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MULTIPLICATION OF A GRANULOSIS VIRUS ISOLATED FROM THE POTATO TUBER MOTH IN A NEW ESTABLISHED CELL LINE OF *PHTHORIMAEA OPERCULELLA*

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SUMMARY

A newly established cell line was obtained from the culture of embryonic cells of the potato tuber moth *Phthorimaea operculella* in low temperature conditions (19° C) using modified Grace's medium supplemented with 10% fetal bovine serum. The population doubling time was about 80 h when cells were cultivated at 19° C and 38 h at 27° C. The cell line had a relatively homogeneous population consisting of various sized spherical cells. The cells were cultivated for more than 25 passages. Their polypeptidic profile was different from profiles of other *P. operculella* cell lines we previously described and from other lepidopteran cells. The new cell line was designated ORS-Pop-95.

The complete replication of the potato tuber moth granulosis virus (PTM GV) was obtained *in vitro* by both viral infection and DNA transfection. PTM GV multiplied at a significant level during several passages of the cell line that was maintained at 19° C. As long as the cells were maintained at 19° C, virus multiplication could also be obtained at the same rate at 27° C. To compare PTM GV multiplied both *in vivo* and *in vitro*, we used morphological identification, serological, DNA probe diagnosis and endonuclease digest profile analysis and confirmed the identity of the virus.

Key words: Phthorimaea operculella; potato tuber moth; cell line; granulosis virus; viral replication.

INTRODUCTION

Most *in vitro* studies on baculoviruses have involved nucleopolyhedroviruses (NPV) for which susceptible cell lines have been available for a long time (8). In contrast, the growth of stable cell lines which support the replication of granulosis viruses (GV) has proved to be difficult and has hindered progress on investigating the molecular biology and genetic manipulation of these viruses. The first report of GV replication in a cell line was by Naser et al. (18) who demonstrated complete replication of *Cydia pomonella* GV (CpGV) in a cell line originating from embryonic cells. Attempts have also been made with other species, but with minor or no multiplication of the virus (7,9,16). All of the above mentioned cell lines lost their susceptibility to the GV after a few months in culture. The only paper reporting cells retaining their ability to replicate a GV was published in 1993 on *Cydia pomonella* cells and GV (29).

Although cell lines have been established from the potato tuber moth (PTM) *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) (by T. D. C. Grace as reported in 10, and 20), no *in vitro* GV multiplication has been reported for this insect. The PTM, a cosmopolitan dominant pest in subtropical and tropical areas, especially in Egypt, is responsible for very large losses in potato production (6). Because the use of chemicals in PTM control is dangerous for humans and generates potatoes unfit for consumption (27), a granulosis virus isolated from this insect is already applied in several countries (21). Establishing an *in vitro* system to study, characterize, and clone the PTM GV is desirable.

Before 1991, a number of cell cultures from *P. operculella* were obtained in our laboratory through various methods of culturing, by cloning, and by selection factors such as media or temperature. Initial results were negative for GV replication (15), but more recently viral DNA multiplication was obtained in cloned cell lines of PTM derived from our first established cell line (ORS-Pop-93) which was maintained at 27° C (13). Because no complete virions or granules were obtained from the PTM GV infections in these cell lines or clones, new cell cultures were initiated and systematically tested against the PTM GV.

MATERIALS AND METHODS

Established cell lines. The ORS-Pop-93 cell line, consisting of heterogeneous cell types (15) and five other cell lines with homogeneous cell types, obtained by selecting (Pop2, Pop3 and Pop4) or cloning (cl1A and cl2B) from embryonic cells of *P. operculella* (13), were cultivated in modified Grace's medium (14) containing 10% fetal bovine serum (FBS) at 27° C. The cells were routinely subcultured every 5 to 7 d and seeded with 1 to 2 × 10^6 cells in 25-cm^2 flasks.

Reference lepidopteran cell lines, *Spodoptera frugiperda* clone 9 (Sf9), *Spodoptera littoralis* (Sl) and *Galleria mellonella* (Gm) were gifts from Dr. J. M. Quiot (St-Christol-lès-Alès, France) and were cultivated under the same conditions just described.

