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# Replication Strategies of the Single Stranded RNA Viruses of Eukaryotes

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# 1 Introduction

Our knowledge of the molecular biology of virus replication has expanded dramatically in the last few years, especially with the advent of rapid techniques for obtaining the nucleotide sequence of viral genomes. Full or partial sequences of virus genomes are appearing monthly, and it seems appropriate at this time to review the subject of the strategies used for replication by RNA animal viruses in the hope of formulating a conceptual framework in which to organize the new sequence information. This chapter will be concerned with the single-stranded RNA viruses which replicate via RNA intermediates and will focus on the animal viruses, but selected plant viruses whose replication strategies are known will also be discussed. The primary topics will be RNA transcription (the production of virus-specific messages), RNA replication (synthesis of viral genomes), and mRNA translation (synthesis and processing of viral proteins).

In terms of replication strategy the RNA viruses can be divided into two groups, the plus stranded viruses and the minus stranded viruses. The plus stranded viruses initiate infection with the translation of the parental genomic RNA to produce the viral replicase/transcriptase enzyme(s). This enzyme complex synthesizes minus strand templates, plus strand genomes, and in many cases plus strand subgenomic messages for virion structural polypeptides. For some viruses, subgenomic messengers are produced for other polypeptides as well. The minus stranded viruses introduce the replicase/transcriptase into the host as a component of the virion and the initial event in virus replication is primary transcription by the parental nucleocapsid to produce messages for all virus-encoded products. Subsequent events include complementary plus strand (antigenome) synthesis, genomic minus strand synthesis, and amplified or secondary transcription. Replication strategies for each group will be discussed in the order of events after infection, starting with translation for plus stranded viruses and with primary transcription for negative stranded viruses.

Replication and transcription of viral RNA involves an initiation event followed by an elongation phase. Temporal and quantitative regulation of transcription versus replication and genome versus antigenome synthesis could reside in the specificity of initiation. We propose that polynucleotide sequences, usually but not always located at the termini of the RNAs, are specifically recognized by the virus-specific replicase/transcriptase enzymes during initiation. Two types of possible recognition signals are described in this review. One is a sequence of 10–20 nucleotides which is highly conserved within a group of related viruses and whose exact RNA sequence may be recognized by the viral enzymes. The second type is a secondary structure composed of up to 200 nucleotides, stabilized by hydrogen bonds in which the structure, and not the sequence, may be recognized. In this case the structure is conserved among related viruses but the primary sequences may diverge. Sequences that may perform these regulatory functions and their significance for replication strategy will be discussed for each virus family.

It has been very difficult to keep the number of literature citations to a manageable number, due both to the scope of this review and to the veritable explosion of published information on viruses in recent years. For this reason we have attempted to make the references selective rather than comprehensive, to cite review articles wherever possible, and to include primarily articles published since 1979.

## 2 The Plus Stranded Viruses of Animals

All the known plus stranded RNA animal viruses with the exception of the nodaviruses contain a nonsegmented genome. The infecting RNA is translated to produce the viral replicase, which in turn copies the parental RNA to produce the minus strand. The minus strand can then be used to produce plus strands. The production of a full length minus strand from the plus strand, and of a full length plus strand from the minus strand, will be referred to as RNA replication, and the enzyme(s) responsible as the viral replicase(s). If one or more subgenomic RNAs are produced, this event will be referred to as transcription and the enzyme(s) responsible as the viral transcriptase(s).

The most characteristic differences in replication strategies of the plus stranded RNA viruses involve the mechanisms used for production and translation of messenger RNAs. Table 1 summarizes the virus groups according to morphology and lists their best-known members and salient features of their replication. Discussion of the virus groups will be organized according to the replication strategies employed by the viruses.

### 2.1 The Picornaviruses

The picornavirus family is made up of nonenveloped virions approximately 22–30 nm in diameter with icosahedral symmetry. The genome is a single RNA molecule of molecular weight $2.5 \times 10^6$ or 7500 nucleotides (7.5 kb) which has a covalently linked protein at the 5′ terminus and a 3′ terminal poly(A) tail of 80–120 nucleotides. The protein shell is made up of 60 copies of each of four virus-specific polypeptides. VP1, VP2, and VP3 have molecular weights between 23 000 and 37 000 (23–37 K) depending upon the virus; VP4 is variously reported to have a molecular weight of 9–15 K (*Rekosh* 1977; *Matthews* 1982). The most recent classification of the picornaviruses divides them into four genera on the basis of buoyant density, stability to acid pH, and structure of the genome: the enteroviruses, cardioviruses, rhinoviruses, and aphthoviruses (*Matthews* 1982). All of these viruses are mammalian pathogens, and the enterovirus and rhinovirus groups contain a number of significant human pathogens. In addition to these four groups there are a number of unclassified picornaviruses of insects.

#### 2.1.1 Translation Strategy

The RNA sequence of the genome of poliovirus has recently been determined in its entirety (*Kitamura* et al. 1981; *Racaniello* and *Baltimore* 1981), and the translation and processing strategy of the virus is becoming clear. The organization of the genome is illustrated schematically in Fig. 1. The RNA is 7433 nucleotides long; translation of the RNA begins at an AUG codon 741 nucleotides from the 5′ terminus and continues for 6621

Table 1. The plus stranded animal viruses

| Virus family[a] | Genera | Representative members | Structure of nucleocapsid or virion | Size of genome (kb) | Terminal modifications of genome | Number of subgenomic mRNAs | Number of polyprotein precursors | Other characteristics |
|---|---|---|---|---|---|---|---|---|
| *Nonenveloped* | | | | | | | | |
| Picornavirus | Enterovirus | Polio, Coxsackie, Echo, SVDV | Icosahedral | 7.5 | 5′ VPg, 3′ poly(A) | 0 | 1 | Internal poly (C) in genome RNA |
| | Cardiovirus | EMC, ME, Mengo | | | | | | |
| | Rhinovirus | > 100 Serotypes of human rhinoviruses | | | | | | |
| | Aphthovirus | FMDV | | | | | | Internal poly (C) in genome RNA |
| Nodavirus | - | Nodamura, black beetle | Icosahedral Two RNAs | 3 1.5 | no poly(A) | 1 | 1 or 2 | Only plus stranded animal virus with segmented genome |
| Calicivirus | - | VESV, SMSV, feline caliciviruses | Icosahedral | 7–8 | 5′ VPg, 3′ poly(A) | 1 or 2 | 1 or more | |
| *Enveloped* | | | | | | | | |
| Togavirus[b] | Alphavirus | Sindbis, SFV, MID, WEE, VEE, EEE, HJ, and others | Icosahedral nucleocapsid | 12 | 5′ cap, 3′ poly(A) | 1 | 2 or more | Replicate in both mammalian host and invertebrate vector |
| | Flavivirus | Yellow fever, JE, SLE, and others | Icosahedral | 12 | 5′ cap, no poly(A) | 0 | 0? | |
| Coronavirus[c] | - | IBV, human coronaviruses, MHV, TGEV, bovine coronavirus, and others | Helical nucleocapsid | 18 | 5′ cap, 3′ poly(A) | 5 (6) | 2 or more | Only plus stranded virus with helical nucleocapsid |

**Abbreviations:** *SVDV,* swine vesicular disease virus; *EMC,* encephalomyocarditis virus; *ME,* Maus-Elberfeld virus; *FMDV,* foot-and-mouth disease virus; *VESV,* vesicular exanthema virus of swine; *SMSV,* San Miguel sea lion virus; *SFV,* Semliki Forest virus; *MID,* Middelburg virus; *WEE,* Western equine encephalitis virus; *VEE,* Venezuelan equine encephalitis virus; *EEE,* Eastern equine encephalitis virus; *HJ,* Highlands J virus; *JE,* Japanese encephalitis virus; *SLE,* St. Louis encephalitis virus; *IBV,* (avian) infectious bronchitis virus; *MHV,* murine hepatitis virus; *TGEV,* transmissable gastroenteritis virus
[a] Matthews (1982); [b] Little is known of the replication of the two other alphavirus genera, the rubiviruses and the pestiviruses; [c] Siddell et al. (1982)

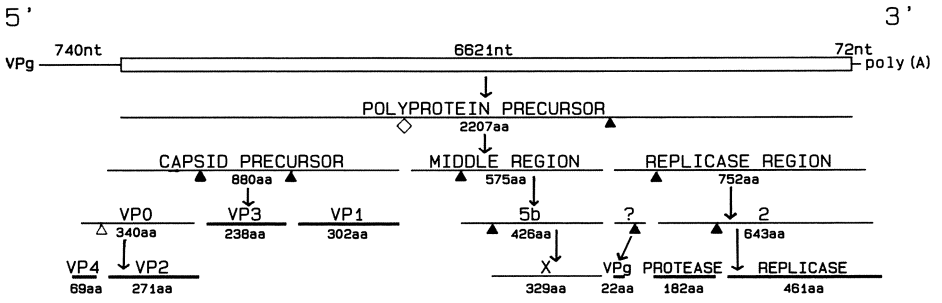


Fig. 1. Translation of poliovirus RNA. The *top line* shows the organization of the genome RNA of the Mahoney strain of poliovirus with a *single line* for untranslated regions at the 5′ and 3′ ends and an *open box* for the translated sequence. Translated products are indicated *below*, with final protein products shown as *heavy lines* and designated according to function (i.e., protease, replicase, etc.). Virion polypeptides are labelled *VP0, VP1, VP2, VP3*, and *VP4. VPg* is the genome linked virus polypeptide. *Solid triangles* indicate cleavages between Gln and Gly and are presumably all due to the virus protease encoded in the replicase region. The *open diamond* on the polyprotein precursor is a Tyr-Gly cleavage and the *open triangle* on *VP0* is an Asn-Ser cleavage. Data for this figure are from *Kitamura* et al. (1981), *Semler* et al. (1981a, b), and *Racaniello* and *Baltimore* (1981). *nt*, nucleotide; *aa*, amino acid

nucleotides to a UAG codon which lies 72 nucleotides from the 3′ terminal poly(A) tract. The polyprotein precursor of 2207 amino acids is organized into three regions. The amino terminal region contains the sequences of the four capsid proteins. The central region contains sequences of various polypeptides of unknown function. The carboxy-terminal region contains the sequences of the viral replicase and of the protease responsible for most or all of the processing of the viral polyprotein.

In the processing of the polyprotein precursor at least ten cleavages are known to occur (reviewed in *Rueckert* et al. 1979). Eight of these occur between Gln-Gly pairs (*Semler* et al. 1981a, b; *Larsen* et al. 1982) and are all almost certainly accomplished by a virus encoded protease (*Korant* et al. 1979). This protease is active not only as a free polypeptide of molecular weight 22 K (which appears to be the form active in producing the individual capsid proteins) but also as a part of various precursor polypeptides (which appears to be the form active in processing the replicase/protease precursors) (*Palmenberg* and *Rueckert* 1982). Many of these cleavages occur fairly slowly, with 15–20 min required for processing.

The first two cleavages in the processing pathway, which separate the polyprotein into the three domains referred to above, occur very rapidly, while the polyprotein is still nascent (reviewed in *Korant* 1979 and *Lucas-Lenard* 1979). In fact the entire polyprotein is produced as such in poliovirus only when processing is inhibited, such as when amino acid analogues are incorporated into the precursor or when protease inhibitors are present. The first of these cleavages, which separates the capsid protein precursor from the rest of the polyprotein, occurs between a Tyr-Gly pair (*Semler* et al. 1981b), and the protease responsible thus has a chymotryptic-like activity. Because this cleavage differs in specificity and kinetics from the remainder, the enzyme responsible may be qualitatively different. Three hypotheses can be proposed to explain this difference:

1. A *host cell protease is responsible for this cleavage. Korant* (1972) treated infected cells with inhibitors of trypsin and chymotrypsin and found a differential effect depend-

ing on the host cell, which was interpreted as evidence that a cellular protease was involved in an early stage of cleavage of the polio polyprotein. These data are in apparent conflict with those of *Summers* et al. (1972) however, and in light of recent discoveries of a number of virus-specific proteases, and in view of the polio RNA and protein sequencing data, it now seems unlikely that a cellular enzyme is involved.

2. The *same virus encoded protease that is active on the Gln-Gly bonds also cuts this Tyr-Gly bond.* This also seems unlikely although the enzyme might possess a broader specificity than apparent at first sight as discussed below.

3. A *second virus-encoded protease is responsible.* In this case the situation might be analogous to the cleavage of the nucleocapsid protein of the alphaviruses from its polyprotein precursor. This alphavirus protease activity appears to reside in the highly conserved carboxyterminal region of the nucleocapsid protein itself, possesses chymotryptic-like activity, is active on the nascent polyprotein, and appears to lose much of its activity once the site-specific cleavage it catalyzes has occurred (see Sect. 2.3.1).

The second nascent cleavage, which separates the middle region of the precursor polyprotein from the carboxyterminal region, occurs between a Gln-Gly pair (*Semler* et al. 1981a), and is presumably catalyzed by the viral protease with this specificity. Why this cleavage is so rapid in comparison to other cleavages is not clear.

Seven of the eight subsequent cleavages which are known to occur take place between Gln-Gly pairs as stated above. The eighth cleavage, which is one of the cleavages in the processing of the capsid proteins, occurs between an Asn-Ser pair (*Larsen* et al. 1982). This cleavage is the last to occur, and appears to coincide with the addition of virion RNA to the procapsid to form the mature virion (reviewed in *Rueckert* 1976). It could provide energy for the formation of the virion, or might activate the virion for subsequent disassembly upon infection. An Asn-Ser pair can be viewed as homologous to a Gln-Gly pair, and it is possible that this cleavage is performed by the same viral protease. The delay in processing could be due to a lower affinity of the enzyme for the Asn-Ser pair, perhaps requiring activation by the presence of RNA in the procapsid. Alternatively, another protease, probably also virus encoded, could catalyze this reaction.

It is unknown at present whether other cleavages occur in the processing of the poliovirus polyprotein, and if so what the enzymatic specificities involved might be. One of the major difficulties in working out the processing scheme is the large number of intermediates with varying half lives present in the infected cell. This situation is made more complex by the fact that alternative pathways of processing appear to exist. With the entire nucleotide sequence of the virus RNA now known, however, the complete details of processing should be known shortly.

The 740-nucleotide segment preceding the start codon of the major polyprotein is remarkably long for a 5′ untranslated region and could conceivably encode one or more small polypeptides not yet identified. Alternatively, the length of this region could relate to the fact that polio mRNA; unlike most eukaryotic mRNAs, lacks a cap structure and initiation of translation must recognize other features of the RNA. In this regard it is noteworthy that during in vitro translation of polio RNA, two different initiation sites appear to be used (*Ehrenfeld* 1979). The significance of this observation and its relation to the known sequence is unclear at present.

The other three groups of picornaviruses, the rhinoviruses, cardioviruses, and aphthoviruses, possess processing pathways which are virtually identical to those of the enteroviruses (*Rueckert* et al. 1980; *Sangar* 1979). Thus, the organization of the genome

and the nature of the proteases responsible for processing of protein precursors are probably the same for all of the picornaviruses. However, the amino acids at the cleavage sites in the capsid precursor of aphthoviruses and of mengovirus, a cardiovirus, are different from those in poliovirus shown in Fig. 1. Thus, whereas the three cleavage sites are Asn-Ser, Gln-Gly, and Gln-Gly for poliovirus, as noted above, they have been found to be Ala-Asp, Glu-Gly, and Gln-Thr, respectively, in one strain of foot-and-mouth disease virus (*Boothroyd* et al. 1981), and Ala-Asp, Gln-Ser, and Gln-Gly, respectively, in mengovirus (*Ziola* and *Scraba* 1976). If the virus-encoded protease is responsible for these cleavages, the enzyme may have a specificity less stringent than appears to be the case from a study of the cleavage sites in poliovirus and/or the specificity of the enzyme(s) may vary from virus to virus. We also note that the cardioviruses and the aphthoviruses differ from the other two groups in having a poly(C) tract of 100–500 nucleotides (*Brown* et al. 1974) in the 5′ untranslated region (*Sangar* et al. 1980) whose function is unknown.

Many virus groups inhibit translation of host mRNAs after infection. Because the picornaviruses lack the 5′ cap structure, they could conceivably interfere with translation of host messenger at the level of cap recognition. Such a mechanism has in fact been proposed for poliovirus (*Trachsel* et al. 1980; *Hansen* and *Ehrenfeld* 1981), although encephalomyocarditis virus seems to inhibit host protein synthesis in a different fashion (*Jen* et al. 1980). Of the other RNA viruses whose 5′ terminal structure has been studied, only the caliciviruses and several groups of plant viruses lack a cap and could also use such a mechanism.

Because the entire genome of the picornaviruses is translated as a continuous polypeptide chain, the only possible mechanism for regulation of the relative amounts of capsid proteins versus nonstructural proteins is premature termination of protein synthesis. Such premature termination is known to occur in vitro (*Ehrenfeld* 1979) and results in capsid proteins being produced in proportionately larger amounts. *Rueckert* (1976) has reviewed the evidence that such a regulatory mechanism operates in vivo as well as in vitro.

### 2.1.2 Replication of Viral RNA

The picornaviruses do not produce subgenomic RNAs and only two enzymatic activities are needed to replicate viral RNA: a minus strand replicase to produce full length minus strands using the plus strand as a template, and a plus strand replicase to produce full length plus strands from a minus strand template (reviewed in *Rekosh* 1977). Because the number of plus strands produced is much greater than the number of minus strands, it is possible that the two activities are not identical.

Although the complete nucleotide sequence has only been reported for poliovirus type 1, a number of authors have determined the 3′ and 5′ terminal sequences of other picornaviruses. *Hewlett* and *Florkiewicz* (1980) examined two strains of poliovirus and one strain of coxsackie virus, and *Nomoto* et al. (1981) examined three strains of poliovirus. The first ten nucleotides from the 5′ terminus are identical in all these viruses and the next ten nucleotides are almost identical in the poliovirus strains, and 50% conserved in coxsackie virus. *Harris* (1980) examined the 5′ sequences of nine aphthoviruses and found that the first 27 nucleotides were highly conserved. As shown in Fig. 2A, this conserved 27 nucleotide stretch of the aphthoviruses is also highly conserved between

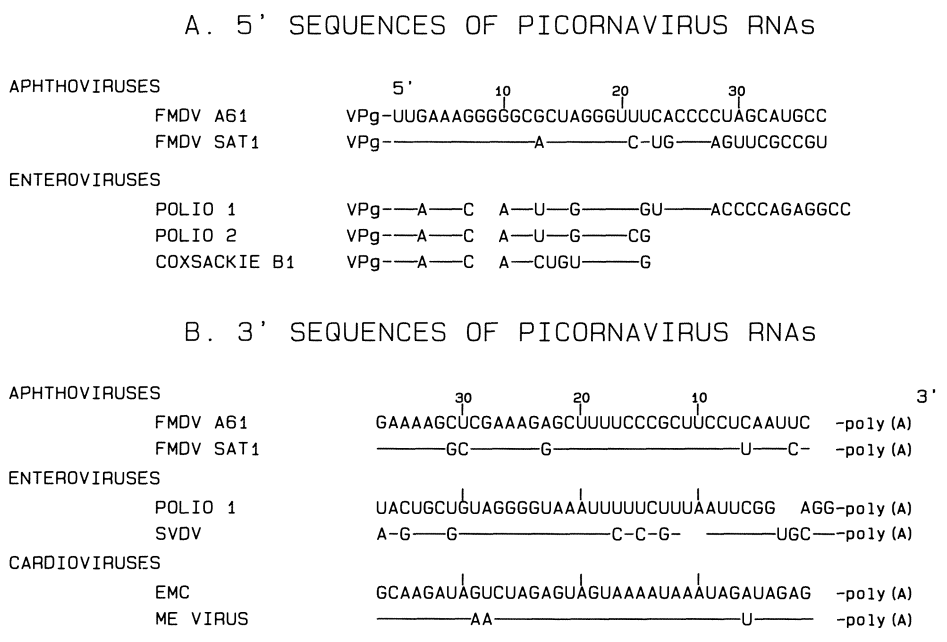


Fig. 2A, B. The 5′ and 3′ terminal sequences of picornaviruses. Sequences are shown from 5′ to 3′ reading left to right. *Horizontal lines* indicate that the nucleotide is identical with the nucleotide in the complete sequence shown above. *Gaps* have been introduced for alignment. Sequencing data are from *Fellner* (1979), *Harris* (1980), *Hewlett* and *Florkiewicz* (1980), *Nomoto* et al. (1981), and *Kitamura* et al. (1981). *FMDV,* foot and mouth disease virus; *SVDV,* swine vesicular disease virus; *EMC,* encephalomyocarditis virus; *ME,* Maus-Elberfeld virus

aphtho and polioviruses: counting deletions as single changes, two-thirds of the nucleotides are conserved.

*Hewlett* and *Florkiewicz* (1980) suggested the conservation they observed was a recognition site for the host translation system, but we feel it likely that the complement of the conserved sequence in the minus strand forms a recognition site for the viral plus strand replicase. We note also that the first 40 nucleotides or so of poliovirus RNA (*Larsen* et al. 1981) and of aphthovirus RNA (*Harris* 1980) can form a stable hairpin structure which could be involved in replication (see also Sect. 2.3.2) or in translation. The size of the hairpin structure and the nucleotides used to form it differ between the polioviruses and aphthoviruses, however, and this structure and the conserved sequence might serve different functions. Thus the hairpin could be involved in translation and the conserved sequence in replication.

The 3′ terminal sequences of the picornaviruses show strong conservation within a genus but no detectable conservation between genera (*Fellner* 1979). Representative data are shown in Fig. 2B. If production of minus stranded RNA from the plus strand involves a recognition sequence, this sequence has diverged markedly among the picornavirus genera. Note that the 3′ terminus has little or no homology with the complement of the 5′ terminus and if these are the initiation recognition signals then the enzyme complex could differentiate between plus and minus strand synthesis.

Initiation of RNA replication, whether plus stranded or minus stranded, is thought to

involve VPg, the 22 residue (in poliovirus) polypeptide covalently linked to the 5′ terminal U of picornavirus RNAs; linkage is through a phosphodiester bond to a tyrosine residue (*Rothberg* et al. 1978; *Ambrose* and *Baltimore* 1978; *Wimmer* 1979). (Several other virus groups are also known to possess a VPg, see Sect. 2.4 and 3.) All nascent RNA strands, both plus and minus, of poliovirus possess a covalently linked VPg (*Petterson* et al. 1978). Initiation of an RNA strand may involve the 85K polypeptide (or perhaps a shortened version of it), encoded in the 3′ part of the poliovirus genome (and identified as "Replicase Region" in Fig. 1), forming a covalent bond with the initiating U through the VPg component. This is followed by or is concurrent with cleavages which result in VPg being formed and the protease activity being released. The 52K replicase component would also be released in this reaction and would elongate the initiated chain (*Palmenberg* et al. 1979). This model would imply that the picornavirus replicase is not a true catalytic enzyme, but rather that each replicase molecule can produce only a single RNA chain. This could explain why complementation between mutants of poliovirus is both very inefficient and asymmetric. In one case where complementation could be demonstrated between a polymerase mutant and a capsid protein mutant, no polymerase mutant genomes were found in the progeny (*Cooper* 1965; *Cooper* 1969). Furthermore, the observation that defective interfering RNAs of poliovirus must be translated to produce the replicase in order to replicate (see Sect. 5) could be explained by such an RNA replication mechanism. The translation strategy of the virus leads to the production of large amounts of replicase, however, and such a mechanism is not only feasible but may be related to the overall replication strategy of this group of viruses.

Results from in vitro experiments are compatible with this model. A soluble RNA-dependent RNA polymerase, which contains primarily a single virus-specific polypeptide variously identified as p63, p58, or p56, has been isolated from cells infected by poliovirus (*Flanegan* and *Baltimore* 1979; *Etchison* and *Ehrenfeld* 1980) or foot and mouth disease virus (*Lowe* and *Brown* 1981). The most highly purified preparations are template dependent but require an oligo(U) primer to initiate replication of picornaviral RNA. However, a host factor has been partially purified which appears to allow initiation of poliovirus RNA by the replicase (*Dasgupta* et al. 1980), although the RNA product has not been characterized. With either mode of initiation the replicase activity is not picornavirus specific but will replicate other poly(A)-containing RNAs as well. Thus any specificity of the initiation event has been lost by the soluble systems isolated to date. The model presented above predicts that specific initiation requires the precursor labeled "replicase region" in Fig. 1 and further efforts to obtain a specific replicase system are clearly needed.

It is of considerable interest that the aphthoviruses have been found to contain three different VPg's which are equally represented in the virion RNA population. These three VPg's are tandemly arranged in the precursor polyprotein (*J.J. Rowlands*, personal communication). The significance of this observation is unclear at present.

The VPg is removed from the RNA destined to become mRNA, apparently by a cellular enzyme (*Ambros* and *Baltimore* 1980). It is unclear whether removal of the VPg is essential for efficient translation. It is also unclear whether VPg has any function in the virus life cycle other than in the hypothetical initiation of RNA replication. It could conceivably have an encapsidation function (since only VPg-linked RNA is encapsidated) and/or could be used to regulate the amount of RNA to be encapsidated rather than translated.

Replication of virus RNA occurs on membranes in factories called replication complexes (reviewed in *Rekosh* 1977). The function of the membrane association is unclear, although we note that a number of other viruses also replicate in association with membranes (see below). Virus replication apparently occurs completely within the cytoplasm, and transcription of the host DNA is not required after infection.

## 2.2 The Flaviviruses

The flaviviruses are a group of enveloped viruses which replicate in both their vertebrate hosts and the arthropod vectors, generally ticks or mosquitoes. The virions consist of an icosahedral nucleocapsid surrounded by a lipoprotein envelope. The capsid contains the genomic RNA, 12 kb in length, complexed with a single species of nucleocapsid protein, V2 or C, which has a molecular weight of 13 K. The viral envelope contains a large glycoprotein, V3 or E, of molecular weight 51–59 K, depending on the virus, and a small (7–8 K) membrane-associated protein, V1 or M, which is not glycosylated (*Westaway* et al. 1980). Although the flavivirus group includes a number of important human pathogens, the molecular biology of their replication is not well understood. This reflects the fact that these viruses do not grow well in tissue culture, that the virions are relatively unstable and difficult to purify, and that many of these viruses are severe pathogens. Some of the members of this group are listed in Table 1. The structure of flaviviruses has been recently reviewed by *Russell* et al. (1980) and the replication of these viruses recently reviewed by *Westaway* (1980). We note that these viruses are classified together with the alphaviruses as togaviruses (Table 1), but that the replication strategies of alphaviruses (Sect. 2.3) and flaviviruses differ significantly. Moreover, the recent discovery of six subgenomic polyadenylated RNAs in cells infected with equine arteritis virus, an unclassified nonarthropod borne togavirus, illustrates the diversity of replication strategies within this taxonomic family (*van Berlo* et al. 1982). Not enough is known of the replication of the other two groups of togaviruses, rubiviruses and pestiviruses, to compare them with the former groups.

The flavivirus RNA is capped but lacks poly(A) (*Wengler* and *Wengler* 1981) and is infectious. The infecting RNA must therefore be translated to produce the viral replicase, but the translation strategy of the flavivirus genome has not been definitively established. No evidence for a subgenomic RNA has been reported and it is generally believed that the viral RNA is the only messenger. *Westaway* (1980) has proposed that the structural polypeptides V1, V2, and V3 as well as nonstructural peptides P20, P27, P37, P71, and P100 are separately initiated and terminated during translation, which would make the flavivirus mRNA unique since most other animal mRNAs studied to date have only one or at most two translation initiation sites; see footnote b in Table 3 (Sect. 4). The data supporting this hypothesis (reviewed in *Westaway* 1980) are (a) no evidence for precursor polyproteins has been found in pulse-chase experiments, and peptide mapping has shown that virtually all of the flavivirus polypeptides found in infected cells are distinct, (b) pactamycin mapping indicates that proteins are completed in order of their size, and (c) reinitiation of protein synthesis following a high salt block results in very rapid labeling of all of the virus proteins. On the other hand, *Wengler* et al. (1979) and *Svitkin* et al. (1981) reported that during translation in vitro only a single initiation site appeared to be used and that only structural protein polypeptides were produced. They

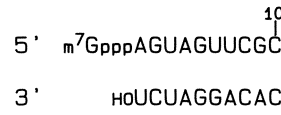


Fig. 3. Terminal sequences of flavivirus RNA. The sequences shown are the 5′ (reading from 5′ to 3′) and the 3′ terminal sequences (reading from 3′ to 5′) of West Nile virus RNA (*Wengler* and *Wengler* 1981)

proposed a genetic map based on these results of 5′-V2-V3-(V1, P20, P27, P37, P71, P100)-3′ and it is of note that these results imply that the structural proteins are encoded in the 5′ end of the genome, as is the case for the picornaviruses. The complete sequence of a flavivirus genome, together with sequence information on the proteins, will probably be required to resolve the situation. In this light it has been found recently that the three structural proteins of Saint Louis encephalitis virus are not blocked and two of them do not begin with methionine (*J.R. Bell, R. Kinney, D.W. Trent, J.H. Strauss,* manuscript in preparation). This suggests that these three proteins are produced, at least in their final form, by posttranslational cleavage.

Details of flavivirus replication have yet to be worked out, and nothing is known about the viral replicase. The 5′ and 3′ terminal sequences of flavivirus RNA are different, implying that the recognition sequences for the plus stranded and minus stranded replicase are different (Fig. 3). The flaviviruses can replicate in arthropod cells (mosquito or tick, depending on the virus) and in a wide range of vertebrate cells. This wide host range implies that any functions supplied by the host during replication must be common to a broad phylogenetic range. It is also known that RNA replication is associated with perinuclear membranes (reviewed in *Westaway* 1980).

## 2.3 The Alphaviruses

Alphaviruses are enveloped viruses, approximately 70 nm in diameter, which replicate in both the arthropod vectors and their mammalian or avian hosts. The virus consists of an icosahedral nucleocapsid surrounded by a lipid bilayer in which are anchored two integral membrane glycoproteins. The three principal virion polypeptides, the capsid protein C, molecular weight 30 K, and the envelope proteins E1 and E2, molecular weights 50–60 K, are present in the virion in equimolar amounts. A third glycoprotein, E3, remains associated with the virion in Semliki Forest virus but is lost into the culture fluid for other alphaviruses. The alphavirus genome is a single stranded RNA of about 12 kb which is capped and polyadenylated, and which is infectious. Most of the molecular biology of these viruses has been determined with either Sindbis virus or Semliki Forest virus, but recent comparative studies with other alphaviruses are giving us better insights into the relationships among the members of this group. An extensive collection of review articles on these viruses has recently appeared (*Schlesinger* 1980).

### 2.3.1 Translation Strategy

The alphaviruses produce two mRNAs after infection (reviewed in *Strauss* and *Strauss* 1977). One is apparently identical to the virion RNA and is translated into the nonstructural proteins of the virus. The second is a subgenomic RNA identical to the 3′ terminal one-third of the genomic RNA which is translated into the structural proteins of the virus.

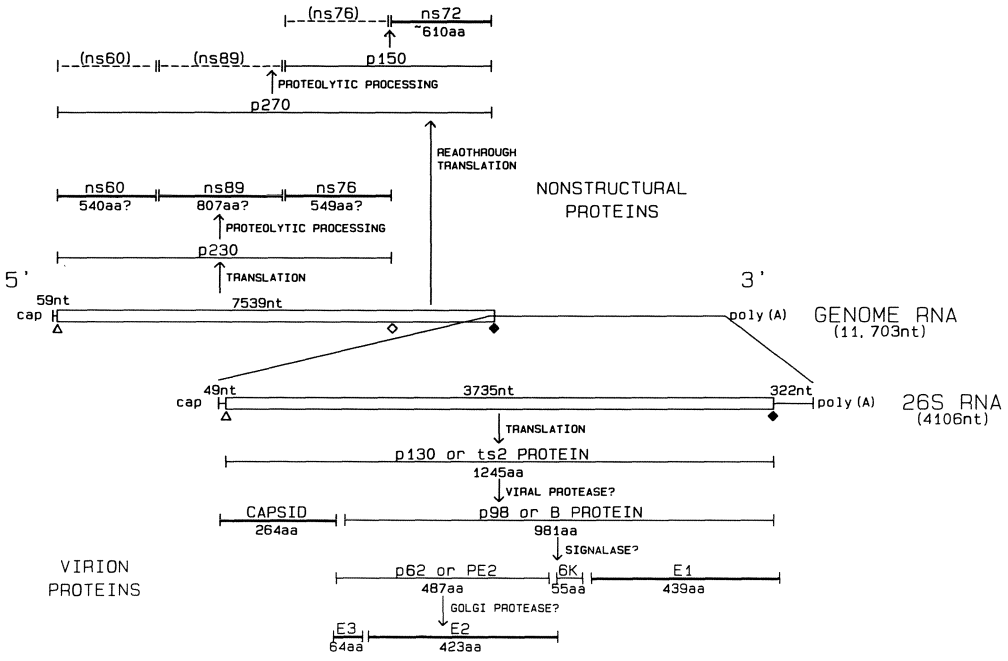


Fig. 4. Replication strategy of Sindbis virus. Untranslated regions of the genomic RNA are shown as *single lines,* and the translated region as an *open box.* The subgenomic RNA region is expanded below using the same convention. Translation products are indicated and the final protein products, both virion and non-structural, are indicated with *heavy lines. Open triangles* are initiation codons, *solid diamonds* are termination codons. The *open diamond* is the UGA codon read through to produce ns72. Data for this figure are from *Ou* et al., 1982a, b, 1983; *Rice* and *Strauss, 1981; Strauss,* et al., 1983a; *E.G. Strauss* unpublished; and *S. Lopez* and *J.R. Bell,* unpublished

Both of these RNAs are capped and polyadenylated. These RNAs and their translation products are illustrated schematically in Fig. 4.

The genomic RNA is a minor message in the infected cell and encodes the non-structural proteins necessary for viral RNA replication. Translation begins at an AUG codon approximately 60–80 nucleotides depending on the alphavirus from the 5'-terminal cap (Ou et al., 1983). As diagrammed in Fig. 4 for Sindbis virus two polyprotein precursors are produced: the major product terminates at an opal codon at nucleotides 5748 to 5750 (open diamond) which interrupts an otherwise open reading frame encoding 2513 amino acids. A minor polyprotein is produced by read through and terminates at multiple in-phase stop codons (solid diamond). The major polyprotein is processed, usually while nascent, to produce the three upstream products, which have been identified following translation in vitro and in extracts of infected cells (reviewed in *Schlesinger* and *Kääriäinen,* 1980; Collins et al., 1982). Genetic analysis had suggested that there were four non-structural polypeptides essential for RNA replication (reviewed in *Strauss* and *Strauss,* 1980). The fourth product, ns72, has recently been identified in Sindbis-infected cell extracts following immunoprecipitation with an antibody directed against a synthetic dodecapeptide with the amino acid sequence of the carboxyl terminus of the 2513 amino acid precursor (*Lopez* and *Bell,* unpublished). The functions of the non-structural polypeptides must include replicase/transcriptase components to replicate the RNA and

transcribe the subgenomic message. In addition, one of the products may be a virus-specific protease, to process these precursors. The read through mechanism allows modulation of the relative amounts of the replicase components; ns72, produced in the smallest amounts, has been suggested to be a regulatory factor for the control of minus strand synthesis (*Strauss* et al., 1983a).

The structural proteins of the virus are translated from a subgenomic messenger. This subgenomic RNA has been completely sequenced in the case of three alphaviruses, Semliki Forest virus (*Garoff* et al. 1980a, b), Sindbis virus (*Rice* and *Strauss* 1981; *Ou* et al. 1982a), and Ross River virus (*L. Dalgarno* et al. 1983 (in press), and corresponds to the 3′ terminal one-third of the genomic RNA. Use of a subgenomic mRNA for the structural proteins allows for amplification of the structural gene products. The subgenomic RNA is produced in about threefold molar excess over the genomic RNA (also see below) and, in addition, much of the genomic RNA is quickly sequestered into nucleocapsids, where it cannot serve as messenger. The result is that 90% of the virus-specific mRNA is the subgenomic species, and only 10% is the genomic RNA; thus a large excess of structural over nonstructural polypeptides is produced (reviewed in *Strauss* and *Strauss* 1977). Because of the use of an infectious genomic RNA and a subgenomic RNA for the structural proteins, the replicase genes are 5′ terminal and the structural protein genes 3′ terminal the inverse order from that of the picornaviruses (see Sect. 2.1.1.).

Translation of the structural proteins from the 4100-nucleotide subgenomic RNA begins at an AUG codon located approximately 50 nucleotides from the 5′ terminal cap (*Ou* et al. 1982a) and proceeds to a termination codon positioned 260–520 nucleotides from the 3′ terminal poly(A) tract (*Rice* and *Strauss* 1981; *Garoff* et al. 1980b; *L. Dalgarno*, et al. 1983 (in press)). Cleavage of the N-terminal capsid protein from the nascent precursor appears to be an autoproteolytic event (reviewed in *Schlesinger* and *Kääriäinen* 1980) and the chymotryptic-like activity, which cuts a tryptophan-serine bond, is thought to reside in the C-terminal region of the capsid protein itself. This cleavage event is not only rapid but quite efficient, and no uncleaved products in found when wild type RNA is translated in vivo or in vitro. Normally the cleavage of the capsid protein from the nascent chain appears to be accomplished by the protease activity in the nascent chain itself, rather than by protease activity in previously released capsid proteins, and much of the proteolytic activity may be lost upon cleavage. At least some proteolytic activity appears to remain, however. Mutants temperature sensitive in the protease activity accumulate large amounts of uncleaved precursor during infection at nonpermissive temperatures. In cells doubly infected with such a mutant, and with mutants defective in the glycoproteins but having a functional protease, the precursor is found in smaller amounts, implying that the mutant polyprotein can be cleaved by a diffusible factor (*Scupham* et al. 1977).

The remaining structural proteins are two integral membrane glycoproteins which traverse the lipid bilayer and are anchored in the bilayer by short hydrophobic stretches found at or near the C-terminus of the proteins (*Garoff* and *Söderlund* 1978; *Rice* et al. 1982). Removal of the capsid from the nascent precursor polyprotein allows a signal sequence of about 19 residues at the N-terminus to function and results in the integration of the first glycoprotein precursor (called PE2, the precursor to glycoprotein E2) into the endoplasmic reticulum, with concomitant core glycosylation (*Garoff* et al. 1979; *Bonatti* et al. 1979; *Bell* et al. 1982). This signal sequence is not cleaved from the precursor at this stage (*Bonatti* and *Blobel* 1979). There is a second, internal signal sequence located

between the two glycoproteins which functions to allow insertion of the second glycoprotein (called E1) into the endoplasmic reticulum (*Hashimoto* et al. 1981), again accompanied by core glycosylation. Removal of this internal signal sequence, which separates the two glycoproteins from one another, requires two proteolytic cleavages, both of which occur after alanine residues. It has been suggested that signalase catalyzes both of these cleavages (*Rice* and *Strauss* 1981).

The glycoproteins, once synthesized and inserted into the endoplasmic reticulum, migrate to the plasma membrane by way of the Golgi apparatus. The cleavage of PE2 to form E2 and E3 has been postulated to occur in the Golgi (*Garoff* et al. 1980b; *Rice* and *Strauss* 1981), catalyzed by the Golgi protease whose specificity is such that it cleaves after clustered basic amino acids, and which cleaves proalbumin, proinsulin, and other precursor proteins (*Dean* and *Judah* 1980). The small glycoprotein produced, E3, is not required for infectivity and may or may not remain associated with the virion. This Golgi protease also appears to cleave glycoproteins of several other enveloped viruses (see below) in addition to the PE2 of alphaviruses.

Thus the cleavage of the alphavirus polyproteins is postulated to require one or more virus proteases active on the nonstructural precursor polyprotein, a virus protease activity present in the capsid protein which acts autoproteolytically, and two cellular proteases, both of which are localized in subcellular organelles.

The alphaviruses inhibit translation of host cell messenger RNAs, apparently by increasing the $Na^+$ concentration and lowering the $K^+$ concentration inside the cell (*Garry* et al. 1979a). The virus messengers are efficiently translated under these altered conditions, whereas most host cell mRNAs are not. The interference with translation is at the level of initiation. The virus structural proteins may be implicated in this inhibition (*Atkins* 1976), and it has been suggested that the altered ionic environment inside the cell results from interference with the $Na^+/K^+$ pump (*Garry* et al. 1979b).

### 2.3.2 Replication and Transcription of the RNAs

During the course of alphavirus replication three RNA synthesis activities are needed: a minus strand replicase to produce full length minus strands using the plus strand as a template; a plus strand replicase to produce full length plus strands using the minus strand as a template; and a transcriptase to produce the subgenomic messenger RNA for the structural proteins, which uses the minus strand as a template. Each of these activities appears to be independently regulated and different recognition sites for the corresponding enzymes are utilized. We postulate that four activities are involved: an elongation enzyme which synthesizes the RNA chain once properly initiated; and three initiation activities which initiate minus strands, plus strands, and the subgenomic mRNA, respectively. These activities could reside in four different polypeptide chains and compose four different complementation groups, or two or more of these activities could reside in the same polypeptide chain. Similarly, the initiation and elongation functions could be expressed as an enzyme which is a functional complex of several polypeptide chains, or the component parts could function as separate enzymes.

Work with temperature-sensitive mutants has supported this concept of several functions involved in RNA synthesis. In the case of Sindbis virus, four complementation groups are required for normal RNA synthesis after infection (*Strauss* and *Strauss* 1980). One group (F) appears to encode an elongation function because, upon shifting cells

infected with temperature-sensitive mutants in this group to the nonpermissive temperature, synthesis of both plus strands and minus strands ceases (*Keränen* and *Kääriäinen* 1979; *Sawicki* et al. 1981a). Complementation group B is required for minus strand synthesis but not for plus strand synthesis (*Sawicki* et al. 1981a), and could be involved in initiation of minus strands. Finally, mutants of complementation groups A and G lead to reduced synthesis of the subgenomic RNA (*Keränen* and *Kääriäinen* 1979), and one or both could be involved in initiation of the subgenomic RNA.

No specific plus strand replicase has been described. The initiation function for plus strands could be present in the F protein (in which case the initiation specificity could be modified by the initiation factors for minus strand or subgenomic RNA synthesis), or

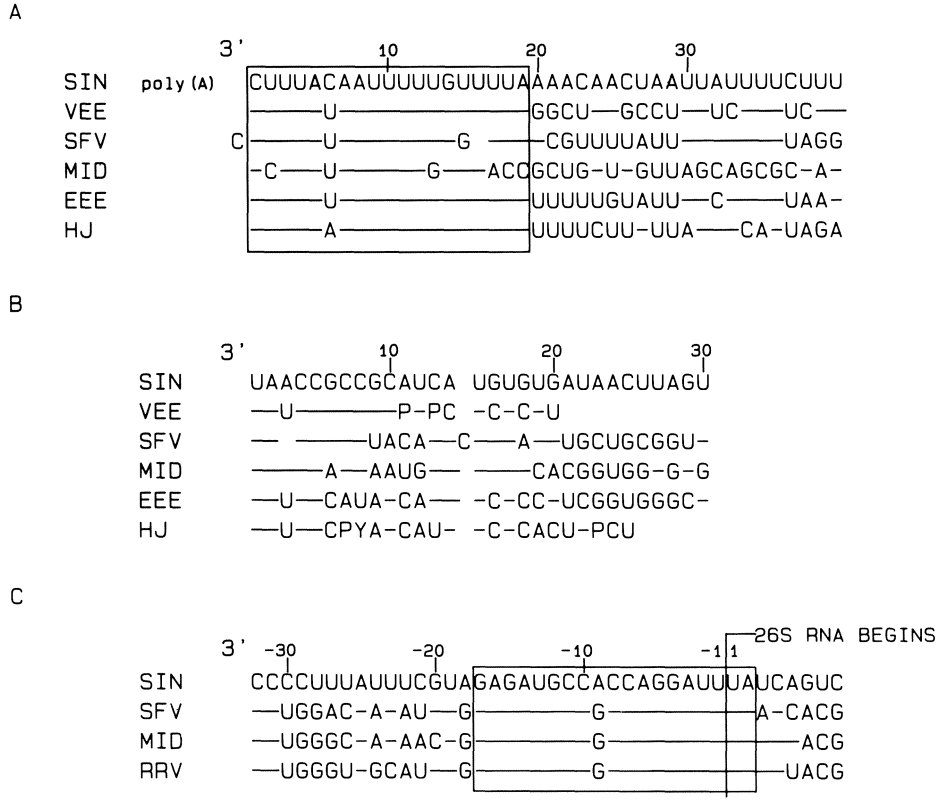


Fig. 5A–C. Conserved sequences in alphavirus RNAs. (A) 3′ terminal sequences of genome RNAs. Sequences read from 3′ poly(A) to 5′ and are shown as the plus strand (genomic RNA) sequence. The *box* encloses a conserved sequence. (B) Sequences complementary to the 5′ terminus of the genomic RNAs. The sequences shown read from 3′ to 5′ on the minus strand (i.e., complementary to the genome RNA). (C) Sequences complementary to the junction region. The sequences shown are the minus strand sequences in the region containing the start of the 26-S subgenomic RNA and are given from 3′ to 5′. The *box* encloses the conserved sequence, presumably a recognition signal for initiation of 26-S mRNA synthesis. In all cases a *horizontal line* indicates that the nucleotide is the same as that in the complete sequence at the head of the group. *Gaps* have been introduced for alignment. *SIN*, Sindbis virus; *VEE*, Venezuelan equine encephalitis virus; *SFV*, Semliki Forest virus; *MID*, Middelburg virus; *EEE*, Eastern equine encephalitis virus; *HJ*, Highland J virus; *RRV*, Ross River virus. Data from *Ou* et al. (1982a, b) and *Ou* et al. (1983 in press)

could be represented by either group A or G. The interpretation of the data is complicated by the fact that defects in the hypothetical protease activity in the polyprotein precursor could have pleiotropic effects.

The minus strand replicase functions early in infection. It may recognize a sequence of 19 nucleotides at the 3′ terminus of the plus strand (*Ou* et al. 1981, 1982b). These 19 nucleotides are highly conserved among alphavirus strains (Fig. 5A), and changes in this sequence appear to affect the ability of RNAs to be replicated by the viral replicases (*Monroe* et al. 1982). There is no evidence that the subgenomic RNA is utilized as a template for production of minus strands, however, which implies that the minus strand replicase must also recognize an additional sequence (or structure) in the genomic RNA, present outside the subgenomic RNA region. The simplest hypothesis is that this sequence is present somewhere near the 5′ end of the genomic RNA, and that cyclization of the molecules (*Hsu* et al. 1973; *Frey* et al. 1979) brings these sequences near the 3′ end where they can interact with the replicase. One possibility for these sequences is a conserved region of 51 nucleotides which begins about 150 nucleotides from the 5′ terminus (*J.-H. Ou* et al., 1983 in press). This sequence, shown in Fig. 6, is able to form a stable double hairpin structure. We postulate that one or both loops form a recognition site for the minus strand replicase, and that it must interact simultaneously with this structure and with the 19-nucleotide recognition sequence at the 3′ end of the plus strand.

Synthesis of minus strands is regulated during infection, and minus strand synthesis normally ceases at 3–4 h after infection (*Sawicki* and *Sawicki* 1980). *Sawicki* et al. (1981b) found that a virus function, represented by complementation group A, must operate to stop minus strand synthesis. In the absence of A function, minus strand synthesis continues. The mechanisms involved are not clear at present, but two hypotheses, which are not mutually exclusive, are appealing. The first is that 26-S RNA, which has the 3′ terminal 19-nucleotide recognition sequence but lacks the second conserved sequence

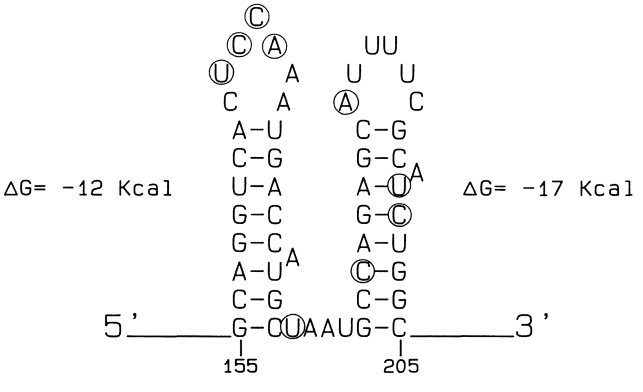


Fig. 6. Conserved structure in alphavirus RNAs. A possible two-dimensional structure in a conserved sequence located near the 5′ end of alphavirus genome RNAs is shown. Sequences of Sindbis virus, Semliki Forest virus, Highlands J virus, and Eastern equine encephalitis virus RNAs were compared. The sequence shown is that of Sindbis virus HR strain and nucleotides are numbered from the 5′ terminus of this RNA. Nucleotides which vary in one or more of the other viruses are *circled*; all others are the same in all four viruses. Data are from *J.H. Ou* et al., 1983 in press). Free energy was calculated according to the method of *Tinoco* et al. (1973)

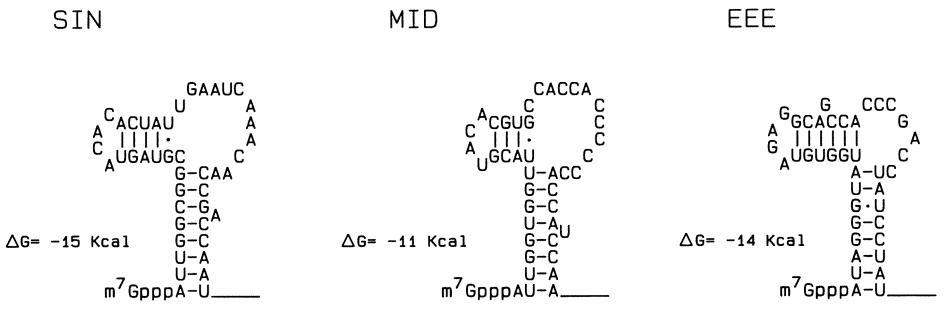


Fig. 7. 5′ Terminal secondary structures in alphavirus RNAs. Moderately stable secondary structures can be formed by the sequences at the 5′ termini of alphavirus genomes. *SIN*, Sindbis virus, *MID*, Middelburg virus, *EEE*, Eastern equine encephalitis virus. Free energies were calculated as in Fig. 6. Data are from *J.H. Ou* et al. 1983 (in press)

found near the 5′ terminus of 49-S RNA, acts as a competitive inhibitor (*Ou* 1982). The second is that one or both of the loops of the 51 nucleotide sequence of Fig. 6 forms a binding site for capsid protein (which then acts as a nucleation site for encapsidation); capsid protein bound to this site would prevent replicase binding.

The minus strand is used as template by the plus strand replicase. Conservation of sequence at the 3′ end of the minus strand (as determined by sequence studies of the 5′ end of the plus strand) is not as striking as at the 3′ end of the plus strand (Fig. 5B). There is, however, a stem and loop structure at the end which does seem to be conserved (Fig. 7). Although hairpins are not uncommon at the 5′ end of mRNAs, and may be involved in translation initiation, a double stem structure as in Fig. 7 is not common, and we propose that either the conserved sequence or the conserved stem and loop structure is recognized by the plus strand replicase to specifically initiate plus strand synthesis. In either event the postulated recognition site is quite different from that used by the minus strand replicase (Figs. 5, 6) which would allow differential regulation of plus strand and minus strand synthesis. It is also of note that no subgenomic minus strand has been demonstrated, and initiation of plus strand synthesis might not require cyclization of the molecule.

Defective interfering viruses (DIs) of alphaviruses are deletion mutants which have lost the ability to replicate in the absence of helper viruses. Sequence studies of DI RNAs of SFV (*Lehtovaara* et al. 1981, 1982) and Sindbis (*Monroe* et al. 1982) have shown that the postulated recognition sequences for both plus and minus strand replication, as well as encapsidation, have been preserved, whereas almost all of the coding sequence is absent. The organization of these DIs will be discussed in more detail in Section 5.

The full length minus strand is also used as the template for synthesis of the subgenomic RNA. There is a conserved sequence of 21 nucleotides around the initiation site of the subgenomic RNA (Fig. 5C) which has been proposed as the recognition site for the transcriptase (*Ou* et al., 1982a). This sequence is different from any of those proposed as recognition sequences for the replicases, and use of it could thus be independently regulated.

Neither the alphavirus transcriptase nor the replicase(s) have been purified to homogeneity and the nature of initiation/elongation factors is unresolved. Partially purified preparations contain a virus-specific polypeptide of molecular weight 70 K, and some

separation of replicase and transcriptase activities has been achieved (*Clewley* and *Kennedy* 1976; *Gomatos* et al. 1980). The question of whether host components are utilized is also unresolved. There is evidence that host-specific functions are required at some stage of the virus replication cycle, however, and one possibility is that host components form part of the viral replicases. We note that alphaviruses replicate in mosquito cells and in a wide range of vertebrate cells, and any host function must be supplied by all these cells. *Kowal* and *Stollar* (1981) isolated two mutants of Sindbis virus which were restricted in their ability to grow in mosquito cells at 34.5°, whereas at this temperature these mutants grew normally in chick cells. They were however temperature sensitive in chick cells, at 40°, and failed to grow. Using complementation analysis, the authors found both mutants belonged to complementation group F, the putative elongation function. This result thus implicates a host cell component in RNA replication. Another argument for the involvement of host components in alphavirus replication is that the time of appearance of DIs during repeated high multiplicity passages is a function of the particular host cell used (*Stark* and *Kennedy* 1978; *Holland* et al. 1980) (see also Section 5).

Other studies which implicate host functions as being necessary for virus production are either inconclusive as to which step of the virus growth cycle requires a host component or suggest that factors are involved at stages other than RNA replication. Thus, although alphavirus replication is insensitive to the addition of RNA synthesis inhibitors at the time of infection, Baric et al. (1983) has shown that pretreatment of cells with either actinomycin D or $\alpha$-amanitin renders the cells unable to replicate Sindbis virus (although the cells are still competent for replication of vesicular stomatitis virus, a negative strand virus). Intriguingly, Baric et al. (1983) have isolated mutants for the virus which are capable of replication in these pretreated cells, and which thus either do not need this host function or can utilize reduced concentrations of the component. These mutants have not yet been classified, however. *Scheefers-Borchel* et al. (1981) showed that Sindbis virus replication in mosquito cells is sensitive to actinomycin D and *Erwin* and *Brown* (1983) found that Sindbis specific antigens but no progeny virions were produced in enucleated mosquito cells, implicating a host function for virus assembly. Finally, *Mento* and *Siminovitch* (1981) have isolated mutants of Chinese hamster ovary cells with a reduced capacity to produce wild type Sindbis virus, but the major effect of one such cell mutant appears to be at the level of mRNA translation.

Alphavirus RNA replication also occurs in association with membranes. In vertebrate cells "cytopathic vacuoles" develop after infection with which RNA replication is associated (*Grimley* et al. 1968), and in mosquito cells virus replication and assembly appears to be associated with vacuoles which also appear after infection (*Raghow* et al. 1973; *Gliedman* et al. 1975). It is also of note that the virus establishes a persistent infection in arthropod cells but is cytocidal in vertebrate cells, implying a differential host response to virus infection (*Stollar* 1980b).

## 2.4 The Caliciviruses

The caliciviruses, whose best known members are San Miguel sea lion virus, vesicular exanthema of swine virus, and feline calicivirus, have been reviewed recently by *Schaffer* (1979) and *Schaffer* et al. (1980a). Originally these nonenveloped viruses were classified as picornaviruses, but on the basis of their morphology with characteristic cup-shaped

indentations on the surface of the virions, and the fact that they possess only a single species of structural protein (molecular weight 65 K), they are now considered a separate family. The icosahedral particle with a diameter of 35–40 nm is made up of 60 morphological subunits, each one consisting of a trimer of capsid protein. The overall replication strategy of this group of viruses is not clear at present, although it appears to resemble that of the alphaviruses. The virion RNA has a size of about 7–8 kb and is presumably translated into the components of the replicase/transcriptase since the deproteinized RNA is infectious (*Schaffer* et al. 1980a). Several nonstructural polypeptides have been described in infected cells (*Black* and *Brown* 1977; *Fretz* and *Schaffer* 1978), and there is preliminary evidence for processing. Thus the replicase/transcriptase appears to be translated as a polyprotein which is cleaved proteolytically.

There is, in addition, at least one subgenomic RNA produced, an mRNA about 3 kb in size. This mRNA is translated in vitro into the single capsid protein (*Black* et al. 1978), a polypeptide of molecular weight 60–65 K. This capsid protein is reported to be derived from a precursor of molecular weight 86 K (*Fretz* and *Schaffer* 1978), which would require virtually the entire coding capacity of a 3-kb mRNA. Thus, in these details, the translation strategy resembles that of the alphaviruses in that the replicase/transcriptase is translated from the genomic RNA and the structural protein from a subgenomic RNA.

