CHAPTER 1

The Genetics of Paramyxoviruses

CRAIG R. PRINGLE

I. INTRODUCTION: THE GENOME STRATEGY OF THE PARAMYXOVIRUSES

The replication of RNA viruses, though sometimes requiring integrity of the cell nucleus, with a single exception takes place in the cytoplasm of the host cell independently of DNA replication. According to taxonomic type, either the positive strand or the negative strand or both may become encapsidated in the virion. The negative-strand viruses include the nuclear hepatitis delta virus and the members of the families *Arenaviridae, Bunyaviridae, Filoviridae, Orthomyxoviridae, Paramyxoviridae,* and *Rhabdoviridae.* Hepatitis delta virus is exceptional in having a genome consisting of a single-stranded, covalently-closed, circular molecule, and although replication competent in some cells, it is dependent on hepatitis B virus for encapsidation and transmission (Wang *et al.*, 1986; Taylor *et al.*, 1987). The linear negative-strand RNA viruses are a diverse collection of viruses replicating in the cytoplasm. They exhibit a genome strategy whereby the noncoding RNA strand is sequestered in the extracellular virion and the positive-strand template RNA and mRNAs are generated by a virion-associated transcriptase/replicase from the complementary template strand when the infectious process is initiated.

The six families of linear negative-strand RNA viruses comprise viruses with circular or linear helical nucleocapsid core structures invested by an envelope composed of viral proteins associated with the host-cell plasma membrane, or an internal membrane in the case of the bunyaviruses. The viruses in these families have relatively large particles, but relatively small genomes ranging between 11,100 and 15,900 nucleotides. The six families are to some extent differentiated by morphology and fall into two distinct catego-

CRAIG R. PRINGLE • Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom.

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ries in terms of genome structure. The arenaviruses, bunyaviruses, and orthomyxoviruses have segmented genomes containing two, three, and seven or eight subunits, respectively, whereas the filoviruses, paramyxoviruses and rhabdoviruses have linear undivided genomes. Segmentation of the genome may be a device to uncouple the transcription of individual viral genes, or an adaptation to compensate for the low fidelity of transcription of RNA polymerases, allowing error correction by reassortment of subunits.

This dichotomy of the negative-strand viruses, whatever its functional significance, has profound genetic consequences. Mutation appears to be the sole means of generation of variation among those viruses with nonsegemented genomes. Unlike the positive-strand viruses, intramolecular recombination has not been detected in viruses with linear negative-strand genomes (see Section III.A). The segmented-genome viruses, on the other hand, can acquire variation by reassortment of genome subunits as well as by mutation, and therefore appear to be inherently more adaptable. In bunyaviruses and orthomyxoviruses, the gene pool may be very large. The propensity of the paramyxoviruses in particular to become persistent may reflect their reduced capacity for variation and their consequent reliance on alternate mechanisms to combat host immune responses. Certain paramyxoviruses associated with recurrent epidemic disease in humans appear remarkably stable. For example, the HN surface glycoprotein gene of parainfluenza virus type 3 and the F protein gene of respiratory syncytial (RS) virus appear to be highly conserved, showing little sequence variation during the course of 20 years since first isolation (Coelingh *et al.*, 1986; Baybutt and Pringle, 1987). However, other genes are less conserved and the apparent stability of the surface glycoproteins may merely reflect the existence of consistent selective forces.

The negative-strand unsegmented genome RNA viruses have many features in common. They are similar in terms of genetic organization (Pringle, 1987), with genes of homologous function transcribed in the same order from a single 3′ terminus-adjacent promoter site. A block of core protein genes is followed by a block of envelope protein genes with the polymerase protein gene located farthest from the promoter and accounting for approximately half the coding capacity of the genome (Fig. 1). The genome of the *Vesiculovirus* genus of the *Rhabdoviridae* is the least complex, comprising five linearly arrayed genes with minimal di- or trinucleotide intergenic junctions and highly conserved gene start and gene stop signals (Fig. 2). The NS (or P) phosphoprotein gene encodes in-frame a second gene product, a feature shared with the homologous P protein gene of some paramyxoviruses. Other rhabdoviruses have genomes of greater complexity, resembling the paramyxovirus pattern. The *G–L* intergenic region of rabies virus has the structure of a pseudogene, and a transcribed gene of unknown function is present between the *G* and *L* genes of the fish rhabdovirus, infectious hematopoietic necrosis virus. The paramyxoviruses and rhabdoviruses differ most with regard to particle morphology and general biology. The paramyxoviruses are pleomorphic, in contrast to the uniform morphology of the rhabdoviruses. The paramyxoviruses are restricted in host range and confined to vertebrates, whereas the rhabdoviruses have a broad host range, infecting vertebrates, invertebrates, and plants. The limited host range and routes of transmission are probably responsible for the strong asso-

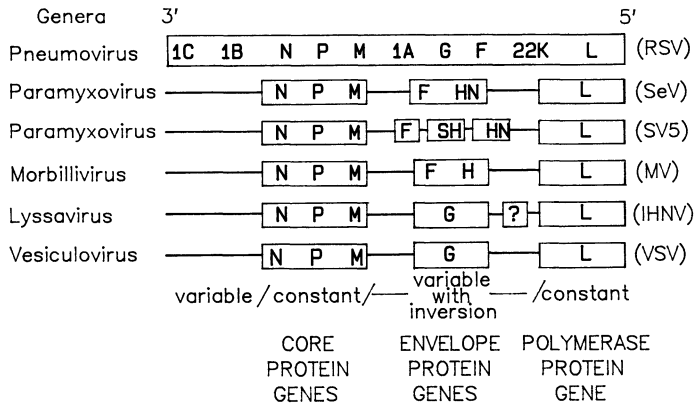

FIGURE 1. Comparison of the linear order of gene functions in the different genera of paramyxoviruses and rhabdoviruses. Negative-stranded (genomic) polarity is depicted. Key: RSV, human respiratory syncytial virus; SeV, Sendai virus; SV5, simian virus 5 (and mumps virus); MV, measles virus; IHNV, infectious hematopoeitic necrosis virus; VSV, vesicular stomatitis virus. 1C and 1B, genes of unknown function; *N*, nucleoprotein gene; *P*, phosphoprotein gene; *M*, nonglycosylated membrane protein gene; 1A, glycosylated membrane protein gene; *G*, *H*, and *HN*, attachment protein gene; *F*, fusion protein gene; SH, small hydrophobic protein gene; ?, transcribed gene of unknown function (pseudogene in rabies virus); *L*, polymerase protein gene.

ciation of paramyxoviruses with respiratory tract infection and disease, or vice versa.

The family *Paramyxoviridae* as presently constituted (Kingsbury *et al.*, 1978) is by no means homogeneous. Two of the three genera, *Morbillivirus* and *Paramyxovirus*, are clearly related viruses by criteria such as nucleotide sequence similarities, gene number and order, and detailed morphology. The third genus, *Pneumovirus*, is distinct. Indeed, the differences distinguishing the pneumoviruses from the other members of the family are greater than those differentiating the rhabdoviruses from the other paramyxoviruses. Evi-

FIGURE 2. Gene start, end, and intergenic consensus sequences. Consensus sequences are given for the start and end regions of the genes of viruses where all or most of the genome has been sequenced. The intergenic junctions are given as consensus sequences or as numbers of intervening nucleotides. Key: N, variable nucleotide; NDV, Newcastle disease virus; PIV3, parainfluenza virus type 3; RSV, human respiratory syncytial virus; PVM, pneumonia virus of mice; VSV, vesicular stomatitis virus. Compiled from data published up to the end of 1988 (except in the case of PVM).

GENE ENDS/ JUNCTIONS /GENE STARTS	
Family Paramyxoviridae	
Genus Paramyxovirus:	
ANUCUUUUUUU/ 1-48 /UGCCCAUCNUN	*NDV*
UNAUUCU/ GNN /UCCCANUUUC	*Sendai*
UUNAAUNNUUUUU (UU) / GAA /UCCNNUUC	*PIV3*
ANNUNUUUUUU (U) / 1-7 /UNCNNNNNU	*Mumps*
Genus Morbillivirus:	
NNNUUUU (UU) / GNA /UCCNNNNUNCN	*Measles*
Genus Pneumovirus:	
UCANNNNNNUU (UU) / 1-52 /CCCNNUUU	*RSV*
UANUUANNN/	*PVM*
Family Rhabdoviridae	
Genus Vesiculovirus:	
AUACU/ NA /UUGUCNNUAG	*VSV*

dence for the apparent uniqueness of the pneumoviruses has come solely from characterization of the bovine and human respiratory syncytial (RS) viruses, although current work on pneumonia virus of mice (PVM), until recently the only other recognized member of the genus, indicates that the structure and function of its genome are virtually identical to those of the RS viruses (Chambers *et al.*, 1990 and unpublished data; Ling and Pringle, 1989a,b). The identification of turkey rhinotracheitis virus (TRTV), a new avian respiratory pathogen, which has similar molecular characteristics to the RS viruses and PVM (Ling and Pringle, 1988; Cavanagh and Barrett, 1988), has increased the biological diversity of the pneumoviruses and strengthened the case for regarding them as a distinct group. Ultimately, the pneumoviruses may be accorded separate taxonomic status; however, for the present they are still ranked as members of the family *Paramyxoviridae.*

Figure 1 compares diagrammatically the structures of the genomes of the rhabdoviruses and the paramyxoviruses. Comparisons of nucleotide and deduced amino acid sequences have confirmed the distinctiveness of the pneumoviruses among the other paramyxoviruses. Alignment of protein sequences suggests possible conservation of cysteine residues and hydrophobic regions in the fusion (F) protein, but little more (Morrison, 1988; Rima, 1989). On the contrary, clear relationships are discernible among all members of the genera *Paramyxovirus* and *Morbillivirus* which have been compared so far.

It is more informative to align and compare amino acid sequences, because the inherent variability of RNA genomes is undoubtedly constrained by the selective forces which maintain protein structure and function. From such comparisons, it has been concluded that the paramyxoviruses can be separated into two distinct groups (Rima, 1989). The first group is fairly homogeneous and comprises the bovine and human strains of parainfluenza virus type 3 and Sendai virus (parainfluenza virus type 1), whereas the second is more heterogeneous and includes Newcastle disease virus (avian parainfluenza virus type 1), simian virus 5 (SV5), mumps virus, and possibly parainfluenza virus type 4 (Komada *et al.*, 1989). This subdivision is reflected in the differing organization of the genome, and supported by immunoprecipitation studies (Ito *et al.*, 1987). The former group is more closely related to the morbilliviruses than is the latter.

In the first group the *P* gene encodes a nonstructural protein in an alternate reading frame. In the second group the internal nonstructural protein gene is encoded in the same reading frame within the *P* gene, and some of these viruses have an additional gene coding for a small hydrophobic (SH) protein inserted between the F and HN envelope glycoprotein genes. Which genome represents the higher form cannot be deduced from such data. On present evidence loss of genes by paramyxoviruses with progression toward the basic pattern of the vesiculoviruses, or acquisition of genes with progression toward the greater complexity exhibited by the pneumoviruses (Fig. 1), may equally be the direction of evolution in the family *Paramyxoviridae.*

Within the morbillivirus group, protein sequence comparisons and monoclonal antibody studies (Sheshbaradaran *et al.*, 1986) suggest that measles virus and rinderpest virus are more closely related than measles virus and canine distemper virus. Norrby *et al.* (1985) have proposed on the basis of monoclonal

antibody studies that rinderpest virus represents the archetypal morbillivirus from which canine distemper virus diverged at an earlier time in evolution than measles virus.

