Nodaviruses of Insects

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I. INTRODUCTION

The study of nodaviruses began with the isolation of nodamura virus (NOV) from mosquitoes in 1956 (Scherer and Hurlbut, 1967; Scherer *et al.*, 1968). The virus drew immediate attention because it uniquely combined the biological property of arthropod transmission to vertebrates with the physical property of resistance to lipid solvents, a characteristic that is now known to indicate the absence of a viral envelope. Molecular studies established that NOV was also unique in its genome structure: two molecules of single-stranded, positive-sense RNA copackaged in spherical virus particles (Fig. 1) (Newman and Brown, 1973, 1977; Clewley *et al.*, 1982). Despite this combination of unusual features, however, the lack of a convenient cell culture system for growing NOV and the absence of antibodies to the virus in human sera, which suggested that it was not naturally transmitted to man, diverted most investigators to more pressing and tractable systems.

When black beetle virus (BBV) and flock house virus (FHV), its close relative, were discovered in black beetles and grass grubs, respectively (Longworth and Archibald, 1975; Longworth and Carey, 1976; Scotti *et al.*, 1983), nodaviruses became easy to study in the laboratory because these viruses grow extremely well in cultured *Drosophila melanogaster* cells (Friesen *et al.*, 1980; Crump and Moore, 1981a, b; Friesen and Rueckert, 1981; Crump *et al.*, 1983; Selling and Rueckert, 1984). At the same time, it became clear that the ability of NOV, the prototype of the virus family, to infect some mammals and possibly birds was not shared by other nodaviruses, and this further diminished the

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FIGURE 1. Schematic representation of the genomic organization and replication strategy of the nodaviruses of insects. After Friesen and Rueckert (1981). The nodavirus particle is an image reconstruction of FHV from Johnson and Reddy, Chapter 7, this volume.

apparent medical importance of the family as a whole. During the last 15 years, however, the abundant replication and very small genomes of these viruses have made them attractive model systems for the study of many basic aspects of molecular virology, including virus structure (Hosur *et al.*, 1987; Fisher and Johnson, 1993; Wery *et al.*, 1994) (see also Chapter 7, this volume); virus assembly (Gallagher and Rueckert, 1988; Schneemann, 1992; Schneemann *et al.*, 1992, 1994; Zhong *et al.*, 1992; Zlotnick *et al.*, 1994); RNA replication (Guarino and Kaesberg, 1981; Gallagher *et al.*, 1983; Saunders and Kaesberg, 1985; Wu and Kaesberg, 1991; Ball *et al.*, 1992, 1994; Wu *et al.*, 1992; Ball, 1994b, 1995; Price *et al.*, 1996; Johnson and Ball, 1997); RNA recombination (Li and Ball, 1993; Ball, 1997); and the control of gene expression (Friesen and Rueckert, 1981, 1984; Friesen, 1983; Zhong and Rueckert, 1993). For earlier reviews of this virus family, the reader is referred to the following papers: Brown and Hull (1973); Longworth (1978); Harrap and Payne (1979); Moore and Tinsley (1982); Moore *et al.* (1985, 1987); Kaesberg (1987); Garzon and Charpentier (1991); Hendry (1991); Ball (1994a); Miller (1996).

Recently, several distinct nodaviruses have been isolated from hatchery-reared fish larvae, where they can cause encephalitis and mass mortality, result-

ing in significant economic losses (Arimoto *et al.*, 1992; Mori *et al.*, 1992; Mushiake *et al.*, 1992; Comps *et al.*, 1994; Munday *et al.*, 1994; Nishizawa *et al.*, 1995; Frerichs *et al.*, 1996; Delsert *et al.*, 1997). While a detailed review of the properties of the nodaviruses of fish is beyond the scope of a chapter on the nodaviruses of insects, they are listed in Table I for the sake of completeness. The reference list for this chapter is comprehensive and includes every paper on nodaviruses that we have been able to find.

II. CLASSIFICATION AND TAXONOMY

The family *Nodaviridae* contains two genera, the alpha nodaviruses, which predominantly infect insects, and the beta nodaviruses, which have been isolated only from fish (Table I). Although the alpha nodaviruses are all antigenically related to one another, individual members of the genus can be distinguished serologically (Reinganum and Scotti, 1976; Reavy *et al.*, 1982; Scotti *et al.*, 1983; Reinganum *et al.*, 1985; Gallagher, 1987; Scotti and Fredericksen, 1987; Hendry, 1991). A more quantitative picture of their interrelationships is provided by pairwise comparisons of the nucleotide sequences of their smaller genome segments (RNA 2) and of the encoded amino acid sequences of their capsid protein precursors (protein α) (Table II).

Comparison of the amino acid sequence of the BBV RNA-dependent RNA polymerase (RdRp) with those of other positive-strand RNA viruses places it in an RNA polymerase group of its own—group 2 in the classification scheme of Koonin (1991). Its closest relatives are the group 3 RdRps of the plant sobemo- and luteoviruses, which together with the picorna-, como-, poty-, and coronaviruses form supergroup I in this classification scheme. The phylogenetic proximity to some plant viruses is interesting in view of the ability of FHV to replicate productively in plant cells (see Section IV.A) (Selling *et al.*, 1990).

III. ISOLATION, ECOLOGY, AND NATURAL INFECTIONS

A. Nodamura Virus

The ecology of the *Nodaviridae* is best understood for NOV, the only member of the family known to be pathogenic for both insects and mammals. Indeed, NOV is highly unusual among viruses in general in being lethal for both insects and mammals. The investigation of NOV ecology was greatly facilitated by the very study that led to its discovery and subsequent isolation. Between 1952 and 1957, now classic ecological studies of Japanese encephalitis (JE) virus, an arthropod-borne flavivirus (Monath and Heinz, 1996), were performed by US Army personnel in Japan (Buescher and Scherer, 1959; Buescher *et al.*, 1959a,b; Scherer and Buescher, 1959; Scherer *et al.*, 1959a–e). During these six yearly surveys, sera were collected from birds (primarily herons and egrets), pigs,

TABLE 1. The Family *Nodaviridae*

Virus	Acronym	Natural host	Reference
Alpha nodavirus genus			
Nodamura virus	NOV	<i>Culex tritaeniorhynchus</i>	Scherer and Hurlbut (1967)
Black beetle virus	BBV	<i>Heteronychus arator</i>	Longworth and Carey (1976)
Flock House virus	FHV	<i>Costelytra zealandica</i>	Scotti <i>et al.</i> (1983)
Boolarra virus	BoV	<i>Oncopera intricoides</i>	Reinganum <i>et al.</i> (1985)
<i>Lymantria ninayi</i> virus (Greenwood)	LN _V G	<i>Lymantria ninayi</i>	Greenwood and Moore (1982)
Manawatu virus	MwV	<i>Costelytra zealandica</i>	Scotti and Frederickson (1987)
New Zealand virus	NZV	Unknown	Scotti and Frederickson (1987)
Also known as <i>Drosophila</i> line 1 virus	DLV		Friesen <i>et al.</i> (1980)
Beta nodavirus genus			
Striped jack nervous necrosis virus	SJNNV	<i>Pseudocaranx dentex</i>	Mori <i>et al.</i> (1992)
Tiger puffer nervous necrosis virus	TPNNV	<i>Takifugu rubripes</i>	Nishizawa <i>et al.</i> (1995)
Barfin flounder nervous necrosis virus	BFNNV	<i>Verasper moseri</i>	Nishizawa <i>et al.</i> (1995)
Japanese flounder nervous necrosis virus	JFNNV	<i>Paralichthys olivaceus</i>	Nishizawa <i>et al.</i> (1995)
Redspotted grouper nervous necrosis virus	RGNNV	<i>Epinephelus akaara</i>	Nishizawa <i>et al.</i> (1995)
<i>Dicentrarchus labrax</i> nervous necrosis virus	DlEV	<i>Dicentrarchus labrax</i> (sea bass)	Comps <i>et al.</i> (1994)
<i>Lates calcarifer</i> encephalitis virus	LcEV	<i>Lates calcarifer</i> (barramundi)	Comps <i>et al.</i> (1994)

TABLE II. Percentage Sequence Identities among Four Nodaviruses^a

	Nucleotide sequence of RNA 2			
	BBV	FHV	BoV	NOV
BBV	100	80	54	53
FHV	87	100	53	52
BoV	52	50	100	48
NOV	51	50	44	100
Amino acid sequence of protein α				

^aData from Kaesberg *et al.* (1990).

Note: Percentage sequence identities are indicated; numbers above the diagonal refer to the nucleotide sequence of RNA 2, while those below refer to the amino acid sequence of protein α .

humans, and wild rodents and analyzed for the presence of neutralizing antibodies against JE virus. These sera provided a valuable resource for ecological analysis of NOV after it was isolated.

Scherer and Hurlbut (1967) provide a delightful description of the isolation of NOV during the course of the JE studies, unique in that it pinpoints the recovery of the virus to the very hours during which the mosquitoes were trapped. Their observations are summarized as follows.

On July 23, 1956, between 8 PM and 7 AM, 5900 female mosquitoes were collected from a pig-baited Magoon trap located on the Sagiyama heronry near Tokyo. 5300 of these mosquitoes were identified as *Culex tritaeniorhynchus* and stored on dry ice in pools of varying sizes. One such pool consisted of 500 *C. tritaeniorhynchus* mosquitoes and was designated Mag 115 (mosquito lot 115 from that particular Magoon trap) (Scherer *et al.*, 1962; Scherer and Hurlbut, 1967). In October of that year, Mag 115 was used to prepare a suspension that was injected into 3-day old suckling mice. Ten out of thirteen mice exhibited hind-limb paralysis and/or death 7 to 14 days after inoculation, suggesting the presence of an infectious virus in the triturated mosquitoes (Scherer and Hurlbut, 1967). Similar results were obtained with the same mosquito suspension 4 months later. In that experiment, brain suspensions from the infected mice induced hind-limb paralysis in second-passage mice. Recognition that the causative agent was a previously unidentified virus (NOV) followed the authors' inability to identify it as one of 88 arboviruses, 47 enteroviruses, or 12 other viruses (Oya *et al.*, 1961; Scherer *et al.*, 1962; Scherer and Hurlbut, 1967). The virus was named after the village of Nodamura where the infected mosquitoes were trapped, and the original Mag 115 strain is available from the American Type Culture Collection (ATCC VR-679).

Relatively little is known about the prevalence of NOV in the wild. The mosquitoes from which NOV was isolated were collected during the peak of the *C. tritaeniorhynchus* population in the summer of 1956 (Buescher *et al.*, 1959b). Between June and October of that year, mosquitoes collected in the same trap

yielded 61 strains of JE virus from *C. tritaeniorhynchus* (Buescher *et al.*, 1959b) and four strains of Sagiyama virus, two of them from *C. tritaeniorhynchus* (Scherer *et al.*, 1962), yet NOV was isolated only once. This suggests that although JE and NOV may share the same source mosquito, the prevalence of NOV in the wild is much less than that of JE.

The single isolation of NOV also may be related to two aspects of the experimental design. First, weanling mice (3 to 4 weeks old) were used for JE virus isolation prior to 1956, whereas in 1956 and 1957 only suckling mice (1 to 4 days old) were used (Scherer *et al.*, 1962). The pathogenicity of NOV for mice is apparently limited to sucklings (Scherer *et al.*, 1968; Ball *et al.*, 1992), suggesting that NOV could have been present in the mosquitoes during the earlier part of the study and remained undetected. Second, pigs were only included in the JE studies starting in 1956. This takes on a new significance in light of the suggestion that pigs may be a natural reservoir for NOV (Scherer *et al.*, 1968).

Several animal species found on farms near Tokyo were tested for the presence of neutralizing (N) antibodies against NOV (Scherer *et al.*, 1968). In 1956 and 1957, a large proportion of pigs were positive for circulating N antibodies: 13 of 16 pigs (81%) in 1956 and 14 of 27 (51%) in 1957, with an age distribution that suggested the pigs had become infected by NOV during the spring or early summer of each year. The apparent seasonal occurrence of swine infection was compatible with transmission by *C. tritaeniorhynchus*, the distribution of which followed a similar pattern. Additionally, pigs attracted and were frequently bitten by large numbers of this mosquito species. It remains unknown whether NOV causes natural disease in pigs. It is also unclear which is the primary host—the mosquito or the pig—although Scherer *et al.* (1968) suggested that pigs might be the source from which the mosquitoes became infected. In contrast, Longworth (1978) suggested that the antibody response in pigs might be due to low-level exposure to NOV rather than true infection and postulated that vertebrates might be incidental hosts for the virus.

