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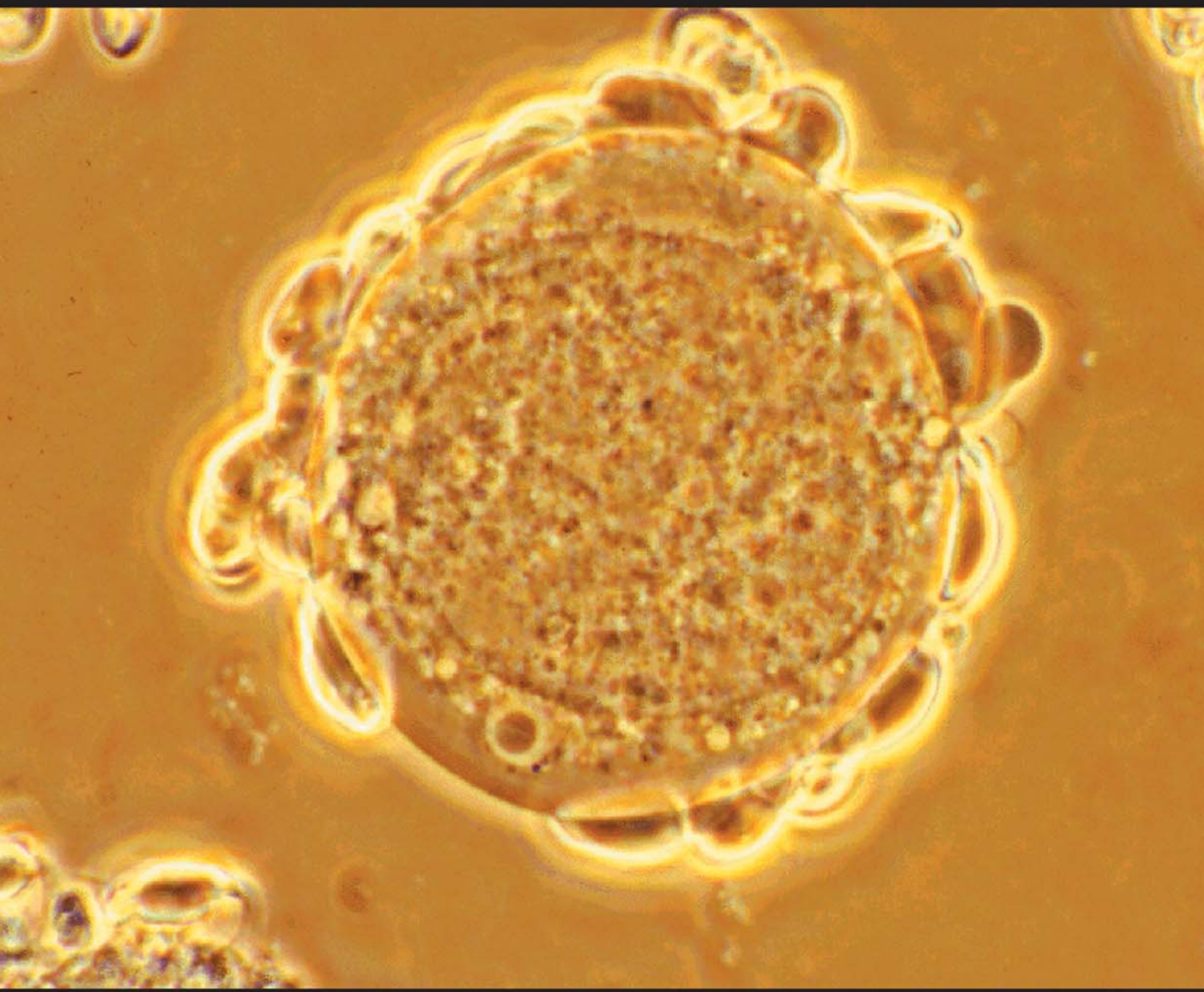

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Growth Characteristics and Expression of Recombinant Proteins by New Cell Clones Derived from *Trichoplusia ni* (BTI Tn5B1-4) High Five™ Cells

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It is well known that the characteristics of a cultured cell line do not always remain stable and may change upon continuous passage. Most continuous cell lines, even after cloning, possess several genotypes that are constantly changing. There are numerous selective and adaptive culture processes, in addition to genetic instability, that may promote phenotypic changes in cell growth, virus susceptibility, gene expression, et cetera. Similar detrimental effects of long term passaging of insect cells have also been reported for continuous cell lines. In this paper, we describe the isolation of cell clones from low passage BTI TN5B1-4 cells (High Five™ Cells), and report their growth characteristics and high level of recombinant protein production.