II. GENOME ORGANIZATION

A. Genome Structure and Function

Details of the structure and function of the genome of paramyxoviruses are discussed elsewhere in this volume and are summarized in Fig. 1. The features of relevance to this chapter are that the paramyxoviruses and morbilliviruses possess six or seven genes and the pneumoviruses possess ten genes. The RS virus genome is unique among paramyxoviruses in that the normally 3′-terminal N-protein gene is preceded by two small nonstructural protein genes, 1C and 1B, of unknown function. In general, gene start and stop signals correspond to consensus sequences which are different for the different genera. Intergenic junctions show more variability among individual viruses and a genus-specific pattern is less evident (Fig. 2). There does not appear to be any temporal control of transcription in paramyxoviruses, but there is a progressive attenuation of transcription with increasing distance from the promoter, so that the 3′-terminus-adjacent *NP* or *N* mRNA is the most abundant species and the L-protein mRNA the least. These attributes are shared in common with the rhabdoviruses, with the exception that in the pneumoviruses the messages transcribed from the 3′-terminal enigmatic 1C and 1B nonstructural protein genes are the most abundant species of viral RNA in the infected cell. Positive-sense and negative-sense leader RNAs have not been unequivocally identified in measles virus-infected cells (Crowley *et al.*, 1988).

The genera are also differentiated by the nature of their surface glycoproteins, as described elsewhere in this volume. All three genera possess a fusion (F) protein gene, and infectivity is probably dependent on proteolytic cleavage of the gene product, although this has not been demonstrated in every case. The functional activities of the attachment protein vary among genera and include hemagglutinin and neuraminidase (HN) activities in the paramyxoviruses, hemagglutinin (H) alone in the morbilliviruses, and neither in RS virus. The pattern in the pneumoviruses is not uniform, however, since PVM can hemagglutinate mouse cells, and this activity has been localized to the putative attachment (G) protein (Ling and Pringle, 1989b). The H protein of morbilliviruses is structurally and in part functionally analogous to the HN protein of parainfluenzaviruses. The G protein of pneumoviruses is structurally different, being a small polypeptide heavily modified by attachment of predominantly O-linked carbohydrate chains. It does resemble the HN attachment proteins of Sendai virus, Newcastle disease virus, and SV5 by its insertion into the lipid bilayer, with the carboxy terminus located externally. The attachment (G) protein and fusion (F) protein genes of RS virus are inverted in the gene order relative to other paramyxoviruses (Fig. 1). This inversion is unlikely to represent a recombinational event, since the block of envelope protein genes in RS virus has been expanded from the three [M, F, H(N)] of

other paramyxoviruses to five [M, 1A, G, F, 22K(M2)]. The pneumoviruses are unique among enveloped RNA viruses in having two additional membrane-associated proteins. The precise function of these additional membrane proteins is unknown, but one of them, the glycosylated 1A protein, appears to contain two overlapping T cell-stimulating sites (Nicholas *et al.*, 1989). Although the genome of RS virus contains more genes than the genomes of the other paramyxoviruses characterized so far, the individual genes are in general smaller. In fact, the sequence of the *L* gene of RS virus has now been determined (P. L. Collins, personal communication), but the size of the complete genome is similar to that of other paramyxoviruses (see p. 7).

The descending order of overall conservation of the structural proteins of paramyxoviruses appears to be L > M > F > N > H(N) > P (Rima, 1989). The *L* genes and proteins of Sendai virus and measles virus exhibit very close similarity. The L proteins of NDV and measles virus are less similar, but there is only a distant resemblance between the L proteins of VSV and measles virus or Sendai virus. However, the L proteins of NDV and VSV do show considerable similarity (Yusoff *et al.*, 1987), and Blumberg *et al.* (1988) have proposed that this represents an evolutionary pathway leading from VSV via NDV to Sendai virus and ultimately to measles virus. Furthermore, they suggest that this pathway is consistent with the progressive narrowing of the host range of these four viruses and that the evolution of two separate glycoproteins of the paramyxoviruses from the *G* gene region of VSV may be part of this process. The L proteins of paramyxoviruses exhibit more extensive similarities, containing conserved sequences common to a number of viral RNA-dependent RNA polymerases, including the Gly-Asp-Asp motif which is considered to represent the nucleic acid recognition or active site of an ancestral RNA polymerase (Kamer and Argos, 1984).

The matrix proteins of paramyxoviruses are hydrophobic in nature, but show little conservation of sequence. Nonetheless, the similarity of the hydropathy plots for the M proteins of measles virus and Sendai virus, and the dissimilarity of those of NDV and mumps virus, reaffirms the close relationship of the morbilliviruses and the Sendai/parainfluenzavirus type 3 subdivision of the paramyxoviruses referred to previously. The overall conservation of cysteine residues, helix-breaking residues, and paired basic residues suggests that a common tertiary structure is maintained (Morrison, 1988; Rima, 1989).

Likewise, among the fusion proteins of paramyxoviruses there is overall conservation of those residues likely to be important determinants of tertiary structure, and the same functional regions (signal region, cleavage site, and membrane anchor region) are preserved throughout the family (Morrison, 1988). Glycosylation sites are not rigidly conserved; in the morbilliviruses there is a relative preponderance of glycosylation sites in the F_1 fragment, whereas in the paramyxoviruses and pneumoviruses the inverse is the case.

The N proteins of parainfluenza viruses appear to possess three distinct domains which may correspond to an amino-terminal RNA-binding region, a central region involved in transcription, and a carboxy-terminal, negatively-charged region possibly exposed on the outside of the nucleocapsid (Buckland *et al.*, 1988). The central region shows the most conservation and the carboxy-terminal region the least.

The attachment proteins of the paramyxoviruses differ in their functional properties: the HN of the paramyxoviruses has hemagglutinating and neuraminidase activities, the H of the morbilliviruses has hemagglutinating activity only, and the G of pneumoviruses has neither in the RS viruses and hemagglutinating activity only in PVM (Ling and Pringle, 1989b). The sequence similarities of the attachment proteins follows the taxonomic subdivision of the family. Within genera there is considerable resemblance, whereas between genera there is little, or none in the case of the pneumoviruses. The cysteine residues are well conserved in H(N) molecules, indicating possibly structural similarity. The G protein of RS virus is exceptional in its rich endowment in O-glycosylation sites.

The P proteins exhibit lesser sequence similarity than the other proteins of paramyxoviruses. They resemble the NS proteins of rhabdoviruses in that the amino-terminal region is hypervariable, conservation being limited to the carboxy-terminal region (Banerjee, 1987).

B. Coding Potential

The size of paramyxovirus genomes is remarkably uniform. The four genomes which have been sequenced completely lie within a few hundred nucleotides of each other; 15,892 nucleotides for measles virus (Crowley *et al.*, 1988), 15,222 for human RS virus (P. L. Collins, personal communication), 15,285 for Sendai virus (Shioda *et al.*, 1986), 15,156 for NDV (Yusoff *et al.*, 1987), and 15,463 for parainfluenza virus type 3 (see Chapter 2 and Appendix to this book). The genomes of the paramyxoviruses are larger than those of the other negative-strand genomes sequenced so far; e.g., the genome of an influenza A virus has been determined as 13,588 nucleotides, that of a bunyavirus as 12,278 nucleotides, and that of the Indiana serotype of VSV as 11,162 nucleotides. By contrast, the circular, negative-stranded hepatitis delta virus has the smallest known RNA genome at 1678 nucleotides.

The coding potential of negative-strand RNA viruses may be extended by a variety of devices, including obligate mRNA splicing, alternate mRNA splicing, and translation from overlapping reading frames. In the paramyxoviruses, the *P* genes of Sendai virus, parainfluence type 3 virus, and measles virus encode a second gene product in an overlapping reading frame which is translated from a functionally bicistronic mRNA. In Sendai virus, a third gene product (C′) of the *P* gene has been identified, which is translated from an anomalous ACG initiation codon upstream from the AUG codons initiating translation of the P and C polypeptides (Curran and Kolakofsky, 1988).

In other paramyxoviruses the *P* gene products appear to be encoded in the same reading frame. The SV5 genome encodes a P protein, which is a virion protein and a component of the transcription complex, and a V protein of unknown function. The P and V proteins are amino-coterminal proteins with 164 amino acids in common, but with different carboxy termini. Thomas *et al.* (1988) have demonstrated that these proteins are translated from two separate mRNAs that differ by the presence or absence of two nontemplated G residues. The presence of these two G residues converts the two overlapping reading frames into a single reading frame encoding a sequence of 392 amino acids. The

mechanism of addition of the two nontemplated nucleotides is unknown, but could be accounted for by reiterative copying at a point where the template has a run of four C residues, and indeed a sequence resembling the polyadenylation signal is located just upstream from the four C residues. The unique C terminus of the V protein has a cysteine-rich region which is present as a highly conserved element of the open reading frame of the *P* gene of other paramyxoviruses, suggesting that it is functionally important. SV5 appears to be unique among paramyxoviruses in having two mRNAs associated with the *P* gene.

In summary, despite the overall similarity of genetic organization of viruses in the paramyxovirus group, there is diversity in the strategy of transcription and in posttranscriptional processing. The overlap of the 22K- and L-protein genes of respiratory syncytial virus may be another unique and as yet unresolved mechanism of transcriptional control (Collins *et al.*, 1987).

III. GENETIC INTERACTIONS

A. Absence of Genetic Recombination

Genetic recombination is now a well-established phenomenon in picornaviruses (King *et al.*, 1982). In laboratory experiments, both intra- and interserotypic recombinants of both poliovirus and of foot-and-mouth disease virus have been isolated in progeny from mixed infections, and recombinant polioviruses are generated in the human intestine during natural infection. It has also been shown that recombination occurs at high frequency in coronaviruses (Lai *et al.*, 1985). However, recombination is not universal in positive-strand RNA viruses, and reports of recombination in alphaviruses have not been confirmed. Recombination appears to be restricted to viruses which lack nucleoprotein, and presumably the investment of the genome by nucleoprotein prevents intramolecular exchange.

There are no confirmed reports of genetic recombination involving negative-strand RNA viruses, other than the reassortment of genome subunits which is universal among the segmented genome viruses. Granoff (1959a,b, 1961a,b, 1962) and Dahlberg and Simon (1968, 1969a,b) demonstrated that presumptive non-*ts* recombinants among the progeny virus from mixed infections of *ts* mutants of Newcastle disease virus (NDV) were in fact complementing heterozygotes. UV irradiation and density gradient electrophoresis were used to investigate the physical structure of these heterozygotes, but the data were conflicting and open to several interpretations (Kingsbury and Granoff, 1970). The pleomorphic morphology of paramyxoviruses suggests that multiploid particles are abundant components of the progeny of all paramyxoviruses. Complementation of *ts* mutants is a special case of the more general phenomenon of phenotypic mixing, and phenotypic mixing involving serotype and thermal stability has been recorded also in paramyxoviruses.

