and it seems to become rate limiting for virus maturation. There is a specific underproduction of M1 in abortively infected cells, which is thought to be responsible for the lack of virus maturation in these cells (Bosch *et al.*, 1978; Valcavi *et al.*, 1978; Lohmeyer *et al.*, 1979). M1 also appears to govern the sensitivity of the virus to the antiviral drug amantidine (Lubeck *et al.*, 1978; Hay *et al.*, 1979). M1 is a type-specific antigen for the influenza A viruses and does not cross-react with M1 of the influ-

enza B viruses (Schild, 1972). With the WSN strain, M1 has been found to be phosphorylated (Gregoriades et al., 1984).

Like hemagglutinin and neuraminidase, M2 is a membrane protein. It is probably not incorporated into virions, but is abundantly expressed at the cell surface (Lamb et al., 1985). M2 has an internal hydrophobic membrane anchorage domain and is associated with the same cellular membrane fractions as the other viral glycoproteins. Of the 97 amino acids of M2, a minimum of 18 amino-terminal residues are exposed at the cell surface and about 50 carboxy-terminal residues are at the cytoplasmic side of the membrane. The available evidence indicates that M2 is not glycosylated, even though the amino-terminal region contains a potential N-glycosylation site. Transport of M2 to the cell surface resembles that of hemagglutinin, but expression studies with the cloned gene indicate that M2 can be processed independently of the other viral membrane proteins (Zebedee et al., 1985).

F. The Nonstructural Proteins NS1 and NS2

The nonstructural proteins NS1 and NS2 are both derived from RNA segment 8 via two different mRNAs. The mRNA of NS1 is a colinear transcript, whereas the mRNA of NS2 is generated by a splicing mechanism (Lamb and Choppin, 1979; Inglis et al., 1979; Briedis and Lamb, 1982). NS1 is synthesized relatively early in infection, whereas NS2 appears later (Lamb et al., 1978). NS1, which is phosphorylated in some strains (Privalsky and Penhoet, 1981), has been detected in the cytoplasm and in the nucleoli (Lazarowitz et al., 1971; Krug and Etkind, 1973). NS2 is found only in the cytoplasm (Lamb et al., 1978). Very little is known about the function of NS1 and NS2, but it has been suggested that NS1 may be involved in the shut-off of host cell protein synthesis and virion RNA synthesis (Wolstenholme et al., 1980; Koennecke et al., 1981). The paracrystalline inclusions of NS1 that are observed in the cytoplasm in some strains late in infection (Morongiello and Dales, 1977; Shaw and Compans, 1978) may have functional consequences, but are more likely simply the result of the abundance of NS1 in the dying cell.

G. The Envelope Glycoprotein of Influenza C Virus

Influenza C virus has a single envelope glycoprotein. The genome of this virus therefore consists only of seven RNA segments. Like the hemagglutinins of influenza A and B viruses, the influenza C glycoprotein is encoded by the fourth RNA segment. It is also anchored in the lipid bilayer at its carboxy-terminal end and undergoes post-

translational proteolytic cleavage, but it shows little sequence homology with the hemagglutinins of influenza A and B viruses (Nakada *et al.,* 1984; Pfeifer and Compans, 1984). The influenza C glycoprotein, in addition to having the properties of a fusion factor that is activated by the proteolytic cleavage (Herrler *et al.,* 1979) and of a hemagglutinin, is also the receptor-destroying enzyme of this virus. The glyocoprotein binds specifically to 9-*O*-acetyl-*N*-acetylneuraminic acid as a receptor (Rogers *et al.,* 1986), and the receptor-destroying enzyme is not neuraminidase but 9-*O*-acetylneuraminate esterase (Herrler *et al.,* 1985a,b).

III. Genetic Reassortment

The particular genomic organization of influenza viruses permits genetic reassortment of the eight RNA segments (genes) when a single cell is infected by two viruses. With a number of conditional lethal temperature-sensitive mutants, eight recombination groups could be defined (Mahy, 1983), and it was found that there is a random reassortment of the different RNA segments with a frequency that is expected for independent segregation of genes, i.e., 50% (Nakajima and Sugiura, 1980). This means that linkage between the RNA segments should not occur. However, of the 254 possible new gene combinations certain combinations of genes derived from a given parent were observed more frequently than expected, or not all of the possible gene combinations could be found (Lubeck *et al.,* 1978; Rott *et al.,* 1976). Furthermore, owing to intrinsic interference between some parent strains, simultaneous double infection might not be possible (Rott *et al.,* 1981). The ability to replicate depends obviously on both of the two virus strains used for reassortment and on the host cell to be infected.

During isolation and purification of virus reassortants random mutations in several viral genes might occur and may influence the phenotype (Scholtissek *et al.,* 1977; Schulman and Palese, 1978; Erickson and Kilbourne, 1980; Ogawa and Ueda, 1981). Such mutational events seem to be responsible, for example, for pathogenicity reactivation of nonpathogenic avian virus reassortants by serial passages under von Magnus conditions (Rott *et al.,* 1983b). However, it is not yet known which viral genes have been mutated under those conditions and how high the reactivation rate is. Restoration of pathogenicity is also possible as a result of extragenic suppression of ts defects in viral genes by reassortment (Scholtissek and Spring, 1981, 1982; Ghendon *et al.,* 1982). In this case the ts phenotype specified by one gene is modified

by substitution of a gene at another locus. It is, therefore, not suffi-
cient to introduce by reassortment a gene with a defect into another
virus strain and then expect this new reassortant to exhibit the corre-
sponding phenotype. The occurrence of suppressor reassortants sug-
gests a close interaction among certain virus-specific proteins. Such
pleiotropic effects have been found among several groups of virus-
specific proteins. Thus, if, for instance, two virus strains exhibit a
relatively low base sequence homology concerning the NS or M gene,
the NS gene segregates preferentially with the HA gene (Scholtissek
et al., 1976).

