STUDIES ON THE SPODOPTERA EXIGUA NUCLEAR POLYHEDROSIS VIRUS INFECTION IN SPODOPTERA EXIGUA CELL LINES

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CHAPTER IV

A Cloned Cell Line of *Spodoptera exigua* with High Susceptibility to the *Spodoptera exigua* Nuclear Polyhedrosis Virus

In the preceding Chapter, a technique for *Spodoptera exigua* nuclear polyhedrosis virus (SeNPV) plaque formation on the *S. exigua* Se3FH cell line was established. This technique enabled characterization of the *in vitro* SeNPV infection and the cloning of the virus. The Se3FH cell line, however, is an uncloned cell line consisting of several heterogeneous cell types. Approximately 70% of the cells of Se3FH supported the replication of SeNPV. It is, therefore, worthwhile to select cell clones supporting high virus production for basic studies as well as for practical applications. Lenz *et al.* (1991) reported that the cloning of *Heliothis zea* cell lines resulted in increased levels of *H. zea* NPV production.

In the present study, a daughter clone was selected from the Se3FH cell line and was examined for susceptibility to a plaque purified SeNPV isolate and the wild isolate, and for its relative capability to serve as an indicator cell for the plaque assay. Also, recombinant *Autographa californica* NPV (AcNPV) encoding ß-galactosidase was tested to determine whether the foreign genes were expressed in *S. exigua* cell lines.

MATERIALS AND METHODS

Cells and Viruses

The continuous cell lines, *S. exigua* Se3FH and *Spodoptera frugiperda* SF9 were maintained as described in Chapter II. The cells were grown in IPL-41 medium with 10% heat-inactivated fetal bovine serum (FBS) at 27°C in 25-cm^2 tissue culture flasks.

The plaque purified isolate, SeNPV-I1, was cloned from SeNPV wild isolate (SeNPV-IW) as described in Chapter III. Recombinant AcNPV encoding a 120K polyhedrin/ß-galactosidase fusion protein under the control of the polyhedrin promoter (Ac360-*501ß-gal*) (Luckow and Summers, 1988) were obtained from Dr. T. Hara, Faculty of Agriculture, Kyushu University, Japan. These viruses were propagated in the Se3FH cell line and stored at -80°C until use.

Cloning of Cells

Cell cloning was done by limiting dilution methods. The Se3FH cells in exponential phase were seeded (0.2 ml/well) in Microtestplate-II (Falcon, 3042) at a density of 5 cells/ml in IPL-41 medium containing 10% FBS and 20% conditioning medium. The conditioning medium was prepared from cell culture fluid of Se3FH cells in exponential phase. The plates were sealed and incubated at 27°C. Only wells that contained single cells 24 h after seeding were screened and approximately 0.1 ml of medium was replaced weekly with fresh medium. Each developing cell colony was detached from the well by using a Pasteur pipette and the cell suspension was divided and delivered into 3

wells each containing 0.15 ml of fresh medium. After reaching confluence, cells were transferred to a multiwell tissue culture plate (Falcon, 3014) in 0.5 ml fresh medium and then the plate was centrifuged at 1,000 rpm for 10 min at 27°C to enhance cell attachment to the well bottom. Six days later, the first passage was performed in a multiwell tissue culture plate. Then, cells which formed a complete monolayer were transferred to a 25-cm^2 tissue culture flasks containing 3 ml of fresh medium, followed by subculture at 4-day intervals. Cloned cell line used in this study has undergone over 100 serial passages.

Kinetics of Cell and Virus Growth

The kinetics of cell growth was analyzed by monitoring the cell densities in 25-cm^2 flasks as described previously. Viable cells assessed with trypan blue (Gibco) were seeded in flasks at a density of 0.2×10^6 cells/ml. Five independent cell counts were made using a hemocytometer on cells sampled from a series of flasks every 24 h for a 7-day period. Cell doubling time was calculated during the exponential growth phase.

The virus growth kinetics were determined as described in Chapter III. Cells in exponential phase (0.6×10^6 cells/ml) were seeded into the wells of a multiwell tissue culture plate (0.4 ml/well). Cells were then inoculated with virus at a MOI of 1.0 PFU per cell. The virus adsorption was performed by centrifuging at 1,800 rpm at 27°C for 1 h. Subsequently, the inoculum was removed and the cells were washed with culture medium, then fresh medium was added. At various time points after inoculation, the medium was removed and centrifuged at 3,000 rpm for 10 min. The supernatant containing the infectious extracellular viruses (ECVs) was stored at -80°C until titration

using the plaque assay. Cell infection rate was determined on the basis of the formation of polyhedral inclusion bodies (PIBs) in 1,000 cells. For quantification of PIBs, 1% SDS was added to infected cultures at 120 h p.i. to release PIBs and incubated at 37°C for 30 min. The released PIBs were quantified by a hemocytometer.

