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REPORT

Two new cell lines originated from the embryos of *Clostera anachoreta* (Lepidoptera: Notodontidae): characterization and susceptibility to baculoviruses

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Abstract Two cell lines designated CAF-Clan I and CAF-Clan II have been established from embryos of *Clostera anachoreta* (Lepidoptera: Notodontidae) in TNM-FH medium containing 10% inactivated fetal bovine serum. CAF-Clan I consists of a mixture of three cell types: spherical cells, spindle-shaped cells, and giant cells. Most of the cultured cells formed a suspension in the medium and were subcultured more than 60 passages. CAF-Clan II mainly consists of spindle-shaped and spherical cells which attached to the culture surface and have undergone more than 40 passages. The cell population doubling time at 27°C of CAF-Clan I at passage 22 and CAF-Clan II at passage 24 was about 68.5 and 38.2 h, respectively. The chromosome number of both cell lines at passage 15 varied from 62 to 100 in the majority of cells, though a few cells exceeded 260 (n=30). DNA amplification fingerprinting–polymerase chain reaction analysis confirmed that the origination of the two cell lines was *C. anachoreta*. The susceptibility of the cell lines to baculoviruses was tested. The results showed that CAF-Clan II was susceptible to infection of *Autographa californica* nucleopolyhedrovirus (AcMNPV) and *Ecotropis oblique* nucleopolyhedrovirus (EoNPV). Occlusion bodies (OBs) production was 129±4 OBs/cell and 124±15 OBs/cell for AcMNPV and EoNPV, respectively. CAF-Clan I was less susceptible to AcMNPV compared with CAF-Clan II, while non-permissive to EoNPV.

Keywords *Clostera anachoreta* · Embryonic cell lines · Baculoviruses · *Autographa californica* nucleopolyhedrovirus · *Ecotropis oblique* nucleopolyhedrovirus

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Since Grace (1962) successfully established long-term cultures of insect cells, more than 500 continuous cell lines have been established from over 100 insect species (Lynn 2001). There are more than 260 lepidopteran cell lines today (Lynn 2007). These cell lines have attracted considerable attention because of their application in diverse areas of research, especially in medicine and agriculture (Sudeep et al. 2002). However, the availability of insect host cell lines is still a limiting factor for the study of some insect viruses (Winstanley and Crook 1993). Moreover, cell lines originating from different insect species tend to differ in their capacity to produce different viruses. Therefore, there is a need to develop more lepidopteran cell lines as substrates for the replication of a variety of baculoviruses (Hink et al. 1991; Iwabuchi 2000).

Clostera anachoreta is a major defoliator pest of poplar and willow trees in China. It is also widely distributed all over the world (Xiao 1992). In this report, we established two cell lines from the embryos of *C. anachoreta* and characterized them. In addition, the susceptibilities of the cell lines to *Autographa californica* nucleopolyhedrovirus (AcMNPV) and *Ecotropis oblique* nucleopolyhedrovirus (EoNPV) were examined. The two cell lines are the first cell lines ever generated from *C. anachoreta* and can thus

be an important tool for the production of novel baculoviruses (for insect pest control purposes) as well as for studying unique host–virus relationships.

Six primary cultures were initiated in May 2007 from 4-d-old eggs in TNM-FH medium with 10% inactivated fetal bovine serum in which penicillin (100 U/ml) and chloromycetin (100 U/ml) were added. The medium pH was adjusted to 6.4 according to the values obtained from the hemolymph of ten fifth-instar *C. anarchoreta* larvae.