Derivation of new cell lines cultivated at 19° C. Eggs from several layings of laboratory reared insects were used for primary cultures as previously described (15). The fragments and cells obtained from embryos were seeded in Nunc tissue culture flasks (25 cm^2) with modified Grace's medium containing 20% FBS and incubated at 19° C. During the first 6 mo., 1 ml of fresh medium was added every month. After this time, the first subcultures were done every month, then every 3 wk at a ratio of 1:2. At the 7th passage, the quantity of FBS was decreased to 15%. From this passage, the cells were subcultured every 15 to 21 d and seeded with 1 to 2 × 10^6 cells per flask. Over subsequent passages, the quantity of FBS was reduced to 10%.

Characterization of cells. Cell morphology was described and photographs of the cultures were taken under light or with a phase-contrast microscope at ×100 to ×500. Growth curves were determined from daily hemocytometer counts of cells from five replicate cultures stained with trypan blue over a period of 20 d. Cells were cultured in petri dishes (10 × 35 mm) seeded with 4×10^5 cells. The population doubling time was calculated at several passages with an exponential formula. Polypeptidic profiles were determined after electrophoresis in 12% polyacrylamide gels with the method of Laemmli (12). Cellular extracts were prepared with a technique of freezing-thawing (15). Electrophoresis was performed with a Biorad Miniprotean II dual slab cell, and the polypeptide profile of all the established cell lines were compared.

Virus. A granulosis virus isolated from the potato tuber moth *P. operculella* (Tunisia isolate), a gift from Dr. El-Bedewi (PTM GV), was used to compare the infectivity of the different cell lines of *P. operculella*. Infected larvae were homogenized and ultrasonicated 1 min in 2 ml serum-free modified Grace's medium. After being centrifuged twice at 5000 ×g for 20 min, the supernatant, diluted in serum-free modified Grace's medium, was filtered (0.45 μm).

Infection and transfection of cells. For the infections, the cell cultures seeded at 2×10^6 cells into 25-cm² tissue culture flasks were infected after 24 h with virions prepared from infected larvae. After 4 h of contact with the cells, the 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 either 27° C or 19° C.

For the transfections, the cell cultures were maintained under the same conditions as just described. They were rinsed with modified Grace's medium twice, then inoculated with 2 μg viral DNA, mixed with 20 μl DOTAP (Boehringer), and diluted in 1.5 ml of serum-free modified Grace's medium. After 6 h contact, the DNA-DOTAP was removed, the cells were rinsed with serum-free medium, and 4 ml of modified Grace's medium containing 10% FBS was added. The cells were incubated at 27° C or 19° C.

New cell cultures were serially infected with infectious supernatants from both infections and transfections to test the sufficiency of the virus to multiply among the passages. Each 25-cm² flask was infected with 0.75 ml of each supernatant diluted with the same volume of modified Grace's medium without FBS according to the procedure just described.

After 2 wk at 27° C and 3 wk at 19° C, the flasks were scraped with a rubber policeman and the cells and media centrifuged 5 min at 400 ×g. The pellet and supernatant were tested by ELISA, immunodiffusion, and DNA probe techniques, then purified.

Purification of the virus. PTM GV-infected or -transfected cells were collected 15 to 20 d posttreatment, scraped from the flasks, and centrifuged 5 min at 400 ×g. The pellet was treated in STE buffer (0.15 *M* NaCl, 0.02 *M* Tris, 1 m*M* EDTA, 0.5% Aprotinin and 0.5% NP40) for 10 min at 0° C under agitation. The treated pellet and supernatant were homogenized with a Potter homogenizer and ultrasonicated. After centrifugation for 30 min at 15 000 ×g, the pellet was resuspended in 0.01 *M* Tris, pH 7.5, deposited on a 30% to 70% (wt/wt) sucrose gradient, and centrifuged 20 min at 30 000 ×g. The band containing granules was collected, concentrated as above, and stored at −20° C. The virus inclusion body concentrations were determined with a spectrophotometer with the relationship OD_{420nm} 0.8 = 0.1 mg of granules/ml and 1 mg granules/ml = 6.0×10^{10} granules (2). The supernatant was ultracentrifuged for 30 min at 100 000 ×g. The pellet was resuspended in 0.01 *M* Tris. pH 7.5, overnight, deposited on a 20% to 50% (wt/wt) sucrose gradient, and centrifuged 1 h at 100 000 ×g. The band containing virions was collected and the particles were concentrated as above and stored at −20° C.

