MULTIPLICATION OF SPODOPTERA LITTORALIS GRANULOSIS VIRUS IN A CELL LINE ESTABLISHED FROM *PHTHORIMAEA OPERCULELLA*

X. LÉRY, O. KHAMISS, A. NOUR-EL-DIN, J. GIANNOTTI, S. ABOL-ELA

Entomovirology Laboratory, ORSTOM, B.O. 26, Giza 12211, Cairo, Egypt

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Summary. – A complete replication of the Spodoptera littoralis granulosis virus (SpliGV) was obtained *in vitro* by both virus infection and DNA transfection in the ORS-Pop-95 (Pop-95) cell line established from embryonic cells of the potato tuber moth, *Phthorimaea operculella.* SpliGV multiplied significantly during several passages in Pop-95 cells at 19°C. When the cells were infected and kept at 19°C for the first 4 hrs and then at 27°C for the rest of the experiment (20 days), the viral multiplication proceeded at the same rate. Comparison of SpliGV progenies, multiplied either *in vivo* or *in vitro*, using electron microscopy and restiction profile analysis, showed their identity.

Key words: *Spodoptera littoralis*; *Phthorimaea operculella* cell line; granulosis virus; virus replication

Introduction

A granulosis virus (SpliGV) was isolated from the Egyptian cotton leaf worm *Spodoptera littoralis* Boisd. (*Lepidoptera, Noctuidae*) more than 15 years ago in Côte d'Ivoire. *S. littoralis* is a very important polyphagous pest in Africa and especially in Egypt. Since 1983 *S. littoralis* larvae can be reared on artificial diet (Baillon, 1983) but the characterization of the SpliGV, a tentative species of the genus *Granulovirus* of the family *Baculoviridae* was conducted only in 1994 (Abol-Ela *et al.*, 1994).

Closely related to SpliGV is the spodoptera littoralis nucleopolyhedrovirus (SpliNPV), a tentative species of the genus *Nucleopolyhedrovirus* of the family *Baculoviridae*. Many studies have been performed on SpliNPV because of the availability of susceptible cell lines (Mialhe *et al.*, 1984; Kislev, 1986). In order to accelerate the progress in investigating the biology of SpliGV, its genetic manipulations and cloning of its different isolates, we had to set an *in vitro* model for its multiplication.

So far, however, the cell-lines obtained from *S. littoralis* are not susceptible to SpliGV (Saldanha and Hunter, 1985). According to recent results obtained with other models on granulosis viruses (Winstanley and Crook, 1993; Léry *et al.*, 1995), their *in vitro* multiplication seems to require low-temperature-established cell lines.

In this paper, we have tested the susceptibility to SpliGV of a cell line established in our laboratory at 19°C from embryonic cells of the potato tuber moth *Phthorimaea operculella* Zeller (*Lepidoptera, Gelechiidae*), which is the usual host of the Phthorimaea operculella granulosis virus (PhopGV) (Léry *et al.*, 1995).

Abbreviations: FBS = foetal bovine serum; MGM = Modified Grace's Medium; PhopGV = Phthorimaea operculella granulosis virus; Pop-95 = ORS-Pop-95; SpliGV = spodoptera littoralis granulosis virus; SpliNPV = Spodoptera littoralis nucleopolyhedrovirus;

Materials and Methods

Established cell line. ORS-Pop-95 (Pop-95), a homogeneous cell line, obtained from embryonic cells of *P. operculella*, was cultivated in Modified Grace's Medium (MGM, Léry and Fédiere,

1990), containing 10% foetal bovine serum (FBS), and incubated at 19°C (Léry *et al.*, 1995). The cells, used between the passages 25-40, were subcultured every 15 – 21 days by seeding 1 –2 x 10^6 cells per a 25 cm^2 flask. Several *S. littoralis* cell lines, established at 28°C, were a gift from Drs J.M. Quiot and G. Groizier, St-Christol-lès-Alès, France. These cells were routinely subcultured every 5 – 7 days in the same way as mentioned above.

