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A New Small RNA Virus Persistently Infecting an Established Cell Line of *Galleria mellonella,* Induced by a Heterologous Infection

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A persistent infection in a *Galleria mellonella* cell line was revealed when infected with a maize stem borer picorna-like virus isolated on *Sesamia cretica* (MSBV). The new virus, completely different from the MSBV, is designated as *G. mellonella* cell line virus (GmclV), induces spectacular cytopathic effects, and is also considered efficient *in vivo*. The GmclV is a 29-nm-diameter isometric virus, with single-strand RNA of 2.9 × 10^6 Da molecular weight with a poly(A) tract. Its capsid is constituted of only two major polypeptides, of 34,500 and 32,500 Da, and no minor bands could be detected. The characteristics of the GmclV do not permit us to classify it with assurance. Even though it has not yet been identified as a picornavirus, it can be classified in the small RNA virus group of the *Picornaviridae*. *G. mellonella* represents a very interesting model, owing to the fact that two different persistent viruses belonging to the same family were isolated *in vivo* and *in vitro,* to further the understanding of the general phenomenon of persistency and induction. © 1997 Academic Press

KEY WORDS: ***Galleria mellonella;*** **Lepidoptera; cell line; small RNA virus.**

INTRODUCTION

Nonevident infections by microorganisms are currently observed in insects (Quiot *et al.,* 1983; Van den Heuvel *et al.,* 1994). Persistent viruses, the rate of multiplication of which stays at a low level that is perfectly tolerated by the cells, are regularly cited in literature (Plus, 1978; Oldstone, 1989; Friesen *et al.,* 1980; Jousset *et al.,* 1993). Generally, the phenomenon of persistence *in vitro* is revealed experimentally after infection with pathogenic homologous viruses and selection by successive passages of the cells, which does not show cytopathic effect (Dasgupta *et al.,* 1994) or is revealed by electron microscopy during routine analysis. Because of the low concentration of the virus in the cells, it is generally difficult to multiply it *in vitro* at a sufficient rate to be purified and characterized and to determine the mode of action with precision. Always, the viruses found in these studies are multiplied on their host to be amplified. In 1981, Kelly *et al.* reported the activation of a persistent Baculovirus in a large amount, during infection with other Baculoviruses.

As part of our laboratory program concerning cellular permissivity, we have revealed such a phenomenon in RNA viruses. During multiple tests of its host range *in vitro,* experimental introduction of the MSBV, a Picorna-like virus isolated on *Sesamia cretica* (Fédière *et al.,* 1991), in an established cell line deriving from ovarioles of *Galleria mellonella* (Lepidoptera: Pyralidae), induces the massive multiplication of another virus which infects persistently this cell line. In this paper, we describe this new virus, designated *G. mellonella* cell line virus (GmclV), and discuss the possibilities that this new model of "latent" virus offers.

MATERIALS AND METHODS

Cell Lines

Three *G. mellonella* cell lines, deriving from avarioles, a gift from Dr. J. M. Quiot (St. Christol-lès-Alès, France), were cultivated in Grace's modified medium (Léry and Fédière, 1990), containing 15% fetal bovine serum (FBS) and incubated at 27°C. The first cell line was cultivated for more than 120 passages before being used in the experiments and was designated Gm120. The second cell line, derived from initial frozen cells of the Gm120, was multiplied for fewer than 85 passages and was designated Gm85. The third cell line, newly established, was used between the 12th and the 15th passages and designated Gm15. The first two cell lines were adherent cell lines while the third one was nonadherent.

Virus

The Picorna-like virus isolated from the maize stem borer *S. cretica* (Fédière *et al.,* 1991) was multiplied in larvae of the host and reared in the laboratory. Purified

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viruses in 0.05 M Tris buffer, pH 7.5, were diluted in Grace's modified medium without FBS and filtered on a 0.45-μm millipore filter.

The *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV), a gift from Dr. Croizier (St. Christol-lès-Alès, France), was multiplied in SF9 cells. The infectious supernatant was filtered on a 0.45-μm millipore filter.