A smaller subgenomic RNA, about 2 kb (18 S) in size, has also been found in infected cells (*Black* et al. 1978). It is unknown whether this RNA is a third messenger or a degradation product. If it is a third messenger, encoding a distinct polypeptide, the relationship of this messenger to the other two messengers is unclear. It could form one of a nested set of RNAs, as in the coronaviruses (Section 2.5), but the coding capacity required by the capsid messenger makes this unlikely. Further work on the structure and function of the viral RNAs will clearly be of great interest in defining the replication/translation strategy of these viruses.

No details of the replication/transcription of the viral RNAs are known. The virion RNA has a small protein (VPg) covalently linked to the 5′ terminus, analogous to the situation with the picornaviruses (*Burroughs* and *Brown* 1978; *Schaffer* et al. 1980b). This VPg presumably serves the same function as in the picornaviruses although, unlike the picornaviruses, it is essential for infectivity of the caliciviruses (*Matthews* 1979). All of the virus specific RNAs are polyadenylated, and none possesses a cap. The structure and replication of caliciviruses are similar to those of the plant virus, southern bean mosaic virus, which possesses a genome-linked VPg, a single species of structural protein and a subgenomic message for that protein (Sect. 3.1.3).

## 2.5 The Coronaviruses

The coronaviruses are a relatively large group of viruses which have been implicated in a number of degenerative diseases in experimental animals and which may serve as model systems for human degenerative diseases of unknown etiology. These viruses include avian infectious bronchitis virus, mouse hepatitis virus, human coronaviruses, and neonatal calf diarrhea virus. The virions are enveloped and have characteristically shaped projections external to the lipid bilayers (a "corona"). The genomic RNA is one of the largest among RNA viruses, approximately 18 kb, and is enclosed in a helical nucleocapsid containing a single species of protein of molecular weight 60 K. The external glyco-

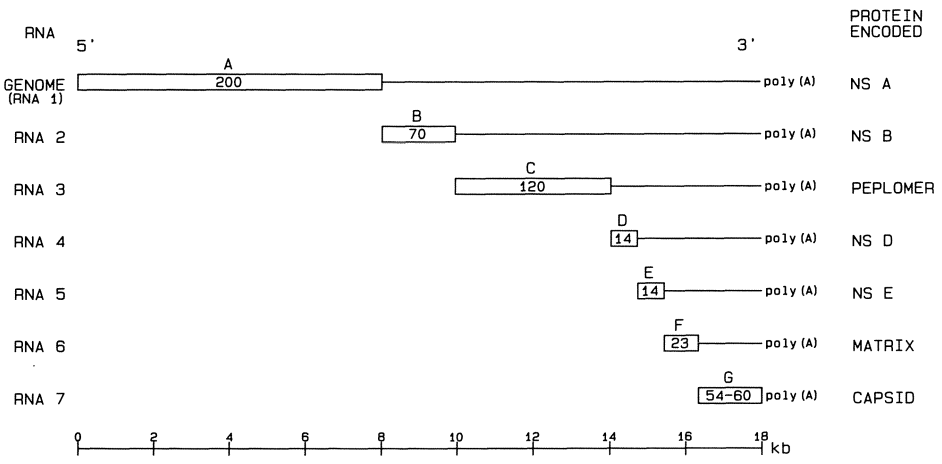


Fig. 8. Replication strategy of coronaviruses. Translated regions of the genome of mouse hepatitis virus and its six subgenomic messages are shown by *open boxes. Numbers inside the boxes* are the size of the protein product in kilodaltons. Data and terminology are from *Siddell* et al. (1982)

protein, variously called E2, the fusion protein, or the peplomer protein, is about 180 K in size, but in many strains is cleaved to two nonidentical species of roughly 90 K. A second glycoprotein, called E1 or the matrix protein, is a transmembrane protein of 25 K molecular weight. The coronaviruses have been recently reviewed by *Tyrrell* et al. (1978), by *Robb* and *Bond* (1979), and by *Siddell* et al. (1982).

The replication strategy of these viruses involves producing five (in the case of avian infectious bronchitis virus) or six (in the case of murine hepatitis virus) subgenomic mRNAs which together with the virion RNA form a nested set (Fig. 8) (reviewed in *Siddell* et al. 1982). Each of these RNAs is capped and polyadenylated, and each presumably has an AUG initiation codon near the 5′ terminus and a stop codon near the junction region of the next member of the set, analogous to the situation with the alphaviruses. The protein translated from the mRNAs has been defined by in vitro translation in the case of the three structural proteins. The nucleocapsid protein is translated from the 3′ terminal mRNA (RNA 7). It is phosphorylated but not otherwise modified after translation. The small virion glycoprotein (matrix protein) is translated from the 3′ penultimate RNA (RNA 6). It is glycosylated in the presence of tunicamycin and is thought to have O-glycosidic linked carbohydrates (*Niemann* and *Klenk* 1981). It is an integral membrane glycoprotein which spans the lipid bilayer but lacks covalently linked fatty acid chains. The large (peplomer) glycoprotein E2 is translated from the third largest RNA (RNA 3). This glycoprotein contains conventional N-glycosidic linked polysaccharide chains and has lipid covalently attached. Most of this protein is found external to the bilayer. It is not known if E2 spans the bilayer, but the presence of lipids suggests it at least penetrates the bilayer. This protein is cleaved posttranslationally into two glycoproteins of molecular weight about 90 K. Details of this processing step are not known, but it may be analogous to the processing of the alphavirus PE2, the influenza HA, and the paramyxovirus F proteins (see also Section 6).

The virion RNA must be translated to form the replicase/transcriptase because the

RNA is infectious. Approximately 300 K daltons of non-overlapping protein could be encoded in this RNA (Fig. 8), and a 200 K polypeptide has been found during in vitro translation of the RNA in a reticulocyte system (*Siddell* et al. 1982). The replicase has not been purified, and its composition remains to be determined.

Thus there are two or three additional nonstructural polypeptides whose function is unknown at present. No evidence for posttranslational cleavage of precursors has been presented except in the case of the structural peplomer glycoprotein, and it is possible that each mRNA is "monocistronic". However, many details of the translation strategy have yet to be worked out and some mRNAs may turn out to be "polycistronic".

The mechanisms involved in transcribing the subgenomic mRNAs are also unknown. UV transcriptional mapping has indicated that each RNA is transcribed independently (the UV dose to inhibit transcription of any of the RNAs is proportional to the size of that RNA) (*Jacobs* et al. 1981). This suggests that a mechanism analogous to the alphaviruses is used, wherein the transcriptase begins transcription at any of six internal sites in the minus strand of murine coronavirus and proceeds to the end of the molecule. The subgenomic RNAs are produced in unequal amounts, but no temporal control of their production has been described. Unequal transcription could be achieved by having transcriptase recognition sites of differing affinity, by using different recognition factors for transcription initiation of the various RNAs, or by secondary structure in the RNA (perhaps stabilized by interaction with capsid or other proteins) leading to unequal use of the initiation sites. The latter two mechanisms would allow temporal regulation of transcription as well. A different mechanism for production of the subgenomic RNAs has been proposed by *Lai* et al. (1982). On the basis of limited sequence data showing an identical tetranucleotide at the 5′ end of all the subgenomic messages, and finding that two or three of the subgenomic RNAs contain a T1 oligonucleotide not present in the genomic RNA, these authors suggested that RNA splicing might be involved in production of the mRNAs. This would conflict with the UV transcription results, and further sequence data is needed to resolve these questions.

Conflicting reports have appeared about the possible involvement of the host cell in replication/transcription of the viral RNA. *Wilhelmsen* et al. (1981) found that murine coronaviruses would replicate in enucleated cells, albeit with reduced yield. These authors used immune fluorescence to show that enucleated cells clearly synthesized virus protein and also quantitated virus yields in enucleated populations. On the other hand *Evans* and *Simpson* (1980) reported that enucleated cells or UV-irradiated cells failed to support the replication of avian infectious bronchitis virus; furthermore, replication of the virus was inhibited by α-amanitin in cells sensitive to the drug, but not in α-amanitin resistant lines. The interpretation of these results is complicated by the fact that enucleation can be accompanied by partial loss of Golgi function, which can result in inhibition of virus maturation (see also Sect. 4.2 and 4.3). However, the situation may be analogous to that with the alphaviruses (Sect. 2.3.2), where sensitivity to α-amanitin or actinomycin D develops after some time, and where different strains of the virus show differing sensitivities to the inhibitory effects of the drugs. Thus it is possible that some component of the host RNA polymerase or of some other host protein is used in the viral replicase/transcriptase, and that the concentrations of the host component required depend upon the virus.

Coronaviruses in general establish cytocidal infections in tissue culture cells, but details on inhibition of host cell functions are lacking. In addition, many coronaviruses

establish persistent infections in their natural hosts, so the responses of the host cell and host organism to virus infection are of considerable interest.

## 2.6 The Nodaviruses

Segmented genomes are common in plant viruses with plus stranded RNA and in animal viruses with negative stranded RNA, but the nodaviruses are the only animal viruses known which have a segmented genome of plus stranded RNA. The two nodaviruses studied, nodamura virus (which grows in a wide range of insects and in mammals) and black beetle virus, contain two RNA molecules in the virion. These RNAs are about 3 kb and 1.5 kb in size respectively and lack poly(A). Virus particles are isometric, approximately 30 nm in diameter, and contain both RNA species encapsidated by a single species of capsid protein of molecular weight 40 K. Both RNAs are required for infectivity. The RNAs have been shown to be active as messengers in vitro (*Newman* et al. 1978; *Guarino* et al. 1981). The large RNA is translated into a polypeptide of molecular weight 105–120 K which is believed to be the replicase of the virus (or a component of the replicase). The smaller RNA is translated into a polypeptide of molecular weight 43–46 K which shares tryptic peptides with the capsid protein of the virion and is believed to be a precursor of it. In vivo synthesized polypeptides of molecular weight 110 K, 40 K, and 8 K have been reported for black beetle virus (*Crump* and *Moore* 1981), which supports the in vitro results. Finally the two RNAs of the virus appear to replicate independently. Thus the replication strategy of these viruses appears to be relatively uncomplicated.

# 3 The Plus Stranded Viruses of Plants

Although this chapter is primarily concerned with replication strategies of animal viruses containing RNA genomes, it seems appropriate to consider a number of plant viruses whose replication strategies resemble those of various groups of animal viruses. Many plant viruses have been described and although viruses infecting plants have been isolated that contain double stranded DNA (caulimovirus), single stranded DNA (geminivirus), and double stranded RNA (plant reoviruses) the vast majority of plant viruses contain single stranded RNA as their genome. Of these, only two groups contain negative stranded RNA and both produce enveloped virions, the plant rhabdoviruses (*Matthews* 1982), and tomato spotted wilt virus (*Mohamed* 1981), which has a segmented genome. The remainder of the plant viruses have a single-stranded plus sense genome, are non-enveloped, and most contain a single species of virus structural protein. Plant viruses have been classified primarily by physiochemical properties, as these viruses are relatively stable to physical and chemical manipulation and could be obtained in sufficient quantities from infected cell sap for examination by electron microscopy and measurements of physical properties. Only recently has sufficient information been assembled to permit grouping by replication strategy. Improvements in in vitro protein synthesizing systems and development of plant cell tissue culture (protoplast) systems, in which intracellular macromolecular synthesis could be studied, have enabled workers in the field to elucidate the translation strategies of a number of these viruses.

The genome of single stranded RNA plant viruses may be segmented or nonseg-

Table 2. The plus stranded plant viruses

| Group | Type virus | Structure | Size of genome (kb) | Transcription | | | Polycistronic messages | Other characteristics | References[a] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Sub-genomic message for coat | Structures at RNA termini | | | | |
| | | | | | 5′ | 3′ | | | |
| *Monopartite Genome Viruses* | | | | | | | | | |
| Potyvirus | Potato Y | Rod | 9–10.5 | No | Cap | Poly(A) | Yes | Cytoplasmic and nuclear inclusions | Dougherty and Hiebert 1980 |
| Tobacco necrosis virus | Tobacco necrosis | Isometric | 4.2 | No | ppApGpU | No poly(A) | Yes | | Salvato and Fraenkel-Conrat 1977 |
| Carnation mottle group | Carnation mottle | Isometric | 4.2 | No | ? | No poly(A) | Yes | | Salomon et al. 1978 |
| Tymovirus | Turnip yellow mosaic | Isometric | 6 | Yes | Cap | $tRNA_{val}$ | Yes | | Mellema et al. 1979; Morch and Benicourt 1980 |
| Sobemovirus | Southern bean mosaic | Isometric | 4.2 | Yes | VPg | No poly(A) no tRNA-like structure | ?; read-through in vitro | Similar to caliciviruses | Mang et al. 1982; Salerno-Rife et al. 1980 |
| Tobamovirus | Tobacco mosaic | Rod | 6 | Yes | Cap | $tRNA_{his}$ [Some strains $tRNA_{val}$] | No; read-through in vitro | Second sub-genomic message | Pelham 1978; Beier et al. 1980; Hirth and Richards 1981 |
| Tombusvirus | Tomato bushy stunt | Isometric | 4.2–6.0 | Yes | ? | ? | No; read-through in vitro | Second sub-genomic message | Dougherty and Kaesberg 1981 |
| Luteovirus | Barley yellow dwarf | Isometric | 16 | No | VPg | No poly(A) | ? | | |
| Potexvirus | Potato X | Flexible rod | 6.3 | ? | Cap | No poly(A) | Probably | | |
| Closterovirus | Sugar beets yellow | Flexible rod | 7.5–13 | ? | ? | ? | ? | | Murant et al. 1981 |
| Carlavirus | Carnation latent | Flexible rod | 7 | ? | ? | ? | ? | | |

Table 2. The plus stranded plant viruses

| Group | Type virus | Structure | Size of genome (kb) | Transcription | | | Polycistronic messages | Other characteristics | References[a] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Sub-genomic message for coat | Structures at RNA termini | | | | |
| | | | | | 5′ | 3′ | | | |
| *Bipartite Genome Viruses* | | | | | | | | | |
| Nepovirus | Tobacco ringspot | Isometric | B RNA=8 MRNA=4 | No | VPg | Poly(A) | Yes | B RNA replicates independently; similar to Comovirus | Chu et al. 1981 |
| Comovirus | Cowpea mosaic | Isometric | B RNA =6.9 M RNA =4.2 | No | VPg | Poly(A) | Yes | B RNA replicates independently; encodes polymerase, protease resembles picornaviruses | Franssen et al. 1982; Goldbach et al. 1982 |
| Tobravirus | Tobacco rattle | Rod | RNA 1 =6.8 RNA 2 =1.5 | No | RNA 2 capped RNA 1 probably capped | ? | No; subgenomic messages for noncoat proteins. Readthrough in vitro | RNA 1 replicates independently; RNA 2 encodes coat protein | Pelham 1979; Bisaro and Siegel 1982; Robinson et al. 1981 |
| Dianthovirus | Carnation ringspot | Isometric | RNA 1 =5 RNA 2 =1.6 | Yes | ? | ? | No | Subgenomic RNA for coat protein from RNA 1 | Morris-Krsinich et al. 1983 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Tripartite Genome Viruses* | | | | | | | | | |
| Bromovirus | Bromegrass mosaic | Isometric | RNA 1 =3.3 RNA 2 =3.0 RNA 3 =2.4 | Yes encapsi-dated | Cap | $tRNA_{tyr}$ | No | | Kaesberg 1976; Ahlquist et al. 1981b |
| Cucumovirus | Cucumber mosaic | Isometric | RNA 1 = 3.8 RNA 2 =3.4 RNA 3 =2.4 | Yes | Cap | $tRNA_{tyr}$ | No | | Dorssers et al. 1981 |
| Ilarvirus | Tobacco streak | Variable sizes Isometric to bacilli-form | RNA 1 = 3.3 RNA 2 = 2.4 RNA 3 =2.0 | Yes | ? | ? | No | | Smit and Jaspars 1980; Koper-Zwarthoff and Bol 1980 |
| Alfalfa mosaic virus group | Alfalfa mosaic | Variable bacilli-form | RNA 1 =3.3 RNA 2 =2.4 RNA 3 =2.0 | Yes | Cap | No poly(A) | No | Coat mRNA or coat protein necessary for infectivity | |
| Hordeivirus | Barley stripe mosaic | Short and long rods | RNA 1 =4.3 RNA 2 =3.6 RNA 3 =3.2 | Yes | Cap | Poly(A) and $tRNA_{tyr}$ | ? | Glycosylated capsid protein; variable num-bers of RNA segments | Gustafson et al. 1981, 1982; Agranovsky et al. 1982 |

[a] In addition to the references listed, much of the information in this table comes from Matthews (as shown) (1982) or Van Vloten-Doting and Neeleman (1980)

mented. In animal viruses, segmented genomes are present within a single virion (for example, nodaviruses, orthomyxoviruses, bunyaviruses, and arenaviruses). In plants a common configuration is to have a segmented plus stranded genome in which the segments are separately encapsidated in two or more different particles, which may or may not be morphologically and physiochemically distinguishable. Simultaneous infection of the same cell by two or more particle types is essential for infectivity.

Table 2 shows a grouping of the plant viruses according to what is known of their replication. Some of these groups will be discussed in more detail below, where parallels can be drawn between their transcription and translation strategies and the better known strategies of animal viruses.

It is noteworthy that a number of plant viruses possess a tRNA-like structure at the 3′ terminus which can be aminoacylated with a specific amino acid by the appropriate aminoacyl-tRNA synthetase. These tRNA-like structures have been reviewed by *Hall* (1979) and are listed in Table 2 for the virus groups in which they occur. Although the integrity of the 3′ terminal structure is essential for infectivity, it is unlikely that its function is involved in protein synthesis, since the amino acid is not donated to a growing polypeptide during translation (*Hall* 1979). These specific secondary structures may act as recognition signals during initiation of RNA replication, and it is not inconceivable that the appropriate host-encoded aminoacyl-tRNA synthetase forms part of the replicase complex. Alternatively this structure, perhaps in the aminoacylated form, could be a recognition sequence for the virus-encoded enzymes. In viruses with multipartite genomes which have tRNA-like 3′ termini, all segments have identical structures, suggesting that these structures are involved in RNA replication and/or encapsidation.

## 3.1 Monopartite Genome Viruses

### 3.1.1 Potyviruses, Tobacco Necrosis Virus, and Carnation Mottle Virus

These various viruses contain a genome which appears to function as one long polycistronic message which is translated into a polyprotein processed by cleavage. The translation strategy is thus formally analogous to that of the picornaviruses. The potyviruses have a genome with a potential coding capacity of about 3500 amino acids. Several virus specific products have been identified in infected plants, including two polypeptides found in nuclear inclusions and another associated with cytoplasmic cylindrical inclusions. Virions contain a single species of capsid protein of approximately 30 K molecular weight. The genomic RNA is capped and polyadenylated and can be translated in a cell-free reticulocyte system yielding products corresponding to four of the virus specified proteins as well as numerous readthrough products (*Dougherty* and *Hiebert* 1980). From these experiments a genetic map for the potyvirus genome has been proposed (Fig. 9). Although the capsid gene is at the 3′ end of the genome, adjacent to the poly(A), there is no evidence for subgenomic messages or "silent cistrons," and the entire genome appears to consist of one long polycistronic message.

Tobacco necrosis virus and carnation mottle virus are two small isometric viruses, whose taxonomic position is unclear, which also appear to have polycistronic messages. The genome of both viruses is roughly 4.2 kb and the three virus-specific proteins which have been identified staturate this genome (*Salomon* et al. 1978; *Salvato* and *Fraenkel-*

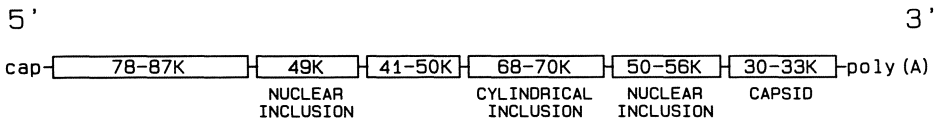


Fig. 9. Genome organization of potyviruses. The gene order for Potato Y virus was determined by in vitro translation in a reticulocyte cell-free system (*Dougherty* and *Hiebert* 1980)

*Conrat* 1977). The genomic RNAs are neither capped nor polyadenylated. They are efficiently translated in cell-free systems, and from such cell-free translation the gene order of carnation mottle virus is thought to be p30, p38 (coat), p77. For tobacco necrosis virus, the predominant product of in vitro translation is the coat protein.

### 3.1.2 Tymoviruses

The genome of the tymoviruses is capped at the 5′ terminus, and the 3′ terminus has the structure of a valine-accepting tRNA (*Hall* 1979). The translation strategy of these viruses is similar to that of alphaviruses. When the genomic RNA is translated in vitro, the nonstructural proteins are produced as a polyprotein precursor which is subsequently processed by proteolytic cleavages (*Mellema* et al. 1979; *Morch* and *Benicourt* 1980). In addition, during translation in vitro in the presence of yeast amber suppressor tRNA, a third polypeptide of 210 K is produced by readthrough of a UAG codon at the terminus of the 195-K protein. This readthrough product is produced by translation of part of the coat protein cistron in another reading frame, but in the absence of in vivo information it is not known whether this 210-K protein plays a role in normal virus infection (*Morch* et al. 1982). The capsid protein is translated from a subgenomic message of 695 nucleotides coterminal with the 3′ end of the genome. This message has been completely sequenced and consists of a coding region of 567 nucleotides flanked by 19 untranslated nucleotides at the 5′ end and 109 untranslated nucleotides at the 3′ terminus. It is interesting that following the open reading frame there are four in-phase termination codons within the next 40 nucleotides of the mRNA (*Guilley* and *Briand* 1978).

### 3.1.3 Sobemoviruses

The genomic RNA of southern bean mosaic virus contains neither a 5′ cap nor 3′ poly(A), but both the genomic message and subgenomic message contain a small protein covalently linked to the 5′ end (*A. Ghosh* et al. 1981) which is essential for infectivity of the RNA (*Veerisetty* and *Sehgal* 1979). The nonstructural proteins are two large proteins of molecular weights 105 K and 75 K, which share many tryptic peptides and are related by readthrough (*Salerno-Rife* et al. 1980; *Mang* et al. 1982). These genes are located near the 5′ terminus of the genome. The capsid protein is translated from a subgenomic message which is coterminal with the 3′ end of the genome (*A. Ghosh* et al. 1981). In addition, however, there is a small 14-K polypeptide which appears to be translated from an internal cistron, but whether this protein is generated by processing of a polyprotein precursor or by independent initiation of translation is unclear at present (*Mang* et al. 1982). With a subgenomic capsid protein message and a 5′ linked VPg, southern bean mosaic virus resembles the caliciviruses.

### 3.1.4 Tobamoviruses and Tombusviruses

These two virus groups have a translation strategy which is reminiscent of that of the coronaviruses, or which can be considered intermediate between the alphaviruses and the coronaviruses. The genomic RNA is translated into one or more nonstructural polypeptides. There are two subgenomic mRNAs produced, one of which is translated into the capsid protein, the other into a different nonstructural protein(s) (Fig. 10).

The genome of tobacco mosaic virus, the type virus of the tobamovirus group, is about 6 kb in length. The RNA has a 5′ methylated cap and a 3′ terminal structure resembling tRNA, which can be enzymatically aminoacylated with histidine for the type virus or valine for the cowpea strain (*Hall* 1979). In vivo or in vitro, the intact genome is

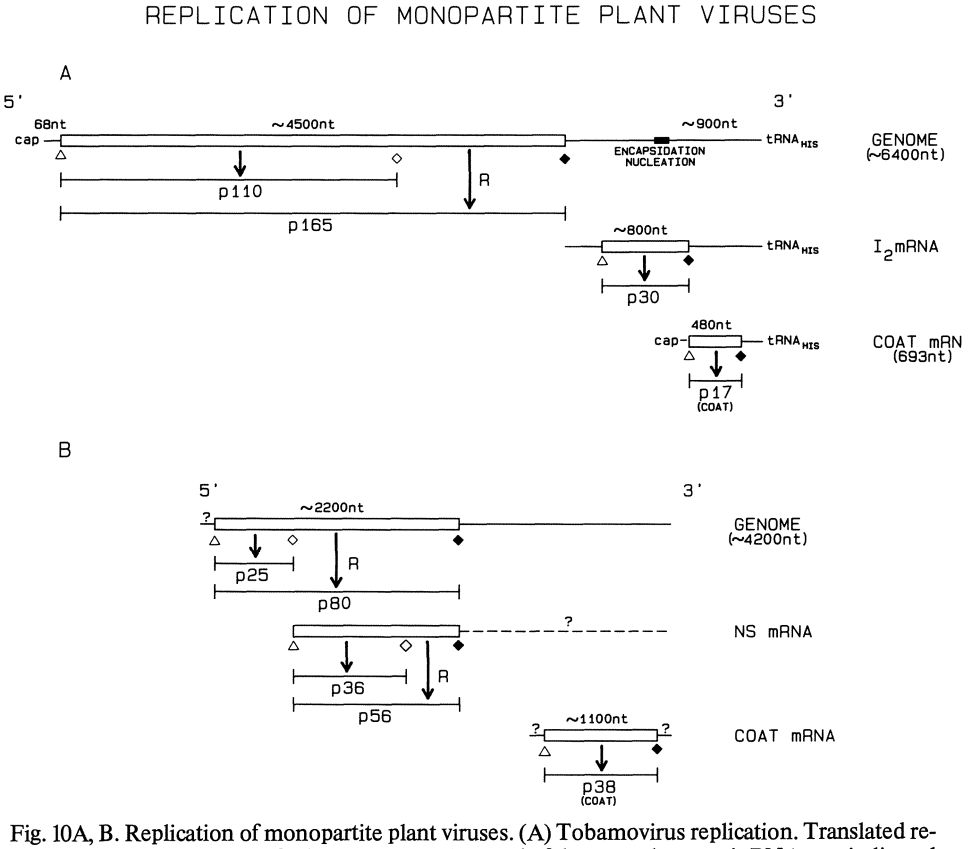


Fig. 10A, B. Replication of monopartite plant viruses. (A) Tobamovirus replication. Translated regions of the genome RNA of tobacco mosaic virus and of the two subgenomic RNAs are indicated by *open boxes*. Protein translation products (*P*) are shown with molecular weights in kilodaltons. The *closed box* in the genome is the sequence for initiation of encapsidation. *Open triangles* indicate initiation codons for translation. *Open diamonds* are suppressible termination codons where readthrough (*R*) occurs in in vitro translation systems; *solid diamonds* are strong termination codons. The 5′ nontranslated region in the coat mRNA is nine nucleotides long. Data from *Hirth* and *Richards* (1981) and *Guilley* et al. (1979). (B) Tombusvirus replication. The translation products of turnip crinkle virus genomic RNA and two subgenomic messages are shown (*Dougherty* and *Kaesberg* 1981). *Symbols* as in Fig. 10A

translated into two large proteins of approximate molecular weights 170 K and 120 K (*Pelham* 1978; *Beier* et al. 1980; *Hirth* and *Richards* 1981). The larger polypeptide is produced by partial readthrough of an amber termination codon. Two subgenomic RNAs are produced which are 3′ coterminal with the genomic RNA and together with it form a nested set of mRNAs (Fig. 10A). The larger subgenomic mRNA is translated into a nonstructural protein 30 K in size, the smaller into the coat protein of the virus. The coat mRNA has been completely sequenced. In the 5′ untranslated region, only nine nucleotides separate the cap from the initiation codon (*Guilley* et al. 1979).

Turnip crinkle virus, provisionally classified as a tombusvirus, has a smaller genome (4 kb), but its replication strategy, insofar as it is known, appears to resemble that of the tobamoviruses. There are only two major nonstructural polypeptides, a 25-K moiety translated in vitro from the 5′ end of the full length genome and a 36-K protein translated in vitro from a subgenomic message. A second subgenomic message is translated into the coat protein. Readthrough products of both the genomic RNA and the intermediate message are produced (Fig. 10B) (*Dougherty* and *Kaesberg* 1981; *Altenbach* and *Howell* 1982). *Russo* and *Martelli* (1982) have argued on the basis of ultrastructure of infected plants that turnip crinkle virus lacks some of the characteristics of other tombusvirus infections, so it is unclear whether the replication strategy presented here is representative of the group as a whole.

## 3.2 Bipartite Genome Viruses

### 3.2.1 Nepoviruses and Comoviruses

Of particular interest are two groups of plant viruses which, although quite distinct in morphology and assembly from the picornaviruses, show remarkable analogies at the level of translation strategies. These are the nepoviruses and the comoviruses (*Matthews* 1982). Both groups form a family of particles consisting of a top component (T particles), which are empty protein shells; a middle component (M) containing M RNA of 4.2–6.6 kb, depending on the species; and a bottom component (B), which contains either two copies of M RNA or one of B RNA (6–8 kb). In addition, some nepoviruses (perhaps due to the presence of satellite viruses, although this is unclear) contain up to five other particle types of varying buoyant densities, each containing multiple copies of a smaller RNA (approximately 1 kb) sometimes in association with M RNA (*Rezaian* 1980; *Gallitelli* et al. 1981; *Rezaian* and *Jackson* 1981). The two virus groups are distinguished from one another by the fact that nepoviruses contain only a single species of coat protein of molecular weight 55–60 K (*Chu* and *Francki* 1979), while all comovirus particles consist of icosahedral shells containing equal numbers of two polypeptide species of molecular weights 22 K and 42 K (*Matthews* 1982).

Both M RNAs and B RNAs of nepoviruses and comoviruses have a 5′-VPg of about 4 K in size, which is necessary for infection for nepoviruses (*Chu* et al. 1981), but not for comoviruses (*Daubert* et al. 1978). Both comoviruses and nepoviruses have a 3′ terminal poly(A) tract of about 120 residues (*Matthews* 1982; *Mayo* et al. 1979). Although both M and B RNAs, or both M and B particles, are required for production of progeny virus and symptomology in the host, it has been shown that the larger RNA (B RNA) of both nepoviruses (*Robinson* et al. 1980) and comoviruses (*Goldbach* et al. 1980; *Rezelman* et al. 1980)

can replicate alone in plant protoplasts. This suggests that B RNA encodes the viral polymerase or some component of it; in addition, the B RNA encodes the VPg (*Robinson* et al. 1980; *Franssen* et al. 1982). Replication and expression of M RNA, on the other hand, is completely dependent upon the presence of B RNA. M RNA encodes the structural proteins of these viruses: it has been shown to encode the serological specificity of nepovirus (*Haber* and *Hamilton* 1980), and to encode the two structural proteins of comoviruses plus one or two nonstructural polypeptides of unknown function (*Hiebert* and *Purcifull* 1981), encoded in cowpea mosaic virus (CPMV) in the order 5′-NS58/NS47-VP37-VP23-3′ (*Franssen* et al. 1982; *Goldbach* and *Rezelman*, 1983).

Recent experiments with CPMV, the type virus of the comovirus group, have shown that protoplasts infected with purified B RNA encode 6 nonstructural proteins varying in size from 32 K to 170 K. Using nonstructural proteins from CPMV-infected leaves as antigens to make antibodies in rabbits, it was possible to elucidate the precursor-product relationships between these products and show that VPg was derived from a 60-K membrane-associated precursor polypeptide (*Goldbach* et al. 1982).

Purified M RNA from CPMV does not produce any detectable virus proteins when inoculated into protoplasts (*Goldbach* et al. 1980; *Rezelman* et al. 1980), although this RNA is an efficient messenger in both reticulocyte and wheat germ cell free protein synthesis systems, producing large products of 95 K and 105 K which share regions of amino acid sequence (*Franssen* et al. 1982; *Rezelman* et al. 1980). B RNA is translated in vivo into a protease which is capable of cleaving the in vitro products of M RNA (*Franssen* et al. 1982). A summary of the cleavage scheme of these polyproteins from B and M RNA is presented in Fig. 11.

The translation and processing scheme of the comoviruses in Fig. 11 is quite similar to that of the picornaviruses (Fig. 1), but with the structural protein genes and the replicase/VPg/protease genes separated into two RNAs in the plant viruses. The case for nepoviruses is not as clear, but their RNAs are also translated into polyproteins and they

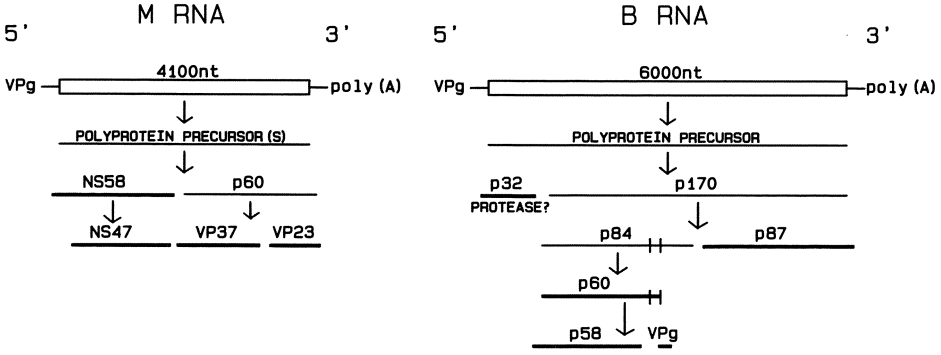


Fig. 11. Translation of comovirus RNAs. Translated regions of the M and B RNAs of cowpea mosaic virus are shown by *open boxes*. Final products, including virion polypeptides *VP23* and *VP37* and the genome linked *VPg*, are shown with *heavy lines*. The processing scheme incorporates data from *Hiebert* and *Purcifull* (1981), *Franssen* et al. (1982), and *Goldbach* et al. (1982). A second pathway has also been found for processing *B* RNA encoded polyproteins, in which p170 is cleaved to p60 and p110 which are further processed to p58, VPg and p87 respectively. Which cleavages are due to the action of the virus-encoded protease, probably p32, is not certain at this time

are probably closely related to the comoviruses. As noted above, another point of similarity to the picornaviruses is the presence of a 5′ VPg on the RNAs as well as 3′ terminal poly(A), which is unusual for plant viruses. Furthermore, the nepoviruses have two species of capsid protein processed from a precursor polypeptide, which is also unusual for plant viruses. Conceivably, both of these virus groups and the picornaviruses could be derived from a common ancestor. Among plant viruses, mechanical transmission with many particles simultaneously infecting a cell is common, and having separately encapsidated sections of genomes does not appear to be evolutionarily disadvantageous.

### 3.2.2 Tobraviruses

Tobacco rattle virus and pea early browning virus contain two separately encapsidated RNAs; RNA 1 is about 7 kb and RNA 2 is about 1.6 kb (*Matthews* 1982). RNA 1 contains the information for RNA replication, while RNA 2 specifies the capsid protein (reviewed in *Bruening* 1977). In vitro translation of RNA 1 produces two proteins related by readthrough, similar to those of tobamoviruses (*Pelham* 1979), but variable amounts and numbers of other subgenomic mRNAs also have been described (*Pelham* 1979; *Bisaro* and *Siegel* 1982). It was originally reported that only RNA 2 was capped (violating the "rule" that all plant virus RNAs of a single species share common termini), but subsequent experiments have shown that in vitro translation of RNA 1 is inhibited by cap analogs, implying that it is also capped (*Pelham* 1979). Since the RNA 2 of the CAM strain of tobacco rattle virus shares sequence homology with RNA 1 (*Robinson* et al. 1981), it is tempting to speculate that this bipartite virus evolved from a monopartite genome by evolving an efficiently encapsidated subgenomic message.

## 3.3 Tripartite Genome Viruses

### 3.3.1 Bromoviruses and Cucumoviruses

The genomes of both bromoviruses and cucumoviruses are composed of three species of RNA of about 3.3, 3, and 2 kb respectively, separately encapsidated into nearly identical icosahedral particles. All three RNAs are required for infectivity, and all are capped at the 5′ terminus and have a structure capable of accepting tyrosine at the 3′ end (*Matthews* 1982; *Kaesberg* 1976). The two larger RNAs (RNA 1 and RNA 2) are monocistronic messages which encode proteins called 1a (120 K) and 2a (110 K), respectively. The third RNA is bicistronic; there is an actively translated cistron for 3a protein which is 5′ proximal and a silent cistron for capsid protein synthesis in the 3′ half of the molecule. Both proteins 3a and 1a may be components of the viral replicase, since temperature-sensitive mutants in RNA 1 and RNA 3 are deficient in RNA synthesis at the nonpermissive temperature (*Dawson* 1981). Capsid protein is translated from a subgenomic message (RNA 4) which is commonly encapsidated but is not required for infectivity (*Kaesberg* 1976). The detailed structure of these RNAs is shown in Fig. 12. One notable feature of RNA 3 is a heterogeneous stretch of internal poly(A), 16–22 nucleotides long, located only 20 nucleotides from the beginning of the RNA 4 sequence (*Ahlquist* et al. 1981b). RNA 4 has a very short 5′ untranslated region, only nine nucleotides plus the cap.

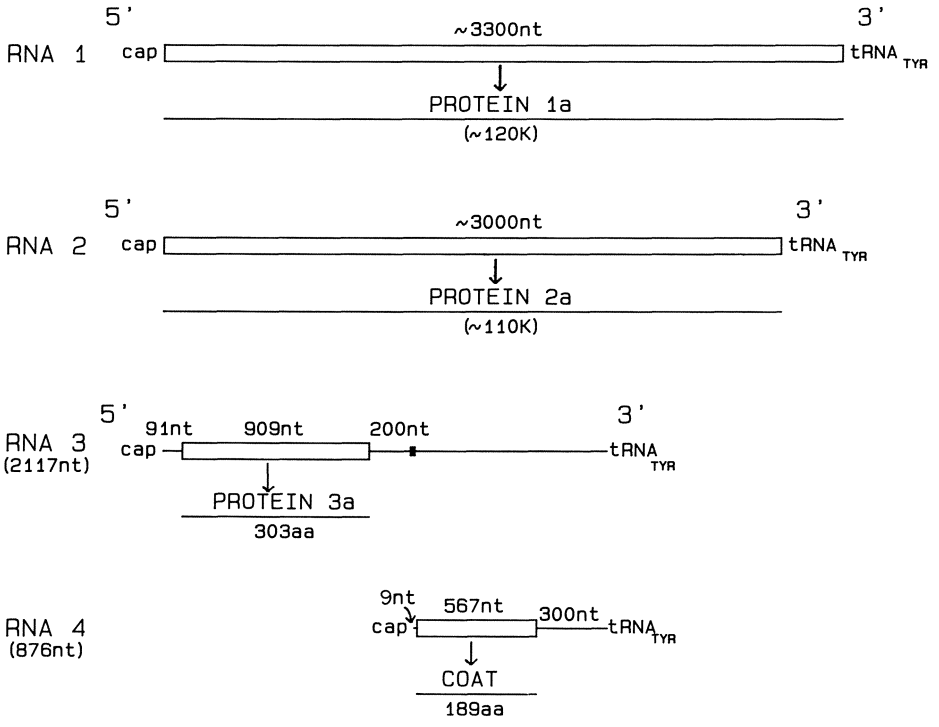


Fig. 12. Translation of brome grass mosaic virus RNAs. *Open boxes* indicate translated regions of the three genomic RNAs and the subgenomic RNA 4. The small *solid box* in RNA 3 represents the poly(A) tract of variable length. Data obtained from *Kaesberg* (1976) and *Ahlquist* et al. (1981b)

The 3′ termini of the three genomic RNAs, as well as of the subgenomic RNA 4, which is 3′ coterminal with RNA 3, contain a highly conserved sequence of about 200 nucleotides which may function as a replicase recognition site. At the level of primary sequence the 3′ termini of the bromoviruses show relatively little homology with the 3′ termini of cucumoviruses. However, stable base paired secondary structures can be constructed from both sequences which are remarkably similar and, as noted above, both sequences are capable of accepting tyrosine (*Ahlquist* et al. 1981a). Thus the functional structure has apparently been conserved between these two groups although the primary sequence has diverged.

Efficient replication of the viral RNAs requires the activity of all three RNA segments (*Takanami* and *Fraenkel*-Conrat 1982), and it is possible that all three noncapsid viral polypeptides are required for a functional replicase (see also Sect. 3.4).

### 3.3.2 Ilarviruses and the Alfalfa Mosaic Virus Group

The ilarviruses and alfalfa mosaic virus are very similar in replication strategy although they are classified as separate genera largely on the basis of virion morphology (*Matthews* 1982). Like the cucumo and bromoviruses, they posess three genomic RNAs with sizes

of about 3.3, 2.4, and 2 kb, plus a subgenomic RNA of about 1 kb which is derived from the smallest genome segment and encodes the capsid protein. However, coat protein or the subgenomic message for coat protein is absolutely required for infectivity; in the absence of RNA 4, some protein must be bound to each of the other three RNA species for infectivity (*Smit* and *Jaspars* 1980). Coat protein binds to the 3′ termini of the RNAs and could conceivably serve as an initiation factor for the replicase. Ilarvirus and alfalfa mosaic virus coat proteins are interchangeable for activation (*Matthews* 1982).

The four RNAs from alfalfa mosaic virus have homologous sequences of 140–150 nucleotides at the 3′ end and lack poly(A), whereas the common sequence of ilarvirus RNAs is only about 45 nucleotides (*Koper-Zwarthoff* and *Bol* 1980). Comparison of the 3′ terminal sequences of two RNAs of tobacco streak virus (an ilarvirus) with alfalfa mosaic virus RNA 3 shows that all three terminate in the same pentanucleotide, -GAUGC-OH. Presumably these conserved sequences serve replication functions. In addition, similar secondary structures can be drawn from the 3′ sequences which contain multiple hairpin loops most of which are preceded by a 5′ flanking sequence -AUGC. This structure has been postulated to be the capsid protein binding site (*Koper-Zwarthoff* and *Bol* 1980).

For both the ilarviruses and alfalfa mosaic virus, the RNAs are separately encapsidated in bacilliform particles of various sizes which can be separated by physical means. Using various mixtures of RNA segments and/or particles, it has been possible to ascertain that the two largest RNAs are required for viral RNA synthesis and that the subgenomic message RNA 4 is not itself replicated, but is transcribed from minus strand complementary to RNA 3 (*Nassuth* et al. 1981). It is clear that production of a subgenomic mRNA for the structural protein(s) is a replication strategy common to many of the plus stranded RNA viruses.

### 3.3.3 Hordeiviruses

The hordeiviruses contain variable numbers of RNA components separately encapsidated in rod-shaped particles of discrete lengths (*Matthews* 1982). They are classfied conventionally as tripartite viruses, although the type strain of barley stripe mosaic virus, contains only two separable segments, RNA 1 and RNA 2. Other strains contain one or two additional RNAs with some homology to RNA 2 and which, therefore, may or may not represent independent genomic segments (*Palomar* et al. 1977; *Boykov* et al. 1981; *Gustafson* et al. 1982). Hordeivirus RNAs are capped at the 5′ end and were initially reported to be 3′ polyadenylated (*Van Vloten-Doting* and *Neeleman* 1980). However, the 3′ termini can be aminoacylated by tyrosine, and it has been suggested that they contain a short poly(A) sequence located between the main body of the genome and the tRNA-like structure (*Agranovsky* et al. 1982). Three polypeptides of molecular weights 25 K, 67 K, and 120 K are synthesized in vitro by polyribosomes from infected plants, and the 25-K protein has been identified as authentic capsid protein (*Gustafson* et al. 1981). Too little is known about the replication strategy of this group to assign it an animal virus counterpart, but it is noteworthy that barley stripe mosaic virus is the only plus stranded RNA plant virus known to have a glycosylated capsid protein, which contains asparagine-linked carbohydrate (*Gumpf* et al. 1977). Comparison of the site and mode of this glycosylation event with the mechanisms of glycosylation of animal virus membrane proteins should prove very interesting.

## 3.4 RNA-Dependent RNA Polymerases

Currently a major controversy in plant virology is whether any or all plant viruses encode virus-specific RNA replicases (reviewed in *Hall* et al. 1982). Although uninfected animal cells are devoid of RNA replicases, RNA-dependent RNA polymerases have been isolated from the cytoplasm of a number of species of "healthy plants" which will transcribe and replicate plant virus RNAs in vitro. The primary polypeptide associated with the polymerase activity is a large protein of molecular weight 130–140 K (*Astier-Manifacier* and *Cornuet* 1978; *Duda* et al. 1973; *Chifflot* et al. 1980). Upon infection with plant viruses these endogenous polymerases are stimulated manyfold in activity, but most of these virus-stimulated enzymes show little template specificity for replicating the RNA of the infecting virus (*Ikegami* and *Fraenkel-Conrat* 1980).

In the case of the comoviruses and the nepoviruses, RNA replication requires the B component of the genome as discussed above (*Robinson* et al. 1980; *Goldbach* et al. 1980). However, purification of the soluble replicase from comovirus-infected cells resulted in a homogeneous protein preparation with a molecular weight of 130 K, lacking specificity for comovirus RNA and lacking any polypeptide components resembling the virus-specific polypeptides which have been identified after comovirus infection (*Dorssers* et al. 1982). Thus it appears that uninfected leaves contain a core enzyme capable of RNA-dependent RNA synthesis whose activity could conceivably be enhanced and amplified by the presence of virus-encoded factors of co-enzymes. The origin of this core enzyme is unclear, for a single genome segment from a bipartite or tripartite heterologous virus could be present without producing any overt symptoms in the "healthy" or uninfected hosts. Subsequently, *Dorssers* et al. (1983) isolated a second RNA-dependent RNA polymerase from a membrane fraction of infected leaves which is specific for comovirus RNA and probably is the true viral replicase.

On the other hand, it has been possible to purify a soluble RNA replicase from cucumber mosaic virus-infected cells which is absent before infection and which contains a major component of 100 K and two minor polypeptides of 35 K and 110 K (*Kumarasamy* and *Symons* 1979). None of these polypeptides, however, appear to be translation products of the viral genome (*Gordon* et al. 1982). Two other examples of isolation of plant virus replicases have been reported. *Mouches* et al. (1981) have isolated an enzyme complex from tymovirus-infected protoplasts which preferentially copies both plus stranded turnip yellow mosaic virus RNA and its complementary minus strand, although other plant virus RNAs are also copied to a lesser extent. Similarly, a crude preparation from brome mosaic virus-infected plants shows a preference for brome mosaic virus RNA as template and appears identical to protein 1a (see Sect. 3.3.1) (*Bujarski* et al. 1982; *Hall* et al. 1982).

By analogy with the replication of the plus stranded RNA animal viruses, we are inclined toward the view that all plant viruses will be found to encode one or more polypeptide components essential for virus-specific RNA synthesis. Whether these virus-specific enzymes will perform the major elongation function, or will be merely necessary for specific initiation has yet to be determined. Sequencing of viral genomes and comparison of deduced polypeptide sequences with the amino acid sequence of RNA-dependent RNA polymerases will be helpful in this regard.

## 4 The Negative Stranded Viruses

Negative strand RNA viruses include two groups whose genome is a single uninterrupted RNA molecule, the rhabdoviruses and the paramyxoviruses; and three groups with segmented genomes, the arenaviruses with two RNAs, the bunyaviruses with three RNA segments, and the myxoviruses with eight genome segments. As the name implies, these RNAs cannot serve as mRNAs in the infected cell, and thus the naked RNA is insufficient to initiate an infection. A summary of negative strand viruses is found in Table 3. These five virus families appear to be more closely related to one another than are the various families of plus strand viruses, and share common characteristics in both structure and replication. All of these viruses contain helical nucleocapsids surrounded by a lipoprotein envelope which is acquired by budding; with the exception of the bunyaviruses, minus strand viruses all bud from the host cell plasma membrane. Infection begins with the entry into the cell of the nucleocapsid which is the functional replicase/transcriptase complex. The nucleocapsid contains one major species of capsid protein, and one or more minor constituents which are thought to possess replicase/transcriptase activity; in orthomyxoviruses the replicase is present as three polypeptides (P1, P2, and P3), but in all the other groups the RNA synthetic activities are found in a large multifunctional protein of about 200 K molecular weight usually called the L protein.

Primary transcription of viral RNA in the infecting nucleocapsid produces the first mRNA molecules. The mRNAs of negative strand viruses are for the most part monocistronic, capped with $m^7GpppA^mp$ at the 5′ end and polyadenylated at the 3′ end. Translation of these mRNAs produces viral proteins, including more polymerase molecules, leading to amplified secondary transcription. Concomitantly, replication begins with the synthesis of full-length complementary RNA strands (antigenomes), which in turn serve as templates for synthesis of negative strand genomic RNA(s). In general the antigenomic RNAs and the mRNAs are not identical.

In all cases where data are available, the 3′ and 5′ termini of the genome or genome segments are largely self-complementary for 11–21 nucleotides. Moreover, in the viruses with segmented genomes these terminal sequences are common to all the segments. These sequences may be necessary for the encapsidation of both genome and antigenome to form the replication template ribonucleoprotein and/or could serve as recognition signals for the initiation of transcription and replication. The fact that the exact 3′ termini of both genome and antigenome are so closely homologous could be a reflection of the fact that both plus strands and minus strands are encapsidated to form the active template, and the primary recognition signal for replication may be the same for both plus and minus strands. However, during replication of all these virus groups, much more minus strand RNA (genome) is produced than plus strand RNA (antigenome template), and the replicase(s) thus synthesize the two strands differentially. In the case of the rhabdoviruses (Sect. 4.1) it is known that sequences located well outside the self-complementary regions, 50–60 nucleotides from the 3′ end, can modulate replicase activity. It is also of note that although the plus strand is encapsidated, it is seldom found in virions except in the paramyxoviruses; thus the plus strand nucleocapsid and the minus strand nucleocapsid can be distinguished at the level of virion assembly. There are many questions still to be answered concerning the recognition signals operative in these processes and their interaction with the components of the functional replication complex, as well as about the control mechanisms which differentiate between the transcription mode and replication mode of the polymerase complex.

Table 3. Negative strand viruses

| Family | Genera | Virus members | Genome (kb) | RNA in genome (*n*) | Transcripts[b] (*n*) | Polycistronic messages (*n*) | Requirement for functional nucleus |
|---|---|---|---|---|---|---|---|
| Rhabdovirus | Vesiculovirus | VSV | 12 | 1 | 5 | 0 | None |
| | Lyssavirus | Rabies | | | | | |
| Paramyxovirus | Paramyxovirus | NDV, Sendai, SV5 | 16–18 | 1 | 6–7 | 0 | Morphogenesis only |
| | Morbillivirus | Measles | | | | | |
| | Pneumovirus | Respiratory syncitial virus | | | | | |
| Orthomyxovirus | Influenza A | | 13.5 | 8 | 10 | 0 | Required for transcription |
| | Influenza B | | 14 | 8 | 10 | 0 | |
| | Influenza C | | 14 | 8 | ? | 0 | |
| Arenavirus | Old World | LCM, Lassa | 10–12 | 2 | ? | ? | Required for transcription |
| | New World | Tacaribe[a] complex | | | | | |
| Bunyaviruses | Bunyavirus | | 14–17 | 3 | ≧ 3 | ≧ 1 | Morphogenesis only |
| | Uukuvirus | | | | | | |
| | Nairovirus | | | | | | |
| | Phlebovirus | | | | | | |

**Abbreviations:** VSV, vesicular stomatitis virus; NDV, Newcastle disease virus; LCM, lymphocytic choriomeningitis virus

[a] Includes Amapari, Junin, Latino, Machupo, Parana, Pichinde, Tacaribe and Tamiami

[b] Another common feature of negative strand virus replication has been recently described: the translation of a single mRNA transcript in two different reading frames to produce two virus-specific polypeptides. The S RNA of bunyaviruses encodes both the capsid protein and a non-structural protein (*Ulmanen* et al. 1981b). The mRNA transcript of segment 6 of influenza B virus encodes both the neuraminidase and the non-structural polypeptide NB (Shaw et al. 1983 in press). The paramyxovirus P protein and the non-structural polypeptide C are also translated from one transcript (*Dethlefsen* and *Kolakovsky,* 1983; and D. Kolakovsky, personal communication)

## 4.1 Rhabdoviruses

The family Rhabdoviridae contains a large number of viruses which share distinct biochemical and morphological characteristics and infect many different hosts (reviewed in Wagner 1975; Bishop and Smith 1977; Matthews 1982). The virions are bullet-shaped or bacilliform, and their distinctive shape makes them useful markers in electron microscopy for a variety of experiments. The helical nucleocapsid is wound into an inner structure exhibiting cross-striations and a central axial channel, and is surrounded by a lipid-containing envelope. Many members replicate in both a primary host (plant or vertebrate) as well as an arthropod vector, although mechanical transmission and congenital infection also occur. Animal rhabdoviruses are grouped into two genera, the vesiculoviruses (vesicular stomatitis virus group) and the lyssaviruses (rabies virus group). Almost all of the molecular biology has been done with vesicular stomatitis virus (VSV), which is easy to work with and for which an extensive catalog of mutants exists. There are also a number of plant rhabdoviruses, whose taxonomy is unclear. In general their replication appears very similar to that of VSV.

### 4.1.1 Overview of VSV Replication

VSV encodes five proteins, all of which are found in the virion, but not in equal amounts. The three major constituents are the G protein (55 K for the polypeptide portion), which is the glycoprotein making up the spikes attached to the lipid bilayer; the M or matrix protein (26 K), which lines the inner surface of the bilayer; and the N or nucleocapsid protein (47 K), which encapsidates the genomic RNA. These molecules are found in about 1800, 4000, and 2000 copies per virion respectively. Two other proteins, the L protein (about 160 K) and the NS protein (25 K), are also associated with the nucleocapsid, but are present in only 50 and 200 molecules per virion respectively. The NS protein has an apparent molecular weight in SDS polyacrylamide gels of approximately 40 K but nucleotide sequence data indicate it contains only 222 amino acids (see Fig. 15). The lyssaviruses also have five proteins but they are called L, G, N, M1 and M2. As described in Section 4.1.5, M2 corresponds to M of VSV and M1 to NS.

The genome of VSV is an uninterrupted single stranded RNA molecule of approximately 11 kb (molecular weight $3.8 \times 10^6$) containing the genes for the five virus-encoded proteins in the linear order 3′-N-NS-M-G-L-5′ (*Abraham* and *Banerjee* 1976; *Ball* and *White* 1976), as shown in Fig. 13. The nucleocapsid contains an RNA-dependent RNA polymerase activity (involving both the NS and L proteins) and disrupted virions will carry out transcription in vitro in the presence of appropriate substrates. Much of what we know about the mechanisms of transcription and replication have been determined from this cell-free system. The genome is transcribed to give five monocistronic messages of sizes 6.5, 1.6, 1.3, 0.8, and 0.8 kb which are translated to produce the L, G, N, M, and NS polypeptides respectively. Each mature message contains an $m^7GpppA^mp$ cap at the 5′ end and a poly(A) tail of variable length at the 3′ end (*Bishop* and *Smith* 1977; *Ball* and *Wertz* 1981). In addition, at the extreme 3′ end of the genome is a sequence of 50 nucleotides (called T in Fig. 13) which is transcribed to give an oligonucleotide of 46 nucleotides called "plus strand leader RNA." At the 5′ end of the molecule is a sequence partially complementary to the leader RNA and designated as R in Fig. 13.

Following adsorption to and penetration of a susceptible cell, the intact VSV nucleo-

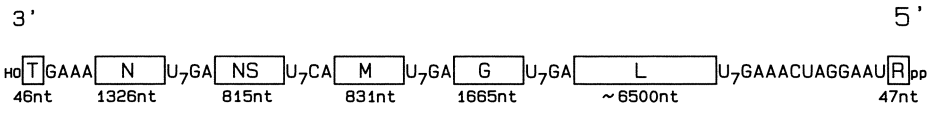


Fig. 13. Genome organization of vesicular stomatitis virus. *Boxes* labeled *T* and *R* are sequences transcribed as the plus strand and minus strand leader sequences (see Fig. 14). *Open boxes N, NS, M, G,* and *L* are sequences transcribed into the five mRNAs. Nucleotides shown are not transcribed into mRNA except that poly(A) is transcribed from the $U_7$ tracts. Data are from *McGeoch* and *Turnbull* (1978), *McGeoch* (1979), *Rose* and *Iversen* (1979), *Rose* (1980), *Schubert* et al. (1980), and *Keene* et al. (1980)

capsid begins primary transcription to produce the five viral subgenomic mRNAs. This transcription is independent of both host protein synthesis and host nuclear functions (*Bishop* and *Smith* 1977). These mRNAs are translated into the five viral proteins which leads to an accelerated synthesis of message known as secondary transcription. Concomitant with secondary transcription, there is also replication of the genomic RNA. This occurs in two steps: first comes the synthesis of a full-length plus strand copy of the genome, which in turn is used as a template for full-length minus strand genomic RNA (*Ball* and *Wertz* 1981; *Lazzarini* et al. 1981). Rhabdovirus multiplication is only slightly inhibited by actinomycin D and normal yields are produced in enucleated cells (*Wagner* 1975; *Pennington* and *Pringle* 1978). Thus it appears that nuclear functions are not needed for any aspect of the production of VSV. For the purposes of this review we shall discuss two aspects of VSV RNA replication in detail: (1) The synthesis of five monocistronic messages and leader RNAs from a single genomic RNA, and (2) the factors which enable the same polymerase complex to synthesize either mRNA ("transcription mode") or full-length, plus strand templates and genomic RNA ("replication mode").