Viruses. SpliGV, isolated from *S. littoralis* in Côte d'Ivoire, originally a gift from Dr. P. Monsarrat, was propagated in the Egyptian *S. littoralis* larvae for several years in our laboratory. This virus was used to test the susceptibility of different cell lines of *S. littoralis*. PhopGV, Tunisian isolate, a gift from Dr. El-Bedewy, International Potato Center, Tunisia, was used as a reference for its ability to multiply in the Pop-95 cells (Léry *et al.*, in press).

Infection and transfection of Pop-95 cells. For the infection, the cell cultures, seeded with 2 x 10^6 Pop-95 cells per a 25 cm^2 tissue culture flask, were infected after 24 hrs with virions prepared from infected larvae. The larvae were homogenized and ultrasonicated for 1 min in 2 ml of MGM without FBS. After 2 centrifugations at 5,000 x g for 20 mins, the supernatant, diluted in MGM, was filtered through a membrane with pore size diameter of 0.45 µm. After 4 hrs of contact with the cells, the virus inoculum (1.5 ml) was replaced by 4 ml of fresh medium containing 10% FBS, and the cells were incubated at 27°C or 19°C. For the transfection, the cell culture in the same conditions as above was rinsed twice with MGM without FBS and inoculated with an inoculum (1.5 ml) containing 2 µg of viral DNA and 20 µl of DOTAP (N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl sulfate) (Boehringer) in MGM used as a cationic liposome-forming compound. After 6 hrs, the inoculum was removed, the cells were rinsed with the medium without FBS and 4 ml of MGM containing 10% FBS was added. The cells were incubated at 27°C or 19°C. For serial virus infections, we used infectious supernatants from both infections and transfections to infect new cell cultures to study changes of the viral infectious activity during passaging. Namely, 0.75 ml of each supernatant was diluted with the same volume of MGM without FBS and used as an inoculum for a 25 cm^2 flask. The infection was performed according to the abovementioned procedure. After 2 weeks at 27°C and 3 weeks at 19°C, the cells were scraped with a rubber policeman into the medium, which was then centrifuged for 5 mins at 400 x g. The cell pellet and the supernatant were then purified.

Purification of the virus. The cells infected or transfected with SpliGV were scraped 15 – 20 days post infection and centrifuged for 5 mins at 400 x g. The pellet was treated with STE buffer (0.15 mol/l NaCl, 0.02 mol/l Tris, 1 mmol/l EDTA, 0.5% Aprotinine and 0.5% Nonidet P-40) for 10 mins at 0°C under agitation. The treated pellet and supernatant were homogenized in a Potter-Elvehjem homogenizer and ultrasonicated. After centrifugation for 30 mins at 15,000 x g, the pellet was resuspended in 0.01 mol/l Tris pH 7.5, layered on a 30% – 70% (w/w) sucrose gradient and centrifuged for 20 mins at 30,000 x g. The band containing the granules was collected and concentrated as above and stored at -20°C. Concentrations of virus inclusion bodies were determined spectrophotometrically using equations $A_{420} = 0.8$ for a concentration of 0.1 mg of granules/ml, and 1 mg of granules = 6.0 x 10^{10} granules (Tchang and Tanada, 1978). The supernatant was ultracentrifuged for 30 mins at 100,000 x g. The pellet resuspended overnight in 0.01 mol/l Tris pH 7.5 was layered on a 20% – 50% (w/w) sucrose gradient and centrifuged for 1 hr at 100,000 x g. The band containing virions was collected and the particles were concentrated as above and stored at -20°C.

Infection of S. littoralis larvae. Second- and third-instar larvae were individually fed on artificial diet in rearing boxes. As a first source contamination, the larvae were inoculated on the head capsule, using a brush with a suspension of 10^8/ml purified granules obtained from cells or larvae. As a second source of contamination, the diet medium was dosed with 10^6 granules for the some origin. The different batches of larvae were kept separately at 25°C.