Plaque Assay

Virus samples were titrated by a plaque assay as described in Chapter III. Logarithmic phase cells were seeded into a multiwell tissue culture plate at a density of 1.0×10^6 cells /ml (0.4 ml/well) in IPL-41 medium supplemented with 10% FBS. Appropriate dilutions of the virus in 0.1 ml of IPL-41 medium were added as inocula. Subsequently, the plate was centrifuged at 1,800 rpm for 60 min at 27°C. The inocula were then removed, and the cells were overlaid with 0.3 ml/well of 0.75% (w/v) Seaplaque agarose in IPL-41 medium containing 10% FBS at 36°C. The plates were incubated for 6 to 7 days at 27°C and the number of plaques were counted employing indirect lighting without staining.

Recombinant Virus Infection and ß-galactosidase Assay

Expressions of both extracellular and intracellular ß-galactosidases were compared on Se3FH, Se301, and SF9 cell lines by infection of a recombinant AcNPV. Cells in the multiwell plate were infected with a recombinant AcNPV containing ß-galactosidase gene at a MOI of 10 PFU per cell, as described in Chapter III. At suitable intervals, the medium was removed, centrifuged at 6,000 rpm for 5 min, and the supernatant was stored at -80°C as samples for extracellular ß-galactosi-

dase assay. Attached cells were washed twice with PBS (Mg^{2+} and Ca^{2+} free) and incubated with lysis buffer (Promega Co.) for 15 min at 27°C. Lysed cells were centrifuged at 6,000 rpm for 5 min to remove cellular debris and the supernatant was stored at -80°C as samples for intracellular ß-galactosidase assay.

An appropriate diluted sample was incubated with assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 50 mM ß-mercaptoethanol, 0.67 mg/ml *O*-nitrophenyl-ß-D-galactopyranoside) (Promega Co.) for 30 min at 37°C. The reaction was stopped with 1 M Na_2CO_3. The absorbance at 420 nm was converted to units per well in 24-well plate of the ß-galactosidase by using standard curve.

RESULTS

Characterization of Cloned Cell Line

A single cell isolated from the Se3FH cell line successfully developed to grow to confluency in a 25-cm^2 tissue culture flask about two months after starting the culture. This cloned cell line, designated Se301, is comprised of adherent spherical cells (Fig. 14A). Cell morphology of the Se301 cell line was considerably uniform in contrast to the parent Se3FH cell line, which consists of spindle-shaped cells and spheroid cells (Fig. 14B). Fig. 15 shows the results of kinetics of cell growth. The initiation of the stationary phase in the Se301 cell line and the Se3FH cell line were day 6 and 5, respectively. Maximal cell density of the Se301 cell line was approximately 3.5×10^6 cells/ml, which was almost the same for the Se3FH cell line. The doubling time of the Se301 cell line (28 h) was 11 h longer than that of the Se3FH cell line.

Comparison of the Cloned Cell Line and the Parental Cell Line as Indicator Cells in a Plaque Assay

Visible plaques were formed on both the Se301 cell line and the Se3FH cell line without using any staining procedures. Plaques were first detected 3 to 4 days in the Se301 cell line, which was 1 to 2 days faster than the Se3FH cell line. The size of the plaques formed in the Se301 cell line ranged from 0.4 to 0.8 mm and was markedly larger than that of the parental cell line (Fig. 14E, F). Figure 16 shows the results of dose-response studies conducted to compare the capability to serve as indicator cells in the plaque assay. A proportional relationship