Infection of P. operculella *larvae.* Third instar larvae were fed on small potatoes (16 g per 25 larvae). The larvae were inoculated on the head capsule by a suspension of 10^8 purified granules per ml (produced in cells or larvae) or the potato was dosed with 10^6 granules. The larvae were kept at 27° C in plastic rearing boxes.

Serological studies. A specific mouse serum was prepared for detecting the viral proteins by injecting the total proteins of granules. The indirect ELISA test (enzyme linked immunosorbent assay) with alkaline phosphatase was conducted according to Kelly et al. (11). Using the same serum, we performed reciprocal comparisons between the virus produced in cells and the PTM GV using a gel immunodiffusion test (19).

Nucleic acid probe detection. Granules or virions purified from infected cells and cellular extracts prepared as follows were used in DNA probe tests. One ml of infected cells and supernatant (1 to 2×10^6 cells) were concentrated by centrifugation for 30 min in a microfuge (13 000 ×g). The pellet resuspended in 100 μl 10 m*M* Tris, 1 m*M* EDTA, pH 8.0 (TE) buffer was incubated for 30 min at 37° C with an equal volume of 0.12 *M* Na_2CO_3, pH 10.9, then treated with 2% sarkosyl and 0.2 mg proteinase K per ml for 2 h at 50° C. The resulting suspension was tested at various dilutions. The digoxygenin-labeled DNA probe was applied according to the protocol recommended by the supplier (Boehringer), and the same protocol was used in the "dot blot" hybridization technique. A DNA probe prepared from the total extract DNA of the PTM GV and a specific DNA probe prepared from a cloned fragment of the same virus were used (unpublished data).

DNA extraction and analysis. Purified granules were suspended in TE buffer and incubated for 30 min at 37° C with an equal volume of 0.12 *M* Na_2CO_3, pH 10.9; the granules and liberated virions were treated with 2% sarkosyl and 0.2 mg proteinase K per ml for 2 h at 50° C. The DNA was extracted by a standard phenol–chloroform–isoamyl alcohol protocol, then precipitated with 100% ethanol (24). The DNA was resuspended in sterilized 0.01 *M* Tris, pH 7.5, buffer for transfection, or TE buffer for restriction endonuclease digestion. The DNA concentration was determined from the OD_{260}. Viral DNA (1–2 μg) was digested for 2 h at 37° C with *Hin*dIII, *Eco*RI, and *Bam*HI restriction endonuclease under the conditions recommended by the suppliers (Boehringer). Electrophoresis of genome fragments for the comparison between larval and cell DNA extracts was carried out on 1% agarose gels in 80 m*M* Tris, 80 m*M* phosphoric acid, and 2 m*M* EDTA, pH 8.0 (TEP), run at 60 V for 2 h. The gels were visualized and photographed on a UV illuminator. The sizes of the fragments were estimated by comparison with λ DNA.

Electron microscopy. Purified virus preparations were negatively stained in 2% uranyl acetate, pH 7.4, and examined with a Zeiss (Labovert) electron microscope. Infected cells were pelleted 5 min at 400 ×g after 10 to 14 d postinfection and resuspended in serum-free modified Grace's medium containing 2.5% glutaraldehyde (diluted in 2.14% cacodylate buffer). After 1 h fixation at 4° C, the cells were pelleted 5 min at 400 ×g and dispersed in 2% low-melting agarose. 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 (23).

Results

Obtainment of a new cell line grown at 19° C. Ten to 20% of the primary cultures that were initiated showed positive results. Most tissue fragments of the embryos began to attach to the culture flask after incubation for 1 or 2 d at 19° C. During the first 6 mo., cells began to multiply from the explants. Multicellular fibers radiated from the aggregates and strongly attached rounded cells began to accumulate. At the end of this period, the flask was completely covered with rounded cells, with some also floating in the medium.

During the first subcultures, the cells multiplied slowly and appeared to be slightly heterogeneous, with a majority being different sized rounded cells and fibroblast-like cells. By the 5th passage, the culture consisted of a relatively homogeneous population of rounded cells. The morphology of these cells did not change among the next passages, even when the quantity of serum was decreased. By the 12th passage, we considered the cells cultivated in 10% FBS were stabilized. When the cells established at 19° C were cultivated at 27° C, there was no apparent difference in morphology but the growth rate increased.