IV. Genome Constellation and Pathogenicity

Through genetic reassortment, biological properties of the virus can
be changed; the advantage this type of genetic interaction offers, in
spite of the exception mentioned above, is the possibility to analyze the
genes or genome constellation responsible for host range and patho-
genicity. Most studies have been performed by reassortment between
avian and mammalian influenza viruses using the easily detectable
and reliable clinical signs that occur in infected chickens. The avian
system offers the advantage of the availability of naturally occurring
avian influenza viruses that differ in pathogenicity, and the addi-
tional advantage of working with the natural host of the virus.

Employing the highly pathogenic fowl plague virus (FPV) in *in vitro*
crosses with nonpathogenic viruses of mammalian or avian origin, it
was demonstrated that pathogenicity is of polygenic nature (Rott *et al.*,
1976). These findings confirmed earlier conclusions of Burnet (1959)
that pathogenicity cannot be confined to a particular viral gene. This
view was reinforced by genetic analysis of a large number of reassort-
ants obtained after mixed infection with *ts* mutants of FPV and differ-
ent human and animal influenza viruses. It could be shown that the
acquisition of single genes not derived from the FPV parent might
restore pathogenicity for chickens. The pathogenic properties of the
reassortants are found to be determined by the particular gene that
was exchanged, as well as by the virus strain from which this gene was
derived. But clearly, pathogenic virus could be rendered attenuated by
a single gene exchange at the seven loci tested (Scholtissek *et al.*,
1977). It should be noted that replacement of the HA gene of a patho-
genic avian virus always led to loss of pathogenicity in chicken, be-
cause of the absence of a "cleavable" HA (see below). With multiple
gene replacements attenuation of pathogenicity for chickens corre-
sponds, in principle, with the number of genes which were replaced

(Rott *et al.,* 1978). The closer the genes or gene products of the parent viruses, which obviously cooperate functionally with each other, are related, the better the replacements of the particular genes are tolerated. When the complete set of genes coding for the polymerase complex (PB2, PB1, PA, and NP) was derived from one or the other avian parent virus, the reassortants were, in general, pathogenic. In contrast, all nonpathogenic reassortants had a mixed polymerase complex. This was the case regardless of whether these genes ultimately came from pathogenic or nonpathogenic virus strains. Reassortment, even between highly pathogenic parent strains, may lead to pathogenic as well as nonpathogenic isolates (Rott *et al.,* 1979).

An increase in pathogenicity following reassortment between nonpathogenic strains is also possible. Certain reassortant viruses derived from crosses between a non- or weakly neuropathogenic FPV and a nonneuropathogenic human influenza virus have been shown to be highly neuropathogenic for mice (Scholtissek *et al.,* 1979). Neuropathogenic reassortants contained a gene constellation which again was dependent on the parent viruses used. Reassortants with a combination of the HA and M genes from FPV and certain polymerase genes from human influenza viruses produced systemic infection in the mouse, with involvement of the central nervous system after intraperitoneal infection (Vallbracht *et al.,* 1980; Reinacher *et al.,* 1983; Bonin and Scholtissek, 1983).

The studies on neuropathogenicity obtained by reassortment showed that neuropathogenic reassortants require a new host cell range *in vitro* that might be an essential property of the virus in the establishment of a systemic infection *in vivo.* Furthermore, it was demonstrated that reassortants between FPV and A/Hong Kong/68 were nonpathogenic for chicken and failed to produce plaques in chicken embryo cells (CECs) in the presence of FPV hemagglutinin, although they could still do so in MDCK cells. Some of the reassortants retained pathogenicity after mating with other influenza viruses, and produced plaques again in CECs (Scholtissek *et al.,* 1978b). Thus, changes in host range can parallel alterations in pathogenicity. There is again no general rule as to which of the eight viral genes cosegregate with a given phenotype for determination of different host range. Attempts to identify viral genes responsible for pathogenicity in humans have verified the concept that specific gene constellations, which depend on the character of the individual parent, are associated with the pathogenic property (reviewed by Beare, 1982). There is, however, some evidence that, with human influenza reassortant viruses, the genes coding for the polymerase complex and the gene coding for HA have a particular significance for pathogenicity.

The common feature in all these studies is that host range and pathogenicity are polygenic and that a gene constellation necessary for optimal viral growth and function in the host has to be achieved. Since the viral phenotype is dependent on the parent strains used for reassortment, the current information does not allow deduction of any rule by which gene replacement ultimately will lead to alterations in biological properties. Replacement of genetically highly related allelic genes might be without any consequence for pathological properties of corresponding reassortants. On the other hand, there is evidence that attenuation or increase in pathogenicity might be determined by one or more genes. A cooperative interplay between genes or gene products might be severely affected when one or the other gene is replaced. It would be very important to define such cooperative effects. But it has also become clear that cleavability of HA in a broad range of host cells, as is the case with certain avian virus strains of the H5 and H7 subtypes, is an important factor for inducing a systemic infection in chickens and mice.

Gene reassortment has been indicated as the most likely mechanism for the origin of "new" influenza viruses in nature. Presumably in naturally occurring reassortants viruses are selected which have an optimally functional genome. In avian viruses a major factor for this selection is the body temperature of the bird, 41–42°C. It has been shown that parental viruses and reassortants pathogenic for chickens replicate efficiently at 41°C. In contrast, nonpathogenic reassortants containing a "cleavable" HA replicate *in vitro* less efficiently at the elevated temperature. As a consequence of double infection *in vitro*, using two different avian influenza viruses at 41°C, reassortants are selected which are exclusively pathogenic for the chicken, if an appropriate hemagglutinin is present in the virus particles. If, on the other hand, mixed infection is performed at 37°C, most progeny are nonpathogenic (Rott *et al.*, 1982). Studies on the temperature-sensitive block of the nonpathogenic reassortants indicate that growth inhibition at 41°C occurs at a late step in virus replication, presumably at virus maturation (Giesendorf *et al.*, 1986).