There was little or no evidence of avian or human infection by NOV during this time. Out of a total of 54 herons and egrets tested for N antibodies in 1956 and 1957, only one positive heron (*Nycticorax nycticorax*) was detected. None of the 42 human subjects tested in 1957 or 1958 were positive, despite the fact that half of the persons tested in 1958 resided on farms where pigs had been positive the previous year (Scherer *et al.*, 1968). Interestingly, these results roughly correlated with those predicted by the distribution of *C. tritaeniorhynchus* mosquitoes at Sagiyama in the summer of 1956: the numbers of mosquitoes collected from traps baited with pigs, herons, or humans were 120381 (95.2%), 5791 (4.6%), and 278 (0.2%), respectively (Scherer *et al.* 1959a). It is also unknown whether wild rodents are involved in the ecology of NOV, because these sera were not tested for NOV antibodies. However, *C. tritaeniorhynchus* feeds poorly on mice (Scherer and Hurlbut, 1967), which suggests that natural infection of mice by NOV might happen infrequently if at all.

A number of animal species support NOV infection in the laboratory. The results of experiments performed with animals are summarized below, while

the results of cell culture experiments will be considered in Section IV. Scherer and Hurlbut (1967) reported results suggestive of NOV infection concurrent with bluetonguelike virus immunization of cattle, and suggested that these cattle had natural infections with NOV either before or concurrent with immunization. In contrast, NOV failed to produce viremia in chicks (Scherer and Hurlbut, 1967). Scherer *et al.* (1968) tested several other vertebrates, including suckling and weanling mice, rabbits, guinea pigs, and Syrian hamsters, and baby chicks and embryonated chicken eggs (Scherer *et al.*, 1968). As in the earlier study (Scherer and Hurlbut, 1967), suckling mice exhibited hind-limb paralysis and death. Weanling mice and rabbits showed no illness or fever, although both had N antibodies 3 to 4 weeks after inoculation, suggesting inapparent infections. No disease was observed in weanling guinea pigs or Syrian hamsters, baby chicks, or embryonated chicken eggs (Scherer *et al.*, 1968). Garzon and Charpentier (1991) reported flaccid paralysis and death in suckling hamsters as well as suckling mice. Ball *et al.* (1992) observed that adult female mice that had eaten virus-infected offspring could confer immunity to their own subsequent litters or by foster-feeding offspring of naive mice; the mothers themselves showed no signs of disease.

In addition to *C. tritaeniorhynchus*, several other mosquito species support NOV infection. Scherer and Hurlbut (1967) showed that *Aedes aegypti* became infected with NOV after feeding on viremic mice, and they could transmit the virus to naive mice. NOV replicated and could be serially passaged in *Culex tarsalis* (Scherer and Hurlbut, 1967), *Aedes albopictus* (Tesh, 1980), and *Toxorhynchites amboinensis* (Tesh, 1980). However, whereas NOV was not reported to be pathogenic for *C. tritaeniorhynchus*, *A. aegypti*, or *C. tarsalis*, intrathoracic injection of NOV into *A. albopictus* or *T. amboinensis* was lethal between 6 and 10 days after inoculation. Shortly before death, these mosquitoes exhibited loss of balance, inability to fly, and eventual paralysis. Infection could also be initiated by feeding or by immersion, but was not fatal by these routes (Tesh, 1980). It is possible that the pathogenicity of the virus or the kinetics by which infection occurs differs by route of infection. This raises the argument that, as Moore and Tinsley (1982) suggested, perhaps the pathogenicity for mosquitoes is an artifact of a nonphysiological route of infection, and that in the natural situation mosquitoes may undergo only inapparent infections like those observed by Scherer and colleagues (Scherer and Hurlbut, 1967; Scherer *et al.*, 1968). Interestingly, NOV failed to replicate in *Culex quinquefasciatus*, showing that its host range is restricted even among mosquitoes (Tesh, 1980).

NOV is also pathogenic for honey bees (*Apis mellifera*) (Bailey and Scott, 1973). Infection of adult bees resulted in death between 8 and 16 days after inoculation, preceded by paralysis of the anterior two pairs of legs a few hours before death (Bailey *et al.*, 1975). Other arthropods studied include soft ticks (*Ornithodoros savignyi*), Indian meal moth larvae (*Plodia interpunctella*), black carpet beetle larvae (*Attagenus piceus*), bedbugs (*Cimex lectularius*), and German roaches (*Blattella germanica*). No illness was observed in any of these host insects; tick and moth larvae supported multiplication of NOV, beetle larvae gave intermediate results, and bedbugs and roaches failed to support viral

replication (Scherer and Hurlbut, 1967). Crickets (*Acheta domestica*) also failed to support NOV replication (Bailey *et al.*, 1975).

NOV infection in wax moth larvae (*Galleria mellonella*) has been studied extensively (Bailey and Scott, 1973; Bailey *et al.*, 1975; Garzon *et al.*, 1978). Bailey and Scott (1973) reported that infected larvae died 7 to 14 days after infection, mostly as pupae. Garzon *et al.* (1978) studied the replication of NOV in *G. mellonella* larvae in greater detail. At four to six days after inoculation, the larvae exhibited paralysis of the posterior five or six segments; paralysis spread to the other segments, resulting in death 15 to 20 days postinfection. Prior to the death of the insects, molting and metamorphosis were inhibited.

B. Other Nodaviruses

1. Black Beetle Virus

Less is known about the ecology of the other nodaviruses. BBV was isolated from scarab beetles, *Heteronychus arator*, collected in a pasture at Wharepapa, near Helensville, New Zealand (Longworth and Archibald, 1975). The virus was present in 25% of the third instar larvae sampled (33 total) and in 7% of the adult beetles sampled (30 total). Infected larvae and prepupae were flaccid and translucent compared with healthy larvae. Larvae of *Pyronota* collected from the same pasture were not infected by BBV, and cattle near the site lacked N antibodies against BBV. BBV was found to be antigenically distinct from NOV (Longworth and Carey, 1976) but related to the other strictly insect nodaviruses as detailed below (Dearing *et al.*, 1980; Friesen *et al.*, 1980; Greenwood and Moore, 1982; Reinganum *et al.*, 1985; Scotti and Fredericksen, 1987).

In the laboratory, injection of BBV into the body cavity of *H. arator* adults failed to cause disease; larvae were not tested because of a decline in population density in the wild. BBV multiplied in larvae of the wax moth and of *Pseudaletria separata*; the infected larvae became inactive, flaccid, and unresponsive to touch, and died 14 to 37 days after infection. BBV was also infectious for larvae of *Aphodius tasmaniae* and *Pericoptus truncatus* (Longworth and Archibald, 1975). Injection of BBV into suckling mice did not result in illness or death (Longworth and Carey, 1976).

2. BBV-W17

In an attempt to isolate a cytolytic variant of BBV that might be able to form plaques, Selling and Rueckert (1984) subjected the virus to 17 serial passages in cultured *Drosophila* (DL1) cells. The virus that emerged (BBV-W17) was indeed more cytolytic than wild-type BBV and formed plaques on monolayers of DL1 cells; but antigenic characterization of the virus and determination of its RNA 2 sequence showed that the capsid protein gene of BBV-W17 was indistinguishable from that of FHV. This virus was the source of the RNA from which the infectious cDNA clones were constructed (Dasmahapatra *et al.*,

1986) and it is now considered to be an isolate of FHV rather than BBV (Gallagher and Rueckert, 1988; Kaesberg *et al.*, 1990).

3. Flock House Virus

FHV was isolated from third instar larvae of the grass grub *Costelytra zealandica*, collected near the Flock House agricultural station, Bulls, New Zealand (Dearing *et al.*, 1980). The infected grubs were alive, but appeared retarded in their development (Scotti *et al.*, 1983). In the laboratory, FHV replicated in the larvae of *C. zealandica* and *G. mellonella*, but not in suckling mice (Scotti *et al.*, 1983). Serological analysis indicated that FHV shared some antigenic determinants with New Zealand virus (NZV) (Friesen *et al.*, 1980) and BBV (Dearing *et al.*, 1980; Scotti *et al.*, 1983).

4. New Zealand Virus

The endogenous *Drosophila* line 1 virus was discovered in the course of investigation of BBV replication in cultured *D. melanogaster* cells (Friesen *et al.*, 1980). These authors showed that uninfected cells from the New Zealand (NZ) subline of *Drosophila* line 1 cells contained endogenous viruslike particles, which they termed DL1 (or DLV) particles. This virus has also been called New Zealand virus (NZV) by Scotti and Fredericksen (1987), and this is the designation we will use here. NZV particles share some antigenic determinants with BBV, but the two viruses are serologically distinct (Friesen *et al.*, 1980). The origin of NZV is unknown; it may have arisen from inadvertent infection of *Drosophila* line 1 cells with BBV and subsequent selection for mutants able to establish a persistent infection (Friesen *et al.*, 1980). No information is available on the host range of this virus.

5. Boolarra Virus

Boolarra virus (BoV) was isolated near Boolarra, Australia, from dead and moribund larvae of the underground grass grub *Oncopera intricoides*, the only known natural host (Reinganum *et al.*, 1985). BoV multiplied in wax moth larvae when inoculated into the hemocoel, with death occurring about 10 days after infection, but it did not cause illness or mortality in suckling mice. Serologically, BoV was found to share antigenic determinants with both BBV and NOV (Reinganum *et al.*, 1985), but not with NZV or Manawatu virus (see next section) (Scotti and Fredericksen, 1987).

6. Manawatu Virus

Manawatu virus (MwV) was also isolated from the New Zealand grass grub *C. zealandica*, from a single larva collected from the same pasture from which FHV was obtained (Scotti and Fredericksen, 1987). No host range studies have been performed with MwV, although it has been analyzed in cell culture (see

Section IV.A). In gel diffusion assays, MwV was found to be serologically distinct from FHV, BBV, and NZV. Immunoprecipitation experiments suggested a closer antigenic relationship between MwV and FHV than between MwV and BBV; no cross-reactivity was seen with BoV antiserum.

7. *Lymantria ninayi* Virus (Greenwood)

Finally, *Lymantria ninayi* virus (Greenwood) (LNV_G) was isolated from diseased or dead moth larvae (*L. ninayi*) in Papua, New Guinea (Greenwood and Moore, 1982). The designation LNV_G was chosen to distinguish this virus from an unassigned tetravirus (LNV) isolated from the same host (Murphy *et al.*, 1995). Larvae of the gypsy moth, *L. dispar*, could be infected with LNV_G by injection or feeding, and exhibited flaccid paralysis and death within 8 to 21 days. In the review by Hendry (1991), this virus was referred to as gypsy moth virus (GMV). LNV_G also replicated in wax moth larvae. A weak serological relationship with BBV was reported.

It is interesting to note that all of the insect *Nodaviridae* identified to date were originally isolated from sources collected from geographical regions that border the Pacific Ocean: Japan, New Zealand, Australia, and New Guinea. In discussing the occurrence of nodaviruses in fish, Munday *et al.* (1994) suggested that the virus group might be endemic throughout the Pacific and associated bodies of water. Perhaps by the same argument the insect nodaviruses are endemic to the land masses bordering the South Pacific.

IV. HOST CELL INTERACTIONS AND CYTOPATHOLOGY

A. Cytocidal Infections

1. Experimental Infection of Animals

We will first consider the cytopathology of the *Nodaviridae* in the affected tissues of the whole animal host, and later discuss the growth of these viruses in cell culture. As described in the previous section, growth in the animal host has been best studied for NOV, both in suckling mice (Scherer *et al.*, 1968; Murphy *et al.*, 1970) and in wax moth larvae (Garzon *et al.*, 1978, 1990; Garzon and Charpentier, 1991). The effects of NOV infection on cellular morphology are remarkably similar in tissues of these two hosts. Each system will be summarized briefly in turn, followed by discussion of the points of similarity between the two.