Kirkegaard and Baltimore (1986) have shown that recombination in poliovirus involves the negative template strand generated during replication, therefore there would appear to be no inherent barrier to recombination in negative-strand RNA viruses. It must be presumed that the rapid association of the nucleoprotein with the negative and positive RNA strands which is charac-

teristic of the replication of all negative-strand RNA viruses prevents interactions between RNA molecules which might result in recombination. There is also no record of rescue into infectious virus of genetic information from the defective-interfering viruses which are present in laboratory stocks of most paramyxoviruses propagated by high-multiplicity cycles of infection.

Drake (1962) and Kirvatis and Simon (1965) observed that high multiplicity of infection with UV-irradiated Newcastle disease virus resulted in a greater number of infected cells or yield of virus than expected from the surviving infectivity. Multiplicity reactivation by a recombinational mechanism cannot account for this phenomenon, since recombinants were not identified. Likewise, production of complementing heterozygotes by UV irradiation is not a sustainable explanation, since a UV-induced lesion anywhere in the genome would abort transcription of the genome. Peeples and Bratt (1982a) showed that at high multiplicity of infection, input virions could supply a UV-resistant function, probably a virion protein, which could rescue other *ts* mutants by complementation. In the paramyxoviruses, therefore, multiplicity reactivation may be another extreme manifestation of phenotypic mixing.

Recently Sakaguchi *et al.* (1989) and Toyoda *et al.* (1989) analyzed the sequences of the hemagglutinin-neuramindase genes of 13 strains of NDV isolated over a period of 50 years and the sequences of the fusion gene of 11 of these strains by a method involving comparison of synonymous nucleotide substitutions in the coding regions. The rate of synonymous substitution can serve as an indicator of evolutionary progression, since it is approximately constant for different genes and greater than the number of amino acid replacements (Miyata and Yasunaga, 1980). Three distinct lineages could be defined which appeared to have cocirculated for a considerable period. Virulent strains were associated with one lineage and avirulent strains with another, whereas the third lineage consisted solely of North American isolates, but contained both virulent and avirulent strains. It was concluded that the different strains appeared to have evolved by accumulation of point mutations and that no gene exchange by recombination had occurred in generation of the three lineages.

Sakaguchi *et al.* (1989) concluded that the evolutionary pattern of Newcastle disease virus with its multiple lineages and absence of progressive antigenic drift more resembled that of the influenza B and C viruses than that of the influenza A viruses, which are characterized by linear evolution and progressive antigenic change.

Comparison of the frequencies of occurrence of monoclonal antibody escape mutants indicated that the inherent genetic variabilities of a paramyxovirus (Sendai virus), a rhabdovirus (vesicular stomatitis virus), and an orthomyxovirus (influenza virus type A) were not markedly different (Portner *et al.*, 1980). Therefore, the occurrence of progressive antigenic drift in influenza virus and its apparent absence in the paramyxoviruses is due to factors other than differences in basic mutation rate.

B. Complementation Analysis with Conditional Lethal Mutants

Genetic analysis in negative-strand RNA viruses with unsegmented genomes is limited to the use of complementation analysis to identify and define

the functions of individual genes of the viral genome. Suppressor-mediated chain-terminating mutations are rare in mammalian viruses, and among negative-strand RNA viruses have only been identified unequivocally in the case of VSV (White and McGeoch, 1987). In most viruses, including the paramyxoviruses, the conditional-lethal mutants employed in genetic analysis are exclusively of the temperature-sensitive type. In both the rhabdoviruses and the paramyxoviruses, collections of temperature-sensitive (*ts*) mutants have been classified into groups containing mutants which do not complement *inter se,* but which complement mutants in other groups. With some minor exceptions, the pattern of complementation is nonoverlapping. In favorable circumstances, the underlying assumption that the complementation groups correspond to the component genes of the viral genome has been substantiated by characterization of representative mutants and identification of the mutational lesion. For example, the 5/6 complementation groups of vesicular stomatitis virus (VSV) have been equated with a fair degree of certainty with individual genes [reviewed in Pringle (1988)].

In the paramyxoviruses and the other rhabdoviruses the assignment of complementation groups is less advanced and is complicated by inability to distinguish between intergenic and intragenic complementation. Where intragenic complementation is prevalent, the number of complementation groups will be overestimated. Intragenic complementation is a consequence of the multifunctional nature of some viral gene products. In the rhabdoviruses the multifunctional role of the L protein appears to be responsible for most intragenic complementation (Gadkari and Pringle, 1980), whereas in paramyxoviruses, as discussed below, the multifunctional properties of the hemagglutinin/neuraminidase (attachment) protein appear to be responsible for most intragenic complementation.

Temperature-sensitive mutants of Newcastle disease virus and Sendai virus (representing the paramyxoviruses), measles virus (representing the morbilliviruses), and respiratory syncytial virus (representing the pneumoviruses) have been obtained by chemical mutagenesis and classified into complementation groups. Five groups have been identified in Newcastle disease virus, seven groups in Sendai virus and measles virus, and eight in human respiratory syncytial virus. The data are summarized in the following four subsections and in Tables I–IV.

1. The Temperature-Sensitive Mutants of Newcastle Disease Virus

a. Complementation Groups

Newcastle disease virus exhibits great variation in its biological and disease-producing properties and it has a long history of genetic analysis, the early stages of which have been reviewed comprehensively by Bratt and Hightower (1977). The most definitive analysis of temperature-sensitive mutants has been carried out by Tsipis and Bratt (1976). Forty-nine mutants were derived from the Australia-Victoria strain of Newcastle disease virus by chemical mutagenesis. Twenty-eight were obtained by nitrosoguanidine treatment, 15 by nitrous acid treatment, and three by growth in the presence of 5-fluorouracil.

TABLE I. The Complementation Groups of Newcastle Disease Virus[a]

Group	RNA phenotype at restrictive temperature (41.8°C)	Gene assignment (tentative)	Nature of evidence
A	–	(*L*)	Largest group (47% of mutants); defective in primary transcription; no rescue by L-deficient *nc* mutants; UV transcription mapping
B/C/BC	+	*HN*	Defective in HN-related functions
D	+	*M*	Electrophoretic migration difference
E	–	*P*	Defective in secondary transcription; complement L-deficient *nc* mutants; UV transcription mapping

[a]For references, see text.

However, five spontaneous mutants were also isolated and the frequency of *ts* mutants in mutagenized stocks was only 3–5% compared with 2% in untreated material. Nine of the mutants were regular temperature-sensitive mutants, whereas the other six mutants plated with equal efficiency at the permissive (36°C) and restrictive (41.8°C) temperatures, but produced only minute plaques at the restrictive temperature. These 15 mutants were classified into five (initially six) complementation groups. Group A contained five mutants with RNA-negative phenotypes; group B, four RNA-positive mutants; group C, one RNA-negative mutant; group D, two RNA-positive mutants; and group E, one RNA-positive mutant. A low level of complementation was observed between some mutants in groups B and C, and a sixth group, BC, containing two mutants was tentatively defined. Subsequent detailed analysis of the phenotypic properties of these and additional mutants added to groups B, C, and BC confirmed one of the several interpretations of these data, namely that the complementation observed between mutants in these groups represented intragenic complementation. Therefore, only four distinct complementation groups are represented in this collection of mutants, i.e., groups A, B/C/BC, D, and E (Table I). The B/C/BC complex clearly represents mutations affecting the HN-protein gene, and the A, D, and E groups have been tentatively assigned to represent the *L*, *M*, and *P* genes, respectively (see below).

b. Assignments

The RNA-negative mutants of group A have been assigned to the L-protein gene (Madansky and Bratt, 1981a,b; Peeples and Bratt, 1982a; Peeples *et al.*, 1982). Four of the seven mutants in group A which were studied in detail were partially or completely defective in primary transcription, as defined by RNA synthesis in the presence of cycloheximide and actinomycin D. Following release of the cycloheximide block, there was no restoration of RNA synthesis during incubation at the restrictive temperature. Similarly, no RNA synthesis

was observed following shift from permissive to restrictive temperature during the first 3 hr of incubation. Except in the case of mutant *ts*A1, which was rapidly inactivated at nonpermissive temperature, shift-up at later times allowed synthesis, suggesting that secondary transcription from progeny genomes was not deficient. Non-*ts* revertants of all the mutants had normal transcriptive ability restored. The group A mutants were assigned to the L-protein gene on the basis of their predominance (47%) and the fact that the L-protein gene accounts for approximately half of the genome, and the observation that the L-protein-deficient, noncytopathic mutants (Madansky and Bratt, 1981a,b), described in Section C.1, complemented the group E RNA-negative mutant, but not the group A mutants. Furthermore, UV inactivation of the ability of mutant *ts*E1 to complement *ts*A1 suggested that the *ts*A1 defect was located furthest from the promoter site, as is the L-protein gene (Collins *et al.*, 1980; Peeples and Bratt, 1982a).

The balance of evidence suggests that the other RNA-negative group, complementation group E defined by the solitary mutant *ts*E1, represents a lesion in the P-protein gene. Complementation of mutant *ts*E1 by *ts*A1 was inactivated by UV irradiation at the same rate as the *P* gene, and, as noted above, all of the noncytopathic putative L-protein mutants complemented *ts*E1. Furthermore, UV-irradiated mutant *ts*B1, which has a non-*ts* mutation affecting the electrophoretic mobility of P, complemented both *ts*E1 and *ts*A1, but did not amplify the expression of P in multiply-infected cells, which is consistent with failure of the irradiated virus to replicate despite the presence of components necessary for the replication of *ts*E1. Mutant *ts*E1 was able to sustain primary transcription at 50% of the wild-type level; however, cycloheximide release and temperature shift-up before 4 hr postinfection suggested that secondary transcription was defective. RNA synthesis was observed on shift-up after 4 hr, suggesting that RNA transcription from progeny genomes was unimpaired. A non-*ts* revertant of *ts*E1 exhibited normal RNA synthesis at the restrictive temperature of 41.8°C.

The assignment of the B, C, and BC groups to the HN-protein gene was confirmed by study of the thermolability of four virion functions associated with the HN protein: hemagglutination, neuraminidase activity, hemolysis and infectivity (Peeples *et al.*, 1983). Some mutants in groups B, C, and BC were much less stable in all four functions than wild-type virus or RNA-positive mutants belonging to group D. Three of four non-*ts* revertants of the most thermolabile mutant exhibited normal HN functions. A large proportion of the non-*ts* revertants of the HN-protein gene (mutants in groups B, C, and BC) appeared to be pseudorevertants. Peeples *et al.* (1988) explored the basis for the decreased incorporation of HN into virions and the temperature sensitivity of the 11 B/C/BC group mutants. The HN of two of the mutants had altered electrophoretic migration rates. In all cases, as much HN protein was synthesized in mutant-infected cells as in cells infected by wild-type virus, but the HN protein of six mutants was rapidly degraded. The HN (and F) protein of NDV normally undergoes antigenic maturation early in infection from a form unable to react with antibodies generated against mature HN to a reactive form. All the mutants, including the six with metabolically unstable HN, were deficient in conversion of the HN protein to the mature antigenically reactive

form, indicating an early block in processing. With the exception of one mutant (BC2), the neuraminidase activity of infected cells was temperature sensitive, but the hemadsorbing properties of infected cells were not. The HN protein of mutant BC2 was extremely thermolabile and both activities were temperature sensitive. These results further substantiate the hypothesis that mutants classified in group B/C/BC have defective HN proteins, and that the complementation observed between these mutants represents intragenic complementation.