V. Changes in Pathogenicity by Mutation

Further evidence that each gene may contribute to pathogenicity comes from analyses of effects of conditional lethal *ts* mutants or from studies of temperature-dependent host-range (*td–hr*) mutants (for review, see Mahy, 1983).

The *ts* mutants, with a mutation in one of the viral genes, were attenuated for experimental animals; *ts* mutants bearing a mutation

in the PB2, PB1, and/or NP genes were observed to be attenuated for humans (Chanock and Murphy, 1980; Ghendon and Markushin, 1980; Scholtissek and Rott, 1984). As one would expect, the level of attenuation at a given locus correlated with the level of temperature sensitivity of replication of the virus *in vitro*. Therefore, the route of infection in experimental animals, intranasally or intramuscularly, might be important. Furthermore, in all these experiments, one has to account for the relatively high reversion rate of *ts* mutants. It was shown that the reversion frequency depended on the gene carrying the ts defect. Reversion to wild-type virus was particularly high in viral genes, the products of which obviously cooperate with each other, such as the proteins for the polymerase complex (Scholtissek and Rott, 1984). Thus, double mutants might be especially stable, if the ts defects are located in genes encoding viral protein products that are expected not to cooperate.

In vitro studies with *td–hr* mutants suggested that mutations in each of the viral genes can alter host range and, conversely, that host factors influence the functioning of each gene (Shimizu *et al.*, 1983). Some of the cold-adapted mutants proved to be attenuated for humans and ferrets. The genetic basis of the attenuation, however, remains unclear (reviewed by Beare, 1982; Murphy *et al.*, 1984).

VI. Virus Components as Determinants of Pathogenicity

The results obtained with viral mutants and reassortants revealed that the expression of virus pathogenicity is dependent on the functional integrity of each gene and on a gene constellation optimal for infection of a given host. Changes that result in alterations in the function of any gene or in genome composition can cause alterations in pathogenicity. Therefore, it is not surprising that several genes or gene products have been described as determinants for host range and virulence of influenza virus. Virulence here, however, often relates to plaque formation in cultured cells, which has not been correlated with the ability to cause disease in a host organism. But in a few exceptions single gene products have been described which may influence pathogenic properties of the virus.

A. Hemagglutinin

As has already been pointed out, hemagglutinin plays a double role in the initiation of infection by binding the virus to neuraminic acid-containing cell receptors and by promoting penetration of the viral genome through membrane fusion. Receptor binding and fusion ac-

tivity depend on an intimate interplay between hemagglutinin and cellular factors, and evidence has been obtained that both functions may contribute to cell tropism, host range, spread of infection, and pathogenicity.

1. The Receptor Specificity of Hemagglutinin

Because influenza viruses bind to neuraminic acid-containing glycoconjugates and because neuraminic acid is found as a cell surface component of all vertebrate species, the presence or absence of receptors has often not been considered a major factor in host range. On the other hand, it has long been known that even closely related influenza virus strains can vary in their receptor specificity. Among the numerous studies dealing with this problem (for review, see Paulson, 1985), the most fruitful approach is based on the use of erythrocytes or tissue culture cells containing enzymatically modified neuraminic acids. A large number of influenza viruses from human and animal origin have been characterized for their receptor specificity by this procedure (Rogers and Paulson, 1983; Higa et al., 1985; Rogers et al., 1986). As can be seen in Table I, influenza A and B viruses bind preferentially to N-acetylneuraminic acid (NeuAc) in the terminal position, but there are variations in the receptor specificity among the individual strains depending on the linkages between the neuraminic acid and the other sugars in the oligosaccharides. In contrast to the influenza A and B viruses, influenza C virus specifically requires 9-O-acetyl-N-acetyl-neuraminic acid (9-O-Ac-NeuAc) for agglutination (Rogers et al., 1986). These observations clearly demonstrate that the specificity of the influenza virus receptor is determined by the structure of the asialooligosaccharide, the linkage between the neuraminic acid and the oligosaccharide, and the substitution of the neuraminic acid.

In the following discussion we will briefly review evidence indicating that variations in receptor specificity, which, as far as is known, are due to structural alterations at the receptor binding site of the hemagglutinin, may be important for the adaptation of a virus from one host system to another one. Early studies by Burnet and Bull (1943) and by Stone (1951) documented a receptor shift following adaptation of human influenza viruses (H1N1) to growth in chicken embryos. The original isolate (O virus) could only be grown in the amnion but after several passages could be propagated in the allantoic cavity as the derived (D) virus. Concomitant with adaptation, the binding properties of the virus changed from preferential agglutination of guinea pig erythrocytes by O virus to agglutination of chick erythrocytes by D virus. More recently, human influenza B viruses isolated and propagated by growth in MDCK cells have been found to undergo

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TABLE I

RECEPTOR SPECIFICITY OF INFLUENZA A, B, AND C VIRUSES[a]

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Erythrocyte preparation	Sialic acid incorporated (nmol/ml)	HA titer[b]							
		C/JHB	PR/8	RI/5+	RI/5−	Mem/102	D/6874	B/HK	B/Eng
Native	—	0	256	32	256	512	32	64	128
Asialo	—	0	0	0	0	0	0	0	0
NeuAc									
α2,3Galβ1,3GalNAc	88	0	256	0	256	0	64	2	64
α2,3Galβ1,4GlcNAc	20	0	128	0	2	0	64	128	256
α2,6Galβ1,4GlcNAc	31	0	128	64	0	1024	2	64	256
NeuGc									
α2,6Galβ1,4GlcNAc	24	0	0	8	0	512	0	2	64
9-O-Ac-NeuAc									
α2,3GalβGalNAc	23	128	0	0	256	0	64	0	0
α2,3Galβ1,4GlcNAc	8	128	0	0	0	0	0	0	0
α2,6Galβ1,4GlcNAc	24	128	0	0	0	0	0	0	0