In the suckling mouse, electron microscopy (EM) was used to detect NOV in brain and heart tissue (Scherer *et al.*, 1968). The observed particles, however, were few in number and larger in diameter (55–75 nm and 60–130 nm) than the 29–31 nm later reported by other groups (Murphy *et al.*, 1970; Newman and Brown, 1973, 1977), now suggesting that at least some of them might not have been NOV. Other tissues were examined by light microscopy: spinal cord

neurons and brown fat exhibited necrosis; limb skeletal muscle showed inflammation and degeneration; and atrophy and degeneration of paravertebral muscles were observed (Scherer *et al.*, 1968). Murphy *et al.* (1970) used EM to detect NOV in the skeletal muscles of the hind limb, in fibroblasts and macrophages in the affected muscles, and in liver tissues. No evidence of viral infection was found in hepatocytes, cardiac muscle, or brain tissue.

In wax moth larvae, the light microscope revealed localized lesions in the cytoplasm of cells of the muscle, the nerve ganglia, and the salivary and molting glands. Some lesions and virions were also observed in the hypodermis, adipose tissue, trachea, and hemocytes. Cells of the silk glands, Malpighian tubes and intestinal epithelia were little affected by the infection (Garzon *et al.*, 1978).

It is on the cellular level that the effects of infection on these hosts are most similar (Murphy *et al.*, 1970; Garzon *et al.*, 1978). Infection is characterized by accumulation of large numbers of smooth membrane vesicles and, in localized areas, of ribosomes and polyribosomes. Garzon *et al.* (1978) suggested that the vesicles are derived from the membranes of the rough endoplasmic reticulum (ER), presumably first stripped of ribosomes, and those of the mitochondria, which were observed in both adipose and muscle tissue to swell and become deformed during infection. These vesicular structures (virogenic stroma) were indistinguishable from those seen in enterovirus-infected cells, where viral replication complexes are associated with virus-induced vesicles (reviewed by Schlegel and Kirkegaard, 1995). Both free virions and large masses in random or crystalline array were sometimes contained in membrane-bound structures. In some cases, virus and virogenic stroma were found within the same vesicles, supporting the idea that the virogenic stroma might represent the sites of viral replication, by analogy with the enteroviruses. These structures were also observed in fibroblasts and macrophages in affected mouse skeletal muscles (Murphy *et al.*, 1970). In the adipose tissue of wax moth larvae, the cytoplasm appeared hypertrophied and rich in mitochondria, lipid droplets and glycogen regressed, and the nuclei became rounded (Garzon *et al.*, 1978).

Garzon *et al.* (1990) further explored the involvement of mitochondria in NOV morphogenesis. In infected *G. mellonella* muscle, numerous mitochondria were clustered together around the nucleus and along the fibrillar spaces. Early in infection, mitochondria in these clusters were elongated and closely interdigitated by a thick electron-dense material, perhaps corresponding to the virogenic stroma. The production of progeny virions apparently from this dense layer eventually resulted in separation of the mitochondria in the clusters and accumulation of virions all along the interfibrillar space. Later, completely disorganized and fragmented muscle fibers were interspersed with progeny virions. These virions were associated with the outer membrane of the degenerated and swollen mitochondria, and sometimes with the inner membrane as well. Such mitochondrial clusters were not seen in infected suckling mice, where cytopathic effects were more variable. In some cases, mitochondria appeared surrounded by virions or by virogenic stroma and progeny virions were scattered between muscle fibers with unaltered morphology, whereas in others, the matrix of some mitochondria was displaced by concentric vesicles

attached to the outer mitochondrial membrane. Virus particles were observed inside the altered mitochondria (Garzon *et al.*, 1990).

The significance of the mitochondrial clusters and their relationship with viral replication remain unclear. In adipocytes and hemocytes, NOV also associates with membranes of nonmitochondrial origin: those derived from the ER (Garzon *et al.*, 1990). These authors suggested that perhaps in tissues in which the ER is not well developed, in muscle cells or plant tissues, for example, mitochondrial, chloroplastic, or peroxisomal membranes could substitute for ER-derived membranes in viral replication.

The morphological changes are particularly striking in infected muscle cells, where they are accompanied by the appearance of interfibrillar spaces and gradual disorganization of muscle fibers, resulting in loss of their characteristic cross-striations. Cytoplasmic retraction, concentration, and disorganization are evident in all areas where virions are found. The intercellular spaces are increased as well, apparently due to formation of edema (Murphy *et al.*, 1970). Late in infection, the cytoplasmic contents of some cells are completely displaced by virions, often in paracrystalline arrays, and the nucleus shows fragmentation and condensation of chromatin (Garzon *et al.*, 1978). By this time, very large numbers of NOV virions are present at multiple sites in the cytoplasm, either dispersed or in paracrystalline arrays. The highest concentration of virus particles can be found near the sarcoplasmic reticulum (SR). Large paracrystalline arrays are found exclusively near the SR, whereas individual virions and small crystalloids are also found interspersed between myofibrils (Murphy *et al.*, 1970).

In the cytoplasm of infected *G. mellonella* cells, isolated virus particles are present along the ER at the apex of the cells, in the intercellular spaces and between the cell and the basal membranes. Ultimately, release of progeny virions can be accomplished by cell lysis, as in the case of the hemocytes, or by the migration of the virus along the ER to the intercellular spaces and basal membranes, as seen in cells of the muscle and trachea.

In liver tissues obtained from mice with muscle lesions, virtually every Kupffer cell contains large paracrystalline arrays of virions in membrane-bound structures, but no free virus particles. Virogenic stroma identical to that found in infected muscles also occurs in membrane-bound structures, either alone or together with virus particles. Murphy *et al.* (1970) postulated that the primary site of NOV infection is in muscle cells and that shedding of virus in intact membrane-bound structures, followed by viremia and phagocytosis, results in the uptake of these structures into cells of the reticuloendothelial system, including macrophages and Kupffer cells.

Garzon *et al.* (1978) also observed accumulation in wax moth larvae of electron-dense filaments 11 nm in diameter, with a recurring helical structure. These filaments are observed primarily inside the basal membranes and in the cytoplasm of cells of muscle and adipose tissue, but not in hemocytes or cells of the molting gland. The filaments appear first in the basal membrane in the middle of the amorphous substance, and then increase in concentration until the basal membrane has degraded, releasing filaments into the cytoplasm of

the neighboring cells. Filaments seem to progress along the basal membranes, causing the dissociation of the tissue or of the bundle of muscle fibers. Similar filaments have been observed in muscle cells of mice infected with coxsackie virus A (Bienz-Isler *et al.*, 1970). Garzon *et al.* (1978) suggested that these filaments might be responsible for the dissociation of the "cellular cement" and for the disorganization of the tissues.

The cytopathology of NOV in mosquitoes was also examined, albeit on a more superficial level. Scherer and Hurlbut (1967) showed that in infected *A. aegypti* mosquitoes, the virus resided primarily in the bodies rather than the heads of the insects. This observation is compatible with passage of virus from bodies through the heads during feeding, resulting in contamination of the head parts, and is consistent with arthropod transmission. In contrast, Tesh (1980) reported that the brains of dead or paralyzed mosquitoes contain large amounts of viral antigen, as measured by an indirect immunofluorescence assay. However, as these studies examined only head squashes, it is not possible to distinguish the specific tissue infected by NOV in these insects.

No data are available on the animal cytopathology of FHV, MwV, LNV_G, NZV, or BoV, although that of BoV has been studied in cell culture. BBV infection was examined by EM of larvae of the black beetle (Longworth and Archibald, 1975) and the wax moth (Longworth and Carey, 1976); in both animals, BBV develops in the cytoplasm of gut and fat body cells. In the wax moth, extensive development of virus in the cytoplasm of midgut columnar cells was observed, but nuclei were unaffected (Longworth and Carey, 1976). Some replication was observed in the fat body as well, but the midgut is apparently the primary target for infection. Late in infection, infected cells contained large numbers of progeny virus particles, some free in the cytoplasm and some in large paracrystalline arrays.

2. Infection of Cells in Culture

A wide range of cultured insect and mammalian cells have been tested for their ability to support nodavirus replication. A hallmark of infection in cell culture is the absence of an obvious cytopathic effect (CPE), even with the most lytic member, FHV. Many attempts have been made to discover a cell culture system that will support NOV infection. Primarily, mammalian cells were tested, although cultured mosquito and *Drosophila* lines were analyzed as well. Bailey *et al.* (1975) successfully infected cells obtained from two mosquito species, *A. albopictus* and *A. aegypti*. No CPE was observed, but infectious virus was detected both in infected cells and in their culture medium, as measured by infectivity assays performed in bees, wax moth larvae, or suckling mice (Bailey *et al.*, 1975). Ajello (1979) observed a low level of NOV in cultured *A. albopictus* cells, but interpreted this as persistence of the inoculum rather than active virus multiplication. Tesh (1980) analyzed the AP-61 mosquito cell line from *A. pseudoscutellaris* for its ability to support NOV infection. Again, no CPE was observed, but the cells were positive for the presence of viral antigen by indirect immunofluorescence.

Cells from several vertebrate species were tested for their ability to support NOV infection: No CPE was detected after infection of primary cultures of kidney cells from Syrian hamster, swine, calf, rabbit, suckling Swiss albino mouse, and cynomolgus monkey, whole chicken embryo, or human amnion, and no plaques were detected in primary cultures of whole chicken or Swiss albino mouse embryos at 36°C or 30°C, respectively (Scherer *et al.*, 1968). Similar negative results were obtained with several continuous cell lines: human HeLa, human liver and conjunctiva, mouse L cells, cottontail rabbit papilloma cells, and fibroblasts derived from normal rabbit skin. However, it is possible that in some of these cells infection was not detected for technical reasons. For example, the lack of CPE is not a reliable means of detecting nodavirus infections, but no other assays for infectivity were performed. In addition, except as noted, these experiments were performed at 36°C, a temperature at which the NOV RNA replicase is severely inhibited, at least in BHK21 cells (Ball *et al.*, 1992).

Similarly, Ball *et al.* (1992) tested a number of cell lines for their susceptibility to NOV infection, using metabolic labeling of viral RNA as an assay: human (HeLa, HEP-2, and 143B) cells; monkey (BSC40) cells; mouse fibroblast (C127) cells; porcine kidney (PK15) cells; primary chicken embryo cells; Syrian hamster kidney (BHK21) and Chinese hamster ovary (CHO) cells; turtle heart cells; and Schneider's *Drosophila* line 1 (DL1) cells. None of these cell types was susceptible by infection by whole virus, even at high multiplicities of infection, although they all supported the replication of transfected virion RNAs, suggesting that the block to viral infection may be at the level of cell entry or uncoating. This finding confirmed that of Gallagher (1987), who also initiated an NOV infectious cycle in DL1 cells by transfection of virion RNAs. In BHK21 cells transfected with virion RNAs, large numbers of progeny virions, sometimes in paracrystalline arrays, were detected in the cytoplasm by EM (Ball *et al.*, 1992). Virions were not detected in the nuclei and there was no evidence of the release of progeny virions from the cells. As seen in NOV infected cells, only mild CPE was observed.

There is some controversy about the infectivity of NOV for hamster kidney cells, in most cases the BHK21 cell line. Neither Scherer *et al.* (1968), who tested primary hamster kidney cells, nor Ball *et al.* (1992), who used BHK21 cells, detected infection, yet two other groups reported infectivity. Bailey *et al.* (1975) detected NOV in infected BHK21 cells and in their culture medium 5 days postinfection in the absence of detectable CPE, using assays for infectivity in bees, wax moth larvae, and suckling mice. Multiplication of NOV in the BHK21 cells was confirmed by metabolic labeling of viral RNAs. Similar results were obtained by Newman and Brown (1973). Recently, Hiscox and Ball (1997) obtained a very low level of infection of BHK21 cells by NOV; infection could also be initiated by transfection of intact NOV virions into BHK21 or *Drosophila* cells.