DNA extraction and endonuclease restriction analysis. The DNA was extracted from purified granules resuspended in TE buffer (Maniatis *et al.*, 1982). After incubation for 30 mins at 37°C with an equal volume of 0.12 mol/l Na_2CO_3 pH 10.9, the granules and liberated virions were treated with 2% Sarkosyl and 0.2 mg/ml proteinase K for 2 hrs at 50°C. The DNA was extracted according to a phenol-chloroform-isoamyl alcohol protocol and then ethanol-precipitated (Summers and Smith, 1987). The DNA was resuspended in sterile 0.01 mol/l Tris pH 7.5 for the transfection or in TE buffer for the restriction endonuclease digestion. The DNA concentration was determined from spectrophotometricaly from A_{260}. Viral DNA (1 – 2 µg) was digested for 2 hrs at 37°C with restriction endonucleases *Hind*III, *Eco*RI, *Bam*HI, *Xho*I, *Bgl*II, *Pvu*I and *Eco*RV using the conditions recommended by the supplier (Boehringer). Electrophoresis of genome fragments was run in 1% agarose gel in 80 mmol/l Tris, 80 mmol/l phosphoric acid and 2 mmol/l EDTA pH 8.0 at 60 V for 2 hrs. The gels were visualized and photographed on a UV-transilluminator. Lambda phage DNA restriction fragments were used as size markers. Viral DNA prepared from PhopGV, Tunisian isolate, and digested with the same restriction endonucleases, was used as reference.

Electron microscopy. Purified virus preparations were negatively stained with 2% uranyl acetate pH 7.4 and examined with a Zeiss electron microscope. Infected cells (10 – 14 days post infection) were centrifuged for 5 mins at 400 x g. The pellet was resuspended in MGM without FBS, containing 2.5% glutaraldehyde made in 2.14% cacodylate buffer. After 1 hr of fixation at 4°C, the cells were pelleted for 5 mins at 400 x g and dispersed in 2% low melting agarose. The cells were then fixed with 1% osmium tetroxide, dehydrated and embedded in pure Epon. Ultratin sections were stained and contrasted according to the method of Reynolds (1963).

Results

Virus multiplication in vitro

After infection of Pop-95 cells, continuously cultivated at 19°C, with SpliGV, no cytopathic effect could be noticed during the first 10 days under light microscope. Then during the next 10 days, some changes occurred in the cells. Some small vacuoles appeared in the cytoplasm and a small zone with nucleus-shaped particles at its extremity could

