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Fish Nodavirus Lytic Cycle and Semipermissive Expression in Mammalian and Fish Cell Cultures

CLAUDE DELSERT,[1]* NATHALIE MORIN,[2] AND MICHEL COMPS[3]

Station Méditerranéenne de l'Environnement Littoral, IFREMER, 34200 Sète,[1] Centre de Recherche en Biologie Macromoléculaire du CNRS, 34033 Montpellier cedex,[2] and Station IFREMER, 34240 Palavas-les-Flots,[3] France

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In this study, *Dicentrarchus labrax* encephalitis virus (DlEV), which causes sea bass encephalitis, was propagated in cell culture, thus allowing study of its lytic cycle. DlEV infection of mammalian and fish cells induced different patterns of expression of capsid proteins, which were assembled as virus-like particles, accumulating in the cytoplasm either as diffuse masses or in vesicles, as shown by electron microscopy. These particles correspond to virions, as shown by their ability to induce secondary infection. Fish cells proved to be more permissive for DlEV than mammalian cells, although virus yield remained low. RNA analysis of infected sea bass cells revealed DlEV RNA3, in addition to genomic RNA1 and RNA2, and the presence of the RNA2 minus strand, thus demonstrating the replication of the DlEV genome. In addition, DlEV RNA-dependent RNA polymerase was associated with mature virions even after purification by a CsCl gradient, but it was dissociated when capsids were destabilized. In addition to providing more information about the relatedness of DlEV to the members of the family *Nodaviridae*, this study shows that fish nodaviruses may not be able to infect as wide a variety of cells as insect nodaviruses can.

The causative agent of a viral disease causing high mortality in a variety of fish species has been identified as a nodavirus based on the organization of its genomic RNA and its physical properties (4, 13). The nodaviruses are a family of small nonenveloped icosahedral viruses; those that infect insects are the best characterized (for reviews, see references 10 and 11). The genome of insect nodaviruses consists of two single-stranded messenger-sense RNAs contained in a single virion (12, 16). Virion RNA1 (approximately 3.1 kb long) codes for protein A (an RNA-dependent RNA polymerase) and protein B (whose function is unknown). Protein B is expressed by means of a subgenomic messenger, RNA3 (approximately 0.38 kb long), which is not encapsidated (8). RNA2 (approximately 1.4 kb long) encodes the virion coat protein precursor, alpha, which is proteolytically processed into the coat proteins beta and gamma (9).

Fish nodaviruses infect a number of fish species, in which they cause an encephalitis characterized by abnormal swimming behavior and nervous necrosis (1, 4, 13–15). Viral particles from infected striped jack (*Pseudocaranx dentex*) (striped jack nervous necrosis virus) (13), sea bass (*Dicentrarchus labrax*) (*Dicentrarchus labrax* encephalitis virus [DlEV]), and barramundi (*Lates calcarifer*) (*Lates calcarifer* encephalitis virus) (4). Their physical properties are similar to those of insect nodaviruses, with a genome organized in two single-stranded RNA segments of about 1.4 and 3.0 kb in length and a nonenveloped icosahedral capsid made of two proteins with similar molecular weights. However, little is known about the biology of these viruses. Availability of a cell culture permissive for DlEV would aid understanding of the fish nodavirus cycle and would greatly assist obtaining virus stocks.

In this report, we describe the screening of various mammalian and fish cell lines for their permissivity for DlEV and their use in the characterization of the DlEV lytic cycle.

* Corresponding author. Mailing address: IFREMER, Station Méditerranéenne de l'Environnement Littoral, 1 quai de la Daurade, 34200 Sète, France. Phone: 4 67 46 33 90. Fax: 4 67 46 02 56. E-mail: cdelsert@univ-montp2.fr.

Expression and titration of DlEV viral stock. Mammalian and fish cell cultures were infected with DlEV for a propagation assay, since permissive cells have not yet been described. Simian Cos1 and human HeLa cells were grown at 37°C, while three fish cell cultures (SBL [sea bass larva], RTG2 [rainbow trout gonad], and BF2 [bluegill fry] cells) were grown at 22°C. Infection was carried out for 1 h on coverslips in 24-well plates with a virus stock purified from diseased sea bass larvae (4). Mammalian cells were further incubated at 28°C for 48 h after infection, while fish cells were maintained at 22°C. The immunofluorescence assay was performed as previously described (6) with a rabbit polyclonal immunoserum raised against purified virions. All positive cells showed cytoplasmic fluorescence, but the intensity of the staining as well as the percentage of infected cells was related to the cell lines used (Fig. 1); uninfected cells remained unstained (Fig. 1f). SBL cells proved to be the most permissive (over 90% DlEV-positive cells [Fig. 1a]), while about 50% of BF2 and RTG2 cells were positive (Fig. 1b and d). Cos1 and HeLa cells exhibited fewer positive cells, which had diffuse cytoplasmic staining and cytoplasmic aggregates (Fig. 1c and e).