Several mammalian and insect cell lines were examined for evidence of BBV infection, as assayed by metabolic labeling of viral RNAs with [³H]uridine

(Friesen *et al.*, 1980). No evidence of multiplication was found in BHK21 or mouse L cells, or in several insect cell lines including those from mosquito, cabbage looper, fall army worm, or the GM1 line of *Drosophila melanogaster* cells. However, the virus multiplied well in Schneider's DL1 cells; yields were unusually high, on the order of 20% of the total cellular protein, yet there was little CPE. The virus remained largely cell-associated, and infected cells remained intact for 4 or more days after infection. Similar results were obtained on transfection of DL1 cells with RNAs isolated from BBV virions (Gallagher *et al.*, 1983). BBV multiplies poorly if at all in a derivative line of DL1 cells, the New Zealand (NZ) subline, later determined to contain endogenous NZV particles. Friesen *et al.* (1980) suggested that NZV may have originated during a persistent infection of the cells by BBV; this will be considered further in Section IV.B.

Drosophila DL1 and DL2 cells also supported infection by the other strictly insect nodaviruses. The original isolation of FHV from infected grass grubs involved passaging in DL1 cells (Dearing *et al.*, 1980), which were clearly permissive for infection, as confirmed by metabolic labeling of infected cells with [³H]uridine. In both DL1 and DL2 cells, FHV infection resulted in a characteristic clumping of the cells, but otherwise only subtle CPE (Scotti *et al.*, 1983). FHV did not cause complete cytolysis of the cells, so that some of the virus remained cell-associated until released experimentally by detergent lysis. These cells produced very high yields of FHV, with an estimated burst size in DL2 cells of 250,000 particles per cell (Scotti *et al.*, 1983). In contrast, Dasgupta *et al.* (1994) observed extensive (99%) lysis of FHV infected DL1 cells within 3 days.

The permissive *Drosophila* cell culture system permitted the first detailed studies of the molecular biology of the nodaviruses, particularly BBV and FHV. Two groups used infected *Drosophila* cells to examine BBV protein synthesis (Crump and Moore, 1981a,b; Friesen and Rueckert, 1981, 1982). Similarly, viral RNA replication was examined in infected (Crump *et al.*, 1983) or virion RNA-transfected *Drosophila* cells (Gallagher *et al.*, 1983). These areas are explored further in Sections VII and IX. Two additional technical advances greatly facilitated the genetic study of nodavirus infection. First, the development of a plaque assay for FHV and BBV in DL1 and DL2 cells (Selling and Rueckert, 1984), which for the first time permitted accurate determination of infectious virus titers. Second, *in vitro* synthesis from FHV cDNAs of infectious viral RNAs made the nodaviruses amenable to reverse genetic approaches (Dasmahapatra *et al.*, 1986). See also Section VIII.C.

Reinganum *et al.* (1985) showed that BoV replicates in DL1 and DL2 cells. Although the cells exhibited no CPE, virus particles could be seen in the culture medium by EM, large aggregates of antibody-complexed particles could be seen by immuno-EM, and RNA replication could be detected by metabolic labeling of the infected cells. The host protein shutoff in BoV-infected DL1 cells is very mild, although some change is evident (Bashiruddin and Martin, 1987).

The *Drosophila* cell culture system also allowed Bashiruddin and Cross (1987) to investigate the ultrastructure of BoV morphogenesis by EM of syn-

chronously infected DL1 and DL2 cells. For the first time, the temporal order of the morphological effects of nodavirus infection could be determined. At no time were morphological alterations to the nuclei of these cells observed. In BoV-infected DL1 cells at 24 hr, regions of virogenic stroma appeared and multilamellar membrane stacks and vesicular bodies were formed. By 48 hr, the virogenic stroma contained arrays of electron-lucent particles. By 72 hr, the virogenic stroma was replaced with particles of two morphologies: electron-dense particles of 30 nm diameter and particles with electron-lucent centers of 16 nm diameter, which the authors postulated might be precursors of mature virions. Both types of particles were found both loose and in paracrystalline arrays. Mature virus was also found outside the cells, in loose aggregates at the cells' edge, attached to the cell membrane in channels, and in channels between adjacent cells.

In both DL1 and DL2 cells, the appearance of vesicular bodies and the disappearance of the mitochondria were simultaneous, and the authors suggested that modified mitochondria may lead to formation of vesicular bodies. Vesicular bodies were distinct in appearance from vesicles and vacuoles in uninfected cells and contained fibril-containing vesicles. Yet the virus can be produced without the involvement of vesicular bodies and without the destruction of all the mitochondria. The EM observations help to explain the absence of a distinct CPE. While large amounts of virus and virus-associated structures could lead to rapid cell disruption, this appears to be averted by the continuous release of virus, allowing longevity of virus formation and ensuring a very large yield. It would also allow lateral transmission early in the infection. BoV is released as single virions early in infection and later as aggregates (Bashiruddin and Cross, 1987).

Isolation of MwV involved three passages of the field isolates in DL1 cells, so MwV is clearly infectious for these cells (Scotti and Fredericksen, 1987). No further analysis of cell culture systems able to support MwV replication has been performed. It is not known whether LNV_G will replicate in DL1 or DL2 cells; in fact, no cell culture systems able to support growth of LNV_G have been described. Reavy *et al.* (1982) reported that LNV_G did not replicate in tissue culture, although no information was provided as to the identities of the cell types tested.

Interestingly, FHV can replicate and produce progeny virions in plants, but only if they are transfected with virion RNA (Selling *et al.*, 1990). FHV infectivity was detected by plaque assay on DL1 cells using as inoculum leaf homogenates from the following transfected plants: barley, chenopodium, cowpea, and *Nicotiana benthamiana*; in contrast, only one of eight homogenates from transfected tobacco plants contained detectable virus. Infectivity was also detected with protoplasts derived from barley leaves. However, in the plant cells, the progeny virions were unable to initiate further infection. Progeny virions were not detected when the same plants were inoculated with whole virions. No time-dependent increase in virions was observed in barley protoplasts infected with virions, but radioactive progeny virions were detected by [³²P]orthophosphate labeling of cells inoculated with unlabeled FHV. No overt

symptoms were detected in any inoculated plants, but there was some evidence of virus spread in *N. benthamiana*. In that case, virions were detected at very low levels in uninoculated leaves, but in the other plant species, virions were detected only in the inoculated leaf.

B. Persistent Infections

The suggestion that nodaviruses might establish persistent infections under certain conditions was made by Friesen *et al.* (1980), who hypothesized that the endogenous *Drosophila* virus NZV might have arisen as the result of a persistent infection of DL1 cells with BBV. The authors proposed that at some point the cells may have been inadvertently infected with BBV, an infection that could have been overlooked in the absence of extensive CPE. Further passaging of the cells might have selected for variants more compatible with continued cell growth, leading to the establishment of a persistent infection, giving rise to the NZ subline, and perhaps accounting for the differences in immunogenicity between NZV and BBV. The ease with which FHV can establish persistent infections in DL1 cells and the observation that the NZ line is resistant to superinfection by BBV lend credence to this idea (Friesen *et al.*, 1980; Dasgupta *et al.*, 1994).

Rueckert and colleagues examined whether nodaviruses could establish persistent infections in cell culture (Selling, 1986; Dasgupta *et al.*, 1994). They found that about 1% of BBV-infected DL1 cells were not killed by the virus but instead became persistently infected and resistant to superinfection. The development of a plaque assay for FHV (Selling and Rueckert, 1984; Selling, 1986) allowed these experiments to be repeated under conditions where both the multiplicity of infection (MOI) and virus yield could be determined (Gallagher, 1987; Gallagher and Rueckert, 1988). Ten separate lines of persistently infected cells produced infectious FHV, with yields between 1.4 and 13% those of newly infected DL1 cells, nearly 2 years after the initial infection. All ten cell lines produced viruses with small plaque phenotypes, due to one or more point mutations in the coat protein coding region of RNA 2.

Similarly, Dasgupta *et al.* (1994) analyzed 20 FHV-resistant cell lines and demonstrated that all 20 were persistently infected in a productive yet nonlytic fashion. These cells were immune to superinfection by BBV and BoV, but not cricket paralysis virus or *Drosophila* X virus. The cells were maintained by passaging at 2-week intervals; an initial tendency of the cells to clump together diminished after the first few passages. This tendency may reflect activation of a cellular defense mechanism against viral replication, or the virus might generate defective RNA templates that interfere with the viral RdRp, a mechanism proposed for persistent infection in many animal viruses. However, this does not appear to be a general requirement for persistence in this system, because defective-interfering RNAs were detected in only 4 out of the 20 persistently infected lines.

Viruses isolated from the persistently infected cultures produced normal-

sized plaques at early passages, but plaque size decreased during later passages. Dasgupta *et al.* (1994) examined the plaque phenotypes as a function of time and showed that establishment of persistence and the appearance of the small plaque phenotype were separate events. No change in plaque size was detected until the fifth passage, when approximately equal numbers of small and normal plaques were observed. The number of small plaque viruses increased during passages seven to nine, resulting in a complete shift to the small-plaque phenotype by the 12th passage. Therefore, the decrease in plaque size must be a secondary event that occurred during the maintenance phase of persistence. Indeed, Dasgupta *et al.* (1994) demonstrated that a single nucleotide change in the capsid coding region is sufficient to alter the plaque size; the role of additional changes in this phenotype is unknown. The authors proposed that the preferential accumulation of mutations in RNA 2 was due to relaxation of selection pressure on the viral capsid protein, which in persistently infected cells is no longer needed for infection of fresh host cells. In contrast, RNA 1 is maintained under continuous selection pressure, because RNA 1 must replicate in order to be maintained as the persistently infected cells divide. The lack of phenotypic changes in the virus during the establishment phase suggests that FHV persistence may be initiated by an unidentified change in the virus-cell relationship, but the exact nature of this change is unknown.

V. VIRION COMPOSITION, PROPERTIES, AND STRUCTURE

As described in Section I, nodaviruses are small, nonenveloped, icosahedral viruses with bipartite positive-strand RNA genomes. NOV was the first arthropod-borne virus discovered whose infectivity was resistant to lipid solvents such as diethyl ether, chloroform, and sodium deoxycholate, indicating that it lacked a lipid envelope (Scherer and Hurlbut, 1967; Scherer, 1968; Scherer *et al.*, 1968). Electron micrographs of negatively stained virus particles confirm the absence of an envelope for NOV (Newman and Brown, 1973), BBV (Longworth and Carey, 1976), FHV (Dearing *et al.*, 1980), BoV (Reinganum *et al.*, 1985), and LNV_G (Greenwood and Moore, 1982).

Nodavirus virions are composed of a protein shell surrounding one copy of each of the two viral genomic RNAs, which are both essential for infectivity and co-packaged into the same particle (Fig. 1) (Newman and Brown, 1973, 1977; Longworth and Carey, 1976; Selling and Rueckert, 1984). The approximate molecular weights of the genomic RNAs are summarized in Table III; many of them have not yet been sequenced, so their exact sizes are unknown. However, the sequence of at least one genomic segment (RNA 2) has been determined for NOV, BBV, FHV, and BoV (Dasgupta *et al.*, 1984; Dasmahapatra *et al.*, 1985; Dasgupta and Sgro, 1989; Dasgupta, 1994), and the sizes of these RNAs and their open reading frames (ORFs) are listed in Table IV.