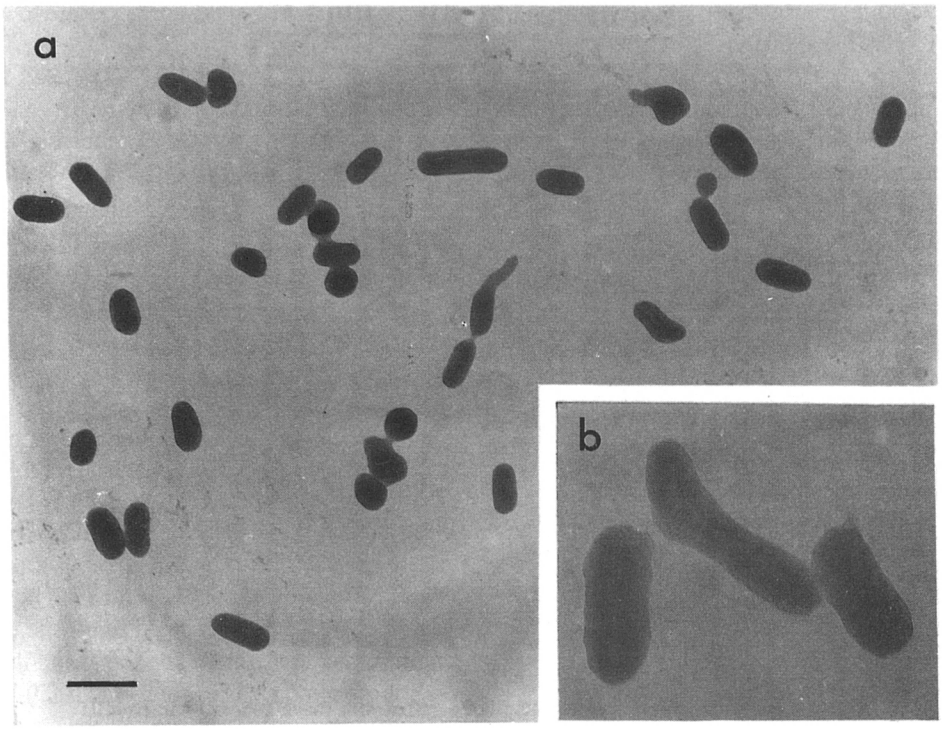

Fig. 1
Electron micrograph of a purified suspension of SpliGV multiplied in Pop-95 cell line
(a) Bar = 500 nm. (b) Additonal magnification 3x.

Table 1. Comparison of the size of restriction fragments of SpliGV and PhopGV (Tunisian isolate) DNA

Fragment	*Eco*RI			*Bam*HI			*Hind*III		
	SpliGV[a]	SpliGV	PhopGV	SpliGV[a]	SpliGV	PhopGV	SpliGV[a]	SpliGV	PhopGV
A 21.0	22.0	20.5	23.0	25.0	26.0	22.0	22.0	21.0	
B	20.0	19.0	20.5	20.0	19.0	17.5	16.0	13.0	14.0
C	19.0	16.5	13.8	16.0	15.5	11.5	12.0	10.0	10.3
D	15.0	13.0	13.8	15.0	–	10.2	8.8	7.5	8.0
E	11.0	10.5	12.8	13.0	12.0	9.9	8.2	7.2	7.0
F	5.9	6.3	9.2	12.0	11.0	8.7	6.3	6.2	7.0
G	4.4	4.4	7.3	7.6	6.5	7.5	5.7	6.0	6.2
H	4.1	4.0	6.4	5.4	5.5	7.5	4.9	4.8	5.8
I 2.8	2.8	3.4	3.8	4.0	6.0	4.6	4.5	4.6	
J	1.7	1.8	3.0	2.6	2.65	2.85	4.4	–	3.9
K 1.6	1.75	2.8	1.7	1.8	2.75	3.5	3.5	3.6	
L	1.0	1.05	1.1	0.92	3.3	3.2	3.4		
M	0.9	0.95	0.45	0.83	3.0	3.0	3.2		
N	0.8	0.9	0.34	0.68	2.8	2.7	3.15		
O 0.58	0.57	–	2.25	2.5					
P	2.1	2.15	2.05						
Q	1.6	1.7	2.05						
R	1.5	1.55	1.5						
S -	1.3	1.5							
T	1.2	1.25	1.3						
U 0.8	0.9								
V 0.75	0.85								
W	0.55								
Total	109.20	105.53	113.50	120.10	104.84	113.30	111.90	105.90	113.75

[a]Data reported by Abol-Ela *et al.* (1994). The size of the restriction fragments is given in kbp.