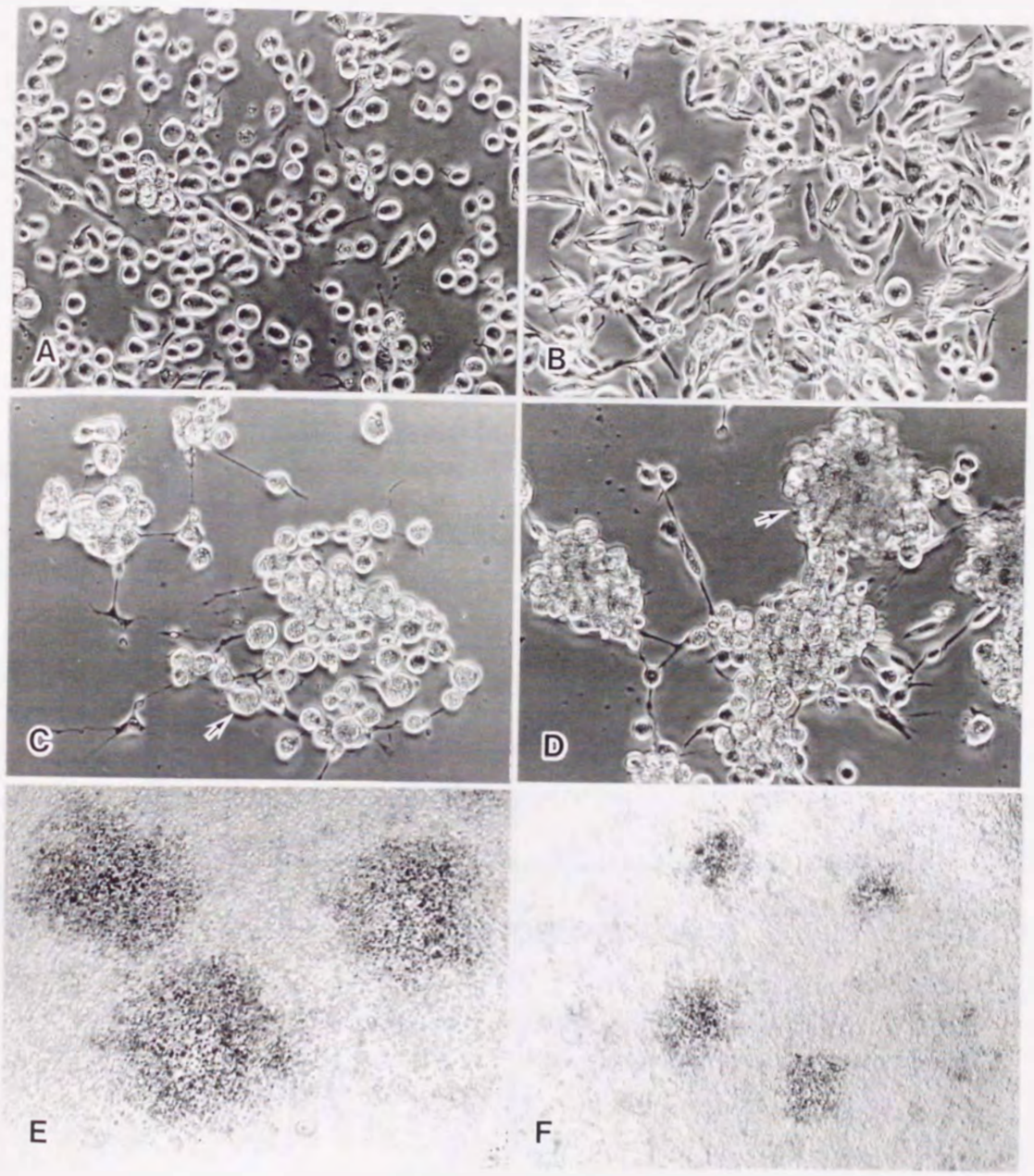

Fig. 14. Phase-contrast photographs of the cloned *Spodoptera exigua* Se301 cell line and its parental Se3FH cell line infected with the plaque purified isolate SeNPV-I1 of *S. exigua* nuclear polyhedrosis virus. A, B, C, D, × 250: E,F, × 40. A, uninoculated Se301 cell line; B, uninoculated Se3FH cell line; C, SeNPV infected Se301 cells 3 days p.i. Most cells contain PIBs (arrow). D, SeNPV infected Se3FH cells 3 days p.i. Note the aggregated cells containing PIBs in the nuclei (arrow). E, SeNPV plaques formed in Se301 cell line; F, A plaque of SeNPV formed in Se3FH cell line.