Characteristics of the ORS-Pop-95 cell line. The new cell line was considered to be established at the 10th passage and had a relatively homogeneous population of various sized spherical cells. The cells attached to the bottom of the flask formed a monolayer. The cell population doubling time at 19° C before the 15th passage and after the 20th passage was 105 h and 80 h, respectively, when 2×10^5 cells/ml were seeded (Fig. 1). If the cells were transferred after the 20th passage to 27° C under the same conditions, the doubling time was further decreased to 50 h. After several passages at 27° C, the cell population doubling time was decreased to 38 h, in contrast to the 34-h doubling time of the cell line established at 27° C (Fig. 1).

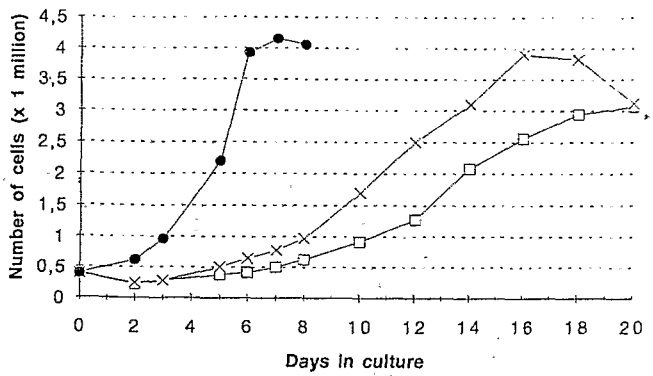

FIG. 1. Growth curves of *P. operculella* ORS-Pop-95 cell line. ● = Cells cultivated at 19° C for 15 passages, then transferred to 27° C. □ = Cells before the 15th passage, always cultivated at 19° C. × = Cells after the 20th passage, always cultivated at 19° C.

The polypeptide profile of the ORS-Pop-95 cell line did not change over the 3 passages tested (10, 15, and 20th), but significant differences were detected between the different established cell lines of *P. operculella*, especially in the range between 14 000 and 50 000 daltons (data not shown).

The cell line has been cultivated for more than 25 passages and can be stored for long periods in liquid nitrogen (−196° C) by suspension in the culture medium containing 10% dimethyl sulfoxide (DMSO) and 20% FBS and progressive freezing in a NICOOL 100 apparatus for 30 min. We have also stored the cells at 4° C for more than 1 mo.

In vitro *viral multiplication.* During the 10 first subcultures, inoculations with PTM GV revealed no cytopathic effect and the response by the ELISA test was not significant. Only the DNA probe prepared from total PTM GV DNA gave a detectable response. At this stage of the cultures, complete granules were identified after purification, but only at a low concentration. A similar level of viral production was obtained after three passages of the virus in the same culture.

After the 11th passage of the cells cultivated at 19° C, the same viral inoculum gave a cytopathic effect 10 d following transformation. Compared to the noninfected cells (Fig. 2 *A*), the rounded cells became fibroblast-like, strongly attached with some dark spots at the tapered ends of the cells, and vacuoles of varied sizes (Fig. 2 *B*). During the next 10 d the size of the vacuoles increased and the cells with a small nucleus zone at their extremity detached from the flask walls and floated in the medium (Fig. 2 *C*). If we attempted viral multiplication at 27° C after a 4-h infection at 19° C, the same phenomenon occurred but more rapidly, with a maximum cytopathic effect after 2 wk. At the end of the infection more than 50% of the cells showed this typical cytopathic effect.

Electron microscopic examinations of infected cells confirmed that the cytopathic effect observed was due to virus. Granules were detected in the cytoplasm associated with active zones, probably viral material in formation, but no preoccluded virions were apparent (Fig. 3 *A*). Later the number of granules increased regularly in the cytoplasm and no transformation in the nucleus could be detected (Fig. 3 *B*). At the end of the infection both cytoplasm and nucleus were degenerating, the number and the size of vacuoles were increased, and many complete granules were observed (Fig. 3 *C*). The same