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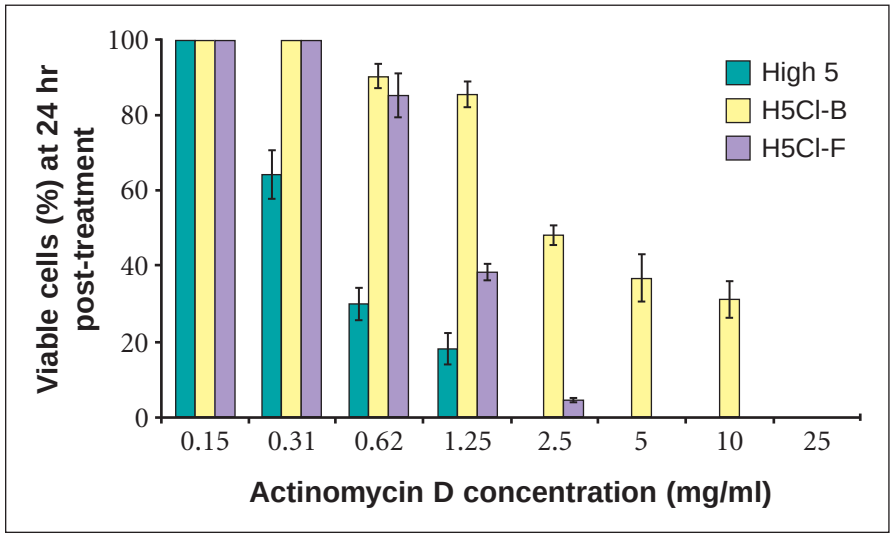

Fig. 2 Resistance of High 5, H5Cl-B and H5Cl-F to Actinomycin D. Wells of a 24-well plate were seeded with each cell line at a concentration of $1x10^5$ cells and cultured for 24 hrs in TNMFH medium containing varying concentrations of Actinomycin D. More than 100 cells in multiple areas of each well were examined under a phase contrast microscope. The test was triplicated and the bar on each histogram indicates +/- SD.

Introduction

BTI Tn5B1-4 (High 5) cells, as well as IPLB-SF21 and its clone (SF9), are the most widely used insect cell lines for the baculovirus expression vector system.[1,2] The maintenance and growth of these, and other, insect cell lines need particular attention since the characteristics of cells may change upon long-term passaging.[3,4,5] In many instances, High 5 cells provide superior production of recombinant proteins when compared to SF9 cells; however, this high productivity may be more evident in low passage cells.[6] Donaldson and Shuler reported that early passage High 5 cells (passage 130) expressed more recombinant secreted alkaline phosphatase (SEAP), and were smaller in size than high passage cells (passage 360).[5] We have seen a similar effect and found that High 5 cells, at passage 85, were superior in expression of both β-galactosidae and SEAP than cells at passage 531 (R. Granados, unpublished). In our laboratory, the lowest passage number of liquid nitrogen-frozen High 5 cells is passage 78. In this paper, we report the cloning of low passage High 5 cells to determine if phenotypic variation occurs at "low" passage, and to maintain the high productivity characteristics of this cell line.

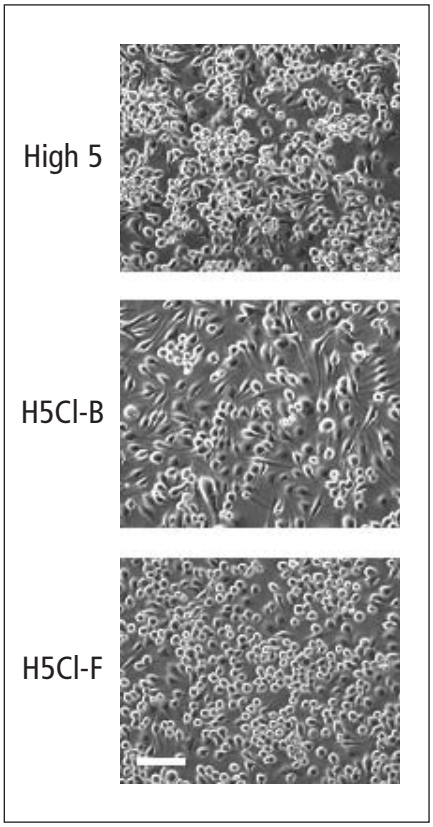

Fig. 1 Phase contrast microscopic photographs of High 5, H5Cl-B, and H5Cl-F cells. Internal scale represents 100 μm.