### 4.1.2 Transcription of mRNAs

It is known that VSV transcription requires an RNA-N protein complex (RNP) as a template, as well as both the NS and L proteins. Both NS and L activities are required because these two polypeptides can be removed from the RNP template, destroying transcriptase activity, and added back to the complex, restoring RNA synthesis. NS protein alone will bind to RNP, but L protein will not bind in the absence of NS (*Mellon* and *Emerson* 1978). NS is a phosphoprotein and is present predominantly in two forms in virions: NS1 (the less phosphorylated) and NS2. NS isolated from infected cell cytoplasm is primarily NS1, as is the NS associated with purified nucleocapsids, but the distribution of these components depends upon the preparation (*Clinton* et al. 1978a; *Kingsford* and *Emerson* 1980). Purified NS1 has little activity in a reconstituted transcriptase assay, whereas NS2 is highly active. However, additional NS1 can further stimulate and activate a transcriptase complex already containing NS2 (*Kingsford* and *Emerson* 1980). Since more NS1 is bound to cores as the pH is raised (*Clinton* et al. 1978a) and more replication takes place at the expense of transcription with increased pH (*Fiszman* et al. 1974), the binding of NS2 may promote transcription and the binding of NS1 may promote replication. It has been shown that NS1 and NS2 are interconvertible in vitro affecting the rate of transcription (C.-H. Hsu et al. 1982) and that a phosphatase activity is present in host cell cytoplasm which might convert NS2 to NS1 in vivo (*Clinton* et al. 1979).

It is generally accepted that mRNA transcription begins with the attachment of the polymerase near the 3′ end of the negative strand. The complex traverses the genome, synthesizing first the leader RNA then the mRNAs in sequential order, N-NS-M-G-L. This was first shown by the fact that it is possible to map the gene order by UV inactivation of transcription (*Abraham* and *Banerjee* 1976; *Ball* and *White* 1976). However, considerable controversy has erupted over the details of this sequential synthesis: whether mRNAs are transcribed as a single polycistronic transcript, including internal poly(A) additions, which is later processed by nucleases and cap additions; or whether the nucleotide chain is reinitiated at the beginning of each gene (reviewed in *Ball* and *Wertz* 1981). For the sake of the following discussion we assume that termination and reinitiation is the case.

A map of the VSV genome, showing which sequences are present in transcripts, is shown in Fig. 13. The leader RNA, whose sequence (*McGeoch* and *Dolan* 1979) is shown in Fig. 14, appears to be composed of two functional domains. The 3′ terminal 18–20 nucleotides are complementary or nearly complementary to the corresponding 18–20 nucleotides at the 5′ end of the genome and may form the primary transcriptase/replicase recognition site. The remainder of the leader sequence contains additional signals which are important in the modulation of transcription and replication. Methylation protection studies have shown that NS protein, which has been proposed to be the initiator protein for transcription, binds to the minus strand between nucleotides 16 and 30 (*Keene* et al. 1981b). The sequences near the end of the leader may contain signals for termination and reinitiation during transcription. If RNA synthesis stops and the leader is released, then the replication complex enters the transcription mode. RNA synthesis reinitiates with a common sequence at the beginning of each gene and terminates with a sequence similar to the termination signal in the leader at the end of each gene. If the leader is not released, the replication mode ensues and a complete plus stranded copy of the entire genome is produced. Studies with defective-interfering particles (DIs) have shown that only those particles retaining the wild-type leader sequence at the exact 3′ terminus of their genomes are capable of transcription, although DIs which contain a sequence complementary to the 5′ terminus in place of the normal 3′ terminus are efficiently replicated (see Sect. 5).

Reading the genome 3′ to 5′ on the minus strand, there are four nucleotides between the leader sequence and the N gene which are not transcribed (*Keene* et al. 1980) (see Fig. 13). The transcriptase apparently initiates transcription of message for N protein (N mRNA) with the sequence AACAG (which is found at the 5′ end of all five mRNAs), transcribes the 1326 nucleotides of the N gene and arrives at a stretch of seven Us. Here the enzyme is thought to hesitate or stutter (in the transcription mode), adding a variable

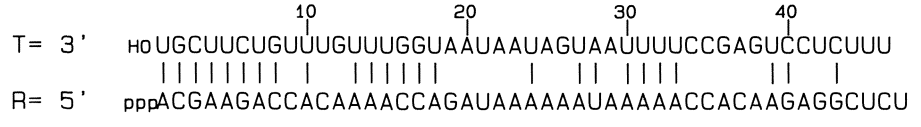


Fig. 14. The 3′ and 5′ terminal sequences of vesicular stomatis virus RNA. *T* and *R* are the sequences of the *boxed regions* labeled *T* and *R* in Fig. 13. Both sequences are shown for the genomic RNA of the Indiana strain. *Vertical lines* indicate complementary nucleotide pairs. Data are from *McGeoch* and *Dolan* (1979), *Schubert* et al. (1978), *Semler* et al. (1979), and *Colonno* and *Banerjee* (1978). In the New Jersey strain of VSV, the first 21 nucleotides of the 3′ and 5′ termini are exactly complementary (*Keene* et al. 1979; *Rowlands* 1979)

number of As at the end of the message. Kinetic analysis of in vitro transcription is in agreement with this hypothesis (*Ball* and *Wertz* 1981; *Iverson* and *Rose* 1981). Capping and methylation appear to be closely synchronized with transcription, and only 10%–15% of in vivo messages lack caps (*Bishop* and *Smith* 1977). Sequencing of intergenic regions of the genomic RNA has shown that the next two nucleotides, GA, are not found in mature transcripts, but that the NS gene mRNA begins with the following nucleotide. Similar events are thought to occur for each gene and the structure at the intergenic region is the same (Fig. 13). The sequence at the 5′ end of each gene (reading 3′ to 5′), $AUACU_7GA$ or $AUACU_7CA$, followed by the UUGUC sequence at the start of each gene, is homologous to the 5′ end of the leader with its eight pyrimidines followed by GA and two nucleotides later by the UUGUC beginning the N gene.

There is a second school of thought which advances the proposal that during RNA transcription there is only a single initiation event and processing is used to release the individual mRNAs from a precursor. Polycistronic transcripts containing the intergenic junctions, complete with poly(A) and the intergenic dinucleotide (*Herman* et al. 1980), and RNA molecules transcribed from the beginning of the leader RNA into the N gene (*Herman* and *Lazzarini* 1981a) have been found and could represent intermediates in such a processing pathway. Alternatively, they could be aberrant products produced by readthrough by the transcriptase.

The mRNAs for the five VSV genes are found in decreasing amounts from N mRNA, the most abundant, through NS, M, and G to L, the least abundant (*Villarreal* et al. 1976). Since this corresponds to the gene order from 3′ to 5′, attenuation at each transcription reinitiation site could account for the distribution of mRNAs found (*Iverson* and *Rose* 1981).

### 4.1.3 Replication of Genomic and Antigenomic RNA

It appears clear that replication of the rhabdovirus genome to produce both full-length plus polarity templates and minus strand genomes employs some or all of the RNA synthetase components involved in transcription, but direct demonstration of this has been difficult. It appears that the RNA synthetic machinery has two modes of action and exists in two functionally distinct forms, the transcription mode discussed above and the replication mode. The replication mode requires the enzyme to initiate at the extreme 3′ end of the genomic RNA and proceed to the 5′ end, faithfully transcribing a plus strand copy of the entire genome and ignoring all internal termination signals, stuttering signals, and possible reinitiation sites. This molecule in turn serves as template for synthesis of full-length minus strands. The primary recognition site for both plus and minus strand synthesis could be the nucleotide sequence at the 3′ terminus because in the case of VSV (New Jersey) the 21 nucleotides at the 3′ end of the minus strand are identical to the 21 nucleotides at the 3′ end of the plus strand, while in VSV (Indiana) 15 of the first 18 are identical (Fig. 14). Other sequences also appear to be involved, such as the binding site for NS protein between nucleotides 16 and 30. Furthermore, the affinity of the template for replication may be modulated by the sequences located up to 55 nucleotides from the 3′ end of the plus strand, since defective-interfering RNAs of VSV containing self-complementary termini 55 nucleotides long will outcompete defective-interfering RNAs containing shorter sequences (*Huang* et al. 1980; see also Section 5).

In vivo, replication begins about 45 min after the onset of infection and, unlike

primary transcription, requires continued protein synthesis. Two viral components have been proposed to be controlling elements for replication: the M protein, which has been shown to inhibit transcription in vitro (*Clinton* et al. 1978b; *Carroll* and *Wagner* 1979; *Wilson* and *Lenard* 1981), and the N protein (*Blumberg* et al. 1981).

Two possible ways of shifting the equilibrium can be suggested. Replication, the direct copying of an RNA strand by a replicase, may occur with the nucleoprotein structure relatively more relaxed than for transcription. As suggested by *Ball* and *Wertz* (1981), chattering to produce poly(A) opposite $U_7$ would involve repetitive breakage of hydrogen bonds and might require a tighter configuration outside the actual polynucleotide addition site to prevent the polymerase from falling off the template. M protein, adapted to interact with RNP at later stages to form the characteristic bullet-shaped core, may relax the structure upon interaction with N protein. M is phosphorylated at tyrosine residues and the degree of phosphorylation may determine the cellular location and regulatory activity of this protein, whether it acts to regulate transcription/replication or acts in morphogenesis and budding. Initially upon infection the M protein from the infecting virion might remain at the surface of the cell, with the tight complex of RNP, NS, and L performing early transcription. Translation of both primary and secondary transcripts increases local M concentration, the system relaxes, and replication is favored over transcription. The idea that the switch from the transcription mode to the replication mode involves a configurational rearrangement of the entire RNA synthetic complex is supported by the facts that replication complexes can be resolved from transcription complexes in Renografin gradients and that some replication complexes appear as circular coiled structures in electron micrographs (*Naeve* and *Summers* 1980).

A second hypothesis has been proposed (*Blumberg* et al. 1981) which involves N protein and the function of leader RNA sequences. Leader RNAs are found in vivo in two forms: plus strand leaders (47 nucleotides complementary to the extreme 3′ terminus of the genomic RNA) and, in much smaller amounts, minus strand leaders (46 nucleotides complementary to the 3′ end of the antigenome) (*Leppert* et al. 1979). This theory suggests that near the end of the sequence of either leader there is a choice point and a termination signal. If the concentration of N protein is low, termination of plus strand leader ensues, the leader is removed, and transcription begins. Similarly, on the antigenome the minus strand leader terminates, but in the absence of other signals for transcription, replication aborts. However, under conditions of continued protein synthesis, ensuring an adequate supply of N protein, the replicase passes through the termination at the end of the leader and proceeds to copy the entire molecule, either genome or antigenome. One problem with this model is that in vivo the majority of leader RNAs are found encapsidated with N protein (*Blumberg* and *Kolakovsky* 1981), but it does appear clear that N protein is required in some way for replication. In vitro replication, as opposed to transcription, can be obtained only in systems where translation is coupled to RNA synthesis (*Hill* et al. 1981). In these systems adequate supplies of N protein are essential, since replication ceases if antibody to N protein is added to the incubation mixture, whereas antibodies to other viral polypeptides are without effect (*D.F. Summers,* personal communication).

Neither of these mechanisms can successfully encompass all the known facts about replication control; indeed, a more complicated scenario involving not only N and M, but also configurational variants of L and variously phosphorylated forms of NS may be involved, as discussed above. Furthermore, host components are probably necessary for

replication, since host range mutants have been isolated which map in the L cistron (*Szilágyi* et al. 1977). In addition, some mechanism must exist to exert temporal control over the synthesis of full-length plus strand templates and minus strand genomes because, early in infection, 40% of the full-length RNA molecules synthesized are of plus polarity, while at later times most of the 42-S RNA produced is genomic (i.e., of negative polarity) (*Simonsen* et al. 1979).

### 4.1.4 Translation Strategy

The monocistronic messenger RNAs from either rhabdovirus-infected cells or from in vitro transcription by disrupted virions can be translated in a variety of cell-free protein-synthesizing systems to yield products chemically and antigenically indistinguishable from native N, NS, L, and M polypeptides. Translation of G mRNA yields a precursor form of the molecule, as discussed below.

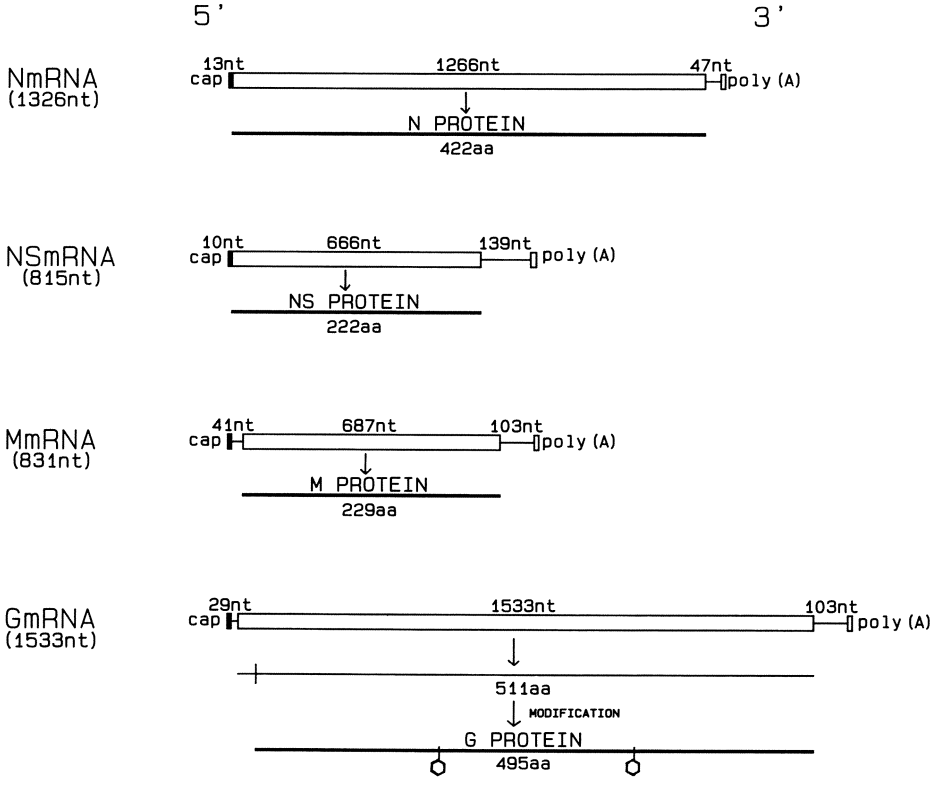


Fig. 15. Translation of VSV mRNAs for the N, NS, M, and G genes. Complete nucleotide sequences for these four mRNAs have been reported in *Gallione* et al. (1981) and *Rose* and *Gallione* (1981). The *solid box* at the 5′ termini indicates the common pentanucleotide AACAG, and the total number of nucleotides preceding the AUG initiation codon is indicated. The *open box* at the 3′ termini represents the common sequence UAUG and the total number of nucleotides untranslated is indicated. Modification of the G protein includes cleavage of the 16 amino acid signal sequence, glycosylation at the two sites shown ( ⏢ ), and esterification with fatty acids at sites which have not been precisely localized

The complete sequences of the mRNA for N, NS, M, and G have been determined (*Gallione* et al. 1981; *Rose* and *Gallione* 1981). The organization of each message and the protein products are shown in Fig. 15. The mRNAs are capped and have a 5′ untranslated region 10–41 nucleotides long, depending on the RNA. The first available AUG codon is used for initiation of translation; it is followed in each case by a single open reading frame for the entire polypeptide which ends with a single termination codon (UAG or UAA) (*Gallione* et al. 1981; *Rose* and *Gallione* 1981). The 3′ untranslated regions of the messages vary from 47 nucleotides in N message to 139 nucleotides in NS.

Three of the proteins are modified posttranslationally. Both M and NS are phosphorylated as noted above. The synthesis and processing of the glycoprotein G has been extensively studied as a model system for other integral membrane proteins, including secretory proteins (reviewed in *Lodish* et al. 1981). The message encodes a polypeptide which begins with a highly hydrophobic 16 amino acid signal sequence which directs insertion through the membrane of the rough endoplasmic reticulum and is cleaved from the protein by signalase in the lumen (*Chatis* and *Morrison* 1979). The protein is glycosylated by transfer of a high mannose oligosaccharide from a dolichol intermediate. The core glycosylated G polypeptide migrates to the Golgi apparatus where further carbohydrate modifications and the covalent attachment of lipids takes place (*H.P. Ghosh* et al. 1981) in a manner analogous to that described for the alphaviruses (Sect. 2.3.1). Although various strains and isolates of VSV show extensive conservation of sequence at the N-terminus of the G protein, there is little or no homology in the signal sequence (*H.P. Ghosh* et al. 1981).

In cells infected with VSV, host protein and RNA synthesis are inhibited (*Wagner* 1975). Neither RNA replication nor production of mature virions is essential for inhibition of host protein synthesis, which apparently is primarily due to competition between cellular and viral transcripts for a limiting number of ribosomes (*Lodish* and *Porter* 1980c, 1981).

### 4.1.5 Lyssaviruses

The second major rhabdovirus group, the lyssaviruses, includes the type virus, rabies virus, and numerous other isolates including several fish viruses (*Matthews* 1982). Molecular characterization of rabies has lagged behind VSV due to its pathogenicity, but several recent articles indicate that its structure and replication are virtually indistinguishable from that of the vesiculoviruses. Lyssavirus particles contain five polypeptides: L, G, N, M1, and M2. The glycosylation of the G protein is strain-dependent and some strains possess two forms, G1 and G2 (*Dietzschold* 1981). L, G, and N correspond to their counterparts in VSV (*Coslett* et al. 1980). M1 and M2 were originally thought to be two forms of matrix protein. However, M2 (25 K) appears to be the true matrix protein and is present in >1000 copies per virion. M1 (39.5 K) is present in about 400 copies, can be found associated with nucleoproteins, exists in two differentially phosphorylated forms (*Cox* et al. 1982), and appears to correspond to the NS polypeptide of VSV.

Five species of mRNA of opposite polarity to virion RNA have been isolated from rabies-infected cells and translated in vitro to produce the five known rabies proteins (*Pennica* et al. 1980). In vivo experiments show that virus-specific proteins are independently translated from these monocistronic messages (*Coslett* et al. 1980). As for vesiculoviruses, there appears to be a polarity of transcription, since UV transcription

mapping gives a gene order of 3′-N-M1-M2-L-5′ (*Flamand* and *Delagneau* 1978), the same relative order found for VSV. The location of G was not determined in these studies, but it is likely that it will be found between M2 and L as in vesiculoviruses.

It is clear from the similarities in their structure and replication that vesiculoviruses and lyssaviruses are closely related. The nucleoprotein antigens retain some cross-reactivity but the external glycoproteins have diverged extensively during viral evolution and appear unrelated by simple inspection. However, using computer programs to search for homologies in amino acid sequences of the G proteins of rabies and VSV, *Rose* et al. (1982) found clear evidence for the common ancestry of these groups.

## 4.2 Paramyxoviruses

The Paramyxoviridae are a family of negative strand RNA animal viruses containing a single molecule of RNA, 16–18 kb in size, as their genome. The RNA is present in a helical nucleocapsid containing primarily a single protein species (NP). The capsid is enveloped by a cell-derived lipid bilayer containing two glycoproteins (HN and F) and an internal nonglycosylated polypeptide (M) (*Choppin* and *Compans* 1975). Three genera have been distinguished on the basis of morphological differences and the activities of the HN protein: the paramyxoviruses, the morbilliviruses, and the pneumoviruses. Paramyxovirus HN protein has both hemagglutinin and neuraminidase activities, morbillivirus HN has hemagglutinin activity only, and the comparable polypeptide in pneumoviruses has neither activity (*Choppin* and *Compans* 1975). Paramyxovirus infection shares many common characteristics with the rhabdoviruses, but much less is known about the molecular biology of paramyxoviruses because they replicate poorly in cell culture.

As is the case for all negative strand viruses, the paramyxovirus nucleocapsid, but not the naked RNA, is infectious. Primary transcription takes place to form at least six known species of capped and polyadenylated plus sense mRNAs even in the presence of protein and DNA synthesis inhibitors. Two size classes of mRNA are made: The 35-S mRNA which is translated to give the L protein, and the 18-S mRNA. For Newcastle disease virus (NDV) it has been shown that the 18-S mRNA can be fractionated by preparative gel electrophoresis into five species, each of which encodes a unique viral protein (*Collins* et al. 1981). Replication of the genome (from a full-length antigenome plus strand template) and secondary transcription both require protein synthesis. Detergent-disrupted virions can be used as an in vitro system to study transcription and in recent experiments it has been possible to transcribe the entire genome of NDV in vitro (*Miller* and *Stone* 1981). The active transcription complex is the nucleocapsid which contains one molecule of RNA; approximately 2600 copies of the nucleocapsid protein NP (60 K); about 300 copies of a phosphorylated minor species, P or NAP (nucleocapsid associated protein) (mol. wt. 53 K); and as few as 30 copies of L, the putative polymerase.

Although little is known about the synthesis of the plus strand messages and their subsequent capping and polyadenylation, it has been shown that transcription initiates at a single promoter in vivo and that the order of the genes can be determined from UV transcriptional mapping. Based on their UV irradiation data, *Glazier* et al. (1977) proposed a gene order of 3′-NP-$F_o$-M-P-HN-L-5′ for Sendai virus. More recently a study of NDV gave the order 3′-NP-P-($F_o$,M)-HN-L-5′ (*Collins* et al. 1980), which for reasons discussed

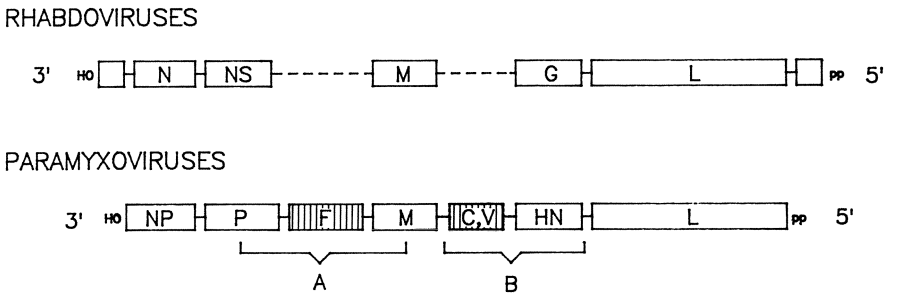


Fig. 16. Genome organization of rhabdoviruses and paramyxoviruses. A schematic representation of the VSV and Newcastle disease virus genomes is shown aligned by functional equivalence of the gene products. *Shaded boxes* indicate paramyxovirus genes which have no VSV equivalent. Both gene orders were determined by UV transcriptional mapping (*Collins* et al. 1980; *Ball* and *White* 1976). *A* and *B* represent regions of UV transcriptional anomalies discussed in the text. The functions of the gene products are discussed in the text

below probably represents the correct placement. In Fig. 16 the genome of NDV is compared with that of the rhabdovirus VSV, and the similarities in terms of the location of functionally analogous polypeptides is striking. From the 3′ end, the first gene encodes the nucleocapsid protein, in both cases the most abundant virus-specific polypeptide in the infected cell. Next is the NS protein in VSV or the P protein of paramyxoviruses. As is the case for NS, the P protein exists in multiple phosphorylated forms (*Smith* and *Hightower* 1981) and plays some role, as yet poorly defined, in regulation of RNA synthesis (*Chinchar* and *Portner* 1981a, b; *Samson* et al. 1981). P protein is the second most abundant virus polypeptide in infected cells, but not in virions, and seems to have a necessary transient association with nucleocapsids (*Portner* and *Kingsbury* 1976). *Collins* et al. (1980) noted that the UV transcription target size covering P, $F_0$, and M (the region marked A in Fig. 16) is too short to encode the apparent polypeptide molecular weights of these three moieties; however, by analogy with the NS protein of VSV (Sect. 4.1.1), it is possible that the molecular weight of P is less than the 53 K estimated from gels.

The next gene of paramyxoviruses, $F_0$, which controls fusion and hemolysis, has no counterpart in VSV. The M or matrix protein follows. In both groups M is crucial for assembly and budding of mature virions, and can be phosphorylated in vivo and in vitro (*Yoshida* et al. 1979). The UV target size of the region marked B in Fig. 16 is too large and for this reason the 22-K nonstructural protein, called C in NDV and V in Sendai, was tentatively located between M and HN (*Etkind* et al. 1980; *Peluso* et al. 1977). However, it is now known that C is translated from P mRNA in a second reading frame (*Kolakovsky,* personal communication) and the anomaly is unresolved.

HN, the surface glycoprotein found in spikes outside the lipid bilayer, can be considered equivalent to the G protein of VSV, although the HN protein appears functionally more complex than G protein. The HN glycoprotein of the paramyxovirus subgroup performs two functions, both of which are essential to virus infection: it is both the hemagglutinin, which binds to erythrocytes and presumably to cellular receptors, and the neuraminidase, which removes sialic acid residues from the complex carbohydrate chains of both cellular and viral glycoproteins. These two activities can be shown to reside in separable domains of the protein through the use of mutants and monoclonal

antibodies (*Portner* 1981; *Yewdell* and *Gerhard* 1981; *Merz* et al. 1981). In addition, this multifunctional polypeptide appears to be required together with the F protein to produce cell fusion (*Seto* et al. 1974; *Ozawa* et al. 1979; *Huang* et al. 1980) and interacts with the M protein in virion assembly (*Yoshida* et al. 1979). Incorporation of such diverse functions in a single polypeptide of 74 K illustrates one approach to economizing on limited genome information. As noted above, the membrane glycoprotein of morbilliviruses corresponding to HN has only the hemagglutinating activity and that of pneumoviruses has neither activity (*Compans* and *Klenk* 1979).

Finally, there is the L gene of both virus groups, a large region (up to 35% of the genome) encoding a very large (200 K) and probably multifunctional protein. The L gene of both VSV and NDV has been implicated in RNA synthesis, as well as in polyadenylation and capping (*Choppin* and *Compans* 1975).

On the basis of the striking similarities in the genome organization, we suggest that rhadboviruses and paramyxoviruses derive from a common ancestral negative strand virus. Indeed, a rhabdovirus genome could be constructed by deletion from a paramyxovirus genome. In this regard it is of interest to compare the nucleotide sequence at the exact 3′ and 5′ termini of the genomes, shown in Fig. 17. Note that seven of the first nine nucleotides are identical between VSV(NJ) and Sendai and that the next seven to ten nucleotides of each represent a polypyrimidine tract. At the 5′ terminus, eight of ten nucleotides are the same between VSV (NJ) and Sendai. Furthermore, five of the Sendai genes terminate in the common sequence 3′-AUUC ($U_5$)–5′ which is very similar to the consensus sequence 3′ AUAC ($U_7$)–5′ terminating the VSV genes (*Gupta* and *Kingsbury*, 1982), confirming the recent evolutionary divergence of these two viruses. We also note that there is no demonstrable homology between these sequences and the comparable sequences at the 3′ ends of other negative strand genomes such as fowl plague virus, an orthomyxovirus (*Robertson* 1979); LaCrosse virus, a bunyavirus (*Obijeski* et al. 1980); or Pichinde, an arenavirus (*Auperin* et al. 1982). Finally, recent results suggest that there is a 3′ leader sequence in Sendai virus (*Leppert* et al. 1979; *Amesse* and *Kingsbury* 1982), and the paramyxoviruses may possess both plus and minus strand leaders analogous to those of the rhabdoviruses. When amino acid sequences of the paramyxovirus proteins become available, either direct or deduced from nucleotide sequencing, it will be very interesting to compare them to the sequences of the functionally equivalent VSV proteins.

Little is known about replication of the paramyxovirus genome. Full-length (50-S) RNA molecules of plus polarity can be isolated from infected cells and these presumably serve as replicative templates for the virion RNA. As is the case for rhabdoviruses, these RNAs are found exclusively in nucleocapsids, rather than as free nucleic acid (*Kingsbury* 1977). Some of these plus strand nucleocapsids are found in mature virions as evidenced

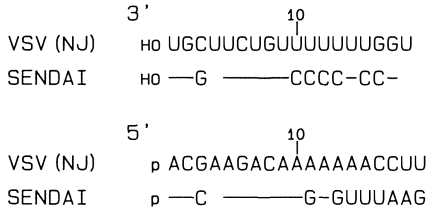


Fig. 17. The 3′ and 5′ termini of Sendai and VSV (New Jersey) RNAs, with sequences aligned for maximum homology. Data are from *Rowlands* (1979) and *Lazzarini* et al. (1981a)

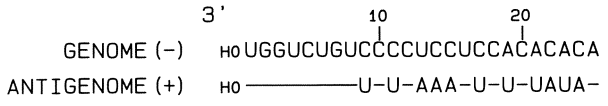


Fig. 18. 3′ Terminal sequences of Sendai virus RNAs. A *horizontal line* in the antigenome sequence indicates that the nucleotide is the same as in the genome sequence above. Note that identity of the 3′ termini of the plus and minus strands is equivalent to self-complementarity of the 3′ and 5′ termini of one RNA molecule. Data are from *Lazzarini* et al. (1981)

by limited self-annealing of RNAs isolated from the virus. As with other negative strand viruses, the 3′ and 5′ ends of the genome show partial complementarity (*Lazzarini* et al. 1981) (Fig. 18), suggesting that the replicase recognition sites for both plus and minus strand synthesis are related and that both strands are made by the same polymerase (see also Sect. 4.1). Replication, unlike primary transcription, requires continued protein synthesis as is the case for VSV and the mechanisms involved in regulation of replication may be similar.

Posttranslational modifications of the structural proteins play an important role in paramyxovirus maturation. The fusion protein is translated as a precursor, $F_o$, which is glycosylated and transported to the cell surface. In permissive cells, this inactive form is cleaved to $F_1$ and $F_2$ which remain covalently linked by disulfide bonds (*Hardwick* and *Bussell* 1979). The orientation of $F_2$ and $F_1$ in $F_0$ have been shown to be $NH_2$-$F_2$-$F_1$-COOH (*Samson* et al. 1980). $F_1$ is known to be the active form of this protein necessary for infection and fusion functions (*Scheid* and *Choppin* 1977; *Samson* et al. 1980). In nonpermissive cell lines, virions are released carrying the inactive $F_0$ form which can be activated by exogeneously supplied trypsin; this observation was the basis for the isolation of a series of Sendai mutants which could be activated by chymotrypsin or elastase rather than trypsin (*Scheid* and *Choppin* 1976). Protein HN is also produced as a precursor $HN_0$ (82 K) which is processed to HN (74 K) with the loss of an amino terminal peptide of molecular weight 8 K (*Nagai* et al. 1976; *Garten* et al. 1980). The cleavage of $F_0$, and perhaps that of $HN_0$ as well, may be catalyzed by the same Golgi protease which is responsible for processing a number of virus glycoproteins. The degree of cleavage of $HN_0$ correlates with virulence. In some cases, such as nonpathogenic strains of NDV, the HN protein is found as the inactive precursor $HN_0$. It is of note that the uncleaved forms of both $F_0$ and $HN_0$ can be utilized normally in the assembly of particles but the resulting particles are noninfectious (*Nagai* et al. 1976). Although both $F_0$ and $HN_0$ are integral membrane proteins, there is as yet no evidence for a cleaved signal peptide at the N-terminus of either one.

In the presence of the drug tunicamycin, unglycosylated forms of F and HN are produced. Unglycosylated $F_0$ is transported to the cell surface, inserted into the plasma membrane, and cleaved to form unglycosylated $F_1$. Unglycosylated HN is also transported to the surface normally, but assumes a different conformation in the plasma membrane from glycosylated HN (*Morrison* et al. 1981).

Paramyxovirus transcription, translation, and replication appear to be wholly cytoplasmic and to be independent of nuclear functions. These viruses replicate in UV irradiated cells, in the presence of nucleoside analogs which block DNA synthesis and in the presence of actinomycin D (*Choppin* and *Compans* 1975; *Kingsbury* 1977). However, although all the specific polypeptides are synthesized in enucleated cells, albeit in

reduced quantities (*Pennington* and *Pringle* 1978), no infectious virions are produced. This is in contrast to VSV replication, which occurs normally in enucleated cells, or influenza replication, in which neither infectious virus nor virus-specific antigens can be detected in the absence of the nucleus (*Kelly* et al. 1974). The exact nature of the requirement for the nucleus for paramyxoviruses maturation is not known but may reflect the fact that enucleation often removes much of the perinuclear membranous structures (such as the Golgi) which are necessary for final maturation of infectious particles (*Pennington* and *Pringle* 1978).

## 4.3 Orthomyxoviruses

The orthomyxovirus group consists of three antigenically separate virus groups: influenza A, influenza B and influenza C. Influenza A encompasses a large number of strains which infect birds, humans, and other mammals, while influenza B is primarily of human origin (*Matthews* 1982). Little is known about the distribution of influenza C, but it is only weakly pathogenic for man. Influenza A viruses have been the object of intensive study for many years because they cause relatively mild seasonal epidemics punctuated every 10–20 years by global pandemics. The antigens of both influenza A and B undergo gradual changes (antigenic drift), but only influenza A viruses exhibit the abrupt changes (or antigenic shift) that lead to the appearance of new strains with entirely unrelated surface markers, which are the cause of the periodic pandemics. Such new strains may arise by recombination between human and animal strains. Large amounts of effort have been devoted to characterizing influenza viruses in order to determine the origin of the new pandemic strains and to devise strategies for effective vaccine production (*Nayak* 1977). Influenza viruses are generally named and characterized by the location and year of the isolate (Hong Kong, Victoria, etc.), the animal of origin, and for human strains by the major serotypes of the hemagglutinin (H0, H1, etc.) and neuraminidase (N1, N2, N3, etc.); for example, A/PR/8/34 (H1N1) is a human influenza A isolated in Puerto Rico in 1934 and has the H1 hemagglutinin and the N1 neuraminidase.

The genome of influenza A consists of eight segments of negative strand RNA, with a total complexity of 13.5 kb (molecular weight $4.5 \times 10^6$), each encapsidated in a separate helical nucleocapsid containing primarily NP protein and some tightly bound matrix protein, as well as catalytic amounts of the transcriptase/replicase components P1, P2, and P3 (*Rees* and *Dimmock* 1981). The segmented nucleocapsids are surrounded by a lipid bilayer of cellular origin which contains the two integral membrane glycoproteins HA (the hemagglutinin) and NA (the neuraminidase) in the form of external "spikes" and a single abundant species of M (matrix protein) lining its inner surface (*Nayak* 1977). Influenza B virions are of a similar architecture (*Racaniello* and *Palese* 1979), whereas influenza C has a somewhat different construction. The number of RNA segments in influenza C may be as many as nine (*Petri* et al. 1979b), and the polypeptide composition is different in that the virions lack neuraminidase activity and possess only a single species of glycoprotein, that corresponding to HA (*Herrler* et al. 1981; *Meier-Ewert* et al. 1981). Unless otherwise stated, the remainder of this section will deal with influenza A only.

### 4.3.1 Overview of Influenza Replication

The general outline of influenza replication resembles that of other negative strand viruses. Nucleocapsids, but not deproteinized genomic RNA, can initiate infection, indicating that virion-associated polymerases are necessary for primary transcription to produce mRNAs complementary to the genome segments. Early studies measured the synthesis of total cRNA, but recent reports indicate that there are two populations of cRNA. The first is made up of mRNAs which are not complete transcripts of the viral RNA, are capped at the 5′ end and polyadenylated at the 3′ end, and are produced by both primary and secondary transcription. The second population is now called A-cRNAs or template RNAs. These are complete transcripts which lack poly(A) tracts and terminate at the 5′ end with a pppA, and which serve as templates for genomic RNA replication (*Smith* and *Hay* 1981; *Hay* et al. 1982). The mechanisms of transcription and replication will be discussed in greater detail in Sect. 4.3.2 and 4.3.3 respectively.

With the exception of the three smallest RNA segments, which encode two polypeptides, each RNA represents a single influenza gene, encoding a single virus-specific polypeptide. The coding assignments are summarized in Table 4. These have been deter-

Table 4. Genome organization of influenza viruses

| RNA segment[a] | Size[b] (nucleotides) | Protein encoded[c] | Linkage group[a] | Function[d] |
|---|---|---|---|---|
| 1 | 2341 | P3 (Ptra) PB2 | I | All RNA synthesis Cap associated |
| 2 | 2341 | P1 (Pol 1) PB1 | III | Initiation of transcription cRNA synthesis |
| 3 | 2233 | P2 (Pol 3) PA | II | vRNA synthesis |
| 4 | 1756 | HA | VI | Hemagglutinin; infection and absorption |
| 5 | 1565 | NP | V | Nucleoprotein; vRNA synthesis |
| 6 | 1413 | NA | IV | Neuraminidase |
| 7 | 1027 | M1, M2 | VII | Assembly for M1 |
| 8 | 890 | NS1, NS2 | VII | vRNA synthesis for NS1 |

[a] Numbering of RNA segments and linkage group designations are for influenza A-WSN strain according to *Palese* (1977). Assignment of polypeptides to RNA segments includes the data of *Inglis* et al. (1977) and *Palese* (1977); [b] Size of segments 1 and 3–8 was determined from complete nucleotide sequencing in the following references: segments 1 and 3 from Fields and Winter (1982), segment 2 from *Winter* and *Fields* (1982), segment 4 from *Porter* et al. (1979) and *Both* and *Sleigh* (1980), segment 5 from *Van Rompuy* et al. (1981) and *Winter* and *Fields* (1981), segment 6 from *Fields* et al. (1981), segment 7 from *Lamb* and *Lai* (1981), and segment 8 from *Lamb* and *Lai* (1980) and *Porter* et al. (1980). [c] P1, P2, and P3 are the designations of *Palese* (1977). A second set of names, in parentheses, was proposed by *Scholtissek* (1979). The recent finding that P1, P2, and P3 can be well separated by two-dimensional electrophoresis into one acidic (P2) and two basic (P1 + P3) proteins (*Horisberger* 1980) suggests the designations PA and PB1 and PB2, where PB2 is the larger of the two basic proteins; [d] Data on functions is a composite of the following references: *Mowshowitz,* 1981; *Shimizu* et al., 1981; *Mahy* et al., 1981; *Scholtissek,* 1979; *Wolstenholme* et al., 1980. vRNA, negative strand genomic RNA; cRNA, complementary (plus strand) RNA, including both mRNA and antigenomic RNA.

mined by a combination of methods including analyses of recombinant viruses (either between naturally occurring strains or temperature sensitive mutants) or by the somewhat different technique of hybrid-arrested in vitro translation. The first method makes use of the fact that for many strains of influenza all eight genomic RNA segments can be distinguished by their electrophoretic mobility in acrylamide gels. When two strains of virus infect a given cell, recombinant viruses are formed with high frequency by reassortment of the RNA segments (*Palese* 1977; reviewed in *Scholtissek* 1979); by analysis of a number of recombinants between two parental viruses, it is often possible to correlate a function with the presence of a particular RNA segment. This type of analysis has also employed catalogs of temperature-sensitive mutants of influenza to assign the functions to the recombination groups (*Shimizu* et al. 1981; *Mahy* et al. 1981).

The method of hybrid-arrested translation complements the genetic results. Mixtures of mRNAs isolated from polyribosomes of infected cells are purified by selection on oligo(dT) cellulose columns. Individual genomic RNA segments (called vRNAs), purified by gel electrophoresis, are hybridized to the mRNA mixture, which is then translated in a cell-free system. By examining the polypeptides produced, and showing that the fragment hybridized is no longer available for translation, the various influenza gene products can be assigned to the vRNA of a particular electrophoretic mobility (*Inglis* et al. 1977).

The fourth column in Table 4 is headed linkage (or reassortment) group rather than complementation group, since intracistronic or intrasegmental complementation is a common phenomenon in influenza. Intrasegmental complementation has been demonstrated for all the polymerase genes, P3 (*Heller* and *Scholtissek* 1980), P1, and P2 (*Shimizu* et al. 1981), which is perhaps a reflection of their multifunctional nature, as well as for the segments encoding neuraminidase and nucleoprotein (*Thierry* et al. 1980). As expected, intrasegmental complementation has also been found between mutants located on segment 8, which encodes two polypeptides (*Wolstenholme* et al. 1980).

There appears to be no permanent or transient linear association of the genomic RNAs, and initiation of transcription is completely independent for each vRNA segment. Attempts to order the genes by UV transcriptional mapping have shown no polarity of target sizes; the UV targets of the mRNAs are proportional to the size of the transcripts (*Abraham* 1979). Similarly, UV inactivation studies have shown that the replication of the genomic segments and their templates is also independent (*Smith* and *Hay* 1982). However, despite the fact that all eight segments are not produced in equimolar amounts in infected cells, they are equally represented in the RNA population isolated from virions, and the mechanism of this selection is not understood.

### 4.3.2 Transcription of Influenza mRNAs

It has been known for many years that influenza virus requires a functional nucleus for replication. Early experiments had shown that neither mature virions nor virus-specific antigens were produced in enucleated cells, under conditions which were permissive for several other virus groups (*Kelly* et al. 1974). The nucleus was essential only early in infection, and enucleation 4 h after infection allowed a low level of production of infectious virus (*Pennington* and *Pringle* 1978). Similarly, UV irradiation or actinomycin D treatment would inhibit replication if administered early in the infection cycle of influenza A and B (*Nayak* 1977), as well as influenza C (*Petri* et al. 1979a). One essential activity was

apparently the cellular DNA-dependent RNA polymerase II since influenza replication was inhibited by α-amanitin (*Rott* and *Scholtissek* 1970; *Mahy* et al. 1972), but virus replication was insensitive to the presence of α-amanitin in host cell lines resistant to the drug (*Lamb* and *Choppin* 1977). Recently, using improved subcellular fractionation techniques and more sensitive probes for newly synthesized RNA species, it has been possible to show that all influenza transcription, both primary and secondary, takes place in the nucleus (*Herz* et al. 1981) using newly synthesized cellular messages as primers (see below). Newly synthesized P protein, NP protein, M protein, and NS1 protein are all transported to the nucleus from their cytoplasmic sites of synthesis early in infection (*Flawith* and *Dimmock* 1979; *Briedis* et al. 1981a), presumably to form more transcription complexes. Both replication and transcription of RNA take place early in the influenza growth cycle; for example, the rate of RNA synthesis, whether vRNA, mRNA, or antigenomic template RNA, reaches a maximum at 2–3 h after infection of mammalian cells by the WSN strain of influenza (*Mowshowitz* 1981; *Smith* and *Hay* 1982) or of chick embryo fibroblasts by fowl plague virus (*Scholtissek* and *Rott* 1970; *Hay* et al. 1977). This explains why inhibitors of RNA polymerase II, enucleation, UV, and actinomycin D only prevent virus replication if they are administered during the first 1.5–2.5 h after infection.

The strategy of RNA transcription by influenza viruses has several aspects which set it apart from the other negative strand viruses. mRNAs isolated from infected cells contain at the 5′ end a cap I structure of the form $m^7GpppA_mpX$ which is followed by 9–15 nucleotides that are heterogeneous and not found in the vRNA sequence, suggesting that host RNAs donate these capped structures to the viral messages (*Krug* et al. 1979; *Dhar* et al. 1980; *Caton* and *Robertson* 1980). Experiments on in vitro transcription of mRNAs by disrupted virions have confirmed the use of host-derived primers (*Plotch* et al. 1979; *Robertson* et al. 1980) and elucidated a number of the details of influenza mRNA transcription. The first step is the cleavage of a host-derived message by a virion-encoded endonuclease to yield an oligomeric primer 10–14 nucleotides in length which has a capped 5′ terminus and a 3′ terminal hydroxyl group. The nuclease which generates the primer has been shown to cleave only molecules containing a methylated cap, to cleave preferentially at locations 10–13 nucleotides from the cap, and to cleave preferentially following purine residues (*Plotch* et al. 1981). The P3 polypeptide (the more rapidly migrating of the two basic polymerase proteins) recognizes the cap I structure and appears to remain associated with it, even during elongation of the message (*Ulmanen* et al. 1981a). It can be cross-linked to the cap and is probably involved in some way with primer generation. The common sequence at the 3′ termini of influenza vRNAs is 3′-UCG . . ., and primers terminating in A are strongly preferred. Transcription is initiated by attaching a G to the primer, complementary to the penultimate nucleotide, C; primers ending in G can also be used to initiate transcription with a C residue, corresponding to the third nucleotide of the virion RNA (*Plotch* et al. 1981). Cross-linking studies have shown that the P1 polymerase can be cross-linked to this first G residue attached to the primer, suggesting that P1 is responsible for initiation (*Ulmanen* et al. 1981a). The fact that the capped oligomers which can serve as primers are predominately 10–13 bases long, while shorter capped structures are not utilized, may reflect a requirement for binding sites for both the P3 and P1 proteins for successful priming (*Plotch* et al. 1981).

After initiation, the transcriptase copies the vRNA exactly up to a tract of 5–7 uridine residues which is located approximately 20 nucleotides from the 5′ end of the segment;

here the enzyme complex appears to stutter, adding variable amounts of poly(A) to the mRNA, and transcription terminates (*Robertson* et al. 1981). The eight primary transcripts corresponding to the eight vRNA segments are used as messengers. In addition two other transcripts are produced from the primary transcripts of segments 7 and 8 by splicing. Since the splice sites are similar to those found in cellular transcripts, cellular enzymes in the nucleus are probably responsible for this processing (*Lamb* and *Lai* 1980; *Lamb* et al. 1981).

Quantification of mRNAs in infected cells has indicated that little temporal control exists, i.e., with the exception of the mRNA for NS2, the relative amounts of the mRNAs corresponding to the eight segments are similar throughout the time course of infection (*Tekamp* and *Penhoet* 1980; *Pons* 1981). In this study, normalizing to the number of copies of segment 4 message, only one-third as many mRNAs are transcribed from each of the three largest segments, the three middle segment mRNAs are present in roughly equal amounts, and mRNAs corresponding to segments 7 and 8 are present in two- to threefold excess. These figures are tantalizingly close to the inverse of the molecular weights of the fragments and would imply that all segments initiate transcription with equal efficiency but that the time required for elongation determines the number of copies produced. However, the relative concentrations of individual vRNA and mRNA segments depend upon the host cells used (*Smith* and *Hay* 1982), and it has been suggested that the relative proportions of genome RNAs available as templates may modulate mRNA synthesis.

### 4.3.3 Replication of the Virion RNA

Much less is known about the details of the replication of the viral RNAs, but in contrast to the transcriptional processes, orthomyxovirus RNA replication appears similar to that of the other negative strand viruses. All eight vRNA segments share common sequences at their 3′ ends, which may represent a replicase binding site (Fig. 19). In addition, the vRNA segments possess partially complementary sequences at the 5′ ends. Thus the 3′ terminal sequences of the template cRNAs are homologous to the 3′ sequences of the vRNAs and could be recognized by the same replicase enzyme. The lengths of these homologous sequences vary from 12 to 16 nucleotides for the different segments. In the case of the rhabdoviruses, (Sect. 4.1) modulation of RNA replication and transcription involves more than the terminal complementary sequences. For influenza the situation is unclear. All eight segments possess the same mismatches between the 3′ terminal sequence of plus and minus strands at positions 3, 5, and 8 which could be responsible for the production of more minus strands than plus strands. Segments 1, 2, and 3 have an additional mismatch at position 4 (Fig. 19) and are produced in smaller amounts in infected cells. Thus the mismatches at positions 3, 4, 5, and 8 could modulate replication.

The eight segments appear to replicate independently, and vRNA segments 5 and 8 are preferentially synthesized early in infection (*Smith* and *Hay* 1982). The cellular site of replication has not been unambiguously determined, but it is quite possible that it occurs in the nucleus as is the case for transcription (*Herz* et al. 1981).

### 4.3.4 Translation

As noted earlier, the polypeptides encoded by the eight segments of the influenza genome have been assigned to their respective segments by genetic and biochemical

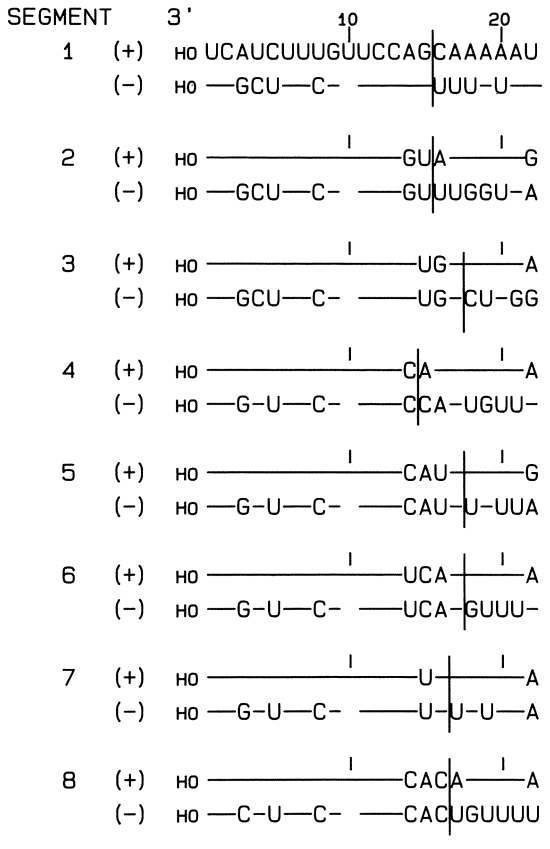


Fig. 19. 3′ Termini of influenza RNA segments. Sequences at the 3′ termini of both genomic (-) and antigenomic (+) RNAs of the eight segments of fowl plague viruses are shown. A *horizontal line* indicates that the nucleotide is identical to that in the plus strand of segment 1. The *vertical lines* indicate the point at which the sequences of a given segment diverge from one another. Nucleotides 16–22 in the plus strand of all segments consist of primarily A residues but the corresponding minus strand sequences show no homology. Data are from *Robertson* (1979)

means. The complete sequences of all of the genome segments, obtained by using recombinant DNA techniques, have now been reported and the amino acid sequences of the encoded polypeptides have been deduced. The transcription and translation of segments 1, 3, 4, 5, and 6 share common features (illustrated by segments 4–6 in Fig. 20). Following the heterogenous host encoded capped primer, each mRNA possesses a short 5′ untranslated region of 20–45 nucleotides beginning with the complement of the common sequences shown in Fig. 19 and continuing with an initiation codon, a single long open reading frame, a termination codon, 4–40 untranslated nucleotides, and a poly(A) tail. As noted earlier, the vRNAs contain a 16-nucleotide common sequence at the 5′ end (closed box) which is lacking in the mRNA transcripts.

The hemagglutinin (HA) has been the most extensively studied influenza protein. Segment 4 RNAs from several strains have been sequenced, primarily to investigate the amino acid changes which occur during antigenic drift of a single serotype and to compare them to the events which signal the emergence of a new serotype during antigenic shifts (*Porter* et al. 1979; *Both* and *Sleigh* 1980; *Gething* et al. 1980; *Min Jou* et al. 1980; *Winter* et al. 1981; *Hiti* et al. 1981; *Fang* et al. 1981). In addition, there has been considerable direct determination of amino acid sequence. The HA polypeptide is translated beginning at the first AUG codon in the mRNA and consists of a hydrophobic signal

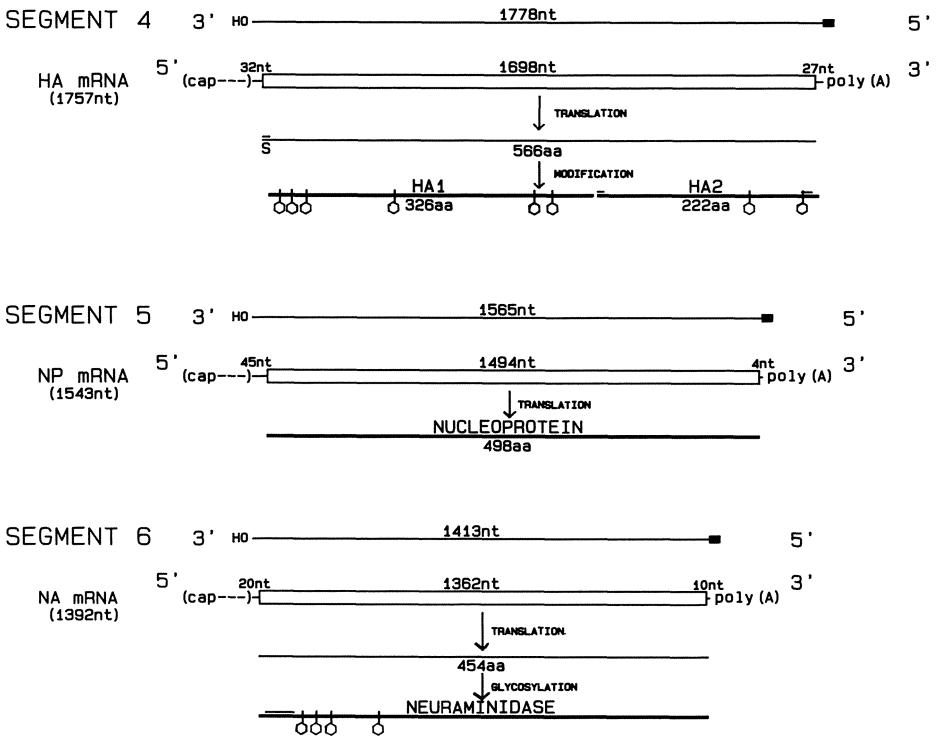


Fig. 20. Transcription, translation, and processing of influenza RNA 4, RNA 5, and RNA 6. The genome RNA (minus polarity) is shown schematically; the *solid box* is the conserved and nontranscribed 5′ terminal sequence. (*Cap*---) indicates the capped primer of variable length which is derived from host mRNA. *Open boxes* indicate the translated region of the mRNA. Modification of the hemagglutinin includes cleavage of the 17 amino acid signal sequence (*S*), glycosylation at the sites shown ( ⬡ ), and cleavage to HA1 and HA2 with the loss of a basic residue from the carboxy terminus of HA1. The neuraminidase is glycosylated at the sites shown. *Overlines* in the HA precursor, in HA2, and in NA indicate regions of hydrophobic amino acids. All data are for the A/PR/8 strain (H1, N1) of influenza A (*Winter* et al. 1981; *Winter* and *Fields* 1981; *Fields* et al. 1981)

sequence, which is removed by signalase and thus not found in the mature HA in virions, followed by 550–570 amino acids depending on the virus (see Fig. 20). There is a second cleavage site near residue 328 which separates $HA_1$ (the external protein) from $HA_2$ (221 or 222 amino acids). Depending upon the virus strain, one, two, three, or more basic residues can be trimmed from the carboxyl terminus of $HA_1$ by a carboxypeptidase of the B type (*Klenk* et al. 1980; *Bosch* et al. 1981). $HA_1$ and $HA_2$ are linked to one another by disulfide bonds in the virion and the functional unit is a trimeric structure (*Wilson* et al. 1981). HA is thus translated $NH_2$-Signal-HA1-HA2-COOH. $HA_2$ possesses a hydrophobic region between amino acids 185–211, which is presumably anchored in the lipid bilayer of the virion, and a short hydrophilic C-terminus, presumably on the interior (cytoplasmic) face of the membrane. The cleavage of HA to $HA_1$ and $HA_2$ may occur, at least in some strains such as pathogenic strains of fowl plague virus, in the Golgi apparatus, catalyzed by a host protease of trypsin-like specificity which may also be involved in the cleavage of other virus glycoproteins. There are variations in the susceptibility of the

hemagglutinins of different influenza virus strains to proteolytic cleavage and these differences appear to be important determinants for the spread of the virus in the organism and for pathogenicity (*Rott* 1979; *Klenk* et al. 1980).