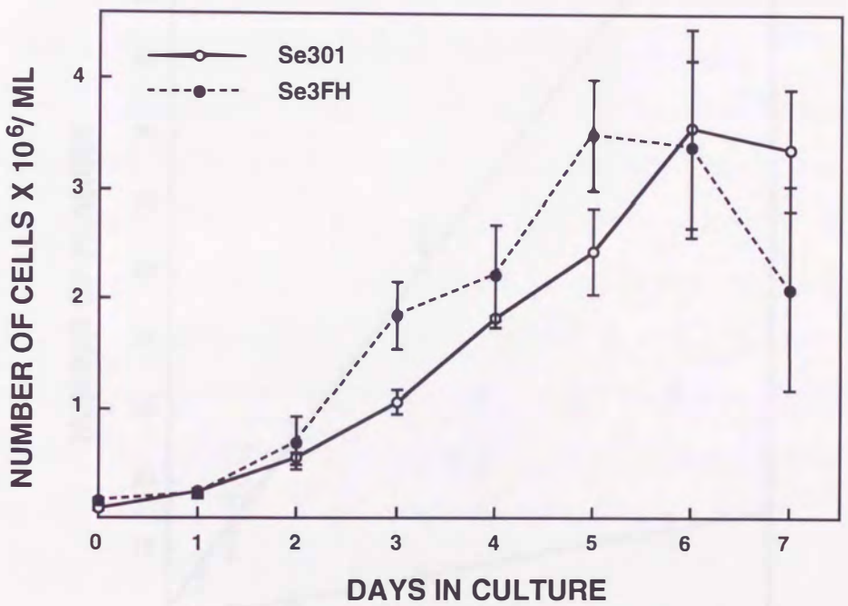

FIG. 15. Growth curves of the cloned *Spodoptera exigua* Se301 cell line and its parental cell line. Data represent means ± SE of a typical experiment (n = 5 / sample).

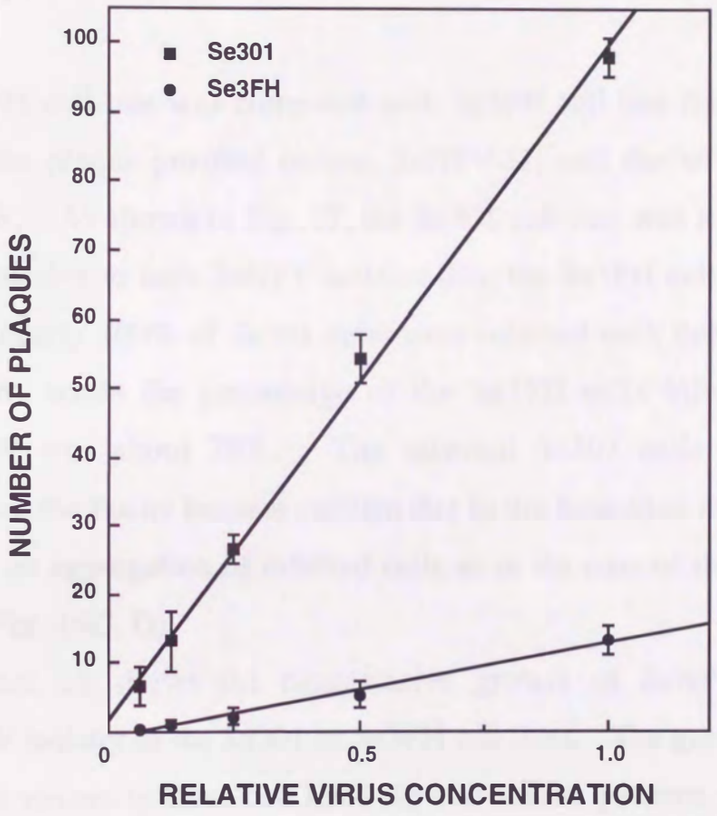

FIG. 16. Dose-response study of the plaque formation of the plaque purified SeNPV-I1 of *Spodoptera exigua* nuclear polyhedrosis virus in *S. exigua* Se301cell line and its parental Se3FH cell line. Data represent means ± SE of a typical experiment (n = 5 / sample).

between the concentration of virus and the number of plaques was observed both in the Se301 and Se3FH cell lines. The number of plaques formed on the Se301 cell line was approximately ten times greater than that formed on the parent Se3FH cell line.