[a] Erythrocyte preparations consisted of unmodified (native) human erythrocytes, sialidase-treated (asialo) human erythrocytes, and human asialo erythrocytes resialylated with CMP–NeuAc, CMP–NeuGc, or CMP–9-O-Ac-NeuAc and one of three purified sialyltransferases to give the sequence Siaα2,3Galβ1,3GalNAc on O-linked oligosaccharides, and the sequences Siaα2,3Galβ1,4(3)GlcNAc and Siaα2,6GalβNAc commonly found on Asn-linked oligosaccharides. The amount of sialic acid transferred to glycoproteins of the erythrocyte membrane for each preparation is expressed as nanomoles of sialic acid per milliliter of packed erythrocytes. Native human erythrocytes contain about 700 nmol of NeuAc/ml of packed cells. Treatment with *Vibrio cholera* neuraminidase removed about 90% of the total sialic acid from native cells. From Rogers *et al.* (1986).

[b] A value of zero indicates a titer of ≤2.

dramatic selection for host variants when adapted to growth in chicken embryos (Schild et al., 1983). Sequence analyses of the HA genes of theses viruses before and after egg adaptation revealed an amino acid substitution resulting in the loss of a carbohydrate attachment site located on the top of the hemagglutinin molecule adjacent to the receptor binding site. Similarly, during egg adaptation of influenza A viruses (H1N1), a variety of amino acid exchanges could be detected, located mostly on the periphery of the receptor binding site (Robertson et al., 1985).

Genetic dimorphism has also been observed in swine influenza viruses whereby cloned viruses exhibited either high yields (H virus) or low yields (L virus) in eggs. Genetic reassortment analysis revealed that the phenotypic properties of the two viruses were carried by the RNA segment coding for hemagglutinin (Kilbourne, 1978). Although both the H and L forms of the virus were found in influenza virus isolates from swine, experimental infections of swine suggested that the H form is more virulent (Kilbourne et al., 1981). The primary structural difference between the H and L hemagglutinins appears to be a single amino acid change of glutamic acid (H) to glycine (L) at residue 155 (Both et al., 1983). It is not clear whether this exchange is associated with quantifiable differences in receptor-binding properties, but it is remarkable that it is located in a region near the receptor binding site.

It is generally accepted that the influenza pandemic in 1968 arose from genetic reassortment of the previously circulating human virus and an avian virus similar to A/duck/Ukraine/1/63, which contributed the H3 hemagglutinin to the new Hong Kong virus (Scholtissek et al., 1978a). On the other hand, it has also been clearly shown that the avian H3 viruses bind preferentially to 2,3-linked neuraminic acid, whereas the human viruses of the same serotype bind to 2,6-linked neuraminic acid. Comparative sequence analyses have revealed that the difference in receptor specificity is due in large part to a point mutation at the receptor binding site involving the exchange of the glutamine residue 226 in the avian viruses for leucine in the human strains (Rogers and Paulson, 1983; Rogers et al., 1983a,b). It is therefore reasonable to assume that generation of the Hong Kong virus involved selection of a receptor-binding variant that allowed propagation in the human tissue. Selective pressure for host adaptation could be exerted either by differential neutralization by serum inhibitors (Choppin and Tamm, 1959, 1960) or by differential adsorption to the cell receptors of the new host.

These observations clearly indicate that the receptor specificity of hemagglutinin is an important determinant for tissue tropism and

host range of influenza virus. It is likely that receptor specificity also controls pathogenicity, but this concept has to be proved by further experimental data.

2. Proteolytic Activation of the Fusion Capacity

Proteolytic activation of hemagglutinin follows a pattern observed with many enzyme and hormone precursors, such as proinsulin, progastrin, and proopiomelanocortin (Docherty and Steiner, 1982). It involves cleavage at an arginine residue by the sequential action of a "trypsinlike" endoprotease and a carboxypeptidase that are both provided by the host. The available evidence indicates that the endoprotease is usually an intracellular enzyme that cleaves hemagglutinin at the late stages of its transport to the cell surface, either in the Golgi apparatus or in vesicles mediating transport between the Golgi apparatus and the plasma membrane (Klenk et al., 1974). Studies on lysates of MDBK cells provided evidence that the enzyme is calcium dependent and has a neutral pH optimum, suggesting that it is not of lysomal origin (Klenk et al., 1984b). However, beyond that, little is known about the nature of the intracellular protease. When a cell does not contain an appropriate enzyme, virus with uncleaved hemagglutinin is released. Under these conditions, trypsin and a whole series of other trypsinlike endoproteases, such as plasmin (Lazarowitz et al., 1973), acrosin (Garten et al., 1981), or bacterial proteases (Klenk et al., 1977; Tashiro et al., 1987a,b), can substitute for the intracellular enzyme.

The carboxypeptidase has been characterized in some detail. It appears to be a host component incorporated into the viral envelope and resembles in many respects carboxypeptidase N. Studies with specific inhibitors have revealed that, unlike the endoprotease, the carboxypeptidase is not necessary for activation (Garten and Klenk, 1983).

Since activation of hemagglutinin is necessary for multiple-cycle replication, it is reasonable to assume that inhibitors of the endoproteases might interfere with spread of infection. By this approach, cleavage of hemagglutinin could be prevented under in vitro conditions (Zhirnov et al., 1982a; W. Garten, E. Shaw, and H.-D. Klenk, unpublished results), and therapeutic effects in infected animals have also been described (Zhirnov et al., 1982b; Tashiro et al., 1987c).