The procedure to initiate primary cultures of *C. anachoreta* embryo cells was modified from Mitsuhashi (2002). Eggs (4 d old) were surface-sterilized with 10% sodium hypochlorite for 5 min, washed with sterile distilled water, then immersed in 75% ethanol for 15 min and rinsed with Ringer's solution. The surface-sterilized eggs were placed in a Petri dish (Corning, NY) containing 1 ml TNM-FH medium, from which the embryos and yolk were released by tearing the chorion with fine forceps. The embryos were collected and transferred to 0.5 ml fresh TNM-FH medium and cut into several pieces. Fragments of the embryos from approximately 40 eggs were transferred to a polystyrene T-25-cm^2 culture flask (Corning, NY) with the culture medium and incubated at 27°C. Twenty-four hours, later 1–1.5 ml medium was added into the flask. Tissue attachment was observed 24 h after the culture was set up. Cell migration from explants started 12 d later and numerous cells attached to the surface appeared to be multiplying. The cells in one of the flasks lately designated CAF-Clan I were arranged as a line 50 d after the primary culture was set up (Fig. 1*a*). Vesicles in CAF-Clan II were observed 20 d after initiation (Fig. 1*b*). During the first 3 mo in culture, half the volume was replaced with fresh medium every 2 wk.

The first attempt to subculture CAF-Clan I was made 44 d after initiation by transferring 1.5 ml contents of the culture flask to a new one containing 1.5 ml fresh growth medium. Prior to cell transfer, it was necessary to pipette the medium against the flask bottom gently to detach cells from the growth surface. The first successful subculture for CAF-Clan I was not achieved until the seventh attempt (92 d after initiation). For CAF-Clan II, the first attempt was made 60 d after initiation. Before the first successful subculture, many unsuccessful attempts, including the usage of trypsin and rubber policeman, were made to