Virus Replication

Se301 cell line was compared with Se3FH cell line for susceptibility to the plaque purified isolate, SeNPV-I1, and the wild isolate, SeNPV-IW. As shown in Fig. 17, the Se301 cell line was found to be more permissive to both SeNPV isolates than the Se3FH cell line. At 96 h p.i., nearly 100% of Se301 cells were infected with SeNPV-I1 or SeNPV-IW, while the percentage of the Se3FH cells infected with SeNPV-IW was about 70%. The infected Se301 cells gradually rounded and the nuclei became swollen due to the formation of PIBs but there was no aggregation of infected cells as in the case of the parental cell line (Fig. 14C, D).

Figure 18 shows the comparative growth of SeNPV-I1 and SeNPV-IW isolates in the Se301 or Se3FH cell lines. The growth kinetics of both viruses in these cell lines showed similar patterns with three phases of development: a latent phase ranging from 0 to 6 h p.i., an exponential phase ranging from 6 to 48 h p.i., and a stationary phase after 48 h p.i. However, for both viruses, the levels of ECV production varied depending on the cell lines used. For example, at 12 h p.i., ECV titer of SeNPV-IW in Se301 cells was 40 times greater than in Se3FH cells, and there was a 20-fold difference in SeNPV-I1 ECV titers between the two cell lines. A difference was also observed in ECV levels in a given cell line between two virus isolates. In Se301 cells, at 24 h

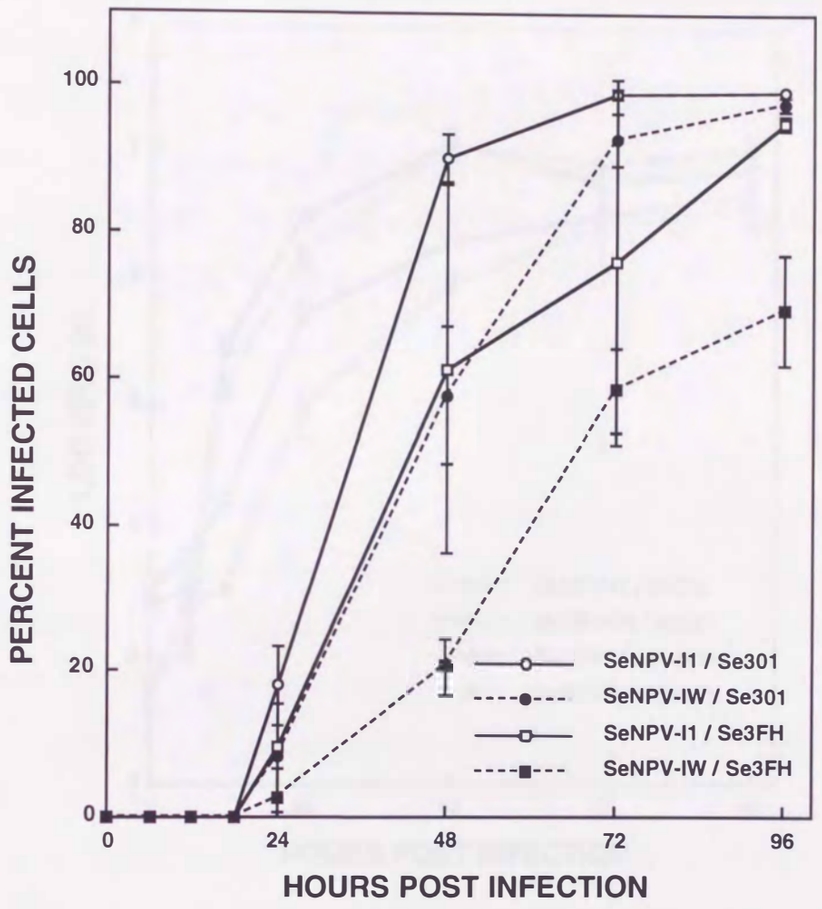

FIG. 17. Rate of virus infection in cells among the cloned *Spodoptera exigua* Se301 cell line and its parental Se3FH cell line inoculated with the plaque purified isolate SeNPV-I1 or the wild isolate SeNPV-IW of *S. exigua* nuclear polyhedrosis virus. Cells in logarithmic phase were infected with virus at a MOI of 1.0 PFU per cell. Cell infection rate was determined on the basis of the formation of PIBs in 1,000 cells. Data represent means ± SE of a typical experiment (n = 5 / sample).