Materials and Methods

High 5 cells at passage 90 were used for cell cloning. Briefly, three rounds of cell cloning were carried out with a 24-well cell cloning plate (Greiner Labotechnik), that consists of 16 grids (each 2 mm square) per well. Seven cell clones were obtained and subjected to preliminary screening and evaluation. This screening consisted of cell selection for rapid growth characteristics, high susceptibility to *Autographa californica* nucleopolyhedrovirus (AcNPV) infection, and high expression of β-galactosidase. Two new clones, High 5 clone B (H5Cl-B) and High 5 clone F (H5Cl-F), were selected for further comparative analysis with the parental High 5 cells. At the start of the analysis, the High 5 cells, and both cell clones, were at passage 161 and 43, respectively. To confirm the genetic origin of cell clones as embryonic cells from *Trichoplusia ni* (*T. ni*), RAPD analysis was carried out with their genomic DNA using a *T. ni* specific primer, 5'ttgctgtcca 3'.[7] The genomic DNA from SF9 cells (negative control) and both High 5 and TnMG1 cells (positive controls) were used for comparative purposes. H5Cl-B and H5Cl-F cells were characterized for viability in phosphate buffered saline (PBS), with pH 7.0 and resistance to Actinomycin D. Viability of the cells was measured at different time points by a trypan blue (Sigma) exclusion test. Recombinant baculoviruses with SEAP and β-galac-

tosidase genes were used for recombinant protein production.[6,8] The cultures were maintained in TNMFH medium containing 10% FBS (GIBCO/Invitrogen), and were subcultured every three to four days.

Results and Discussion

Both the High 5 Cells and the two clones grew as anchorage dependent cells in T-flasks. The morphology of High 5 cells, grown in TNMFH medium, generally consists of spindle cells with the presence of some round cells (Fig. 1). The combined size of these cells was 16.6 x 38.4 mm. The H5Cl-B cells were comprised of mainly spindle shaped cells with a size of 17.9 x 48.8 µm. The H5Cl-F cells consisted of slightly smaller spindle and round cells, which measured 17.2 x 37.8 µm. RAPD analysis of the genomic DNA of both cell clones confirmed their genetic identity as *T. ni* cells (data not shown). One-step growth curves showed a similar pattern and population doubling times: 26.7 hrs, 26.3 hrs, and 25.4 hrs for High 5, H5Cl-B and H5Cl-F cells, respectively (data not shown).

To characterize the physiological phenotype of both H5Cl-B and H5Cl-F cell clones, we examined their resistance to nutritional stress in PBS and Actinomycin D, which are known as typical elicitors of apoptosis of cultured cells.[9] When the cell clones were exposed to PBS for one to four days, H5Cl-B showed high resistance to nutrient stress, in comparison with H5Cl-F and High 5 cells. All of the cells in treated cultures of High 5, H5Cl-F, and H5Cl-B died within two, three, and five days, respectively. Cell viability after one day in PBS was over 60% with H5Cl-B, but only 4% and 11% with High 5 and H5Cl-F, respectively (data not shown). When the cell clones and High 5 cells were exposed to an Actinomycin D concentration of 1.25 mg/mL for 24 hrs, cell viability was 18, 38, and 85% for High 5, H5Cl-F, and H5Cl-B cells, respectively (Fig. 2). At a concentration (2.5 mg/mL) that killed all the High 5 cells in 24 hrs, 5% of H5Cl-F and 48% of H5Cl-B cells were still viable. In these assays, it was demonstrated that both clones were more tolerant to cell stress than the parental High 5 cells, and that H5Cl-B cells were more resistant to cell stress than H5Cl-F.

The susceptibility of both clones and High 5 cells to wild-type AcNPV was approximately the same at four days post infection (p.i.) with an infection of over 95% of the cells. At four days p.i. with AcNPV, the H5Cl-B cells exhibited cell lysis and approximately 50% of the cells released many polyhedra into the culture medium (data not shown). However, the H5Cl-F and High 5 cells showed little evidence of lysis at this time point. The production of budded virus by the clones and High 5 cells was lower (average of $1.8x10^7$ $TCID_{50}$ /mL) in comparison to the higher level produced in SF9 cells ($1.5x10^8TCID_{50}$/mL; data not shown). In contrast, the production of occlusion bodies (OBs) by both the clones and High 5 cells was high (average of 100 OBs/cell), and outperformed SF9 cell production of OBs by a factor of 2.5-fold.