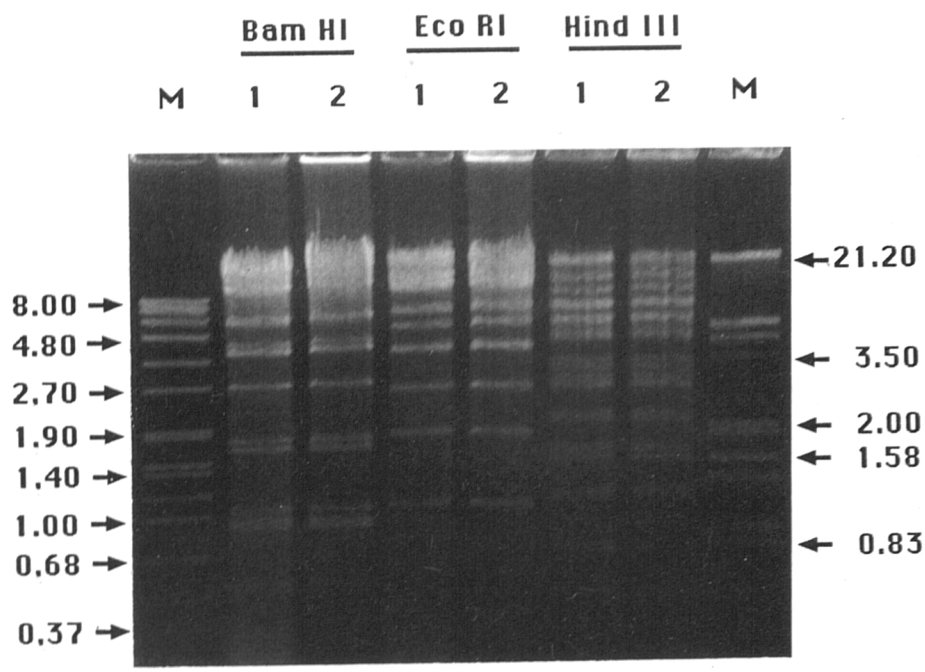

Fig. 2
Agarose gel electrophoresis of SpliGV DNA digested with *Bam*HI, *Eco*RI and *Hind*III
SpliGV grown in *S. littoralis* larvae (1) and Pop-95 cells (2). Molecular size markers (M) with their kbp values.

be seen in some cells. At the end of the infection, after 20 days, the cells attached to the flask walls kept the appearence of viable cells, which was confirmed by a trypan blue staining test. If the cells were infected at 19°C for 4 hrs and then incubated at 27°C, the same phenomenon could be observed.

The examination of infected cells under electron microscope confirmed the slight modifications observed, but, moreover, it revealed the presence of complete granules in the cytoplasm during the first 10 days. The number of granules increased later regularly.

After infection with virions orginating from infected larvae or after transfection with purified DNA, the same results were obtained.

Whether virus multiplication was initiated by infection or transfection, both granules (Fig. 1) and virions were obtained. Based on A_{420} values, the yields were of 2×10^8 – 5×10^8 granules/ml, corresponding to 200 to 500 granules/cell. The number of free virions obtained was relatively low.

The number of granules, produced in the cells during the first two passages of the virus using an infectious supernatant inoculum, increased to $1-2 \times 10^9$ granules/ml (approximately 1000 to 2000 granules/cell). As 90% of the cells appeared to be infected, the number of granules produced was nearly 2000/cell. The amount of free virions also increased and could be easily maesured after purification, giving $A_{260} = 0.2$ for 10^6 infected cells. After several passages of the virus, the quantity of both granules and virions did not change.

In comparison, using the same tests, negative results were always obtained when SpliGV was grown in different cell lines of *S. littoralis* established and cultivated at 27°C.

Restriction profile analysis

The purified virus multiplied in Pop-95 cells gave a positive reaction in the gel immunodiffusion test with the serum prepared with SpliGV (data not shown). All the restriction endonucleases tested gave the same profiles with the DNAs obtained from either infected cells or larvae (Fig. 2). The *Eco*RI, *Bam*HI and *Hind*III restriction profiles of viral DNAs obtained from *S. littoralis* cells and larvae show that there was a normal replication of SpliGV. The restriction profile of DNA of the virus used as the initial infectious inoculum was identical to that of the virus produced by passaging in the cell culture. Table 1 shows that SpliGV and PhopGV, both grown in Pop-95 cells, gave different restriction fragment data and thus represent two different viruses. On the other hand, the restriction fragment data, obtained for the SpliGV presently studied by us and those reported for SpliGV by Abol-Ela *et al.* (1994) are except some slight differences identical.

Multiplication of SpliGV granules in vivo

Using various batches of 10 mixed second- and third-instar larvae, fed on artificial diet, the infections with granules purified from both infected cell cultures and larvae gave 100% mortality in 15 days. In the controls, all the non-infected larvae became pupa and then adult. The number of granules produced in infected larvae was the same, namely $2.0 - 2.5 \times 10^{10}$ granules/larva.

