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A new *Bombyx mori* larval ovarian cell line highly susceptible to nucleopolyhedrovirus

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Abstract

Lepidopteran cell lines constitute the backbone for studying baculoviral biology *in culturo* and for baculovirus vector based recombinant protein expression systems. In the present study, we report establishment of a new continuous cell line designated as DZNU-Bm-1 from larval ovaries of the silkworm, *Bombyx mori*. The cells were grown in MGM-448 insect cell culture medium supplemented with 10% fetal bovine serum (FBS) and 3% heat inactivated *B. mori* haemolymph at 25 ± 1 °C. A large number of attached epithelial-like and round refractive cells migrated from the explants and multiplied in the primary cultures. Both type of cells were subcultured initially for a few passages but after 10 passages the round refractive cells dominated the population, which could be subcultured continuously using MGM-448 medium with 10% FBS. The population doubling time of cell line was about 42 h at 25 ± 1 °C. The cell populations were largely diploids and triploids, while a few tetraploids and hexaploids were also observed. DNA profiles using Inter Simple Sequence Repeat (ISSR)-PCR and Simple Sequence Repeat (SSR) loci established the differences between DZNU-Bm-1 cell line and most widely used BmN cell line and the *B. mori* W-chromosome specific sequences confirmed the origin of DZNU-Bm-1 cell line to be from female silkworm. When cells were infected with free nonoccluded *B. mori* nucleopolyhedrovirus (BmNPV), the cell line was found to be highly susceptible with 92–94% of the cells harbouring BmNPV and having an average of 20–23 OBs/infected cell. We suggest the usefulness of this cell line in BmNPV based baculoviral expression system and also for studying in culturo virus replication.

Keywords: *Bombyx mori*; Ovarian cell line; DZNU-Bm-1; DNA fingerprinting; BmNPV infection; Baculoviral expression system

1. Introduction

Insect cell lines have become important tools in biological, biotechnological, biopharmaceutical, and biopesticide research. This is true with lepidopteran cells. They are commonly used to study insect viruses (Blissard, 1996) and have been considered for production of certain virus species as biopesticides or for recombinant proteins. Two cell lines from *Spodoptera frugiperda* (fall armyworm) and *Trichoplusia ni* (cabbage looper) are currently in use with baculovirus expression vector (BEV) derived from *Autographa californica* nucleopolyhedrovirus (AcNPV). However there are alternate baculoviruses, which are suitable for cloning of heterologous genes. *Bombyx mori* nucleopolyhedrovirus (BmNPV) is one such baculovirus, which is being used in BEV system for expression of recombinant proteins in susceptible cell lines (Maeda, 1987, 1989; Maeda et al., 1991). Further, cell lines derived from *B. mori* could be genetically engineered to continuously express high levels of foreign protein *in vitro* (Farrell et al., 1998, 1999). These studies show that, like AcNPV–*S. frugiperda*/*T. ni* system, BmNPV–*B. mori* cell lines are equally useful for development of BEV.

There are some established cell lines of *B. mori* originated from embryonic tissues (Chen et al., 1988; Imanishi et al.,

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1988; Inoue and Mitsuhashi, 1984; Ninaki et al., 1988; Pandharipande, 1994) and larval and pupal ovarian tissues (Grace, 1967; Quiot, 1982; Sudeep et al., 2002) but only a few can support replication of BmNPV. Agathos (1993) in his review reported that there is an urgent need for more efficient and more widely applicable cell lines as host for baculovirus replication and foreign gene expression. As it is almost a few decades since the establishment of most commonly used BmN cell line, there is a necessity to introduce a new cell line derived from a commercially successful variety of the silkworm. Such a cell line will be useful in study of BmNPV replication vis-à-vis BmN cell line with respect to effect of strains and effect of the age of the cell lines. Since BmNPV can infect larval ovary of *B. mori* and also transmits to next generation (Khurad et al., 2004), in the present study, a new cell line was developed from ovarian tissue of a commercial variety of the silkworm. We confirmed the origin of the cell line to be silkworm specific by SSR markers and a *B. mori* W-chromosome specific sequence (Nagaraja et al., 2005; Prasad et al., 2005; Reddy et al., 1999). We have also shown that this new cell line is highly susceptible to BmNPV infection.