Interestingly, infected cells displayed no cytopathic effect, although numerous cytoplasmic vesicles were observed in SBL cells at higher magnification (Fig. 1a, inset). However, we observed that infected cells became round 6 days postinfection (p.i.) (data not shown).

Virus stock titration was carried out on SBL, BF2, RTG2, and Cos1 cells. The results of three independent experiments are summarized in Table 1. These results show that SBL cells were the most permissive, with a titer of 3×10^8 focus-forming units (FFU) per ml of virus stock, while Cos1 cells were the least permissive cells, with a 15-fold-lower titer (Table 1).

Expression of viral proteins in infected mammalian and fish cells. Capsid protein expression was examined with SBL cells infected as described above (1 FFU/cell) and harvested at 96 h. Western blot analysis of the postnuclear supernatant of infected SBL cells revealed a 43- to 45-kDa protein (Fig. 2A, lane 1), which was identical on a shorter exposure to the doublet observed with mature virions purified from diseased

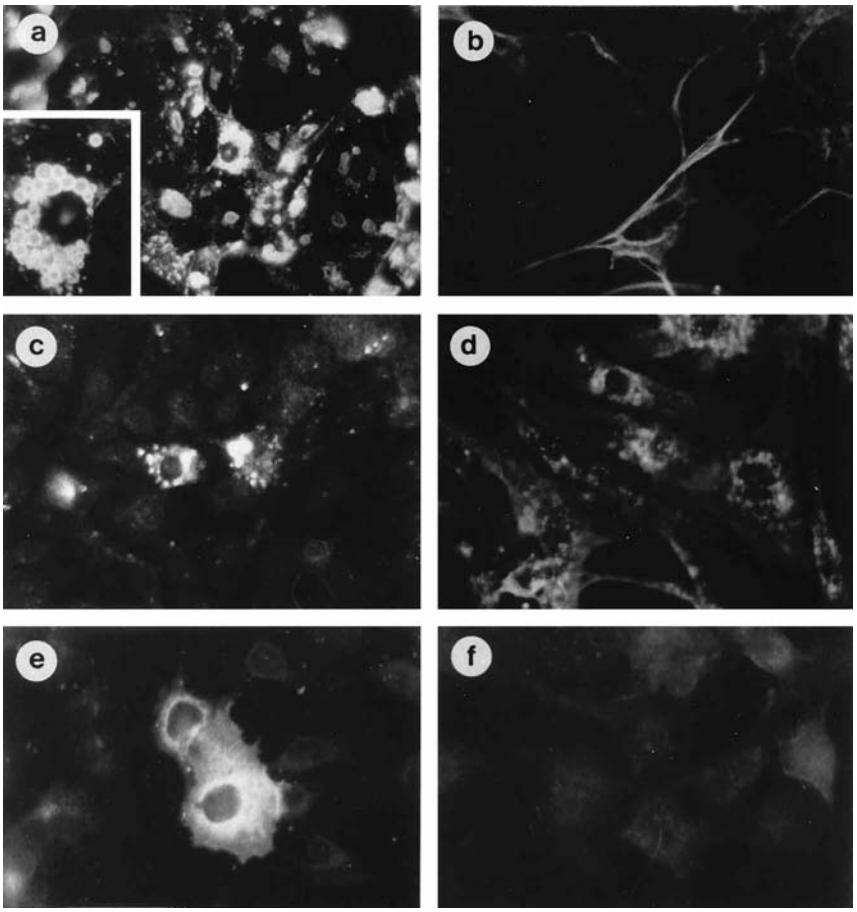

FIG. 1. DlEV immunodetection in mammalian and fish cell cultures. Cells were grown to near confluence on coverslips in 24-well plates at 22 and 37°C for fish and mammalian cells, respectively. Cells were infected for 48 h. Note that mammalian cells were shifted to 28°C, a temperature that proved to be permissive for DlEV. Cells were processed for immunofluorescence with nodavirus antibodies as described in the text. Cytoplasmic fluorescence was observed in all cell cultures except mock-infected cells (f). (a) SBL cells (the inset shows detail of cytoplasmic vesicles at higher magnification); (b) bluegill fry (BF2) cells; (c) Cos1 cells; (d) rainbow trout gonad (RTG2) cells; (e) HeLa cells.