Infection of Cells

Viral suspensions of MSBV at a concentration of 0.1 OD/ml were used to infect 24-hr cultures of *G. mellonella* cell lines, seeded at 2×10^6 cells, and grown in 25-cm^2 tissue culture flasks. After 2 hr contact, 1.5 ml of viral suspension was removed and replaced by 4 ml of fresh medium containing 10% FBS and the cells were incubated at 27°C. The inoculum was kept for testing and for an eventual evaluation of the quantity of virus which enters the cells. After 5 to 8 days postinfection, the flasks were scraped with a rubber policeman and the cells and medium were tested by ELISA test or used for purification. Three successive passages of the virus were performed to evaluate its infectivity, using 0.75 ml of the infectious supernatant diluted with the same volume of Grace's modified medium without FBS, under the same conditions used for primary infection.

Fifty microliters of AcMNPV infectious SF9 supernatant at more than 10^8 PFU/ml (0.1 m.o.i./cell) was used to infect 2×10^6 cells, grown in 25-cm^2 tissue culture flasks. The infection was performed as described above.

Morphological and Ultrastructural Cell Studies

Cells experimentally infected with MSBV and noninfected cells were observed under a normal light microscope or with a phase-contrast inverted microscope at magnification of 100 to 500×. The cells were observed for possible cytopathic effect or changes.

For electron microscopic studies, the cells were pelleted for 5 min at 400*g* after 5 days and resuspended in Grace's modified medium without FBS, containing 2.5% glutaraldehyde (diluted in cacodylate buffer). After a 1-hr fixation at 4°C, the cells were pelleted for 5 min at 400*g* and dispersed in 2% low melting agarose at 38°C. The cells were postfixed in 1% osmium tetroxide, dehydrated, and then embedded in pure epon. Ultrathin sections were stained and contrasted according to the method of Reynolds (1963).

ELISA Test

For detecting the viral proteins, a specific rabbit serum was prepared by injecting purified virus as previously described (Fédière *et al.*, 1991). The indirect method of ELISA (enzyme-linked immunosorbent assay) using phosphatase alkaline was conducted according to Kelly *et al.*, (1978).

Purification of the Small Virus

When a cytopathic effect was observed and in all cases, between 5 to 8 days postinfection, the cells (approximately 5×10^6 cells/flask) were scraped from the boxes and centrifuged for 5 min at 400*g*. The pellet was treated in STE buffer (0.15 M NaCl; 0.02 M Tris; 10^{-3} M EDTA; 0.5% Aprotinine; and 0.5% NP-40) for 5 min at 4°C under agitation. The treated pellet and supernatant were homogenized with Potter and ultrasonicated. After centrifugation for 30 min at 15,000*g*, the supernatant was ultracentrifuged for 1 hr 30 min at 100,000*g*. The pellet resuspended in 0.05 M Tris, pH 7.5, overnight, was deposited on a 15 to 45% (W/W) sucrose gradient, and centrifuged for 2 hr 30 min at 120,000*g*. The viral band was collected and the viruses were concentrated as above and stored at −20°C.

Characterization of the Virus

Electron microscopy. Purified virus preparation was negatively stained in 2% (W/W) uranyl acetate, pH 7.4, and examined in an electron microscope. The viral particle size was calculated by measuring diameter of more than 10 closed particles.

Spectrophotometric analysis. UV absorption of purified virus was examined using a wavelength of between 320 and 220 nm. The average ratio of optical densities at 260 and 280 nm (maximum absorption of nucleic acid and proteins, respectively) was measured.

Nucleic acid analysis. Nucleic acid was extracted from purified virus by a simplified procedure as follows. One milliliter of 3.5 OD virions was treated twice by saturated phenol in Tris (10^{-2} M)–EDTA (10^{-3}M), pH 7.8, for 10 min and was centrifuged at 12,000*g* for 5 min, then treated twice with chloroform:isoamyl (24:1). Finally, RNA was precipitated by cold ethanol (2 volumes) and 10% ammonium acetate overnight at −20°C. The type and strandedness of the nucleic acid were established by treatment with DNase (Sigma) and RNase A (Sigma) and then electrophorized. The RNA size was estimated by electrophoresis in formaldehyde denaturating agarose gels (1%) and the retention of the RNA by an oligo(dt) cellulose column was used to detect the presence of poly(A), according to the methods recommended by Maniatis *et al.* (1989).