Both H5Cl-B and H5Cl-F cells showed higher levels of β-galactosidase production in comparison to High 5 cells. At six days p.i., H5Cl-B and H5Cl-F cells produced 30 and 45% more β-

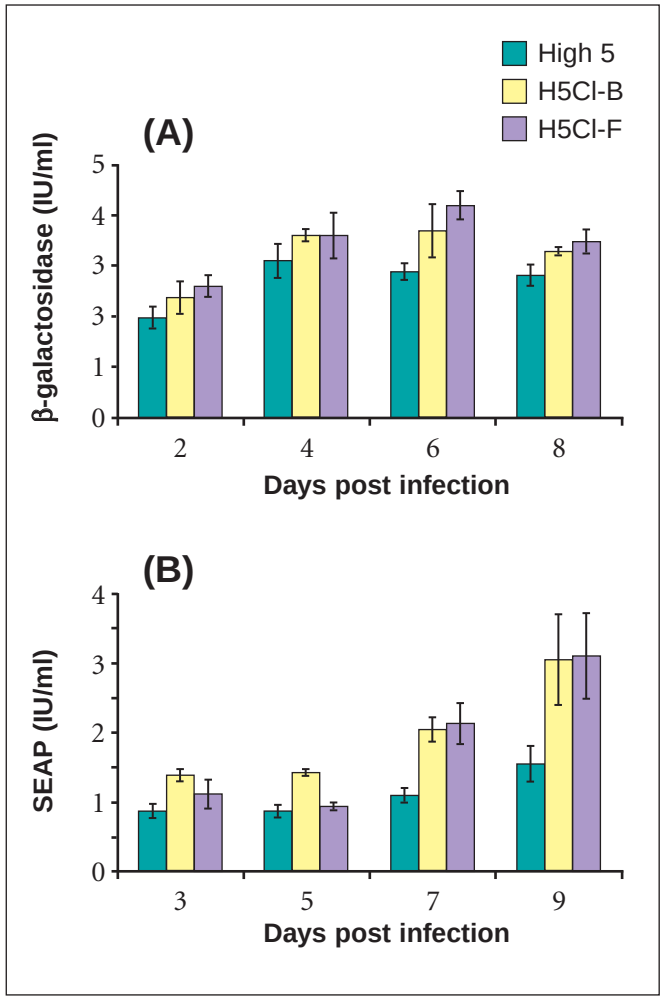

Fig. 3 Recombinant β-galactosidase (A) and SEAP (B) production in High 5, H5Cl-B and H5Cl-F cells. Log phase cells, at a concentration of $1.0x10^5$ cells, were seeded in each well of a 24-well plate. They were then inoculated with a recombinant AcNPV that carried the β-galactosidase or SEAP genes, under control of the polyhedrin promoter, at a multiplicity of infection of 10 $TCID_{50}$ units/mL. After allowing the virus to adsorb onto the cells for one hour, the cells were washed once with fresh medium, and then maintained for eight days for β-galactosidase, and nine days for SEAP production.

galactosidase than High 5 cells, respectively (Fig. 3A). Similarly, both H5Cl-B and H5Cl-F cells produced significantly more secreted SEAP than High 5 cells (Fig. 3B). Starting at seven days p.i., both clones produced more secreted SEAP than High 5 cells, and at nine days p.i., the clones expressed two-fold more SEAP as compared to High 5 cells.

In this article, we report the successful isolation and characterization of two cloned lines from High 5 cells at passage 90. This outcome is a relatively low passage of the High 5 cells, and it is likely that we isolated high expression-type cell clones present in these cells, and that genetic variability already existed in the High 5 cells at passage 90. We cannot exclude the possibility that other high-producing cells may be obtained from higher passage High 5 cells, or the possibility that the cell cloning process changed the phenotype of the cells. At this time, it is difficult to estimate at which passage these cells may begin to lose some of their ability for high expression of recombinant proteins, since there may be many unknown culture factors and mutation events that cannot be predicted. However, even at "high" (+300) passages (Granados, unpublished), the High 5 cells are still very high producers when compared to other available cell lines.[7] Both cell clones, H5Cl-B and H5Cl-F, are higher producers of β-galactosidase and SEAP than the parental cells. In particular, the clones showed a significant increase in the secretion of SEAP into the culture medium. Also, the high viability of these cells under conditions of physiological stress may be an excellent characteristic for scale-up of the cultured cells. Since High 5 has been considered as the highest producer among currently available lepidopteran cell lines, these distinct characteristics with the H5Cl-B and H5Cl-F clones could provide significant application in large-scale production of recombinant proteins.

Conclusions

Two clonal cell lines were obtained from the parental cell line, BTI Tn5B1-4 (High 5) at passage 90. Both clones, designated as H5Cl-B and H5Cl-F, exhibited a distinct morphology and a similar growth rate in serum-containing TNMFH medium. Clone H5Cl-B was remarkably resistant to nutrient stress in phosphate buffered saline while both clones were highly resistant to Actinomycin D. Both H5Cl-B and H5Cl-F clones produced 30 and 45% more recombinant β-galactosidase than the parental High 5 cells. Similarly, both clones produced 100% more secreted alkaline phosphatase than the parental cells. Continued vigilance in the culture of cells, and in the maintenance of frozen stocks of early passage cells of any insect cell lines, is highly recommended.

Acknowledgements

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