sea bass larvae (lane 3) and to the products of in vitro translation in rabbit reticulocyte lysate of viral RNAs (lane 5) or of gel-purified RNA2 (lane 4), while the absence of a band with the mock-infected extract (lane 2) confirmed the high specificity of the anti-DlEV immunoserum. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell-free translation extracts carried out in the presence of [^{35}S]methionine revealed an identical protein doublet when the extracts were programmed either with viral RNA1 and RNA2 (Fig. 2B, lane 2) or with gel-purified RNA2 (lane 1), while no band was observed in an unprogrammed extract (lane 3).

TABLE 1. Immunotitration of a virus stock in different cell lines

Cell line[a]	DlEV titer (FFU/ml)[b]	% of titer in SBL cells
SBL	3×10^8	100
BF2	1.5×10^8	50
RTG2	1.5×10^8	50
Cos1	2×10^7	6

[a] SBL, BF2, and RTG2 are fish cell lines; Cos1 is a mammalian cell line.
[b] Mean of results from three independent experiments.

Products of in vitro translation programmed with viral RNAs in the presence of [^{35}S]methionine revealed by SDS-PAGE an additional protein of approximately 110 kDa (Fig. 2B, lane 2). The additional band was not detected when translation was programmed with gel-purified RNA2 (lane 1) or was unprogrammed (lane 3). Interestingly, Western blot analysis revealed the same additional band of about 110 kDa in an infected SBL cell extract (Fig. 2A, lane 1), in mature virions (lane 3), and in an in vitro translation extract programmed with viral RNA1 and RNA2 (lane 5) but not in an extract programmed with purified RNA2 (lane 4) or in a mock-infected SBL cell extract (lane 2). Together, these data indicate that an RNA1-encoded protein is recognized by the rabbit anti-DlEV immune serum raised against purified mature virions.

The accumulation of capsid proteins was monitored during the course of infection by Western blot analysis of identical amounts, as determined by the Bradford assay (2), of post-nuclear cell extracts of infected SBL and Cos1 cells at various times p.i. The results indicate that the capsid protein steady-state level visibly increased during the course of infection in SBL cells (data not shown); by contrast, only a very slight increase was observed in Cos1 cells (data not shown). Densitometric analysis of the signal for capsid protein indicated that the amount of capsid proteins at 96 h was approximately 5 and

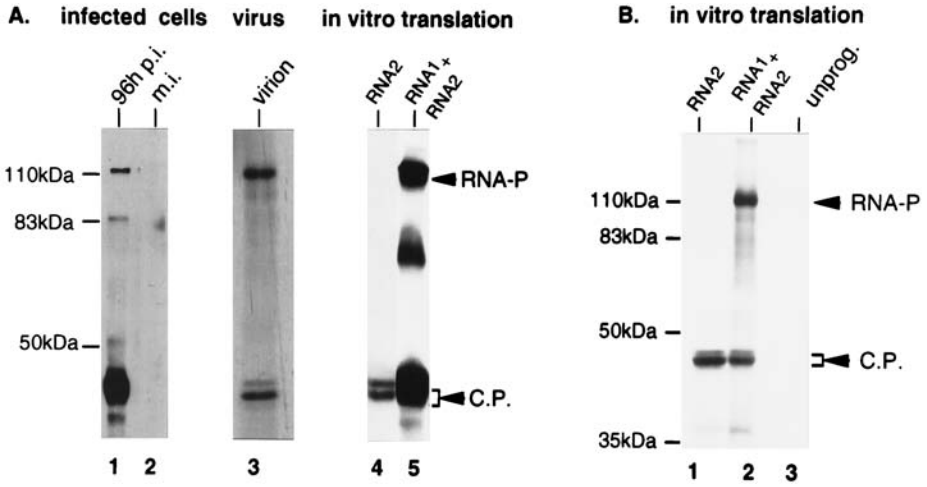

FIG. 2. Analysis of DlEV capsid proteins in infected cells, mature virions, and cell free translation extracts. (A) Postnuclear supernatants of SBL cells either mock infected (m.i.) (lane 2) or infected for 96 h (lane 1) were loaded on SDS-polyacrylamide gels and immunoblotted with polyclonal antibodies raised against purified mature DlEV virions. Also, purified virions (lane 3) and in vitro translation products from either total viral RNAs (RNA1 and RNA2) (lane 5) or gel-purified RNA2 (lane 4) were analyzed in parallel. (B) In vitro translation, carried out in the presence of [^{35}S]methionine, was programmed with viral RNAs (lane 2) or with purified RNA2 (lane 1); lane 3 shows unprogrammed (unprog.) extract. Proteins were analyzed by SDS-PAGE and autoradiographed after En^{3}Hance treatment. Numbers on the left indicate molecular mass markers. C.P., capsid proteins; RNA-P, RNA-dependent RNA polymerase.