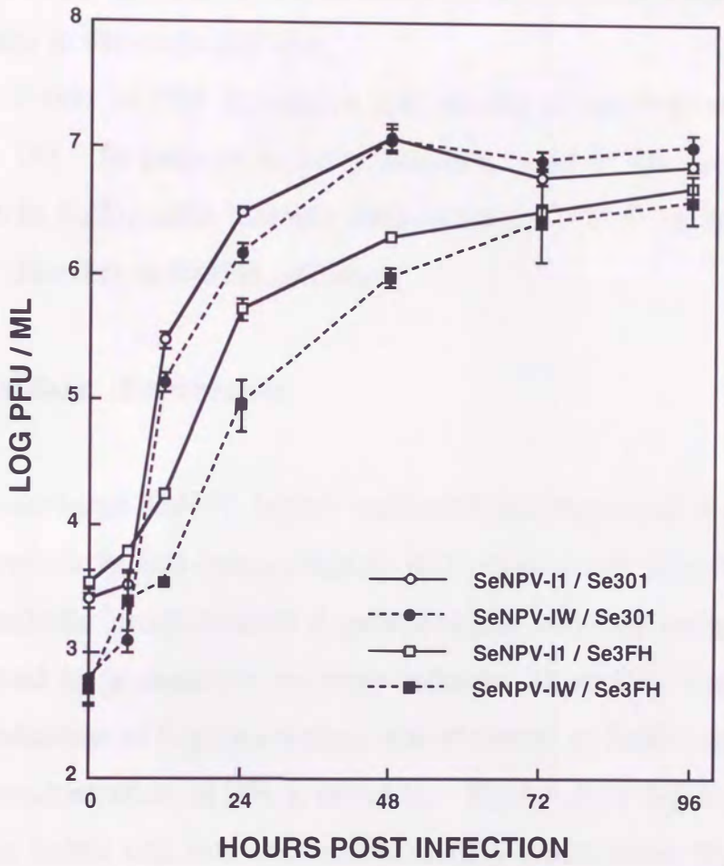

FIG. 18. Replication of the plaque purified isolate SeNPV-I1 and the wild isolate SeNPV-IW of *Spodoptera exigua* nuclear polyhedrosis virus in the cloned *S. exigua* Se301 cell line or its parental Se3FH cell line. Cells were infected with the virus at a MOI of 1.0 PFU per cell. The extracellular virus production was monitored by plaque assay using the Se301 cell line as indicator cells. Data represent means ± SE of a typical experiment (n = 5 / sample).

p.i., the ECV level of SeNPV-I1 was two times higher than that of SeNPV-IW. Similarly, in Se3FH cells, the level of SeNPV-I1 was four times higher than that of SeNPV-IW at 24 h p.i. A significant difference, however, was not observed in the maximal titer between two virus isolates in the same cell line.

The levels of PIB formation also varied depending on the cell lines (Fig. 19). In contrast to the production of ECV, the level of PIB production in Se301 cells infected with either SeNPV-I1 or SeNPV-IW was lower than that in Se3FH cell line.

ß-galactosidase Expression

Recombinant AcNPV highly replicated and expressed ß-galactosidase intracellularly and extracellularly in *S. exigua* cell lines (Fig. 20a, b). Intracellular production of ß-galactosidase was first detected 24 h p.i., followed by a dramatic increase between 48 and 72 h p.i. The highest production of ß-galactosidase was observed in Se301 cell line 72 h p.i at a concentration of 201 ± 10 units. The level of ß-galactosidase activities in Se301 cell line was approximately seven times higher than that in SF9 cell line in both intracellular and extracellular phases.