Discussion

For the first time, a complete replication of SpliGV was obtained *in vitro*. All the characterization methods which were used, namely the morphological identification, serological test and DNA restriction endonucleases digestion profile indicated that the granulosis virus multiplying in the Pop-95 cell line was identical to the wild virus introduced. This demonstrates that the *in vitro* multiplication of a granulovirus in a heterologous cell line is possible. The use of tissue extract as virus inoculum (Chen and Dai, 1987) is probably a good way to initiate a first complete multiplication of a granulovirus, when compared with the negative results obtained with other inocula as hemolymph or purified virions. But as during the first passages of the virus *in vitro* the use of infectious supernatants as inocula increased the production of both granules and free virions, this indicates the limit of such a viral source. This effect may be due to a cellular adaptation of the virus. The infectivity of the virus produced *in vitro* was preserved and the 100% mortality observed in the larvae infected with an *in vitro* produced virus inoculum indicates that an amplification of the virus grown in cells could be easily realized. Thereafter, no modification occurred in the amount of granules and in the number of free virions produced during the next passages of the virus, indicating that the viral process became stabilized.

The low temperature used during the establishment of the cell line has probably lead to the selection of a specific cell population, different from that selected at 27°C, confirming the differences obtained in our model between the non-permissive cell lines established at 27°C (Léry *et al.*, 1995; Léry *et al.*, in press) and the permissive cell line established at 19°C in the same conditions. The fact that a heterologous cell line multiplied SpliGV confirmed the strong effect of the temperature on the susceptibility of the cell lines to granuloviruses.

The temperature, however, is not the only factor responsible for the success of the granulovirus multiplication. Some authors have already obtained interesting results using cell lines established at 27°C, even when the multiplication was low or stopped after few passages (Chen and Dai, 1987). Also we have demonstrated earlier that an incomplete multiplication could be obtained with a cloned cell line continuously grown at 27°C (unpublished data). This indicates that the selection of a specific cell type, as far as the conditions of the culture are appropriate (temperature, medium, etc.), could increase considerably the potential of a cell line. However, all the results obtained by us with the *S. littoralis* cell lines established and cultured at 27 – 28°C indicated that there was no multiplication of SpliGV, confirming the results reported earlier by Sadanha and Hunter (1985). In the present study, we demonstrated that there are no more barriers to multiply granuloviruses *in vitro*, even if a specific homologous cell line does not exist. It is now possible to imagine, as it is well known with nucleopolyhedrovirus models, cell lines with a large spectrum of susceptibility to granuloviruses.

The slight differences observed between the restriction fragment profiles obtained with SpliGV in our study and those with the SpliGV reference isolate already described (Abol-Ela *et al.*, 1994) are probably due to differences in the method of calculation used, the number of electrophoreses performed, the resolution of the gels and the quantity of DNA used. These differences could also be considererd significant and the two viruses could represent two different genotypes or variants, as observed with other granuloviruses (Smith and Crook, 1993; Vickers *et al.*, 1991).

We have obtained a significant and complete replication of SpliGV. The complete *in vitro* multiplication of this important virus, already used for microbial control, could allow us to clone its genome and complete its characterization, to provide the users with a more completely known potential biological agent. It also opens the way for comparing this granulovirus to other baculoviruses and finally to manipulate them to increase their potential. The fact that in cell lines of *S. littoralis* established at 27°C, the spodoptera littoralis nucleopolyhedrovirus (SpliNPV) but not SpliGV was multiplied, and that in Pop-95 cell line established at 19°C, a transfection with SpliGV DNA but not with SpliNPV DNA (unpublished data) was obtained, allow us to consider that some biotechnological studies could be performed on these baculoviruses.

In this study, we tested only the Côte d'Ivoire isolate of SpliGV, but there exist also other strains of this virus. The susceptibility to these strains of the Pop-95 cell line could be tested to compare their pathogenicity *in vitro*. The study of possible recombination between the different strains of SpliGV and/or between SpliNPV and SpliGV could be of great interest.

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