Two determinations of the NP gene, segment 5, have been performed (*Winter* and *Fields* 1981; *van Rompuy* et al. 1981). The mRNA again has a short 5′ untranslated region, an open reading frame of 480–500 triplets, and a short 3′ untranslated region (Fig. 20). One of the reported sequences contains, relative to the other, a deletion of 46 nucleotides in one location and an insertion of two nucleotides at another site, which results in a phase shift in the deduced amino acid sequence. It seems likely that the difference is due to a sequencing artifact, and direct amino acid sequence determination of the NP protein would be useful. The protein is quite basic, as would be expected considering its major role in interacting with the RNA to form nucleocapsids. Clustering of basic residues occurs, although not nearly to the extent seen in the alphavirus capsid protein (*Garoff* et al. 1980a; *Rice* and *Strauss* 1981), and there is speculation that NP interacts with up to 20 or 30 nucleotides of RNA along its length.

Segment 6, the neuraminidase gene, has also been sequenced in its entirety for two influenza strains with the N1 serotype (*Fields* et al. 1981; *Hiti* and *Nayak* 1982). Although NA is a membrane-associated glycoprotein, there is no evidence for an N-terminal signal sequence. No "unprocessed" form of NA has been seen and the N-terminal sequence contains both proline and charged residues. Potential glycosylation sites are noted in Fig. 20, and all fall within the N-terminal half of the polypeptide. There is a strongly hydrophobic region between amino acid residues 7 and 35 which has been postulated to be the membrane-associated anchor of the protein. Pronase digestion of intact virions releases partial NA molecules in a tetrameric association called "neuraminidase heads", which lack the N-termini of NA (deduced from the nucleic acid sequence) and retain the C-termini as predicted this hypothesis (*Blok* et al. 1982). This orientation is unusual among virus glycoproteins, most of which have their hydrophobic roots located at or near the C-terminus. It is noteworthy that partial sequencing of RNA segment 6 from eight of nine available serotypes of NA indicates that the N-terminal eight to ten amino acids are strongly homologous to N1, but that there is little or no homology among strains for amino acids 12–20 (*Air* et al. 1981; *Blok* et al. 1982). This may reflect the nonconservation of sequence which is often found in strongly hydrophobic domains of proteins. RNA segment 6 of influenza B, but not influenza A, encodes both NA and a nonstructural protein NB of mol. wgt. 11,200, which are translated in two different reading frames from a single bicistronic mRNA (*Shaw* et al. 1983 in press).

For RNA segments 7 and 8 the situation is more complex, as it has been shown that each of these segments encodes two proteins and that these proteins are translated from different mRNA species. As shown in Fig. 21, three mRNAs have been found which will hybridize with segment 7. The largest is M1 mRNA, 1005 nucleotides long, which contains all of the coding capacity of segment 7 and encodes M1, the matrix protein of the virion. Protein M1 is 252 amino acids long, and there are 224 nucleotides untranslated at the 3′ end of M1 mRNA. The two smaller messages are M2 mRNA, 322 nucleotides in length, and M3 mRNA, containing 278 nucleotides (*Lamb* et al. 1981; *Inglis* and *Brown* 1981). M2 RNA is 5′ coterminal with the M1 RNA for 51 nucleotides, which include the 5′ untranslated region and a coding region for nine amino acids. The sequence is interrupted for 689 nucleotides and spliced to the remainder of segment 7, where translation resumes in a different reading frame for 88 residues. Of these 88 triplets, 15 overlap the

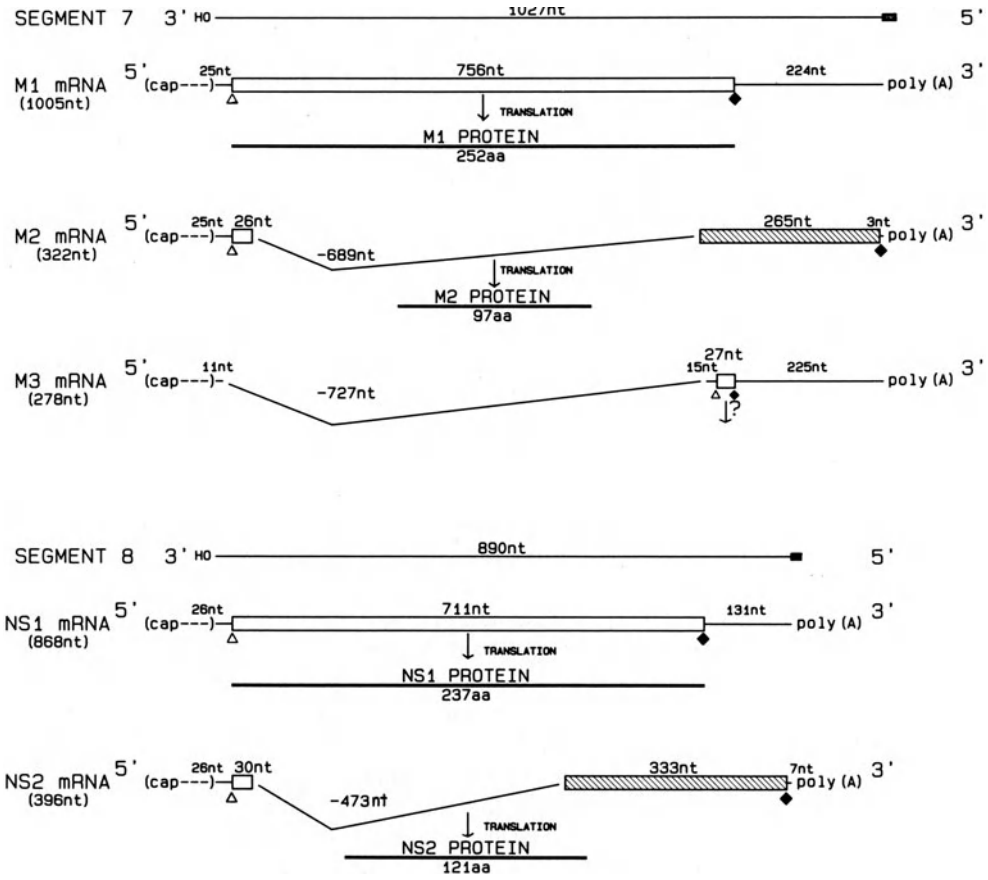


Fig. 21. Transcription and translation of influenza RNA 7 and RNA 8. Applicable conventions are the same as for Fig. 20. Initiation codons (AUG) are shown as *open triangles*; termination codons are shown as *solid diamonds*. Nucleotides excised in the spliced messages for M2, M3, and NS2 are shown by *Vs* joining the two halves of the message. *Shaded boxes* are translated in a different reading frame from *open boxes*. Data for segment 7 are from *Lamb* and *Lai* (1981), and *Lamb* et al. (1981) for the Udorn strain. Comparable sequences of influenza segment 7 have also been obtained for the genome RNA of A/PR/8 (*Winter* and *Fields* 1980) and its small mRNAs (*Inglis* and *Brown* 1981). The sequence of segment 8 shown is for the Udorn strain of influenza (H3N2) from *Lamb* and *Lai* (1980). Similar results have been obtained for fowl plague virus segment 8 (*Porter* et al. 1980) and for duck influenza and A/PR/8 (*Baez* et al. 1981)

M1 coding region and the last 73 are in the 3′ untranslated region of M1 mRNA. A minor protein species corresponding to this message has been found in infected cells (*Lamb* and *Choppin* 1981). The function of the M3 mRNA is unknown, since its splice point precedes the common initiation codon used for M1 und M2. The only translatable sequence would encode an oligopeptide nine amino acids long, identical to the C-terminus of the M1 polypeptide (*Lamb* et al. 1981).

Comparison of the M1 and M2 polypeptides from an H1N1 influenza strain (*Allen* et al. 1980; *Winter* and *Fields* 1980) with the matrix protein from an H3N2 isolate revealed that the M1 sequence was conserved with only seven changes in 252 amino acids while

M2 was much more variable, with ten changes in 97 amino acids (*Lamb* and *Lai* 1981). Segment 7 of influenza B has a similar organization, with two reading frames, and there is significant homology between the deduced amino acid sequences of the M1 proteins of influenza A and B (*Briedis* et al. 1982).

The smallest virion RNA, segment 8, has been known for some time to encode two nonstructural proteins, NS1 and NS2. These two polypeptides are the only influenza proteins with a definite temporal control. NS1 is an early protein, a product of primary transcription, which migrates to the nucleus (particularly the nucleolus) and is implicated in vRNA synthesis. NS2, on the other hand, appears late in the infection cycle and is not made in the absence of host protein synthesis (*Lamb* et al. 1978). The structure of the NS gene, as deduced from sequencing of the virion RNA and the mRNAs, is shown in Fig. 21 (*Lamb* and *Lai* 1980; *Porter* et al. 1980). The overall strategy is identical to segment 7. The vRNA is 890 nucleotides long. The NS1 message is the entire transcript (except for the 5′ terminal 22 nucleotides of the vRNA) and contains a host cap and primer at the 5′ end and poly(A) at the 3′ end. The first initiation codon at nucleotide 26 precedes an open reading frame of 711 nucleotides encoding the NS1 polypeptide of 237 amino acids. In this reading frame there is a moderately long (131 nucleotides) 3′ untranslated region. The NS2 message is 396 nucleotides long [exclusive of primer and poly(A)] and begins with 26 nucleotides of untranslated sequence followed by 30 nucleotides which encode the same ten amino acids which form the N-terminus of NS1. Following this, 437 nucleotides are spliced out and translation resumes in a second reading frame for 111 amino acids. This includes 210 nucleotides which overlap for NS1 and NS2 and most of the remainder of the segment 8 genome. The NS2 polypeptide is thus 121 amino acids long. As expected, the number of nucleotide changes found between strains is much higher in the regions encoding only NS1 or NS2, while much more conservation of nucleotide sequence is present in the overlap region where two frames are being used for protein information (*Baez* et al. 1981). We note that both NS1 and NS2 are blocked, and thus no direct amino acid sequence data exists to confirm these conclusions, which are based upon the nucleotide sequence. Segment 8 of influenza B virus and its messenger RNAs have also been sequenced and show a very similar organization, with an unspliced mRNA encoding NS1 and a spliced mRNA for NS2 (*Briedis* et al. 1981b; *Briedis* and *Lamb* 1982).

The presence of spliced messengers is unique to the myxoviruses among all RNA viruses examined to date, although it is conceivable that a similar strategy might be employed by the arenaviruses (vide infra) where there appears to be nuclear involvement in replication. Splicing appears to be restricted to the nucleus and thus only viruses whose transcription occurs in the nucleus can make use of splicing, at least using the host system. Splicing allows ready use of more than one reading frame, which increases genomic information without increasing genome size.

It is known that the various influenza specific proteins are made in differing molar amounts, but it is not clear how their synthesis is modulated. The concentration of the most abundant virus polypeptides is roughly 50-fold that of the least abundant (*Tekamp* and *Penhoet* 1980) and the relative proportions in mature virions are even more disparate (*Nayak* 1977).

### 4.4 Arenaviruses

The arenaviruses are a group of viruses whose primary host is usually a single species of rodent. They normally cause a chronic disease in this rodent host, but some can also cause serious or fatal human diseases such as hemorraghic fevers and aseptic meningitis. The family is divided into two serologically distinguishable groups: the Old World arenaviruses, which include various isolates of lymphocytic choriomeningitis (LCM) virus, Lassa fever virus, and Mozambique virus; and the New World arenaviruses, also known as the Tacaribe complex, which include Junin (causative agent of Argentine hemorraghic fever), Machupo (Bolivian hemorraghic fever), Amapari, Latino, Parana, Tamiami, Tacaribe and Pichinde viruses (reviewed in *Matthews* 1982; *Pederson* 1979; *Rawls* and *Leung* 1979).

The arenaviruses contain five species of single stranded RNA, of which three are of host cell origin, the 28-S and 18-S ribosomal RNAs and a heterogeneous collection of small RNAs of 4–5 S. The two true genomic RNA segments are called L (31 S), with a size of 6.3–8.4 kb for various members of the group, and S (23 S), of 3.3–3.9 kb (*Matthews* 1982; *Rawls* and *Leung* 1979; *Ramsingh* et al. 1980). These enveloped viruses contain cellular ribosomes giving them the grainy appearance in the electron microscope which led to the name arenavirus (Latin *arena*, sand). Although the presence of ribosomes gives the members of this group their distinctive morphology, it has been shown that functional ribosomes are not necessary for infectivity of the virions and they are currently considered to be adventitious inclusions (*Leung* and *Rawls* 1977). The structure of the nucleocapsid of the virus is ill-defined and nucleocapsids have been described as heterogeneous circular or hairpin-like structures whose lengths do not correlate well with the RNA sizes. Recent experiments have indicated that the nucleocapsid exists as a discrete core structure which can be dissociated into strands 10–12 nm in diameter that have a beaded appearance resembling that of nucleosomes (*Vezza* et al. 1978; *Young* et al. 1981).

As is the case for enveloped viruses in general, intact arenavirus particles have surface projections external to the lipid bilayer which can be removed by chymotryptic digestion leaving spikeless particles that lack most or all the major glycoprotein (see below).

The polypeptide composition of arenavirus particles and the apparent molecular weights of the structural proteins on acrylamide gels appear somewhat variable for different members of this family. The major protein species in arenaviruses is the nucleoprotein of molecular weight 63–72 K, depending upon the virus. In addition, one or two species of glycoprotein are present. Pichinde and LCM viruses possess two envelope glycoproteins (*Harnish* et al. 1981a; *Young* et al. 1981; *Buchmeier* et al. 1981), one of molecular weight 34–38 K and a larger one of 45–70 K. Tacaribe and Tamiami viruses, on the other hand, appear to have only a single species of glycoprotein of molecular weight 42–44 K (*Gard* et al. 1977; *Rawls* and *Leung* 1979). This glycoprotein may be present as up to ten species resolvable by isoelectric focussing, but all these forms give identical patterns after limited digestion with the V8 protease from *Staphylococcus aureus,* suggesting that the heterogeneity may be due to the carbohydrate moiety (*Compans* et al. 1981). The nucleoprotein may also be present as a number of species of differing isoelectric points. Numerous minor components have also been described, notably a 72–77-K polypeptide associated with the nucleocapsid fraction (*Rawls* and *Leung* 1979; *Vezza* et al. 1977; *Harnish* et al. 1981b; *Young* et al. 1981) and several minor glycoproteins in Junin virus (*de Martinez*

*Segovia* and *de Mitri* 1977). The nucleoprotein (N) possesses group specific antigens which cross-react serologically with all members of the group, while the glycoprotein antigens are more species-specific (*Buchmeier* et al. 1981).

Little is known about the replication of the arenavirus RNA. The two segments contain unique sequences and one RNA is not a subgenomic fragment of the other. This has been shown on a molecular level by hybridization to cDNA probes (*Leung* et al. 1981); and on a genetic level by the fact that temperature-sensitive mutants of Pichinde virus can be grouped into two linkage groups, which show the high frequency of recombination characteristic of reassortment of segmented genomes (*Vezza* et al. 1978). The genomic RNAs are neither capped nor polyadenylated and are not themselves infectious. Furthermore, RNA isolated from polyribosomes from arenavirus-infected cells hybridizes efficiently to genomic RNA. For these reasons arenaviruses have been classified as negative strand viruses. An RNA dependent RNA-polymerase activity as well as poly(A) and poly(U) polymerases have been isolated from purified Pichinde virions (*Leung* et al. 1979). The poly(U) and poly(A) polymerase activities are similar to activities found in uninfected cells and may represent activities normally associated with ribosomes which were incorporated into virions nonspecifically during morphogenesis (*Matthews* 1982). Recently it has been shown that 17 of the first 19 nucleotides at the 3′ termini of L and S RNAs from Pichinde virus are identical (Fig. 22). The sequences for the two segments then diverge completely (*Auperin* et al. 1982b). The 3′ terminal 19 nucleotides of the S RNAs of Tacaribe virus and LCM virus are identical to those shown for Pichinde virus; the L RNA terminal sequence for LCM differs from that of Pichinde virus and Tacaribe virus by substitutions at positions 9 and 17 (*Auperin* et al. 1982a). This conserved terminal sequence may represent a replicase binding site and/or a common sequence for nucleation with N protein to form the nucleocapsid.

Details of the transcription and translation strategies of these viruses are unclear at present. Two potential initiation sites for translation are found in the first 50 nucleotides of the L segment, whereas the first initiation codon on S is at nucleotides 84–86 (*Auperin* et al. 1982). No protein sequence exists for comparison with the RNA sequence, however. The major virus-specific protein produced in Pichinde-infected cells is the N protein, as shown by immunoprecipitation of extracts from pulse labeled infected cells with anti-Pichinde antiserum (*Harnish* et al. 1981b). Also present in these experiments were up to six smaller polypeptides related to N by peptide mapping and which are probably degradation products. Similarly, both immunological and biochemical experiments have shown that a 79-K glycoprotein (called GPC or cell-associated glycoprotein) is present which is the precursor of the mature virion glycoproteins GP1 and GP2 of LCM virus (*Buchmeier* et al. 1981) and Pichinde virus, respectively (*Harnish* et al. 1981b). In the case of Tacaribe, the cell-associated glycoprotein GP70 has been shown to be the precursor of the single virion glycoprotein of 42 K; presumably the other portion of this pre-

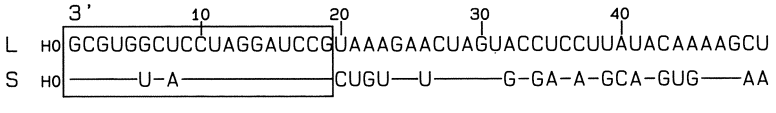


Fig. 22. 3′ Termini of arenavirus RNA segments. The sequence at the 3′ termini of the L and S segments of Pichinde virus are shown. The conserved sequence of 19 nucleotides is *boxed*. There is a second short conserved region between nucleotides 24 and 31; thereafter the sequence becomes divergent. Data are from *Auperin* et al. (1982b)

cursor is lost during maturation (*Saleh* et al. 1979). This is a clear-cut example of post-translational processing in this system. The N protein of Tacaribe (*Compans* et al. 1981) and the minor P polypeptide (79 K) share oligopeptides and may be another example of processing. So far, however, no larger precursor of N protein has been found (*Saleh* et al. 1979) which contains the amino acid sequence of all three structural polypeptides, although there is evidence that N and GPC are both encoded by the S genomic RNA (see below). It is therefore unclear whether these two major products are translated from a single species of messenger RNA and processed so efficiently that their common precursor is not detected, or whether separate messages are transcribed for N and GPC.

Recently the presence of a large polypeptide of 200 K was demonstrated in the cytoplasm of Pichinde-infected cells and was also shown to be a component of Pichinde virions (*Harnish* et al. 1981b). This is thought to be the viral replicase, although the data so far are inferential only. In size it is similar to the replicases of VSV and NDV. In addition, two nonstructural polypeptides called NS1 (79 K) and NS2 (105 K) were isolated by precipitation of Tacaribe-infected cells with hyperimmune serum (*Compans* et al. 1981).

Because of the efficient reassortment of the genomic segments and the presence of naturally occurring variants and laboratory-derived temperature-sensitive mutants, it has been possible to map various arenavirus functions to one or the other of the genomic segments. The S segment of molecular weight $1.1–1.3 \times 10^6$ has been shown to encode the N and GPC polypeptides (*Vezza* et al. 1980; *Harnish* et al. 1981a). These two polypeptides (79 K and 63 K) would saturate its nominal coding capacity of 130 000 daltons of protein if both were read in the same reading frame. With intertypic LCM recombinants it could be shown that the pathogenicity for guinea pigs depended upon S RNA gene products (*Kirk* et al. 1980), implicating structural proteins in this phenomenon. Group I temperature-sensitive mutants of Pichinde also map on the S RNA segments (*Vezza* et al. 1980).

The L segment (molecular weight $2.1–2.8 \times 10^6$) has a coding capacity of about 280 000 and is the only segment large enough to encode the L protein. Group II Pichinde mutants map to this segment. In addition, for both Pichinde and LCM the L segment determines the plaque morphology of the virus (*Kirk* et al. 1980; *Vezza* et al. 1980). Thus the genome organization appears to be that the structural genes are encoded in one RNA segment and the replicase genes in the second segment.

Finally, several lines of evidence suggest that arenavirus replication is dependent upon host cell factors and there is some indication of nuclear involvement. Pichinde will not replicate in enucleated cells, nor are virus-specific antigens synthesized (*Banerjee* et al. 1976). α-Amanitin inhibits Pichinde replication but α-amanitin-resistant cells with an altered RNA polymerase II will produce arenavirus in the presence of the drug. Actinomycin D also prevents the production of infectious Pichinde virus but does not prevent antigen synthesis, implying a late block in virus maturation (*Rawls* and *Leung* 1979). The nature of the nuclear involvement in arenavirus replication is unclear but it is possible that the arenaviruses may have characteristics in common with the myxoviruses.

## 4.5 Bunyaviruses

The Bunyaviridae form a large and diverse group of viruses of worldwide distribution which on the bais of serological considerations have been divided into four genera: *Bunyavirus, Nairovirus, Phlebovirus,* and *Uukuvirus.* A number of members of this group

Table 5. Bunyaviridae

| Genus | Subgroups | Members[a] |
|---|---|---|
| Bunyavirus | Bunyamwera group | Bunyamwera, Guaroa |
| | California group | California encephalitis, Inkoo, LaCrosse, snowshoe hare, Tahyna, Trivittatus |
| | Patois group | Pahayokee, Patois, Shark River |
| Uukuvirus | - | Uukuniemi |
| Nairovirus | - | Crimean hemorrhagic fever, Qalyub |
| Phlebovirus | - | SF-Sicilian |

[a] Type virus of the genus or subgroup and/or viruses mentioned in the text

are listed in Table 5. Originally classified as arboviruses, most members infect both vertebrate and invertebrate hosts. The viruses are roughly spherical and enveloped. The envelope surrounding the three circular helical nucleocapsids has protein spikes made up of two glycoproteins. The nucleocapsids contain one major nucleocapsid protein N and three species of genomic RNA called L (large), M (medium), and S (small) with approximate sizes of 7.5–9 kb, 5.5.–7 kb, and 0.9–1.5 kb respectively. Serological groupings of these viruses are complicated by the fact that some members are serologically related by hemagglutination inhibition or neutralization tests (reflecting similar glycoprotein antigens in the outer envelope) while showing no relatedness by complementation fixation, which is an attribute of the N protein. As seen below, the glycoproteins and the nucleocapsid protein are encoded on separate genome segments and this complex serological situation indicates that reassortment of these segments has occurred during evolution, increasing the diversity of this group (*Bishop* and *Shope* 1979; *Bishop* et al. 1980). The existence of naturally occurring reassortment viruses has recently been confirmed by studies of two viruses of the Patois subgroup whose L and S RNA segments are nearly identical by oligonucleotide mapping, whereas their M segments differ markedly (*Ushijima* et al. 1981).

The Bunyaviridae contain RNA which is not infectious and possess a virion-associated RNA-dependent RNA polymerase. mRNA from polysomes of infected cells hybridizes to virion RNA. For these reasons Bunyaviridae are classified as negative stranded viruses and, although the details of their replication cycle are not well documented at present, they appear to share many aspects of replication strategy with other negative strand viruses. The organization of the genome is similar to that of the arenaviruses, but with the S segment of the arenaviruses divided into the M and S segments of the bunyaviruses (see below).

It has been shown that bunyavirus virions contain the following polypeptide components: the nucleocapsid protein N (molecular weight 19–26 K), present in more than 2100 copies; two glycoproteins G1 and G2, present in approximately 630 copies each; and a minor component L, of molecular weight 120–200 K, which is internal and present in only 20–25 copies. Among the bunyaviruses, G1 is variously reported to have a molecular weight of 85–120 K, and G2 of 30–50 K. In Uukuniemi virus (the type Uukuvirus) the two glycoproteins are much more similar in size, roughly 75 K and 63 K respectively (*Bishop* and *Shope* 1979; *Bishop* et al. 1980).

The coding relationships are that the smallest RNA segment encodes N (*Gentsch* and *Bishop* 1978), the M RNA encodes G1 and G2 (*Gentsch* and *Bishop* 1979), and the largest segment must encode the L protein. The assignments of polypeptides to S and M were originally determined genetically and have been confirmed (see below) by in vitro translation of the corresponding mRNAs. The assignment of L protein to the L segment remains inferential only. In addition, the L segment contains sufficient excess coding capacity to encode other minor nonstructural polypeptides which are occasionally observed in bunyavirus-infected cells (*Ushijima* et al. 1981).

Messenger RNA species complementary to L and M, isolated from infected cells, appear to correspond to full-length transcripts of their respective genomic segments, although small size differences would not have been seen. It is unknown whether the mRNAs are capped at the 5′ end, but they appear to lack polyadenylate tracts at their 3′ termini (*Ulmanen* et al. 1981b). Messenger RNA complementary to S RNA appears smaller than S RNA by approximately 20% for both Uukuniemi virus (*Ulmanen* et al. 1981b) and snowshoe hare virus (*Cash* et al. 1979). Oligonucleotide protection analysis suggests that the S mRNA is complementary to the exact 3′ terminus of the genomic S RNA (*Cash* et al. 1979; *Clerx-van Haaster* and *Bishop* 1980). S mRNA of Uukuniemi virus translated in vitro produces two polypeptides. One is identical in size to the nucleocapsid protein N (25 K) and is immunoprecipitable by antiviral antiserum. The second polypeptide is larger (30 K) and does not appear to share any antigenic determinants with N (*Ulmanen* et al. 1981b). No precursor containing both of these polypeptides has been identified, and furthermore, the coding capacity of the S mRNA is insufficient to encode both polypeptides without overlap. The sequence of the genome segment contains two possible open reading frames (see below) which could either be read from one mRNA using different initiation codons or be translated from two different messages nearly identical in size. Genetic evidence supports the idea that S encodes two functional polypeptides in vivo. Mutants of Bunyamwera viruses can be grouped into three groups by high-frequency recombination, which occurs by reassortment of genome segments. Group I corresponds to S RNA, encoding the N protein, Group II to M RNA, specifying the glycoproteins, and Group III to L RNA, encoding the replicase (*Pringle* and *Iroegbu,* 1982). However, complementation has been observed between two non recombining mutants of Group I complement indicating that the S segment may encode more than one function (*Iroegbu* and *Pringle* 1981). Furthermore, in the case of snowshoe hare virus a nonstructural protein with a molecular weight of 7.4 K has been identified and assigned to the S RNA by analysis of recombinant snowshoe hare/LaCrosse viruses (*Fuller* and *Bishop* 1982). The relation of this protein to the 30-K protein produced by translation of Uukuniemi S mRNA discussed above is not clear.

The sequence at the 3′ terminus of each of the three RNA segments of three bunyaviruses, LaCrosse, snowshoe hare, and a LaCrosse variant, has been determined for approximately 200–240 nucleotides by the dideoxy chain termination method (*Clerx-van Haaster* et al. 1982). For the L and M segments there is only one open reading frame of any length which continues to the end of the sequenced region. For both segments, the open reading frame starts with an AUG codon at nucleotides 62–64; for the M segments; this is the first AUG, but for the L segments it is the second AUG in the sequence. In contrast, the S segment has two open reading frames, one beginning at nucleotide 82, the other at nucleotide 101. It appears that the open reading frames in L and M and both open reading frames in S are in fact coding. In the case of the L segment of the three viruses,

there were 28 nucleotide changes between nucleotides 62 and 200. Twenty-three of these were silent changes, i.e., nucleotide substitutions which would not result in a changed amino acid; four of the remaining five would result in the conservative substitution of Tyr for Phe, or Lys for Arg. Such conservation of encoded amino acid sequence while the nucleotide sequence varies strongly implies that these sequences are in fact translated into protein. In the case of M RNA, there were a number of nonsilent alterations between the two virus strains in the region which would encode the N-terminal 18 amino acids of the protein, but not thereafter. This region is highly hydrophobic and probably represents a signal sequence. Comparable studies with VSV G protein have shown numerous differences in the amino acids within the signal sequence of different virus strains, but strict conservation of the mature G protein sequence was observed (*H.P. Ghosh* et al. 1981). Again this implies that this sequence is translated.

Finally, in contrast to the L and M segments, the S RNA shows very high conservation of nucleotide sequence. If the two open reading frames found are both translated, such sequence conservation would be required in order to conserve amino acid sequences. As noted above, S mRNA is translated in vitro into two distinct polypeptides.

In vitro translation of M mRNA in a reticulocyte system produces a precursor to the glycoproteins of approximately 110 K which has not been observed in vivo. However, if dog pancreas microsomes are added to the system, only the mature forms of G1 and G2 can be found (*Ulmanen* et al. 1981b) indicating that the glycoproteins are processed efficiently during membrane insertion, and suggesting that signalase may be responsible for the processing as has been suggested for the alphaviruses (Sect. 2.3.1). In addition, a nonstructural polypeptide of approximately 11 K has been mapped to this segment (*Fuller* and *Bishop* 1982). It has not so far been possible to translate the L mRNA in vitro. Thus the bunyavirus family appears to have a complicated translation strategy with one genome segment producing a polycistronic message while the mRNA from another segment produces proteins from two different reading frames.

Nothing is currently known about the synthesis of these mRNA species, but in contrast to orthomxyoviruses, bunyavirus replication is insensitive to α-amanitin, rifampin, and actinomycin D, indicating that transcription does not require host nuclear function (*Bishop* and *Shope* 1979). Bunyavirus virions are not produced in enucleated cells, but all of the polypeptide species can be found in these cells, and the effect of enucleation appears to be on a late morphogenetic step probably involving perinuclear membranes (*Pennington* and *Pringle* 1977) (see also Sect. 4.2). Viral polypeptide synthesis is also temporally controlled, with L and N being early proteins and G1 and G2 late proteins. N protein can be detected as early as 2 h post infection, and early N synthesis may be required in order to form nucleocapsid complexes of full-length plus strand RNA to serve as replication templates. Most intracellular RNA is found as ribonucleoproteins containing both minus strand and plus strand RNA in the ratio of 4:1 (*Bishop* and *Shope* 1979).

Genetically, the bunyaviruses undergo a high frequency of recombination which is characteristic of reassortment of segmented genomes. Indeed, analysis of recombinant viruses of closely related strains, such as LaCrosse virus and snowshoe hare virus, have led to some of the initial assignments of the polypeptides to particular segments noted above (*Gentsch* and *Bishop* 1978, 1979). The genetic reassortment appears to be under certain constraints, however, and members of the different genera will not exchange genomic segments. In addition, it has been shown that within the genus Bunyavirus, members of the California subgroup (LaCrosse, snowshoe hare, Tahyna, California

encephalitis, and Trivittatus) will form recombinant viruses with one another, but that none of these will exchange genomic segments with Guaroa virus of the Bunyamwera subgroup (*Bishop* et al. 1981).

As mentioned above, RNA sequences adjacent to the 3′ ends of the RNA segments have been determined for a number of bunyaviruses and several sequences are compared in Fig. 23. In all cases the L, M, and S RNAs of a particular virus possess a conserved sequence of 11–13 nucleotides at the 3′ end. For LaCrosse and snowshoe hare viruses (both California subgroup), this 11-nucleotide sequence is identical. Two other viruses, Shark River and Pahayokee, members of the Patois subgroup of bunyaviruses, also have 11 identical 3′ terminal nucleotides, which differ from the LaCrosse/snowshoe hare

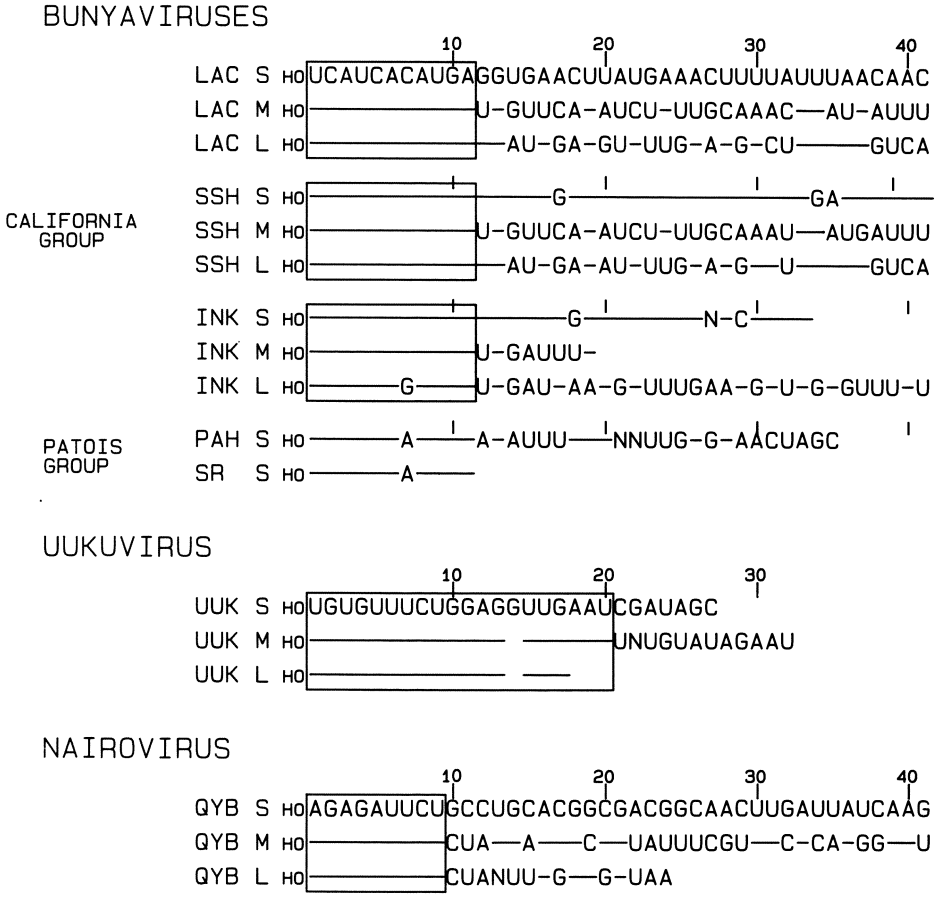


Fig. 23. 3′ Terminal sequences of Bunyaviridae. Sequences have been aligned for homology. A *horizontal line* indicates that the nucleotide is identical to the complete sequence shown at the head of the group. *Boxes* indicate sequences shared by all three segments of a given virus. Data are from *Obijeski* et al. (1980), *Parker* and *Hewlett* (1981), *Bishop* et al. (1981) and *Ushijima* et al. (1981). *LAC*, LaCrosse virus; *SSH*, snowshoe hare virus; *INK*, Inkoo virus; *PAH*, Pahayokee virus; *SR*, Shark River virus; *UUK*, Uukuniemi virus; *QYB*, Qalyub virus. *S, M,* and *L* are the small, medium, and large RNA segments respectively

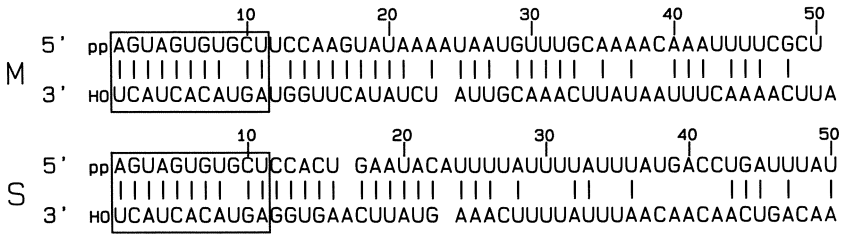


Fig. 24. 3′ and 5′ termini of LaCrosse virus segments. The first 50 nucleotides from the 3′ and 5′ ends of the small (*S*) and medium (*M*) segments of LaCrosse virus RNA are shown. *Gaps* have been introduced for alignment; *vertical lines* indicate complementary nucleotides. The conserved sequence at the termini of the RNAs is *boxed*. Data are from *Obijeski* et al. (1980)

sequence by a single A to C change at nucleotide 7 (Fig. 23) (*Ushijima* et al. 1981; *Clerx-van Haaster* and *Bishop* 1980). In contrast, Uukuniemi virus L, M, and S segments have a conserved stretch of 13 nucleotides at the 3′ terminus which is unrelated to the sequences described above. Of these 13 nucleotides, 11 are complementary to the first 11 nucleotides at the 5′ end (*Parker* and *Hewlett* 1981), as is characteristic of minus strand viruses. With LaCrosse virus, the M and S RNAs have been sequenced for 200 and 240 nucleotides respectively from the 3′ ends (*Clerx-van* Haaster et al. 1982) and 60 and 90 nucleotides respectively from the 5′ end (*Obijeski* et al. 1980). The 5′ termini of all three segments contain an 11-nucleotide common sequence which is complementary to the common 3′ termini described above. Although the M and S sequences diverge at this point, each segment has largely self-complementary regions of 50–60 nucleotides and thus could form stable circles with panhandles (Fig. 24). These are the longest cyclization sequences which have been identified in standard (nondefective) virions and may explain the circular nucleocapsids isolated from virus particles (*Obijeski* et al. 1980).

The extreme conservation of sequence at the termini of the RNAs implies that, as appears to be the case for all minus strand viruses, the 3′ termini of the RNAs interact specifically with the virus-encoded proteins for replication and transcription and/or encapsidation. The various genera of Bunyaviridae have evolved different terminal sequences, as shown in Fig. 23. If these are indeed initiation sites for the virus-encoded replicase, these viruses have probably evolved group-specific proteins to interact with these altered sequences. Viruses with identical or nearly identical 3′ termini undergo reassortment to produce hybrid viruses, whereas viruses with nonhomologous sequences at the 3′ termini appear to be prohibited from such genetic exchange, as would pe predicted by the hypothesis that these sequences form replication recognition sites.

## 5 Defective-Interfering Viruses

It has been known for many years that successive passages of viruses at high multiplicity leads to the accumulation in the virus stock of defective or incomplete virus particles. This phenomenon was first recognized for influenza viruses by *von Magnus* (1954) and has been seen to a greater or lesser degree in almost all animal virus groups studied. (For general reviews see *Huang* 1973; *Huang* and *Baltimore* 1977; *Perrault* 1981.) Defective-interfering (DI) particles share the following characteristics:

1. They lack part of the standard viral genome, from as little as 10% to as much as 90%, depending on the group.
2. They are defective, in that they cannot replicate alone, but require a helper virus (standard virus). In many cases there is no expression (translation and/or transcription) of the DI genome.
3. The DI particle contains the same complement of structural proteins as the standard virus.
4. They interfere with the replication of homologous standard virus, resulting in greatly reduced yields of infectious virions.
5. With successive passages, they form a progressively greater proportion of the virus yield; this is sometimes referred to as enrichment.

These characteristics taken together indicate that the deleted DI RNA genomes must retain any signals required for replication of both the plus strands and the minus strands of RNA, as well as any encapsidation signals necessary for specific association with viral proteins. Furthermore, if the DI is to be enriched, the recognition and binding sequences must have a selective advantage over those in standard virus. The mode of generation of DIs and the structure of their remaining genome is thus important for our understanding of normal viral replication, and, as might be expected, the DIs of plus stranded viruses such as picornaviruses and togaviruses are quite different from those of negative strand viruses.

Defective-interfering particles have been readily demonstrated for all the groups of negative strand viruses. Interference phenomena have been noted for rhabdoviruses, paramyxoviruses, and influenza viruses after as little as two or three high multiplicity passages in tissue culture cells. DIs are a major source of low virus yields in these systems. The most extensively studied DIs are those of rhabdoviruses, where the shortened genomic RNA leads to the production of truncated (T) virions which are physically separable from standard virus (*Huang* and *Baltimore* 1977). This means that it is possible to isolate a pure DI population to analyze their genomic organization and to infect cells with known ratios of DI and standard virus to study interference. As recently reviewed by *Lazzarini* et al. (1981) and *Perrault* (1981), VSV DIs can be organized in several ways (see Fig. 25), but in all cases the exact 5′ terminus of the genome is retained. Only one DI has been described which retains the original 3′ terminal sequence of the VSV genome. This 3′ DI RNA appears to be a simple deletion of most of the L gene. Nucleocapsids of the 3′ DI can be transcribed in vitro to produce plus strand leader RNA and mRNAs for N, NS, M, and G (*Colonno* et al. 1977). Unlike other VSV DIs, in vivo the 3′ DI produces functional viral proteins in the absence of helper virus (*Johnson* et al. 1979). Although the deletion is entirely within the L gene, an aberrant G mRNA is made which contains the remnant of the L gene covalently attached (*Herman* and *Lazzarini* 1981b). This DI interferes with both homologous and heterologous VSV strains, presumably at the level of both transcription and genome replication (*Bay* and *Reichmann* 1982).

In all other VSV DIs which have been described, the 3′ terminus of VSV RNA has been replaced with a sequence of 45–70 nucleotides which is exactly complementary to the 5′ terminus of the genome and which enables the RNA to cyclize, forming small panhandles that can be visualized by electron microscopy (*Perrault* 1981). The majority of these DIs have lost most or all of the coding sequences in the 3′ half of the VSV genome and only retain portions of the L gene. These 5′ DIs lack the region of the genome where transcription initiates, and therefore none of the DI genes are expressed. One compound

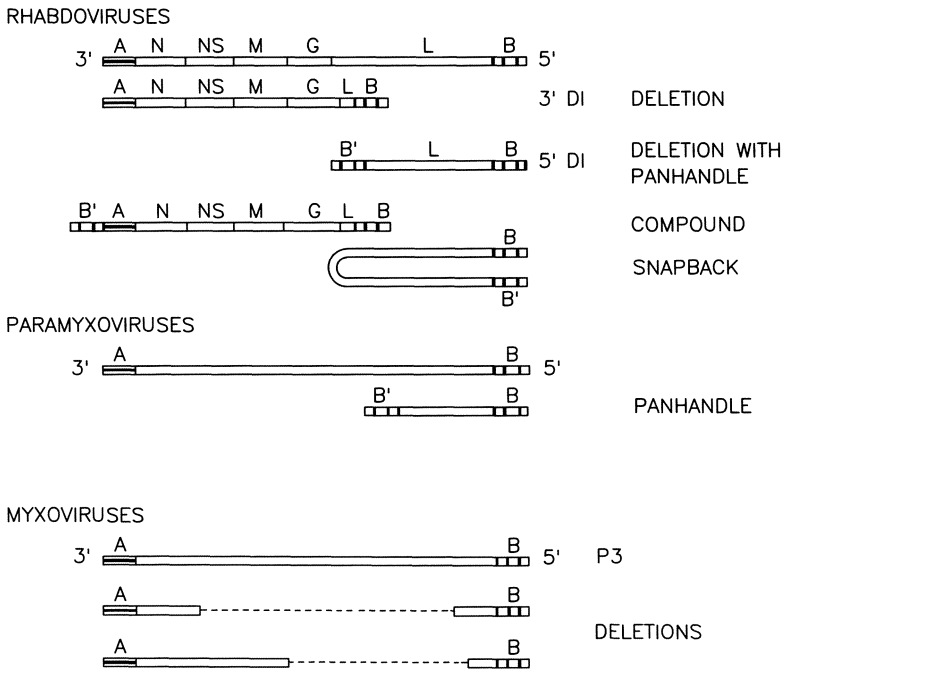


Fig. 25. Genome organization of DI particles. Schematic representation of DI genomes from rhabdoviruses (VSV), paramyxoviruses (Sendai) and myxoviruses (influenza). VSV genes are labeled as in Fig. 13. *A* and *B* in all cases represent terminal sequences present in the standard virus genome. *B'* represents the complement of *B*. The 3' DI deletion of VSV is a transcribing DI and produces mRNAs for N, NS, M, and G. The remaining classes of VSV DIs are nontranscribing. Genome organization of rhabdovirus DIs is from *Perrault* (1981), *Lazzarini* et al. (1981). Paramyxovirus DIs are described in *Leppert* et al. (1977), and the influenza deletion DIs in *Nayak* et al. (1982)

DI, called DI-$LT_2$, has been isolated from the same DI population as the 3' DI described above. It contains 70 nucleotides at its 3' end which are complementary to the 5' terminus of VSV RNA, followed by the 3' leader sequence, most of the region encoding N, NS, M, and G, and the VSV 5' terminal sequence. Although it appears to be the same as 3' DI, with the exception of the 70 nucleotides at the 3' terminus, transcription does not occur since the normal plus strand leader is not at the exact 3' terminus (*Keene* et al. 1981a). However, both 5' DIs and compound DIs transcribe an small abnormal leader RNA from the new 3' terminus of the DI genome (labeled B' in Fig. 25) (*Schubert* et al. 1978; *Leppert* et al. 1979). Finally, a class of snapback DIs have been isolated which are self-complementary along their entire length. They have been used as model systems to study interferon induction (*Schubert* and *Lazzarini* 1981).

The presence of the 5' terminal sequence of VSV RNA at the 5' terminus of all the DIs and the replacement of the normal 3' terminus with the complement of the 5' terminus in almost all DIs emphasizes its importance in controlling replication and transcription. As might be predicted from the fact that only one DI has been described which contains the original 3' sequence, this 3' DI is effectively outcompeted by the DI-$LT_2$ described above (*Perrault* and *Semler* 1979). Similarly, in a study of two 5' DIs derived

from the same population which differed in the length of their self-complementary termini (derived from the 5′ terminus of VSV RNA), a DI possessing a 55 base pair stem and 50% of the virus genome outcompeted a DI with a 45 base pair stem and 33% of the coding region (*Huang* et al. 1980). This result suggests that the sequence found between 45 and 55 nucleotides from the 5′ terminus (and located between the leader and the L gene) is important in the control of RNA replication. It should be noted that the difference in competitive advantage is only seen when two DIs are introduced into the same cell and that both interfere to the same extent with the standard VSV used as helper virus (*Rao* and *Huang* 1982).

The DIs of paramyxoviruses appear to be mainly of the panhandle variety (Fig. 25) (*Leppert* et al. 1977). Although the original 3′ and 5′ termini of the nondefective Sendai virus are largely self-complementary for 12 nucleotides, this degree of complementarity is insufficient to permit the RNA to cyclize. DIs of Sendai virus, on the other hand, have self-complementary regions of 150–200 bases and the isolated RNA will readily from panhandle structures visible in the electron microscope. DIs of different total lengths have panhandles of comparable lengths whose sequences have been shown to contain the 5′ end of the genomic RNA and its complement as well as portions of the L gene. Paramyxovirus DI genomes are very small, containing only 8%–30% of the standard virus complexity.

A second series of ten Sendai DIs have been recently isolated and characterized by T1 oligonucleotide mapping. These DIs possessed common 5′ terminal sequences derived from the L gene. In addition, however, they also had one or more oligonucleotides from the 3′ leader region of the genome (*Amesse* et al. 1982). Sequence analysis will be required to elucidate their genome organization, which may be different from any class of VSV DIs described to date. In view of the strong similarities in genome organization and replication strategy between the rhabdoviruses and the paramyxoviruses, it seems probable that DIs corresponding to all the classes of VSV DIs will eventually be isolated for paramyxoviruses.

Defective-interfering viruses of influenza have been reviewed by *Nayak* (1980). Defective particles are not physically separable from standard virus and have been difficult to study, since all populations contain some proportion of the helper virus. They were originally thought to lack one or more genomic segments, but recent analysis has shown that various DIs have apparently replaced one of the three segments encoding polymerase proteins (segment 1, 2, or 3) with a small DI RNA containing the ends of the respective P gene but with most of the coding region deleted (*Davis* et al. 1980; *Nayak* et al. 1982). The unequal amounts of various RNA segments originally reported for influenza viruses may reflect the presence of DIs in virus stocks. How these small RNAs can cause interference with the replication of the other seven influenza genes is unclear at this time. If similar deletion mutants for the other genomic segments exist, they apparently do not display the classical phenotype of interference. A single influenza DI has been found in which the small DI RNA retains the 3′ and 5′ terminal sequences from segment 3, which it replaces, and also contains three other short domains from segment 3 as well as nucleotides 30–89 from the 3′ end of segment 1 (*Moss* and *Brownlee* 1981; *Fields* and *Winter* 1982). The domains have been rearranged such that sequences originally near the 5′ terminus are adjacent to regions derived from the 3′ terminus.

DIs have also been reported for arenaviruses (*Gimenez* and *Compans* 1980) and bunyaviruses (*Kascsak* and *Lyons* 1978). In these cases smaller RNA species than the genomic

RNAs are produced, but sequence data are lacking. For example, DI particles of bunyamwera virus are reported to have only 16-S RNA and to lack 26-S and 33-S RNA, but whether this represents overproduction of the S RNA or deleted forms of M and L RNA is not known (*Kascsak* and *Lyons* 1978).

Neither the mechanisms by which DIs interfere with the production of infectious standard virus nor the role of these particles in establishing or maintaining persistent infections and in influencing replication in infected hosts is clearly understood. In negative strand viruses, interference by DI particles primarily affects viral RNA replication rather than transcription or translation. This could occur by direct competition for limiting virion-encoded products: either a limiting supply of polymerase molecules or the major nucleocapsid protein, since functional templates are all encapsidated. Among the negative strand viruses, polymerase components appear to exchange freely between standard and DI genomes, and DI genomes are packaged as particles containing L polypeptides encoded by the helper virus. Host cell factors also seem to be involved, since certain hosts favor the rapid generation of DIs while others do not (*Holland* et al. 1976; *De* and *Nayak* 1980). As has been noted earlier, host cell proteins may form components of some virus replicases, and such host components could affect the fidelity of replication and thus DI generation.

Defective interfering viruses have been described also for two plus stranded virus groups, the picornaviruses (*Cole* et al. 1971) and the togaviruses (reviewed in *Stollar* 1980a). Notably, no DIs have been reported for any of the plus stranded plant viruses (*Huang* 1973). Defective viruses are much more difficult to generate for plus stranded viruses than for negative strand viruses and generally require many more passages at elevated multiplicities.

Defective-interfering particles of togaviruses have repeatedly been isolated from both Sindbis virus and Semliki Forest virus (reviewed in *Stollar* 1980a). In addition to increased interference with successive passages, in some cases alphavirus DI RNAs appear to go through sequential reduction in size with the DI-specific RNAs appearing to be roughly half, one-third and then one-quarter the size of the genome RNA, etc. The alphavirus DI particles are generally not physically separable from standard virions and the roughly fractional sizes of the RNAs may reflect a constraint of having two molecules of half size, three of one-third, etc., so that the content of RNA per particle remains relatively constant. *Kääriäinen* et al. (1981) determined that the small (18-S) SFV DI RNA found after numerous passages at high multiplicity evolved with continued passage to larger DI RNAs (24 S and 33 S). The sequence complexity of the larger DI RNAs was progressively less than that of the small RNA, however, and these larger DI RNAs possessed a selective advantage over the small RNA. The selection for the larger RNAs appears to occur at the stage of encapsidation and might signify that it is more difficult to package multiple copies of smaller RNAs then fewer copies of a larger RNA. In any event it appears clear that alphavirus DI RNAs continue to evolve during many passages at high multiplicity and in the process attain an increasing selective advantage over the standard virus.

Recent sequence analyses have shown that the events which occur during the generation of alphavirus DIs are quite complex. In one case, two DI RNAs from a single population were cloned as cDNA and the sequences determined (*Lehtovaara* et al. 1981, 1982). One DI, 1652 nucleotides long (compared to standard SFV RNA of about 12 000 nucleotides), retained the 106 nucleotides adjacent to the 3′ poly(A), lacked almost all of

the sequences of the 26-S subgenomic RNA encoding the structural proteins, and contained three tandem repeats of 484 nucleotides, one of which had an extra 60-nucleotide segment. Because the complete sequence of the genomic RNA has not yet been determined, the origin of the entire 484 nucleotides of the sequence cannot be unambiguously stated. However, it is known that large regions of the 5′ terminal genome sequence are present in this repeat. The second cloned DI contained two tandem repeats of sequences overlapping, but not identical to, those in the first clone. Another analysis, by direct sequencing of DI RNA or of cDNA synthesized by reverse transcriptase, of a population of Sindbis DIs gave similar results. The 3′ end of the RNA (50 nucleotides in one case and 73 in another) and a short sequence from the structural protein region of the genome were present, as well as what appeared to be multiple copies of sequences that originated from 5′ end of the genome RNA (*E.G. Strauss*, unpublished work; *Monroe* et al. 1982). Thus alphavirus DIs contain not only multiple deletions, but also duplications and rearrangements.

It was postulated earlier that synthesis of full-length plus and minus strand alphavirus RNAs require different recognition signals for initiation. The conserved 3′ terminal sequence is thought to be a recognition site required for minus strand synthesis and is strictly conserved in the DI RNA. However, the 5′ terminal sequence is somewhat variable both among DIs and among standard alphaviruses (*Petterson* 1981; *J.-H. Ou, E.G. Strauss, J.H. Strauss,* in preparation) as might be expected if it is a structure rather than a sequence that forms the recognition signal for plus strand synthesis (see Sect. 2.3). Furthermore, we suggested that replication of full-length minus strand templates involves cooperation of the two ends of the 49-S genome, by cyclization, since minus strands corresponding to the subgenomic 26-S RNA are not found. The putative 5′ sequence required for replication does occur in DIs but has been transposed in one DI to a location adjacent to the 3′ end. DI RNAs cyclize much less readily than does the genomic RNA (T. Frey, personal communication), and it is possible that early passage DI RNAs must still cyclize in order to replicate, but as DIs evolve, the translocation of the recognition sequence to a position near the 3′ end obviates the necessity for cyclization. Such a DI RNA might be able to replicate more rapidly. The competitive advantage of alphavirus DIs for replication thus appears to involve: (a) duplication to form multiple tandem repeats of the 5′ genomic sequence, which is probably involved in both replication and encapsidation; (b) strict conservation of the 3′ terminal region; and (c) rearrangements and deletions which bring these important regions into closer juxtaposition as well as removing most of the coding sequences.

Alphavirus DIs have also been isolated from a clone of *Aedes albopictus* cells. These DIs from the invertebrate host interfered with standard virus on subsequent infection of mammalian cells. This illustrates that if host cell factors are involved in conveying replication advantage to alphavirus DIs, these factors must have a wide phylogenetic distribution (*Logan* 1979).

Picornavirus DIs with a number of characterisitics in common have been independently isolated in several laboratories (*Cole* et al. 1971; *Nomoto* et al. 1979; *Lundquist* et al. 1979; *McClure* et al. 1980). These characteristics are very different from those of other virus DIs and reflect differences in the mode of replication of this group of viruses. Picornavirus DIs have relatively small internal deletions of 4%–13% of the genome and these deletions are always located between the site for initiation of translation, at nucleotide 741, and the end of the region encoding the capsid proteins. They can replicate their

RNA in the absence of helper viruses, i.e., they can be translated to produce functional replicase, but require the helper to make particles. Compared to other DIs, they interfere poorly with standard virus replication. These multiple constraints on DIs – that the deletion must be less than a certain size, must not affect initiation of translation, and must allow translation of the polymerase region in the normal reading frame – help to explain why picornavirus DI generation is a rare event. The fact that the DI must encode its own functional polymerase suggests that the poliovirus polymerase is primarily *cis*-active on its own RNA. These results, as well as the fact that the initiation of RNA synthesis and the processing of the replicase from the precursor appear to be coupled, support the hypothesis that the picornavirus replicase is not a catalytic enzyme (see Sect. 2.1.2).

It is clear that the study of DI particles and their modes of replication and interference can tell us a lot about the replication of standard virus. It has also been shown that the presence of DI particles promotes the establishment of persistent infection in culture. However, DI particle generation has only been observed in viruses passed at high multiplicity in tissue culture and DIs as such could be considered laboratory artifacts. Whether these particles can be generated in vivo and what role they could play in attenuation or amelioration of disease in animals is unclear at this time.

## 6 Assembly and Morphogenesis

In the preceding sections, "replication strategy" has been somewhat narrowly defined to include transcription of viral mRNAs, synthesis of antigenomic template RNAs, synthesis of the viral genomes, and translation and primary processing of viral polypeptides. In this section an attempt will be made to summarize the additional strategies employed to assemble the nucleic acid with its protein coat into either a virion or a nucleocapsid structure and, for those viruses that possess an envelope, the posttranslational modification and transport of the virus envelope glycoproteins and the processes involved in the budding event itself.

Two types of organization of identical protein subunits with RNA are possible in which each unit occupies an equivalent or quasi-equivalent position relative to both the RNA and neighboring units. These are the helix and the icosahedron (*Caspar* and *Klug* 1962). Both of these plans are used by the RNA viruses, both for assembly of virions (rod shaped and isometric plant viruses and the isometric picornaviruses) and for nucleocapsids of enveloped virions (helical nucleocapsids in negative strand viruses and coronaviruses and isometric capsids in togaviruses). The assembly of these ribonucleoprotein structures appears to be rapid and complete and to occur in almost all cases by self-assembly, i.e., direct interaction of the nucleic acid with a site or sites on the proteins without enzymatic intervention by nonstructural polypeptides.