Since the activating proteases are cellular enzymes, the infected cell type determines whether or not hemagglutinin is cleaved (Klenk et al., 1975; Klenk and Rott, 1980). The hemagglutinins of the mammalian influenza viruses and the nonpathogenic avian influenza viruses, which cause a local infection, are susceptible to proteolytic cleavage only in a restricted number of cell types. On the other hand, the

hemagglutinins of pathogenic avian viruses among the H5 and H7 subtypes, which cause a systemic infection, are cleaved by proteases present in a broad range of different host cells (Bosch et al., 1979). Thus, there are differences in host range resulting from differences in hemagglutinin cleavability which can be correlated with the pathogenic properties of the virus. The differences in cleavability are due to differences in the structure of the cleavage site of the hemagglutinin (Bosch et al., 1981; Garten et al., 1981). Sequence analyses have revealed that the HA1 and HA2 fragments of the hemagglutinin molecule of the apathogenic avian and of all mammalian influenza viruses are linked by a single arginine. This is in contrast to the pathogenic avian strains, which have a sequence of several basic amino acids at the cleavage site with the common denominator lysine–arginine or arginine–arginine (Table II). Although the hemagglutinins of all influenza viruses are cleaved by the same general mechanism, resulting in the elimination of the basic amino acids, it has to be assumed that differences exist in the specificities of the proteases, which recognize either a single arginine or the paired basic residues lysine–arginine and arginine–arginine (Garten et al., 1982; Garten and Klenk, 1983).

It is reasonable to assume that such differences in cleavability may be generated by mutation. Recent results with a human influenza virus have revealed that susceptibility of hemagglutinin to cleavage is indeed not a fixed entity of the molecule. In that case hemagglutinin could be altered by adaptation of the virus to a novel host cell, previously nonpermissive to the wild-type virus. Cleavability of the hemagglutinin of the adapted virus in the new cell type was attributed to a single amino acid substitution close to the cleavage site. The adapted virus still contained a single arginine at the cleavage site, and the mutation did not result in a general increase in the susceptibility of the hemagglutinin to activating proteases. By the amino acid substitution the hemagglutinin was activated only by the enzymes present in the particular host cell to which the virus was adapted, in addition to the original permissive cell types (Rott et al., 1984). Preliminary studies with a nonpathogenic avian influenza virus (A/turkey/Oregon/71, H7N3) have shown that, following adaptation to chicken fibroblasts, variants could be obtained, the hemagglutinin of which became activated in a broad range of different cell types, similar to the pathogenic avian viruses. With some of the variants, the increase in cleavability was correlated with an aggravation in pathogenicity for chickens (R. Rott and M. Orlich, unpublished results). Sequence analysis of hemagglutinin will have to be carried out to throw light on the structural basis for this alteration. A single point mutation in hemagglutinin indeed appears to be responsible for the

TABLE II

Cleavage Sites of the Hemagglutinins of Mammalian and Avian Influenza A Viruses

Virus	Serotype	Pathogenicity for fowl	Cleavage site[a]	References
A/Puerto Rico/8/34	H1	—	-Pro-Ser -Ile -Gln-Tyr ——————ARG-∇Gly-Leu-	Winter et al. (1981)
A/Japan/305/57	H2	—	-Pro-Gln-Ile -Gln-Ser ——————ARG-∇Gly-Leu-	Gething et al. (1980)
A/Memphis/102/72	H3	—	-Pro-Glu-Lys -Gln-Thr ——————ARG-∇Gly-Leu-	Sleigh et al. (1980)
A/duck/Ukraine/1/63	H3	Apathogenic	-Pro-Glu-Lys -Glu-Thr ——————ARG-∇Gly-Leu-	Fang et al. (1981)
A/chick/Penn/1370/83	H5	Pathogenic	-Pro-Gln-LYS——LYS-LYS————ARG-▼Gly-Leu-	Kawaoka et al. (1984)
A/seal/Mass/1/80	H7	—	-Pro-Glu-Asn -Pro -Lys -Thr ——————ARG-∇Gly-Leu-	Naeve and Webster (1983)
A/FPV/Rostock/34	H7	Pathogenic	-Pro-Glu-Pro -Ser -Lys -Lys -Arg -Glu -LYS-ARG-▼Gly-Leu-	Porter et al. (1979)
A/FPV/Rostock/34	H7	Pathogenic	-Pro-Glu-Pro -Ser -LYS-LYS-ARG-LYS -LYS-ARG-▼Gly-Leu-	Garten et al. (1985)
A/FPV/Dutch/27	H7	Pathogenic	-Pro-Glu-Leu -Pro -LYS-LYS-ARG-ARG-LYS-ARG-▼Gly-Leu-	Pritzer (1985)
A/chick/Germany/49	H10	Apathogenic	-Pro-Glu-Val -Val -Gln -Gly ——————ARG-∇Gly-Leu-	Feldmann et al. (1988)

[a] ∇, Cleavage site of endoproteases recognizing a single arginine; ▼, cleavage site of endoprotease recognizing pairs of basic residues. Amino acids eliminated by the sequential action of endoprotease and carboxypeptidase are indicated by capital letters.

drastic increase in pathogenicity that occurred within a series of outbreaks of avian influenza in Pennsylvania in 1983. Comparison of different isolates which all belonged to the H5N2 subtype revealed that virus from an early outbreak had low pathogenicity and contained a hemagglutinin with restricted cleavability, whereas virus from a late outbreak was highly pathogenic and had a hemagglutinin that was always cleaved. Sequence analysis revealed that, as shown in Table II, the pathogenic variant had a cleavage site of the structure lysine–lysine–lysine–arginine, i.e., a cleavage site susceptible to ubiquitous proteases. The apathogenic precursor strain had the same cleavage site. But here it was masked by an adjacent oligosaccharide which was subsequently lost by point mutation (Kawaoka *et al.*, 1984; Kawaoka and Webster, 1985).