The group D mutants exhibited normal hemagglutinating and neuraminidase activities, but had lower specific infectivities and hemolytic activities, indicating mutation in a gene for a protein required for membrane fusion and infectivity (Peeples and Bratt, 1982a,b). Unlike the thermostability of the hemagglutinating and neuraminidase activities, the thermostabilities of infectivity and hemolytic activity were salt dependent. Two of the three group D mutants exhibited increased thermolability in low-salt buffer, which is consistent with the assignment of this group to a gene other than the HN-protein gene. These lower activities were correlated with decreased amounts of the cleaved fusion ($F_1 + F_2$) glycoprotein in virions of group D *ts* mutants of Newcastle disease virus propagated in eggs. In fact, incorporation of $F_1 + F_2$ into virions was diminished at both permissive and restrictive temperatures when the group D mutants were propagated in cultured chick embryo cells, yet infectivity was correlated with the amount of $F_1 + F_2$ in virions. One of the group D mutants, *ts*D1, produced an M protein which migrated faster in SDS-polyacrylamide gel, and the M protein of three of four non-*ts* revertants of this mutant exhibited the electrophoretic mobility of the wild-type M protein. In the case of these three mutants, and also three non-*ts* revertants of both *ts*D2 and *ts*D3, there was coreversion of the low-specific-infectivity and reduced-hemolytic-activity phenotypes to near normal levels and normal incorporation of $F_1 + F_2$ into virions. These observations indicate that the group D mutants represent lesions of the M protein gene and that a defective M protein is responsible for the $F_1 + F_2$ related phenotypes (Peeples and Bratt, 1984). The group D mutants imply a specific interaction between the M and F proteins of Newcastle disease virus which has not been detected by other means. At least seven of the ten revertants of *ts*D mutants examined were considered to be pseudorevertants, since their heat stability was significantly different from both their parents and the wild-type virus.

c. Other Temperature-Sensitive Mutants of Newcastle Disease Virus

Dahlberg and Simon (1968) obtained 48 temperature-sensitive mutants of the Beaudette-C strain of Newcastle disease virus using a restrictive temperature 42.5°C and nitrous acid mutagenesis. These mutants were classified into complementation groups; five groups were described by Dahlberg and Simon (1968) and nine by Dahlberg (1968). These mutants were employed subsequently in the study of multiploid particles (Dahlberg and Simon, 1969a,b). The properties of six of these mutants from different complementation groups and non-*ts* revertants of each mutant were studied by Sampson *et al.* (1981). Two-dimensional electrophoresis was employed to discriminate

changes in size and/or isoelectric point of individual proteins which might occur as a result of missense mutation. One of the six mutants appeared to be a double mutant with a temperature-sensitive lesion affecting the P protein and a non-temperature-sensitive lesion involving the HN protein. This mutant had an RNA-negative phenotype at the restrictive temperature, suggesting involvement of the P protein in RNA polymerase activity. Further characterization of these mutants has not been reported.

Preble and Youngner (1973a) isolated four spontaneous and four nitrous acid-induced *ts* mutants of the Herts strain of NDV using a restrictive temperature of 43°C. All were RNA-negative. Preble and Youngner (1972, 1973b, 1975) also described RNA-negative temperature-sensitive variants isolated from a persistently infected line of L cells which had been initiated by infection with the same strain of Newcastle disease virus. Two types of variant were defined, one which continued to synthesize RNA for some time after shift-up from permissive to restrictive temperature and one which did not. Three other temperature-sensitive variants isolated from these cells possessed virion transcriptases which were less stable than the wild-type enzyme when assayed *in vitro* at 42°C (Stanwick and Hallum, 1976).

These mutants were used by Kowal and Youngner (1978) to analyze the functions required for induction of interferon by Newcastle disease virus. Newcastle disease virus is a poor inducer of interferon in unprimed chick embryo cells. However, when infectivity is inactivated by UV irradiation, Newcastle disease virus can become a potent inducer of interferon in unprimed cells. From their analysis they concluded that no single virus function appeared to be responsible for interferon induction. At the restrictive temperature, the interferon-inducing capacity of some UV-irradiated mutants was dependent on prior exposure of the cells to interferon, and one mutant (*ts*100) was defective at permissive temperature as well as restrictive temperature. The interferon-inducing ability of this mutant could be partially restored by coinfection with heavily-irradiated wild-type virus. The molecular basis of interferon induction has not been resolved.

A variety of other variants of Newcastle disease virus have been recovered from persistent infection of cultured cells and this work has been comprehensively reviewed by Bratt and Hightower (1977).

2. The Temperature-Sensitive Mutants of Sendai Virus

a. Complementation Groups

Ten temperature-sensitive mutants of the Enders strain of Sendai virus have been described by Portner *et al.* (1974). These mutants were isolated following exposure to *N*-methyl-*N*-nitro-*N*-nitrosoguanidine or growth in the presence of 200 μg/ml 5-fluorouracil. The parental and mutant viruses were grown in chick embryo lung (CEL) fibroblasts at 30°C with a restrictive temperature of 38°C, some 5–6 deg below that employed with NDV. The ten mutants were placed in seven nonoverlapping complementation groups, five of the groups (A, D, E, F, and G) being represented by single mutants and groups B and C by two and three mutants, respectively (Table II). Since the mutants in

TABLE II. The Complementation Groups of Sendai Virus[a]

Group	RNA phenotype at restrictive temperature (38°C)	Gene assignment (tentative)	Nature of evidence
A	−	None	—
B	−	None	—
C	−	None	—
D	−	(*N*) or (*L*) or (*P*) defect	Nucleocapsid assembly
E	−	None	—
F	−	None	—
G	+	*HN*	Production of HN-deficient particles; enhanced transcription (due to absence of HN-mediated inhibition)

[a]For references, see text.

six of the seven groups had RNA-negative phenotypes and individual complementation values ranged between 2 and 30,000, it is likely that the number of discrete complementation groups has been overestimated due to inability to discriminate intergenic and intragenic complementation.

b. Assignments

Biochemical characterization of the ten mutants distinguished at least four phenotypic groups. Temperature-shift experiments revealed three phenotypes among the RNA-negative mutants. Mutants in groups A, B and C appeared to be defective in an early function which was not required throughout the growth cycle, whereas the group D mutant was defective in RNA synthesis throughout the entire cycle, with the mutants in groups E and F intermediate in behaviour.

Mutant *ts*271, the single RNA-positive mutant representing group G, was defective in hemagglutinating activity. Portner *et al.* (1975) subsequently showed that noninfectious particles which lacked both the M_r 70,000 virion envelope protein and hemagglutinin and neuraminidase activities were released from *ts*271-infected CEL cells maintained at the restrictive temperature. These M_r 70,000 protein-deficient particles failed to attach to susceptible cells, thereby confirming that the HN protein was the attachment protein. The HN polypeptide was synthesized in infected cells at the restrictive temperature and was presumed to be unable to adopt a functional configuration. This conclusion was supported by the fact that mutant virions released from cells maintained at the permissive temperature exhibited a reversible ability to hemagglutinate erythrocytes on temperature shift to 38°C and back to 30°C. The neuraminidase activity of the protein was unaffected by temperature shift, indicating that the hemagglutinating and neuraminidase activities of the molecule were functionally separate. The behavior of mutant *ts*271 confirmed that morphogenesis of Sendai virus particles did not depend

on maintenance of the structural and functional integrity of the HN protein, and that neuraminidase played no role in any virus-specified event between eclipse and release. Because of the absence of cell killing at the restrictive temperature, it was concluded that the native HN protein was a determinant of cytopathogenicity. The HN-deficient particles exhibited sevenfold enhanced *in vitro* transcriptase activity, which correlated with previous observation of an inhibitory effect of HN protein on *in vitro* transcriptase activity (Marx *et al.*, 1974). No revertants of *ts*271 could be isolated and comparison of the nucleotide sequence of the HN genes of *ts*271 and wild type revealed three amino acid replacements, two in close proximity at positions 262 and 264 and one distant at position 461. The two adjacent replacements are close to antigenic site I as defined by neutralization escape mutants (Fig. 2) and may be responsible for the temperature-sensitive phenotype (Thompson and Portner, 1987).

Only one of the RNA-negative, temperature-sensitive mutants of Sendai virus, mutant *ts*105 of group D, has been examined in detail (Portner, 1977). The *ts*105 mutation appeared to involve a nucleocapsid protein, possibly P or NP, which is involved in the synthesis or stability of viral RNA. There was no evidence of any defect in viral mRNA translation, but on shift to restrictive temperature there was a time-dependent loss of ability of the NP protein to assemble into nucleocapsids. NP synthesized at restrictive temperature, on the other hand, could assemble into nucleocapsids on temperature down-shift. Association of the P protein with nucleocapsids was unaffected by the temperature of incubation. However, since the behavior of the L protein was not determined in these experiments and revertants were not studied, the temperature-sensitive phenotype could not be associated with any specific core polypeptide. Interestingly, replicative RNA synthesis was favored over transcription on resumption of RNA synthesis on shift-down to permissive temperature.

3. The Temperature-Sensitive Mutants of Measles Virus

a. Complementation Groups

A total of five (possibly seven) complementation groups have been defined by analysis of three independently isolated series of temperature-sensitive mutants. Temperature-sensitive mutants of the Rapp strain were isolated by Yamazi and Black (1972), of the Edmonston strain by Bergholz *et al.* (1975) and Ju *et al.* (1980), and of the Schwarz vaccine strain by Haspel *et al.* (1975). In each series a temperature of 39°C was used as the restrictive temperature.

Yamazi and Black (1972) obtained seven *ts* mutants following mutagenesis with proflavine or 5-fluorouracil. Complementation was observed with some combinations of mutants and the results were interpreted as indicative of the existence of three groups. Temperature-shift experiments were carried out which identified mutants with early and late functional defects (Yamazi *et al.*, 1975), but further characterization of these mutants has not been reported.

Bergholz *et al.* (1975) obtained nine mutants from an unattenuated Edmonston virus grown in the presence of 100 μg/ml 5-fluorouracil, and these mutants were classified into three complementation groups. Group A (*ts*1) and

group B (*ts*2, *ts*3, *ts*4, *ts*5, *ts*6, and *ts*7) contained RNA-negative mutants, and group C (*ts*9) contained a single RNA-positive mutant. Mutant *ts*8 complemented the two mutants representing groups A and C and accordingly was classified in group B, but it was distinguishable from the other mutants of group B by its intermediate phenotype and its propensity to interfere with their multiplication. These *ts* mutants were attenuated in their ability to induce fatal illness following intracerebral inoculation of hamsters, and animals surviving infection exhibited no sequelae for up to 12 months following recovery.