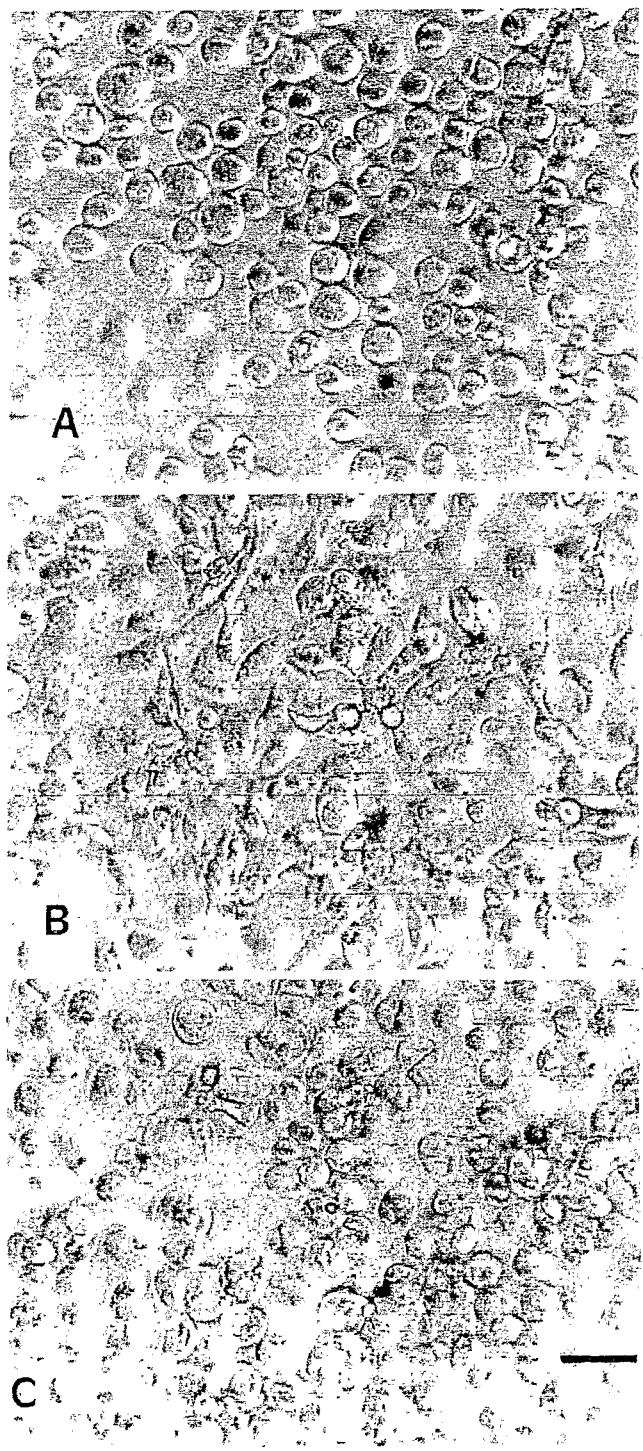

FIG. 2. Cell cultures of *P. operculella* observed in phase contrast at the 15th passage. *Bar* represents 25 µm; both panels are at same magnification. *A*. Noninfected cells after 7 d. *B*. Cells infected with PTM GV, 8 d postinfection. *C*. Cells infected with PTM GV, 15 d postinfection.