These data indicate the important role of proteolytic activation of hemagglutinin for pathogenicity. If the hemagglutinin is cleaved in a restricted number of cell types, the infection will be confined to localized areas of the host. In mammals this type of infection affects the respiratory tract, whereas in birds it is likely to be clinically inapparent. On the other hand, cleavability of hemagglutinin in a wider range of different host cells, as is the situation in the pathogenic H5 and H7 viruses, permits a rapid production of infectious virus particles in all organs and thereby spread in the organism, resulting in a systemic fatal disease (Rott *et al.*, 1980; Klenk *et al.*, 1984a; Rott and Klenk, 1986).

3. Cleavage Activation by Bacterial Proteases

There is now evidence that proteolytic activation of hemagglutinin is a pathogenicity determinant not only for avian viruses, but also for mammalian influenza viruses. Combined viral–bacterial pneumonia in man is considered to be three times more common than primary viral pneumonia (Stuart-Harris *et al.*, 1985). In addition to a number of other bacteria, *Staphylococcus aureus* is commonly involved. It is generally thought that virus infection in a given tissue favors growth conditions for bacteria. On the other hand, it was recently found that *Staphylococcus* exert a decisive influence on influenza virus replication in the respiratory tract and promote the development of influenza pneumonia. Some *S. aureus* strains have been shown to secrete a protease capable of activating hemagglutinin by proteolytic cleavage *in vitro*. The presence of the bacterial enzymes in cell culture media enabled the virus to undergo multiple growth cycles. Thus, coinfection of mice with *Staphylococcus* enhanced the virus titer in the lung enormously, resulting in a fatal disease with extended lesions in lung tissue (Tashiro *et al.*, 1987a,b) (Fig. 2). These findings may explain the
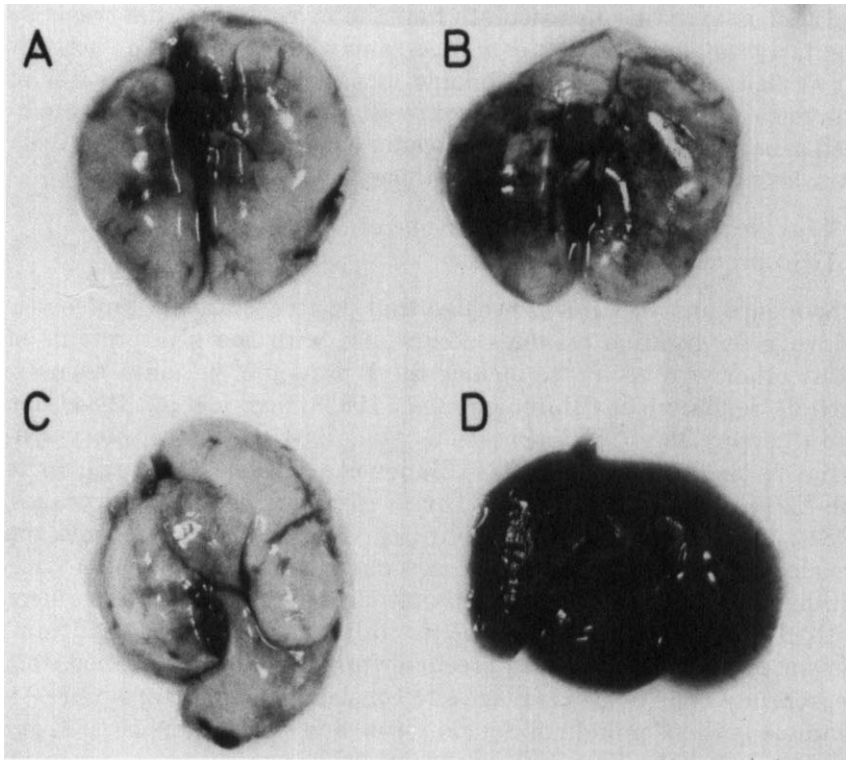
FIG. 2. Pathological alterations in the lungs of mice after double infection with influenza virus and *Staphylococcus aureus*. A, Mock infected; B, intranasally infected with A/swine/1976/31 (H1N1); C, infected with *S. aureus* Wood 46; and D, coinfected with virus and *S. aureus*, respectively. The lungs were taken 5 days after infection. Doses of inoculum were 10^2 plaque-forming units of virus and 10^6 colony-forming units of bacterium (Tashiro *et al.*, 1987a; reprinted by permission).

high fatality rate in humans seen after coinfection with *S. aureus* (Robertson *et al.*, 1958; Stuart-Harris *et al.*, 1985). *Staphylococcus aureus* is most likely not the only microorganism that can provide suitable proteases. One of several other candidates is *Haemophilus influenzae*, which was regularly isolated during the devastating influenza pandemics of 1918/1919.

A similar pathogenic mechanism may have been responsible for an influenza epizootic observed in harbor seals on the New England coast in 1980. Since the influenza virus isolated from the dead animals had a hemagglutinin (H7) of low cleavability with a single arginine at the cleavage site (Naeve and Webster, 1983), and since it showed only low pathogenicity in seals after experimental reinoculation (Webster *et al.*,

1981), it is tempting to speculate that the mycoplasma that was also regularly isolated (Webster *et al.*, 1981) may have provided an activating protease. Thus, it is reasonable to assume that severe cases of influenza in man and in animals are often caused by protease-mediated synergism between an apathogenic virus and a second relatively harmless and ubiquitous microorganism.