Haspel *et al.* (1975) obtained 24 mutants after mutagenesis with 5-fluorouracil, 5-azacytidine, and proflavine from a derivative of the Schwarz vaccine strain. A wild-type stock of this strain (designated CC) able to grow at 39°C was established by cocultivation of persistently infected hamster cells and uninfected BS-C-1 cells. Three complementation groups were defined: Group I contained 21 mutants of RNA-negative phenotype, group II contained 2 mutants of RNA-positive phenotype, and group III contained a single mutant of RNA-negative phenotype. Twenty-three of the 24 mutants were derived from the CC wild type and one (*ts*A) was derived directly from the Schwarz vaccine strain.

Breschkin *et al.* (1977) carried out cross-complementation experiments to establish the homologies of the groups. Groups I and B of the two series did not cross-complement and were considered to be homologous, whereas the other groups of each series did cross-complement and therefore appeared to be unique. Consequently, five complementation groups of measles virus were identified and the groups were redesignated as A, B, C, D, and E (Table III). Group B contained 28 of the 33 *ts* mutants isolated.

Two more complementation groups were identified tentatively by Ju *et al.* (1980), and the phenotypic properties of these mutants are given in Table III. Ju *et al.* (1978) obtained their *ts* mutants without the use of mutagen from human lymphocytes persistently infected with the Edmonston strain. Eighty percent of the clones isolated from these cells were temperature sensitive in Vero cells. Twenty-one of the mutants were classified into the four groups A, B, C, and D by complementation with the standard groups defined by Breschkin *et al.*

TABLE III. The Complementation Groups of Measles Virus[a]

Group[b]	RNA phenotype at restrictive temperature (39°C)	Gene assignment (tentative)	Nature of evidence
A (A)	−	None	—
B (B or I)	−	(*L*)	Largest group (85% of mutants)
C (III)	−	None	—
D (C)	+	(*F*)	Hemolysin defect
E (II)	+	None	—
F	+	None	—
G	+	None	—

[a]For references, see text.
[b]Earlier designation in parentheses.

(1977). Group E could not be determined directly in this way, due to leakiness of the group E prototype stock, but two mutants complemented groups A, B, C, and D were presumed to represent group E, since they had phenotypic properties in common with other group E mutants. Two additional groups (F and G) were identified tentatively on the basis of their RNA-positive phenotypes. However, many (6/21) of the isolates obtained from persistently infected lymphocytes appeared to be multiple mutants, with mutation of the gene corresponding to group B the most common (17/22).

b. Miscellaneous Phenotypic Properties

The interferon-inducing properties of the mutants isolated by Haspel *et al.* (1975) and Bergholz *et al.* (1975) were investigated by McKimm and Rapp (1977). Unlike the three wild types, all the mutants tested and a revertant of one (*ts*G3), were incapable of inducing interferon at either permissive or restrictive temperature. Ability to induce interferon was not restored by complementation of different mutants. Coinfection of a noninducing mutant and wild type had no inhibitory affect on interferon induction. The most likely explanation of the failure of all the mutants to induce interferon was that the three wild-type stocks were heterogeneous for this property, and the mutants were derived from a predominant noninducing type.

Mutant *ts*G3 exhibited reduced neurovirulence in intracerebrally inoculated newborn hamsters and induced a high frequency of hydrocephalus (Breschkin *et al.* 1976; Haspel and Rapp, 1975; Haspel *et al.* 1975). Significantly, however, lower titers of infectious virus and less viral antigen were detected in the brain of animals infected with mutant *ts*G3. Antigen was predominantly localized to the meninges, and hydrocephalus was presumed to be a consequence of occlusion of the subarachnoid space and closure of the foramena of the fourth ventricle, rather than as a result of infection of ependymal cells. The pattern of immunofluorescence suggested that measles virus spread perivascularly and via the meningeal surface (Woyciechowska *et al.*, 1977).

Chui *et al.* (1986) described a temperature-sensitive mutant (*ts*38) of the LEC strain of measles with an unusual property. This mutant had been derived by 5-fluorouracil mutagenesis and possessed an RNA-negative phenotype at a restrictive temperature of 39°C. NP protein presynthesized in *ts*38-infected cells at 32°C was transported into the nucleus when the temperature was raised to 39°C. Nuclear accumulation of the NP protein is usually associated with isolates of measles virus obtained from SSPE patients by biopsy or from postmortem material, although it can be observed in both lytic and persistent infections. Robbins (1983) proposed that the extent of viral nuclear invasion was a reliable indicator of morbidity. Consequently, mutant *ts*38 may be useful in elucidating the role of nuclear invasion in measles virus pathogenesis.

Mutant *ts*38 has also proved a useful experimental tool in analysis of measles virus-induced immunosuppression. Measles virus can infect and multiply in stimulated human peripheral blood mononuclear cells, but at 37°C no infectious virus was released from *ts*38-infected cells, although virus protein synthesis was detected by immunofluorescence and RNA synthesis by means

of an *N* gene-specific probe detecting positive-sense RNA (Vydelingum *et al.*, 1989). The viability of *ts*38-infected cells was unaffected, but their response to mitogens and antigens was severely diminished. This experiment suggests that the immunosuppression observed during measles virus infection is not a consequence of destruction of immune cells. Antibodies to alpha-interferon partially reversed the virus-associated inhibition of lymphocyte mitogenesis, indicating that alpha-interferon may play a role in immunosuppression.

c. Persistent Infection

Haspel *et al.* (1973) and Armen *et al.* (1977) reported that a high proportion of the virus released from persistently infected cells was temperature sensitive, although in the former case Fisher and Rapp (1979a,b) found that defective virus rather than temperature-sensitive virus was released at later passages. This particular persistent infection had been initiated by infection of hamster embryo fibroblasts with a derivative of the Schwarz vaccine strain and during the initial phase of propagation did not spontaneously release infectious virus, although virus could be recovered by cocultivation with susceptible cells (Knight *et al.*, 1972). Subsequently, Fisher (1983) initiated persistent infections of Vero cell with the Schwarz vaccine strain, the Halle SSPE strain, the Edmonston strain, and mutant *ts*841 derived from the Edmonston strain. She found that only the cells infected with *ts*841 released temperature-sensitive virus. Wild and Dugre (1978), on the other hand, could find no involvement of either temperature-sensitive virus or defective virus in a persistent infection of BCG cells initiated by infection with the Halle strain. They concluded that a host-cell factor played the major role in restricting virus replication.

4. The Temperature-Sensitive Mutants of Human Respiratory Syncytial Virus

a. Complementation Groups

Induced temperature-sensitive mutants of the A2 (Gharpure *et al.*, 1969), the RSN-2 (Faulkner *et al.*, 1976), and the RSS-2 (McKay *et al.*, 1988) strains of human respiratory syncytial virus have been isolated from wild-type viruses propagated in secondary bovine kidney cells, BS-C-1 cells, and MRC-5 human diploid cells, respectively. The mutagens employed were 5-fluorouracil and 5-fluorouridine for the A2 strain, and 5-fluorouracil for the RSN-2 and RSS-2 strains. In the case of the A2 and RSS-2 strains, second-stage mutations were induced using nitrosoguanidine (L. S. Richardson *et al.*, 1977) or the acridinelike compounds ICR340 and ICR372 (McKay *et al.*, 1988), respectively. The restrictive temperature was 37°C for the A2 strain and 39°C for the other two strains.

Seven temperature-sensitive mutants of the A2 strain were classified into three complementation groups and the RSN-2 strain mutants into six groups. Although it was determined subsequently that the A2 strain belonged to the A and the RSN-2 strain to the B antigenic subgroups of respiratory syncytial virus, interstrain complementation was observed and the homology of the

TABLE IV. The Complementation Groups of Human Respiratory Syncytial Virus[a]

Group	RNA phenotype at restrictive temperature (37°C, 39°C)	Gene assignment (tentative)	Nature of evidence
A	+	(*F*)	F_0 mobility difference
B	(+)	*G*	G processing defect[b]
B′	−	(*L*)	Majority (45%) group
C	+	None	—
D	+	*M*	M instability and apparent electrophoretic mobility difference[c]
E	−	*P*	Loss of monoclonal antibody reactivity: Serine (AGU) present at position 172 in this mutant, and glycine (GGU) in the wild type and a non-ts revertant
F	(+)	None	—
G	(+)	None	—

[a]Compiled from Pringle *et al.* (1981) and unpublished data of C. Caravokyri.
[b]This mutant also exhibits a non-ts defect involving the F protein.
[c]The P protein of this mutant has a non-ts mutation at position 217 which affects its electrophoretic mobility. Asparagine (AAU) in the wild type is replaced by aspartic acid (GAU) in the mutant.

groups could be established. The conditions for successful complementation were critical and strain dependent (Gimenez and Pringle, 1978). Seven (Gimenez and Pringle, 1978) and subsequently eight (Pringle *et al.*, 1981) distinct complementation groups were defined. Complementation group A was common to both strains, groups B and C were unique to the A2 strain, and groups B′, D, E, F, and G were unique to the RSN-2 strain (Table IV). Six of the eight groups are represented by single mutants; hence it is not possible in these cases to discriminate between intergenic and intragenic complementation. However, since the genome of the A2 strain of respiratory syncytial virus is composed of at least ten genes (Collins *et al.*, 1984), it is likely that the complementation groups correspond to individual genes, since two of the single mutant groups have been successfully assigned (see below).

b. Assignments

The temperature-sensitive lesions in mutants *ts*1 and *ts*19 of the RSN-2 strain representing groups D and E involve the M and P proteins, respectively. The group D mutant has an M protein which is synthesized, but rapidly degraded, at restrictive temperature. This mutant can be complemented by a vaccinia virus recombinant expressing the RS virus M protein (C. R. Pringle, unpublished data). The group E mutant does not react with a unique anti-P monoclonal antibody, and non-*ts* revertants regain reactivity.

Mutant *ts*2 (A2 strain) of group A appears to have a temperature-sensitive mutation affecting the G protein and a non-temperature-sensitive mutation affecting the F protein (C. Caravokyri and C. R. Pringle, unpublished data). The dual lesion in *ts*2 correlates with the complex phenotype of this mutant, which appears to have a temperature-sensitive defect in attachment/adsorption and a non-temperature-sensitive lesion of the fusion function (Belshe *et al.*, 1977).

c. Vaccine Potential

Temperature-sensitive mutants of the A2 strain have been extensively evaluated as potential live vaccines [reviewed in Chanock (1982) and McIntosh and Chanock (1985)]. These mutants are restricted at 37°C and it was anticipated that they would replicate sufficiently in the upper respiratory tract where the temperature is in the range 32–34°C to induce a local immune response, but not penetrate into the lower respiratory tract. Mutant *ts*1 (complementation group A) was administered intranasally to seropositive adults and seropositive and seronegative children. No disease occurred, nor was there any indication of reversion of mutant *ts*1 during multiplication *in vitro* in cell culture, or *in vivo* in hamsters and adults. A significant immune response was observed in previously seronegative children, but sporadic mild disease was observed and there was evidence of loss of temperature sensitivity during replication in fully susceptible individuals. An attempt was made to obtain greater genetic stability and reduction of residual virulence by remutagenization with a different mutagen (NTG) and selection of more temperature-sensitive virus (L. S. Richardson *et al.*, 1977). However, the result was overattenuation (Belshe *et al.*, 1978) and the development of a *ts*1-based vaccine was abandoned despite its early promise.