2. Materials and methods

2.1. Silkworm

Healthy female fifth instar larvae of day one of 'Swarnandhra' variety were collected from the rearing stock maintained at Centre for Sericulture and Biological Pest Management Research (CSBR) of Nagpur University. This is a commercial variety reared in many parts of Andhra Pradesh, Karnataka, and West Bengal, India.

2.2. Primary cultures

About 10–15 female larvae were used to initiate primary culture in a 25 cm^2 Falcon culture flask. Each of the larvae was surface sterilized by submersion in 70% Ethyl alcohol for 2–3 min and dissected under a stereoscopic binocular microscope. The rudiments of ovary from the seventh and eighth segment were removed, washed in Carlson's fluid (Carlson, 1946) thrice and transferred into a cavity block containing culture medium. About 10–15 pairs of pooled ovaries were cut into small pieces and fragments were explanted in culture flask with culture medium. The culture medium used was the MGM-448 (Mitsuhashi, 1984) enriched with 10% fetal bovine serum (FBS) and 3% heat inactivated *B. mori* haemolymph (60 °C for 30 min). The cultures were maintained at 25 ± 1 °C in conditioned medium by replenishing half of the medium once a week.

2.3. Subculturing

The cells were detached by flushing the medium over the monolayer with Pasteur pipette and split into two flasks. Suspension containing cell masses and single cells were subcultured with split ratio of 1:2 using plastic (Falcon) and glass (Abico, Japan) culture flasks.

2.4. Growth analysis

Growth of the cell line was determined at 25th passage by seeding each culture flask with 2×10^5 cells/ml. On alternate day cell suspension was sampled from two culture flasks and cell number was counted using haemocytometer. Cell population doubling time was calculated using the exponential formula of Hayflick (1973). The viability was determined by dye exclusion test using Trypan blue stain. The cells were also adapted to MGM-448 with 10% fetal bovine serum (FBS) and cell growth was estimated.

2.5. Chromosome analysis

Demicolcine at a final concentration of 1 μg/ml was added to the cultures to halt divisions in metaphase and after 24 h cells were harvested, treated with hypotonic solution (0.6% KCl) and fixed in 50% glacial acetic acid for 10 min. Cell smears were stained with Acetorcein. Chromosome number and range were determined by counting 100–150 chromosome spreads.

For Giemsa staining, the cells were lysed in hypotonic solution (0.6% NaCl) and washed repeatedly in fixative (methanol:acetic acid in 3:1 ratio). After the final wash, cell smears were stained with Giemsa (0.7% (w/v) Giemsa in 1:1 methanol:glycerol) for two min, washed in distilled water and air-dried. Chromosome preparation was analysed using Cytovision software (Zeiss).

2.6. DNA profiling

DNA was isolated from DZNU-Bm-1, BmN and NIAS-MaBr-92, *Mamestra brassicae* (Mitsuhashi and Shozawa, 1985) cell lines by standard protocol of Nagaraja and Nagaraju (1995), with the exception that Proteinase K treatment was not given. The quality and quantity of the isolated DNA was confirmed by agarose gel electrophoresis.

SSR- and ISSR-PCR were carried out according to Nagaraju et al. (2001). The details of the primers used are given in Table 1. Briefly, SSR-PCR was performed using a Perkin–Elmer 9700 Thermal cycler in a typical PCR reaction of 10 μl containing 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl and 0.1% Triton X-100), 100 μM of each dNTPs, 400 nM of SSR primers, 1 unit of Taq polymerase (Perkin–Elmer Cetus) and 20 ng of template DNA with different annealing temperature and $MgCl_2$ concentration (Table 1). Bands were then separated by 2.0% agarose gel electrophoresis. Gel images were captured in Biorad gel documentation system.

For ISSR-PCR, reactions were carried out in a volume of 10 μl containing 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl and 0.1% Triton X-100), 100 μM of each dNTPs, 400 nM of ISSR primers, 1 unit of Taq polymerase (MBI Fermentas), 2.5 mM $MgCl_2$ and 20 ng of template DNA