The structure of tobacco mosaic virus, a simple rod-shaped virus containing one molecule of RNA and 2200 identical subunits of protein, has been examined in detail for many years. The RNA-protein complex is wound as a helix containing 16 1/3 protein molecules per turn with each protein molecule interacting with three nucleotides. In the fully assembled rod the RNA is completely protected by protein, illustrating that the interaction of capsid protein and triplet is not sequence dependent. Elegant studies have shown that the RNA and protein can be separated and reconstituted to form infectious

virions (reviewed in *Richards* and *Williams* 1976). Depending upon concentration, temperature, and pH, the purified protein exists in solution in a number of oligomeric associations, which can be described as A protein (dimers, trimers, and higher multimers), disks (constisting of two rings of 17 subunits each opposed in a polar fashion), and RNA-free helices with an organization identical to virions. TMV protein will self-assemble into rods with certain heterologous RNA species, but at an efficiency far below that for the homologous genome, indicating that a specific nucleation sequence in the TMV RNA to which the first protein binds is required for efficient encapsidation. Similarly, it has been determined that assembly preferentially begins with binding of a disk to the RNA, suggesting that initial contact involves 17–34 protein subunits binding to >51 nucleotides (*Zimmern* 1977). Nucleation begins at a specific nucleotide sequence approximately 150 nucleotides long located 900–1350 nucleotides from the 3′ end of the genome, in a region encoding a minor protein of 28 K. From this position elongation of the rod proceeds bidirectionally to both ends of the molecule (*Otsuki* et al. 1977), but the rate of elongation toward the 5′ terminus is ten times that toward the 3′ terminus (*Fukuda* et al. 1978). The nucleation sequence is homologous to, but by no means identical to, a portion of the coat protein cistron, which may explain the encapsidation in some TMV strains of the subgenomic coat message which lacks the primary nucleation site (*Otsuki* et al. 1977).

The fact that nucleation of TMV RNA by its capsid protein involves multimers of the capsid protein which attach to a moderately long sequence, found neither at the 3′ terminus nor at the 5′ terminus of the genome, means that it differs in important details from other helical self-assemblies such as the helical nucleocapsids of the negative strand viruses. In the case of rhabdoviruses and paramyxoviruses and probably of influenza virus, it is clear that protein-RNA association begins at or near the 5′ terminus of the RNA, most likely concomitant with replication. The leader RNAs of VSV, of both positive and negative sense, consisting of the terminal 46 nucleotides of the genome, are efficiently encapsidated (*Blumberg* and *Kolakovsky* 1981), as are the DI particles of paramyxoviruses, which consist of the 5′ termini of the RNA and its complement, but have lost most of the internal cistrons of the genome (*Leppert* et al. 1977). In the case of influenza virus all eight segments of the genome are equally encapsidated, and the most likely candidate for an encapsidation signal is the common sequence of 13 nucleotides present at the extreme 3′ and 5′ termini of the genomic (and antigenomic) RNAs (*Robertson* 1979). The presence of the host-derived caps and primers at the 5′ termini may prevent encapsidation, as influenza mRNAs are not found in RNP complexes.

The self-assembly of icosahedral protein-RNA particles is exemplified by the picornaviruses and the small spherical plant viruses and by the icosahedral nucleocapsids of the togaviruses. As reviewed by *Brown* (1980), the assembly of alphavirus nucleocapsids appears rapid and efficient and no intermediates have been identified. No empty shells of protein are seen during normal infection, nor are condensed RNA cores found in the absence of capsid protein synthesis. It has been postulated (Sect. 2.3.2) that nucleation or encapsidation begins at a specific sequence near the 5′ terminus of the genomic RNA, since neither the negative strand template nor the subgenomic message is encapsidated. Furthermore, as previously described (Sect. 5), alphavirus DI particles contain amplified copies of this region of the genome. Comparison of the amino acid sequences of the capsid proteins of two related alphaviruses (Sindbis and Semliki Forest viruses) reveals that the N-terminal half of both proteins contains clusters of basic residues and proline, reminiscent of the composition of histones, but that there is little direct homolo-

gy in this region (*Garoff* et al. 1980a; *Rice* and *Strauss* 1981). This indicates that while the nucleation event may be quite specific, the general protein-RNA interaction appears to be an electrostatic attraction between positively charged polypeptide domains and the negative charged nucleic acid. The structure of the nucleocapsid could involve the C-terminal domains of the proteins, which are highly homologous, forming the shell of the capsid while the N-terminal domains penetrate into the interior and interact with the RNA in a nonspecific fashion. The conserved C-terminal domains have also been postulated to be the site of interaction with the envelope proteins to facilitate budding (*Strauss* and *Strauss* 1977; *Rice* and *Strauss* 1981), and this region could be involved in the formation of a nucleation complex as well. The absence of either free genomic 49-S RNA or free capsid protein suggests that the nucleocapsid formation is concomitant with RNA replication (the initiation complex model).

Self-assembly of picornaviruses follows a somewhat different pathway, which may be summarized in the following steps: (a) synthesis of the polyprotein containing the amino acid sequences of the four coat proteins (see Sect. 2.1.1); (b) association of pentameric subassemblies of this polyprotein; (c) cleavages in the polyprotein to give VP0, VP3, and VP1; (d) assembly of a procapsid containing 12 pentamers; (e) addition of the RNA to the exterior of the procapsid; and (f) final proteolytic cleavages to the mature VP1, VP2, VP3, and VP4 with concomitant internalization of the genome RNA. This process is reviewed in some detail in *Rueckert* (1976) and illustrates the other general pathway proposed for icosahedral viruses, in which the protein shell is formed first and in one way or another filled with RNA to form the mature virion (the preformed shell model).

Icosahedral plant virus assembly has been reviewed by *Hung* (1976), who points out that for different viruses the relative contributions to virion stability of protein-protein interactions, as opposed to protein-RNA interactions, vary widely. The in vivo or in vitro presence of protein shells lacking RNA is an extreme example of protein-protein stabilization, but it is unknown whether such shells are intermediates in virion assembly, later filled with RNA, or merely very stable abortive products. The two alternative models of particle assembly, the initiation complex model (exemplified by the assembly of alphavirus nucleocapsids) and the preformed shell model (employed by the picornaviruses), have both been proposed for assembly of these viruses. In general the in vivo assembly of plant viruses is little understood and either or both of these models may be used by isometric plant viruses.

Reassembly of small icosahedral plant viruses from the RNA and the capsid protein has been attempted in a number of different systems with varying degrees of success. Some of the particles produced are morphologically very similar under the electron microscope to authentic virions, but the conditions of pH and ionic strength necessary for artificial reassembly are very different from the physiological parameters of the host cytoplasm and the reassembled viruses are generally less stable to subsequent disassembly (*Hung* 1976).

In the case of the enveloped viruses, which include all of the minus stranded viruses, the togaviruses, and the coronaviruses, there is an additional step in virus assembly, the acquisition of the envelope around the nucleocapsid. This involves the synthesis and modification of membrane glycoproteins and their transport to the site of virus assembly on one of the host cell membranes. Viruses use the normal cellular mechanisms for these processes, and the study of enveloped viruses has been a useful tool for the study of

membrane biogenesis (reviewed in *Compans* and *Klenk* 1979; *Lodish* et al. 1981; *Simons* and *Garoff* 1980).

The insertion of the glycoproteins into the endoplasmic reticulum and the core glycosylation of many of these proteins have been studied in vivo and in vitro. The insertion is a cotranslational process and may utilize either N-terminal signal sequences, as in the case of the G protein of VSV (reviewed in *Lodish* et al. 1981) and the PE2 protein of alphaviruses (*Garoff* et al. 1978); or internal signal sequences, as in the case of the alphavirus E1 (*Hashimoto* et al. 1981; *Garoff* et al. 1980b; *Rice* and *Strauss* 1981; *Welch* et al. 1981) and possibly the neuraminidase of influenza virus. These signal sequences may (e.g., VSV G protein, *Chatis* and *Morrison* 1979) or may not (e.g., alphavirus PE2, *Bonatti* and *Blobel* 1979; *Bell* et al. 1982) be removed by signalase during synthesis of the polypeptide. Glycoproteins are glycosylated while nascent by the transfer of high-mannose carbohydrate chains from a dolichol intermediate (*Sefton* 1977; *Rothman* and *Lodish* 1977). Following synthesis and core glycosylation the glycoproteins are transported to the virus assembly site, which is specific for the virus group, and are usually further modified in the process.

For the coronaviruses, assembly takes place at the rough endoplasmic reticulum (reviewed in *Siddell* et al. 1982). Coronaviruses have two glycoproteins. One appears to be the equivalent of a matrix protein (see below) and is one of only two examples of polysaccharides linked to virus proteins through O-glycosidic linkages. It is tempting to speculate that the O-glycosidic linkage of the polysaccharide is related to the site of virus assembly. The second glycoprotein or peplomer protein contains complex carbohydrates as well as covalently attached lipids, both modifications which normally occur in the Golgi apparatus. In addition, the peplomer glycoprotein is cleaved into two components in most coronavirus strains, a modification which probably also occurs in the Golgi apparatus in the case of many viruses. Thus even if virus assembly occurs at the rough endoplasmic reticulum, there may be Golgi involvement in the final maturation of the virion.

For the flaviviruses (reviewed in *Westaway* 1980) and the bunyaviruses (reviewed in *Bishop* and *Shope* 1979), assembly takes place at the smooth endoplasmic reticulum or within the Golgi apparatus. No budding figures have been observed for flaviviruses, and how these viruses acquire their membrane is unclear. It is noteworthy that no preformed nucleocapsids have been isolated from flavivirus infected cells, whereas preformed nucleocapsids can be isolated from cells infected with most enveloped viruses. Bunyaviruses, on the other hand, have been reported to bud into Golgi vesicles. In the case of both viruses the Golgi apparatus appears to be the final destination of the glycoproteins.

Alphaviruses, rhabdoviruses, myxoviruses, paramyxoviruses, and arenaviruses bud from the host cell plasma membrane (reviewed in *Compans* and *Klenk* 1979). The virus glycoproteins are synthesized and core glycosylated, as described above, and transported to the Golgi. Transport of the VSV G protein, and presumably of all the virus glycoproteins, from the endoplasmic reticulum to the Golgi has been found to be mediated by clathrin-coated vesicles (*Rothman* and *Fine* 1980). In the Golgi the attached carbohydrate moieties are trimmed and some are modified to complex polysaccharide chains by the addition of galactose, fucose, and sialic acid. Some glycoproteins are further modified by covalent attachment of fatty acids (*Schmidt* et al. 1979; *Schmidt* and *Schlesinger* 1979) to their hydrophobic membrane-spanning regions (*Rice* et al. 1982; *Capone* et al. 1982). This event also appears to take place in the Golgi, but the exact location of the enzymes responsible is not known. Attachment of fatty acids is a late modification of viral glyco-

proteins; a mutant of VSV G protein has been isolated which is blocked at a late stage of maturation and contains complex carbohydrates with galactose and sialic acid, but lacks fatty acids and fucose (*Zilberstein* et al. 1980). Lipids have been found attached to the G protein of rhabdoviruses, both glycoproteins of alphaviruses, the F protein of paramyxoviruses, and the HA2 polypeptide of influenza (*Schmidt* 1982). Other membrane glycoproteins, such as the HN protein of paramyxoviruses and the neuraminidase of influenza, lack attached lipids (*Schmidt* 1982), and it is unclear what role these moieties play in viral membrane assembly and virion morphogenesis.

Another modification of membrane glycoproteins which is believed to occur in the Golgi is a proteolytic cleavage event (*Garoff* et al. 1980b; *Rice* and *Strauss* 1981) catalyzed by an enzyme which recognizes a sequence of at least two basic amino acids (*Dean* and *Judah* 1980). This cleavage is followed by a trimming event in which the basis residues are removed, either by the same Golgi enzyme or by a virus-specified protease (*Bosch* et al. 1981). For paramyxoviruses (Sect. 4.2) and influenza (Sect. 4.3.4) this processing is not necessary for virion assembly, since normal yields of particles, albeit noninfectious, are produced containing uncleaved $HN_0$, $F_0$, or HA polypeptides (reviewed in *Klenk* and *Rott* 1980). On the other hand, the cleavage of the precursor to produce E2 and E3 of the alphaviruses is essential for virion maturation, since no virus particles are formed in the absence of cleavage (reviewed in *Brown* 1980).

Following modification, the mature glycoproteins of alphaviruses, rhabdoviruses, myxoviruses, paramyxoviruses, and arenaviruses are transported, apparently in clathrin-coated vesicles, from the Golgi apparatus to the plasma membrane (*Rothman* and *Fine* 1980). The role of glycosylation in transport and function of membrane proteins appears to be at the level of overall conformation of the particular polypeptide, rather than the presence of the carbohydrate per se. For the alphaviruses, nonglycosylated forms of the glycoproteins are not incorporated into virions, and no budding virus is observed when infection is carried out in the presence of tunicamycin (*Leavitt* et al. 1977). Normal yields of infectious virus are obtained in mutant cells that lack *N*-acetylglucosaminyltransferase activity, however, indicating that complex oligosaccharide chains are not required (*Schlesinger* et al. 1976). For some influenza strains particles of low specific infectivity are produced in reduced yield in the presence of tunicamycin, indicating that carbohydrate is not absolutely required for transport and assembly but does affect function (*Compans* and *Klenk* 1979). As noted above for paramyxoviruses (Sect. 4.2), the nonglycosylated form of one protein, F, is inserted normally into the plasma membrane, but the HN protein lacking carbohydrate assumes a different configuration at the cell surface from the glycosylated moiety; normal yields of particles are released which are not infectious (*Morrison* et al. 1981). For rhabdoviruses the situation is more complex, and different strains of VSV are affected differently by growth in tunicamycin (*Gibson* et al. 1979). Furthermore, different G protein mutants vary in their requirements for carbohydrate; some require glycosylation for proper insertion into the plasma membrane while others are insensitive to the presence of polysaccharide chains (*Chatis* and *Morrison* 1981). Thus the requirement for carbohydrate chains in order to form a functional protein depends upon the virus strain and can even be affected by point mutations in the polypeptide.

Although the glycoproteins of all five of these groups of viruses are targeted for the plasma membrane, it is noteworthy that the G protein of VSV appears to be addressed differently from those of other groups of viruses. In cultured epithelial cells which retain an apical and a basolateral domain, VSV buds from the basolateral surface whereas

alpha-, myxo-, and paramyxoviruses bud from the apical surface (*Rodriguez-Boulan* and *Sabatini* 1978), and the distribution of virus glycoproteins in the plasma membrane mimics this distribution (*Rodriguez-Boulan* and *Pendergast* 1980). This asymmetry of maturation is even preserved when cells are mixedly infected with both VSV and influenza virus (*Roth* and *Compans* 1981). This implies that the surface from which the virus buds is determined by the address carried on the protein moiety of the virus glycoproteins. This hypothesis is supported by work with temperature-sensitive mutants of an alphavirus, Semliki Forest virus. At the nonpermissive temperature certain of these mutants make glycoproteins which are not transported to the plasma membrane but which instead remain in the endoplasmic reticulum or in the Golgi apparatus. In the case of one such mutant, the glycoprotein becomes functional upon shiftdown to the permissive temperature and the accumulated protein in the Golgi apparatus leads to virus budding into the Golgi immediately upon shiftdown. Somewhat later after shiftdown, budding virus can be seen at the plasma membrane as the protein is transported to the cell surface (*Saraste* et al. 1980).

The nature of the address signal in a virus glycoprotein is unknown at this time. It seems likely that the address resides in the conformation of the protein, or of some section of the protein, rather than in a single linear sequence of amino acids. We have recently sequenced two temperature-sensitive mutants of Sindbis virus with defects in glycoprotein E1, together with revertants of these mutants. These mutants are of the thermoreversible type described above, wherein mutant glycoprotein accumulates inside the cell at the nonpermissive temperature but is transported to the plasma membrane upon shiftdown to the permissive temperature. One of these mutants has two mutations in E1, one at residue 106 and the other at residue 267, both of which must revert in order to restore the wild-type phenotype and permit migration of the protein to the cell plasmalemma at the elevated temperature. The other mutant has a single change at residue 176 (*C. Arias-Ortiz* et al. 1983 in press). The scattered nature of these changes supports the hypothesis that the conformation of the protein is important for transport of the glycoprotein to the proper organelle within the cell.

The final stage in the assembly of enveloped virions involves an interaction between the nucleocapsid and the virus-modified membrane. This interaction has been studied indirectly in a variety of ways, such as studies on phenotypic mixing between related and unrelated viruses, the degree of exclusion of host membrane proteins from virions, and isolation of mutants whose budding is abnormal. Two types of interaction exist: direct binding of the nucleocapsid to the cytoplasmic domains of transmembranous glycoproteins (exemplified by the alphaviruses), or an interaction through a matrix protein which lines the cytoplasmic face of the lipid bilayer (exemplified by the rhabdoviruses, orthomyxoviruses, and paramyxoviruses).

Direct binding is a very specific event, and the equimolar ratio of glycoprotein and nucleocapsid protein suggests that for alphaviruses each glycoprotein subunit interacts with a single capsid protein molecule (reviewed in *Strauss* and *Strauss* 1977). Studies of Sindbis temperature-sensitive mutants have shown that in some cases, if the glycoproteins are present in the plasma membrane then the nucleocapsids will line up along the cytoplasmic face of the plasmalemma, even at the nonpermissive temperature when no budding can take place (*Brown* 1980). Moreover, during wild-type infection host glycoproteins are rigorously excluded from mature virus particles (*Strauss* 1978). Phenotypic mixing experiments have shown that alphaviruses such as Sindbis and Western equine

encephalitis virus can exchange glycoproteins during budding (*Strauss* et al. 1983 in press), but that alphaviruses cannot use the glycoproteins of unrelated viruses such as VSV to form virions (*Zavadova* et al. 1977).

For virus groups which have a matrix protein, the interaction of the glycoproteins with the nucleocapsid is much less specific. Rhabdoviruses, for example, will form phenotypically mixed particles which contain VSV nucleocapsids, VSV matrix protein, and envelope glycoproteins contributed by a variety of heterologous viruses, including alphaviruses, paramyxoviruses, orthomyxoviruses, retroviruses, and herpesviruses (*Pringle* 1977). As noted above, however, phenotypic mixing between alphaviruses and VSV is asymmetric, and particles containing Sindbis capsids with rhabdovirus surface antigens are not found (*Zavadova* et al. 1977). It is of interest that although VSV will incorporate glycoproteins from many viruses during assembly, most host glyproteins are excluded (*Lodish* and *Porter* 1980b); thus there is some specificity in the interaction of the matrix protein with the glycoproteins, but much less than in the case of the alphaviruses.

Although the interaction of the VSV matrix protein with its glycoprotein shows relatively low specificity, the interaction of matrix protein with the nucleocapsid shows much higher specificity. The ratio of matrix protein to nucleocapsid is constant, whereas the relative proportion of G can vary over a sixfold range (*Lodish* and *Porter* 1980a). It has also been found in phenotypic mixing experiments that VSV nucleocapsids are always associated with VSV matrix protein, implying that the interaction is specific. Moreover, functional M protein is essential for the maturation of VSV virions, since M protein mutants produce no particles at the nonpermissive temperature (*Weiss* and *Bennett* 1980).

Myxoviruses and paramyxoviruses will also phenotypically mix (*Granoff* and *Hirst* 1954), although host proteins are not incorporated into the virions of influenza (*Holland* and *Kiehn* 1970) and the interaction of the matrix protein of these viruses with the glycoproteins appears to have a specificity resembling that of VSV. The influenza matrix protein contains two hydrophobic domains buried in the lipid bilayer (*Gregoriades* and *Frangione* 1981) and can be incorporated in vitro into preformed liposomes (*Bucher* et al. 1980). Exogenously supplied neuraminidase is capable of associating more readily with such liposomes than with liposomes without matrix protein (*Davis* and *Bucher* 1981).

In summary, it appears that the matrix proteins of negative strand viruses interact very specifically with their respective nucleocapsids. Although the interaction between it and the glycoproteins is less specific, the M protein probably plays a role in selecting virus-encoded polypeptides and excluding host components. The arenaviruses and the bunyaviruses apparently lack matrix proteins, and the organization of their envelopes is unknown.

The glycoprotein of coronaviruses is intermediate in characteristics between transmembranous glycoproteins such as those of alphaviruses or VSV and the matrix proteins of VSV or influenza. A small portion of the polypeptide, containing the carbohydrate, is external to the bilayer, but the bulk of the protein is on the cytoplasmic side (*Siddell* et al. 1982). The characteristics of the interactions during assembly may also be intermediate, but no phenotypic mixing experiments between coronaviruses and heterologous viruses have been reported.

## 7 Concluding Remarks

It is clear that the positive strand RNA viruses exhibit a much greater degree of diversity than the negative strand viruses. This is true both in terms of morphology and in terms of replication strategies. The plus stranded plant viruses range from small icosahedral particles to long and flexuous rods. The plus stranded animal viruses include both non-enveloped icosahedral forms and enveloped viruses which may have either cubic or helical nucleocapsids. In contrast, all negative strand viruses, plant or animal, are enveloped, somewhat similar in size and complexity, and possess helical nucleocapsids. Plus strand viruses also display a remarkable divergence in genome structure, reflecting the dual role of the RNAs as both genomes and functional mRNAs. The 5′ terminus of the genomic RNA may be unmodified as in some plant viruses, or may possess one of two types of modification: a genome-linked protein (thought to be important in RNA replication) or a cap (important in translation). The 3′ terminus of these viruses may consist of a poly(A) tract, as do a number of eukaryotic mRNAs; may form a tRNA-like structure in many plant RNAs, which is thought to be necessary for RNA replication; or may exhibit neither of these properties. The genomic RNAs of the minus strand viruses are unmodified at their termini, although the mRNAs produced by most of these viruses are capped and polyadenylated. Furthermore, the plus stranded viruses appear to have evolved unrelated sequence signals for transcription, replication of antigenome templates, and replication of genomes. The minus stranded viruses seem to use the same primary recognition sequence for these activities; for all three activities, the RNA template is present as a ribonucleoprotein complex or nucleocapsid structure, and all minus strand viruses except the myxoviruses have a replicase protein of about 200 000 daltons. These characteristics suggest that the plus stranded viruses are the more ancient group evolutionarily, whereas the negative strand viruses arose more recently and still show considerable evidence of a common ancestral heritage. Of great interest in this regard is a recent analysis which established that two of the more dissimilar negative strand virus groups are distantly related: computer analysis of the primary amino acid sequence of the matrix proteins of VSV and influenza (*Rose* et al. 1982) showed that they were derived from a common ancestor.

Within the negative strand viruses, the remarkable similarity in genome structure of the paramyxoviruses and rhabdoviruses makes it likely that they are fairly closely related. Orthomyxoviruses appear to be more distantly related because of their unusual mode of producing messenger RNAs. The situation with the bunyaviruses and arenaviruses is less clear. These two virus groups have a similar genome organization and differ from the other groups of viruses in certain important details, such as the lack of a matrix protein. Sequence studies of these viruses and computer searches for homologies such as those mentioned above will be of great interest in working out the evolutionary history of these viruses.

Similarities in the genome organization of a number of plant and animal viruses have been referred to in this review. It is unclear at present whether this results from convergent evolution or descent from a common ancestor. Again, sequence studies of these viruses will be of considerable interest in determining whether the viruses had a common origin and how closely related they are.

Throughout this review we have postulated that the RNA viruses, both plus stranded and minus stranded, use sequences of about 20 nucleotides in length as recognition

signals for replicases and transcriptases; the exact nucleotide sequence could determine the interaction with the enzymes. A second type of replication element may also be used, although less frequently; namely a secondary structure in the RNA which could be recognized by the replicase. Such structures are found most often in the plant viruses, but the alphaviruses also may use such signals. Further studies with DI RNAs and with virus variants whose sequences differ in these key regions will be of great value in defining these sequence elements and structures.

We began this review with the premise that with the available nucleotide and protein sequence data about viruses expanding at a very rapid rate, it might be timely to consider the relationship of sequence and genome organization to replication strategy. Nucleotide sequencing of a virus genome or of one its genes can elucidate the translation strategy (initiation codons, termination codons, number of open reading frames, etc.) as well as provide a deduced amino acid sequence of the proteins. When the sequence of the genome is augmented by direct protein sequence of its virus-encoded products and sequence of mRNA transcripts, accurate models for both transcription and protein processing can be drawn. Genetics takes on a new dimension when the amino acid changes responsible for a lesion in a mutant can be identified and correlated with the function of a virus gene. For determining common sequence elements necessary for replication, and for a better understanding of the functional domains of virus proteins, however, the study of a number of related viruses is invaluable. Comparative sequence analysis can identify regions of conservation of amino acid sequence and regions of conservation of nucleotide sequence. Because of the rapid evolution of RNA viruses (*Holland* et al. 1982), these two are not equivalent; the degeneracy of the code allows nucleotide sequence divergence even when amino acid sequence is conserved (*Ou* et al. 1982a, b). The significance of conserved nucleotide sequences as control elements can be tested directly using the methods of modern genetic engineering; synthetic viruses, or more specifically synthetic DIs, can be constructed containing primarily these hypothetical replication signals and tested for selective advantage and interference with standard virus. It may be possible to use such artificial virions as cloning vectors by fusing recognition elements to heterologous genes. Furthermore, this approach may be usable for production of vaccine strains of pathogenic viruses by manipulating the genome to produce a mutant containing the recognition signals and therefore efficient for replication, but lacking the sequences responsible for pathogenicity. Such chemical recombination experiments will be of great help in our understanding of the molecular mechanisms involved in the replication of RNA viruses.

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# Hepadna Viruses: Hepatitis B and Related Viruses

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## 1 Introduction

The discovery of Australia antigen in human serum by *Blumberg* et al. (1965) and its subsequent association with viral hepatitis led to the identification and characterization of one of the most common and interesting human hepatitis viruses, hepatitis B virus (HBV). Australia antigen is now known to be a viral-specified antigen found on the surface of hepatitis B virions and it is currently known as hepatitis B surface antigen (HBsAg). The development of serologic tests for HBV antigens and their respective antibodies made possible the recognition that HBV has a worldwide distribution and that infection rates in some parts of the world are extremely high.

Investigation of HBV during the early and mid 1970s revealed unique antigenic, ultrastructural, molecular and biological features which distinguished it from members of all recognized virus groups. One of the most notable features of this virus was its DNA structure. Virions contain small, circular DNA molecules (*Robinson* et al. 1974) that are partly single-stranded (*Summers* et al. 1975; *Landers* et al. 1977; *Hruska* et al. 1977), and a DNA polymerase in the virion (*Kaplan* et al. 1973; *Robinson* and *Greenman* 1974) can repair the DNA to make it fully double-stranded. Among the unique biological features are its liver tropism and the common occurrence of persistent infection, with viral antigen (*Bayer* et al. 1968) and infectious virus (*Barker* and *Murray* 1972) in high concentrations remaining in the blood for years. This pattern of infection accounts for the com-

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mon transmission of HBV by percutaneous transfer of serum and serum-containing material. Several disease syndromes in addition to acute hepatitis may occur during acute and chronic HBV infection. Included are a serum-sickness-like syndrome, membranous glomerulonephritis, and necrotizing vasculitis (polyarteritis) (*Gocke* 1975), all probably related to viral antigen–antibody-complex-mediated tissue injury. In addition, persistent infection can be associated with near-normal liver or with chronic hepatitis. The latter may be severe and progressive and lead to cirrhosis and, in some cases, hepatocellular carcinoma (*Szmuness* 1978). This wide spectrum of disease manifestations appears to involve several different pathogenetic mechanisms. Its narrow host range-confined to man and a few higher primates- and failure so far to infect tissue culture cells have made investigation of some questions about HBV difficult.

Although for several years HBV was considered to be a unique virus, similar viruses have recently been found in three different animal species. The first was found in 1978 in sera of eastern woodchucks (*Marmota monax*), members of the Sciuridae or squirrel family, by *Summers* et al. (1978). Robert Snyder had been observing the diseases of woodchucks at the Penrose Laboratory of the Philadelphia Zoo since 1960. The most frequent cause of death in these animals was hepatocellular carcinoma accompanied by a particular form of hepatitis with degenerative and regenerative changes. The same chronic hepatitis was found in some of the other animals without hepatocellular tumors. As a result of these studies, *Snyder* (1968) proposed a viral cause of the woodchuck hepatitis which *Summers* et al. (1978) showed to be correct. The sera of animals with chronic hepatitis were found to have an HBV-like virus form containing DNA polymerase activity and a circular, partially double-stranded DNA similar in size to that of HBV. The virus was called woodchuck hepatitis virus (WHV).

Discovery of the third member of this virus family, ground squirrel hepatitis virus (GSHV), was reported in 1980 by *Marion* et al. (1980a). This virus was found in the Beechey ground squirrel (*Spermophilus beecheyi*), another genus of the Sciuridae family, during a search for an HBV-like virus in California relatives of the eastern woodchuck.

The observation of frequent hepatomas in a species of domestic ducks in the People's Republic of China led to the discovery of the fourth member of this virus family. Some sera from ducks of this species were found by Summers, London, Sun, Blumberg et al. (unpublished data) to contain an HBV-like virus. Attempts to passage this virus in eggs from Pekin ducks (*Anas domesticus*) from the United States led to the discovery by *Mason* et al. (1980) that approximately 10% of Pekin ducks in some commercial flocks in this country carry a similar virus, called duck hepatitis B virus (DHBV). It is not yet known whether the virus from Chinese ducks is the same as that from domestic Pekins in the United States, but the Pekin duck is of Chinese ancestry, having been introduced to this country from China in the nineteenth century.

HBV and the three related viruses of lower animals may be only the first discovered members of a larger group of related viruses, now called the hepadna (for "hepatic" and "DNA") viruses (*Robinson* 1980). The goal of this review is to examine the similarities and the range of differences of the known members of this unique virus family.

## 2 Virus Morphology and Antigenic Structure

The hepatitis B virion was first observed in electron micrographs in 1970 as a spherical particle approximately 42 nm in diameter (*Dane* et al. 1970). It has an electron-dense,

Table 1. Physical properties of hepadna viral forms

| | Virion | | Surface | | Core particle | | |
|---|---|---|---|---|---|---|---|
| | Diameter (nm) | Buoyant density in CsCl (g/ml) | Diameter (nm) | Buoyant density in CsCl (g/ml) | Diameter (nm) | Buoyant density in CsCl (g/ml) | Filaments |
| HBV | 42 | 1.24 | 22 | 1.19–1.20[a] | 27 | 1.34 | Present |
| WHV | 45 | 1.225 | 20–25 | 1.18[b] | 27 | 1.34[b] | Present |
| GSHV | 47 | 1.24[c] | 15–25 | 1.18[a] | 30 | 1.34[d] | Present (long, abundant) |
| DHBV | 40–45 | 1.16 | 40–60 | 1.14 | 27 (Spikes) | 1.34[c] | Absent |

[a] *Gerlich* et al. (1980); [b] *Werner* et al. (1979); [c] P. Marion, unpublished observations; [c] *Feitelson* et al. (1982a)

spherical inner core with a diameter of approximately 27 nm and an outer shell or envelope approximately 14 nm in thickness. The lipid-containing envelope bears the hepatitis B surface antigen (HBsAg) (*Dane* et al. 1970; *Almeida* et al. 1971) which is contained in a viral genome-specified polypeptide to which virus-neutralizing antibody (anti-HBs) is directed. The inner core particles which can be released from virions by detergent treatment bear the hepatitis B core antigen (HBcAg) (*Almeida* et al. 1971) and contain the viral DNA (*Robinson* et al. 1974), the DNA polymerase activity (*Kaplan* et al. 1973; *Robinson* and *Greenman* 1974), and a protein kinase activity which phosphorylates the viral genome-specified major polypeptide of the core (*Albin* and *Robinson* 1980).

The virions of WHV (*Summers* et al. 1978), GSHV (*Marion* et al. 1980a), and DHBV (*Mason* et al. 1980) have ultrastructural features similar, but not identical, to those of HBV, and each virus appears to have surface and core antigens analogous to those of HBV. Table 1 lists some of the ultrastructural similarities and differences of the four hepadna viruses. The virion morphologies of the three mammalian viruses are more similar to each other than to the duck virus, which is more pleomorphic and whose core is covered with spike-like projections not apparent on the cores of the mammalian viruses. Similarly, the virion buoyant densities of the three mammalian viruses are closer to each other than to that of the DHBV, which is significantly lower (*Mason* et al. 1980). Core particles of the GSHV contain a protein kinase activity which phosphorylates the major core polypeptide (*Feitelson* et al. 1982b) analogous to the enzyme in HBV cores (*Albin* and *Robinson* 1980). No information is yet available about such enzyme activity in the other viruses.

In addition to virions, there are more numerous particulate forms which bear HBsAg in serum of HBV-infected patients (*Bayer* et al. 1968; *Dane* et al. 1970; *Almeida* et al. 1971). These are small (16–25 nm in diameter) spherical particles and filamentous forms 22 nm wide and varying in length up to several hundred nm. These particles are composed of lipid, protein, and carbohydrate and lack HBcAg, nucleic acid, or other virion core components. Thus, they are considered to be incomplete viral forms. Similar particles, which apparently carry the respective surface antigens, are associated with infection with the other three hepadna viruses (*Summers* et al. 1978; *Marion* et al. 1982a; *Mason* et al. 1980), except that the filamentous forms have not been described for the

DHBV. The filamentous forms in ground squirrel serum are particularly long and numerous. The spherical particles associated with DHBV are larger and more pleomorphic than those of the mammalian viruses.

The third HBV antigen, the hepatitis B e antigen (HBeAg), was first detected as a soluble antigen in serum of HBV-infected patients (*Magnius* and *Espmark* 1972). The soluble HBeAg is antigenically and physically distinct from HBsAg particles described above. HBeAg also appears to be present in a cryptic form in the virion core and can be detected only after disruption of core particles, as for example, by detergent treatment (*Takahashi* et al. 1979). The major polypeptide of virion cores appears to manifest HBeAg specificity when isolated from cores (*Takahashi* et al. 1979). Antibody reacting with HBeAg has been found in sera of some infected woodchucks (*Lutwick* et al. 1981), and antigen reacting with anti-HBe is found in the sera of infected ground squirrels (*Marion* et al., unpublished observations), indicating that antigens related to HBeAg are associated with WHV and GSHV infections. There is no information of this kind about the DHBV.

The antigenic structure of HBsAg has been analyzed in much more detail than that of the surface antigens of the other viruses. HBsAg is a complex antigen with an *a* determinant which is found in all HBsAg preparations and two sets of subtype determinants (*d/y, w/r*) (*Courouce-Pauty* and *Solier* 1974). HBsAg preparations contain determinants *d* or *y* and *w* or *r*. Antigenic variation of the *w* determinant and additional minor determinants (*Courouce-Pauty* et al. 1976) add to the complexity of HBsAg. Similar antigenic variation of the surface antigens of the other hepadna viruses has not been described, although this question has not yet been investigated in detail.

Several laboratories have attempted to determine the relatedness of the virion antigens of the different hepadna viruses, but it is difficult to compare results because diverse methods have been used in different laboratories to quantitate serological relatedness. Despite these reservations, some conclusions can be made from the existing data. Antibody to HBsAg (anti-HBs) does bind to both WHsAg and GSHsAg, with a reported cross-reactivity of 1% with WHsAg (*Werner* et al. 1979) and 48% with GSHsAg (*Gerlich* et al. 1980), using different assay methods. Two studies measuring the binding of HBsAg by anti-GSHs and anti-WHs showed less binding than did the reverse experiments with anti-HBs, using the same methods. Whether certain antigenic determinants of HBsAg cross-react more strongly than others with the surface antigens of the other mammalian hepadna viruses is not known, but tryptic peptide mapping (described below) suggests that a common amino acid sequence is found in HBsAg of all subtypes, and in GSHsAg and in WHsAg polypeptides (*Feitelson* et al. 1981). This could represent the *a* determinant of HBsAg. Comparison of GSHsAg and WHsAg has shown them to be closely related but not identical (*Feitelson* et al. 1981). While investigators in this country (*Mason* et al. 1980; *Marion* et al. unpublished data) have not detected any serological cross-reactivity between the duck and mammalian virus surface antigens, Chinese workers have described such an interaction with the strains of HBV and the duck virus obtained in China (*Chou* et al. 1980). They observed an inhibition of the binding rate of HBsAg and anti-HBs with the duck virus and aggregates visible with electron microscopy following incubation of three virus-containing duck-serum samples with anti-HBs. Further studies will be necessary to determine whether the Chinese finding of surface-antigen relatedness is due to the particular strains of either the human or duck viruses, or to the serological methods used.

*Werner* et al. (1979) found a high degree of cross-reactivity between WHcAg and HBcAg, using counterelectrophoresis (12.5%–25%). They concluded that since the cross-

reactivity between human and animal virus core antigens was apparently so much greater than that between the surface antigens of these species (1%), the hepadna core antigens might serve as "group-specific" antigens. In support of this, the DNA sequence data of *Galibert* et al. (1982) indicate that there is a greater amino acid-sequence homology between virion core polypeptides (73%) than between surface antigen polypeptides (62%) of HBV and WHV. GSHcAg and WHcAg have not been directly compared. No comparison of the core antigen polypeptides of DHBV with any of the mammalian virus core antigens has been reported.

## 3 Virion Polypeptides

Information on the virion polypeptides of the three mammalian viruses is summarized in Table 2. Multiple polypeptides have been found in purified surface antigen particles of HBV, WHV, and GSHV. A major pair of polypeptides with apparent molecular weights of 25 000 (P-25) and 29 000 (P-29) and a variable number of minor components with apparent sizes up to 100 000 daltons have been repeatedly observed in HBsAg preparations (reviewed by *Robinson* 1977). Most of these react with antibody raised against intact HBsAg particles and against the major polypeptides, indicating that they must share at least some amino acid sequences. This conclusion has been confirmed by tryptic peptide mapping of the different polypeptides (*Feitelson* et al., unpublished results). P-25 and P-29 share amino acid sequences at their carboxy and amino termini (*Peterson* et al. 1977), but only P-29 is glycosylated, suggesting that the two may have identical primary sequences, and differ in electrophoretic mobility only because of the carbohydrate in P-29. The multiple polypeptides of WHsAg and/or GSHsAg may also possess common antigenic determinants, but no data are yet available about this. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) shows that the two major polypeptides of WHsAg and GSHsAg are each smaller than the corresponding polypeptides of HBsAg, and both share a similar tryptic peptide map homology with the HBsAg polypeptides (*Feitelson* et al. 1981). The apparent size difference in the polypeptides of HBsAg and WHsAg determined by SDS-PAGE is not substantiated by the theoretical molecular weights calculated from the nucleotide sequence of HBV and WHV DNA (*Galibert* et al. 1982), suggesting that posttranslational processing, or some other mechanism, may be responsible for the difference in electrophoretic mobility. Evidence for a close relatedness of HBsAg, WHsAg, and GSHsAg comes not only from their serologic cross-reactivity but also from tryptic peptide mapping of their polypeptides. More than 50% of the tryptic peptides of the major nonglycosylated polypeptides of WHsAg and GSHsAg are identical. (*Feitelson* et al. 1981). Of the tryptic peptides of the major nonglycosylated polypeptides of HBsAg (of subtypes *adw, adr,* and *ayw*), GSHsAg, and WHsAg, 25% are identical, indicating that a significant region of this polypeptide has been conserved during the evolution of these viruses. A 66% homology has been found in the nucleotide sequences of the genes for the major surface antigen polypeptides of HBV and WHV (*Galibert* et al. 1982).

Virion cores of HBV, WHV, and GSHV all contain a single major polypeptide of approximately 20 000 daltons (but not identical in electrophoretic mobility) and several minor polypeptides with larger apparent sizes (*Feitelson* et al. 1982a) (Table 3). The nature of the DHBV core polypeptides is unknown. The major core polypeptide of HBV after isolation by SDS-PAGE neither reacts with anti-HBc nor induces anti-HBc in rabbits

Table 2. Polypeptides of mammalian hepadna viruses

| Virus | Theoretical mol. wt. of major sAg unglycosylated polypeptide (K) | Apparent mol. wt. of 2 major sAg polypeptides (K) | Apparent mol. wt. of minor sAg polypeptides (K) | % tryptic map sAg homology to HBsAg | Theoretical mol. wt. of major cAg polypeptide (K) | Apparent mol. wt. of major cAg polypeptide (K) | Apparent mol. wt. of minor cAg polypeptides (K) | % tryptic map homology to HBcAg |
|---|---|---|---|---|---|---|---|---|
| HBV | 25.4[a] | 25, 29[b] | 31, 49, 54, 56, 63, 72[b] | 100 | 21.2[d] | 18[e] | 11, 12, 14, 27.5, 30.5, 34.5, 40, 48, 53, 68, 100[e] | 100 |
| WHV | 25.6[a] | 23, 27[c] | - | 25[c] | 22.0[d] | - | - | ND |
| GSHV | - | 23, 27[b] | 34, 39, 43, 70, 75[b] | 25[c] | | 17.5[e] | 11, 14, 15.5, 26, 28[e] | 56[e] |

[a] *Galibert* et al. (1981); [b] *Gerlich* et al. (1980); [c] *Feitelson* et al. (1981); [d] *Galibert* et al. (1982); [e] *Feitelson* et al. (1982a, b)

Table 3. Characteristics of the DNA of hepadna viruses

| | Size (bp) | Circular | Virion DNA contains single-stranded region | Cohesive ends | Closed circular form in liver |
|---|---|---|---|---|---|
| HBV | 3182 | yes | yes | yes | ? |
| WHV | 3308[a] | yes[b] | yes[c] | yes[b] | yes[d] |
| GSHV | 3250–3300[e] | yes[f] | yes[f] | yes[f] | yes[g] |
| DHBV | 3000[h] | yes[h] | a minority only[c] | yes[k] | yes[k] |

[a] *Galibert* et al. (1982); [b] *Summers* et al. (1980); [c] *Summers* et al. (1978); [d] Summers, unpublished observations; [e] *Siddiqui* et al. (1981); [f] *Marion* et al. (1980); [g] Marion, Rogler, Robinson and Summers, unpublished observations; [h] *Mason* et al. (1980); [i] *Mason* et al. (1981); [j] *Summers* (1981); [k] *Mason* et al. (1982);

(*Hruska* and *Robinson* 1977). It has, however, been reported to react with anti-HBe (*Takahashi* et al. 1979). The antigenic specificity of the polypeptides of GSHV and DHBV cores has not been studied. Approximately 56% of the tryptic peptides of the major core polypeptides of HBV and GSHV are identical, as might be expected in view of the serologic relatedness of HBcAg and GSHcAg (*Feitelson* et al. 1982a). Approximately 62% homology has been calculated for the nucleotide sequences of the genes apparently coding for the core antigens of HBV and WHV (*Galibert* et al. 1982), further establishing their relatedness.

Specific polypeptides with the DNA polymerase and protein kinase activities found in virion cores have not been identified, and the protein covalently linked to the 5′ end of the long strand has not been sized or further characterized.

## 4 Physical and Genetic Structure of the Viral DNA

The virions of all four hepadna viruses contain small circular DNA molecules (see Table 2) that are partially double stranded. In HBV DNA, the DNA which has been studied in greatest detail, the single-stranded portion varies in length from approximately 15% to 60% of the circle length in different molecules (*Summers* et al. 1975; *Landers* et al. 1977; *Hruska* et al. 1977). Thus the DNA consists of a long strand, *L*, of constant length (3182 bases) in all molecules and a short strand, *S*, which varies in length between 1700 and 2800 bases in different molecules. The DNA polymerase activity in the virion (*Kaplan* et al. 1973; *Robinson* and *Greenman* 1974) repairs the single-stranded region in the viral DNA to make fully double-stranded molecules of approximately 3200 base pairs (bp). DNA synthesis is initiated for this reaction at the 3′ end of the short strand, which occurs at different sites within a specific region (50%) of the DNA in different molecules. DNA synthesis is terminated when the uniquely located 5′ end of the short strand is reached. The long strand is not a closed circle, but a nick exists at a unique site approximately 300 bp from the 5′ end of the short strand (*Summers* et al. 1975; *Sattler* and *Robinson* 1979; *Siddiqui* et al. 1979). The circular DNA can be converted to a linear form with single-stranded cohesive ends by selectively denaturing the 300-bp region between the 5′ end of the short strand and the nick in the long strand by heating under appropriate condi-

tions (*Sattler* and *Robinson* 1979). The linear form can be recircularized by reassociation of the complementary single-stranded ends. Although they have the same basic structures as HBV DNA, the DNAs of WHV (*Summers* et al. 1978), GSHV (*Marion* et al. 1980a), and DHBV (*Mason* et al. 1980) are not identical.

The DNAs of the four viruses appear to vary somewhat in length, from approximately 3 000 bp for DHBV DNA to 3 308 bp for WHV DNA, as shown in Table 3. Although the DNAs of all the viruses have a single-stranded region which can be made double-stranded by the endogenous DNA polymerase, the single-stranded region is smaller, on average, in the duck virus and many fully double-stranded molecules can be recovered from duck hepatitis B virions (*Mason* et al. 1981). The DNAs of the three animal viruses appear to have cohesive ends, as does HBV DNA, since they can be made linear by heating. The 5′ ends of both the long and short strands of HBV DNA appear to be blocked in a manner which prevents phosphorylation with polynucleotide kinase (*Gerlich* and *Robinson* 1980). The chemical nature of the blocked 5′ end of the short strand is unknown. Recent evidence indicates that a polypeptide is covalently attached to the 5′ end of the long strand of the DNA isolated from virions and this undoubtedly prevents phosphorylation of this strand (*Gerlich* and *Robinson* 1980). The DNA of the DHBV has recently been shown to contain a similar covalently attached protein (*Molnar-Kimber* et al. 1982). Although a function for this polypeptide has not been shown, it seems likely that it could have a primer activity as do the similar polypeptides in polio and adenoviruses, and be involved in DNA replication as they are (reviewed by *Wimmer* 1982).

The DNAs of all four viruses have been cloned in bacterial cells (Table 2), and the complete nucleotide sequences of HBV (*Galibert* et al. 1979) and WHV (*Galibert* et al. 1982) have been determined. Both HBV and WHV DNAs have four open reading frames in the complete, or long, DNA strand and these have similar locations in each virus with respect to the cohesive ends of the DNAs. The genes for the two major virion polypeptides have been identified. Open reading frame 2 (ORF-2, Fig. 1) contains the S-gene specifying the major surface antigen reactive polypeptide of each virion. Computer analysis shows that the base sequences within the open reading frames of the two viruses are 62%–70% homologous, and there is lower homology in regions that are apparently noncoding. The ATG initiation codon corresponding to the N-terminus of the P-25 surface antigen polypeptide is the third ATG in this reading frame and the nucleotide sequence coding for this polypeptide constitutes only the distal half of ORF-2. This coding sequence in HBV DNA is 681 nucleotides long and specifies a polypeptide of 25 422 daltons. In WHV DNA this sequence is 669 nucleotides, and the coded polypeptide, 25 645 daltons. The nucleotide sequence homology between the S-gene of HBV and WHV is 74%, and the amino acid sequence homology of the polypeptides of the two viruses is 62%. The sequences upstream from the initiation codon for this polypeptide in ORF-2 are called the pre-S region, and if translated in vivo could give rise to a large P-25 precursor with C-terminal sequence identical to that of P-25. Whether such a precursor is actually made in vivo or whether this pre-S region has some other function remains to be determined. Open reading frame 3 (Fig. 1) contains the C-gene which appears to specify the major polypeptide of the virion core. The C-gene of HBV codes for a polypeptide of 183 amino acids and that of WHV a polypeptide of 188 amino acids. The C-genes of the two viruses share 66% nucleotide sequence homology and the coded polypeptides share 73% amino acid sequence homology, reflecting the percentage of silent base differences between the

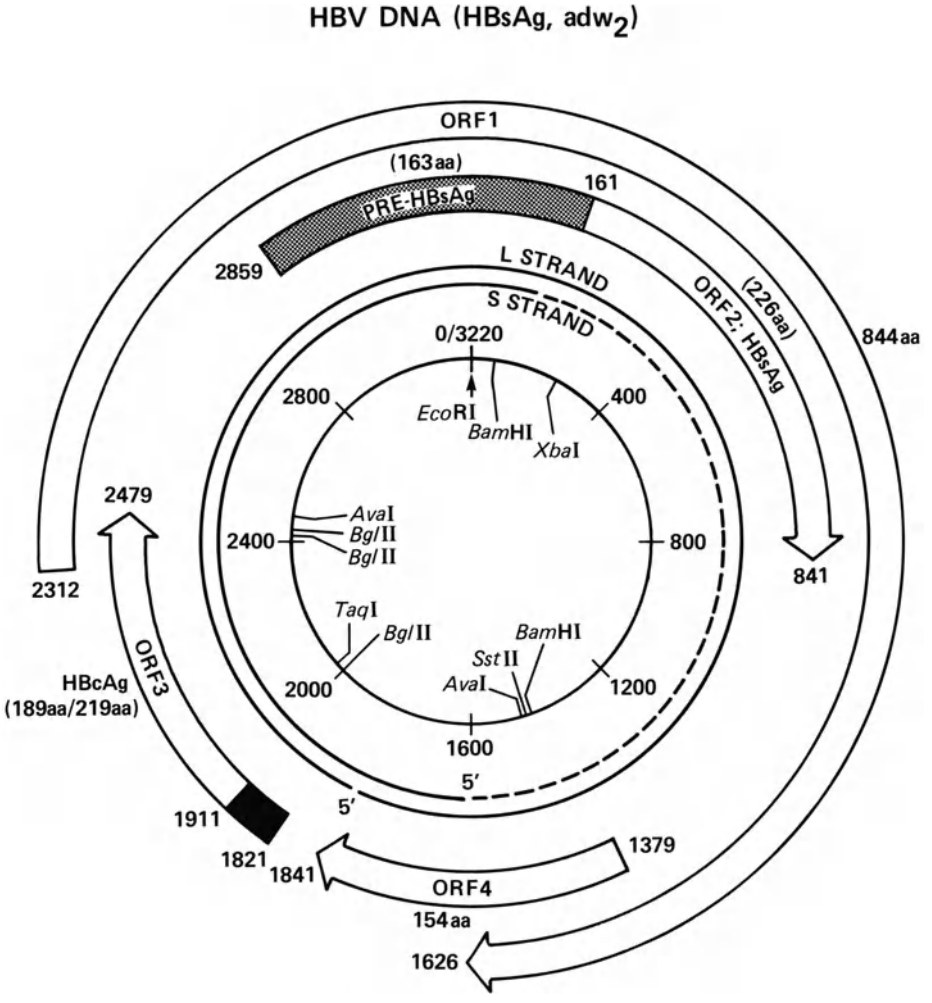


Fig. 1. Physical map of HBV DNA. The DNA strands are represented by the lines labeled *L strand* for long strand and *S strand* for short strand. The *dotted line* represents the region of the short strand synthesized by the virion DNA polymerase reaction, and this region differs in length in different molecules. The arrows labeled *ORF 1–4* represent the positions of open reading frames in the L strand. The inner circle represents a scale of DNA length in base pairs (bp) starting from the single restriction endonuclease Eco*RI* site. Other restriction endonuclease cleavage sites are indicated by the names of the enzymes

C-genes of the two viruses two times higher than that found in the S-genes. Polypeptides coded by the two other open reading frames on the long DNA strand (ORF-1 and 4; Fig. 1) have yet to be identified. ORF-1 completely overlaps ORF-2, which contains the S-gene and could potentially code for a polypeptide of 95 000 daltons. ORF-4 is the smallest potential coding sequence in the long strand and could specify a polypeptide of approximately 16 000 daltons (154 amino acids in HBV and 141 amino acids in WHV).

*Galibert* et al. (1982) have noted that both HBV and WHV DNAs have a sequence within the smallest open reading frame on the long strand (ORF-4) and near the nick in the

strand which can form a stable hairpin structure and which might function as the origin of replication.

Although the DNA sequence of the GSHV is not available, cross-hybridization studies indicate significant homology between HBV and GSHV DNAs. Hybridization with two restriction fragments of HBV DNA containing the coding sequence for the major HBsAg polypeptide (S-gene) and the major core particle polypeptide coding sequence (C-gene) with restriction fragments of GSHV DNA, respectively, revealed separate homologous regions in the two viral DNAs (*Siddiqui* et al. 1981). Each homologous region had approximately the same location with respect to the cohesive ends in the two viral DNAs. Thus all results to date suggest that the S- and C-genes of the three mammalian viruses have similar locations with respect to the unique physical features of the virion DNAs.

There has been no apparent homology detected between DHBV DNA and the DNAs of the mammalian viruses (*Mason* et al. 1980; *Marion* et al., unpublished observations), and nucleotide sequence data are not yet available for DHBV DNA.

## 5 Virus Replication

Relatively few details about molecular events in hepadna virus replication are available because of the difficulties in using liver tissue infected in vivo for such studies and the lack of a more useful system such as infected cells in culture. Several studies have examined the state of hepadna viral components in hepatic tissue during acute and chronic infection and in hepatoma tissue and cell lines isolated from hepatomas. Viral DNA has been found in several forms in infected liver. Southern blot analysis (*Southern* 1975) of DNA as isolated from human (*Brechot* et al. 1981a, b; *Shafritz* et al. 1981; *Brechot* et al. 1982; *Shafritz* 1982), woodchuck (*Ogston* et al. 1981), ground squirrel (*Marion* et al., unpublished data), and duck (*Mason* et al. 1982) liver, each productively infected with the respective homologous virus, has revealed the predominant forms of viral DNA to have electrophoretic behavior of closed circular and relaxed circular unit length (3200 bp) molecules. In addition such analyses of infected human liver have sometimes revealed HBV DNA base sequences in one or more distinct DNA fragments with electrophoretic mobilities corresponding to those of linear molecules of greater than unit length virion DNA (*Brechot* et al. 1982). Because these high-molecular-weight forms have been detected only in liver DNA digested with a restriction enzyme (HindIII) for which HBV DNA contains no sites, and not in undigested DNA, they have been considered to be viral DNA sequences integrated at specific sites in host DNA. The fragment number and sizes vary in different patients, suggesting that the integration site must be variable in different patients. If these high molecular weight bands do represent sites of HBV DNA integration, HBV must differ from other DNA viruses and retroviruses, which integrate at random sites so that no integration site is detected as a defined band in Southern blot analysis, except when the cells analyzed are of clonal origin as in virus-induced transformed cells or tumors.

Southern blot analysis of livers of infected woodchucks (*Summers* et al. unpublished data), ground squirrels (*Marion* et al., unpublished data), and ducks (*Mason* et al. 1982) has failed to demonstrate such high-molecular-weight viral DNA forms. On the other hand, high-molecular-weight forms of viral DNA have been found in productively in-

fected woodchuck (Rogler and Summers, 1982 and ground squirrel (*Marion* et al. 1982) liver when the much more sensitive method of DNA cloning in the lambdoid vector Charon 30 was used. The high-molecular-weight viral DNA forms that have been isolated in this way and analyzed to date appear to be oligomeric, or longer than unit-length viral DNA with deletions and rearrangements. In the woodchuck system, at least, these oligomeric forms have been found integrated into host DNA sequences. The finding of these forms in infected ground squirrel and woodchuck liver raises the question of whether they also exist in HBV-infected human liver and whether the high-molecular-weight DNA bands containing viral sequences detected in Southern blots of HBV-infected liver represent such oligomeric forms alone rather than integrated viral DNA. Proof of the nature of the high-molecular-weight fragments in infected human liver will require a detailed analysis that is most feasible only after the fragments are cloned.

More definitive evidence for integration of viral DNA has been obtained in hepatoma tissue from infected humans and woodchucks and in cell lines isolated from hepatomas. Numerous studies have demonstrated high-molecular-weight DNA fragments containing viral DNA sequences after HindIII digestion of tumor (*Shafritz* et al. 1981; *Brechot* et al. 1982; *Shafritz* 1982; *Chen* et al. 1982) or cultured tumor cell (*Marion* et al. 1980b; *Chakraborty* et al. 1980; *Brechot* et al. 1980; *Edman* et al. 1980) DNA by Southern blot analysis. The specific number and size of high-molecular-weight HBV sequence-containing DNA fragments are not the same in different hepatomas, indicating that the integration sites are different in different tumors. A direct demonstration of viral DNA integration has been done in the woodchuck system. High-molecular-weight DNA fragments from woodchuck hepatomas of the size seen on Southern blots have been cloned in bacterial cells and shown to contain both WHV and host DNA (*Ogston* et al. 1982).