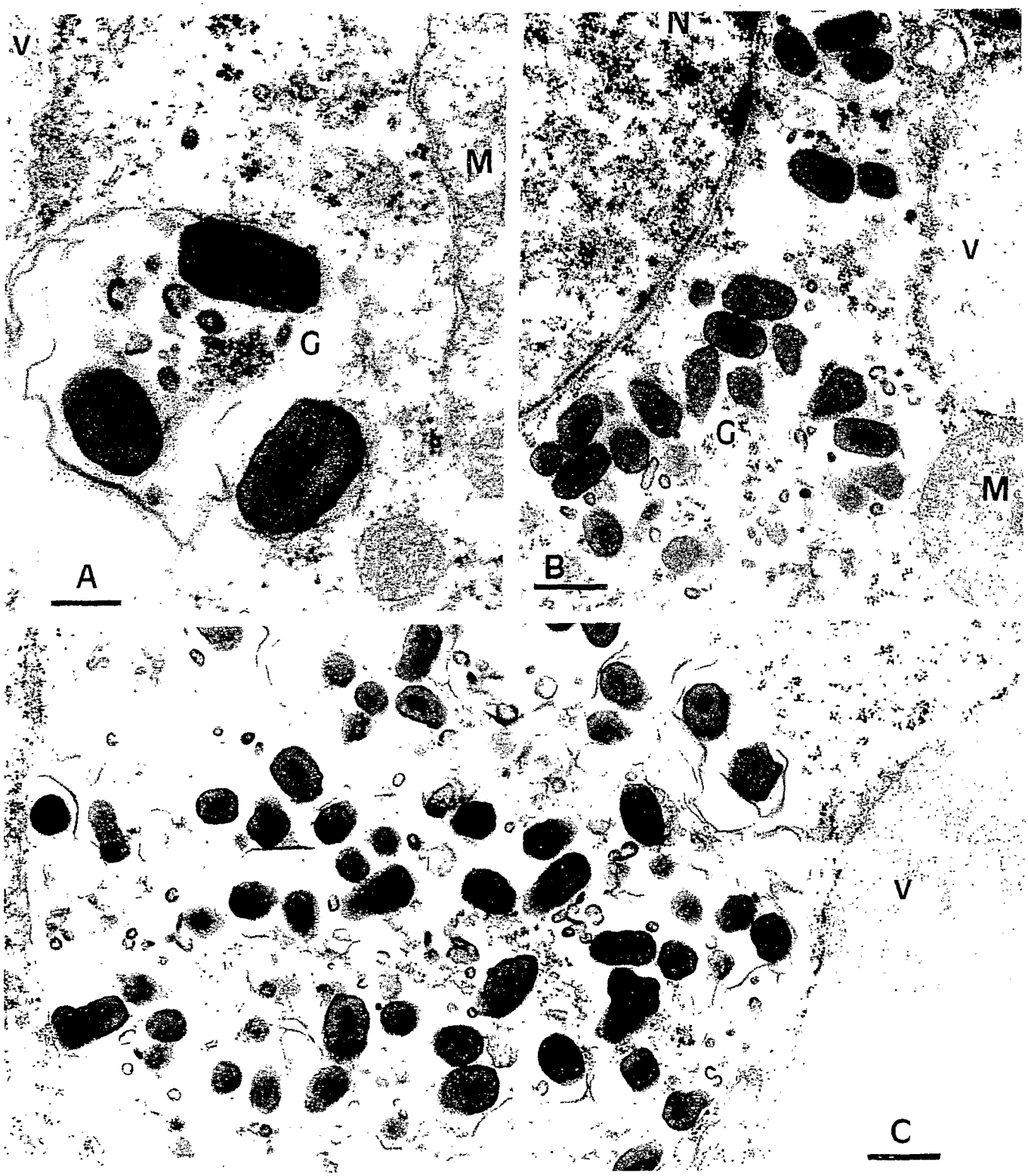

FIG. 3. Electron micrographs of *P. operculella* cells infected with PTM GV. *A*, Granules in an active zone of the cytoplasm near a vacuole, 10 d postinfection (*bar* represents 200 nm). *B*, Granules synthesized in the cytoplasm without disruption of the nuclear membrane, 16 d postinfection (*bar* represents 400 nm). *C*, Presence of many granules late in infection, near a vacuole (*bar* represents 400 nm). G, granules; V, vacuole; N, nucleus; M, mitochondria.

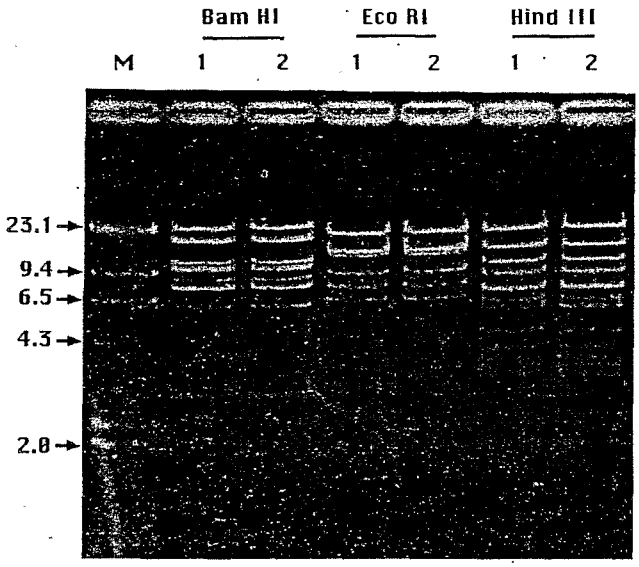

FIG. 4. Comparison of the electrophoretic profiles in 1% agarose gel of restricted genomic DNA from PTM GV multiplied in larvae (1) and the ORS-Pop-95 cell line (2), by three restriction endonucleases. M represents DNA molecular weights.

results were obtained in infections with virions originating from either infected larvae or transfection with purified DNA.