1.5 times the amount present 30 min p.i. in SBL and Cos1 cells, respectively.

SBL cells were infected and incubated for 1 h at various times p.i. with [^{35}S]methionine before being harvested. Identical amounts of cell extract, as determined by the Bradford assay, were subjected to SDS-PAGE and autoradiography. The results show that the pattern and the intensity of labelled bands are essentially the same at 30 min and 24, 48, and 96 h p.i. (data not shown). These results indicate that cellular protein synthesis was not affected by DlEV infection and show that capsid proteins accumulate during the course of infection even as late as 96 h p.i.

Viral RNA replication in Cos1 and SBL cells. SBL cells were infected as described above, and total RNA was extracted as previously described (3) at 24 and 96 h. Northern blotting (7) was performed on 20 μg of total RNA, and the RNA was hybridized to three radiolabelled double-stranded DNA fragments: probe A, a 1,200-bp fragment that specifically hybridizes to RNA2 (5), and probes B and C, 650- and 450-bp fragments, respectively, that hybridize to RNA1 (5). The results show the detection of two RNAs of approximately 3,000 and 1,400 nucleotides (nt). These RNAs correspond to the expected sizes for DlEV RNA1 and RNA2 (5), respectively, in infected SBL cells at 24 and 96 h (Fig. 3, lanes 1 and 2); no signal was detected in mock-infected cells (lane 3). Interestingly, probes B and C revealed an additional smaller band, approximately 400 nt long, in RNA extracted at 96 h (lane 2). This band is likely to correspond to RNA3, a subgenomic RNA produced during RNA1 replication in insect nodaviruses (8).

In order to detect the DlEV genomic RNA2 plus strand and its replicative form, the RNA2 minus strand, an RNase protection assay was performed as previously described (7) with specific probes uniformly labelled with [α-^{32}P]UTP. Analysis of 20 μg of total RNA of infected SBL cells with a large excess of riboprobe provided a signal for the RNA2 plus strand, but no signal was detected with mock-infected SBL cells (data not shown). Quantitation by densitometric analysis indicated that the signal provided by the RNA extracted 96 h p.i. is about 2.5-fold more intense than the signal provided by the RNA extracted 24 h p.i. This result indicates a moderate accumulation of the RNA2 plus strand 96 h p.i., thus implying that some DlEV RNA replication had taken place in SBL cells. An RNase protection assay using the RNA2 minus-strand-specific probe on total RNA of infected SBL cells at 96 h yielded a weak signal (data not shown), indicating the presence of the RNA2 minus strand 96 h but not 24 h p.i. A similar experiment done with RNA extracted from infected Cos1 cells at 24 and 96 h did not produce any detectable signal for the RNA2 minus strand (data not shown).

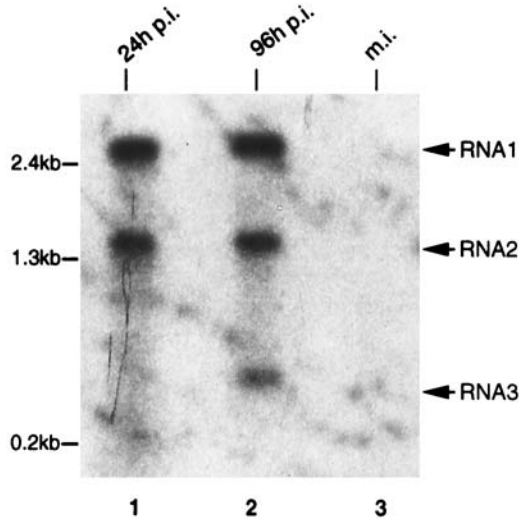

FIG. 3. Expression of RNA3 in infected SBL cells. Northern blot analysis was carried out on 20 μg of total RNA as previously described (7). Probes were produced by random-primed labelling of purified DNA fragments after *Xho*I and *Eco*RI digestion of the following plasmid recombinants (5): probe A, a pC12-derived 1,200-bp fragment, for detection of RNA2, and probes B and C, which are pC2- and pC13-derived 650- and 450-bp fragments (5), respectively, for detection of RNA1. The probes revealed RNA1, RNA2, and RNA3, a nonencapsidated subgenomic RNA, in RNA extracted at 96 h (lane 2). RNA3 was not present at 24 h (lane 1). RNA from mock-infected (m.i.) cells is shown in lane 3. Molecular size markers are shown on the left.