Recent findings by *Summers* and *Mason* (1982) suggest that at least the duck virus DNA may replicate in a unique and interesting way. *Mason* et al. (1982) noted that a large amount of viral DNA from infected duck liver and pancreas migrated on agarose gels as a diffuse band, moving faster than the supercoiled form. Its electrophoretic mobility did not vary with agarose gel concentration or heating as expected for double-stranded DNA, and its sedimentation rate in sucrose gradients was markedly sensitive to salt concentration. These characteristics indicated that this was single-stranded DNA, and its mobility was found to be the same as that of denatured 3000 bp DHBV DNA, indicating that most of the single strands were full length. Single-strand-specific probes prepared in the bacterophage vector M13mp7 demonstrated that the single-stranded DNA present in infected duck liver consisted predominantly of minus strands, i. e., complementary to polyadenylated messenger RNAs found in both duck liver and pancreas. The minus strand in DHBV DNA has been determined to be the complete strand of the virion DNA (*Mason* et al. 1982), as appears to be the case for HBV and WHV (*Galibert* et al. 1982).

*Summers* and *Mason* (1982) then isolated subviral particles resembling virion core particles from infected duck liver. These particles were shown to incorporate deoxynucleoside triphosphates into single-stranded viral DNA (both plus and minus strands). Newly synthesized plus strands were nuclease $S_1$ resistant in contrast to the nascent minus strands which were sensitive and thus probably single-stranded. Plus-strand and not minus-strand synthesis was sensitive to actinomycin D, consistent with plus-strand synthesis on a DNA template and minus-strand synthesis on an RNA template. Further evidence for minus-strand synthesis on an RNA template was the observation that one-half of newly synthesized minus strand, while still hydrogen-bonded to its template,

appeared at buoyant densities intermediate between RNA-DNA hybrids and RNA in equilibrium $Cs_2SO_4$ density gradient centrifugation. The size of the RNA strands in the complexes appeared to vary inversely with the size of the DNA strand. This observation plus the S1 sensitivity of nascent minus strands suggested degradation of the template RNA by an RNase H-like activity as minus-strand synthesis progressed.

These findings, with the earlier observation by *Mason* et al. (1982) that a full-length (3Kb) polyadenylated viral specific RNA is a major species in DHBV livers, indicate a novel method of DNA replication for DHBV. Based on these findings, Summers and Mason have proposed the following model for DHBV DNA replication. Following infection with DHBV, a genome-length plus strand RNA is synthesized on a viral DNA template that may be virion or closed circular DNA. Minus-strand DNA is then synthesized by a reverse transcriptase utilizing that RNA as a template with a simultaneous degradation of the template. The protein known to be covalently attached to the 5′ end of the minus strand probably serves as a primer for synthesis of this strand. A DNA polymerase, possibly that associated with the virion, could then synthesize the plus strand. Packaging and release of virions would occur only after the plus-strand synthesis was at least 50% complete.

It has yet to be shown that replication of other hepadna virus DNAs involves similar steps. Because these viruses cannot be studied in tissue culture, many details of their replication cycle are not yet understood. For example, the function of the supercoiled DNA forms in hepadna-infected liver and the role of the protein covalently attached to the 5′ end of the long DNA strand are still not known. Finally, little is known about virus transcription and translation for expression of HBsAg and HBcAg polypeptides and other viral gene products. It is clear, however, that at least HBsAg can be expressed when only integrated HBV DNA and no episomal DNA is present. No synthesis of core protein has been observed from such integrated DNAs.

Only a little is known about the cellular sites of synthesis and assembly of different virion components. Unit-length forms of the viral DNAs (single- and double-stranded) appear mostly in cytoplasmic extracts (*Mason* et al. 1982). Immunofluorescent staining of liver tissue infected with HBV shows HBcAg in cell nuclei and HBsAg in cell cytoplasm and in outer membrane (*Barker* et al. 1973; *Gudat* et al. 1975; *Ray* et al. 1976). Studies localizing the antigens of the other hepadna viruses have not been published.

## 6 Course of Infection and Host Response

Primary HBV infections in adults are most often self-limited and are resolved within six months. However, 5%–10% of immunologically normal adults (*Redeker* 1975; *Hoofnagle* et al. 1978) and a much higher fraction of newborn infants (*Schweitzer* et al. 1973; *Tong* et al. 1981) fail to resolve primary infections and become persistently infected. Such patients remain infected with HBsAg in the blood (chronic HBsAg carriers) for many months or years. Approximately 1% of such chronic carriers in the United States have been shown to clear the infection (become HBsAg negative) each year (*Scullard* et al. 1981). Almost all patients during acute infection and some with persistent infection have detectable HBeAg- and DNA-containing virions (detected by assay for specific DNA polymerase or for viral DNA by hybridization) and infectious virus in their blood. Approximately 10%–20% of HBsAg carriers with detectable HBeAg- and DNA-containing virions in the blood

clear these markers each year and frequently develop anti-HBe but continue to be HBsAg positive, although at lower titer (*Scullard* et al. 1981). In at least some such carriers (HBsAg positive; HBeAg and virion negative), infectious HBV is undetectable when tested by chimpanzee inoculation (*Scullard* et al. 1982). Recent experiments indicate that liver tissue of HBsAg carriers with DNA-containing virions in the blood contains abundant unit-length (3200 bp) viral DNA. Interestingly, liver tissue of at least some HBsAg carriers without detectable DNA-containing virions in the blood contain no detectable unit-length viral DNA, and only viral DNA sequences apparently integrated in cellular DNA can be detected (*Brechot* et al. 1981a, b; *Brechot* et al. 1982). It is clinically useful to view HBsAg carriers as falling into two groups. Those that are HBeAg and virion positive have been shown to be highly contagious (*Alter* et al. 1976; *Okada* et al. 1976; *Perrillo* et al. 1979; *Tong* et al. 1981).Those that are HBeAg and virion negative and anti-HBe positive have low or no contagiousness. It also appears that persistent HBV infections slowly wind down with time, and many patients in the highly contagious group (HBeAg and virion positive) lose their contagiousness as HBeAg and virions disappear from the blood and HBsAg titers fall. Liver cells in at least some patients in the latter group appear to have lost the replicating forms of viral DNA. The incomplete HBsAg particles that continue to be produced and appear in their blood seem to be the result of expression of viral DNA sequences (HBsAg genes) integrated in host DNA.

The prevalence of HBsAg carriers in different populations varies from approximately 0.1% in the United States and Northern Europe to more than 10% in parts of Asia and Africa (reviewed by *Szmuness* 1978). The geographic areas with very high carrier rates are those in which infections frequently occur at early ages, usually by transmission of virus from carrier mothers to newborn infants or young children. It has been estimated that there are more than 170 million HBsAg carriers in the world today (*Szmuness* 1978).

There is a wide spectrum of disease associated with HBV infection. Primary infections are most often associated with mild, clinically inapparent hepatitis, but some patients have more severe, clinically apparent hepatitis, and a few have fulminant disease with extensive hepatic necrosis and high mortality (*Peters* 1975). Persistent infection can be associated with near-normal liver histologically, but more often such patients in the United States have at least a mild, nonprogressive lesion, termed chronic persistent hepatitis, with normal or mildly abnormal liver function (*Redeker* 1975; *Peters* 1975). A significant number of patients have a more severe lesion, termed chronic active hepatitis, which may be associated with symptoms and progress to cirrhosis. Long-standing carriers, mostly those with cirrhosis, develop hepatocellular carcinoma with an incidence more than 300 times greater than that in HBsAg-negative controls (*Beasley* et al. 1981). Hepatomas develop in more than three cases per year per 1000 male HBsAg carriers aged 50 years or older in Taiwan and 43% of all male HBsAg carriers above age 40 die of hepatoma (*Beasley* et al. 1981).

Acute and chronic infection may also be associated with extrahepatic disease. A serum-sickness-like syndrome with fever, rash, and arthralgias occurs during the late incubation period of acute hepatitis B in 15%–20% of cases (*Gocke* 1975). Infrequently, acute and/or persistent HBV infection is accompanied by polyarteritis or membranous glomerulonephritis (*Gocke* 1975). Reasonable evidence suggests that all of these syndromes are caused by pathogenic effects of HBsAg-anti-HBs complexes.

Much less is known in detail about the course of hepadna virus infection in woodchucks, ground squirrels, and ducks. The best studied of the three is WHV in *M. monax*.

There is a high prevalence of persistent infection in wild populations of these animals in regions of the eastern United States (*Tyler* et al. 1981). Experimental infections, on the other hand, are most often transient and often without detectable WHsAg in the blood *Summers* et al. 1980; *Wong* et al. 1982). Only very mild hepatitis has been observed during such primary experimental infections. On the other hand, significant hepatitis has been observed in wild-caught animals that are persistently infected, and this hepatitis may be severe and progressive possibly leading to death.

A histological study by Snyder of the livers of 139 wild-caught woodchucks that died in captivity showed that some form of hepatitis was present in 75% of the animals, although only 35% had markers of active WHV infection, suggesting that factors other than WHV infection may be responsible for at least some mild hepatitis in these animals (*Snyder* and *Summers* 1980). More severe hepatitis described by Snyder as chronic active hepatitis (CAH) was observed in 28% of the animals. Degenerative and regenerative changes with a minimal spilling of inflammatory exudate over the limiting plate and fewer necrotic cells were seen as compared with CAH in humans. Ten animals had large zones of necrotic cells between portal tracts or between portal tracts and central veins. A greater inflammatory reaction was present. Two of these livers contained large amounts of WHV DNA, and the serum of these animals contained complete virions. Although some cases of hepatitis in woodchucks may be severe and progressive, the disease does not appear to lead to cirrhosis. Among the most interesting associations, however, is the development of hepatocellular carcinomas which appear in up to one-third of persistently infected animals per year but not in uninfected animals.

These tumors in woodchucks have been described as multiple, circumscribed nodules involving all or most lobes of liver (*Snyder* 1968; *Snyder* and *Summers* 1980). The tumors are white to beige in color and often have hemorrhagic or softened interiors. The tumor cells are most commonly arranged in plates (trabecular) and resemble hepatocytes with both hyperchromatic nuclei and large, prominent nucleoli. Metastases were rare or absent.

More recently *Popper* et al. (1981) described the histologic features of hepatitis and hepatomas in woodchucks and noted several differences between these tumors in woodchucks and in man. As woodchuck hepatomas develop, they have a more conspicuous plasma-cell reaction and more acute and chronic inflammation than is observed in man. There is often a focal accumulation of hemopoietic cells in tumor tissue and elevated serum gamma globulin levels in woodchucks, and these are not commonly seen in man. Cirrhosis is present in three-fourths of humans with hepatoma but has not been observed in woodchucks. Carcinoma develops during active acute and chronic inflammation in woodchucks, with high levels of viral DNA in the serum, while in HBV-associated tumors evidence of viral replication is low or absent and active inflammation is rare.

These investigators felt that HBV-associated carcinomas arise either from dysplastic hepatocytes which produce excess HBsAg and sometimes other phenotype markers, or from hyperplastic nodules similar to those which appear to be the precursors in experimental chemical carcinogenesis. Woodchuck hepatomas appear to arise from the latter precursor alone, raising the possibility that a cocarcinogen may interact with virus to induce these hepatomas.

As described in Sect. 5, "Virus Replication", HBV DNA has been found integrated in host DNA in many HBV-associated hepatomas (*Shafritz* et al. 1981; *Brechot* et al. 1982; *Shafritz* 1981; *Chen* et al. 1982). Recent studies by *Ogston* et al. (1981) and *Summers* et al.

(to be published) of WHV-associated woodchuck hepatomas showed that 15 of 16 tumors did have high-molecular-weight WHV-specific DNA detectable by blot hybridization. The sizes of these virus-specific DNA-containing fragments, when isolated with restriction enzymes that do not cut viral DNA, varied from tumor to tumor, as do those from human hepatomas. Therefore, in the woodchuck system as in the human, there is no evidence for specific host sites of integration. Although no high-molecular-weight DNA was observed by blot hybridization in a liver from a chronically infected animal, integrated viral DNA forms were found in such livers by cloning liver DNA into a lambdoid vector (*Ogston* et al. 1982). It is not yet clear from this work whether there is a site or region specific for attachment and integration on the viral DNA. Indeed, just what the relationship may be between integrated viral DNA and tumor formation is intriguing, but unknown.

GSHV-infected ground squirrels have a distinctly different disease response. While the rate of persistent infection is very high in endemic areas (up to 52%) and the titer of virus (measured by virion DNA polymerase assay) is unusually high (*Marion* et al. 1980a), little or no hepatitis occurs in the infected animals. None of the 25 infected captive animals followed for over two years in this lab has developed significant hepatitis, cirrhosis, or hepatomas. Only the mildest form of hepatitis in some carrier animals has been detected on histologic examination (*Marion* et al., submitted for publication). Some animals with high titers of virus in their sera show no histological abnormalities in their livers. The mild hepatitis is characterized by mild inflammation and intact lobular structure, although the hepatocyte plates appear irregularly bent. Mobilization of PAS-positive sinusoidal cells, focal necrosis, and a mild degree of portal inflammation are also seen. As in woodchucks, some mild hepatitis is also seen in animals without GSHV, although in these animals there are fewer or no acidophilic bodies and fewer Shikata staining cells than in carrier animals and in WHV- or HBV-infected liver.

The differences in both pathogenicity and tumor formation associated with infection by these closely related viruses is very intriguing. Whether these differences reside in the pathogenicity of the viruses, or in genetic differences in the hosts, or are due to environmental influences, remains to be determined.

Again, DHBV has been studied less than the other hepadna viruses, and infected animals have been observed for shorter periods. The presence of DHBV DNA in serum decreases much more rapidly with time than does GSHV DNA in ground squirrels (Marion et al., unpublished data). No clinically apparent hepatitis, hepatoma, or cirrhosis has yet been associated with DHBV infection in American ducks. No study of liver histology has yet been reported for this virus.

In summary, persistent infection is common for all members of the hepadna virus family, although the range of virus titers in serum varies among the four virus-host systems. Only WHV in woodchucks and HBV in man have been clearly associated with hepatocellular carcinomas. Hepatomas have not been found in American GSHV- or DHBV-infected animals. Cirrhosis, a significant sequela of chonic HBV infection, has not been observed in the three animal models.

## 7 Natural Geographic Distribution

There is much less information about the geographic distribution of WHV, GSHV, and DHBV compared with the large body of information about the human virus (reviewed by

*Szmuness* 1978). Both woodchucks and ground squirrels must be trapped in the wild and bled at many locations to determine the geographic distribution of these viruses. Studies on the distribution of duck virus in wild ducks will be similarly difficult. Such information can be much more easily obtained in domestic ducks.

WHV has been found in the subspecies *Marmota monax monax* on the East Coast of the United States in Delaware, Maryland, New Jersey, and Pennsylvania (*Tyler* et al. 1981). The virus was not detected in a study of Iowa woodchucks (*Lutwick* et al. 1981), nor has it been observed in a colony of animals from Vermont and Massachusetts (reviewed by *Summers* 1981). A study by *Tyler* et al. (1981) showed that 23% of the adult animals trapped in endemic areas were carriers ($n = 115$), while 36% had detectable anti-WHs, indicating past infection. Animals less than 8 months of age showed much less evidence of infection: 2 of 51 were WHsAg positive and only 16% had detectable anti-WHs. No sex difference in WHV markers has been observed.

As of this writing, the distribution of GSHV is even more localized. The virus has thus far been observed only in one area of northern California (*Marion* et al. 1980a), although a systematic screening of Beechey ground squirrels in the state and of the many species of ground squirrels throughout the country has not been reported. Of the squirrels in one portion of the endemic area, 52% were found to be carriers ($n = 60$), while 33% exhibited antibody to viral antigens. In another area, 3 of 17 were carriers (18%), while 41% had virus-specific antibody. Like the woodchuck population, the incidence of all virus markers is less in young animals (under 5 months old) than in adults, and no significant difference in sex distribution has been observed in either carrier or convalescent populations.

In the United States DHBV has been found in 5%–10% of animals in some flocks, while other flocks appear to be virus-free (reviewed by *Summers* 1981; Marion et al., unpublished observations). Virus has been found in embryos, hatchlings, and adults (*Mason* et al. 1981). Limited data indicate no sexual differences in carrier rates. No data are yet available on the percentage of animals with virus-related antibody. The distribution of DHBV in ducks in China has not been published.

In summary, there is some evidence that in wild populations of woodchucks and ground squirrels, as in worldwide human populations, there are locations where hepadna viruses are very common with a high incidence of carriers, and others where the virus is not readily detectable. The host ranges of the viruses of the lower animals have not been clearly determined.

## 8 Transmission of Hepadna Viruses

### 8.1 Natural Transmission

In man HBV is often transmitted from infected mothers to infants at the time of birth, by intimate contact such as that between infected mothers and infants or young children, by heterosexual and homosexual contacts, and by percutaneous transfer of blood and possibly other body fluids (e. g., saliva), by, for example, therapeutic use of blood products or "sharing" needles during illicit drug use (reviewed by *Robinson* 1980). These modes of transmission are facilitated by the continuous presence of high concentrations of infectious virus in blood and lower amounts in other body fluids such as saliva and semen

(*Alter* et al. 1977) in most individuals with acute and persistent infections. The virus has not been found in feces, and there is no evidence for fecal-oral transmission via food or water as is common for hepatitis A virus. It also appears that transplacental or intrauterine infections are rare, if they occur at all.

Little is known of the natural routes of transmission of WHV and GSHV due to the difficulty of breeding the animals in captivity. These viruses are continuously present in blood during active infections so that some of the routes of transmission known for HBV in man might be expected in these animals. Serum samples from both woodchucks and ground squirrels in the wild have shown that young animals (less than 8 months for woodchucks and 5 months for ground squirrels) have fewer markers of hepadna virus infection Than adults (*Tyler* et al. 1981; Marion et al., unpublished data). Therefore, perinatal or postnatal infection is most likely. Ground squirrels, at least, live in large colonies and have abundant contact while competing for burrows and mates and defending their young. At least 4 of 25 animals, without any serological markers of GSHV and over 1 year of age, that were trapped in the endemic area developed infection from 1 to 4 months following capture; two remained persistently infected and two exhibited only production of antibody to core and surface antigens (Marion et al., unpublished results). Therefore, horizontal transmission of GSHV does not seem to be uncommon in ground squirrels.

The natural transmission of DHBV in flocks of Pekin ducks in the United States would appear to be primarily vertical, since embryonated eggs of infected females frequently contain virus (*Mason* et al. 1980, 1981). The infection rate in breeding-age animals, determined by presence of viral DNA in sera, is the same as that in day-old hatchlings, suggesting little evidence of horizontal spread of the virus. Since there are not yet adequate measurements for antibody to DHBV antigens, the prevalence of antibody indicating past infection is not known. We have observed that the level of DHBV DNA in many ducks falls to undetectable levels as the ducks get older, suggesting that there may be significant horizontal transmission in ducks to maintain the rate of detectable active infections in adults at the same level as that in 1-day-old hatchlings. Such transmission could happen during contact among animals in flocks.

## 8.2 Experimental Transmission

Good studies of experimental transmission depend upon the availability of adequate serologic methods to detect viral antigens and antibodies. In the case of hepadna viruses, other markers of virus in blood and liver such as virion DNA polymerase and viral DNA are also useful. Such testing has been best developed for HBV, less well for GSHV and WHV, and least well for DHBV, for which virion antigens are not yet well characterized.

Experimental transmission of HBV to volunteer human subjects and chimpanzees has yielded much information about the course of HBV infections. In one large study 70% of 149 susceptible subjects became HBsAg positive between 3 and 11 weeks after inoculation and remained HBsAg positive for 1 to 22 weeks (*Hoofnagle* et al. 1978). Only 90% of these subjects developed anti-HBs; 5% of the patients developed persistent infection and 23% developed primary anti-HBc and anti-HBs responses, indicating HBV infection without HBsAg being detected when weekly sera were tested. These basic patterns of primary HBV infection (HBsAg-positive and -negative transient infection and HBsAg-positive persistent infection) have been repeatedly documented in both experimental

and natural infections in man and chimpanzees. Serial dilutions of sera containing HBV up to $10^{-7}$ (*Barker* and *Murray* 1972) and $10^{-8}$ (*Scullard* et al. 1982; *Shikata* et al. 1977) have successfully infected human volunteers and chimpanzees, indicating the high titers of infectious virus that may be present in serum of some infected patients. The transmission studies have suggested that the higher the infecting dose of virus, the shorter the incubation period and the more severe the initial hepatitis (*Barker* and *Murray* 1972; *Shikata* et al. 1977; *Scullard* et al. 1982). When low virus doses are given the incubation period is long, the hepatitis mild and subclinical, and persistent infection is more likely to develop.

Less information is available concerning experimental infection of the lower animals. *Summers* et al. (1980) injected five young woodchucks (4–8 months) with varying amounts of WHV-containing sera. All animals developed antibody to both WHsAg and WHcAg, with four of the five exhibiting brief antigenemia ranging in duration from 1 to 3 weeks. The onset of antigenemia varied from 1 week after intravenous inoculation of 3 ml to 8 weeks following $10^{-4}$ ml of serum given by intramuscular injection. None of the animals developed persistent infection.

*Wong* et al. (1982) inoculated two woodchucks intravenously with 1 ml of a $10^{-3}$ dilution of WHV-positive serum and observed WHsAg-positive infection in one animal and only appearance of anti-WHs in the other. Antibody to core antigen was not assayed. WHsAg was detected in the first animal at 5 weeks following inoculation and was detectable for only 5 weeks thereafter.

Experimental infection of ground squirrels with GSHV in this lab has resulted in more prolonged or persistent infections than those observed so far in woodchucks. When five animals (1 year old) were inoculated with various dilutions of virus and serum tested every 2 weeks, three exhibited viremia (virion DNA polymerase and GSHsAg positive) and three developed only anti-GSHs and anti-GSHc, suggesting that they were infected. A brief viremia, however, could have been missed, since testing was done only every other week. Of the three proven viremic infections, one became persistent and has continued for 17 months.

In our lab we have attempted to transmit GSHV to more than 22 seronegative animals of varying ages. Undiluted sera of actively infected ground squirrels or various tenfold dilutions up to $10^{-8}$ served as the infectious inoculum. Animals were infected by dilutions up to $10^{-7}$ showing that a high titer of virus was present in the serum used for inoculation. Nine animals had primary GSHsAg and virion DNA polymerase-positive infections. Six of these were self-limited or transient with onset of antigenemia between 6 and 14 weeks after inoculation and duration from 1 to 18 weeks. Three of the primary infections became persistent. Twelve animals developed only anti-GSHs and anti-GSHc responses, suggesting primary infection without detectable antigenemia (or too transient to be detected by bleedings at 2-week intervals). Both patterns of primary infection (GSHsAg-positive and -negative) and both transient and persistent infections occurred with high- and low-titer virus inocula. However, the incubation periods (time between inoculation and appearance of GSHsAg), which were between 6 and 14 weeks, varied inversely with the virus dose.

Inoculation of DHBV into chorioallantoic veins of 15- to 17-day incubated embryonated duck eggs resulted in a high percentage of infection (Mason et al., personal communication). It is not known how many of these infections become persistent. Inoculation of 3-month-old ducklings with DHBV-containing sera did not result in detectable viremia during a 6-month observation period (*Marion* et al., unpublished results).

In summary, experimental transmission has been possible with all four hepadna viruses. There appears to be a variation in duration of viremia in ground squirrels and woodchucks and apparent age dependency in ducks. It is difficult to compare these results quantitatively with those of studies with HBV in humans because of the small numbers of animals used so far, and because the starting inocula have not all been of known titer. Our data show that ground squirrels, at least, show the same range of serological responses to inoculation with GSHV as do humans with HBV. However, natural transmission of virus in ground squirrels and woodchucks appears to result in a much higher ratio of persistent to self-limited or transient infections than does experimental transmission. Further research is needed to determine what factors lead to chronic infection by hepadna viruses.

## 9 Summary and Conclusions

The four viruses described here share features which place them in the same virus family and distinguish them from viruses of other families. These include the characteristic virion ultrastructure, antigenic structure, and molecular features already detailed. The partly single-stranded, circular virion DNA and DNA polymerase which repairs the DNA are features unique to this group. Similarly, several common biological features distinguish this group: the striking tropism for hepatocytes, and the persistent infection with very high concentrations of virus and incomplete viral antigen particles continuously in the blood.

Although knowledge of the hepadna viruses is not at all complete, at this time it appears that they share some features with retroviruses. Among these features are similarities in genome structure, although the nucleic acid type in virions is different, i. e., DNA in the case of hepadna viruses and RNA in retroviruses. Separation and repair of the cohesive ends of hepadna virus DNAs results in linear molecules with inverted, repeat terminal sequences of approximately 300 bp, similar to those of retroviruses. In addition, at least the duck virus DNA appears to replicate through an RNA intermediate utilizing a reverse transcriptase as described, a mechanism with some analogy to retrovirus replication. A third similarity is that viruses of both groups appear to readily integrate in cellular DNA. A fourth similarity is tumor formation during infection by at least some members of each virus group. The clear association between HBV and WHV infections and hepatocellular carcinoma is among the more intriguing features of these viruses. It will be of great interest to investigate in more detail their role in formation of these tumors. It is important to determine whether they integrate in sites adjacent to oncogenes and function as retroviruses are thought to function in cell transformation and tumor induction (*Hayward* et al. 1981).

Although the four hepadna viruses share many common features, they are not identical viruses. The three mammalian viruses are clearly more similar to each other than to the DHBV in ultrastructure, antigenic specificity, and DNA homology. There are also striking differences in virus-associated disease in different animals. For example, the frequent and often severe hepatitis and hepatoma formation in woodchucks is in sharp contrast to the relatively disease-free state of infected ground squirrels. While cirrhosis is not an infrequent sequela of chronic active hepatitis B in man, this has not been observed in

woodchucks. The roles of virus, host, and environment must all be considered in investigating these differences in disease response.

Because natural infections with hepadna viruses are unusually silent for long periods of time, it seems likely that other members of this virus family will be found in unsuspected animal species and that they could be widespread in nature.

The three lower animal-virus systems described here are important as experimental models to investigate mechanisms of persistent infection and disease pathogenesis for viruses of this family in ways not possible with HBV in man. They also represent models in which antiviral drug testing and other therapeutic approaches can be investigated in attempts to terminate or modify infections by these viruses.

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# Rotaviruses: A Review

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## 1 Introduction

The *Rotavirus* genus of the Reoviridae consists of a number of antigenically related and morphologically identical viruses that cause enteritis, primarily in young mammals and avians. The genus was established by the International Committee on Taxonomy of Viruses at the Fourth International Congress for Virology in 1978 (*Matthews* 1979). Type species, such as human and bovine rotavirus, have been proposed, but because there are multiple serotypes and cross-infection of heterologous species may occur these names may be difficult to formalize. It is clear that an acceptable nomenclature for rotavirus species is needed.

We support the scheme suggested by *Rodger* and *Holmes* (1979), which is based on the nomenclature for influenza viruses. This scheme would utilize a cryptogram and would include the following information: species of animal from which the virus was isolated/geographical origin (country, state, city) of virus/strain identification number/year of isolation/serotype number/electropherotype letter.

Synonyms in the literature for viruses that have previously been a taxonomic problem and are now known to be rotaviruses are: murine rotavirus – epidemic diarrhea of infant mice (EDIM); bovine rotavirus – Nebraska calf diarrhea virus, neonatal diarrhea reovirus, and Nebraska or neonatal calf diarrhea (scours) virus; and human rotavirus – orbivirus, orbivirus-like, duovirus, reo-like agent, and infantile gastroenteritis virus. For convenience in this review, rotaviruses will be named after the mammalian or avian species from which they were isolated.

The earliest reported work on what was probably rotavirus gastroenteritis was done by *Light* and *Hodes* (1943, 1949). They isolated a filterable agent from stools of infants with gastroenteritis and showed that it produced diarrhea in newborn calves. Subsequent electron microscopic (EM) analysis of a specimen of calf feces from these early experiments that had been lyophilized and stored since 1943 revealed morphologically typical rotavirus, but it could not be established whether the virus was of human or calf origin because the isolate was not infectious after so long a period of storage (*Hodes* 1977). After the work of Light and Hodes, other viruses with characteristics of the Reoviridae were isolated, including the viruses causing epizootic diarrhea of infant mice (EDIM, murine rotavirus) (*Cheever* and *Mueller* 1947; *Kraft* 1957; *Adams* and *Kraft* 1967; *Banfield* et al. 1968), simian virus SA11, the "O" agent (*Malherbe* and *Strickland-Cholmley* 1967), and bovine rotavirus (calf diarrhea, scours) (*Mebus* et al. 1969). These viruses were subsequently shown to be rotaviruses.

In 1973 medical researchers recognized that a virus similar to the one described by Mebus and colleagues in calves caused diarrhea in young children. The virus was detected in Australia by *Bishop* et al. (1973) when they examined duodenal biopsies from children ill with gastroenteritis by thin-section EM. They reported finding orbivirus-like particles in mucosal epithelial cells. Shortly thereafter in England, *Flewett* et al. (1973) detected the virus in fecal extracts by negative-contrast EM. Subsequent reports of rotavirus infections of humans in Canada (*Middleton* et al. 1974), the United States (*Kapikian* et al. 1974), and Australia (*Holmes* et al. 1974; *Bishop* et al. 1974) showed that rotavirus was a major cause of enteritis among young children throughout the world (*Editorial* 1975). A virus similar in morphology to the one described from calves in the United States (*Mebus* et al. 1969) was also isolated from calves in Great Britain (*Woode* et al. 1974). Rotaviruses have now been isolated from many mammalian and some avian species. The

increasing number of reports associating rotaviruses with new clinical situations (*Halvorsrud* and *Örstavik* 1980; *Holzel* et al. 1980; *Linhares* et al. 1981; *Yolken* et al. 1982) emphasizes the fact that important unanswered questions about the epidemiology, infection, transmission, and significance of the rotaviruses remain.

Because of the established importance of the rotaviruses as human and veterinary pathogens, a global effort has emerged to develop effective methods of disease treatment and prevention. This need has stimulated basic studies on the molecular biology of the rotaviruses required to understand the replication strategies of these viruses. Our knowledge of the mechanisms of replication and infection and of the origin, characteristics, and functions of the rotavirus proteins in infection and immunity has increased rapidly in the last few years. Several other reviews have discussed the clinical and epidemiological aspects and preliminary biochemical characterization of the rotaviruses in the past several years (*Flewett* and *Woode* 1978; *Walker-Smith* 1978; *McNulty* 1978; *Estes* and *Graham* 1979; *Holmes* 1979; *Steinhoff* 1980; *World Health Organization* 1980; *Greenberg* et al. 1981b; *Kapikian* et al. 1980, 1981b). In this review we shall emphasize recent studies which serve to bring into focus important areas for future research efforts. These studies suggest that continued research on the rotaviruses will not only help develop preventive measures but will also reveal novel virus-host interactions and virus replication mechanisms.

Viruses that resemble rotaviruses in size, morphology, number of genome segments, and tropism for enterocytes, but which are antigenically distinct, have recently been reproted in swine (*Saif* et al. 1980; *Bridger* 1980; *Bridger* et al. 1982; *Bohl* et al. 1982), chickens (*McNulty* et al. 1981), and humans (*Rodger* et al. 1982; *Nicolas* et al. 1983; *Dimitrov* et al., to be published). These antigenically distinct rotaviruses may eventually constitute separate subgroups within the *Rotavirus* genus. While the origin, prevalence, and significance of the antigenically distinct rotaviruses remain unclear, their description is importent in the interpretation of all studies that have diagnosed rotaviral infections by EM alone because in these studies antigenically distinct rotaviruses would have been called rotaviruses. Although these new rotaviruses will not be discussed further in this review, their origin and importance clearly are of interest. It should be emphasized that until immunologic reagents are available to detect this new group of rotaviruses, their presence will be recognized only if researchers screen stool samples by both electron microscopy and an immunologic assay and pursue those viruses that are rotaviruses by EM but are not detectable by the immunologic assay.

## 2 Prevalence of the Virus in Nature and Host Range

Rotaviruses have been isolated from mice (*Kraft* 1957; *Adams* and *Kraft* 1967), monkeys (*Malherbe* and *Harwin* 1963; *Malherbe* and *Strickland-Cholmley* 1967; *Stuker* et al. 1979, 1980), calves (*Mebus* et al. 1969; *Woode* et al. 1974), humans (*Bishop* et al. 1973; *Flewett* et al. 1973), foals (*Flewett* et al. 1975; *Dickson* et al. 1979), sheep (*McNulty* et al. 1976c; *Snodgrass* et al. 1976a, b, 1977a), goats (*Scott* et al. 1978), rabbits (*Bryden* et al. 1976; *Petric* et al. 1978), pigs (*Lecce* et al. 1976; *Lecce* and *King* 1978; *Woode* et al. 1976a; *McNulty* et al. 1976f), deer (*Tzipori* et al. 1976), pronghorn antelopes (*Reed* et al. 1976), apes (*Ashley* et al. 1978), impala, Thompson's gazelles, and addax (*Eugster* et al. 1978), dogs (*Eugster* and *Sidwa* 1979; *England* and *Poston* 1980), kittens (*Snodgrass* et al. 1979), turkeys (*Bergeland*

et al. 1977; *McNulty* et al. 1979), and chickens (*Jones* et al. 1979; *McNulty* et al. 1979). The "O" agent (*Malherbe* and *Strickland-Cholmley* 1967) is an unclassified rotavirus that was isolated from intestinal washings of sheep and cattle.

In addition to those viruses that have been either isolated and characterized or directly visualized, there is serological evidence indicating that guinea pigs and bears (*Panel Report* 1978), ducks (*McNulty* et al. 1979), and other mammalian and avian species can also be infected. Preliminary studies (*Gary* et al. 1980b) indicate that some wild birds in the United States have rotavirus antibody. We expect future reports of rotavirus isolation and suspect that rotaviruses will be shown to infect virtually every mammalian and avian species.

The significance of these multiple strains of virus remains unknown. It is now evident that many of the viruses isolated from different mammalian and avian species can be distinguished by neutralization tests and that these viruses represent different serotypes (*Woode* et al. 1976b; *Thouless* et al. 1977b; *Estes* and *Graham* 1980a; *McNulty* et al. 1980; *Hoshino* et al. 1981; *Wyatt* et al. 1982). Many rotavirus serotypes, therefore, apparently exist. The present data suggest that under natural conditions these viruses are species specific, although interspecies infectivity has been demonstrated for some strains by experimental inoculation. For example, experimental inoculation has shown that human rotavirus infects conventional (*Middleton* et al. 1975; *Davidson* et al. 1977) and gnotobiotic (*Middleton* et al. 1975; *Bridger* et al. 1975; *Torres-Medina* et al. 1976) piglets, monkeys (*Wyatt* et al. 1976b; *Mitchell* et al. 1977; *Majer* et al. 1978), lambs (*McNulty* et al. 1976c; *Snodgrass* et al. 1977b), calves (*Mebus* et al. 1976, 1977), and dogs (*Tzipori* 1976), but apparently not suckling mice (*Bridger* et al. 1975; *Noble* et al. 1980) or gnotobiotic rabbits (*Petric* et al. 1978; E. Palmer, unpublished data). Bovine rotavirus has been found to infect piglets (*Woode* et al. 1974, 1975; *Hall* et al. 1976) and mice (*Noble* et al. 1980). Simian rotavirus causes infection in the rhesus monkey, chimpanzee, and baboon (*Soike* et al. 1980) and has been suggested to be pathogenic for gnotobiotic piglets and young calves (*Rodger* et al. 1977), but it does not infect lapine species (*Petric* et al. 1978).

The recent reports that the simian, rhesus and canine rotaviruses are serologically similar and that they are included in one of the human rotavirus serotypes (*Wyatt* et al. 1982; *Gaul* et al. 1982) provide further indirect evidence for the hypothesis that the rotaviruses are transmitted across species boundaries.

It should be emphasized that, while infection of heterologous animals can be demonstrated by recovery of virus or detection of viral antigens in intestinal contents, clinical illness is rarely observed following experimental inoculation of conventional animals. This observation therefore suggests that the current lack of direct evidence of cross-species infection in nature is not surprising. While the question of whether domestic or non-domestic animals serve as reservoirs of infection for man remains unanswered, the segmented nature of the rotavirus genome suggests the possibility that animals could be a source of new virus serotypes through genetic recombination (gene reassortment) as is now recognized for the influenza viruses (*Webster* and *Laver* 1980) and has been suggested for the more closely related orbiviruses (*Gorman* 1979; *Sugiyama* et al. 1982). The potential and preliminary evidence for the emergence of new strains of virus by genetic recombination has been demonstrated in the laboratory (*Matsuno* et al. 1980; *Greenberg* et al. 1981a; *Clarke* and *McCrae* 1982).

# 3 Characteristics of the Virus

## 3.1 Morphology

When seen by negative-stain-contrast electron microscopy, rotaviruses can be distinguished from reoviruses and orbiviruses (*Flewett* et al. 1973; *Middleton* et al. 1974) by

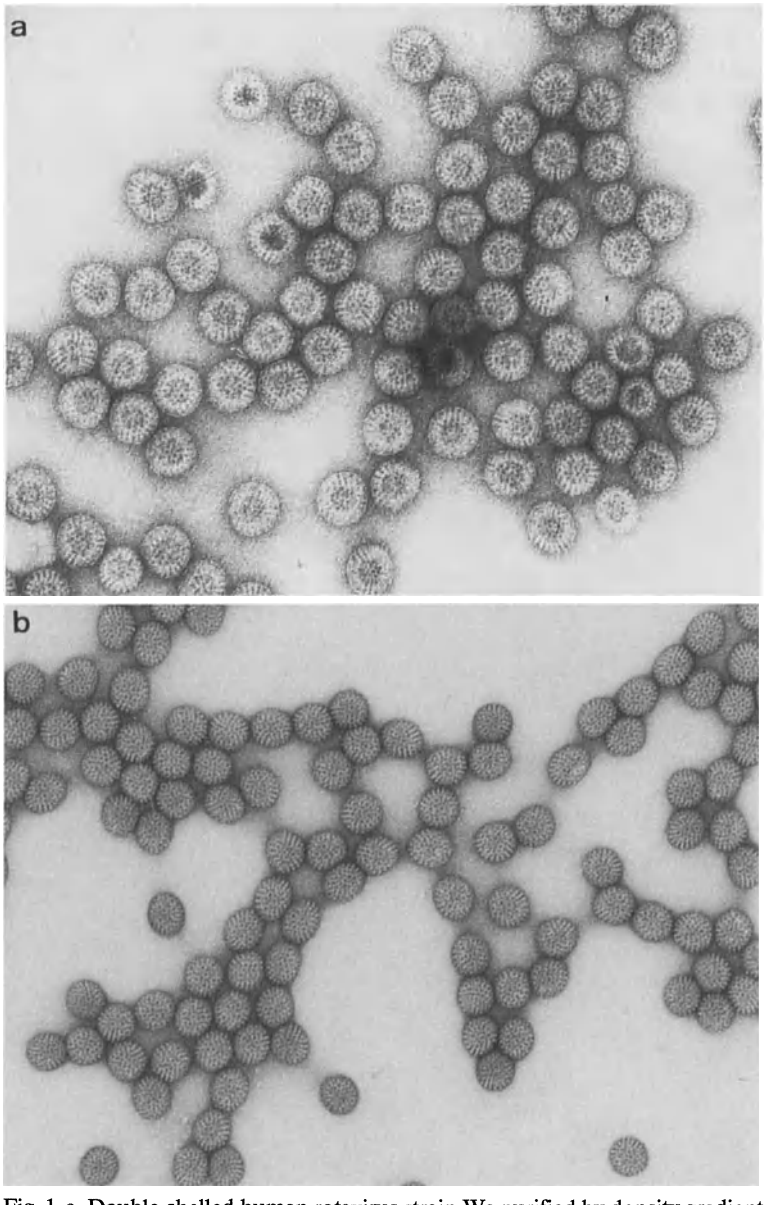


Fig. 1. a, Double-shelled human rotavirus strain Wa purified by density gradient centrifugation in glycerol-potassium tartrate gradients. b, Single-shelled human rotavirus strain Wa purified by density gradient centrifugation in glycerol-potassium tartrate gradients

their sharply defined outer layer. The morphology of intact particles is distinctive and suggestive of a wheel (from the Latin *rota*, a wheel) with the core forming a hub, capsomeres radiating from the core forming spokes, and the precisely marginated outer layer forming the rim (*Flewett* et al. 1974). Rotaviruses from various mammalian species are morphologically indistinguishable from each other and, like other members of the Reoviridae, they have a double-shelled capsid composed of an outer layer, an inner capsid, and an icosahedral core. Both double- and single-shelled particles (Fig. 1a, b) are seen in most preparations (*Bridger* and *Woode* 1976). The complete human rotavirus particle was found to be 67–68 nm in diameter when measured in relation to a catalase crystal internal calibration standard (*Palmer* et al. 1977).

The inner capsid is composed of capsomeres that radiate from an icosahedral core approximately 38 nm in diameter (Fig. 2) (*Palmer* et al. 1977). As with the reoviruses, the arrangement of these capsomeres and the definition of the capsomere unit itself have not

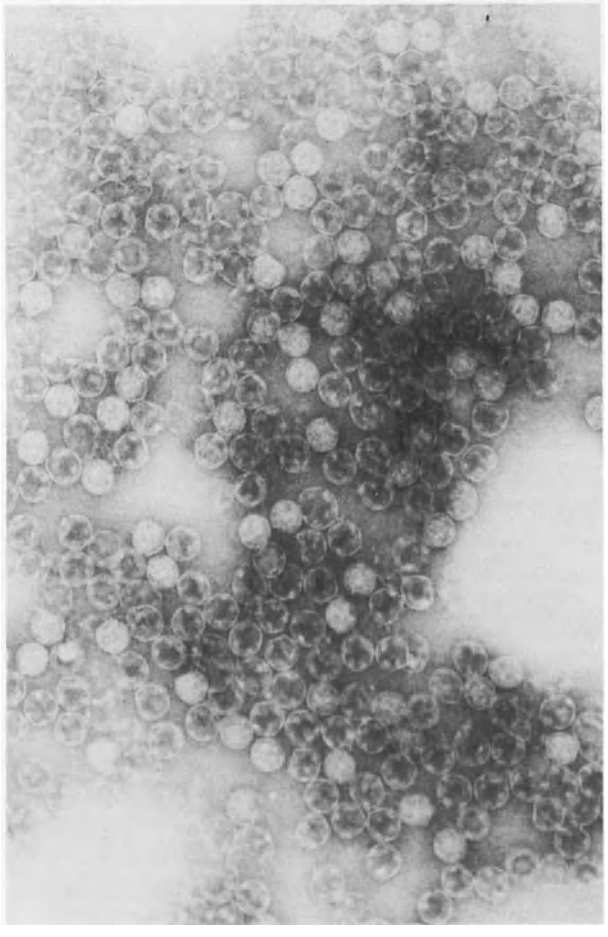

Fig. 2. Core particles produced by treatment of purified single-shelled simian rotavirus SA11 with 1.5 *M* $CaCl_2$, followed by purification through a sucrose cushion as in *Bican* et al. (1982). (Courtesy of B. Petrie) × 99 000

been firmly resolved. The structure of rotavirus has been difficult to define because capsomeres are formed by the sharing of subunits. Definition of actual vertex points used to determine T number is necessary for a clear understanding of the structure of icosahedral viruses. With viruses such as herpesvirus, distinct columnar capsomeres can be seen aligned in a row between two neighboring vertex capsomeres. On the other hand, actual vertex points used to determine T number for viruses of the Reoviridae are holes around which are arrayed trimeric subunits. These subunits form large "capsomeres," two subunits of which are always shared by the adjacent capsomeric structure. This sharing of subunits gives the surface of the virus a honeycomb-like appearance.

*Martin* et al. (1975) concluded that the capsomere surface of human rotavirus single-shelled particles conformed to a trimer surface arrangement of a T = 9 icosadeltahedron composed of 540 structural units arranged as 180 wedge-shaped trimer subunits. These subunits were, in turn, clustered into 32 morphological units or capsomeres arranged as T = 3 and which, at low magnification, appeared to be ring-shaped. The capsomeres were formed by sharing of subunits. *Palmer* et al. (1977) found that this type of surface arrangement was common to both rotavirus and orbivirus single-shelled particles. However, *Stannard* and *Schoub* (1977) interpreted the large ring-like structures described by *Martin* et al. (1975) on the inner capsid as moiré-pattern artifacts; they disregarded these and considered the smaller units as true capsomeres. They proposed that the inner capsid was an icosahedron consisting of 180 morphological units arranged in an open lattice formation with the vertex spaces surrounded by five capsomeres.

*Esparza* and *Gil* (1978) calculated a different number of subunits for the rotavirus capsid and therefore derived a different T number, T = 16. They presented a model in which five holes exist on each edge of the virus icosahedron and calculated the total number of holes in the virus capsid to be 162, formed by the lattice-like arrangement of 320 subunits. They found the subunits to be trimeric structures, thereby giving a total of 960 protomeres per virion.

*Kogasaka* et al. (1979) noted that peripheral capsomeres were enhanced by 10 or 12 rotations, according to the direction of view. These represented 20 or 24 capsomeres, and they noted one sixfold axis between two fivefold axes. They therefore proposed a capsomere number of 42, T = 4, for rotavirus.

*Roseto* et al. (1979) examined rotaviruses with freeze-fracture techniques instead of negative staining and obtained good preservation of rotavirus particles. Superimposition of images was also avoided. Two fivefold axes could be seen on the surface of many single-shelled particles, and the minimum number of sixfold axes between two vertices was 3. The sixfold axes were not symmetrical to the edges, but were just to the center of them. They proposed that single-shelled particles corresponded to an icosadeltahedron of the skew class (T = 13) with 132 capsomeres.

The outer layer of rotavirus was described by *Flewett* et al. (1973, 1974) as a layer of short T-shaped capsomeres attached directly to the ends of the capsomeres of the inner capsid. *Palmer* et al. (1977) did not discern individual capsomeres but described the layer as a covering attached to the main capsid layer. The outer layer was depicted by *Stannard* and *Schoub* (1977) as a three-dimensional honeycomb layer, 3.5–4.0 nm in diameter, in which septa of the honeycomb meet the capsomeres of the inner capsid and the spaces coincide. *Esparza* and *Gil* (1978) proposed that the outer layer is more or less planar and suggested that a free-lying lattice arrangement with large 8-nm holes seen in rotavirus preparations may represent the outer coat of the virion. The spacing of the holes of this

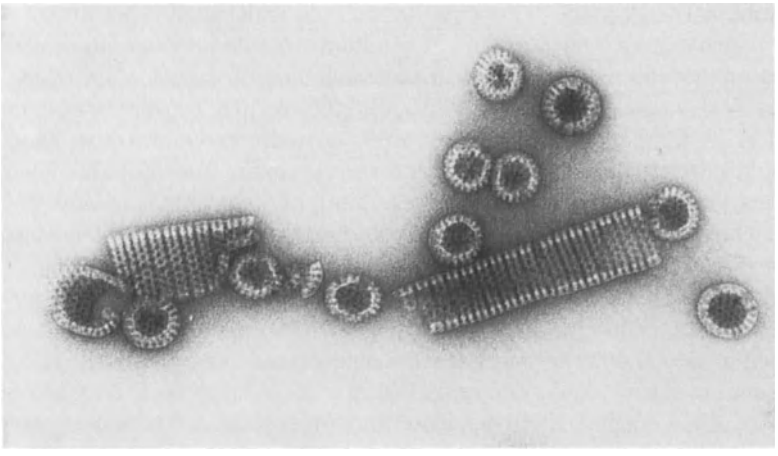

Fig. 3. Tubular structures which are frequently found in rotavirus preparations. Subunits are in lattice-like arrays.

type of lattice, like the honeycomb model of Stannard and Schoub, would correspond to that of the holes of the inner capsid. *Roseto* et al. (1979) showed micrographs of freeze-etch-prepared, double-shelled particles which indicate that the outer layer is a shell with small holes that correspond one-by-one with those of the inner capsid. They were 3 nm in diameter and were regularly organized around five- and sixfold axes. These data are consistent with the smooth surface seen by negative-stain EM. More recently *Palmer* and *Martin* (1982) have shown that the capsomeres of the outer layer are shaped like the type of pin (pushpin) commonly used to mark locations on a map. These are covered by a thin layer of glycoprotein so that the capsomeres cannot be seen unless particles are degenerated.

Free-lying lattice sheets with 10-nm lattice spacing, the same as that of the virion, are frequently seen in rotavirus preparations (Fig. 3). These lattices, which vary in size, may result from the fragmentation of rotavirus particles during specimen preparation. They may also be formed by the reassembly of subunits after the disruption of rotavirus particles (*Esparza* and *Gil* 1978). Tubular forms of rotavirus also occur in fecal material (*Kimura* and *Murakami* 1977), and they have been reported in infected tissue cultures (*Adams* and *Kraft* 1967; *Banfield* et al. 1968; *Altenburg* et al. 1980). *Holmes* et al. (1975) initially described these as 54–100 nm in diameter with a 10-nm lattice spacing. The significance of these tubular forms is not known, but both the tubular forms and the lattice sheets appear to be composed of viral subunits arranged in a hexagonal pattern.

Immunoelectron microscopic studies of tubules 50 nm in diameter and larger ones 75–80 nm in diameter in fecal extracts from children with diarrhea have shown these tubules to be antigenically related to the inner capsid of the virion (*Kimura* 1981). Additional types of rotavirus particles (including enveloped particles) have been described in studies on the morphogenesis of rotaviruses in cell culture (see Sect. 10.2), and these morphogenetic intermediates can be detected in samples of crude virus.

## 3.2 Physicochemical Properties

Early information about the chemical and physical properties of rotavirus was obtained with virus purified from feces. Purification procedures involved dilution and homogenization of the starting material and removal of gross material by low-speed centrifugation. A fluorocarbon extraction was usually necessary to remove lipid and other debris which entrapped virus particles. Viruses could be concentrated by high-speed centrifugation or, if large volumes were used, by polyethylene glycol precipitation. Final purification involved banding of the virus in CsCl density gradients.

It is now apparent that all the mammalian rotaviruses have similar physicochemical properties, although no extensive comparative studies have been performed. Double-shelled particles have a buoyant density of 1.36 g/ml in CsCl, and single-shelled particles have a density of 1.38 g/ml in CsCl (*Petric* et al. 1975; *Kapikian* et al. 1976a; *Bridger* and *Woode* 1976; *Elias* 1977b). Double-shelled particles have a sedimentation coefficient of 500–530 S (*Petric* et al. 1975; *Newman* et al. 1975), and single-shelled particles have a sedimentation coefficient of 380–400 S (*Tam* et al. 1976). The icosahedral core (*Palmer* et al. 1977) has recently been isolated from bovine rotavirus and has a buoyant density of 1.44 g/ml in CsCl and a sedimentation coefficient of 280 S (*Bican* et al. 1982).

## 3.3 Virus Stability and Inactivation

It is anticipated that, being enteric viruses, the rotaviruses would be relatively stable entities. Virus stability has been evaluated by monitoring virus morphology (*Palmer* et al. 1977) and infectivity (*Malherbe* and *Strickland-Cholmley* 1967; *Welch* and *Thompson* 1973; *Estes* et al. 1979a; *Shirley* et al. 1981).

The infectivity of simian rotavirus SA11 is stable to low pH (pH 3.5), ether, chloroform, Genetron, freeze-thawing, and sonication. However, infectivity is labile to pH values above 10.0 and to treatment with low concentrations (5 m*M*) of the chelating agents ethylenediamine tetra-acetic acid (EDTA) or ethyleneglycobis (β-aminoethyl-ether)-*N,N'*-tetra-acetic acid (EGTA), or with high concentrations (2 *M*) of calcium chloride. Infectivity is also lost by heating at 50 °C in 2 *M* $MgCl_2$, but it is stabilized by heating in 2 *M* $MgSO_4$ (*Estes* et al. 1979a).

A study of human rotavirus demonstrated that virus infectivity is stabilized by low levels (0.15–1.5 m*M*) of calcium chloride and strontium chloride (1.5 m*M*), but not by magnesium chloride; magnesium sulfate was not tested (*Shirley* et al. 1981). A role for calcium in maintaining virus integrity has been implicated from these studies, and this hypothesis is supported by the demonstration that the outer capsid glycoproteins are removed by calcium chelation (*Cohen* et al. 1979). These studies are consistent with the observation that only double-shelled particles are infectious (*Elias* 1977b).

Viral infectivity is enhanced by treating the virus with the proteolytic enzymes pancreatin, trypsin, or elastase, and this treatment has been required for successful cultivation of these relatively fastidious viruses (see Sect. 11).

The morphology of rotavirus does not appear to be altered by heat, and it is also stable to treatment with most proteolytic enzymes except in the presence of EDTA (*Palmer* et al. 1977). Virus morphology is degraded by chaotropic agents such as sodium thiocyanate (*Almeida* 1979; *Almeida* et al. 1979) and high concentrations ($>1$ *M*) of calcium chloride,

which remove the inner capsid of the particle and produce viral cores (*Bican* et al. 1982). Simian and bovine rotaviruses are rapidly degraded by high concentrations of alcohol (*Bishai* et al. 1978; *Bastardo* and *Holmes* 1980), and a comparative study of the effects of common disinfectants on the infectivity of SA11 concluded that 95% ethanol is an effective skin disinfectant for handwashing (*Tan* and *Schnagl* 1981). *Brade* et al. (1981) have confirmed the effectiveness of ethanol as a disinfectant against SA11 and also found a phenol compound to be useful. In a smaller study, *Snodgrass* and *Herring* (1977) found formalin, lysol, and an iodophor preparation (but not chlorine) to be useful disinfectants, particularly if a relatively long exposure time (2 h) was possible. Purified simian rotavirus SA11 is rapidly inactivated by chlorine treatment (R. Floyd, personal communication). This suggests that mechanisms such as virus aggregation or virus adsorption to solids must be important for rotavirus survival in sewage treatment plants, or in chlorinated tapwater (*Smith* and *Gerba* 1982), or in the disinfectant study mentioned above.

## 4 Rotavirus Serotypes and Viral Antigens

Recent studies have shown that serotypes of rotaviruses can be discriminated by serum neutralization tests. Plaque-reduction assays are able to distinguish bovine, canine, feline, human, simian, and porcine rotaviruses that are cultivable (*Estes* and *Graham* 1980a; *Hoshino* et al. 1981; *Murakami* et al. 1981; *Wyatt* et al. 1982), and neutralization of fluorescent cell-forming units has been used for rotaviruses that are not capable of sufficient replication in culture to permit plaquing (*Flewett* et al. 1978; *Thouless* et al. 1978; *Zissis* and *Lambert* 1978; *Beards* et al. 1980; *McNulty* et al. 1980). These studies have demonstrated that there are at least four, and possibly more, serotypes of human rotavirus, two serotypes of bovine rotavirus, and three serotypes of avian rotavirus. More complete antigenic characterization will be possible when a large number of isolates are cultivated in tissue culture. Knowledge of whether a finite number of rotavirus serotypes exists is critical for vaccine development, and the recent success of culturing many human rotavirus

Table 1. Human Rotavirus Serotypes

| Serotype[a] | Species of isolation | Strains[b] | Subgroup[c] | RNA pattern[d] |
|---|---|---|---|---|
| 1 | humans | Wa, KU, K8, Birmingham I, Fh | 2 | long |
| 2 | humans | DS-1, S2, KUN, HN-126, Birmingham II | 1 | short |
| 3 | humans | M, P, YO, MO, 14, Birmingham III | 2 | long |
| | animals | rhesus, SA11, canine rotaviruses | 1 | long |
| 4 | humans | St. Thomas 4 | 2 | long |
| - | humans | Hochi, Ito, Hosokawa, Nemoto[e] | NT[f] | NT |

[a] Serotypes as designated by *Flewett* et al. (1978); [b] Compiled from *Flewett* et al. (1978), *Gaul* et al. (1982), *Sato* et al. (1982), *Thouless* et al. (1982), *Urasawa* et al. (1982), *Wyatt* et al. (1982) and *R. Wyatt* (personal communication); [c] Data from *Greenberg* et al. (1983); [d] Refers to the overall migration of the 11 genome segments in polyacrylamide gels (*Kalica* et al., 1981); [e] These four strains from Japan have been distinguished by plaque reduction assays from serotype 2 (Wa) and serotype 3 (SA11) viruses. They have not been compared to viruses of the other 2 serotypes (*Sato* et al., 1982); [f] NT, not tested

strains (*Sato* et al. 1981a; *Urasawa* et al. 1981) will clearly help answer this question. Table 1 presents a summary of the current classification of a limited number of human rotavirus strains into serotypes.