Mutant *ts*2 (complementation group B) of the A2 strain was found to be avirulent in primates and defective in adsorption/penetration in cultured cells at 37°C. However, this mutant proved to be poorly infectious both in adult volunteers and in seronegative children, and consequently unsuitable for vaccine development.

The A2 strain mutants were isolated and propagated in bovine secondary cells and hence were vulnerable to contamination with extraneous agents. More recently, single-stage *ts* mutants restricted at 39°C and two-stage *ts* mutants restricted at 38°C have been derived from the RSS-2 strain entirely in human diploid cell culture. One of the two-stage mutants (*ts*1B) was tested in adult volunteers and proved to be almost as immunogenic as wild-type virus, but with greatly reduced disease-producing potential (McKay *et al.*, 1988; Watt *et al.*, 1990).

These independent vaccine trials, together with other studies of cold-temperature-adapted virus, establish that a substantial immune response can be generated by RS virus restricted to multiplication in the upper respiratory tract. The real value of these experiments is that they demonstrate that vaccination can be achieved without the exacerbation of disease encountered in the earlier trials of formalin-inactivated vaccine (McIntosh and Fishaut, 1980).

C. Other Mutants

1. The Noncytopathic Mutants of Newcastle Disease Virus

Noncytopathic (*nc*) mutants of the Australia-Victoria strain of NDV do not induce plaque formation in chick embryo fibroblasts, although near normal amounts of infectious virus are produced by infected cells. The *nc* mutants were detected because red blood cells adsorb to the surface of infected cells.

These mutants can be cloned and titrated by scoring hemadsorbing foci on infected chick embryo fibroblast monolayers. All the *nc* mutants were associated with extended mean embryo death times, and the mutants with the smallest foci exhibited the greatest prolongation of mean embryo death time. These observations suggested that the *nc* mutants have reduced virulence for chickens (Madansky and Bratt, 1978, 1981a,b).

Viral RNA and protein synthesis were reduced in *nc*-infected cells, and there was a disproportionate reduction in the amount of L protein in infected cells, though not in virions. Inhibition of host macromolecule synthesis was also diminished. Plaque-forming revertants of *nc* mutants exhibited coreversion, regaining plaque-forming ability, normal viral RNA and protein synthesis, and normal host protein synthesis inhibition. A new protein (X) identified as an altered form of the P protein was present in cells infected with two of the mutants, *nc*4 and *nc*16, and also in released virions. A subclone of *nc*4 which produced larger hemadsorbing foci had the normal form of the P protein restored (Madansky and Bratt, 1981a,b). Thus, the lesion in the P protein did not appear to be a primary determinant of cytopathogenicity. Similarly, a lesion in the F protein affecting its cleavability by trypsin was associated with mutant *nc*7, but did not appear to be a determinant of cytopathogenicity, since a plaque-forming revertant of *nc*7 still exhibited the F protein defect of the mutant. Thus, the P and F protein lesions in mutants *nc*4, *nc*7, and *nc*16 were genetically distinct from the noncytopathogenic phenotype. Nonetheless, the P protein and F protein lesions caused an extension of the mean embryo death time, and similar phenotypes appear in naturally avirulent strains. Consequently, the determination of virulence *in vivo* is likely to be polygenic in nature.

2. The Plaque Morphology Mutants of Newcastle Disease Virus

Although there is a positive correlation between plaque size and virulence of Newcastle disease virus in chickens (Schloer and Hanson, 1968), the ability to spread in one cell type *in vitro* cannot be assumed to correspond to replicative ability in the tissues of the natural host. Indeed, Granoff (1961a) reported that certain mutagen-induced small-plaque mutants isolated in chick embryo cell cultures exhibited enhanced neurovirulence in mice. Estupinan and Hanson (1971a) distinguished six plaque morphology mutants present in the Delaware-Hickman strain of Newcastle disease virus, which differed in their virulence for chickens. A large, clear, plaque-forming virus was the most virulent, and a small, red plaque-former the least virulent. The spectrum of chicken virulence was paralleled by the mean embryo death time and rapidity of cytopathic effects in chick embryo fibroblast monolayers. Minor antigenic differences were observed between the mutants as measured by neutralization test and resistance to challenge. These different mutants also differed in immunogenicity as measured by their inability to induce good immunity to themselves. In general, the most virulent mutants were the best antigens, even after inactivation. Virulence decreased with diminishing plaque size in the case of both clear and red plaques. Ability of mutants to induce interferon was not related to virulence in chick embryos, nor were there any correlations

among virulence, rate of release of virus, or neuraminidase or hemagglutinin activities. Virulence was correlated, however, with a shorter lag period during single-cycle growth (Schloer and Hanson, 1971).

The rate of mutation of red to clear plaque type was high in this and other strains of Newcastle disease virus (Granoff, 1961a, 1964; Thiry, 1964), whereas the reverse was rare (Estupinan and Hanson, 1971b). Schleor and Hanson (1971) estimated by the fluctuation test that the mutation rate from red to clear plaque was 3.3×10^{-6} and that from large to small plaque was 1×10^{-7} per particle per generation.

Estupinan and Hanson (1971a,b) observed that a heterogeneous population could be restablished within a few passages from any of the red plaque types present in the original Delaware-Hickman isolate, whereas the clear mutants appeared to be genetically stable, and it was apparent that components of the original isolate were soon lost during propagation in cultured cells. Consequently, the apparent genetic homogeneity of the common laboratory stocks of Newcastle disease virus may not represent the true genetic potential of this virus, and can complicate the analysis of virulence and pathogenesis. The genetic heterogeneity of field isolates of Newcastle disease virus, on the other hand, is particularly obvious.

Lomniczi (1975) characterized plaque morphology mutants of the mesogenic Herts vaccine strain and discounted the possibility of strain heterogeneity in the original isolate. Small plaque-forming mutants isolated from this strain lacked neurovirulence, contrary to previous reports that lentogenic viruses did not form plaques on chick embryo cells.

The general conclusion from these and similar studies is that there are multiple determinants for both plaque size and virulence.

3. Monoclonal Antibody-Resistant Mutants of Newcastle Disease Virus and Sendai Virus

Monoclonal antibodies have been used extensively to map antigenic sites and epitopes in Newcastle disease virus and Sendai virus. Usually, neutralization-resistant ("escape") mutants have been used for this purpose (e.g., Abenes *et al.*, 1986; Nishikawa *et al.*, 1983; Portner, 1981, 1984; Portner *et al.*, 1987a,b; Iorio and Bratt, 1983; Toyoda *et al.*, 1987). Iorio and Bratt (1985) described the isolation of unique nonneutralizable mutants with lesions located in the hemagglutinin-neuraminidase protein gene of Newcastle disease virus. A high proportion of anti-HN protein monoclonal antibodies do not neutralize the infectivity of Newcastle disease virus, although they bind to virions. Nevertheless, Iori and Bratt (1984a) were able to define four antigenic sites by competitive binding and additive neutralization assays. Antibodies to all four sites were required for maximum neutralization (Iorio and Bratt, 1984b). Persistent nonneutralizable fractions and enhanced neutralization following addition of rabbit anti-mouse IgG were further indications of the prevalence of nonneutralizing monoclonal antibodies. Iorio and Bratt (1985) developed a protocol which enhanced the recovery of neutralizing monoclonal antibodies. Pretreatment of monoclonal antibody-exposed virus with rabbit anti-mouse immunoglobulin prior to plating reduced the amount of infectious virus with bound

nonneutralizing monoclonal antibodies. Two types of mutant were isolated from the residual infectious virus; typical "escape" mutants resistant to neutralization by the monoclonal antibody used in their selection, and unique mutants binding the selecting antibody without loss of infectivity. Both types of mutant have value in the fine mapping of epitopes and the methodology is applicable to other viruses.

Three antigenic determinants have been identified on the F protein of Newcastle disease virus (Abenes *et al.*, 1986; Toyoda *et al.*, 1987). All the monoclonal antibodies used in defining these sites had neutralizing, hemolysis-inhibiting, and fusion-inhibiting activities, but none could recognize either the nascent polypeptide or the denatured F protein, suggesting that the three antigenic determinants, though functionally related, were dependent on protein folding. Sequencing of the F genes of a series of escape mutants confirmed that the three sites were located at a distance from the fusion-inducing N terminus of the F_1 subunit. Sites I and III were located in the F_1 subunit and site II in the F_2 subunit. Therefore, either the three antigenic sites are located adjacent to the fusion-inducing domain in the folded protein and inhibition is by steric interference, or more than one domain in the F protein is involved in membrane fusion.

Monoclonal antibodies directed against the HN protein of Newcastle disease virus have been classified into three groups according to their biological activities (Nishikawa *et al.*, 1983). Data on the frequency of isolation of escape mutants and competitive binding assays indicated that two of the three sites were topologically distinct and nonoverlapping.

Only one epitope has been defined on the F protein of Sendai virus (Portner *et al.*, 1987b). The sequences of the F protein gene of three escape mutants have been determined. In all three mutants there was a single amino acid substitution (Pro → Glu) at a site (residue 399) remote from the putative fusion-inducing F_1 N terminus. The loss of a proline could affect protein folding and result in loss of antigenic recognition.

By contrast, four antigenic sites have been topographically mapped on the HN protein of Sendai virus by competitive binding assays (Portner *et al.*, 1987a). Antibodies to the different sites had different biological activities. Antibodies to sites I, III, and IV inhibited hemagglutination and neuraminidase, hemagglutination alone, or neuraminidase alone, respectively, whereas antibodies to site II inhibited hemolysis, an F-protein function, suggesting either steric interference or a direct involvement of the HN protein in membrane fusion. The combined result of the mapping neutralization escape mutants and *ts* mutants of Sendai virus is illustrated in Fig. 3.