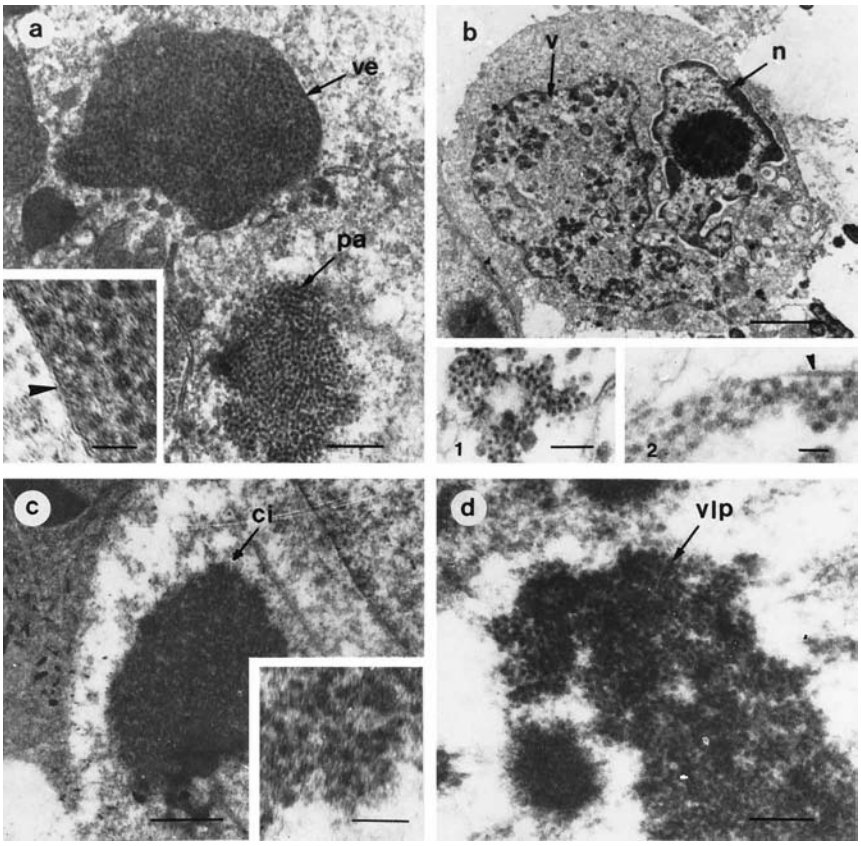

FIG. 4. Electron microscopy of infected cells and tissues. (a) DlEV-infected sea bass larva. A section through the brain showing nervous cells containing virus particles free in the paracrystalline array (pa) or enclosed in membrane-bound vesicles (ve) is shown. Bar = 200 nm. (Inset) At higher magnification, the vesicle membrane appears as a trilaminar element (arrowhead). Bar = 50 nm. (b) A DlEV-infected SBL cell. A large vesicle (v) containing numerous virus-like particles assembled in the cytoplasm of the infected cell is shown. n, nucleus. Bar = 2 μm. (Inset 1) aggregate of virus-like particles enclosed in a vesicle. Bar = 200 nm. (Inset 2) Detail of the membrane delimiting a vesicle. Bar = 50 nm. (c) A DlEV-infected HeLa cell. A finely granular, electron-dense inclusion is present in the cytoplasmic inclusion (ci). Bar = 1 μm. (Inset) Cytoplasmic inclusion showing electron-dense virus-like particles at higher magnification. Bar = 100 nm. (d) A DlEV-infected Cos1 cell. Electron-dense virus-like particles (vlp) are packed together in cytoplasmic diffuse regions. Bar = 200 nm.

Expression of virus-like particles in the cytoplasm of infected cells. Electron microscope examination of brain tissues of diseased sea bass larvae revealed electron-dense inclusions in the cytoplasm of infected nervous cells (Fig. 4a). These inclusions consisted of virus-like particles either arranged in paracrystalline arrays or enclosed in vesicles bounded by a plasma membrane (Fig. 4a, inset).