Protein analysis. Polypeptidic profiles were analyzed by SDS–PAGE on 15, 12, and 9% gels by the method of Laemmli (1970). Ten different gels were analyzed to determine the molecular weight of the polypeptides.

Serological test. Reciprocal comparison was performed between the MSBV and GmclV, using the gel immunodiffusion test (Ouchterlony, 1948).

Infection in Vivo

Twenty-five larvae of *G. mellonella* were infected by injection of 8×10^{-3} OD/larve of purified GmclV diluted in Tris, pH 7.5, buffer. The dead larvae were collected from the third day postinfection, when symptoms were observed.

RESULTS

Morphological and Serological Studies

Three days after the infection of the Gm120 cell line by MSBV, modifications occurred in the cells. The cells began to enlarge and detach from the flask walls, and then they burst one by one and floated in the supernatant, representative of the typical cytopathic effect of this infection, compared with noninfected cells (Fig. 1). Six to 7 days postinfection, more than 75% of the cells were affected. Morphological changes could be observed in these cells, e.g., a condensation of the chromatin, suggesting nuclear disintegration and cytoplasm transformations. Electron micrographs of virus-infected cells are shown in Fig. 2. First, the virus accumulated in bags in the cytoplasm (Fig. 2a); at the end of the infection, the bags burst to liberate the virus into the cytoplasm (Fig. 2b). Finally, the cells became full of vacuoles, corresponding to the cytopathic effect observed under the light microscope.

Examination of infected cells or infectious supernatant by the ELISA test, using the MSBV-specific serum, revealed negative results. After purification of the multiplied virus, the same test was always negative and a serological study showed no relationship between the virus produced by the *G. mellonalla* cell line and MSBV.

Since this virus was consistently inducible, it was probable that the virus existed in the cells in a latent or persistent state. Careful examination of noninfected cells of the same cell line processed for electron microscopy showed that cells containing the virus could be detected (Fig. 2c). The percentage of cells showing the presence of the virus was relatively low.

Using the two other cell lines, Gm85 and Gm15, no induction of latent virus was observed using the MSBV and no virus was multiplied.

The same results were obtained for the three cell lines, using AcMNPV. The multiplication of the latent virus was induced in the Gm120, but no viral multiplication in Gm85 and Gm15 was revealed.

Characterization of GmclV

Examination of purified viral suspension by electron microscope revealed a large number of isometric particles, 29 nm in diameter (Fig. 3). UV extinction spectra of the virus showed a maximum absorption at 260 nm and a minimum at 240 nm, which characterize the viral nucleoproteins. The average ratio of extinction at 260 nm to that at 280 nm was 1.5. The electrophoresis of the extracted nucleic acid after treatment with DNase and RNase indicated its RNA nature. Under denaturating conditions, the RNA consisted of a single band, with an estimated molecular weight of 2.9×10^6 Da. The presence of a poly(A) tract was revealed after RNA fragments were bound to an oligo(dt) column and then eluted and precipitated. Electrophoresis of the viral proteins in 9 and 12% polyacrylamide gels revealed the presence of two major bands, 34,500 and 32,500 Da (Fig. 4). No minor bands could be detected. The electrophoretic profiles of MSBV and GmclV were completely different.

Pathogenicity of GmclV

The pathogenicity of the GmclV was tested against *G. mellonella* cells and larvae. The superinfection of the cells with the homologous persistent virus resulted in the multiplication of the virus in the same proportion as when it was induced by the *S. cretica* virus, 1 OD/flask. The same cytopathic effects were observed. After three successive passages of the infected supernatant, no significant decrease in the production of the virus was noted. Five days postinfection, all the larvae of *G. mellonella,* injected with GmclV at a concentration of 8×10^{-3} OD/larve, were dead. After purification of the virus multiplied on these larvae, production of 0.45 OD/larve occurred, and all the methods used to characterize the GmclV indicated that it was the same virus.