The ELISA response was not significant when infectious supernatant and infected cells were used, because of a nonspecific reaction in the control. The DNA probe prepared with total DNA gave a positive reaction with infected cells, but a small nonspecific response was also observed in the control. This false positive response was eliminated when a specific probe prepared with a cloned fragment of the PTM GV DNA (unpublished data) was used.

Purification of the virus from cells following infection or transfection yielded both granules and virions. Measuring the OD at 420 nm gave amounts of 10^8 to 2×10^9 granules/ml, corresponding to 100 to 200 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 when using infectious supernatant inoculum was used was increased to 10^9 granules/ml (approximately 1000 granules/infected cell). On the basis of electron microscopic examination, 1% of the infected cells yield 10^4 granules each whereas the remaining cells produce fewer than 100 granules each. The number of free virions also increased in the same proportion among the first passages and 10^6 infected cells gave purified virion suspensions at 0.2 OD_{260}. After several passages of the virus, the quantity of both granules and virions produced did not change. The same results were obtained where cells were maintained at 19° C or 27° C during the viral infection.

Identification of progeny virus. The purified virus from cell cultures gave a positive reaction in a gel immunodiffusion test with serum prepared with PTM GV. The *Eco*RI, *Bam*HI, and *Hind*III restriction fragment profiles of viral DNA from *P. operculella* cells and larvae showed that there was normal replication of PTM GV. The DNA profile of the virus used to obtain the initial infectious inoculum was identical to that of the virus produced after passaging in cell culture (Fig. 4).

Multiplication of granules in vivo. With different batches of 25 third instar larvae fed on small potatoes, the infections with granules purified from infected cell cultures and infected larvae both produced 100% mortality in 10 d. In the controls, all the larvae became pupae and then adults. The number of granules produced in larvae infected with either virus source was the same, 2.4 to 2.7 $\times$ 10^{10} granules/larva.

DISCUSSION AND CONCLUSION

The new cell line ORS-Pop-95 derived from embryos of the potato tuber moth is the first cell line of *Phthorimaea operculella* established at a low temperature (19° C). The cultures were composed of various cell types, principally rounded and fibroblast-like cells during the first subcultures as commonly described for insect cells (7,17,20). After the 5th passage, only rounded cells of varied sizes were observed. The morphology of the ORS-Pop-95 cell line is similar to the cell lines previously established at 27° C (13,15,20). Increasing the temperature to 27° C does not affect the morphology of the cells established at 19° C. Winstanley and Crook (29) observed that a cell line established from embryos of *Cydia pomonella* at 19° C changed morphology when cultivated at 27° C. Thus, they decided to select different cultures to obtain more homogeneous cell lines. We consider our cell line to be homogeneous and stable without further selection.

Even though morphology of the cell line is not affected by temperature, growth rate is. The doubling time of the cells established at 19° C is 80 h when maintained at 19° C, but decreases to 38 h when transferred to 27° C during several passages. This was comparable to the growth rates of cell lines established at 27° C which had a doubling time of 34 h. This result corresponds to those generally obtained when one tests the effect of temperature on the multiplication of cells (22). Even though similar cell morphology is observed in different established cell lines, this does not reflect their homology because differences can be identified in their polypeptide profiles and growth curves.

For the first time, complete replication of PTM GV was obtained *in vitro*. All the controls used—morphological identification, serological test, DNA probe, and digestion with DNA restriction endonucleases—indicate that the granulosis virus grown in the ORS-Pop-95 cell line is identical to the wild virus introduced. Over the initial passages of the virus, use of infected supernatants increases both granules and free virion production. This may be due to cellular adaptation of the virus. No change occurred in the amount of granules and in the number of free virions produced during the subsequent passages of the virus, indicating that there was stability in the viral process. Virus produced *in vitro* is infectious because 100% mortality is observed in the third and fourth instar larvae inoculated with virus produced *in vitro*.