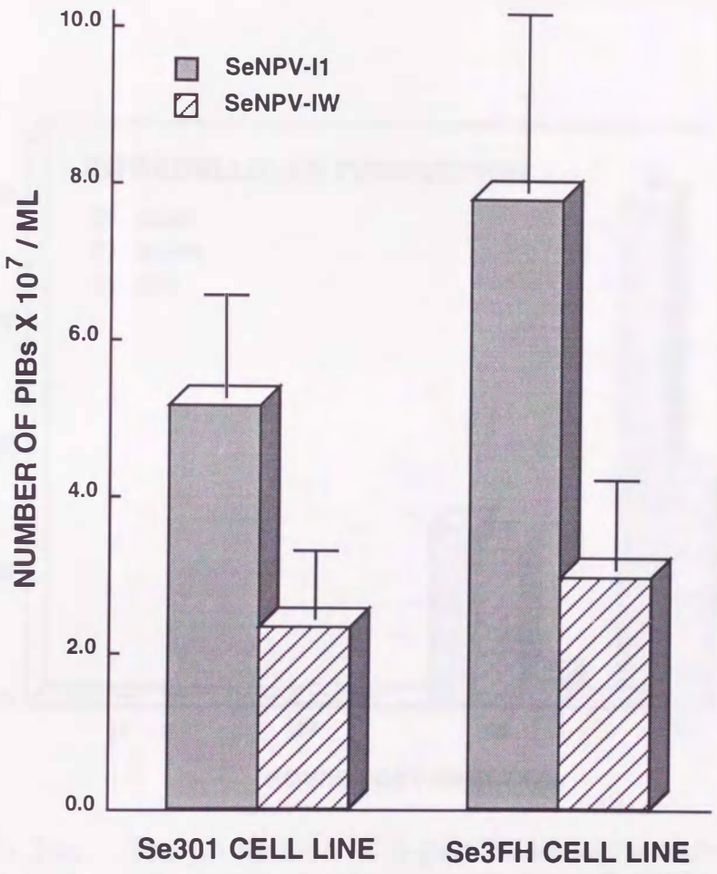

FIG. 19. Polyhedral inclusion body (PIB) production by the plaque purified isolate SeNPV-I1 and the wild isolate SeNPV-IW of *Spodoptera exigua* nuclear polyhedrosis virus in the cloned *S. exigua* Se301 cell line or its parental Se3FH cell line at 120 h p.i. Data represent means ± SE of a typical experiment (n = 5 / sample).

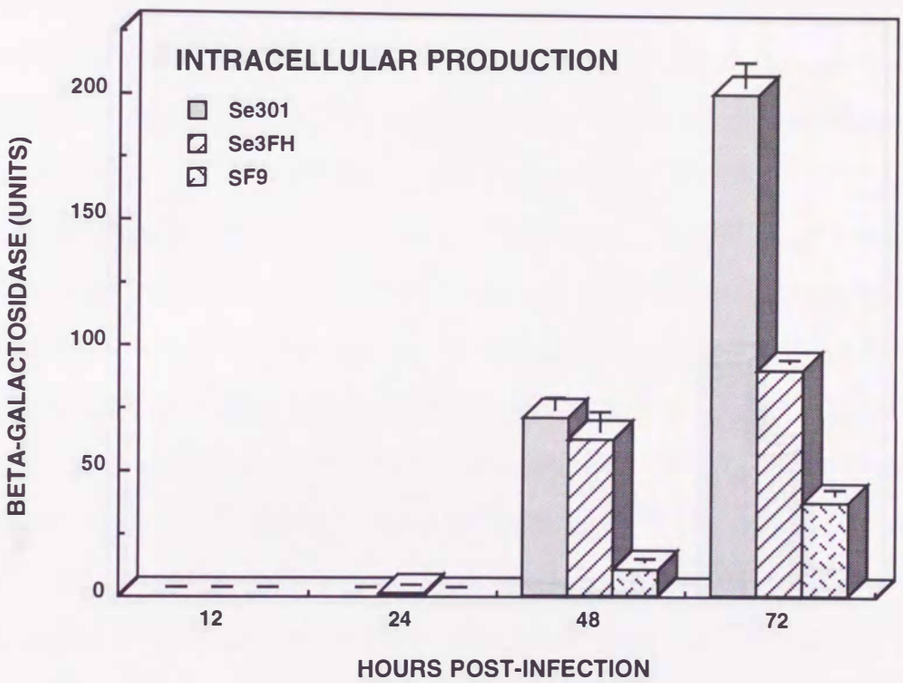

FIG. 20a. The production of ß-galactosidase (units/well in 24-well plate) in intracellular fluids of three cell lines, Se3FH and Se301 from *Spodoptera exigua* and SF9 from *Spodoptera frugiperda*. Data represent means ± SE of a typical experiment (n = 3 / sample).