4. Significance of Proteolytic Cleavage of Glycoproteins of Other Viruses

Sequence analyses have revealed that posttranslational proteolytic cleavage at arginine residues occurs also with the glycoproteins of many other viruses. These include the F protein of paramyxoviruses, such as Sendai virus (Blumberg *et al.*, 1985; Hidaka *et al.*, 1984; Hsu and Choppin, 1984), SV5 (Paterson *et al.*, 1984), and respiratory syncytial virus (Collins *et al.*, 1984; Elango *et al.*, 1985); the precursor to the E2 protein of alphaviruses (Garoff *et al.*, 1980; Rice and Strauss, 1981); the E2 protein of coronaviruses (Binns *et al.*, 1985); and the envelope glycoprotein of retroviruses, such as murine leukemia virus (Shinnick *et al.*, 1982) and HIV1 (Ratner *et al.*, 1985). In agreement with the observations made on the influenza virus hemagglutinin, virions containing uncleaved precursor proteins can be obtained from appropriate cells if the cleavage site consists of a single arginine. An example is the F protein of Sendai virus, and it should be pointed out that this was the first viral glycoprotein shown to be activated by proteolytic cleavage (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). Newcastle disease virus, another paramyxovirus, comprises a whole series of strains which, like the avian influenza viruses, differ widely in pathogenicity for chickens. Differences in pathogenicity can be correlated to the cleavability of the F protein (Nagai *et al.*, 1976; Nagai and Klenk, 1977; Garten *et al.*, 1980), and recent sequence analyses have revealed that the apathogenic strains have single arginine residues and the pathogenic strains have paired basic residues at their cleavage sites, exactly as has been observed with the avian influenza viruses (Table III) (Toyoda *et al.*, 1987). Finally, it should be mentioned here that the infectivity of rotaviruses, which do not contain an envelope, is activated by trypsin cleavage of one of its capsid proteins, VP3 (Kalica *et al.*, 1983). In genetic studies, VP3 was identified as a virulence marker (Offit *et al.*, 1986). Since rotavirus strains differ from each other by single arginine residues or by paired basic amino acids at the cleavage site of VP3 (Lopez *et al.*, 1986), it is tempting to speculate that cleavability of this surface protein determines pathogenicity, although such a correlation has not been demonstrated yet. Thus, evidence is increasing that proteolytic activation of functionally impor-

TABLE III

CLEAVAGE SITES OF THE F PROTEIN OF DIFFERENT STRAINS
OF NEWCASTLE DISEASE VIRUS[a]

Virus	Pathogenicity for fowl	Cleavage site[b]
Miyadera	Pathogenic	-Gly-Gly-Arg-Arg-Gln-ARG-ARG-▼-Phe-Ile-
Herts	Pathogenic	-Gly-Gly-Arg-Arg-Gln-ARG-ARG-▼-Phe-Ile-
Italien	Pathogenic	-Gly-Gly-Arg-Arg-Gln-ARG-ARG-▼-Phe-Ile-
Victoria	Pathogenic	-Gly-Gly-Arg-Arg-Gln-LYS-ARG-▼-Phe-Ile-
La Sota	Apathogenic	-Gly-Gly-Gly-Arg-Gln-Ser-ARG-▽-Phe-Ile-
D26	Apathogenic	-Gly-Gly-Gly-Lys-Gln-Gly-ARG-▽-Leu-Ile-
Queensland	Apathogenic	-Gly-Glu-Gly-Lys-Gln-Gly-ARG-▽-Leu-Ile-
Ulster	Apathogenic	-Gly-Gly-Gly-Lys-Gln-Gly-ARG-▽-Leu-Ile-

[a] From Toyoda et al. (1987).
[b] ▽, Cleavage site of endoprotease recognizing a single arginine; ▼, cleavage site of endoprotease recognizing pairs of basic residues. Amino acids eliminated by the sequential action of endoprotease and carboxypeptidase are indicated by capital letters.

tant proteins may be a rather common determinant of virus pathogenicity.

B. Other Viral Components

It has been proposed that the nature of the second virus glycoprotein, neuraminidase, determines whether cleavage of HA may occur (Schulman and Palese, 1978). These authors showed that A/WSN/33-A/HK/68 or WSN-A/FM/1/47 reassortants that derived only the NA gene of the WSN virus did produce infectious virus with cleaved HA in MDCK cells, whereas reassortants which were identical to WSN except for its NA gene, did not. Similar results were obtained among reassortants for A/turkey/Ontario/7732/66 (H5N9) and WSN virus. Again, WSN neuraminidase determined whether the infectious virus was produced in chicken embryo cells. Interestingly, reassortants were found to replicate in neuroblastoma cells in vitro with cleaved HA (Nakajima and Sugiura, 1980) and exhibited neuropathogenicity for mice (Sugiura and Ueda, 1980) only when the NA was derived from the WSN parent virus in conjunction with M and NS proteins. However, reassortants between WSN and other avian influenza viruses or a number of other reassortants with different HA–NA combinations did not show these characteristics (Rott et al., 1983a; unpublished results). Thus, the role of neuraminidase for HA cleavage, and therefore the

initiation of viral replication leading to pathogenic effects, may be restricted to an interaction between a specific HA–NA combination. The mechanism by which a viral NA in some instances may facilitate cleavage of HA is not understood. It is, however, possible, as suggested by Schulman (1983) that "different neuraminidases may differently activate or inactivate the required host proteases." In this context it should be remembered that different proteases differ from each other in their capacity to cleave and activate the HA of different virus strains (Tashiro *et al.*, 1987b).

During evolution of the three human subtype viruses known so far, the PB2, NP, M, and NS genes have been retained within all subtypes. Therefore, it is probable that these genes are essential for the host range specificity of these viruses. Particular emphasis was placed on the examination of the NP gene and its protein product. Scholtissek *et al.* (1978a, 1985) have shown that the NP of human and some swine influenza viruses, all of the H3N2 subtype, in contrast to avian viruses, could not rescue *ts* mutants of FPV with defects in the NP gene following mixed infection of chicken embryo cells, although they were able to do so in MDCK cells. Furthermore, the phosphorylation pattern of NP appears to determine the extent to which a given cell type may support virus growth (Kistner *et al.*, 1985). In terms of these parameters and genetic relatedness of the NP genes, H3N2 viruses derived from different hosts of origin could be discriminated (Scholtissek *et al.*, 1985). The interpretation of these results was that NP is a determining factor of host specificity, although other factors cannot be excluded with certainty.