Early reports suggested the ability to classify virus serotypes by non-neutralization assays such as complement-fixation and enzyme-linked immunosorbent assay (ELISA) with selected postinfection sera (*Yolken* et al. 1978b; *Zissis* and *Lambert* 1978, 1980, 1981). Further analyses have revealed that these tests do not recognize the same antigens detected in serum neutralization tests and therefore they do not identify serotypes (*Kapikian* et al. 1981a). It has now been reported that ELISA and an immune adherence hemagglutination assay (IAHA) can be used to distinguish a subgroup antigen that is encoded by the sixth genome segment (*Kalica* et al. 1981b), and two subgroups have been characterized with the use of selected convalescent antisera. It should be clearly recognized that ELISA and IAHA tests are nonspecific tests based on the detection of any antigen-antibody reaction, and their ability to act as specific tests depends solely on the specificity of the antisera used. The initial report described IAHA-ELISA subgroup 2 as consisting of two strains of human rotavirus (D and Wa), while one other human rotavirus strain (DS-1), two bovine (UK and NCDV), one porcine (OSU), three simian (SA11, rhesus 1 and 2), and the "O" agent belong to IAHA-ELISA subgroup 1. The murine rotavirus (EDIM) could not be typed in this test. These results have emphasized the importance of following standard classification methods to define virus serotypes. Knowledge of the biological or epidemiological significance, if any, of the subgroup antigen(s) will require further evaluation with multiple virus isolates. This task will be simplified by the recent development of monoclonal antibodies with defined specificities for the two subgroup antigens (*Greenberg* et al. 1983). Until further antigenic characterization is reported, the epidemiological studies that have characterized disease outbreaks or serologic responses reportedly to serotypes 1 and 2, which are now subgroups 1 and 2 (*Zissis* and *Lambert* 1978, 1980; *Yolken* et al. 1978e; *Rodriguez* et al. 1978; *Brandt* et al. 1979), must be interpreted with caution.

In addition to their type-specific or neutralizing antigens, mammalian and avian rotaviruses share common antigens that can be detected by a variety of serological tests, including complement fixation, immunofluorescence, gel diffusion, immune electron microscopy, IAHA, ELISA, and radioimmunoassays. The common rotavirus antigens are not shared with viruses of the other genera of the Reoviridae (*Kapikian* et al. 1976b; *Woode* et al. 1976b; *McNulty* et al. 1979). The common antigens are located on the inner capsid of the virus, whereas the type-specific antigens are located on the outer capsid of the virus, as demonstrated by the agglutination of only single-shelled particles with antisera prepared against heterologous viruses (*Bridger* 1978). The existence of the common antigens has made it possible to use cultivable animal rotaviruses (or antisera to them) as substitute reagents in diagnostic tests for human rotaviruses in stools or in serological tests to detect evidence of infection (*Kapikian* et al. 1976b; *Matsuno* et al. 1977a; *Schoub* et al. 1977; *Bishai* et al. 1979; *Brade* and *Schmidt* 1979).

Recent efforts have attempted to define the polypeptides that possess type-common antigenic determinants. Using monospecific antisera prepared against purified SA11 structural polypeptides, preliminary experiments have demonstrated that several of the capsid polypeptides possess common determinants as detected by immunofluorescence assays and immunoprecipitation experiments performed with cells infected with homologous and heterologous viruses (*Ester* et al., unpublished data). Monoclonal anti-

bodies are also being produced (*Roseto* et al. 1982; *Greenberg* et al. 1983). These antibodies should ultimately be useful as diagnostic reagents and as powerful research tools to probe antigenic diversity and virus morphogenesis. Since oral administration of rotavirus antibody has resulted in resistance to rotavirus challenge in various animal models (*Snodgrass* et al. 1977b; *Bridger* and *Brown* 1981), and since it was reportedly effective in stopping chronic excretion of rotavirus in a limited number of immunosuppressed children (*Saulsbury* et al. 1980), neutralizing monoclonal antibodies might also prove useful as passive disease therapy in children.

## 5 Hemagglutination

Some bovine rotavirus isolates, simian rotavirus (SA11), one human rotavirus isolate, and chicken and turkey rotaviruses hemagglutinate (HA) human "O," guinea pig, and chicken erythrocytes (*Spence* et al. 1976; *Inaba* et al. 1977; *Fauvel* et al. 1978; *Kalica* et al. 1978a; *Burtonboy* et al. 1978; *Shinozaki* et al. 1978; *Bishai* et al. 1978; *Hancock* et al. 1983), and various other erythrocytes. Treatment of erythrocytes with receptor-destroying enzyme blocks HA by simian rotavirus SA11, suggesting that the virus interacts with neuraminic acid-containing receptors (*Bastardo* and *Holmes* 1980). The HA of bovine rotavirus is inactivated by heat up to 45 °C, pH 2.0, freeze-thawing, and chloroform without discernable changes in virus morphology (*Bishai* et al. 1978).

*Sato* et al. (1978) found that serum albumin was indispensable for effective hemagglutination of one strain of bovine rotavirus, but this has not been a general observation. Hemagglutination inhibitors present in culture fluids can be abolished by treating preparations with fluorocarbon (*Inaba* et al. 1978). Others have not had this problem with simian rotavirus (*Kalica* et al. 1978; *Martin* et al. 1979). HA activity is associated with the double-shelled particles, and it appears to detect a type-specific antigen, although this has not been rigorously tested (*Spence* et al. 1978). Hemagglutination-inhibition (HI) tests can distinguish between bovine and simian viruses, and hyperimmune serum to one human virus isolate did not inhibit HA by simian or bovine viruses (*Spence* et al. 1978). HI and neutralizing activities of sera show good correlation (*Inaba* et al. 1978), so the HI test may prove useful in differentiation of rotavirus serotypes. Again, this remains to be demonstrated experimentally.

*Spence* et al. (1976) found that some sera which had complement-fixing (CF) antibody did not react in HI, so they suggested that HI was more specific than CF. An alternative explanation is that HI using bovine rotavirus antigen is less sensitive than CF in detecting seroconversion to human rotavirus. We have attempted to demonstrate HA activity with several human rotavirus isolates, including the tissue-culture-adapted Wa virus, with negative or equivocal results (*Palmer* et al., unpublished data). *Shinozaki* et al. (1978) found an isolate of human rotavirus that agglutinated chicken red blood cells, and they were able to demonstrate seroconversion with several acute and convalescent illness-phase sera using HI.

Recent studies suggest the outer capsid VP3 is the polypeptide with hemagglutinating activity, since gene four encodes the hemagglutinin of rhesus rotavirus (*H. Greenberg*, personal communication).

## 6 The Rotavirus Genome

Preliminary characterization of bovine, murine, and human rotaviruses showed that these viruses contained RNA (*Welch* 1971; *Much* and *Zajac* 1972; *Petric* et al. 1975) which was double-stranded (*Welch* and *Thompson* 1973; *Petric* et al. 1975; *Todd* and *McNulty* 1976). Subsequent studies by *Rodger* et al. (1975) and *Newman* et al. (1975) confirmed those data by separating bovine rotavirus RNA into eight bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One band (6) had a molar ratio indicating that it contained multiple RNA species. It was concluded that the genome of bovine rotavirus consists of 11 segments ranging in molecular weight from $2.2 \times 10^6$ to $0.2 \times 10^6$, with a total molecular weight of $11\text{–}12 \times 10^6$.

It should be noted that all of these determinations of RNA molecular weights were performed under experimental conditions where secondary structure of the RNA molecules could remain. Only one study has been performed under totally denaturing conditions by using methyl mercury-agarose gel electrophoresis (*Bernstein* and *Hruska* 1981). They compared the rotavirus RNA segments to ribosomal RNAs and reported the molecular weights of the segments were $2.8\text{-}0.78 \times 10^6$ and $3.4\text{-}0.80 \times 10^6$ for the bovine and SA11 rotavirus respectively, values higher than those previously reported. These molecular weight determinations show a large discrepancy for the smaller RNA segments when compared with previous reports. It is possible that the migration of the smaller RNA transcripts in this study was nonlinear, but that this was not recognized due to the use of larger ribosomal RNAs as molecular weight markers.

Electrophoretic analyses of a number of rotavirus strains have shown that all rotaviruses contain 11 segments of double-stranded RNA. These segments can be divided into four size-classes (groups I–IV) based on contour-length measurements of the RNAs by EM and electrophoretic patterns in PAGE (*Kalica* et al. 1976; *Barnett* et al. 1978). Rotavirus strains can be distinguished on the basis of differences in the migration patterns of the genome segments by PAGE (the RNA electropherotype) (*Kalica* et al. 1976), as illustrated in Fig. 4. A classification scheme to analyze isolates has been proposed by *Lourenco* et al. (1980); it compares the relative migration of RNA bands within each group by a small letter, and patterns observed to date have been described (*Lourenco* et al. 1981). The electropherotype of an isolate can be expressed as the combination of its patterns, e.g., an isolate might demonstrate the pattern Ia, IIa, IIIa, IVa. This system has the advantage of avoiding the tedious comparison of the migration of each segment relative to the migration of the segments of a standard such as simian rotavirus SA11. Acceptance of this classification system should help investigators in widely separate regions determine whether isolates are similar.

Extensive genome diversity of rotavirus isolates from man and animals has been demonstrated in studies in Australia (*Schnagl* and *Holmes* 1976; *Rodger* and *Holmes* 1979; *Rodger* et al. 1981; *Schnagl* et al. 1981), England (*Clarke* and *McCrae* 1981, 1982), France (*Verly* and *Cohen* 1977; *Lourenco* et al. 1981), Ireland (*Todd* et al. 1980), Mexico (*Espejo* et al. 1979, 1980a), New Zealand (*Croxson* and *Bellamy* 1979), Scotland (*Cash* 1982), and the United States (*Kalica* et al. 1978b, c; *Dimitrov* et al., to be published). Nineteen and 29 different human rotavirus electropherotypes respectively were detected in a 6-year study in Australia (*Rodger* et al. 1981) and in a 1-year study in France (*Lourenco* et al. 1981). The most comprehensive study (*Rodger* et al. 1981) extended the earlier observations of *Espejo* et al. (1980a) that certain rotavirus electropherotypes may persist for more than 2 years.

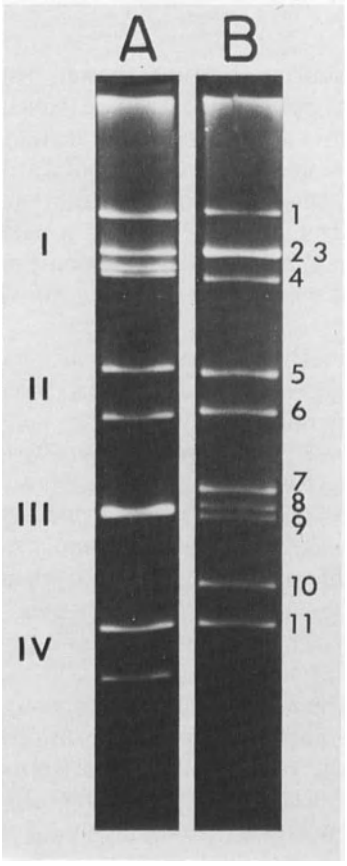


Fig. 4. Comparison of the RNA genomes of (*A*) simian rotavirus SA11 strain SI/S. Africa/H96/58 and (*B*) a human rotavirus strain Hu/Texas/81 by electrophoresis in a 10% Laemmli polyacrylamide gel. Migration was from *top* to *bottom*, and the RNA segments were visualized after staining with 1 μg/ml ethidium bromide. RNA segments are numbered from the *top* and, in the case of SA11, segments 7, 8, and 9 comigrated. For this human strain segments 2 and 3 comigrated. The RNA electropherotype for SA11 is called a "long" pattern, while the RNA pattern for the human strain is called a "short" pattern. (Courtesy of D. Dimitrov)

These studies did not confirm the speculation that rotavirus electropherotypes cycle over a 3- to 4-year period (*Kalica* et al. 1978b). In contrast to the diversity of electropherotypes from children and adults, rotavirus specimens from neonates were limited to two electropherotypes and one of these persisted for 4 years (*Rodger* et al. 1981). No explanation for the persistence of a few virus strains in the neonatal nurseries has emerged, but the observation is intriguing, especially since rotavirus infection in at least one nursery is seasonal.

The demonstration that a number of electropherotypes may be present concurrently within a city has suggested that dual infections could result in the appearance of new virus strains by reassortment of genes. This suggestion has been supported by the observation that stools from some children have revealed genome profiles with more than 11 genome segments (*Lourenco* et al. 1981; *Rodger* et al. 1981). Evidence for the occurrence of genome segment reassortment between rotaviruses in nature has been obtained from one-dimensional structural analyses of individual genome segments of bovine virus field isolates (*Clarke* and *McCrae* 1982).

In addition to describing the rotavirus genome diversity, these studies have emphasized the usefulness of studying genome variation to obtain detailed epidemiologic information on rotavirus disease outbreaks and to help monitor modes of disease trans-

mission. More comprehensive molecular epidemiological studies of RNA profiles of rotaviruses may now be possible by direct extraction of double-stranded RNA from stools with optimized methods of extraction and detection (*Espejo* et al. 1978; *Clarke* and *McCrae* 1981; *Croxson* and *Bellamy* 1981; *Theil* et al. 1981). A method using 3′ terminal labeling of extracted RNA with $^{32}P$ and $T_4$ RNA ligase has been developed, permitting analysis of genomes directly from feces without a virus purification step (*Clarke* and *McCrae* 1981). This method reportedly can be used to obtain RNA profiles on a 1-ml sample containing as little as $2–5 \times 10^9$ particles/ml.

The high level of genome variation observed for the rotaviruses is analogous to the heterogeneity of genome profiles observed in influenza virus and reovirus isolates (*Palese* 1977; *Hrdy* et al. 1979). In influenza viruses, gene reassortment between two viruses upon infection of a single animal is one mechanism that explains genome variation and accounts for the rapid appearance of new pandemic strains of virus (*Webster* and *Laver* 1980). It should be emphasized that the rotavirus genomic variation does not necessarily reflect antigenic variation. There have been some attempts to correlate rotavirus serotypes with electropherotypes, but this area has not been thoroughly investigated, due primarily to the difficulty in propagating these fastidious viruses. Since it has been clearly demonstrated with the related reoviruses and orbiviruses that different electrophoretic patterns of double-stranded viral genomes do not necessarily reflect changes in serotypes (*Hrdy* et al. 1979; *Gorman* et al. 1981), studies to correlate the electropherotype(s) with serotypes are important.

One interesting observation resulting from the analysis of RNA electropherotypes is that some human viruses exhibit an altered migration of RNA segments 10 and 11. Two overall patterns of migration of the 11 rotavirus genes have been detected, and they have been termed "long" and "short" electropherotypes (see Fig. 4). All animal viruses analyzed to date [except EDIM virus (*Smith* and *Tzipori* 1979; *Smee* et al. 1982)] exhibit long electropherotypes. Hybridization analysis and in vitro translation of genes 10 and 11 from human viruses with long and short patterns have demonstrated that these patterns result from a change in the migration of gene 11 (*Dyall-Smith* and *Holmes* 1981a; G. Flores, personal communication). Based on the size of the primary polypeptide product they encode, the short pattern is the pattern that corresponds to gene products being produced in the "correct" order. The significance of these gene alterations remains unknown, but it has been reported that the long and short electropherotypes of a virus correlate with the subgroup antigen detected in the ELISA-IAHA test (*Kalica* et al. 1981a; *Rodger* et al. 1981; *Kutsuzawa* et al. 1982). Since genome segment 6 codes for the subgroup antigen (*Kalica* et al. 1981b), there is no obvious reason why this association exists. It is possible that with further testing of more strains the association will not hold up. Characterization of a limited number of recently cultivated human isolates with the short RNA electropherotype suggests these are distinguishable by serum neutralization from viruses with long RNA electropherotypes. Although it is now known that viruses with long electropherotypes represent more than one serotype, not enough isolates with short electropherotypes have been examined to conclude that all viruses with a short electropherotype will represent a single serotype (*Kalica* et al. 1981a; *Wyatt* et al. 1982).

The recent observation of unusual short RNA patterns in this laboratory from samples of human rotavirus collected in South America (*Dimitrov* et al., to be published) and the demonstration that the murine rotavirus RNA exhibits unique migration patterns for gene segments 10 and 11 (*Smith* and *Tzipori* 1979; *Smee* et al. 1982) suggest that

further investigation will continue to detect more genome diversity. Again, the remaining critical question concerning RNA electropherotypes is whether the diversity reflects antigenic diversity caused by gene reassortment. If it does, this will have a profound effect on the strategies to be used to prevent rotavirus disease.

# 7 Rotavirus Proteins

## 7.1 Viral Structural Polypeptides

The protein composition of rotavirus particles was first examined in 1975 by two groups of workers (*Rodger* et al. 1975; *Newman* et al. 1975). These and many subsequent studies (*Bridger* and *Woode* 1976; *Todd* and *McNulty* 1977; *Monette* et al. 1979; *Obijeski* et al. 1977; *Rodger* et al. 1977; *Cohen* et al. 1979; *Kalica* and *Theodore* 1979; *Matsuno* and *Mukoyama* 1979; *Thouless* 1979; *Espejo* et al. 1980b, 1981; *Mason* et al. 1980; *Smith* et al. 1980; *Estes* et al. 1981a; *McCrae* and *Faulkner-Valle* 1981; *Novo* and *Esparza* 1981; *Bican* et al. 1982) described the rotavirus structural polypeptides following electrophoresis of the polypeptides in polyacrylamide gels containing sodium dodecyl sulfate. These studies analyzed the polypeptides of rotavirus particles of several species obtained both from stool samples and from tissue culture cells infected with cultivable rotaviruses.

These numerous studies yielded conflicting data with respect to the number (5–10) of polypeptide species in rotavirus particles and to the apparent molecular weights of the structural polypeptides. The reported discrepancies were thoroughly summarized by *McNulty* (1979), and we shall emphasize the unifying data from recent studies. Most of the early discrepancies probably reflect differences in methodology rather than actual large differences in the polypeptide composition of different rotavirus strains. The methodology differences included: (a) the diversity in the resolution of the PAGE sys-

Table 2. Polypeptide composition of simian rotavirus SA11 particles[a]

| Double capsid particles cultivated without proteolytic enzymes | Double capsid particles exposed to trypsin during cultivation or in vitro | Polypeptides (mol. wt. $\times 10^3$) |
|---|---|---|
| VP1 | VP1 | 125 |
| VP2[b] | VP2 | 94 |
| VP3[c] | VP3 | 88 |
| | VP4*[d] | 84 |
| | VP5*[d] | 60 |
| VP6[b] | VP6 | 41 |
| VP7[c, e] | VP7 | 38 |
| | VP8*[d] | 28 |
| VP9[c, f] | VP9 | 27 |

[a] Modified from data in *Estes* et al. (1981a); [b] Major polypeptides; [c] Outer capsid polypeptides; [d] Asterisk denotes a cleavage product; [e] Glycosylated; the occurrence of one or two glycoproteins and the molecular weight (VP7, VP7a) of the outer capsid glycoprotein(s) can vary from 36 K to 38 K, depending on the plaque isolate and passage history of the virus analyzed (*Estes* et al. 1982); [f] A very minor component, also reported for SA11 by *Rodger* et al. (1975) but not by *Espejo* et al. (1981)

tems used, (b) the failure to use molecular weight standards, or (c) the analysis of heterogeneous virus preparations obtained from either fecal specimens or tissue cultures where the virus had different exposure to proteolytic enzymes before analysis, resulting in an alteration of the polypeptide pattern.

The simian rotavirus SA11 has been the most thoroughly studied. The polypeptide composition of purified SA11 particles (*Estes* et al. 1981a) is summarized in Table 2. This table emphasizes that the number of polypeptides detected may vary, depending on whether virus has been exposed to trypsin or proteolytic enzymes during cultivation or purification. Although no consensus on rotavirus polypeptide nomenclature yet exists, we followed the original suggestion of *Rodger* et al. (1975) and termed the structural polypeptides of the virus as viral polypeptides (VP), with VP1 representing the largest, and VP2 and VP6 the major polypeptides (*Mason* et al. 1980). Our original studies examined virus, produced in the presence of trypsin, and VP1–VP8 were characterized; the minor component VP9 was not detected. These studies compared the SA11 structural polypeptides with polypeptides synthesized in an in vitro translation system derived from rabbit reticulocyte lysates and programmed with rotavirus mRNA. Several of the viral structural polypeptides (VP4, and VP5, and the glycosylated VP7) were not synthesized in vitro as primary gene products, which was the first evidence for post-translational processing of some structural polypeptides (*Mason* et al. 1980). Subsequent studies demonstrated that VP4*, VP5*, and VP8* were proteolytic cleavage products, and cleavage products are now denoted by an asterisk (*).

Detailed comparisons of rotavirus polypeptides synthesized in infected cells and those synthesized in vitro from virus-specific RNA transcripts have established that post-

Table 3. Summary of SA11 polypeptides and their modifications[a]

| Genome segment[b] | Primary product | Designation | Modification | Modified product(s) |
|---|---|---|---|---|
| S1 | 125 K | VP1 (inner) | None | None |
| S2,3 | 94 K | VP2 (inner) | Cleaved | VP3*, VP4* (88 K, 84 K) |
| S4 | 88 K | VP3 (outer) | Cleaved | VP5*, VP8* (60 K, 28 K) |
| S5 | 53 K | Nonstructural | None | None |
| S6 | 41 K | VP6 (inner) | None | None |
| S7 | 34 K | Nonstructural | None | None |
| S8 | 35 K | Nonstructural | None | None |
| S9 | 37 K[c] | VP7 (outer precursor) | Glycosylated and trimmed | VP7[d] (38 K) |
| S10 | 20 K[e] | Nonstructural (precursor) | Glycosylated and trimmed | 28 K, 29 K |
| S11 | 26 K | VP9 (outer precursor) | Yes, type unknown | ~ 27 K |

[a] Modified from *Ericson* et al. (1982); [b] Gene assignments from *Mason* et al. (1981, 1983); [c] Contains a signal sequence (*Ericson* et al. 1983); [d] The molecular weight of the structural glycoprotein can vary from 36 K to 38 K, depending on the plaque isolate of virus analyzed (*Estes* et al. 1982); [e] Contains a noncleavable signal sequence (*Ericson* et al. 1983)

translational modification (glycosylation and/or proteolytic cleavage) of the rotavirus primary gene products occurs (*McCrae* and *Faulkner-Valle* 1981; *Arias* et al. 1982; *Ericson* et al. 1982). Our current understanding of the relationships between the primary gene products detected in SA11-infected cells and their subsequent modifications and final products is summarized in Table 3.

It should be noted that other terminologies for the structural polypeptides in the literature have included: (a) designating the polypeptides in the outer and inner capsids as "O" and "I" respectively (*Thouless* 1979; *Smith* et al. 1980), (b) designating the polypeptides simply by their apparent molecular weights (*Cohen* et al. 1979; *Novo* and *Esparza* 1981), or (c) a combination, designating each primary gene product as VP and the capsid polypeptides as "I" or "O" and cleavage products as "c" (*McCrae* and *Faulkner-Valle* 1981). Most of the confusion in the studies utilizing the O and I terminology has come about because the polypeptide designated O1A detected in infected cells was a nonstructural polypeptide and was not analogous to VP5* detected in purified virus. In addition, when O1A was found as a cleavage product in purified virus, its precursor (VP3) was not detected as a major outer capsid polypeptide (*Thouless* 1979; *Smith* et al. 1980; *Dyall-Smith* and *Holmes* 1981a, b).

Two major polypeptides – VP2 and VP6 – have been consistently observed in all strains analyzed to date. These two proteins, both of which are found in single-shelled particles, have been reported to comprise about 80% of the total virus protein composition, based on densitometer tracings of stained viral proteins (*Obijeski* et al. 1977) and 99% of the protein was in single-shelled particles (*Novo* and *Esparza* 1981). The largest viral polypeptide, VP1 (molecular weight 125 000; 125 K), is a minor protein associated with single-shelled particles and viral core particles. It remains controversial whether a fourth high-molecular-weight polypeptide (that is not a proteolytic cleavage product) is associated with single-shelled particles. The existence or fate of the fourth high-molecular-weight primary gene product, which is predicted from the four high-molecular-weight genome segments, has not been clearly demonstrated in any studies to date.

A few studies have attempted to localize the polypeptides in the capsid. It has been suggested from iodination experiments with bovine rotavirus particles that the VP2 (91 K) polypeptide is the most exposed polypeptide of the inner capsid (*Novo* and *Esparza* 1981). This conclusion has been challenged by the demonstration that single-shelled particles can be converted into icosahedral core particles by treatment with high concentrations of chaotropic agents such as $CaCl_2$ or sodium thiocyanate (*Almeida* 1979; *Bican* et al. 1982); this conversion results in the selective loss of VP6, suggesting that VP6 and not VP2 may be the most externally located polypeptide in single-shelled particles (*Bican* et al. 1982; *Estes* and *Cohen*, unpublished observations). One plausible explanation for these apparently different results is that VP6 contains a low number of tyrosine residues or inaccessible tyrosine residues that would not be iodinated by the lactoperoxidase method. The inner core particles derived from single-shelled particles contain a minor amount of one high-molecular-weight polypeptide in addition to VP1 and VP2 (*Bican* et al. 1982). It remains to be established whether this additional polypeptide represents the "missing" fourth high-molecular-weight polypeptide or whether it is a cleavage product of VP2 that has been described previously (*Estes* et al. 1981a).

The polypeptides of the outer capsid are of particular interest due to their possible roles in virus infectivity and virulence, and as antigens that might elicit neutralizing antibodies for disease protection. Three to five proteins have been associated with only the

outer capsid, a variation that is explained in part by the fact that the outer capsid high-molecular-weight polypeptide (VP3) is susceptible to proteolytic cleavage, yielding the cleavage products VP5* (60 K) and VP8* (28 K) (*Espejo* et al. 1981; *Estes* et al. 1981a; *Clark* et al. 1981). This cleavage apparently is required for viral infectivity (*Estes* et al. 1981a) (see Sect. 11).

One or two outer capsid polypeptides have been reported to be glycosylated (*Rodger* et al. 1977; *Cohen* et al. 1978, 1979; *Matsuno* and *Mukoyama* 1979; *Estes* et al. 1981a). Recent studies have demonstrated heterogeneity in the simian rotavirus SA11 glycoprotein VP7. The molecular weight (36 000–38 000) and the ability to detect one or two glycoproteins vary according to the virus stock analyzed (*Estes* et al. 1982). This heterogeneity apparently reflects mutations in the glycoprotein gene, but whether heterogeneity in the glycoprotein has any biologic significance, such as antigenic drift in influenza viruses (*Webster* and *Laver* 1980), remains unknown. The heterogeneity is of interest, however, since it helps explain discrepancies in the reported polypeptide patterns.

The final outer capsid polypeptide (VP9) has not been consistently found by all groups (*Arias* et al. 1982). Definitive proof of the relationship of VP9 to a primary gene product is lacking, although one study in our laboratory suggests that it is modified from the 26-K primary gene product in an unknown manner (*Ericson* et al. 1982). Others have speculated that this minor outer capsid polypeptide is glycosylated (*Dyall-Smith* et al. 1981a).

This review of the rotavirus structural polypeptides has summarized and focused on the studies with SA11 and the bovine rotavirus where the conclusions of the number of structural polypeptides in purified virus have been similar. It should be mentioned that, after extensive studies with the United Kingdom strain of the bovine rotavirus, *McCrae* and *Faulkner-Valle* (1981) and *McCrae* and *McCorquodale* (1982) concluded that 10 of the 11 primary gene products were viral structural polypeptides. Whether this result reflects differences between the UK strain and SA11 remains to be determined.

It is clear that some of the structural proteins of rotaviruses isolated from different species, and even polypeptides from different virus strains of the same species, may have different molecular weights (*Thouless* 1979; *Espejo* et al. 1980b), so strain differences still may explain some of the discrepancies in the reports on polypeptides.

## 7.2 Rotavirus Polypeptides in Infected Cells

In addition to the structural polypeptides, several other polypeptides are detectable in cells infected with rotaviruses. These polypeptides represent nonstructural polypeptides or precursors or intermediates to structural or nonstructural polypeptides. We have defined nonstructural polypeptides by their absence in purified virus preparations, by their inability to be immunoprecipitated with hyperimmune antisera prepared against purified virus, and by comparison of their partial protease maps with those generated from polypeptides of similar molecular weight synthesized in vitro from viral RNA transcripts (*Ericson* et al. 1982). We suggest designating the nonstructural polypeptides as NS, followed by their molecular weight (i.e., NS53, NS35, NS34, gNS29, and gNS28 for the SA11 nonstructural polypeptides; Table 3). Other designations in the literature have included NS1–4 (*Thouless* 1979; *Smith* et al. 1980; *Dyall-Smith* and *Holmes* 1981a, b), and NCVP for noncapsid viral polypeptides 1–3 (*Matsuno* and *Mukoyama* 1979; *Matsuno* et al. 1980), and NCVP 1–6 (*Arias* et al. 1982).

The pattern of polypeptide synthesis reported for SA11 (*Ericson* et al. 1982) is in general agreement with other recent studies of the polypeptides in SA11 or bovine rotavirus-infected cells (*Matsuno* and *Mukoyama* 1979; *Thouless* 1979; *Carpio* et al. 1981a; *McCrae* and *Faulkner-Valle* 1981; *Urquidi* et al. 1981; *Arias* et al. 1982). Rotavirus infection (at high multiplicity and in conjunction with actinomycin-D) greatly decreases host cell protein synthesis, which allows easy detection of virus-specific polypeptides. Post-translational modifications (glycosylation and proteolytic cleavage of polypeptides) are readily demonstrated by using sugar labels, by performing pulse-chase experiments, and by using inhibitors of glycosylation and amino acid analogs (*Ericson* et al. 1982). Detection of all the precursors to the glycoproteins requires the use of the antibiotic tunicamycin, which blocks glycosylation.

These studies have revealed a second virus-specified glycoprotein (molecular weight 29 000), synthesized in SA11-infected cells from a 20 000 molecular weight precursor, in addition to the structural glycoprotein VP7, which is synthesized from a precursor of 35 500 molecular weight. Both these glycoproteins are glycosylated co-translationally and then post-translationally modified. The final post-translational polypeptide modifications result from carbohydrate trimming (*Ericson* et al. 1983).

These results with SA11 have been extended further by examining the polypeptide(s) synthesized in MA-104 cells infected with bovine (Lincoln strain) and porcine (OSU strain) rotaviruses in the presence and absence of tunicamycin (Ericson and Estes, unpublished data). These studies confirm SA11 as a general rotavirus model. The molecular weights for the individual polypeptides differ for the different virus strains, thus agreeing with other reports (*Rodger* et al. 1977; *Thouless* 1979), and the relative migration of the precursor to the structural glycoprotein may differ both between and within virus strains. In some strains the glycoprotein precursor migrates more slowly than the 35-K nonstructural protein, and in other strains it migrates between the 35-K and 34-K nonstructural polypeptides. The different migration of these precursors may explain the reports that the hypothesized precursor to the bovine virus glycoprotein (UK strain) migrates more rapidly than the bovine proteins designated VP8 and VP9 by *McCrae* and *Faulkner-Valle* (1981), and that the precursor to the SA11 glycoprotein co-migrated with the nonstructural NCVP3 in a study by *Arias* et al. (1982).

Our present understanding of rotavirus polypeptide maturation is summarized in Table 3. It remains to be determined whether the extensive modifications of the rotavirus polypeptides (*McCrae* and *Faulkner-Valle* 1981; *Arias* et al. 1982; *Ericson* et al. 1982) modulate or affect the biological activities of these proteins. Although our knowledge of the molecular biology of the rotaviruses and the events regulating virus synthesis is still in its infancy, it is apparent that some of the differences in the amounts of the polypeptides synthesized in vivo and in vitro which have been interpreted as gene regulation are the result of post-translational modifications.

## 7.3 Protein-Coding Assignments of the Rotavirus Genome Segments

The proteins encoded by each of the 11 segments of the rotavirus RNA genome have been investigated by biochemical techniques. Rotavirus polypeptides have been synthesized in vitro from virus-specific RNA transcripts (*Mason* et al. 1980, 1981, 1983; *Estes*

Table 4. Rotavirus gene-coding assignments based on in vitro translation of isolated denatured genome segments

| Genome segment | Virus | | |
|---|---|---|---|
| | Simian[a] | Simian[b] | Bovine[c] |
| 1 | 113 (I1) | VP1 (I) | VP1 (I) |
| 2 | 96 (I2) | VP2 (I) | VP2 (I) |
| 3 | 91 (I3) | VP3 (O) | VP3 (I) |
| 4 | 84 (I4) | NCVP1 + 13 K | VP4 (O) |
| 5 | 57 (O1A) | NCVP2 | VP5 |
| 6 | 42 (I5) | VP6 | VP6 (I) |
| 7 | 34 (O2)[d] | pVP7[d] | VP8 (I) |
| 8 | 33 NS1[d] | NCVP3[d] | vpr7 (O) → VP7c |
| 9 | 31 (NS2)[d] | NCVP4[d] | VP9 nonstructural |
| 10 | 21 (NS3) | pNCVP5 → NCVP5 | VP12 → VP10 → VP10c |
| 11 | 25 (O4) | ND[e] | VP11 → VP11c |

[a] *Smith* et al. (1980) and *Dyall-Smith* and *Holmes* (1981a); [b] *Arias* et al. (1982); [c] *McCrae* and *McCorquodale* (1982); [d] Individual assignments from genes 7–9 have not been made; [e] No products detected

et al. 1981b) or from denatured double-stranded genome RNA (*Smith* et al. 1980; *Dyall-Smith* and *Holmes* 1981a; *Arias* et al. 1982; *McCrae* and *McCorquodale* 1982). The synthesized products have been identified as virus specific by immunoprecipitation and by comparison of peptide maps with those generated from authentic viral polypeptides from purified virus. The protein-coding assignments for the SA11 genome as defined by *Mason* et al. (1981, 1983) are also summarized in Table 3. Different coding assignments for SA11 and for the UK strain of bovine rotavirus, reported by others and based on the translation of denatured isolated genome segments, are shown in Table 4. Controversy persists over the SA11 coding assignments of genome segments 2 and 3. Although we have detected only one definitive gene product from transcripts of these two segments, others have reported that segments 2 and 3 encode two inner capsid polypeptides (*Smith* et al. 1980) or one inner and one outer capsid polypeptide (*Arias* et al. 1982). Gene segment 4 has also been reported to encode an inner capsid polypeptide (*Smith* et al. 1980) or a nonstructural polypeptide and a low-molecular-weight polypeptide (*Arias* et al. 1982). The complete protein-coding assignments of the UK strain of bovine rotavirus have recently been reported (*McCrae* and *McCorquodale* 1982). The assignments for the bovine rotavirus agree, in general, with our assignments for SA11 and extend them by suggesting that segment 3 encodes a polypeptide that is translated poorly and is cleaved, but whose identity in infected cells remains undetermined. In addition, protein assignments were made for the UK gene segments 7–9. Segment 7 encoded a polypeptide VP8 which is a structural polypeptide of the inner capsid, segment 8 encoded the precursor to the viral structural glycoprotein VP7, and segment 9 encoded the only gene product VP9 described as being nonstructural in this system. Although exact correlations between these two viral systems may not be possible, comparisons of the polypeptide patterns for SA11, the bovine (Lincoln) and porcine (OSU) strains (Ericson and Estes, unpublished data) predict that VP8 and VP9 of the UK virus should be analogous to the SA11 NS35 and

NS34. Completion of the SA11 gene map should determine whether the SA11 and UK protein-coding assignments are identical.

Identical protein-assignment maps between rotavirus strains cannot be assumed. This has already been emphasized in the rotavirus system by the demonstration that the proteins encoded by genes 10 and 11 of viruses with a long electropherotype (i.e., SA11 and human strain Wa) were NS3 and O4, while the proteins encoded by a human strain (M) with a short electropherotype were of the opposite assignment (*Dyall-Smith* and *Holmes* 1981a). These results would suggest that the rotavirus gene maps may not be universal.

The ability to distinguish rotavirus strains by RNA electropherograms and polypeptide patterns has stimulated comparisons of the RNA electropherotypes and of the structural polypeptides of different rotavirus strains in an attempt to determine which RNA segments code for the polypeptides that represent type-specific antigens (*Kalica* et al. 1978b; *Rodger* and *Holmes* 1979). No such correlation has been achieved, and the existing mapping studies suggest that these attempts to use relative migration patterns on polyacrylamide gels as a method of relating a viral polypeptide to the "coding" RNA genome segment are doomed because of oversimplification. For example, the initial failure of investigators to recognize that several of the viral structural polypeptides are not primary gene products led to mismatching of polypeptides and genome segments. In addition, this approach was unsuccessful because the size of the polypeptide encoded by an RNA segment cannot be predicted by direct comparison of migration patterns. Although direct coding assignments have been made for a few rotavirus strains, caution must be exercised in generalizing between gene maps for different virus strains because of the possibility of gene rearrangements, as has been demonstrated for the reoviruses (*Sharpe* et al. 1978).

Confirmation of the rotavirus gene map by the genetic approach has made slower progress. A series of temperature-sensitive mutants of SA11 (*Ramig* 1982) and the bovine UK virus have been isolated (*Greenberg* et al. 1981a; *Faulkner-Valle* et al. 1982), but these mutants have not yet been exploited to their full potential. Preliminary studies with reassortants between temperature-sensitive mutants of the UK virus and noncultivable human rotaviruses have indicated that the neutralization gene for the Wa strain of human rotavirus is gene segment 9, gene segment 8 for human rotavirus strain DS-1, and gene segment 8 or 9 for the UK virus (*Kalica* et al. 1981b; H. Greenberg, personal communication). The assignment for the UK neutralization gene is consistent with biochemical assignment of gene 8 as encoding the precursor to the structural glycoprotein (*McCrae* and *McCorquodale* 1982). The assignment of different genes of human rotavirus strains as neutralization genes suggests that switching in the migration of RNA species in the 7–9 complex may be a common phenomenon, and it emphasizes the importance of performing complete and critical analyses on several rotavirus strains before general conclusions about the rotavirus gene maps are made.

## 7.4 Rotavirus Glycoproteins

The recognition that the rotaviruses code for two glycoproteins (one structural and the other, tentatively, a nonstructural glycoprotein) is of interest since these viruses are not enveloped. The occurrence of glycoproteins in nonenveloped viruses is rare, so the rotaviruses may represent novel models for probing glycoprotein biosynthesis and func-

tion. The fact that the rotaviruses acquire a transient envelope as they bud into the lumen of the endoplasmic reticulum (*Saif* et al. 1978; *Altenburg* et al. 1980) is consistent with the hypothesis that the precursor to the structural glycoprotein is co-translationally glycosylated, and it suggests that the glycoprotein(s) may play a role in viral morphogenesis.

This is also supported by the observation that the structural glycoprotein VP7 is localized by immunoelectron microscopy at the endoplasmic reticulum with monospecific antisera to VP7 (*Petrie* et al. 1982). Since the transient envelope does not appear to be essential for viral infectivity, and since preparations of nonenveloped particles exhibit specific infectivities similar to most mammalian viruses (50–100 physical particles: 1 plaque-forming unit), a role for the envelope remains to be defined.

The rotavirus glycoproteins are also of interest because they represent a class of relatively few viral glycoproteins that do not contain oligosaccharides of the complex type (*Kornfeld* and *Kornfeld* 1980). Both of the rotavirus glycoproteins have been characterized biochemically as containing N-linked high-mannose oligosaccharides based on their sensitivity to digestion by endo-β-*N*-acetylglucosaminidase H and by the fact that their synthesis is blocked by treatment with tunicamycin (*McCrae* and *Faulkner-Valle* 1981; *Arias* et al. 1982; *Ericson* et al. 1982; *Ericson* et al. 1983). These results are supported by the observations that; (a) these glycoproteins incorporate radiolabeled mannose and glucosamine, but not galactose, fucose, or glucose; (b) the structural glycoprotein VP7 is not altered by treatment with neuraminidase (*Ericson* et al. 1982); and (c) agglutination of bovine rotavirus particles with various lectins suggested mannose as a terminal residue (*Cohen* et al. 1978).

The structural glycoprotein (VP7) is also of interest because it is thought to elicit neutralizing antibody. This hypothesis is supported by genetic studies with the human Wa strain and with the bovine UK strain that assign the neutralizing gene to a genome segment of the 7–9 complex (*Kalica* et al. 1981b), and by biochemical studies with SA11, showing that these genome segments encode the precursor to the viral structural glycoprotein and two nonstructural polypeptides (*Arias* et al. 1982; *Ericson* et al. 1982; *Mason* et al. 1983). Additional evidence to support this hypothesis is the fact that monospecific antisera prepared against VP7 neutralize viral infectivity (*Bastardo* et al. 1981; *Killen* and *Dimmock* 1982). It must be recognized, however, that the necessary comprehensive studies with proven monospecific antisera to the other outer capsid polypeptides to determine whether or not other virus capsid polypeptides can also elicit neutralizing antibodies have not been performed.

Knowledge of the gene (8 or 9 in the few strains examined to date) coding for the structural glycoprotein that elicits neutralizing antibodies will undoubtedly be applied through recombinant DNA technology in attempts to develop modern vaccines.

## 8 Virion Enzymes

Rotavirus particles contain an RNA-dependent RNA polymerase, which is consistent with their classification as members of the Reoviridae. The polymerase was first demonstrated in bovine rotavirus by *Cohen* (1977). He showed that the RNA synthesized in vitro was single-stranded, based on its sensitivity to pancreatic RNase, and that it represented the entire genome because it hybridized completely with double-stranded virion RNA. Purified virus banding at a density of 1.359 g/ml (double-shelled virus) had low poly-

merase activity, but enzymatic activity was stimulated by heat-shock (56 °C for 30 min) or treatment of the particles with 1 m*M* EDTA, which also resulted in a shift in the density of the particles to 1.378 (single-shelled virus). Optimal polymerase activity was found between 45° and 50 °C at pH 8 in the presence of 8–10 m*M* magnesium ions. *Hruska* et al. (1978) later confirmed the presence of polymerase activity in single-shelled bovine rotavirus particles (density 1.38) purified from tissue culture fluids and in human rotavirus samples derived from stool extracts. Activation of the polymerase was further characterized when *Cohen* et al. (1979) showed that the cation $Ca^{++}$ plays a major role in stabilizing the outer capsid of the virus at near-neutral pH values. At very low calcium ion concentration, or after treatment of the virus with chelating agents, the external glycoproteins (two in their bovine virus) were solubilized (molecular weights 34 and $31 \times 10^3$) and the polymerase activity was stimulated. Although proteolytic enzymes play a role in activating viral infectivity (see Sect. 11), they have not been shown to stimulate polymerase activity.

The properties of the RNA polymerase of human rotavirus (*Spencer* and *Arias* 1981) are similar to those of the bovine virus. Optimum polymerase activity requires all four ribonucleoside triphosphates, a preference for magnesium over manganese as the divalent cation, and a pH of 8.5. Polymerase activity is inhibited by sodium pyrophosphate but not by actinomycin-D, α-amanitin, or rifampin (*Cohen* 1977; *Spencer* and *Arias* 1981). An obligate requirement for a hydrolyzable form of ATP was also reported for transcription with human viruses, and it has been suggested that ATP is important not only for polymerization but also for initiation and elongation of RNA molecules (*Spencer* and *Arias* 1981).

Studies of the enzymes in related reovirus and cytoplasmic polyhedrosis virus suggest that rotavirus particles may contain other enzymes (nucleotide phosphohydrolase, guanylyl transferase, and methyl transferases) required for the formation of virus-specific messenger RNAs with a 5′-terminal cap (*Shatkin* 1976). To date, there have been no reports of these enzymes in rotavirus particles and no direct demonstration of capping of rotavirus messenger RNAs. Localization of the polymerase activity was addressed by examining the core particles prepared from single-shelled particles by treatment with chaotropic agents (*Bican* et al. 1982) and no activity was found. Whether the lack of polymerase activity in the cores was due to its inactivation by the $CaCl_2$ or due to structural changes caused by the removal of VP6 was not clear, but purified VP6 did not possess detectable polymerase activity. The proteins involved in this enzymatic function therefore remain obscure.

A poly(A) polymerase activity has been detected in human rotavirus particles (*Gorziglia* and *Esparza* 1981). Interestingly, this enzymatic activity was associated only with complete double-shelled particles, in contrast to the RNA-dependent RNA polymerase. The function of the poly(A) polymerase in particles and its possible relation with the virus-associated polymerase remain unknown.

## 9 Transcription of Rotavirus RNA

Our current knowledge of rotavirus RNA transcripts comes from a limited number of studies on the transcripts synthesized in vitro with the endogenous RNA polymerase. The initial studies (*Cohen* 1977) showed that the polymerase products synthesized in vitro with the bovine rotavirus were sensitive to ribonuclease (i.e., single-stranded), and they

hybridized to viral RNA. In later experiments, *Cohen* and *Dobos* (1979) showed that the transcripts hybridized to all 11 of the genome segments, and that they were active in a messenger-dependent, in-vitro translation system derived from rabbit reticulocytes. Only the smaller molecular weight (< 40 000) polypeptides were synthesized in these experiments.

The products of the rotavirus polymerase in the simian rotavirus SA11 were further characterized by *Mason* et al. (1980), who analyzed the transcripts by sedimentation in sucrose gradients and by electrophoresis in acid-urea-agarose gels. These studies demonstrated the importance of adding an RNase inhibitor to the polymerase reaction mixture to increase the synthesis of the larger transcripts. They also demonstrated that the transcripts are separable into nine distinct groups when analyzed on acid-urea-agarose gels (Fig. 5), but they are not resolved into discrete peaks, as are reovirus transcripts, when analyzed on sucrose gradients. The SA11 transcripts were active in a messenger-dependent, cell-free translation system derived from rabbit reticulocyte lysates, and 11 polypeptides were synthesized ranging in molecular weight from 20 000 to 125 000 (*Mason* et al. 1980).

Translation of all the primary gene products was stimulated by the use of transcripts synthesized in the presence of *S*-adenosyl methionine (SAM), suggesting that rotavirus transcripts might contain a 5′-methylated cap. SAM did not significantly stimulate the polymerase activity and, with the exception of their more efficient translation, no differences in the transcripts synthesized in the presence or absence of SAM were detected (*Mason* et al. 1980). Whether the increased translational efficiency of the RNA transcripts synthesized in the presence of SAM reflected an increased stability of the transcripts or more efficient initiation of translation remains unknown.

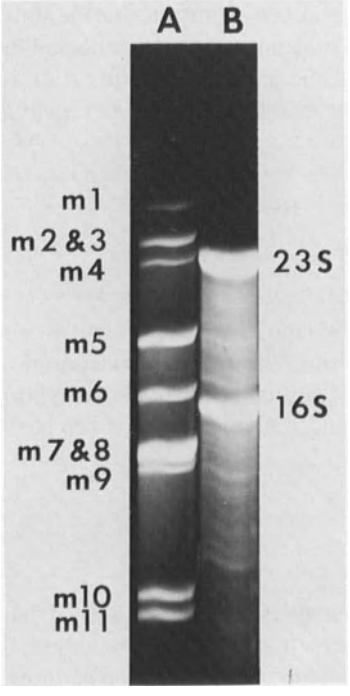


Fig. 5. Agarose gel electrophoresis of simian rotavirus SA11 transcripts synthesized in vitro. SA11 transcripts were subjected to electrophoresis in a 2% acid-urea-agarose gel, according to *Mason* et al. (1980). Migration was from the *top* to the *bottom,* and the bands were visualized after staining with ethidium bromide. The second and sixth transcript bands each contained transcripts from two segments. (Courtesy of B. Mason)

Comparison of these proteins synthesized in vitro from the transcripts with the virus structural polypeptides and with the polypeptides synthesized in infected cells by molecular weights, by partial protease maps, and by immunoprecipitation with hyperimmune antiserum to purified SA11 has produced our current understanding of the rotavirus primary gene products, as outlined in Sect. 7.3. Further translation of individual transcripts isolated from the acid-urea-agarose gels and identification of the segment that encodes them by hybridization to viral RNA has generated the gene-coding assignments for SA11 (*Mason* et al. 1980, 1983; see Table 3).

*Bernstein* and *Hruska* (1981) have also shown that the in-vitro-synthesized transcripts are of one polarity, they are identical in size to the denatured parental double-stranded RNA segments and they are not polyadenylated.

The transcripts synthesized in vitro have also been used to probe the genetic relatedness among rotavirus strains (*Matsuno* and *Nakajima* 1982) and to determine the parental origin of reassortant viruses constructed in the laboratory (*Greenberg* et al. 1982; *Flores* et al. 1982). Genetic relatedness was examined by annealing double-stranded genome RNA of two human strains of rotavirus (Wa and TK80), of a bovine strain (NCDV) of rotavirus, or of SA11 with $^{32}$P-labeled transcripts synthesized in vitro from each virus strain. Genetic homology was detected between the two strains of human rotavirus at the level of 88%–100% of the homologous reaction, but it was not known if these two human strains represented the same or different serotypes. No transcripts from the Wa virus hybridized with genome RNA from NCDV or SA11. Similarly, transcripts from NCDV did not hybridize with double-stranded RNA from human rotavirus (strain Wa), but they did hybridize with double-stranded RNA from SA11 at a level of 30% of the homologous value, suggesting a closer relationship of SA11 to NCDV than to human rotavirus (*Matsuno* and *Nakajima* 1982). These preliminary results indicate that hybridization may be a useful tool for identifying closely related viruses. They also demonstrate that the antigenic similarity observed between all rotavirus strains may not always be reflected in homology in the nucleotide sequence as detected by nucleic acid hybridization. A similar discrepancy between genetic and serologic results has been found in reovirus (*Gaillard* and *Joklik* 1980).

## 10 Rotavirus Replication Cycle

Biologically, the rotaviruses are fastidious agents. Until 1981 most human rotaviruses had not been successfully cultivated in vitro in most laboratories. Virus cultivation is described later in Sect. 11, but first we will consider the essential features of the rotavirus replication cycle as determined from studies with the bovine and simian viruses – which have been excellent models for studying rotavirus replication.

### 10.1 Growth

Several studies of the growth of animal rotavirus strains in tissue culture have shown that these viruses have a fairly rapid ($\cong$12 h) replication cycle. It is not known whether all strains (e.g., the still fastidious human strains) will exhibit the same replication patterns,

but the animal strains examined in different cells under conditions of high multiplicity of infection (MOI) have shown similar growth properties. The viral eclipse phase is approximately 2–3 h, with maximal virus production in 12 h at 37 °C. Proteolytic enzymes enhance viral infectivity, and the virus growth curves may be altered by the failure to pretreat virus inocula with proteolytic enzymes prior to infection of cell cultures. It should be recognized that most plaque assays for rotavirus include proteolytic enzymes in the overlay as facilitators. The results or titers obtained from these assays therefore represent the amount of activated virus in a stock. This titer will be artificially high unless virus stocks are activated with enzymes prior to inoculation of cell cultures. For example, in the absence of preactivation of virus, the input MOIs calculated on plaque assay results may be two- to tenfold higher than that observed experimentally by immunofluorescence assays that monitor the percentage of cells infected at different calculated MOIs (*Graham* and *Estes* 1980; *Estes* et al. 1981a). If cells are inoculated with trypsin-activated virus at high multiplicity (10–20 plaque-forming units/cell), the replication cycle is completed in 10–12 h at 37 °C; otherwise, a slow increase in virus yield may be observed up to 20 h. Viral protein synthesis is maximal at 3–5 h postinfection (*McCrae* and *Faulkner-Valle* 1981; *Ericson* et al. 1982), which is consistent with viral antigens being first detectable by the fluorescent antibody technique by 4–6 h postinfection (*Estes* et al. 1979b).

Replication occurs in the cytoplasm of cells, and viral antigens detected in the cytoplasm are often seen as discrete perinuclear granules. Late in infection, diffuse antigen is generally present throughout the entire cytoplasm. Different cell cultures can exhibit a marked variability in their susceptibility to rotavirus infection, and observable cytopathic effect correlates with the percentage of infected cells in a culture (*Estes* et al. 1979b). Some of the variability in cell susceptibility is overcome if viruses are activated with trypsin prior to culture inoculation and if trypsin is maintained in the media throughout the infection (*Graham* and *Estes* 1980). Progeny genome RNA synthesis is first detected during the virus latent period at 2–4 h postinfection, and it continues throughout the replication cycle without significant differences in the kinetics of production of the various genome segments (Estes, unpublished observation; *McCrae* and *Faulkner-Valle* 1981).

## 10.2 Morphogenesis

Rotavirus replication has been observed by thin-section EM both in intestinal samples from infected animals and in cultured cells inoculated with various strains of rotavirus (*Adams* and *Kraft* 1967; *Banfield* et al. 1968; *Lecatsas* 1972; *Bishop* et al. 1973; *Stair* et al. 1973; *Holmes* et al. 1975; *Hall* et al. 1976; *Chasey* 1977; *Saif* et al. 1978; *McNulty* 1978; *McNulty* et al. 1976d, 1979; *Pearson* and *McNulty* 1977; *Altenburg* et al. 1980; *Esparza* et al. 1980; *Carpio* et al. 1981a; *Petrie* et al. 1981, 1982; *Suzuki* et al. 1981). Several common features of morphogenesis have emerged from these studies, and *Chasey* (1977) has described five (I through V) different types of rotavirus particles that can be seen in thin sections of rotavirus-infected cells (see Figs. 6–8).

The few studies of the early stages of infection suggest that incoming virions enter the cell by endocytosis and are sequestered into lysosomes (Fig. 6a) (*Petrie* et al. 1981). Uncoating probably occurs in lysosomes, producing Chasey's type IV, 50-nm subviral particles (Fig. 7b) (*Petrie* et al. 1981). After the eclipse phase, virus particles consisting of a dense nucleoid (25–35 nm) surrounded by a layer of less-dense material (corresponding

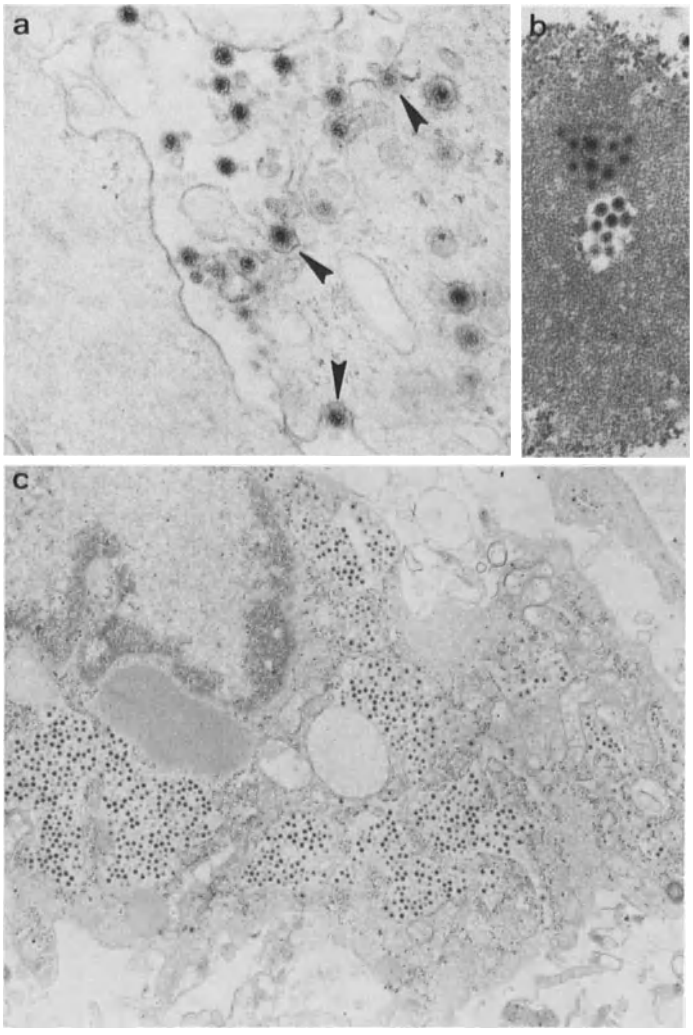


Fig. 6. a, SA11 rotavirus particles in close proximity to a CV-1 cell, with some in various stages of being phagocytized (arrowheads) and single particles enclosed in phagocytic vesicles within the cytoplasm. × 55 100 b, Dense core structures (type I) within an area of viroplasm. × 42 000. c, Portion of a cell with viroplasm in a juxtanuclear position, and distended cisternae of the endoplasmic reticulum and large vacuoles filled with vast quantities of SA11 particles, mostly type III. × 30 000 Rotavirus thin section EM. (Courtesy of A. Harrison)

to Chasey's type I particles) are formed near the periphery of large, granular cytoplasmic inclusions (viroplasms) (Fig. 6b), where newly synthesized viral proteins and possibly RNA have accumulated (*Altenburg* et al. 1979; *Petrie* et al. 1982).