The viral capsid consists of 180 protomers arranged with icosahedral symmetry on a $T = 3$ surface lattice (Hosur *et al.*, 1987). The major protein produced

TABLE III. Physicochemical Properties of the *Nodaviridae*

	NOV ^a	BBV ^b	FHV ^c	BoV ^d	MwV ^e	NZV ^f	LN _{V_G} ^g
Particle size (nm)	29	30	29	30	ND ^h	ND	32
Sedimentation coefficient	135	137	142	140	142	137	ND
Density (g/ml)	1.34	1.335 (1.352) ^e	1.351 (1.372) ^e	1.34	(1.366) ^e	(1.373) ^e	1.3
Mol. wt. of RNAs (× 10 ⁶)							
RNA 1	1.15	1.12	1.10	1.03	1.10	1.12	0.95
RNA 2	0.46	0.46	0.46	0.47	0.46	0.46	0.47
Mol. wt. of proteins (× 10 ³)							
Protein α	43	44	47	40.6	ND	ND	34
Protein β	40	40	43	38	40	40 (43) ^e	31

^aMurphy *et al.* (1970); Newman and Brown (1973, 1977).^bLongworth and Carey (1976); Friesen *et al.* (1980); Friesen and Rueckert (1981); Scotti and Fredericksen (1987).^cDearing *et al.* (1980); Scotti *et al.* (1983); Scotti and Fredericksen (1987).^dReinganum *et al.* (1985).^eScotti and Fredericksen (1987).^fFriesen *et al.* (1980); Scotti and Fredericksen (1987).^gGreenwood and Moore (1982); Reavy *et al.* (1982).^hND, not determined.

during viral infection is the capsid protein precursor, α (Friesen and Rueckert, 1981), which is autocatalytically cleaved following viral assembly, producing proteins β and γ (Hosur *et al.*, 1987; Gallagher and Rueckert, 1988). In the mature virion, 10 to 25% of protomers contain uncleaved α, while the remainder have one copy each of β and γ, both of which remain associated with the virion (Hosur *et al.*, 1987). This maturation cleavage is required for infectivity (Schneemann *et al.*, 1992). The molecular weights of each nodaviral protein β (and its precursor, α) are listed in Table III; values were estimated from migration in sodium dodecyl sulfate–polyacrylamide gels. Note that the molecular weight of LN_{V_G} β protein is only 31,000, which may account for its limited serological cross-reactivity with other nodaviruses (Greenwood and Moore, 1982; Reavy *et al.*, 1982). The sizes of the protein α ORFs of NOV, BBV, FHV, and BoV have been determined as 399, 407, 407, and 403 amino acids, respectively, based on the published sequences for these RNA 2 segments (Table IV) (Kaesberg *et al.*, 1990).

The three-dimensional structures of BBV, FHV, and NOV have been solved crystallographically at high resolution (Hosur *et al.*, 1984, 1987; Fisher and Johnson, 1993; Cheng *et al.*, 1994; Johnson *et al.*, 1994; Wery *et al.*, 1994; Zlotnick, 1994) and the FHV structure may be viewed at the following web-site, constructed by J.-Y. Sgro: <http://www.bocklabs.wisc.edu/fhv.html>. Nodavirus structures are reviewed in detail in Chapter 7, this volume, and therefore will not be discussed further here.

Some physicochemical properties of the nodaviruses are summarized in Table III. Nodavirus particles are 29 to 32 nm in diameter (Murphy *et al.*, 1970;

TABLE IV. Sequenced Genomic and Subgenomic RNAs of BBV, FHV, BoV, and NOV

Virus	RNA	Size (nt)	ORF length(s) (codons, protein)	Accession number (GenBank)	Reference
BBV	RNA 1	3106	998, protein A	X02396	Dasmahapatra <i>et al.</i> (1985)
	RNA 3	389 (?) ^a	102, protein B1 106, protein B2		Guarino <i>et al.</i> (1984)
BBV	RNA 2	1399	407, protein α	X00956	Dasgupta <i>et al.</i> (1984)
FHV	RNA 1	3107	998, protein A	X77156	Dasgupta (1994)
	RNA 3	387	102, protein B1 106, protein B2		
FHV	RNA 2	1400	407, protein α	X15959	Dasgupta and Sgro (1989)
BoV	RNA2	1305	403, protein α	X15960	Dasgupta and Sgro (1989)
	RNA 3	387	106, protein B2		Harper (1994)
NOV	RNA 2	1335	399, protein α	X15961	Dasgupta and Sgro (1989)

^aSee text.

Longworth and Carey, 1976; Dearing *et al.*, 1980; Greenwood and Moore, 1982; Reinganum *et al.*, 1985). The diameter of NOV, which is now known to be 29 nm, was originally reported as 55 to 75 nm, based on filtration studies and EM analysis (Scherer *et al.*, 1968); the reasons for the discrepancy are unclear. From the results of crystallographic analysis, Hosur *et al.* (1987) reported a diameter of 31.2 nm for BBV, which is slightly larger than the 29 nm measured previously (Murphy *et al.*, 1970; Newman and Brown, 1973, 1977). The sedimentation coefficients were determined by centrifugation of virions in sucrose gradients, compared with standards of known sedimentation properties, and virus buoyant densities were measured by centrifugation in cesium chloride gradients (Table III). The values reported by Scotti and Fredericksen (1987) (in parentheses in Table III) are higher than in previous reports because of a change in methodology (Scotti, 1985). Both values are presented in an effort to provide a comprehensive list.

The stabilities of several of the *Nodaviridae* have been determined under a variety of conditions. Hendry (1991) noted that all these viruses except BoV are stable in 1% SDS. In several cases, extraction of the RNA from virions requires the use of phenol-SDS at 45°C (Longworth and Carey, 1976; Newman *et al.*, 1978; Gallagher *et al.*, 1983). The two genomic RNAs were released sequentially from NOV when the virions were treated with phenol alone, which released RNA 1, followed by phenol-SDS treatment, which released RNA 2 (Newman and Brown, 1973). These results were originally interpreted to mean that the genomic RNAs were packaged into separate particles; the discovery that the RNAs are co-packaged (Longworth and Carey, 1976; Newman and Brown, 1977; Selling and Rueckert, 1984) suggests that RNA 2 may simply be more tightly associated with the capsid proteins than is RNA 1. This idea may be related to the observation that RNA 2 contains a bulged stem-loop structure that functions as a packaging signal (Zhong *et al.*, 1992) (see Section VII.D).

Gallagher and Rueckert (1988) reported that mature FHV virions were stable in deoxycholate (1%), urea (1 M), EDTA (20 mM), or SDS (1%) in the presence of magnesium or calcium ions (10 mM). However, immature particles known as provirions in which most of protein α remained uncleaved were very sensitive to these denaturants (Gallagher and Rueckert, 1988). Provirions could also be dissociated by freezing at pH 9.0 in a buffer containing 0.1 M NaCl, 0.1 M urea, and 10 mM EDTA, whereas mature virions remained completely stable under these conditions (Schneemann *et al.*, 1994).

NOV and FHV were tested for their stability upon heating. A crude extract of NOV-infected cells exhibited a 1000-fold reduction in titer after 2 hr at 50°C, but only if the virus particles were first diluted to remove cellular factors (Murphy *et al.*, 1970). FHV was stable for 10 min at 40°C, but 91% of its infectivity was lost at 53°C, and 99% at 58°C (Scotti *et al.*, 1983). Cheng *et al.* (1994) observed by EM an apparent single-site release of RNA after heating FHV for 10 min at 65°C.

NOV, BBV, and FHV are stable at pH 3.0; the pH ranges tested were 3.0 to 7.7 (Murphy *et al.*, 1970; Newman and Brown, 1977), 3.0 to 10.0 (Longworth and Archibald, 1975; Longworth, 1978), and 2.5 to 8.4 (Scotti *et al.*, 1983), respectively. Finally, NOV is unstable in solutions containing chloride ions, except near its isoelectric point of pH 3 to 4 (Newman and Brown, 1977). BBV and LNV_G are unstable when stored in cesium chloride (Longworth and Carey, 1976; Greenwood and Moore, 1982), whereas FHV is stable under these conditions (Scotti *et al.*, 1983).

VI. GENOME ORGANIZATION AND MOLECULAR BIOLOGY

A. Viral RNAs

The nodavirus RNA species whose sequences have been determined are listed in Table IV. The larger RNA (RNA 1) contains about 3.1 kb (Table IV) and encodes the entire viral contribution to the RdRp that replicates both viral genome segments (Gallagher *et al.*, 1983; Gallagher, 1987). The smaller RNA (RNA 2) contains about 1.4 kb and encodes protein α , a precursor by proteolysis to the two virion structural proteins, β and γ (Dasgupta *et al.*, 1984; Dasmahapatra *et al.*, 1985; Hosur *et al.*, 1987). The genes for intracellular replication of the viral RNAs and for extracellular spread of infection are therefore segregated between the two genome segments.

In BBV, both virion RNAs have cap(0) structures and the sequence ^m7G5'-ppp5'GpUp ... at their 5' ends, but like the RNAs of NOV (Newman and Brown, 1976), their 3' ends are not polyadenylated (Dasgupta *et al.*, 1984). Indeed, the precise structure of the 3' ends of nodaviral RNAs remains a mystery because the RNAs fail to react with RNA ligase or poly(A) polymerase, enzymes that specifically recognize free 3'-OH groups on RNA molecules (Guarino *et al.*, 1984; Dasmahapatra *et al.*, 1985). This observation led to the original suggestion that nodaviral RNA 3' ends might be covalently blocked in some way, perhaps

by a protein (Kaesberg, 1987), but no evidence for the presence of such a protein was provided by direct amino acid analysis of purified FHV RNA, by sequential Edman degradation, or by metabolic incorporation of radiolabel from a mixture of amino acids. In addition, FHV RNAs failed to show anomalous electrophoretic migration, even under conditions that gave single-nucleotide resolution (L. A. Ball, unpublished results). These results, together with the observation that the RNA 3' ends are fully reactive in double-stranded versions of the viral RNAs (Guarino *et al.*, 1984; Dasmahapatra *et al.*, 1985), where intramolecular secondary structure would be eliminated, suggest that the block to reactivity is more likely due to an unusually strong secondary structure than the covalent attachment of a protein. However, the attachment of a small, non-protein-blocking group cannot be excluded.

In addition to RNAs 1 and 2, a third positive-sense viral RNA (RNA 3) accumulates to high levels in infected cells, although it is not packaged into virus particles (Friesen and Rueckert, 1982; Gallagher *et al.*, 1983). RNA 3 corresponds to the 3' end of RNA 1, contains a 5' cap and an unreactive 3' end like RNAs 1 and 2 (Guarino *et al.*, 1984), and functions as mRNA for one or two small nonstructural proteins (see Sections VI.B and VI.C). The sequence of BBV RNA 3 was compiled by applying direct and indirect sequencing methods to both single- and double-stranded versions of the molecule isolated from infected cells. These approaches led Guarino *et al.* (1984) to conclude that the BBV RNA 3 positive strand contained 389 nucleotides (nt) and initiated at nt 2718 in RNA 1 with the sequence $m^7GpppUCGUUACC \dots$. However, more recent examination of the 5' end of positive-sense FHV RNA 3 by primer extension indicates that it starts at nt 2721 in RNA 1 (which is equivalent to nt 2720 in BBV RNA 1) with the sequence $m^7GpppGUUACC \dots$, and would thus contain only 387 nt (Fig. 2) (Ball, 1995). It is unlikely that this reflects a difference between BBV and FHV, because their RNA 1 sequences are identical over the 3' 569 nt (Dasgupta, 1994). Rather, these results imply that the synthesis of the major species of positive-sense RNA 3 initiates with a purine (as do almost all

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FHV/BBV    ...GACGAAUCGUUACCAUGUUAACGAUGCCAAGCAAACUCGCGCUAAUCCAGGAACUU...
Protein A   ....D E S L P M L N D A K Q T R A N P G T...
Protein B1          M L N D A K Q T R A N P G T...
Protein B2                      M P S K L A L I Q E L...