Shioda *et al.* (1988) sequenced the *M, F,* and *HN* genes of three laboratory strains derived from a wild strain of bovine parainfluenza virus type 3 which differ in their syncytium-inducing activity. No amino acid substitutions were identified in the deduced F proteins. A syncytium-formation-deficient variant (MR) was isolated from the strain with the most pronounced syncytium-inducing activity (M). The single amino acid change identified was located not in the F protein, but in the HN protein, where phenylalanine at residue 193 in the M strain was replaced by leucine in the MR variant. These observations also suggest that the HN protein may contribute to syncytium formation in addi-

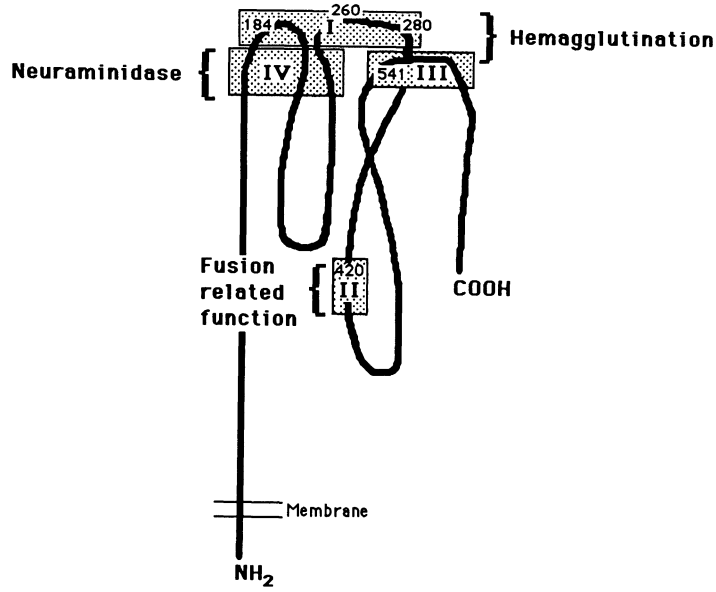

FIGURE 3. Hypothetical model of the HN glycoprotein of Sendai virus. [Reproduced with permission from Thompson and Portner (1987).] The boxes represent antigenic sites on the HN protein. Sites I, II, and III have been located by mapping of antigenic and temperature-sensitive mutants. Site IV has not yet been located. The function associated with each antigenic site is indicated.

tion to the F protein. An alternative explanation, however, is that any mutation affecting maturation may simply enhance syncytium formation by increasing the amount of F protein in the plasma membrane.

4. A Neuraminidase Activity-Deficient Variant of Mumps Virus

Adaptation of mumps virus to growth in cultured cells is usually accompanied by development of a syncytial cytopathic effect. Waxman and Wolinsky (1986) have shown that the neuraminidase protein plays a role in cell-to-cell fusion, and that fusion is not simply (as in Sendai virus) a consequence of mutation affecting proteolytic processing of the fusion (F) protein. By propagation of the nonfusing O'Take strain of mumps virus in CV-1 cells in the presence of the neuraminidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, a syncytium-forming variant was obtained. This variant had no detectable neuraminidase activity. Hemagglutinin activity was unaffected and as a consequence the syncytium-forming variant could agglutinate red blood cells, but, unlike wild-type virus, was unable to elute once adsorbed. The isolation of this variant demonstrates that the viral neuraminidase modulates the cytopathogenicity of mumps virus, and may be a determinant of neurovirulence, since a correlation has been observed between fusogenic ability in cell cultures and neurotropism in newborn hamsters.

Löve *et al.* (1985) isolated four mutants of the neurotropic Kilham strain of mumps virus which have enhanced neuraminidase activity. These mutants

were obtained by passage in the presence of neutralizing anti-HN monoclonal antibody. The four mutants had markedly different biological properties in addition to alteration of the target epitope. One of the four mutants had reduced neurovirulence, again implicating the surface glycoproteins of mumps virus as major determinants of pathogenicity and virulence.

5. Plaque Morphology Mutants of Canine Distemper Virus

Cosby *et al.* (1981) described a stable, small-plaque-forming variant isolated from the large-plaque-forming Onderstepoort vaccine strain of canine distemper virus, which had altered neurovirulence for weanling hamsters. The large-plaque-former induced acute neurological illness and little neutralizing antibody, whereas the small-plaque-former induced high titers of neutralizing antibody, but no disease. By altering the levels of antibody early in infection, the outcome could be modified, showing that the major factor in determination of virulence was the differing immunogenicity of these viruses (Cosby *et al.*, 1983). Other small-plaque mutants were isolated from a culture of Vero cells persistently infected with the large-plaque form of canine distemper virus and shown to have phenotypic properties similar to the original small-plaque isolate; i.e., enhanced immunogenicity and decreased neurovirulence.

Plaque morphology mutants induced by 5-fluorouracil treatment, however, exhibited a spectrum of plaque types and disease-producing potential. The mutagen-induced variants did not show the same stability on passage as the small-plaque type derived previously. It was concluded that the small-plaque type was probably generated during high-multiplicity passage and was probably the result of multiple mutations (Cosby *et al.*, 1985).

6. The Protease Activation Mutants of Sendai Virus

The orthomyxoviruses and the paramyxoviruses proper adsorb to neuraminic acid-containing receptors. Since neuraminic acid-containing glycoproteins and glycolipids are ubiquitous components of the cell membrane of vertebrates, the availability of surface receptors may play a lesser role in determining host range and tissue tropism than it does in other enveloped viruses, such as the retroviruses.

In the case of the paramyxoviruses, and perhaps also the morbilliviruses and pneumoviruses, the second surface glycoprotein which mediates fusion and hemolysis is a major determinant of host range and tissue tropism. Cleavage of the F protein is essential for activation of infectivity and membrane fusion, and this specific cleavage is mediated by a host protease (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). Consequently, the host range, tissue tropism, and pathogenicity of paramyxoviruses are dependent on the availability of the appropriate cellular protease.

Some unique genetic studies by Scheid and Choppin (1976) provided the essential experimental confirmation of the predominant role of cellular proteases in activation of infectivity. The F_0 protein of wild-type Sendai virus is cleaved by trypsin and in the presence of this protease, plaques are formed on monolayers of the otherwise nonpermissive MDBK line of bovine kidney cells.

Other proteases, such as chymotrypsin and elastase, did not promote plaque formation. However, when MDBK cells were infected in the presence of chymotrypsin with Sendai virus mutagenized by treatment with 1 *M* sodium nitrite at pH 4.4, a cytopathic effect was observed in a small proportion of cultures. Two protease activation mutants, *pa*-c1 and *pa*-c2, were isolated from these cultures, which were able to form plaques on MDBK cell monolayers in the presence of chymotrypsin. Similarly, a series of elastase-activated mutants, *pa*-e1 to *pa*-e8, were obtained from mutagenized virus by incubation of infected cells in the presence of elastase. Mutants activated by plasmin or thermolysin were also obtained. Some of these *pa* mutants retained the propensity to be activated by trypsin, whereas others did not. Analysis of the polypeptide composition of *pa* mutants grown in different host systems confirmed the association of F protein cleavage and activation of infectivity.

The importance of these protease activation specificities in determining host range in the whole organism was demonstrated by injecting *pa* mutants into the allantoic sac of the chick embryo. Mutant *pa*-c1 was unable to undergo multiple cycles of replication in the allantoic sac unless chymotrypsin was injected simultaneously, and similarly, mutant *pa*-1e required elastase for activation in the allantoic sac. In acquiring their new specificity, both of these mutants had become resistant to cleavage by the proteases normally present in the allantoic sac of the chick embryo. These mutants of Sendai virus provide sensitive probes for identifying the proteases present in tissues.

An important implication of these results is that when a paramyxovirus is isolated from a living organism by inoculation into cultured cells, the virus which grows out may be one which is susceptible to cleavage by the protease present in the cell membrane of the detector system. Consequently, the isolation procedure may introduce a bias by selecting a minor component or rare mutant which is not typical of the virus predominating in the organism. This may be one reason why virus propagated in culture rarely has the disease-producing potential of the original isolate. Similarly, the rare progression of disease by invasion of a normally inviolate tissue may be due to the appearance of a novel *pa* mutant. So far, however, protease activation mutants have only been described for Sendai virus, and Sendai virus is the only paramyxovirus known which produces noninfectious virions containing uncleaved F_0 protein.

The sequence of 60–70 amino acids around the cleavage site has been derived from the mRNA sequence for five of the *pa* mutants, revealing in each case one or two amino acid replacements near or at the cleavage sites (Hsu *et al.*, 1987). Figure 4 shows that in three instances the change in protease specificity appeared to be due to a replacement at the cleavage site, whereas in the remaining two it was more remote. Itoh and Homma (1988) confirmed independently that the replacement of Arg by Ile at position 116 is responsible both for the loss of trypsin sensitivity and gain of chymotrypsin sensitivity, although amino acid sequence analysis showed that chymotrypsin cleaved between residues 114 and 115, whereas trypsin cleaved between residues 116 and 117. In the work of Itoh *et al.* (1987), the complete *F* gene nucleotide sequence was determined and amino acid changes at more remote sites in the F protein could be excluded. Protease activation mutants derived by passage in the presence of chymotrypsin independently in different laboratories had identical

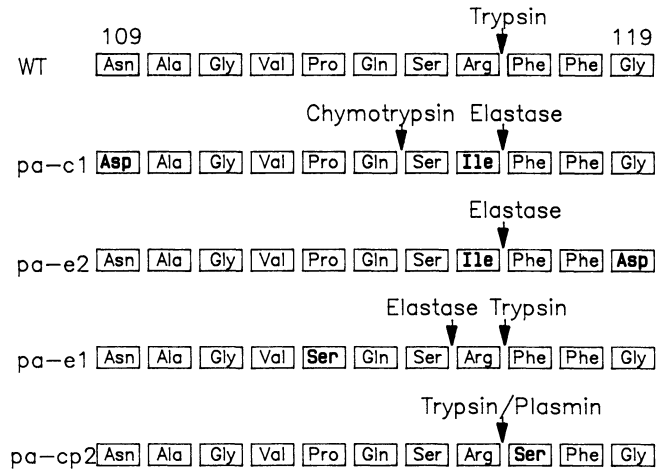

FIGURE 4. Amino acid sequences around the cleavage activation site of the F protein of wild-type and four protease activation (*pa*) mutants of Sendai virus. The cleavage site of each protease is indicated by an arrow. The amino acids are numbered from the amino terminus of the F protein. Mutant *pa*-c1 was selected with chymotrypsin, mutants *pa*-e1 and *pa*-e2 with elastase, and mutant *pa*-cp2 with chicken plasmin (see text).

mutational changes at positions 109 (Asn → Asp) and 116 (Arg → Ile). The Asp at 109 may be essential for chymotrypsin sensitivity, or alternatively Asn may be dispensable, but neither is sufficient, since Asn was unchanged in a chymotrypsin-resistant revertant TSrev-58.

Although the chick embryo is not the natural host of Sendai virus, Tashiro and Homma (1983), using the trypsin-sensitive wild type and a chymotrypsin-sensitive, trypsin-resistant mutant (TR-2), showed that a trypsinlike activity in the bronchial epithelium of the mouse was responsible for activation and multiplication of Sendai virus in the mouse lung. A trypsin-sensitive revertant (TSrev58) of trypsin-resistant mutant TR-5 had the pneumopathogenicity of the wild-type virus restored (Mochizuki *et al.*, 1988). The amino acid sequences around the cleavage sites of TR-2, TR-5, and TR-rev58 were predicted from the nucleotide sequence. In mutants TR-2 and TR-5 there are two replacements, one at the cleavage site (Arg → Ile at 116) and another at position 109 (Asn → Asp). In the revertant, there was a single reversion of Ile → Arg at position 116, restoring the wild-type amino acid, leaving the Asp at 109 unchanged (Itoh *et al.*, 1987; Itoh and Homma, 1988). It is clear that a single nucleotide change in the F protein gene in certain circumstances can alter the specificity of protease activation and thereby affect the host range and tissue tropism of the virus.