Within these inclusions, the viral RNA is packaged into 50-nm "core" particles, and viral capsid proteins assemble around the "core." Virus particles bud through ribosome-free areas of the rough endoplasmic reticulum into swollen vesicles, becoming enveloped in the process. Perhaps the most characteristic features of rotavirus morpho-

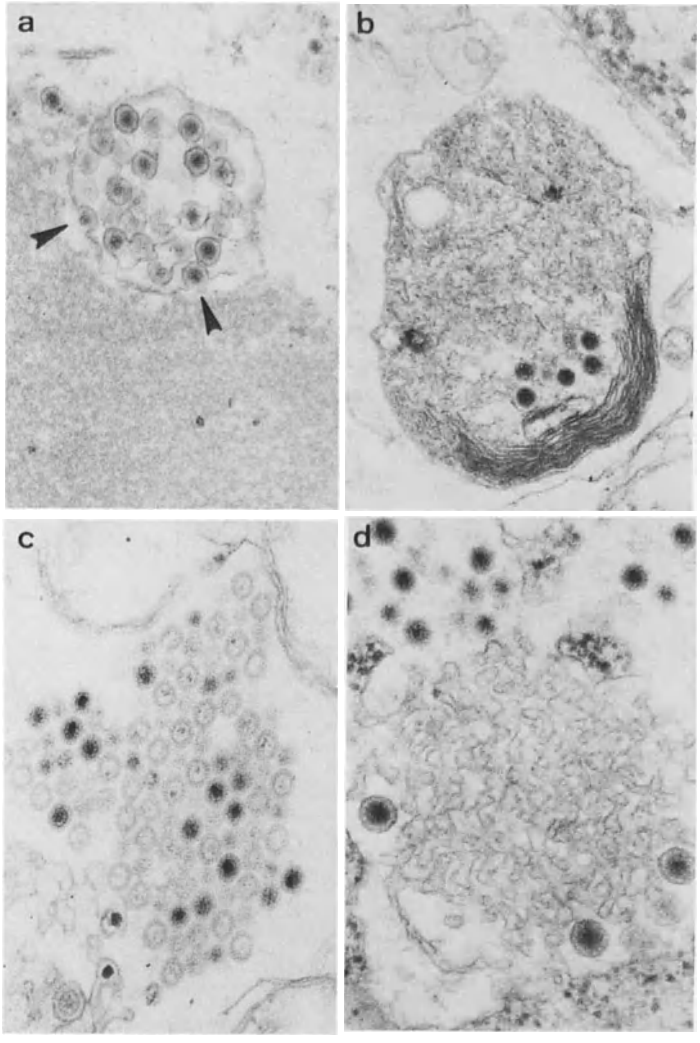


Fig. 7. a, Particles budding through an intracytoplasmic membrane (*arrowhead*) and enveloped particles (type II) within a vacuole. × 52 500 b, Type IV particles within a lysosome-like body. × 85 000 c, A rare paracrystalline array of particles with dense or hollow cores and an electron-lucent envelope in a rarified area of a necrotic cell. × 53 000 d, Mass of convoluted smooth membrane and both type II and III virus particles in close proximity. × 76 000

genesis are the distended cisternae of the rough endoplasmic reticulum and large vacuoles filled with vast quantities of virus particles (Fig. 6c). Most of these particles resemble Chasey's type III particles, having a dense central core with a less-dense halo and measuring 57–63 nm in diameter (Fig. 6c). Less numerous larger particles, 74–79 nm in diameter (Chasey's type II), possess a well-defined envelope derived from budding through the membrane of the endoplasmic reticulum (Fig. 7a). The envelope is apparently lost as virus particles continue to mature.

Other viral forms seen are similar to Chasey's type V but, like those described by

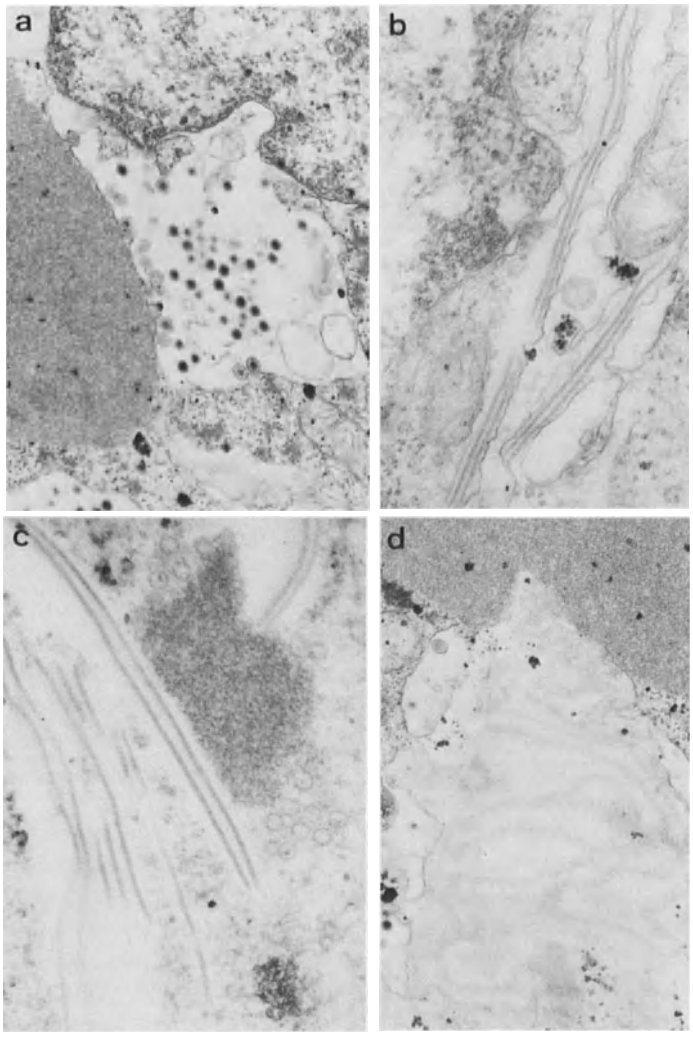


Fig. 8. a, Wide separation of nuclear envelope with accumulation of virus particles between the lamellae. × 29 800 b, Filaments in the cytoplasm. × 37 350 c, Filaments in a rarified area of the nucleus accompanied by viroplasm and hollow particles. × 64 800 d, Tangentially cut filaments producing a moiré pattern. × 64 800

*Snodgrass* et al. (1977b) in the villous epithelial cells of lambs, they lack the dense outer profile seen by Chasey. These particles, 51–57 nm in diameter, consist of a moderately dense core surrounded by an electron-lucent halo with fine, filamentous projections interconnecting the particles, and are found in association with the two more common types of particles.

A recent study addressed the question of which forms of virus particles seen in thin sections of infected cells correspond to the double- and single-shelled particles observed by negative-contrast EM. The results suggested that Chasey's type III particles correspond to the double-shelled particles, the single-shelled particles correspond to Chasey's

type V particles, enveloped virus particles correspond to Chasey's type II particles, and subviral particles produced after viral uncoating correspond to Chasey's type IV particles (*Petrie* et al. 1981).

A second type of cytoplasmic inclusion seen in rotavirus-infected cells consists of masses of convoluted smooth membranes (Fig. 7d). Since these have been seen in other viral infections [mouse hepatitis virus (*David-Ferreira* and *Manaker* 1965), rubella (*Kim* and *Boatman* 1967), St. Louis encephalitis (*Murphy* et al. 1968)] and in a number of pathologic tissues (*Chandra* 1968), they may be a secondary effect of viral alteration of cell metabolism and synthetic capacity rather than a specific accumulation of rotavirus products.

Nuclear changes are also prominent late in infection. Irregular, and frequently wide, separation of the nuclear envelope is common, with accumulations of virus particles between the lamellae (Fig. 8a). Filaments are seen both in the cytoplasm (Fig. 8b) and in rare field areas of the nucleus, where they are occasionally accompanied by viroplasm and hollow particles 53–56 nm in diameter (Fig. 8c). When sectioned in a favorable orientation, cross striations may be seen in the filaments and, when cut tangentially, can produce a moiré pattern (Fig. 8d).

Mitochondria containing virus particles have also been observed (*Altenburg* et al. 1980; *Tektoff* et al. 1980). Concomitant with the changes caused by virus replication on cellular integrity as monitored by electron microscopy, biochemical evaluation of the effects of viral infection on macromolecular synthesis has shown that viral infection inhibits host cell functions. Macromolecular synthesis is inhibited as measured by a decreased incorporation of $^{3}$H-thymidine, $^{3}$H-uridine, and $^{3}$H-amino acids into cellular macromolecules (*Carpio* et al. 1981a; *McCrae* and *Faulkner-Valle* 1981; *Ericson* et al. 1982). Eventually, virus particles are expelled from the cells either by a process of exocytosis or by rupture of the plasma membrane.

The viral specificity of many of these ultrastructural changes has been shown by immunocytochemical electron microscopy using polyvalent antisera prepared against purified double-shelled particles (*Altenburg* et al. 1979; *Chasey* 1980) or monospecific antisera against purified viral structural polypeptides (*Petrie* et al. 1982). These studies have shown that rotavirus inner capsid proteins are synthesized throughout the cytoplasm and become concentrated in viroplasmic inclusions, while the outer capsid glycoprotein is synthesized only in ribosomes of the rough endoplasmic reticulum. Thus, the outer capsid layer appears to be acquired during viral budding into cisternae of the endoplasmic reticulum. Although antisera to all the viral capsid polypeptides have not yet been tested, these studies begin to offer an understanding of how these viruses are assembled in cells (*Petrie* et al. 1982).

## 11 Cell Culture Propagation and Assay

Simian rotavirus SA11 and the "O" agent were propagated in cell culture in the 1960s (*Malherbe* and *Strickland-Cholmley* 1967), but at that time these viruses were not known to be associated with clinical illness. Bovine rotavirus, the cause of neonatal calf diarrhea (*Mebus* et al. 1969), was subsequently grown in calf kidney cells (*Mebus* et al. 1971a). Until 1981 only a limited number of strains of bovine and porcine rotavirus (*Fernelius* et al. 1972; *Welch* and *Twiehaus* 1973; *Woode* et al. 1974; *McNulty* et al. 1967b, 1977; *L'Haridon* and

*Scherrer* 1976; *Theil* et al. 1977; *Babiuk* and *Mohammed* 1978), avian rotavirus (*McNulty* et al. 1979), and human rotavirus (strain Wa) (*Drozdov* et al. 1979; *Wyatt* et al. 1980) could be grown in cell culture. The investigation of rotaviruses isolated from various animals and birds has been hampered by their fastidious growth properties in cell cultures. Recognition that pancreatic proteolytic enzymes enhance viral replication was a major breakthrough for rotavirus detection, isolation, and propagation in cell culture (*Babiuk* et al. 1977b; *Babiuk* and *Mohammed* 1978; *Theil* et al. 1977, 1978b; *Bryden* et al. 1977; *Almeida* et al. 1978; *Barnett* et al. 1979; *Clark* et al. 1979; *Schoub* et al. 1979; *Graham* and *Estes* 1980; *Theil* and *Bohl* 1980).

During the past year, successful cultivation of several strains of human rotavirus (*Sato* et al. 1981a; *Urasawa* et al. 1981) and the routine cultivation of strains of bovine rotavirus (*Fukusho* et al. 1981; *Bachmann* and *Hess* 1981) have been reported, using a combination of trypsin treatment of particles prior to inoculation of cells and the inoculation of cells in tubes maintained as rolling cultures with trypsin being additionally present in the culture medium during the entire course of infection. The stools that allowed successful isolation were not selected for extremely high concentrations of virus or for a high concentration of double-shelled virus particles. The infected cell culture lysates were, however, concentrated by centrifugation prior to the second and third passages in culture. Several human virus isolates have been subsequently adapted to growth in stationary cultures, and a plaque assay has been developed for most strains. There is variability in the amount of cytopathic effects produced by individual strains (*Urasawa* et al. 1981), and it remains to be determined how many strains can be readily cultivated. This successful cultivation of the human rotaviruses has clearly been a major advance in our technology, and important basic data required to develop strategies for disease prevention will undoubtedly now rapidly appear.

Although a variety of primary cells or epithelial cell lines (primarily of kidney origin) support the growth of rotaviruses isolated from different animal strains, a line of fetal rhesus monkey kidney cells (MA-104 cells, Microbiological Associates) has been shown to support the replication of virus from almost all strains of animals, either for initial isolation or following adaptation of these strains to growth in tissue culture of other cell lines. This fact and the ability to plaque rotaviruses from different strains in one cell line (*Estes* and *Graham* 1980a) should facilitate future attempts to differentiate serotypes and to study the replication and genetics of these viruses.

It has been suggested (*Hoshino* et al. 1981) that, based on their characteristics in cell culture, rotaviruses can be divided into two groups: (a) those that can only grow very poorly without proteolytic enzyme treatment (human, porcine, chicken, turkey, and some strains of bovine rotavirus) and (b) those that can undergo multiple rounds of replication in cell cultures without the aid of proteolytic enzymes (feline, canine, and simian rotaviruses, the UK strain of bovine rotavirus). Since viruses in the second group cannot undergo multiple cycles of replication in some cell lines (e.g., SA11 in Vero cells) (*Graham* and *Estes* 1980) without proteolytic enzymes, this classification may be artificial.

The basis for the enhancement of viral infectivity by proteolytic enzymes has been examined with SA11 and a bovine rotavirus strain, and it should be emphasized that both these viruses can replicate to some extent without the aid of proteolytic enzymes. These studies have shown that a major outer capsid polypeptide (VP3) is cleaved by trypsin (*Clark* et al. 1981; *Espejo* et al. 1981) or by trypsin or elastase, but not by chymotrypsin (*Estes* et al. 1981a). A correlation of the cleavage of VP3 with enzymes that enhance infec-

tivity (trypsin and elastase), but not with chymotrypsin, which does not enhance infectivity, suggests that the proteolytic cleavage of VP3 activates infectivity. Cleavage of a second polypeptide, VP2, was also detected in the studies with SA11 (*Estes* et al. 1981a). Although the cleavage of VP2 appeared to be a secondary cleavage, it remains to be definitively proven whether the cleavage of VP3, VP2, or both, is the critical molecular event that activates infectivity (*Estes* et al. 1981a). It also remains to be determined whether this is a common mechanism for all virus strains. If it is, the molecular basis for the difficulty in propagating some of the rotavirus strains remains to be determined.

The gene coding for VP3 is genome segment 4 for SA11 and the UK virus (*Mason* et al. 1980; *Estes* et al. 1981b; *McCrae* and *McCorquodale* 1982; *Mason* et al. 1983), and these data are consistent with the observation that gene 4 is the segment of the UK virus that is able to rescue noncultivable human rotaviruses by gene reassortment (*Greenberg* et al. 1981). The observation that trypsin treatment cleaves a major outer capsid polypeptide is also consistent with earlier biologic studies suggesting that trypsin exerts its effect on the virus and not on the cells (*Barnett* et al. 1979; *Graham* and *Estes* 1980) and that trypsin treatment can allow viruses to undergo multiple rounds of replication in normally restrictive cells (*Graham* and *Estes* 1980). It has been suggested that proteolytic enzyme treatment allows enhanced penetration of virus into cells (and does not enhance adsorption or more efficient propagation once the virus has penetrated the cell), but this has not been rigorously proven. It is anticipated that, with the ability to cultivate a large number of virus strains, comprehensive studies on the molecular biology and on the antigenic variation of these viruses will be forthcoming.

It is of interest that although both the bovine and simian rotaviruses can be grown in cell culture in the absence of proteolytic enzymes, they cannot produce detectable plaques in the absence of trypsin. Quantitative plaque assays have now been reported for bovine, human, porcine, and simian rotaviruses (*Matsuno* et al. 1977b; *Ramia* and *Sattar* 1979; *Smith* et al. 1979; *Estes* and *Graham* 1980a; *Urasawa* et al. 1981; *Faulkner-Valle* et al. 1982; *Wyatt* et al. 1982). Plaque formation for all these viruses, with the exception of the UK strain of bovine rotavirus (*Faulkner-Valle* et al. 1982), requires the presence of pancreatin or trypsin in the agar overlay. A requirement for diethylaminoethyl(DEAE)-dextran as a second facilitator has not been found in all cases. DEAE-dextran enhances the size of the plaques when added to an overlay containing agar (*Smith* et al. 1979), but it is generally not required when purified agar or agarose overlays are used (Graham and Estes, unpublished observation). It is possible that the fastidious human rotaviruses may be more susceptible to inhibitors in agar, since *Urasawa* et al. (1981) reported they would only form plaques in purified agar. Although they have had only limited application to date, plaque-reduction assays could also be used to detect serum antibodies (*Matsuno* et al. 1977b; *Sato* et al. 1981b). These assays must be carefully interpreted, however, for us to be confident that neutralizing activity detected in sera at low dilutions does not represent nonspecific reductions of infectivity caused by anti-protease activity in serum (*Estes* and *Graham* 1980a; *Graham* and *Estes* 1980). A plaque assay for the recently cultivated human rotaviruses has also been developed; it utilizes acetylated trypsin (3 μg/ml), DEAE-dextran (50 μg/ml), and 0.6% purified agar for optimal plaque formation (*Urasawa* et al. 1981). These workers found that agar was inhibitory to plaque formation.

Because it has not been possible to apply the plaque assay to all viruses, viral infectivity has also been quantitated by fluorescence focus assays (*Banatvala* et al. 1975; *Bryden* et al. 1977; *Schoub* et al. 1979; *Clark* et al. 1979; *Barnett* et al. 1979). With this assay,

detection of human rotavirus is increased by low-speed centrifugation of the inoculum onto cell cultures (*Banatvala* et al. 1975; *Bryden* et al. 1977). A fluorescence-focus neutralization test has been used to compare serotypes of human rotavirus (*Thouless* et al. 1977b; *Flewett* et al. 1978; *Beards* et al. 1980). This method will continue to be useful for monitoring infections in experimental conditions where viruses replicate poorly, or where they may not induce cytopathic effects, or where abortive infections occur.

Several unusual virus-host interactions have been reported, but their significance remains undetermined. The establishment of persistent infections in both permissive (*Misra* and *Babiuk* 1980) and nonpermissive (*Estes* and *Graham* 1980b) cells has been reported, but the virus or cellular mechanisms influencing these interactions remain undefined. It has been suggested that cell permissiveness to rotavirus infection may vary depending on the physiological state of the host cell (*Bégin* 1980). These virus-cell interactions are of interest, however, because they suggest a potential for viral persistence in nature which could explain some of the unusual epidemiologic features of rotavirus disease and disease transmission.

The synthesis of large numbers of coreless, probably defective, particles has also been seen when ovine and porcine rotaviruses not adapted to serial growth in cell cultures were examined by electron microscopy (*McNulty* et al. 1978). Similarly, infections of human embryonic kidney cells with human rotaviruses resulted in poor replication (< 1% of the cells were infected), and the inoculated cells mainly synthesized particles lacking the outer capsid layer (*Wyatt* et al. 1974, 1976a). Even when up to 60% of the cells in a culture could be infected with human rotavirus, a large majority of the particles synthesized appeared defective (*Esparza* et al. 1980). These studies emphasize that the particular defect preventing normal replication of rotaviruses remains to be elucidated. It should be recognized that until this effect is understood routine cultivation of rotaviruses may remain elusive to the standard virology laboratory, particularly because it takes several passages to detect virus by cytopathic effects. One must also wonder if the current successful methods of cultivation are merely selecting cultivable mutants.

## 12 Rotavirus Disease in Humans

### 12.1 Significance

Epidemiological studies on rotavirus infections have shown these ubiquitous agents to be the major cause of gastroenteritis in children hospitalized with diarrhea in developed countries (*Kapikian* et al. 1976c; *Tallett* et al. 1977; reviewed in *Kapikian* et al. 1981b). Although gastroenteritis is no longer a major cause of death in developed countries, this illness remains a major cause of morbidity in infants and young children. Recent studies have expanded our understanding of this disease by evaluating the types of pathogens causing diarrhea in nonhospitalized patients in developed countries. One prospective study of diarrhea in infants and young children in Canada supported the data from the previous studies of hospitalized cases of diarrhea, and showed that rotaviruses are the most common enteropathogen in young children who are outpatients (*Gurwith* et al. 1981). In this study, only 3 of 72 children with rotavirus infection were hospitalized, while 23 required some kind of medical attention.

Another recent study has evaluated the economic impact of rotavirus gastroenteritis

in the United States (*Rodriguez* et al. 1980). It was estimated that rotavirus gastroenteritis annually accounts for 220–370 hospitalizations per 100 000 children less than 2 years of age. Based on an estimation of 6 600 000 children less than 2 years old in this country, the annual cost for hospitalization alone is $ 27 million. While this is significant, the total economic impact is much greater since the majority of cases of rotavirus diarrhea in this country receive outpatient care. The additional impact of rotavirus gastroenteritis that is also difficult to measure is the time parents lose from work when they stay home to care for their sick children.

While it is recognized that diarrhea, especially in children, is a major cause of morbidity in developing countries, only recently has the importance of various bacterial, viral, and parasitic agents been carefully assessed. A study of enteric pathogens associated with diarrhea at a diarrhea treatment center in rural Bangladesh revealed that rotavirus and enterotoxigenic *Escherichia coli* (ETEC) respectively were isolated from approximately 50% and 25% of patients less than 2 years of age, while ETEC was the most frequently identified pathogen for patients of all ages. In agreement with earlier studies in both developed and developing countries, rotavirus was identified as the most common pathogen for children less than 2 years of age who visited a treatment center for diarrhea (*Black* et al. 1980). In a 1-year study in a rural village of Bangladesh (*Black* et al. 1981), rotaviruses were the third most commonly detected enteropathogen. Rotavirus and ETEC were found in 31% of the diarrhea episodes experienced by children less than 2 years of age and in 70% of the episodes associated with dehydration. Lastly, these two pathogens were identified in the stools of 77% of young children with life-threatening dehydration seen at a diarrhea treatment center. This study suggested that these two pathogens may be the etiologic agents that are most likely to result in death, and this fact focuses efforts of vaccine development against ETEC and rotaviruses.

The need for immunoprophylaxis against the rotaviruses has been recognized (*Kapikian* et al. 1980; *Chanock* 1981), but a better understanding of the biology and epidemiology of the viruses and of host factors involved in protection from disease are required for this need to be filled. The clinical diagnosis and epidemiology of rotavirus gastroenteritis have been recently reviewed in detail (*Kapikian* et al. 1981b), so we will only highlight the main features of the disease in humans.

## 12.2 Incidence

In temperate climates, rotavirus gastroenteritis shows a definite seasonal pattern, with peak incidence during the winter months (*Bryden* et al. 1975); in tropical climates, rotavirus infections occur throughout the year (*Hieber* et al. 1978; *Soenarto* et al. 1981). In spite of these seasonal differences, infections do occur throughout the year in temperate, tropical, and subtropical climates. Figure 9 shows the typical seasonal pattern of disease observed during 1975–1976 and 1976–1977 among children who were admitted to the Hospital for Sick Children, Toronto, Canada, with rotavirus gastroenteritis or who acquired the disease in the hospital. During both years, peak infection rates occurred from December through March, when it is coldest in that part of the western hemisphere.

Nosocomial infections are well documented and have been reported to cause gastroenteritis in 10%–20% of control children admitted to hospitals (Fig. 9) (*Ryder* et al. 1977; *Soenarto* et al. 1981).

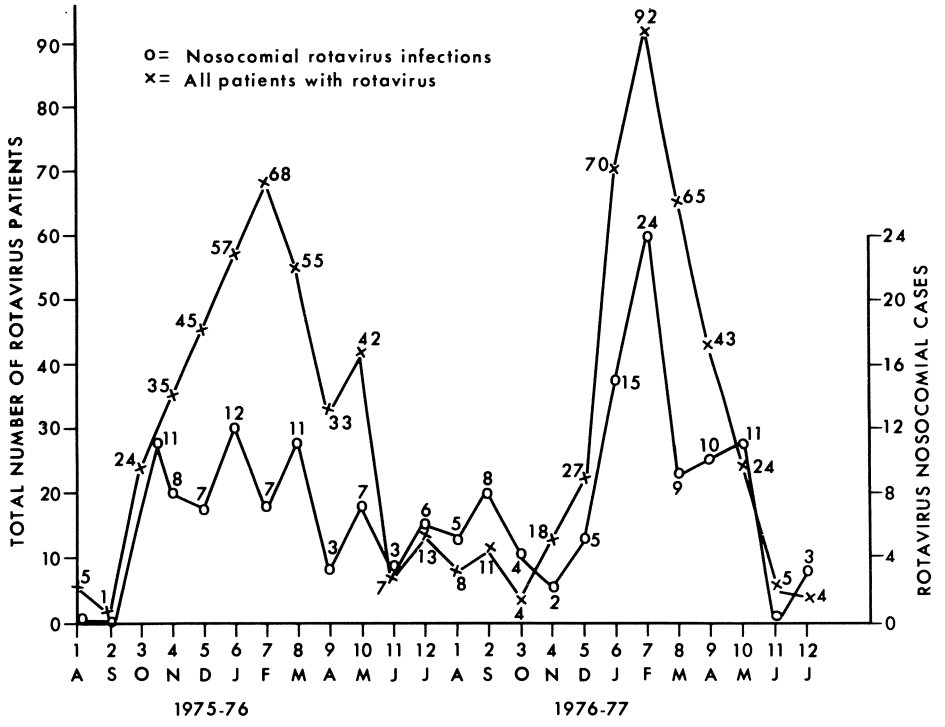


Fig. 9. Influence of season of the incidence of rotavirus during 1975–1976 and 1976–1977 among children who were admitted to the Hospital for Sick Children, Toronto, Canada, with rotavirus gastroenteritis or who acquired the disease in the hospital (Courtesy of P.J. Middleton, Hospital for Sick Children, Toronto, Canada)

Rotavirus is the most frequent cause of nonbacterial gastroenteritis among young children between 6 months and 2 years of age and is less frequent among children more than 5 years old. By 5 years of age, most children have acquired circulating antibody to the virus. The incubation period is 1–3 days and virus is, in general, excreted for 5–7 days (*Davidson* et al. 1975a). The infection can be mild or severe, or it can result in death (*Carlson* et al. 1978). Most children suffer from vomiting, followed by diarrhea and an associated fever (*Middleton* 1978). *Lewis* et al. (1979) performed a prospective study of 152 children with diarrhea, 74 of whom excreted rotavirus. They found a respiratory illness or otitis media in 66% of these children. Vomiting was also common, preceding diarrhea, and lasted between 1 and 3 days. Prolonged diarrhea was rare, and children less than 2 years old were most often affected. Persistent vomiting or dehydration occurred in many children, with dehydration being most severe in infants 12–18 months old. This is now recognized as "rotavirus syndrome" (*Editorial* 1979). *Foster* et al. (1980) suggested that human rotavirus might have been spread by the respiratory, as well as the fecal-oral, route during an epidemic which occurred on Truk Island in the 1960s. However, no direct evidence of rotavirus replication in the nasopharynx has been demonstrated (*Goldwater* et al. 1979), and a recent prospective study comparing the clinical symptoms of children with diarrhea caused by rotavirus or other pathogens found no difference in the

occurrence of respiratory symptoms (*Maki* 1981). The possibility that respiratory symptoms might arise from aspiration of vomitus should be considered.

Rotavirus infection has been described in a limited number of children with intussusception (*Konno* et al. 1978), exanthem subitum (*Saitoh* et al. 1981), and in two children with central nervous system disorders, including fatal Reye's syndrome and encephalitis (*Salmi* et al. 1978). A recent study failed to find an etiologic role for rotavirus in intussusception (*Mulcahy* et al. 1982), but the role of rotavirus in the other conditions remains unknown.

## 12.3 Occurrence in Neonates

One of the more intriguing features of rotavirus-host interactions is that, while neonates are highly susceptible to rotavirus infection, many neonatal infections are asymptomatic (*Chrystie* et al. 1975, 1978; *Madeley* and *Cosgrove* 1975). In a 1-year study of 1056 5-day-old infants, 343 (32.5%) were found to be excreting rotavirus, but of 189 babies on one ward, only 15 (8%) were symptomatic (*Banatvala* and *Chrystie* 1978; *Chrystie* et al. 1978). *Murphy* et al. (1977) found that 220 of 304 neonates were asymptomatic, which is a higher percentage than that noted by Banatvala and colleagues. Nevertheless, it is clear that a large percentage of infected neonates are asymptomatic. One important question which arises from these observations is whether rotavirus-infected newborns are "immunized" as a result of infection. Recent studies suggest that neonatal infection may not confer lasting immunity, based upon the inability to detect serum antibodies in these children 3–6 months after their neonatal infection (*Crewe* and *Murphy* 1980; *Bryden* et al. 1982). However, since the presence of serum antibodies may not correlate with protection from infection, these results do not rule out the possibility that these children remain "immune" to reinfection. This is an important question that needs to be answered.

Rotavirus infections in premature infants can also occur, and these infections have been reported to be mild (*Van Renterghem* et al. 1980) or associated with mild-to-severe gastroenteritis (*Rocchi* et al. 1981; *Thomson* et al. 1981). *Bishop* et al. (1979) noted that babies requiring special care are much more likely to develop symptomatic illness after rotavirus infection than are full-term babies. They also found that breast-feeding did not always protect infants from diarrhea, which was confirmed by *Crewe* and *Murphy* (1980), who reported that the infection rate of neonates did not differ between breast-fed and bottle-fed babies. The role of maternal antibody in preventing symptomatic rotavirus gastroenteritis in infants has not been adequately evaluated. Nevertheless, the existing data suggest breast-feeding does have a positive value in preventing symptomatic infections. As early as 1929, *Zahorsky,* writing on hyperemesis hiemis or winter vomiting disease of young children (now thought to have been rotavirus gastroenteritis), noted that breast-fed babies were generally immune to symptomatic disease. *Banatvala* and *Chrystie* (1978) also noted a lower incidence of infection in their study of rotavirus among breast-fed neonates.

Another study reported that breast-fed babies are less likely to become infected with rotavirus, and the presence of antibodies and trypsin inhibitors in human milk can be associated with the protection of neonates against rotavirus infection in the first 5 days of life (*McLean* and *Holmes* 1981). Studies in both humans and animals have shown that rotavirus antibody is often detectable in colostrum and milk (*Ellens* et al. 1978; *Cukor* et

al. 1978b, 1979; *Inglis* et al. 1978; *Simhon* and *Mata* 1978), and virus can be neutralized by immunoglobulins or other factors in milk and colostrum (*Thouless* et al. 1977a; *Otnaess* and *Örstavik* 1980; *Palmer* et al. 1980). It has also been clearly demonstrated that specific antibody titers in colostrum and milk can be elevated by vaccinating pregnant animals (ewes or dams) and that the passive administration of colostrum or milk containing antibody will protect newborn animals from challenge with virulent virus (*Snodgrass* and *Wells* 1976, 1978; *Snodgrass* et al. 1977b, 1980; *Wells* et al. 1978; *Saif* and *Bohl* 1980; *Bridger* and *Brown* 1981).

Further study is needed to determine whether these mechanisms are significant in preventing symptomatic rotavirus gastroenteritis in humans and whether they can be exploited in strategies to prevent human disease.

## 12.4 Occurrence in Adults

Although rotavirus enteritis is primarily a disease of young mammals and avians, there is ample evidence that the human virus can infect adults. Older children may also be infected (*Hara* et al. 1976), and it is known that adult cattle can be infected with bovine rotavirus (*Woode* and *Bridger* 1975). In adult humans the symptoms are generally mild and do not usually cause the patient to seek treatment leading to microbiological testing. *Middleton* et al. (1974) initially demonstrated with a volunteer experiment that human rotavirus can infect adults. *Örstavik* et al. (1976) later noted two adult cases which they thought resulted from reinfection. Child-to-mother transmission of disease as well as transmission to other adult family contacts has been demonstrated (*Zissis* et al. 1976; *Kim* et al. 1977), as has adult epidemic rotavirus gastroenteritis (*von Bonsdorff* et al. 1976, 1978; *Lycke* et al. 1978). In a prospective family study of rotavirus infection, *Wenman* et al. (1979) found that infection occurred in 36 of 102 adults whose children had rotavirus infection. Serum antibody did not correlate with a reduced risk of infection or symptomatic disease. They concluded that rotavirus is a mild but common infection in parents of young children. In contrast, *Halvorsrud* and *Örstavik* (1980) diagnosed 92 cases of rotavirus gastroenteritis among 256 individuals in a nursing home for the elderly. Most of the patients were between 70 and 90 years of age, and the outbreak was extensive and severe, with 1 death. They suggested that this may have been the result of lowered immunity against rotavirus among the elderly. Rotavirus infections have also been confirmed by serological studies and/or detection of virus in fecal material from ill adults (*Kapikian* et al. 1976c; *Örstavik* et al. 1976; *von Bonsdorff* et al. 1976; *Meurman* and *Laine* 1977; *Gomez-Barreto* et al. 1976). An outbreak of rotavirus diarrhea among a nonimmune, isolated South American Indian community again demonstrated that all age groups can be susceptible. In that study, the proportion of clinically apparent infections was 70% or more in all age groups (*Linhares* et al. 1981). Another outbreak among adults in a cardiology ward reported that disease occurred in four patients and 11 members of the nursing and medical staff. Disease was mild and short-lived (<4 days), except for one 83-year-old adult who exhibited symptoms for 9 days (*Holzel* et al. 1980).

An outbreak of rotavirus infection in an obstetric unit reportedly resulted in illness in 30 patients, staff, and hospital workers. None of the seven babies suffered more than mild disease, but the severity of disease in the adults varied, and two suffered severe dehydration (*Hildreth* et al. 1981).

These reports demonstrate that rotavirus causes outbreaks of diarrhea in adults in hospitals and, since excretion of virus in the absence of symptoms can be common in adults, isolation procedures may be required for adequate management of such outbreaks.

The recognition that rotavirus infections may cause illness in other specialized situations continues to become apparent. Rotaviruses have been associated with traveler's diarrhea, normally attributed to ETEC (*Bolivar* et al. 1978; *Echeverria* et al. 1981; *Sheridan* et al. 1981; *Ryder* et al. 1981), and with gastroenteritis in bone-marrow-transplant recipients (*Yolken* et al. 1982). Prolonged rotaviral disease and virus excretion has also been documented in immunosuppressed children (*Saulsbury* et al. 1980).

## 13 Immunity

The ubiquitous nature of the rotaviruses can also be demonstrated by measurement of the prevalence of immunity in the population. By 6 years of age, 60%–90% of children have serum antibody titers (*Elias* 1977a; *Ghose* et al. 1978) and antibodies persist, as measured by virus neutralization, immunofluorescence tests, complement-fixation or ELISA assays (*Elias* 1977a; *Yolken* et al. 1978c). Neutralizing antibodies were detectable in 67% of 27 sera of people aged 50–59 years and in 31% of 26 sera from those aged 70–79 years (*Elias* 1977a). The protective nature of the circulating antibodies remains unclear, since it is known that both humans and animals can become infected even when they possess detectable serum antibodies (*Kapikian* et al. 1974, 1981b; *Woode* 1978). Local immune factors, such as secretory IgA or interferon, appear to be important in protection against rotavirus infection. Alternatively, recurrent infections in the presence of circulating antibodies may reflect the presence of multiple serotypes of virus. Recurrent disease has been documented, but unfortunately the ELISA test used in these studies recognized subgroup antigens and not serotypes (*Fonteyne* et al. 1978; *Rodriguez* et al. 1978; *Yolken* et al. 1978e; *Simhon* et al. 1981).

Studies in animals have clearly demonstrated that antibody present in the lumen of the small intestine appears to be the prime mediator of protection against rotavirus infection (*Woode* et al. 1975; *McNulty* et al. 1976e; *Snodgrass* and *Wells* 1976, 1978). There is good experimental data suggesting that animals can be effectively immunized against disease induced by rotaviruses of their own species, at least for a limited time. At present it appears that rotavirus infection in mammals results in the development of immunity to illness and rotavirus shedding, and that for vaccines to be effective they will need to protect against each serotype that can infect the particular mammalian species under consideration. Obviously, if an infinite number of serotypes exists, and if new recombinants appear through gene reassortment, effective vaccination may be impossible.

Recent reports of rotavirus infections in immunosuppressed children (*Saulsbury* et al. 1980) and bone-marrow transplant recipients (*Yolken* et al. 1982) reemphasize our lack of specific knowledge about the role of local and/or cellular immune mechanisms in protection from enteric disease. The results of a study that evaluated the development of serum and intestinal antibody responses to rotavirus after naturally acquired infections in man suggested that there may be a relationship between the severity of the rotavirus infection and the nature of the systemic and secretory antibody responses (*Riepenhoff-Talty* et al. 1981). For example, it was noted that the titers of IgG rotavirus antibody in con-

valescent specimens of serum were lower in patients with severe or prolonged rotavirus infection than in specimens from subjects with mild or moderate disease. With the increasing support of the Diarrhoeal Disease Control Programme of the World Health Organization, some of these important questions should begin to be answered.

## 14 Laboratory Diagnosis of Rotavirus Infection

### 14.1 Detection of the Virus

Diagnosis of rotavirus infection is usually made by detection of virus or viral antigen in feces and a variety of methods are available (see Table 5). These methods have recently been described in detail in several reviews (*Kapikian* et al. 1981b). Serological diagnosis can be made using a variety of tests when acute and convalescent illness phase sera are available, but detection of virus by electron microscopy is the basis of rotavirus diagnosis. EM examination of biopsy material from children with acute nonbacterial gastroenteritis reveals virus particles within epithelial cells from duodenal mucosa (*Bishop* et al. 1973). Negative-contrast EM of fecal extracts from children with rotavirus gastroenteritis reveals virus particles with the distinctive morphology of rotavirus (*Flewett* et al. 1973). Virus is usually present in very large numbers, often $10^{10}$–$10^{11}$ particles per gram of stool, and can in most instances be seen by direct EM examination of fecal specimens. Some investigators have concentrated virus by ultracentrifugation (*Bishop* et al. 1974; *Middleton* et al. 1974; *Kapikian* et al. 1974; *Davidson* et al. 1975a) prior to EM examination, whereas others have had equally good success using the pseudoreplica technique fully described by *Smith* (1967), *Gomez-Barreto* et al. (1976), and *Portnoy* et al. (1977). A sero-

Table 5. Methods of detecting rotavirus

| Methods | Samples for analysis | | |
|---|---|---|---|
| | Intestinal contents and feces | Biopsy specimens | Cell culture |
| EM | ● | ● | ● |
| IEM | ● | | |
| CF | ● | | ● |
| CIEOP | ● | | |
| Gel diffusion | ● | | |
| IF | ● | ● | ● |
| ELISA | ● | | |
| RIA | ● | | |
| Plaque assay | ● | | ● |
| HA | | | ● |
| CPE | | | ● |
| PAP | | ● | ● |

EM, electron microscopy; IEM, immunoelectron microscopy; CF, complement fixation; CIEOP, counter-immunoelectroosmophoresis; IF, immunofluorescence; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; HA, hemagglutination assay; CPE, cytopathic effect; PAP, peroxidase-antiperoxidase

logical trapping method which involves coating EM grids with protein A and specific rotavirus antiserum is also useful in EM (*Nicolaieff* et al. 1980).

Because rotavirus is usually present in stools in large numbers, it has been possible to develop alternative diagnostic techniques to EM for use in laboratories where electron microscopes are not available. The advantages and sensitivity of each test vary as described below. Virus in fecal samples may be covered with antibody (*Watanabe* et al. 1978), and the influence of this possibility should be evaluated in all tests developed to detect virus in fecal samples. One of these, a fluorescent virus precipitin test (FVPT), is a diagnostic test where free virus is reacted with antibody and the resulting virus-antibody aggregates detected by immunofluorescence. *Foster* et al. (1975) used the method to detect bovine rotavirus and found it to be as sensitive as immune EM. Similar results were obtained by *Peterson* et al. (1976). *Yolken* et al. (1977b) later incubated stool filtrates with pig antihuman rotavirus serum, then with antipig serum conjugated with fluorescein isothiocyanate. Aggregates were centrifuged and pellets applied to slides and examined by the fluorescent antibody test. Of 30 EM-positive stools, 29 were also positive by the FVPT, and testing of 30 human stools showed three that were EM negative but positive by the FVPT.

Rotavirus diagnosis by detection of viral RNA by PAGE has also been used as a simple method of low cost, excellent specificity, and good sensitivity (*Espejo* et al. 1978; *Avendano* et al. 1982). Although it remains unclear whether rotavirus electropherotyping will ultimately be useful in distinguishing serotypes, this method does provide useful information about rotavirus epidemics, since patterns of disease outbreak and transmission can be analyzed by changes in electropherotypes (see Sect. 6).

Immunofluorescent (IF) staining of cell cultures inoculated with fecal preparations has also been done (*Barnett* et al. 1975; *Bridger* and *Woode* 1975). There have been problems with IF tests for human rotaviruses because virus derived directly from stool extracts replicates poorly in cell cultures. However, *Banatvala* et al. (1975) found that the sensitivity of the test could be increased by centrifuging inocula at 3000 g to enhance virus-cell attachment. *Bryden* et al. (1977) developed this system in microtiter plates using LLC-MK2, HEK, and CK cells. They centrifuged human fecal samples onto monolayers and detected fluorescence after adding antibovine rotavirus conjugate. Of 35 specimens positive by EM 31 were also positive by IF. The LLC-MK2 cultures showed more infected cells than the other cultures. Rotavirus antigen can also be detected in infected cells or in histologic tissue sections using indirect immunoperoxidase tests or the peroxidase-antiperoxidase (PAP) test. The advantages of these tests are that they are read with a regular light microscope, and permanent slides are produced (*Graham* and *Estes* 1979). These methods are also easily applicable to immunoelectron microscopy (*Altenburg* et al. 1979; *Petrie* et al. 1982).

The CF test has been used as a method for detecting rotavirus in fecal extracts (*Spence* et al. 1975; *Tufvesson* and *Johnsson* 1976b; *Middleton* et al. 1976). Opinions on the sensitivity of CF tests differ, and problems with anticomplementary (AC) activity of stool suspension have not made the test popular, although *Zissis* et al. (1978) have had success with a modified test. They compared CF tests with EM and immunoelectron microscopy (IEM) and used 2 units of complement instead of the usual 5 units. They also used fetal calf serum to adsorb stool suspensions to remove AC activity and found the test as sensitive as EM for detecting virus. *Zissis* and *Lambert* (1980) also used a modified CF test in serotyping human rotavirus which was about as sensitive as most ELISA systems but, as

discussed in Sect. 4, it currently appears that this test measures subgroups and not serotypes (*Kapikian* et al. 1981a).

Counter-immunoelectroosmophoresis (CIEOP) has been used to detect rotavirus with varying results. *Middleton* et al. (1976) used guinea pig antihuman rotavirus serum and stained precipitates with tannic acid, but found the method less sensitive than EM for antigen detection. In contrast, *Spence* et al. (1977) and *Tufvesson* and *Johnsson* (1976a) found the method to be almost as good as EM. *Grauballe* et al. (1977) used crossed CIEOP in two dimensions with rabbit antiserum produced to immunoprecipitates of human rotavirus and with bovine antiserum to bovine rotavirus. They found 61% of 87 fecal samples of infants and children with gastroenteritis to be positive, whereas EM showed 50% positive. In two cases a positive EM diagnosis could not be confirmed by CIEOP. Similar results have been reported by *Muchinik* and *Grinstein* (1980). The test does have the advantage of allowing large numbers of specimens to be examined rapidly and may be of value in field studies.

There is an antigen subunit associated with the inner capsid of human and calf rotavirus particles which reacts with specific antiserum in immunodiffusion tests (*Mathan* et al. 1977). Use of the antigen in other tests, however, apparently has not been evaluated. *Matsuno* and *Nagayoshi* (1978) used an immune adherence hemagglutination (IAHA) test to titrate human rotavirus and found a good correlation between this test and EM in detecting virus. This test has been modified by using the cultivable human Wa virus as an antigen, and it is proposed as a sensitive method for seroepidemiological studies of human rotavirus infections (*Matsuno* et al. 1982).

Solid-phase radioimmunoassay (RIA) (*Middleton* et al. 1977; *Kalica* et al. 1977; *Cukor* et al. 1978a) is comparable to EM for detection of rotavirus in stools. The test is useful in high-volume operations where equipment for counting radioisotopes and radiolabel are available. The ELISA (*Ellens* and *de Leeuw* 1977a, b; *Yolken* et al. 1977a; *Payment* et al. 1979; *Scherrer* and *Bernard* 1977) is also as sensitive a test as EM, and is currently considered the most useful method for screening large numbers of specimens. *Zissis* and *Lambert* (1980) found that the ELISA was more sensitive and easier to read visually when the test used two type-specific hyperimmune sera, that is, antibody to two specific human serotypes rather than antibody to two different species. *Birch* et al. (1979) compared RIA, ELISA, EM and IF for practicability, speed, and sensitivity in the detection of human rotavirus. IF was the least sensitive, and they concluded that in laboratories doing routine analysis of fecal specimens ELISA and RIA are the most useful alternatives to EM. In another comparison of ELISA with EM, CIEOP, and IF for detection of rotavirus in stools, ELISA was found to be simple, rapid, reliable, and sensitive (*Grauballe* et al. 1977, 1981).

*Yolken* and *Stopa* (1979) developed an enzyme-linked fluorescence assay (ELFA) and found that the use of a substrate that yielded a fluorescent product markedly increased the sensitivity of the test but noted that tests could not be read visually. The ELFA appears to be useful where maximum sensitivity is required. *Bradburne* et al. (1979) used a solid-phase aggregation-coupled erythrocyte (SPACE) method for virus quantitation. The method used chromic-chloride-linked, antibody-coated erythrocytes to detect virus and was about as sensitive as either IF or EM. The method appears very useful for specimens that have high background contamination.

Two commercial rotavirus diagnostic kits that are enzyme immunoassays are now available. One kit (Rotazyme), sold by Abbott Laboratories, North Chicago, Ill., is per-

formed with polystyrene beads in plastic test tubes. The second commercial kit (Enzygrost), produced by Behring-Werke, Marburg, F.R.G., is performed on microtiter plates, so a larger number of samples is optimally assayed at one time. An evaluation of these commercial kits has shown them to be highly efficient for the detection of rotavirus when compared with EM (*Yolken* and *Leister* 1981; *Rubenstein* and *Miller* 1982).

## 14.2 Detection of Antibody

Numerous serological tests have been used to demonstrate rotavirus infection. Among the tests, CF was initially widely used for the detection of rotavirus antibody (*Kapikian* et al. 1974, 1975, 1976b; *Blacklow* et al. 1976; *Tufvesson* and *Johnsson* 1976b; *Gomez-Barreto* et al. 1976; *Tufvesson* et al. 1977; *Gust* et al. 1977). *Kapikian* et al. (1976b) found that human rotavirus from fecal extracts was the most efficient virus for detection of homologous CF antibody, followed by the "O" agent, SA11, and then bovine rotavirus. Immunoelectron microscopy (*Kapikian* et al. 1974), immunofluorescence (*Davidson* et al. 1975b), neutralization (*Thouless* et al. 1977b; *Flewett* et al. 1974), and CIEOP (*Middleton* et al. 1976; *Cook* et al. 1978) also proved to be useful in initial studies of rotavirus infection. Newer tests, such as ELISA (*Scherrer* and *Bernard* 1977; *Yolken* et al. 1978a, c, d; *Ghose* et al. 1978), solid-phase RIA (*Babiuk* et al. 1977a; *Watanabe* and *Holmes* 1977), and ELISA blocking assay (*Yolken* et al. 1978c), are more versatile and have expanded the range of possible rotavirus serological studies, such as evaluation of the IgG, IgM, and IgA immune response to infection by RIA (*Sarkkinen* et al. 1979) or ELISA (*Gary* et al. 1980a). Antirotavirus IgM has been measured by FA (*Davidson* et al. 1975b; *Morishima* et al. 1976; *Örstavik* et al. 1976), CF of sera treated with mercaptoethanol (*Konno* et al. 1977; *Abe* and *Inouye* 1979), ELISA (*Yolken* et al. 1978a; *Gary* et al. 1980a), and RIA (*Sarkkinen* et al. 1979). RIA and ELISA have also been used to measure antirotavirus IgA in serum (*Sarkkinen* et al. 1979; *Gary* et al. 1980a).

*Martin* et al. (1979) compared CF, ELISA, and HI for detection of antibody to human rotavirus present in sera known to contain either high or low levels of antibody (*Foster* et al. 1980). Convalescent illness phase sera from 104 patients were tested with simian virus as HA antigen in the HI test, with bovine virus in ELISA, and with human virus in CF tests, and the results showed that all three tests detected antibody in all sera. No test was significantly more sensitive than the others, and when antibody levels were relatively low by one test they were also correspondingly low by the other two tests. Although the ELISA test was not more sensitive, titers obtained were 10–13 times higher than with CF, which is about the same as reported by *Ghose* et al. (1978), who found ELISA titers to be about 16 times higher than CF titers. Results were more variable with sera that were obtained from individuals who had not recently been ill with rotavirus gastroenteritis and who had low levels of antibody. In these sera, some differences were noted using the three tests, but these only occurred when titers were very low ($<8$ CF; $<10$ HI; $<64$ ELISA). HI is the easiest test to perform and the test which requires the least amount of reagents. It will be of interest to determine the value of the HI tests in rotavirus serology if the human viruses adapted to tissue culture (*Wyatt* et al. 1980; *Sato* et al. 1981a; *Urasawa* et al. 1981) can eventually be used as HA antigen, because the test is easily performed and could easily be standardized worldwide.

It is not known whether the various serological tests for detection of rotavirus anti-

body during serum surveys, which do not use immunoglobulin class-specific reagents, measure the same type of antibody, and this should be remembered when interpreting and comparing the results of different studies that have used these tests.

## 15 Genetics and Virus Variants

The development of a genetic system for the rotaviruses will offer investigators the opportunity to confirm the gene-polypeptide assignments by an independent method, to probe the functions of the rotavirus polypeptides, and to probe the viral genes involved in virus virulence and pathogenesis. The potential of this approach has been eloquently demonstrated in the reovirus system (see reviews by *Fields* 1982, and *Sharpe* and *Fields* 1982).

The beginnings of the needed genetic systems are reported in the isolation and characterization of temperature-sensitive (*ts*) mutants of the simian rotavirus SA11 (*Ramig* 1982) and of the UK strain of the bovine rotavirus (*Greenberg* et al. 1981a; *Faulkner-Valle* et al. 1982). These mutants have been classified into groups between which recombination occurs at high frequency. Five recombination groups have been reported for both SA11 and the UK strain of bovine rotavirus (*Ramig* 1982; *Faulkner-Valle* et al. 1982), while four recombination groups have been reported by *Greenberg* et al. (1981a). It is not yet known whether the UK groups are the same, and the gene locations of the *ts* lesions of the mutants are not yet mapped. The high frequency of recombination observed between these mutants suggests that recombination in rotaviruses occurs by reassortment of genome segments during mixed infection, as has been shown to occur with the related reoviruses and orbiviruses.

Gene reassortment has been demonstrated directly following mixed infections in cell cultures and the isolation of a recombinant between simian and bovine rotavirus (*Matsuno* et al. 1980) and of recombinants between the *ts* mutants of the bovine rotavirus and noncultivable human rotavirus strains (*Greenberg* et al. 1981a). Characterization of a series of such reassortants between the human and bovine *ts* mutants has allowed the assignment of the biological properties of neutralization to gene 9 of the Wa virus (*Kalica* et al. 1981b) and to gene 8 of the DS-1 strain of human rotavirus (H. Greenberg, personal communication), and the gene coding the subgroup antigen has been identified as gene 6 (*Kalica* et al. 1981b). These studies have also suggested that gene 4 is responsible for the restriction of growth of the human viruses in cell culture (*Greenberg* et al. 1981a; H. Greenberg, personal communication).

A series of plaque isolates of the simian rotavirus SA11 picked independently from uncloned virus stocks yielded viruses which exhibit heterogeneity in the structural glycoprotein (*Estes* et al. 1982). These isolates are not *ts*, but they may be useful in probing the genetic structure of the glycoprotein gene. One isolate produces a polypeptide that is not glycosylated, and this isolate should be useful in determining the role of the carbohydrate in virus replication and antigenicity.

A series of bovine rotavirus isolates which exhibit different patterns of virulence in ligated intestinal loops has also been described. Attempts were made to correlate polypeptide patterns with differences in virus virulence. The virus that was nonpathogenic and another isolate whose virulence was not tested exhibited a polypeptide pattern different from that of three reportedly virulent strains (*Carpio* et al. 1981b). We now

recognize that the difference in the polypeptide pattern of the nonvirulent strain was that it exhibited the pattern of trypsin-treated virus. Unfortunately, not enough isolates were available for similar analyses, but this result is intriguing. Although the phenomenon is reversed, it is known that the virulence of paramyxoviruses and orthomyxoviruses correlates with their protease sensitivity (*Nagai* et al. 1976; *Rott* et al. 1980). It will be exciting to follow the anticipated studies that will probe the genes for rotavirus virulence and pathogenesis.

## 16 Pathogenesis and Animal Models

Our knowledge of the histopathology and pathophysiology of rotavirus infections has come from analyses of infections in animals and from limited studies of mucosal biopsy specimens from infected children. The general pattern of infection involves virus penetration and infection of the differentiated enterocytes in the villi of the small intestine (*Mebus* et al. 1971b, 1977; *McNulty* et al. 1976a; *Wyatt* et al. 1976b; *Mebus* and *Newman* 1977; *Theil* et al. 1978a). Rotaviruses multiply in the cytoplasm of these cells and damage the absorptive cells, resulting in damage to both the digestive and the absorptive functions. The available evidence suggests that such damaged cells are sloughed into the small intestine and that lysis of these cells releases the virus into the intestine, resulting in the large quantities of virus detected in the stools of infected subjects. Recent studies suggest that the diarrhea caused by rotavirus infection is an osmotic diarrhea due to nutrient (carbohydrate) malabsorption (*Graham* et al. 1982). The highly differentiated absorptive villous cells are replaced by immature crypt cells that are not able to immediately compensate for the absorptive defect (*Middleton* 1978).

One difficulty facing researchers seeking therapeutic evaluation, new treatments, vaccine development, or a method to elucidate the genetic determinants of virus virulence is the lack of a convenient, inexpensive, small animal model. Pathogenesis studies in animals have shown that inoculation of most conventional animal species with heterologous rotaviruses results in subclinical infections (see Sect. 2). The inoculation of gnotobiotic animals may produce severe disease, but these animals clearly cannot be used to study immune responses. A limited number of studies have suggested a promising homologous animal model may be EDIM virus infections in mice (*Noble* et al. 1980; *Wolf* et al. 1981; *Little* and *Shadduck* 1982). However, this imposes the technical limitation of working with newborn mice, and the fact remains that EDIM virus has not been successfully cultivated in tissue culture.

Miniature swine offer an excellent, although expensive, animal model to study viral pathogenesis and the effects of the disease and treatment on gastrointestinal physiology. These animals are particularly suitable to studies of gastrointestinal disease due to similarities of the human and porcine diet and gastrointestinal tract (*Graham* et al. 1982). Their major limitation is that they do not share the feature of cross-placental transfer of immunoglobulins, so comparative studies of immunity may be limited. They do offer some immunologic similarity to humans because the protective antibody delivered to piglets in porcine colostrum is IgA and not IgG as in calves (*Saif* and *Bohl* 1980). Clearly, the discovery of a small animal model that is susceptible to a variety of human rotavirus strains is one area where progress needs to be made in order to facilitate the conquest of rotavirus disease. Fortunately, while methods for immunoprophylaxis are being developed, the

worldwide mortality and morbidity from rotaviral diarrheal disease can be effectively reduced by oral rehydration therapy (*Sack* 1982; *Santosham* et al. 1982).

## 17 Conclusions

It is clear from this review that once clinical and epidemiologic studies established the rotaviruses as significant human and veterinary pathogens an enormous amount of basic research rapidly followed. Our current understanding of the molecular biology of the rotaviruses has revealed these nonenveloped viruses as excellent models for the study of glycoprotein biosynthesis. The recognition that protease cleavage of a viral protein is important for viral infectivity raises the question of whether this is a generalized mechanism affecting viruses that replicate on mucosal surfaces. Future studies will certainly address the question of whether protease activation influences rotavirus virulence, as is now recognized for the reoviruses or the orthomyxoviruses and paramyxoviruses, and whether this event might represent a potential target for preventing virus replication.

The recently gained ability to cultivate the human rotaviruses should allow the critical question – whether there are a finite or infinite number of virus serotypes – to be answered and so clearly facilitate the development of strategies for immunoprophylaxis or disease prevention. Even if a limited number of serotypes exist, a major challenge facing these practical efforts will be to overcome the problems of developing methods to induce an intestinal immune response and protection in children prior to their peak susceptibility between 6 and 24 months of age.

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