BoV        ...GACGAAUCGUUAUUAAGCUUAACGAUGCAAAGCAAACUCGCGCUAAUUCAGGAACUU...
Protein A   ....D E S L L K L N D A K Q T R A N S G T...
Protein B2          M Q S K L A L I Q E L...

```

FIGURE 2. Nucleotide sequences in RNA 1 of BBV, FHV, and BoV surrounding the 5' ends of RNA 3. The underlined regions are represented in the corresponding RNA 3 molecules and the predicted partial amino acid sequences of proteins A, B1, and B2 are shown. The BBV sequence was from Dasmahapatra *et al.* (1985), as corrected by Kaesberg (1987); the FHV sequence was from Dasgupta (1994); and the BoV sequence was from Harper (1994).

RNAs) and that its first two nucleotides (GU) are the same as those of RNAs 1 and 2. It remains a possibility that the positive strand in dsRNA 3 (which is not capped) is two nucleotides longer at its 5' end.

Negative-sense versions of RNAs 1, 2, and 3 occur in infected cells but not in virus particles. After deproteinization under nondenaturing conditions, these RNAs are found as double-stranded molecules with apparent sizes of about 3.1, 1.4, and 0.39 kilobase pair (kbp), respectively. A 1.75-kbp dsRNA of unknown origin has also been observed in BBV-infected cells (Guarino *et al.*, 1984). When examined under denaturing conditions, by formaldehyde-agarose gel electrophoresis and hybridization of RNA blots with strand-specific probes, the predominant negative-strand species of RNAs 1 and 2 in BHK21 cells migrate with apparent sizes of 6.2 and 2.8 kb, respectively, as if they were covalently linked dimers (Ball, 1992, 1994b). The 2.8-kb species was also observed as a minor component after glyoxal denaturation of FHV RNAs replicating in *Drosophila* cells or in yeast (see Section IX.B), but it was interpreted by Price *et al.* (1996) as an undenatured duplex of 1.4-kb single-stranded RNAs. Whatever their true sizes and structures turn out to be, negative-sense RNAs accumulate to only a few percent of the level of the corresponding positive strands.

B. Open Reading Frames

The complete sequence of RNA 1 is currently known only for BBV and FHV. These sequences are 99% identical and contain an ORF that extends for 998 codons from nt 39 to 3032 in BBV or from nt 40 to 3033 in FHV. Partial sequences of BoV and NOV RNA 1 indicate that these RNAs have diverged by about 15% and 50%, respectively, from BBV/FHV (R. Dasgupta, personal communication; L. A. Ball, unpublished results). In the original published sequence of BBV RNA 1, nt A2689 was overlooked, which truncated the ORF after 897 codons (Dasmahapatra *et al.*, 1985). The 998-residue ORF encodes a polypeptide of 112,290 Da (protein A), whose predicted sequence contains clear polymerase motifs, including at amino acids 691 to 693 the GDD sequence characteristic of RdRps, strongly suggesting that protein A is the catalytic subunit of the RdRp. No sequence motifs characteristic of helicases, guanylyltransferases, or methyltransferases, however, have been identified in protein A.

Near its 3' end, ORF A overlaps a 106-codon ORF in the +1 reading frame that extends from nt 2737 to 3054 (BBV) or 2738 to 3055 (FHV) (Fig. 2). Not unexpectedly, this downstream ORF is not expressed from RNA 1 itself, but rather from RNA 3, which directs abundant synthesis of the corresponding 11,633-Da protein, B2 (Friesen and Rueckert, 1982; Guarino *et al.*, 1984). The BBV and FHV B2 proteins (which are identical in sequence) fail to show significant homology with any protein in the database except the B2 protein of BoV. However, BoV B2 shows distant homology with a *Caenorhabditis elegans* protein that contains sequence motifs characteristic of tyrosine-specific protein kinases (L. A. Ball, unpublished results). The significance of this is unclear. In

BBV and FHV, RNA 3 also directs the synthesis of a second small protein called B1, whose initiating AUG precedes that of B2 and whose sequence corresponds to the C-terminal 102 amino acids of protein A; but BoV RNA 3 encodes only protein B2 (Fig. 2) (Harper, 1994).

The nodaviral RNA 2 sequences each contain a single ORF that occupies about 88% of their length and encodes a proteolytic precursor (protein α) to the virion structural proteins β and γ . Virion RNAs 1 and 2 direct vigorous synthesis of proteins A and α , respectively, in a variety of cell-free translation systems, including those from *Drosophila* cells, HeLa cells, rabbit reticulocytes, and wheat germ (Newman *et al.*, 1978; Crump and Moore, 1981a,b; Guarino *et al.*, 1981; Friesen and Rueckert, 1984). After incubation for 24 to 48 hr, FHV protein α made in reticulocyte lysates is replaced by a protein that comigrates with protein β (Blackhurst and Hendry, 1987), suggesting the possibility of proteolytic processing in the cell-free system. No particle formation was detected under these conditions, however, and protein α , containing a mutation at N363 that renders it resistant to cleavage *in vivo*, nevertheless undergoes the same mobility shift in reticulocyte lysates, so the significance of the reaction is unclear (J. A. Hiscox and L. A. Ball, unpublished results).

C. Nonstructural Proteins

Three nonstructural proteins—A, B1, and B2—that correspond to the ORFs in RNAs 1 and 3 have been detected in FHV-infected cells and in cells transfected with FHV RNA 1 alone (Friesen and Rueckert, 1981; Gallagher *et al.*, 1983; Guarino *et al.*, 1984; Harper, 1994). Protein A and some of protein B2 form a complex that co-purifies with the RdRp activity, although the enzyme has not yet been purified to homogeneity (Guarino and Kaesberg, 1981; Saunders and Kaesberg, 1985; Wu and Kaesberg, 1991; Wu *et al.*, 1992). Frameshifts and some missense mutations in the A ORF eliminate RNA replication, but mutations that prevent the synthesis of B2 do not, at least in mammalian cells (Ball, 1995). In contrast, eliminating the B2 ORF severely inhibits RNA replication in *Drosophila* cells transfected with mutant RNA 1, and attempts to generate infectious B2-minus mutants of FHV in *Drosophila* cells succeeded only in recovering revertants that had restored the B2 ORF (Harper, 1994). These results indicate that B2 is required for some stage of the overall process of virus replication in *Drosophila* cells, although the mammalian cell results suggest that B2 may be an accessory factor rather than an essential subunit of the RdRp or that its function can be complemented by the host cell under some circumstances. Protein B1 on the other hand is not essential for virus replication: BoV does not encode this protein (Fig. 2); it is made in small amounts and is turned over rapidly in FHV-infected cells (J. A. Hiscox and L. A. Ball, unpublished results); and viable B1-minus mutants of FHV have been isolated (Harper, 1994). What role, if any, this minor protein plays during BBV and FHV infection is obscure.

D. Structural Proteins

Virions of BBV and FHV are composed of protein α , which contains 407 amino acid residues, and the two products of its autocatalyzed proteolysis, proteins β (residues 1 to 363) and γ (residues 364 to 407). Although some cleavage of α is required for infectivity (Gallagher and Rueckert, 1988; Schneemann *et al.*, 1992), the process is incomplete, and 10 to 25% of the protomers remain uncleaved in most virus preparations. The structural and functional significance of these uncleaved protomers and their distribution within or between virus particles is unknown. The N-terminus of proteins α and β is chemically blocked and invisible in the virion crystal structures, so it is unclear whether the initiating methionine residue is retained in the mature proteins (Hosur *et al.*, 1987; Gallagher and Rueckert, 1988; Schneemann *et al.*, 1992; Fisher and Johnson, 1993; Cheng *et al.*, 1994; Wery *et al.*, 1994). The N-terminus of γ (A364 in BBV and FHV), which is generated by post-synthetic cleavage, is unblocked. The three-dimensional structures of BBV, FHV, and NOV are described in detail in Chapter 7, this volume.

VII. VIRUS REPLICATION CYCLE

A. Virus Entry and Uncoating

Receptors for nodaviruses have not yet been identified, but by analogy with other animal viruses, it is likely that molecules exist on the surface of susceptible cells that mediate virus attachment and participate in the process of entry. Entry must achieve transfer of the viral genome either directly across the plasma membrane or across the membrane of an endocytic vesicle, but this process is poorly understood for any nonenveloped virus. For the nodaviruses, Johnson and colleagues (Cheng *et al.*, 1994; Johnson, 1996) have suggested a mechanism by which the viral RNAs enter the cytoplasm through a *trans*-membrane channel composed of a pentameric bundle of γ peptides (see also Chapter 7, this volume). Since the viral genome segments are fully functional mRNAs, it is feasible to suppose that translation facilitates the passage of RNA from the interior of the virion into the cytoplasm. Such cotranslational disassembly is well documented for some plant RNA viruses as well as for the nucleocapsids of some alphaviruses (Ulmanen *et al.*, 1976; Shaw *et al.*, 1986).

In accordance with this proposal, Hiscox and Ball (1997) found that virions of FHV or NOV that are transfected into cells lacking receptors or introduced directly into cell-free protein-synthesizing systems spontaneously release their RNA to ribosomes. This reaction is all the more remarkable in view of the extreme stability of FHV and NOV particles, which can resist both ribonuclease and 1% SDS without losing their infectivity. These results by no means discount the participation of receptors in nodavirus attachment and entry, but

they do imply that the conditions or components of the cytoplasm may mediate a virion structural transition that exposes the viral genome segments to the translational machinery of the cell.

B. RNA Translation

Despite the simplicity of their genomes, nodaviruses regulate gene expression at both the transcriptional and translational levels. Synthesis of proteins A and B2 takes place early in infection and then declines abruptly, whereas synthesis of protein α starts somewhat later but then continues unabated throughout the rest of the infectious cycle (Friesen and Rueckert, 1981; Ball *et al.*, 1992). This differential gene expression occurs despite the continued presence and ongoing synthesis of equimolar proportions of RNAs 1 and 2, the messengers for proteins A and α , respectively.

Friesen and Rueckert (Friesen, 1983; Friesen and Rueckert, 1984) duplicated the repression of RNA 1 translation by RNA 2 in mRNA-dependent lysates of *Drosophila* cells, and showed that it was due simply to the ability of RNA 2 to outcompete RNA 1 for some unidentified, limiting component of the cell's translational machinery. The AUG codon that starts the protein α ORF occurs in an optimal context to initiate translation, which probably helps RNA 2 dominate the translation of RNAs 1 and 3, whose initiation codons are in less favorable contexts (Kozak, 1989). Accordingly, when RNA 1 replicates in the absence of RNA 2, synthesis of proteins A and B2 is not shut off. However, the increase of protein A synthesis under these latter conditions is less than might be expected, considering that no RNA encapsidation will occur in the absence of protein α , so all the RNA 1 molecules should remain available as templates for replication and translation. The constant rate of protein A synthesis seen during replicative amplification of RNA 1 in the absence of RNA 2 suggests that the segregation of RNA 1 molecules between replication and translation may be subject to additional regulatory mechanisms that remain unexplored.

C. RNA Replication

RNA replication occurs in the cytoplasm and starts within 2 to 3 hr after infection of cells in culture. As with other RNA-dependent RNA syntheses, the reaction is insensitive to actinomycin D, which inhibits DNA-dependent RNA synthesis, and therefore it can be conveniently assayed by following the incorporation of radiolabeled uridine in the presence of the drug. RNAs 1 and 3 are made first, followed closely by RNA 2. Once RNA 2 replication begins, the synthesis of RNA 3 is strongly suppressed but RNA 1 synthesis continues, so at later times in infection the entire capacity of RNA replication is devoted to synthesis of the two viral genome segments, which are produced in equimolar amounts.

1. RNA 1 Replication

Since RNA 1 encodes the entire viral contribution to the RNA replicase, it functions as both message and template for the RdRp, and thus constitutes an autonomous RNA replicon (Gallagher *et al.*, 1983). Mutants of FHV RNA 1 in which one or the other function of the molecule has been selectively destroyed are unable to replicate autonomously, but mixing them restores replication because the functional message directs the synthesis of protein A that replicates the functional template (Ball, 1995). These results demonstrate both that protein A is required for replication and that it can act *in trans*, even on RNA-1-derived templates.

2. RNA 3 Synthesis and Suppression

The mechanism of RNA 3 synthesis is unclear, but in the absence of evidence for nucleolytic cleavage of RNA 1, it is thought to be initiated by partial transcription from a putative internal promoter site on the RNA 1 negative strand, similar to the mechanism used for subgenomic mRNA synthesis by alphaviruses and bromoviruses (Buck, 1996). In FHV RNA 1, mutation of the nucleotide that corresponds to the 5' end of RNA 3 (G2721) severely inhibits RNA 3 synthesis, but does not interfere with the replication of RNA 1 itself, at least in mammalian cells (Ball, 1995). However, the other structural features of RNA 1 that are required for RNA 3 synthesis appear to be complex: the RNA 1 negative strand must itself be replicable, indicating that the synthesis of RNA 3 is tightly coupled to RNA 1 replication. Some internally deleted FHV RNA 1 replicons retain the ability to direct RNA 3 synthesis but others do not, despite containing the entire RNA 3 region and several hundred nucleotides upstream of the putative internal promoter. These results suggest that some global feature of the RNA 1 negative strand, such as its three-dimensional structure, may determine its ability to function as a template for RNA 3 synthesis. Whether the RNA 3 negative strand serves as a template for the synthesis of the corresponding positive strand remains unresolved (Wu and Kaesberg, 1991; Zhong, 1993; Zhong and Rueckert, 1993).