The biological importance of the specific cleavage of the F protein of paramyxoviruses is emphasized on the one hand by the conservation of sequence of the N termini of the F_1 proteins and on the other hand by the inhibitory activity of oligopeptides which structurally mimic this region (Choppin and Scheid, 1980). C. D. Richardson *et al.* (1980) established that the most inhibitory oligopeptide was a heptapeptide with the same sequence as the N terminus of Sendai virus. The presence of a carboxybenzoxy group on the

N terminus enhanced activity, but the correct peptide sequence was more important. This was verified by isolation of a mutant of measles virus resistant to the inhibitory action of carbobenzoxy-phenylalanine-phenylalanine–nitro-arginine [Z-Phe-Phe–L(NO2)Arg] by growth in its presence, which remained sensitive to the oligopeptide Z-Phe-Phe-Gly. Sequencing of the *F* gene of this mutant revealed that mutations were present at residues 338, 352, and 460 in the deduced F_0 protein sequence and not in the cleavage site region (Hull *et al.*, 1987). It is likely, therefore, that conformation of the F protein is important in functionally orientating the fusion sequence.

IV. ANALYSIS OF GENE FUNCTION

Although genetic analysis of paramyxoviruses is limited by the inability to achieve successful marker rescue (see Section III), the methodology of recombinant DNA technology has a role in analysis of gene function.

A. Transport and Glycosylation of the G Glycoprotein of Respiratory Syncytial Virus

Three C-terminally-deleted mutants of the G glycoprotein of the A2 strain of respiratory syncytial virus have been described (Vijaya *et al.*, 1988; Olmsted *et al.*, 1989). Plasmids expressing truncated products containing the N-terminal 71, 180, and 230 amino acids of the 298-amino acid G glycoprotein were constructed by mutagenesis which introduced a restriction enzyme site followed by an in-frame termination codon and another restriction enzyme site after proline residues 71, 180, and 230. The 71-amino acid product reacted poorly with specific antiserum and a reporter sequence was added which consisted of a cysteine and the 12 C-terminal amino acids of the 1A glycoprotein of RS virus. This chimera could be detected with a 1A-specific C-terminal peptide antiserum. The truncated proteins expressed by all three constructs contained O-linked carbohydrates like the complete G protein, although interpretation of data for the 71-amino acid protein was compromised by the addition of the reporter sequence. The 180- and 230-amino acid proteins also contained N-linked carbohydrates and had M_r values estimated from electrophoretic mobility that were approximately twice the values calculated from their composition. Experiments with the inhibitor monensin suggested that this discrepancy was due to posttranslational addition of carbohydrates or dimerization in or beyond the Golgi apparatus, or both. All three truncated proteins were transported to the cell surface and had their C termini located extracellularly, like the complete G protein. Thus, the N-terminal 71-amino acid sequence contained all the structural information required for transport and membrane insertion.

Cotton rats were immunized with vaccinia recombinant viruses expressing these truncated proteins. The 230-amino acid truncated protein induced levels of protection and specific antibodies approaching those induced by the complete G protein, whereas the two shorter proteins failed to induce either

response. The positive results with the 230-amino acid fragment indicate that the C-terminal 68-amino acid region of the 236-amino acid ectodomain does not contain major epitopes responsible for induction of protective immunity. The 230-amino acid product is the only one of the three which contains the highly conserved cysteine-rich region, indicating that this region of the molecule plays a key role in the antigenicity and function of the protein.

The tolerance of the G protein to drastic mutational trauma contrasts with the common experience that the introduction of mutations into integral membrane proteins adversely affects their transport to the plasma membrane. The efficient transport of truncated G proteins suggests that the conformational state of the G protein is not critical. The considerable antigenic and sequence variability of the G protein of respiratory syncytial virus (Johnson *et al.*, 1987) is another indication of the tolerance of the ectodomain of this protein to mutational change.

This tolerance of the G protein to genetic manipulation has been utilized to design a carrier to facilitate transport of a foreign peptide sequence to the plasma membrane (Vijaya *et al.*, 1988). The rationale was based on the assumption that protein folding begins during synthesis. If so, conformational disturbance should be minimized by attaching a foreign peptide to the C terminus of a protein with an uncleaved signal-anchor domain. The truncated G-protein genes described previously were flanked by vaccinia virus DNA to permit cloning and expression in a vaccinia virus vector. A sequence encoding four copies of the four-amino acid immunodominant repeating epitope of the circumsporozoite of the malaria parasite *Plasmodium falciparum* was attached to the truncated G gene and recombined into the vaccinia virus genome. A chimeric protein was expressed which was transported to the plasma membrane and exposed on the external surface of the cell.

B. Membrane Interactions of the F_1 Polypeptide of SV5

The hydrophobic N termini of the F_1 proteins of paramyxoviruses are implicated in the process of membrane fusion. The inactive F_0 precursor, however, is translocated across the cell membrane and subsequently is activated by proteolytic cleavage to disulfide bond-linked F_1 and F_2 polypeptides with loss of an interconnecting peptide of one to 5 basic amino acids. The exposed hydrophobic N terminus of the F_1 polypeptide is highly conserved in all paramyxoviruses. Paterson and Lamb (1987) examined the effect of the position of this hydrophobic region on its function by *in vitro* mutagenesis. Plasmids were constructed which expressed hybrid proteins comprising the hydrophobic N terminus of the F_1 protein, with or without the F_1/F_2 interconnecting peptide, translocated to the HA protein of influenza A virus in place of its own membrane anchorage region. The HA–F_1 hybrid protein without the cleavage activation region behaved as an integral membrane protein, but was not transported beyond the Golgi apparatus. The HA–F_1 hybrid protein which did contain the interconnecting peptide of five basic residues did not behave as an integral membrane protein and was exported from the cell. These observations were interpreted in terms of differential hydrophobicity thresholds according

to location; when located internally in its normal location, the N terminus of F_1 did not exceed the threshold of hydrophobicity to function as an anchor sequence, whereas, when located terminally, the critical threshold was exceeded. Since the five-residue interconnecting peptide was found not to be necessary for transport of the F_0 protein of SV5, it was concluded that its presence in the HA–F_1 hybrid protein lowered its hydrophobicity below the threshold.

C. Gene-Specific Hypermutation in Measles Virus

It is well established that under conditions of persistent infection, mutations accumulate in the genomes of negative-strand RNA viruses primarily as a consequence of both the inherent low fidelity of transcription of RNA polymerases consequent upon the lack of a proofreading mechanism, and an apparent relaxation of selective pressure in nonlytic infection (Domingo *et al.*, 1978, Holland *et al.*, 1979; Rowlands *et al.*, 1980; Steinhauer and Holland, 1987). Although genomic variability is intrinsically high, many paramyxoviruses exhibit stable biological properties and in general there is no progressive antigenic variation in response to immunological pressure. It is likely that a stable consensus sequence is established in RNA virus populations maintained under constant selective pressure, masking the inherent variability of the genome.

Cattaneo *et al.* (1988) attempted to estimate the mutational load accumulated by measles virus in the course of persistent infection of human neural tissue by cloning full-length transcripts of measles virus genes directly from affected tissue obtained at autopsy. Approximately 2% of the nucleotides of the genome had undergone mutation, with 35% of these changes resulting in amino acid replacements. cDNAs were obtained from the brains of individuals with subacute sclerosing panencephalitis (SSPE) and a single individual with measles inclusion body encephalitis (MIBE), as well as from the lytic Edmonston strain. Since the original infecting viruses were undefinable and the Edmonston strain inappropriate because of its complicated passage history, a consensus sequence was derived to identify the mutations accumulated in these viruses during persistent infection of neural tissue. The consensus sequence represented the most frequent nucleotides in nine independently determined sequences (from three lytic and six defective viruses), and deviations from this consensus were designated mutations. Remarkably, mutations were not distributed randomly, but were more abundant in the M protein gene from the MIBE case. Moreover, the mutations were predominantly U → C transitions. In fact, 132 of the 266 U residues in the consensus sequence were changed to C residues in this gene. The number of U → C transitions in the *M* gene from the MIBE case was 20-fold greater than that of other mutations, whereas in the corresponding genes from the SSPE cases the U → C transitions were not more frequent than other mutations. The high frequency of U → C transitions was confined within the boundaries of the M protein gene. These mutational changes introduced a frameshift which created a termination codon resulting in a truncated gene product.

A significant feature of this phenomenon was that A → G changes corresponding to the U → C mutations in the other genomic strand were not in-

creased, indicating that the U → C transitions were introduced exclusively into one strand. This suggests either a singular hypermutational event or repetitive cycles of strand-specific restricted mutation. Cattaneo *et al.* (1988) concluded from the apparent conservation of changes in different clones from the same source that a singular mutational event was the more acceptable explanation, and they inclined to the view that the phenomenon was mediated by an aberrant RNA polymerase. (This gene-specific hypermutation is discussed in greater detail in Chapter 12).

Lamb and Dreyfuss (1989) put forward an explanation of the hypermutation phenomenon in the M protein that is based on the finding of an ATP-dependent unwinding activity which covalently modifies double-stranded RNA molecules by converting adenosine residues to inosine residues (Baas and Weintraub, 1988). If, during transcription of the *M* gene of the SSPE virus, the mRNA transcript fails to separate from the genomic template, the dsRNA unwinding/modifying activity might change A residues to I residues in the transcript. In the next round of transcription of mRNA, these I residues would be converted to C residues. There is little evidence that double-helical RNA structures are normal intermediates in the replication of paramyxoviruses, and hypermutation in the *M* gene may represent a rare failure of separation of template and progeny strands. Indeed, the absence of double-stranded RNA structures in the replication cycle of single-stranded RNA viruses may be a requirement for protection from potentially lethal hypermutational events (Weissmann, 1989).

V. PROSPECTS

Full application of genetic approaches to the study of paramyxoviruses awaits development of techniques which will enable genetic information manipulated *in vitro* to be rescued into infectious virus. The lack of this experimental facility is a barrier to progress. It is a general problem for genetic analysis of all negative-strand RNA viruses, and represents one of the remaining major technical limitations of recombinant DNA technology.

Interim expedients may be the use of virus vectors carrying individual paramyxovirus genes to confirm and extend the assignment of temperature-sensitive mutations, and refinement of mapping by the RNase A mismatch cleavage procedure (Lopez-Galindez *et al.*, 1988). In the immediate future existing methods of genetic analysis will have particular application in the elucidation of the functions and homologies of nonstructural proteins and in the unraveling of the determinants of virulence.

Note added in proof: The first significant steps towards this end have now been taken. Enami *et al.* (*Proc. Natl. Acad. Sci. USA* **87**:3902–3905, 1990) have succeeded in rescuing infectious influenza virus from cells transfected with RNAs derived from specific recombinant DNAs following addition of purified influenza virus polymerase complex. Ballart *et al.* (*EMBO J.* **9**:379–384, 1990) have been able to generate the infectious measles virus from cloned measles virus cDNA by the microinjection of committed transcription complexes into

the cytoplasm of helper cells which supply the appropriate proteins required for encapsidation and transcription/replication. These developments make the site-specific mutagenesis of negative strand RNA virus a reality, though not yet a universally applicable technique.

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