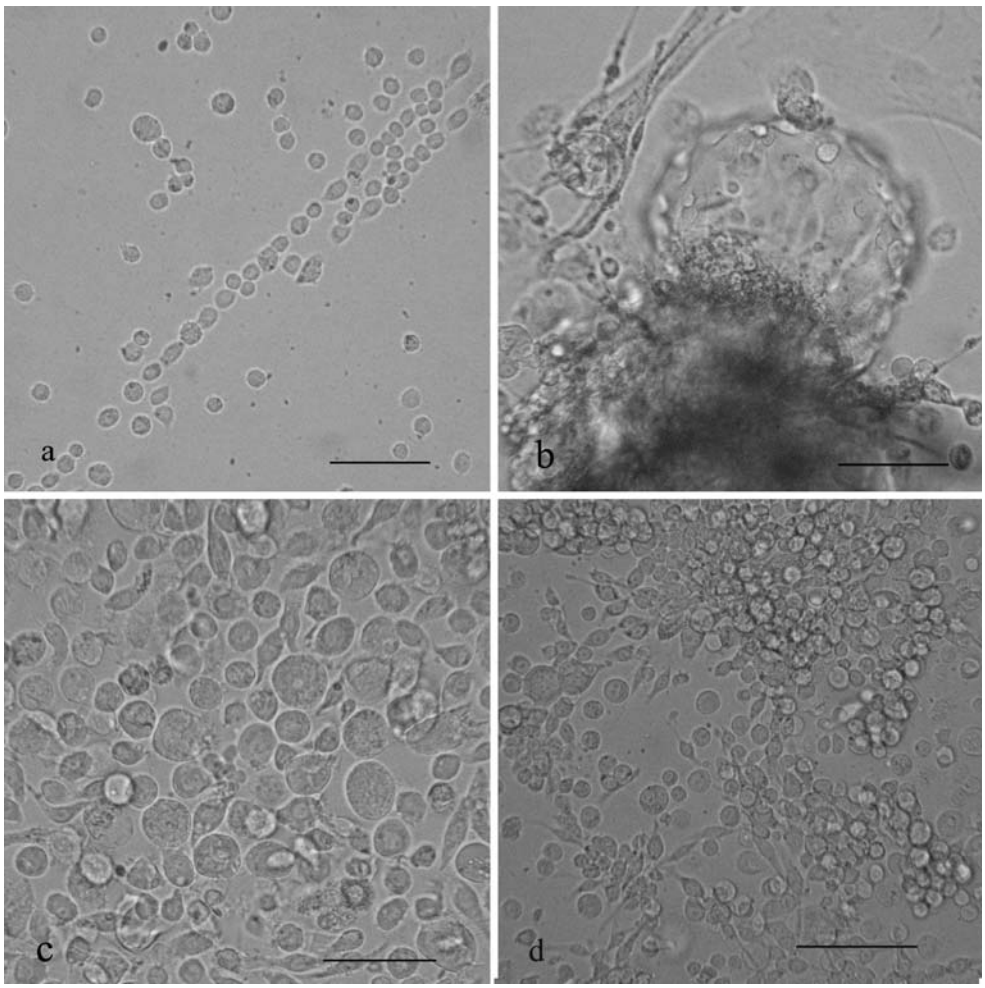

Figure 1. Photomicrograph of CAF-Clan I and CAF-Clan II. *a*, The cells in one of the flasks which become CAF-Clan I finally were arranged as a line 50 d after the culture was set up. *b*, Vesicle was observed 20 d after initiation in the other flask in which the cell become CAF-Clan II finally. *c*, The 40th passage CAF-Clan I showed a heterogeneous population constituted of round cells, fibroblast-like cells and giant cells. *d*, The CAF-Clan II cell population at passage 20 remains of two dominant morphologies: round cells and fibroblast-like cells. The *bar* is 100 μm.

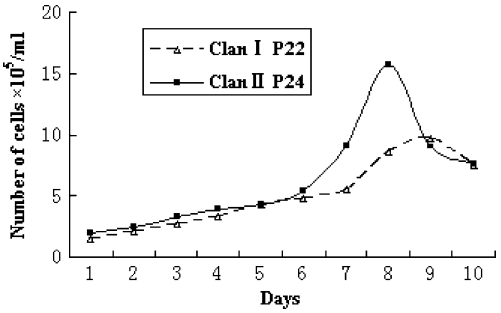

Figure 2. Growth curve for CAF-Clan I and CAF-Clan II cell lines. The population doubling time of CAF-Clan I at passage 22 and CAF-Clan II at passage 24 was about 68.5 and 38.2 h, respectively.

subculture the line. As a result, its first successful subculture was made 5 mo after the establishment of the primary culture. Subculturing in the early stages was carried out at intervals of 14–20 d. However, after the fourth passage, the cells were found to multiply quickly and the intervals between subcultures were shortened to 5–7 d. Employing a split ratio of 1:5, CAF-Clan I and CAF-Clan II have undergone more than 60 and 40 passages, respectively. The cell lines are now stored either in liquid nitrogen or in a freezer at −80°C. These two methods have proven to be equally effective in conserving the viability of the cell lines.

The cultured cells were observed with a Motic inverted microscope. Cell sizes were measured and converted to actual size according to a calibrated magnification factor. Average cell dimensions were determined from measurements of 30 cells. Most of the CAF-Clan I cells grow as a suspension culture. CAF-Clan I in its 40th passage showed a heterogeneous population that comprised round cells (77.5%), fibroblast-like cells (20%), and giant cells (2.5%; Fig. 1*c*). The round cells have a mean diameter of 21.96± 5.57 μm. The fibroblast-like cells measured 58.05± 12.66 μm in length and 14.4±3.84 μm in width. The giant cells measured 60.3±9.2 μm in diameter. Most of the CAF-Clan II cells grew as a monolayer attached to the flask bottom. The cells frequently formed multicellular aggregation 7–8 d after subculture. The CAF-Clan II cell population at passage 20 showed two dominant morphologies: round cells (60%) and fibroblast-like cells (40%; Fig. 1*d*). The mean size of the round cells was 14.94± 5.1 μm in diameter and the fibroblast-like cells measured 65.84±32.31 μm in length and 10.26±1.79 μm in width.

Growth curves were determined at the 22nd passage for CAF-Clan I and the 24th passage for CAF-Clan II. Cells were seeded at a density of 2×10^5cells/ml into 48 ml medium using two 24-well plates (Corning, NY). Viable cell counts were made every 24 h using a hemocytometer and Trypan blue staining (three counts) for 10 d. The population doubling time was calculated based on the regression equations during the exponential growth phase of the cells (Mcintosh and Ignoffo 1983). The population doubling time of CAF-Clan I at passage 22 and CAF-Clan II at passage 24 was 68.5 and 38.2 h, respectively (Fig. 2). When cell density was 0.9×10^6cells/ml, CAF-Clan I reached its stationary growth phase, whereas CAF-Clan II reached its stationary phase at 1.6×10^6 cells/ml.