Suppression of RNA 3 synthesis requires the replication but not translation of RNA 2 and specifically affects production of the positive strand (Zhong, 1993; Zhong and Rueckert, 1993). These results point to a mechanism of suppression that involves direct RNA–RNA interactions between the negative-sense template for RNA 3 synthesis and one or other strand of RNA 2. Indeed, three short regions of the positive strand of BBV RNA 2 show partial complementarity with regions of the RNA 1 negative strand that lie immediately upstream of the start site for RNA 3 (Dasmahapatra *et al.*, 1985). However, since the original suggestion was made, the significance of this tantalizing observation has become less clear: correction of the BBV RNA 1 sequence to include A2689 interrupts one of the proposed regions of complementarity, and one of the other two is not conserved in FHV (Dasgupta and Sgro, 1989). The two regions that are conserved

FIGURE 3. Possible interactions between the positive strand of FHV RNA 2 and the negative strand of FHV RNA 1 near the site of initiation of RNA 3 synthesis. The original proposal of Dasmahapatra *et al.* (1985) for BBV has been modified to reflect only the regions of complementarity that are also present in FHV. All nucleotide numbers refer to the position in the positive strand.

in both BBV and FHV are shown in Fig. 3. Perhaps the sequences of BoV and NOV RNA 1 will help to resolve this issue when they become available.

3. RNA 2 Replication

RNA 2 is replicated by the same RdRp that replicates RNA 1 and the two genome segments are made in equimolar proportions throughout most of the infectious cycle. Considering that in addition to their roles as templates for replication, these RNAs are being translated and encapsidated, the results imply the existence of a regulatory mechanism that ensures equimolar RNA synthesis. The first evidence for such a mechanism comes from the observation that the RdRp must replicate RNA 1 or an RNA-1-derived replicon before it can accept RNA 2 as a template (Ball *et al.*, 1994). This conclusion was drawn from experiments in which wild-type protein A was expressed from a version of RNA 1 that lacked part of the 3' noncoding region and was therefore not able to direct its own replication. This enzyme could replicate full-length RNA 1 and some RNA 1 molecules with internal deletions, but it was unable to replicate RNA 2 unless an RNA-1-derived replicon was replicated concurrently. These results suggest that, in order to be replicated, RNA 2 requires not only the RdRp but also a *trans*-acting factor produced during RNA 1 replication. Proteins A, B1, B2, and RNA 3 were all eliminated as possibilities, leaving the RNA 1 negative strand as the most likely candidate for the required factor. However, this identification remains to be established, as does the overall mechanism of coupling between the replication of RNAs 1 and 2. A requirement for a viral

RNA component in assembling a functional RdRp has also been noted in the case of the plant virus brome mosaic virus (Quadt *et al.*, 1995). Interestingly, cells infected with reassortant nodaviruses whose genomes consist of RNA 1 from FHV and RNA 2 from BoV (see Section VIII.A) also produce equimolar yields of RNAs 1 and 2 (Gallagher, 1987).

Cis-acting signals in the RNA 2 positive strand that allow it to be recognized and replicated by the RdRp are located within 60 nt of the 3' end and unexpectedly in an internal region between nt 520 to 720. The importance of the internal region was confirmed by its retention in each of 36 different spontaneous deletions of RNA 2 (Ball and Li, 1993; Li and Ball, 1993). Also surprising is the observation that only two nucleotides (GU) are required at the 5' end of the positive strand, since this region complements the 3' end of the negative strand where the more active origin of replication was expected to lie. Examination of the *cis*-acting signals in the RNA 2 negative strand confirmed that only a few 3'-terminal nucleotides are required for replication (Ball, 1994b). These results imply that RdRp recognition occurs elsewhere in the molecule and, together with the possible dimeric structure of the RNA 2 negative strand (see Section VI.A), they raise the possibility that RNA 2 replicates by a mechanism that is more complex than simple end-to-end displacement of monomeric complementary strands.

D. Virus Assembly

The only intermediates so far identified in the assembly pathway are provirions, which consist of 180 molecules of protein α surrounding one molecule each of RNAs 1 and 2. Protein α is incorporated into provirions within minutes of synthesis, but incorporation of newly made RNA takes several hours (Gallagher, 1987; Gallagher and Rueckert, 1988). Provirions differ from mature virions in that they contain only uncleaved protein α rather than mostly its cleavage products β and γ (see Sections V and VI.D); they also are not infectious (Schneemann, 1992; Schneemann *et al.*, 1992) and they are less stable to denaturants (Schneemann *et al.*, 1994) (see Section V). Although the maturation cleavage of α is autocatalytic, it occurs very slowly, if at all, in intact cells, perhaps because of the low intracellular concentration of calcium ions that occupy key positions in the mature capsid (see Chapter 7, this volume). However, once the provirions are released from infected cells, cleavage of α occurs over a period of several hours and confers stability and infectivity on the mature virion (Gallagher and Rueckert, 1988).

In contrast with many other icosahedral viruses, no empty capsids are found in cells infected with BBV or FHV. Even when FHV protein α is expressed alone from a recombinant baculovirus, the viruslike particles it forms contain RNA (Fisher *et al.*, 1993; Schneemann *et al.*, 1993). The structure of mature virions provides a satisfying explanation for this observation, because it shows that regions of duplex RNA are located at the 30 twofold symmetry axes and play an indispensable role in dimensioning the $T = 3$ particle (Fisher and John-

son, 1993). Interestingly, the only candidates for empty particles that have been detected during nodavirus infections are the electron-lucent particles that accumulate in BoV-infected cells (Bashiruddin and Cross, 1987). Unlike the 30-nm mature BoV particles, these are 16 nm in diameter, and thus the right size to have the $T = 1$ structure that might be predicted to form in the absence of encapsidated RNA.

Zhong *et al.* (1992) identified a 32-nt region in FHV RNA 2, within the protein α ORF, which is both necessary and sufficient for encapsidation of the RNA into virus particles. As predicted from its sequence, this region forms a bulged stem-loop of a size that could be accommodated in one of the duplex RNA-binding sites visible in the virus particle structure (Zhong, 1993). It is unclear what features of this encapsidation signal confer its unique ability to nucleate the process of assembly, or what other RNA structural requirements must be satisfied to propagate the formation of a correctly dimensioned capsid. Moreover, it is not known whether RNA 1 contains an independent encapsidation signal or "piggybacks" on RNA 2 for assembly. However, a promising region of complementarity between the positive strands of BBV RNAs 1 and 2, which was suggested to mediate direct RNA-1–RNA-2 interaction (Dasmahapatra *et al.*, 1985), is not conserved in FHV.

Under conditions of mild denaturation, purified provirions disassemble to yield monomeric protein α and RNAs 1 and 2 (Schneemann, 1992; Schneemann *et al.*, 1994). Remarkably, removal of the denaturant allows the particles to reassemble spontaneously, undergo cleavage of protein α , and acquire substantial infectivity. However, only abnormal, noninfectious particles form if the protein and RNA components are physically separated before reconstitution, raising the possibility that molecules other than RNAs 1 and 2 and protein α may be needed for authentic assembly. The "pocket factor" detected in the nodavirus crystal structure is one candidate for such a molecule (see Chapter 7, this volume), although other explanations for the aberrant reconstitution have not been eliminated (Schneemann *et al.*, 1994).

VIII. VIRAL GENETICS

A. Reassortment of Genome Segments

Together with the bipartite tetraviruses (see Chapter 9, this volume), the nodaviruses are the only known animal viruses with segmented, positive-strand RNA genomes. As with other viruses having segmented genomes, viable reassortants containing genome segments from different parents can be readily isolated and can provide useful genetic tools. All six possible reassortants between the RNAs of BBV, FHV, and BoV are infectious and their properties show that RNA 1 determines the time course of infection and the viral CPE, whereas RNA 2 determines virus yields and is the major determinant of plaque sizes (Gallagher, 1987). In contrast, none of these viruses, whose host range is

limited to insects, can exchange RNAs with NOV because its RdRp has a distinct template specificity that does not overlap those of BBV, FHV, and BoV. The structural features of the viral RNAs and RdRps that determine this template specificity are not known.

B. RNA Recombination

In addition to exchanging intact RNA segments, nodaviruses readily undergo RNA recombination that results in the covalent juxtaposition of sequences that were not adjacent in the parental molecule(s). Most frequently, this process produces deletion mutants of RNAs 1 and 2, which can interfere with the replication of the full-length genome segments and severely reduce virus yields. Such defective interfering RNAs can contribute to the establishment and maintenance of persistent infections, at least in cultured cells (see Section IV.B) (Dasgupta *et al.*, 1994).

Recombination in this and other RNA replication systems is thought to occur when the RdRp switches templates during RNA synthesis (reviewed by Nagy and Simon, 1997). In FHV RNA 2, sequences at the crossover points show that template-switching occurs predominantly during synthesis of negative strands and is influenced by features of both the primary and secondary structures of the RNA template. This aspect of nodavirus biology is reviewed in more detail in Ball (1997).

C. Reverse Genetics

Reverse genetics is the experimental approach of creating a mutant gene and then examining its phenotypic effects and the properties of its encoded protein. The construction of full-length cDNA clones of FHV RNAs 1 and 2 and their transcription to produce infectious viral RNAs (Dasmahapatra *et al.*, 1986) permitted the application of this powerful approach to FHV. Examples include analysis of the autocatalytic cleavage mechanism of protein α (Schneemann *et al.*, 1992, 1994; Zlotnick *et al.*, 1994) (see also Chapter 7, this volume), and examination of the *cis*-acting signals and *trans*-acting factors that are involved in the replication of FHV RNAs 1 and 2 (Ball, 1992, 1994b, 1995; Ball and Li, 1993; Ball *et al.*, 1994). The latter approach is described further in Section IX.A.

IX. EXPERIMENTAL RNA REPLICATION SYSTEMS

Several aspects of nodavirus RNA replication make it an attractive system for study. These include its unusually vigorous activity and the abundance of its replication products, the apparent structural simplicity of the RdRp, and its compatibility with a wide range of intracellular environments. These features

have been exploited in studies of RNA replication in mammalian cells, yeast, and cell-free systems.

A. Replication of DNA Transcripts in Mammalian Cells

Of several cultured vertebrate cell lines tested, BHK21 cells support the most abundant RNA replication when transfected with FHV and NOV RNAs (Ball *et al.*, 1992). These cells are also susceptible to infection with vaccinia virus (VV) and its recombinants, which provide convenient and versatile vectors for the transcription of foreign cDNAs in the cytoplasm of infected cells. It is thus possible to reconstruct FHV RNA replication from cDNA clones of RNAs 1 and 2 expressed in recombinant VV-infected BHK21 cells (Ball, 1992). Two such systems have been explored: VV recombinants that express FHV RNAs 1 and 2 themselves (Ball *et al.*, 1994), and the VV recombinant that expresses bacteriophage T7 DNA-dependent RNA polymerase (Fuerst *et al.*, 1986) to drive the transcription of plasmids introduced by transfection (Ball, 1992, 1994b, 1995; Ball and Li, 1993; Ball *et al.*, 1994). The former system allows replicable RNAs to be introduced into every cell in a culture as well as into experimental animals, whereas the latter system allows multiple different transcripts to be tested for their ability to replicate, both alone and in novel combinations.

Application of these systems to FHV showed that terminal extensions on the viral RNAs severely inhibit their activities as templates for the RdRp, so for successful replication it is necessary to engineer the cDNA-derived transcripts with much greater precision than is required for mRNA translation. Correct 5' ends can usually be created by promoter positioning, and correct 3' ends were achieved by incorporating the cDNA of a *cis*-acting ribozyme (Dzianott and Bujarski, 1988, 1989) immediately downstream of the nodaviral cDNA (Ball, 1992, 1995). Although the hammerhead ribozyme from satellite tobacco ringspot virus RNA yields FHV transcripts that replicate despite having 12 additional 3' nucleotides, optimum replication can be achieved by using the antigenomic ribozyme of hepatitis delta virus, which cleaves the primary transcript to generate a perfect 3' end (Pattnaik *et al.*, 1992; Ball, 1995). Interestingly, 5' terminal extensions on primary transcripts (of either polarity) are more detrimental to RNA replication than are 3' extensions, even though they are corrected during the replication process. This may be due to inhibition of replication *in trans* by RNAs with 5' extensions, which may form nonproductive complexes with the RdRp.