Transmission electron microscopy did not detect virus-like particles in infected SBL cells at 24 h (data not shown), although a large amount of these particles was found at 72 h (Fig. 4b), either as cytoplasmic aggregates (inset 1) or mainly as membrane-bound vesicles (inset 2).

By contrast, infected HeLa (Fig. 4c) and Cos1 (Fig. 4d) cells at 72 h did not contain vesicles but exhibited only diffuse regions of aggregated capsids. At higher magnification (Fig. 4c, inset), the capsids observed in the infected cells were no different in shape or in size from the capsids examined in fish brain sections (4).

In order to determine whether virus-like particles produced in cell cultures had the physical properties and composition of mature virions, a cytoplasmic extract of infected SBL cells harvested 96 h p.i. and mature virions purified from infected larvae were fractionated on 5 to 20% sucrose gradients (9). Western blot analysis of gradient fractions using the anti-DlEV immunoserum revealed a peak of capsid proteins at the bottom of both gradients and centered on fraction 3, showing that virus-like particles sedimented as mature virions (data not shown). Essentially no capsid protein was found at the top of the gradients (data not shown), indicating that capsid proteins synthesized in infected SBL cells were efficiently assembled as virus-like particles. To further characterize these virus-like particles, an aliquot of fraction 3 of both gradients was subjected to Northern blot analysis as described above. Hybridization to probe A revealed a 1,400-nt band which had the same intensity for both samples (data not shown) and corresponded to RNA2 (5); no signal was detected in RNA extracted from mock-infected SBL cells. Thus, the virus-like particles assembled in infected SBL cells sediment as mature virions and contain genomic RNA.

In addition, Western blot analysis revealed an upper band migrating at approximately 110 kDa in fraction 3 of both gradients (data not shown); this band was similar to that observed with mature virions (Fig. 2A, lane 3). This additional band most likely corresponds to the RNA-dependent RNA polymerase.

Western blot analysis of mature virions after pretreatment in 1% SDS and sedimentation on a 5 to 20% sucrose gradient revealed capsid proteins in most of the gradient, although a smaller peak was present in the bottom fractions of the gradient (data not shown). Interestingly, the capsid protein peak was no longer associated with a band of 110 kDa as described above; instead, it was detected in the top gradient fraction

(data not shown). The biological significance of the association of RNA-dependent RNA polymerase with mature virions remains to be determined. Nevertheless, its normal presence, indirectly demonstrated by the anti-RNA-dependent RNA polymerase reactivity of an immune serum raised against mature virions purified by a CsCl gradient, raises the possibility that it has a definite function, such as acting as a scaffolding protein, or that it reflects the simultaneous occurrence of genome replication and capsid assembly.

SBL cell culture is semipermissive for DlEV. Virus yield was determined with SBL cells, since they were found to be more permissive, with more positive cells and a stronger signal, than the other cell types used in this study. Cells were infected at a multiplicity of infection of 1 FFU/cell for 1 h and incubated with a 1:250 dilution of anti-DlEV immunoserum in phosphate-buffered saline. The cells were further incubated for 96 h, rinsed several times with cold phosphate-buffered saline, and lysed by repeated freeze-thaw shocks. Serial dilutions of infected SBL cell lysate were used for secondary infection of SBL, BF2, and Cos1 cells. Cos1 cells were further incubated at 28°C, while SBL and BF2 cells were maintained at 22°C. Immunostaining was performed 48 h p.i., and the number of fluorescent foci was determined for each cell culture. The results of three independent experiments indicate that the SBL culture yielded about 3 FFU/cell when infected with 1 FFU/cell. The mean virus yields were 1.2×10^7 FFU for SBL cells, 6×10^6 FFU for BF2 cells, and 1×10^6 FFU for Cos1 cells. The results of the titration indicate that SBL cells were twice as permissive as BF2 cells and 12-fold as permissive as Cos1 cells. Therefore, the observed rate of amplification of the DlEV stock in these cells corresponds to a semipermissive level of expression of DlEV.

The restricted permissivity of mammalian and fish cells for DlEV is the most striking characteristic of a virus belonging to the family *Nodaviridae*. Indeed, insect nodaviruses are known to infect a wide spectrum of cell cultures from various organisms, including plants (17). Moreover, while insect nodaviruses were found to have infected a variety of tissues in diseased insects (10), DlEV expression is confined to neuronal cells, as previously shown by in situ hybridization.

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