DISCUSSION AND CONCLUSION

Our results show that a virus which apparently infects a *G. mellonella* cell line in a persistent manner (GmclV) can be efficiently induced to replicate by the MSBV and also by the AcMNPV. During this process, no trace of MSBV or AcMNPV could be detected, indicating that they were not multiplied. The GmclV appears to be completely different than the inducing viruses and does not reflect a selection of a minor component of the viral inoculum. When it was induced, the GmclV appears to be pathogenic. The cells were completely destroyed 7 days postinfection. Many changes occurred in the cells during the viral multiplication: modifications in the cytoplasm where the virus multiplied and disintegration of the nucleus. GmclV is also pathogenic for the homologous larvae.

The characteristics of the GmclV are Picornavirus-like (Francki *et al.,* 1991). The two principal members of this group are represented by the cricket paralysis virus (CrPV) and the *Drosophila* C virus (DCV) (Scotti *et al.,* 1981). Like the two reference viruses, GmclV

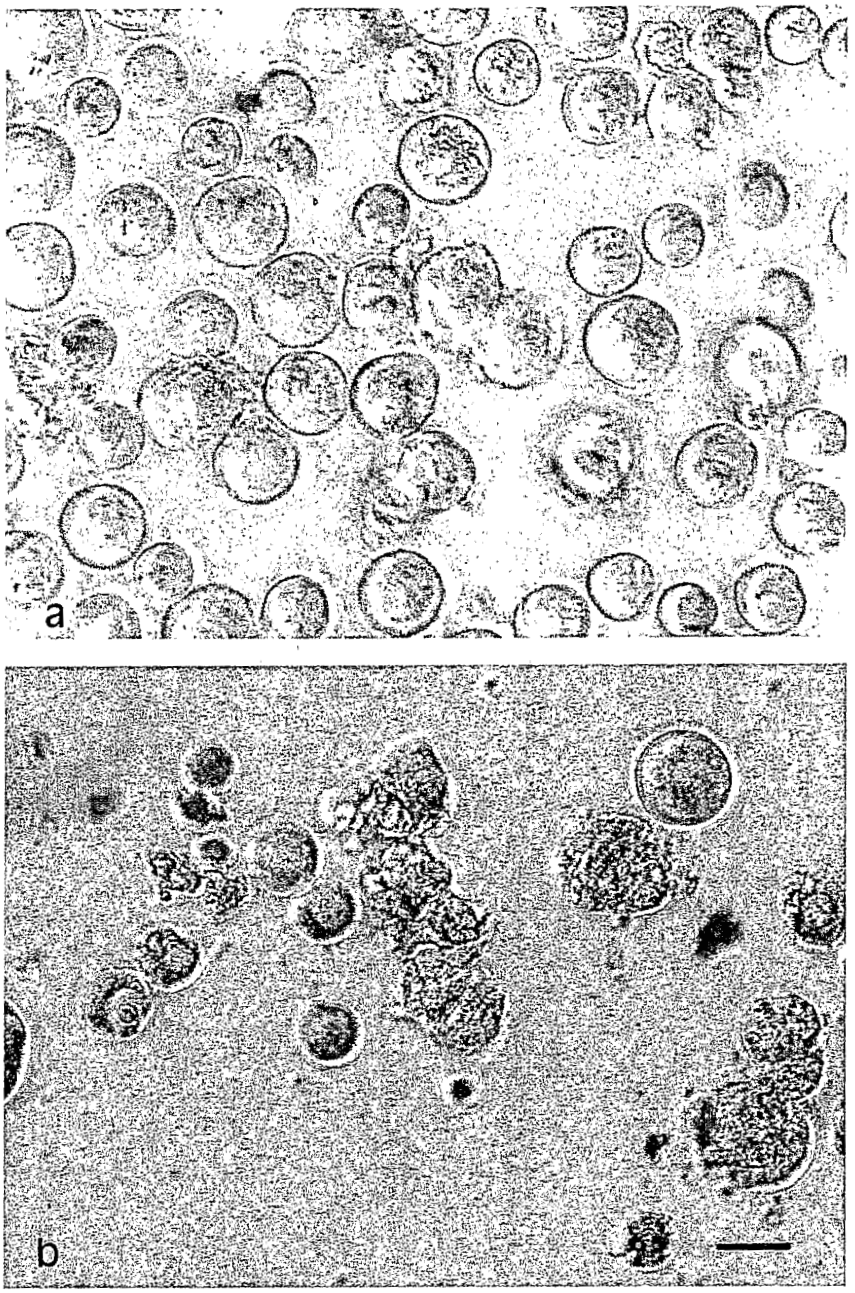

FIG. 1. Cell cultures of *G. mellonella* after 5 days. (a) Noninfected cells; (b) cells infected by the MSBV. Bar represents 25 μm (both panels at the same magnification).