FHV replication launched from cDNAs in BHK21 cells reconstructs many of the features of authentic virus replication. These include the balance between RNAs 1 and 2, the suppression of RNA 3 synthesis by RNA 2, and even the production of virus particles that are infectious when assayed on *Drosophila* cells. These results indicate that despite the heterologous nature of the reconstructed system, the mechanisms of RNA replication and encapsidation are sufficiently authentic that their study will yield meaningful information.

For example, it is reassuring that an internal region that is needed for efficient replication of FHV RNA 2 transcripts when they are expressed from plasmids in VV-infected BHK21 cells (Ball and Li, 1993; Li and Ball, 1993) (see Section VII.C) is also required in *Drosophila* cells transfected with transcripts made *in vitro* (Zhong, 1993).

VV is cytotoxic, so the two expression systems described above provide only a finite experimental window during which RNA replication can be examined. However, substituting a promoter that is recognized by cellular RNA polymerase II creates plasmids able to launch RNA replication independently when they are transfected into normal cells (Johnson and Ball, 1997). Both constitutive and inducible polymerase II promoters have been tested, and stable cell cultures have been prepared in which functional RdRp and autonomous RNA replication can be induced over an extended period of time (K. L. Johnson and L. A. Ball, unpublished results). These experiments are described further in Section X.A.

B. Replication in Yeast

The complete replication cycle of FHV can be recreated not only in mammalian cells but also in the yeast *Saccharomyces cerevisiae* (Price *et al.*, 1996). Yeast spheroplasts transfected with FHV genomic RNAs support robust RNA replication and the exponential production of infectious virions in about the same abundance as infected *Drosophila* cells. Moreover, as in VV-T7-infected mammalian cells, autonomously replicating RNA 1 can be expressed from a DNA plasmid that contains full-length FHV 1 cDNA followed by the hepatitis delta virus ribozyme sequence. The RdRp expressed in this way can drive the replication of an RNA that contains both the *cis*-acting signals necessary for RNA 2 replication and the ORF for a yeast selectable marker, the *URA3* gene. The *URA3* protein expressed from the replicated RNA can complement a genomic *ura3*-minus mutation and confer stable uracil prototrophy on the recombinant strain. Since growth in the absence of uracil is dependent on RNA replication catalyzed by the FHV RdRp, this approach renders the host cell functions that are necessary for RNA replication accessible to yeast genetics. Further examination of this novel experimental system and an analogous yeast strain that supports brome mosaic virus RNA replication (Janda and Ahlquist, 1993; Quadt *et al.*, 1995; Ishikawa *et al.*, 1997) should provide unique insights into the mechanisms of RNA replication.

C. Replication in Cell-Free Systems

Abundant RdRp activity can be detected in cell-free extracts of DL1 cells that have been infected with BBV (Guarino and Kaesberg, 1981; Saunders and Kaesberg, 1985) or FHV (Wu and Kaesberg, 1991; Wu *et al.*, 1992; Ahlquist *et al.*, 1994). As in other positive-strand RNA virus systems (Buck, 1996), the enzyme

is membrane-bound but it can be released by sonication as a 120S complex that contains the viral RNAs (Guarino and Kaesberg, 1981). The mechanism of membrane binding is not clear, since neither protein A nor B2 contains obvious *trans*-membrane anchor domains. Nevertheless, the RdRp can be solubilized in an enzymatically active form by treatment with detergents such as dodecyl- β -D-maltoside, and rendered template dependent by micrococcal nuclease digestion of the endogenous viral RNAs (Saunders and Kaesberg, 1985). When supplied with exogenous positive-sense RNA 1 or 2, this enzyme catalyzes the synthesis of the corresponding full-length complementary strand(s), although the product RNAs remain as double-stranded complexes with their templates.

In its membrane-bound form, the crude FHV RdRp can use its endogenous templates to synthesize positive- and negative-sense RNAs in a reaction that produces both double- and single-stranded products. When this preparation is made template dependent by nuclease treatment, it acquires the ability to copy exogenous FHV RNAs 1 and 2, but can no longer produce the true products of replication: single-stranded positive sense RNAs. However, if any of several different glycerophospholipids are included in the reaction mixture, both double- and single-stranded products are made in response to exogenous templates; the former contain newly synthesized positive- and negative-sense RNAs, while the latter are exclusively positive sense (Wu and Kaesberg, 1991; Wu *et al.*, 1992; Ahlquist *et al.*, 1994). The reaction continues unabated for at least 8 hr and accurately reproduces many of the features of authentic RNA replication. The only other eukaryotic, positive-strand RNA viruses for which bona fide RNA replication has been achieved in a cell-free system are poliovirus (Molla *et al.*, 1991) and cucumber mosaic virus (Hayes and Buck, 1990).

It is not clear how glycerophospholipids promote the second step of RNA replication, namely the synthesis of single-stranded RNA of the same (positive) sense as the input template, but the stoichiometry of the dependence suggests some direct or indirect interaction with the RNA replicase itself rather than with its template. Wu *et al.* (1992) suggested that continuous synthesis or modification of cellular membranes may be required to support nodavirus RNA replication, and although this question has yet to be examined for the nodaviruses, inhibition of phospholipid biosynthesis and membrane rearrangement interferes with viral RNA replication in eukaryotic cells infected with two other positive-strand RNA viruses, poliovirus and Semliki forest virus (Guinea and Carrasco, 1990; Perez *et al.*, 1991; Maynell *et al.*, 1992). These results suggest that membrane dynamics may play an essential role in positive-strand RNA replication in several different eukaryotic virus systems.

X. TECHNOLOGICAL APPLICATIONS OF THE *NODAVIRIDAE*

A. RNA Replication

The same properties that make the nodaviruses attractive systems with which to study RNA replication also make them powerful tools for the ampli-

fication of heterologous RNAs. The VV-T7 and VV-FHV recombinant systems described in Section IX.A provided crucial information on the *cis*-acting requirements for FHV RNA replication (Ball and Li, 1993; Ball, 1994b, 1995; Ball *et al.*, 1994). In particular, the 5' and 3' termini must closely resemble those of authentic FHV RNAs in order to serve as templates for the viral replicase. As discussed earlier, this was achieved by precisely positioning the promoter at the 5' end of the viral cDNA and by RNA cleavage at the 3' end of the primary transcript by the antigenomic hepatitis delta virus ribozyme (Ball, 1992, 1995; Ball and Li, 1993). Although the VV-based RNA replication systems are ideal for some experimental purposes, they are limited by the cytotoxic nature of VV infection, and thus are not appropriate for long-term studies. We therefore turned to cellular polymerase II (pol II) to direct the synthesis of primary transcripts capable of autonomous replication (Johnson and Ball, 1997). Plasmids were constructed that contained the constitutive SV40 early or Rous sarcoma virus long terminal repeat (LTR) promoters that had been resected to position the first nucleotide of the FHV cDNA at the putative transcriptional start sites. Transfection of pol II-FHV1 plasmids into mammalian cells at 28°C resulted in replication of RNA 1 and synthesis of the subgenomic RNA 3. Cotransfection of similar plasmids containing the RNA 2 cDNA resulted in replication of RNA 1 and RNA 2 and the suppression of RNA 3 synthesis, an expected consequence of RNA 2 replication (Zhong and Rueckert, 1993). Relative to the VV-based systems, the constitutive pol II system exhibited a 2- to 3-day lag prior to detection of RNA replication, a possible consequence of nuclear synthesis of primary transcripts, which must be transported without splicing into the cytoplasm before RNA replication can occur. These data and those of others suggest that this is an inefficient process (Herweijer *et al.*, 1995; Dubensky *et al.*, 1996; Johnson and Ball, 1997). The VV-based systems synthesize primary transcripts in the cytoplasm and exhibit no such lag. More recent studies using FHV1 plasmids that contain an inducible pol II promoter suggest that the lag also might be related to the level of primary transcription from the DNA template (K. L. Johnson and L. A. Ball, unpublished observations).

B. Expression of Heterologous RNAs

Replication and expression of heterologous RNA sequences in the nodavirus system must rely on the *cis*-acting signals from one of the viral genomic RNAs. In theory, these signals could be derived from either RNA 1 or RNA 2. There are, however, several technical considerations. In the case of RNA 1, the options are rather more limited than for RNA 2, due to the requirement of an intact RNA replicase ORF. For this reason, cloning of a heterologous RNA sequence directly into the subgenomic RNA 3, which overlaps the protein A coding region, would be impossible. In the alphavirus-based vector systems, heterologous RNAs have been cloned directly into the subgenomic RNA region of the alphavirus cDNA clone, under a duplicate subgenomic promoter (reviewed by Liljestrom, 1994). By the same token, it should be possible to intro-

duce a duplicate copy of the RNA 3 promoter and a heterologous RNA sequence downstream of a full-length copy of RNA 1. However, the subgenomic promoter for RNA 3 has not yet been identified and may be complex, as discussed in Section VII.C.

A more attractive target would be RNA 2, which contains the protein α coding region, is dispensable for RNA 1 replication (Gallagher *et al.*, 1983), and is naturally replicated *in trans*. Zhong (1993) demonstrated that, in the presence of FHV RNA 1, the *cis*-acting signals of FHV RNA 2 supported the replication of RNAs containing the coding region for chloramphenicol acetyl transferase (CAT) in cultured *Drosophila* cells; production of CAT protein was detected by an enzymatic assay. In addition, the CAT-containing transcripts were packaged into infectious virions when protein α was provided *in trans*. Similarly, an RNA 2 derivative that contains the yeast *URA3* ORF can be replicated by the FHV RdRp in yeast cells, as described in Section VIII.B (Price *et al.*, 1996).

C. Epitope Presentation

FHV RNA 2 has also been used as the basis for an epitope-presenting system, which allows the presentation of heterologous epitopes in multiple sites in protein α (Tisminetzky *et al.*, 1994; Scodeller *et al.*, 1995; Buratti *et al.*, 1996, 1997; Schiappacassi *et al.*, 1997). This system relies on the finding by Schneemann *et al.* (1993) that cultured *Spodoptera* cells infected with recombinant baculoviruses containing the FHV protein α coding region produced high yields of viruslike particles with the same morphology as authentic FHV. These particles, which packaged the capsid protein mRNA, matured in a fashion similar to that of the authentic virus (Schneemann *et al.*, 1993). The three-dimensional crystal structure of FHV (Fisher and Johnson, 1993) reveals the presence of five different positions on the surface of protein α into which foreign viral epitopes might be inserted (Buratti *et al.*, 1996). Chimeric FHV α -foreign epitope cDNAs were constructed and expressed in recombinant baculoviruses; the foreign epitopes were presented in conformations that varied depending on where in the FHV capsid they were inserted. The conformations could be determined by computer prediction, allowing some selectivity in where in the FHV capsid the epitopes could be inserted to give optimal immunoreactivity.

This system has been used to present in a conformationally specific manner the V3 loop epitope of human immunodeficiency virus-1 (HIV-1) gp120 (Tisminetzky *et al.*, 1994; Scodeller *et al.*, 1995; Schiappacassi *et al.*, 1997), two neutralizing epitopes of HIV-1 gp41 (Buratti *et al.*, 1996), and three epitopes from the hepatitis C virus core protein (Buratti *et al.*, 1997). The resulting viruslike particles were used in one case as immunogens to elicit a neutralizing response in guinea pigs (Scodeller *et al.*, 1995), but were used primarily to screen sera from patients infected with the virus from which the epitopes were derived.

XI. CONCLUSIONS AND SUMMARY

The major importance of the insect nodaviruses lies in their value as model systems for studying basic processes that are of general interest in molecular virology. The simplicity of nodavirus genomes and their ease of manipulation, coupled with the stability of the virus particles and their robust replication in cell culture offer unique experimental opportunities to examine the mechanisms of translation, replication, and encapsidation of the viral RNAs, the detailed structures of virions, and their pathways of assembly and disassembly. In many cases, the nodaviruses allow these processes to be studied at higher levels of resolution than can routinely be achieved with other animal viruses. Despite the lack of major economic or medical impact of the insect nodaviruses, these considerations fully justify future research, which can be expected to expand our understanding of the basic biochemistry of RNA virus replication.

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