Karyologic analysis of the two cell lines was carried out at passage 15 according to Takahashi et al. (1980). The chromosome number in CAF-Clan I and CAF-Clan II at passage 15 varied from 62 to 100 in the majority of cells, though a few cells exceeded 260 (n=30; Saitoh 1960), as shown in Fig. 3. A chromosome count of 140 cells revealed that the cell line CAF-Clan I consisted mostly of diploid cells (66%), although there were also polyploid and heteroploid cells.

Identification of the cell lines was carried out using DNA amplification fingerprinting (DAF) employing polymerase chain reaction (PCR; McIntosh et al. 1996). PCR conditions and primers were performed as described by Zhang et al. (2006). The CAF-Clan I and CAF-Clan II were confirmed to have originated from *C. anachoreta* by DAF-PCR analysis as shown in Fig. 4. Major bands in agarose

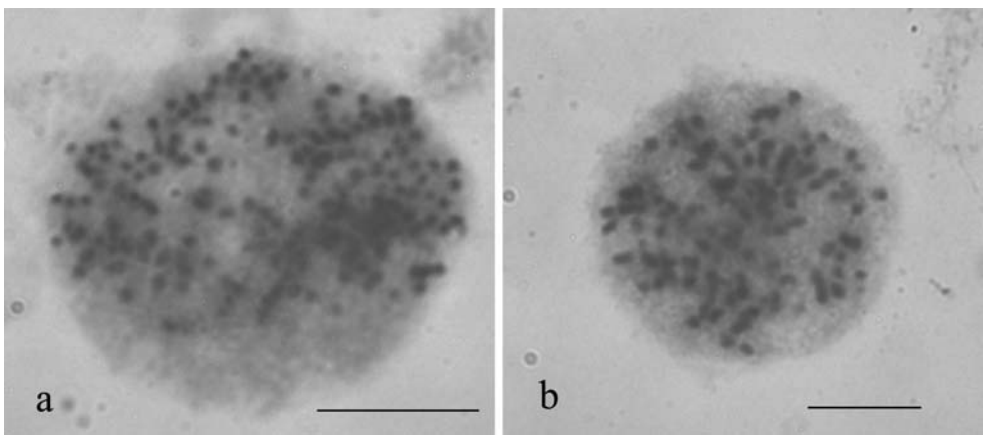

Figure 3. Karylogy of CAF-Clan I (*a*) and CAF-Clan II (*b*) showing a typical lepidopteron pattern. The *bar* is 10 μm.

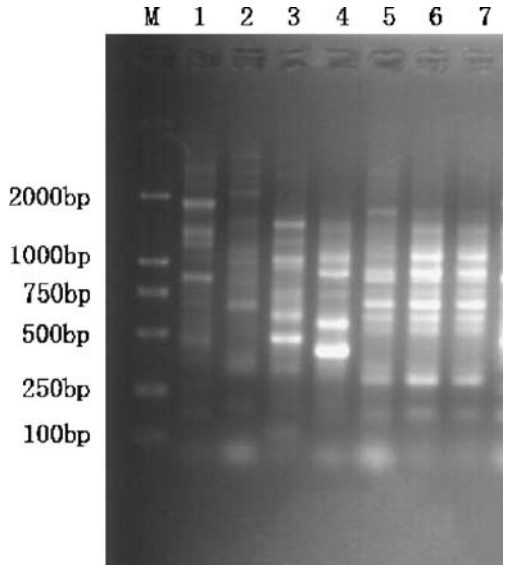

Figure 4. DAF-PCR profiles of CAF-Clan I and CAF-Clan II cell lines (*lanes 6*, *7*) and *C. anachoreta* eggs (*lane 5*) and other cell lines in our laboratory: Sl2, Sf9, BCRIL-HzAM1, IOZCAS-Spex II (*lanes 1–4*). Major bands identified are shared between the cell lines and their host while, the other cell lines show different profiles.

electrophoresis gel were shared by the two cell lines and their host, while the other cell lines showed a different profile. The DNA profiles of the CAF-Clan I and the CAF-Clan II were identical.