contains a single RNA species with a poly(A) tract and a molecular weight of 2.9×10^6 Da. Only the number of polypeptides differs. CrPV and DCV are considered to be constituted of three major bands, VP1, VP2, and VP3, of molecular weight around 30,000 Da and a VP0 band near 39,000 Da, the precursor of VP2 and VP4, which was not always found (Scotti *et al.,* 1981). We found only two major bands, near 30,000 Da. In Picornaviruses, the capsid assembly is via pentameric intermediates composed of copies of VP1, VP3, and a precursor protein. VP0, which gives VP2 and VP4 upon cleavage, in one of the final steps of capsid assembly, may be related to the encapsidation of the nucleic acid and to stabilization of the mature particle (Stanway, 1990).

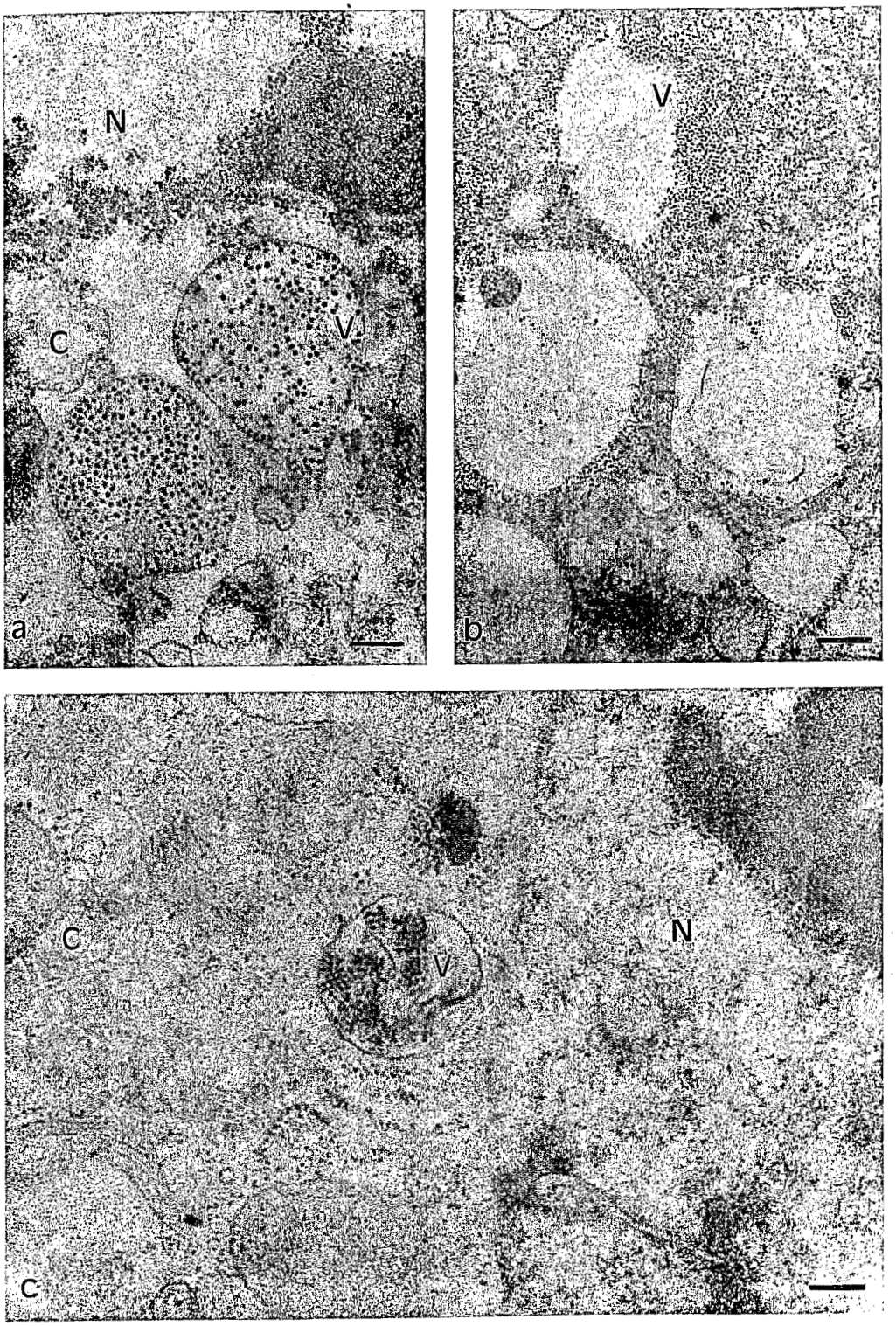

FIG. 2. Electron micrographs of *G. mellonella* cells. (a) Cells infected by the MSBV 3 days postinfection. Bar represents 200 nm. (b) Cells infected by the MSBV 6 days postinfection. Bar represents 350 nm. (c) Nonexperimentally infected cells after 5 days of culture. Bar represents 200 nm. V, virus; C, cytoplasm; N, nucleus.

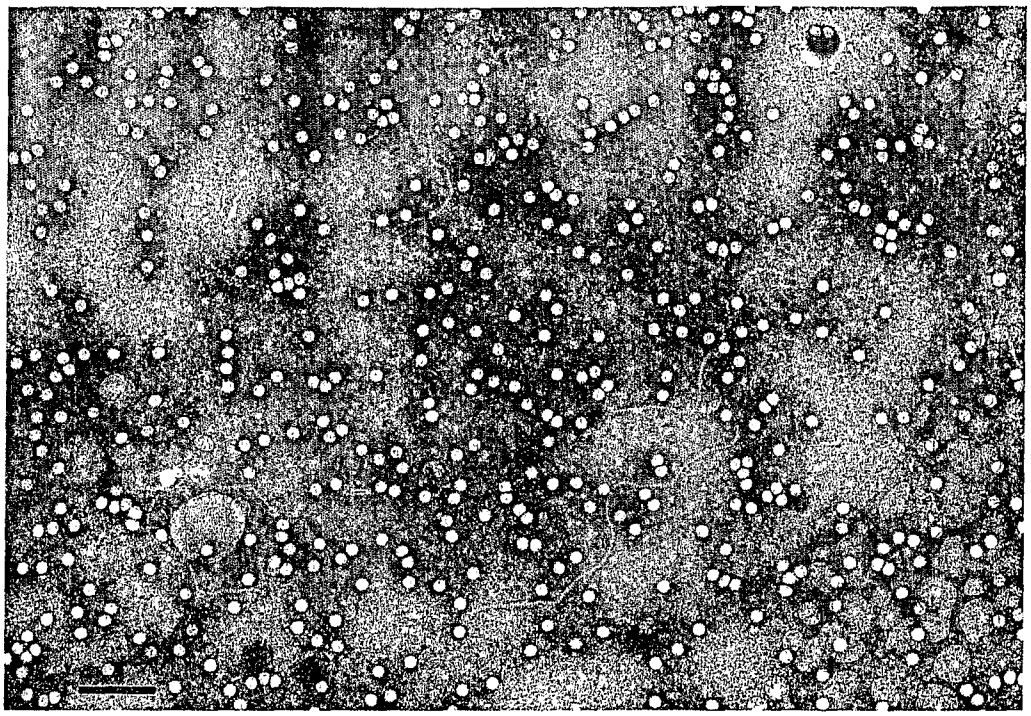

FIG. 3. Electron micrograph of GmclV purified as described under Materials and Methods, negatively stained in uranyl acetate. Bar represents 200 nm.