Observations of infected cells by electron microscopy revealed an atypical process in the replication of the PTM GV *in vitro*. Generally, GV replication begins in the nucleus and then, after disruption of the nuclear membrane, continues in the mixed nuclear and cytoplasmic components (25,29). In our study, no apparent cytopathological changes could be detected in the nucleus in the early and medium phase, and replication took place only in the cytoplasm as rarely observed (5). Only in the very late phase, corresponding to the cytopathological effect observed under phase-contrast light microscopy, was destruction of the cells visible. Differences can be noticed between the *Cydia pomonella* system described by Winstanley and Crook (29) and our system after electron microscopic studies because of the absence of the nucleic phase of the GV replication. However,

the cytopathic effects observed under light microscopy look similar in the two cases, indicating that the cytopathic effect reflects primarily cytoplasmic changes.

If we compare the results obtained before and after the 10th passage of the ORS-Pop-95 cell line at 19° C, we can consider that the low multiplication of the PTM GV during the first passages is possibly related to incomplete stabilization of the cell population. In this case, the increase in viral multiplication and the presence of a cytopathic effect after the 10th passage may indicate the selection of specific cell types more susceptible to the PTM GV. This result is in relative contradiction with the ideas generally accepted for the multiplication of the GVs *in vitro*. Many authors have considered that the unsuccessful multiplication of the GV *in vitro* was related to selection among the passages of a cell population that became unsusceptible to the GV, even though multiplication occurred in the early passages (7,10). The differences obtained by these authors and our results are probably due to the culture conditions we used. The low temperature used during the establishment of the cell line has probably led to selection of a specific cell population different from that selected at 27° C as previously described (29). This is also consistent with the differences obtained in our system between the nonpermissive cell lines established at 27° C (13,15) and the permissive cell line established at 19° C under otherwise similar conditions.

Temperature is not the only factor in the success of GV multiplication. Some authors have already obtained interesting results using 27° C established cell lines, even when multiplication was low or stopped after a few passages (3). We have also demonstrated that incomplete multiplication could be obtained on a cloned cell line always multiplied at 27° C (13). This suggests that the selection of a specific cell type could considerably increase the potential of a cell line for growing virus. In our study, 60% of the cell population of the ORS-Pop-95 cell line could be infected by the PTM GV and the number of granules per cell obtained (500 to 1000) was relatively low, when electron microscopic observations have shown some cells with thousands of granules (approximately 1% cells with 10^4 granules). We believe that cloning the cell line could considerably increase the multiplication rate of the virus. Cloning might be impractical because only cells with a rapid doubling time, impossible to obtain with cells cultivated at 19° C, are easily cloned. In 1994, Winstanley (28) reported the possibility of conserving susceptibility to the CpGV obtained at 19° C when transferred to 27° C using medium supplemented with dimethyl sulfoxide or retinoic acid. This could also be a way to adapt our cell line to 27° C and then to clone the viral susceptible cell population.

We have obtained significant and complete replication of the PTM GV with a significant cytopathic effect. The complete *in vitro* multiplication of this important virus could permit us to clone the PTM GV and complete its characterization. This would provide investigators a completely known pathogen for use as a biological agent. This will also allow comparison of this GV with other baculoviruses and perhaps to allow us to manipulate them to increase their potential as biocontrol agents. In previous studies, we have shown that the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) can be multiplied in *P. operculella* cell lines (15). This NPV represents the most characterized virus of this group (4). The possibility of transfecting GV DNA (1) and obtaining complete replication of the virus permits us to consider that some biotechnological studies could be carried out between these two baculoviruses. In fact, recent results indicate that the AcMNPV also multiplies on the ORS-Pop-95 cell line even when cultivated at 19° C.

In this study, we have tested only the Tunisia isolate of PTM GV, but different strains have been described (26). When ORS-Pop-95 was available, its susceptibility could be tested against these isolates to compare their pathogenicity *in vitro*. The study of possible recombinations between the different strains of PTM GV and/or between AcMNPV and PTM GVs to obtain recombinant viruses is now possible.

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