The M1 protein seems to be essential for virus assembly by initiation of budding. There is a specific underproduction of M1 protein in different abortively infected cell lines, where budding is rarely (if ever) seen (Bosch *et al.*, 1978; Valcavi *et al.*, 1978; Lohmeyer *et al.*, 1979). A marked reduction in the synthesis of M1 protein has also been found in mouse astrocytes infected *in vitro* with nonneurovirulent influenza viruses (PR8 or WS), in contrast to astrocytes infected with the neurovirulent WSN virus. On the other hand, in oligodendrocytes production of M1 protein was the same for each virus (Bradshaw and Schlesinger, 1986). Immunohistological examinations have shown positive reactions of ependymal and parenchymal cells with anti-NP serum in mouse brain infected with either WSN or PR8, but similar reactions with anti-M1 serum could only be seen in WSN-infected but not in PR8-infected cells of the brain (Bradshaw and Schlesinger, 1987). Therefore, one could assume that neurovirulent and nonneurovirulent virus strains differ in expression of M1 protein in infected brain cells, which is rate limiting for the production of infectious virus. The information available, however, does not allow an

explanation for the control of M1 protein synthesis in a permissively or an abortively infected cell. It seems likely that host cell factors are involved in this process.

VII. Genetic Resistance to Influenza Virus Infection

The Zürich group of virologists has made remarkable contributions concerning the mechanism of host-determined differences in susceptibility of mice to influenza virus infection. It could be shown that resistance of two inbred mouse strains, A2G and SL/NiA, and several laboratory-reared offspring of wild mice (Lindenmann, 1964; Haller *et al.*, 1986) is inherited by a single dominant gene, designated Mx^+, located on mouse chromosome 16 (Staeheli *et al.*, 1986a). *In vitro* and *in vivo* studies revealed that resistance selectively affects influenza viruses, including pneumotropic, neurotropic, and hepatotropic strains (Lindenmann, 1964; Haller *et al.*, 1979). The expression of the resistance phenotype is mediated by the action of interferon-α and -β (IFN-α; IFN-β), but not IFN-γ (Haller *et al.*, 1979, 1980). Murine IFN induces in cogenetic Mx^+ cells, but not in Mx^- cells, preferentially a 72,000-Da polypeptide, the Mx protein, which concomitantly protects Mx^+ cells against infection (Horisberger *et al.*, 1983; Staeheli *et al.*, 1986). The Mx protein, which accumulates in the nucleus of IFN-treated Mx^+ cells (Dreiding *et al.*, 1985), inhibits influenza virus replication, presumably by affecting viral mRNA synthesis (Krug *et al.*, 1985).

A cDNA encoding the Mx protein has been cloned and sequenced (Staeheli *et al.* 1986b). The Mx protein, as deduced from the nucleotide sequence, contains 631 amino acids. It is highly hydrophilic and contains an unusually high percentage of charged amino acids, some of which are clustered. A stretch of carboxy-terminal basic amino acids is assumed to be responsible for its nuclear localization. Transfection of the Mx protein encoding cDNA in Mx^- mouse cells led to expression of this protein and consequently conferred to these cells resistance against influenza virus infection. Southern blot analyses of chromosomal DNA revealed that Mx^- mice carry deletions at the Mx locus (Staeheli *et al.*, 1986b).

All in all, these data clearly show that the specific resistance of mice to influenza virus infection is solely due to IFN-α/β-induced expression of the cellular Mx protein, which inhibits virus replication at a very early step. Similar inheritable events might be responsible for some of the unknown complex set of virus–host interactions, in which host-determined differences are also of crucial importance. Appropri-

ate examples of this could be that avian influenza viruses are not able to cross the species barrier directly to infect man (reviewed by Rott and Klenk, 1987) and that avian viruses, highly pathogenic for chickens and turkeys, do not produce disease in other avian species (Slemons and Easterday, 1972; Tashiro *et al.*, 1986).

VIII. CONCLUDING REMARKS

It is an old and generally accepted concept that the pathogenicity of a virus is of polygenic nature. Because of their segmented genome and because of the large variety of different strains available, influenza viruses provide a suitable system to prove this concept. The numerous studies employing virus mutants and reassortants have indicated that pathogenicity depends on the functional integrity of each gene and on a gene constellation optimal for the infection of a given host. As a consequence, virtually every gene product of influenza virus has been reported to contribute to pathogenicity, but evidence is steadily growing that a key role has to be assigned to hemagglutinin.

As the initiator of infection, hemagglutinin has a double function: first, promotion of adsorption of the virus to the cell surface, and then penetration of the viral genome through a fusion process between viral and cellular membranes. Adsorption is based on the binding to neuraminic acid-containing receptors, and different virus strains display a distinct preference for specific oligosaccharides. Fusion capacity depends on proteolytic cleavage by host proteases, and variations in amino acid sequence at the cleavage site determine whether hemagglutinin is activated in a given cell. Differences in cleavability and presumably also in receptor specificity are important determinants for host tropism, spread of infection, and pathogenicity. The concept that proteolytic activation is a determinant for pathogenicity was originally derived from studies on avian influenza viruses, but there is now evidence that it may also be relevant for the disease in humans, since bacterial proteases have been found to promote the development of influenza pneumonia in mammals. Moreover, proteolytic activation has been reported for a whole series of other viral surface proteins, and it appears that it may be quite a common determinant of virus pathogenicity.

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