VP4, linked to myristic acid, and VP2 may play a role in the uncoating of the nucleic acid (Ratka *et al.,* 1989). One possibility, if we consider that the virus persistently infects the cell line, is to consider a repression of the VP0, and the products of its cleavage, VP2 and VP4. Then, the two major bands of the GmclV could be considered VP1 and VP3. This will be in contradiction with the great infectivity of the purified GmclV *in vivo,* which is a prerequisite for the cleavage of VP0. But it must be considered that the larvae of *G. mellonella* are also known to contain another latent virus, different from the virus isolated from our cell cultures (unpublished data). Another possibility is, considering Moore and Eley (1991), that two bands of the virus were so close, that they could not be separated by the techniques used or that the two bands represent in reality (VP0 + VP1) and (VP2 + VP3). Finally, we can also consider the GmclV as a new member of the small RNA viruses, nonclassified. Unfortunately, sera from the CrPV and DCV were unavailable, so a serological community could not be performed to begin to answer this question.

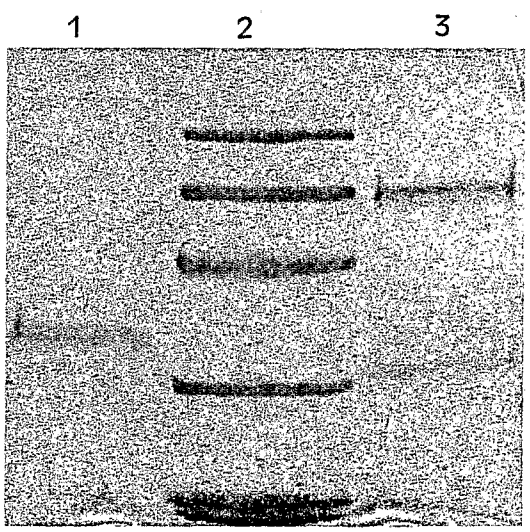

FIG. 4. Comparison of the electrophoretic profiles of GmclV and MSBV in a 12% polyacrylamide gel. Lane 1: GmclV; lane 2: marker (reference proteins: 94,000; 67,000; 43,000; 30,000, and 20,100 daltons); lane 3: MSBV.

Even if the placing of GmclV in the classification of viruses cannot be achieved with assurance, the question of the latency phenomenon observed arises. On *G. mellonella* larvae, another virus is known to be chronically persistent (Zeddam, personal communication; Durandel, 1994). The virus described as persistently infecting the larvae is different from the GmclV that we induced in the cell line. In rearings of *G. mellonella* obtained from Egypt, we have revealed the same persis-

tent virus that was described by these authors using a heterologous virus (unpublished data). These results ask the problem of the origin of these two viruses. Is the latency due to a genetic modification of the virus, a cellular reaction, or adaptation? Some authors have tried to answer this question. Dasgupta *et al.* (1994), studying 20 cell lines experimentally persistently infected by baculoviruses, indicated that the establishment of persistent infection is not due to a mutation in the virus genome, but suggested the possibility that the first event in establishment of persistent infection is a change in the expression of one or more cellular genes. This conclusion may be considered with regard with the positive results we have obtained after several passages of the infectious supernatant *in vitro,* which indicate that once the virus was induced, it keeps its faculty to replicate. We can imagine that when the heterologous virus induced the multiplication of the persistent virus, probably due to a derepression of the cellular system, too much virus is present in the cells to stop it. The inoculum used for the passage contained large amount of viruses (0.2 OD/flask), and the repression cellular system was not efficient. To study this point, different concentrations of inoculum could be used.

The general phenomenon of induction of a persistent virus in an insect cell line is of interest because it mimics in part the situation found in some insects, in which infection of a particular insect species by a virus from a heterologous insect species results in the test insect succumbing to a disease caused by a virus typical of the homologous species (McKinley *et al.,* 1981). The model we have described represents a new model of a complex of RNA viruses that persistently infected the host and a homologous cell line. As the activation of latent virus *in vivo* represents a focus of interest regarding the biological controls (Kelly *et al.,* 1981), the *in vitro* and *in vivo* models on *G. mellonella,* with two different viruses belonging to the same insect group, could contribute to the study of the replication of the induced virus and their possibilities.

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