CAF-Clan II was found to be susceptible to AcMNPV and EoNPV. CAF-Clan I was slightly susceptible to AcMNPV, while non-permissive to EoNPV. The budded viruses (BVs) of EoNPV were prepared in *E. oblique* late third-instar larvae by feeding the occlusion bodies [1×10^7 occlusion bodies (OBs)/ml] on contaminated diets. Four days later, the larvae were surface-sterilized and bled to obtain the hemolymph containing BVs. In brief, one of the larval prolegs was cut with sterile scissors and the hemolymph was collected in a centrifuge tube containing 1 ml TNM-FH medium with phenylthiourea to prevent hemolymph melanization. The mixture was centrifuged at 800×*g* for 5 min and the supernatant was filtered with a 0.22-μm filter. The inoculum constituted the first passage of BVs and was either used immediately or stored at −20°C. The BVs of AcMNPV was prepared in the larvae of *Helicoverpa armigera* by the above method.

Cells was seeded into T-25 flasks with approximately 3×10^6 cells/flask and infected with 0.2 ml of the respective virus suspensions after 24 h. The cells were incubated at 27°C for 2 h, gently shaken at 15-min intervals, and fed with 5 ml fresh medium after the third wash with TNM-FH medium. The cultures were observed every 2 d for OBs under a microscope. On the third day post-infection, 10% and 30% CAF-Clan II cells contained AcMNPV and EoNPV OB, respectively, which increased to 75% and 90% on the tenth day post-infection. The cells began to aggregate in the late infection and numerous OBs were released into the medium. The cells detached from the growth surface and floated in the medium when infected with EoNPV on the 18th day post-infection (Fig. 5). CAF-Clan I cells infected with AcMNPV became round in shape, with a large number of OBs showing Brownian motion on the eighth day post-infection. After the cells were lysed with 1% sodium dodecyl sulfate for 1 h at room temperature, the number of OBs from the cell lines were counted using a hemocytometer (three counts), and OBs/cell were calculated according to the virus yield and the density of the cells (Granados et al. 1994). OB production of CAF-Clan II was 7.5×10^7–8×10^7 OBs/ml (129±4 OBs/cell) and 6.4×10^7–8.25×10^7 OBs/ml (124±15OBs/cell) for AcMNPV and EoNPV, respectively. The cell line CAF-Clan I yielded 1.85×10^7–2.75×10^7 OBs/ml (36±8 OBs/cell) on the 18th day post-infection.

Examination of the susceptibility of the cell lines to other viruses is still being performed. Attempts should be made in the future to produce more homogeneous cell lines which might increase the multiplication rate of selected virus and remain permanently virus-susceptible by selectively cloning permissive cell types. Cloned cell lines also can provide significant assistance in the large-scale production of recombinant proteins.

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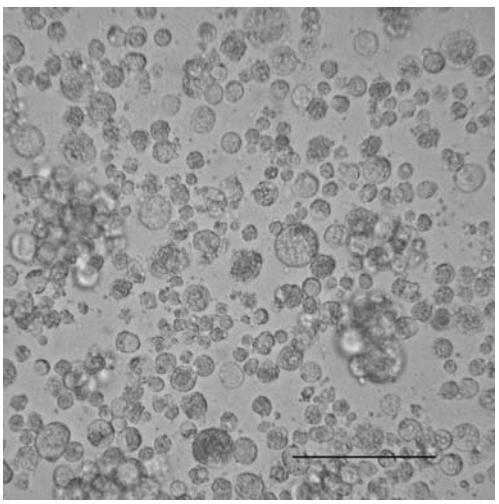

Figure 5. CAF-Clan II cells detached from the flask bottom and floated in the medium when infected with EoNPV on the 18th day post-infection. The *bar* is 100 μm.

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