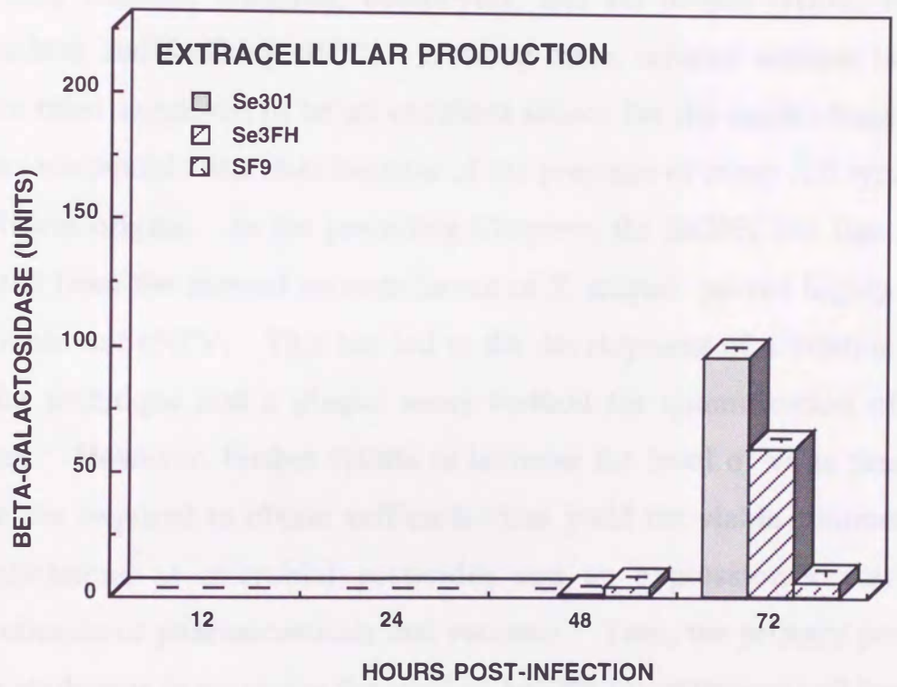

FIG. 20b. The production of ß-galactosidase (units/well in 24-well plate) in extracellular fluids of three cell lines, Se3FH and Se301 from *Spodoptera exigua* and SF9 from *Spodoptera frugiperda*. Data represent means ± SE of a typical experiments (n = 3 / sample).

DISCUSSION

In general, NPV has a relatively narrow host range both *in vitro* and *in vivo* and is specific for continuous cell lines derived from the original host (Granados, 1976; Granados and Hashimoto, 1989). Continuous cell lines have been established from a variety of tissues including ovaries, embryos, hemocytes, and fat bodies (Hink, 1972; Knudson and Buckley, 1977). Among them, minced neonate larvae have been suggested to be an excellent source for the establishment of virus susceptible cell lines because of the presence of many cell types of different origins. In the preceding Chapters, the Se3FH cell line generated from the minced neonate larvae of *S. exigua* proved highly susceptible to SeNPV. This has led to the development of a dilution end point technique and a plaque assay method for quantification of the virus. However, further efforts to increase the level of virus production are required to obtain sufficient virus yield for viable commercial applications as microbial pesticides and an expression vector for production of pharmaceuticals and vaccines. Thus, the primary goal of this study was to maximize the yield of SeNPV in continuous cell lines.

In this Chapter, a daughter cell clone, designated Se301, was successfully isolated from the parent Se3FH cell line. The results show that the Se301 cell line is 10 times more susceptible to SeNPV than the parent cell line and has an increased ability to support plaque formation of the virus. In this cell clone, SeNPV forms visible plaques with increased sizes more rapidly and clearly than in the Se3FH cell line. The increase in the plaque size is probably due to the highly enhanced levels of ECV production, leading to the more rapid spread of infection among the cell populations.

Interestingly, the cloning of this cell line resulted in the reduction in the number of PIBs produced. Lenz *et al.* (1991) reported that not all of the cloned *H. zea* cell lines, which produce high titers of ECV for *H. zea* NPV, generated high levels of PIBs. A similar observation was also made by Rice *et al.* (1989) in cloned cell lines of *H. zea* where there was no correlation in the production levels between ECV and PIB for *H. zea* NPV. It is likely that the yield of ECVs and PIBs vary depending on the source of tissues used for establishment of the cell line. The parental Se3FH cell line is from minced neonate *S. exigua* larvae and contains many cell types of different origins (Chapter I). In this study, therefore, the cell type producing a high titer of ECV with decreased level of PIB production may have been isolated from Se3FH cell line.

Corsaro and Fraser (1987) observed a difference in cell doubling time between the *H. zea* cloned cell lines and the parental cell line. Volkman and Summers (1975), in their study on the replication of AcNPV in cloned cell lines of *Trichoplusia ni*, showed that there was little correlation between the virus production and rates of cell growth. Brown and Faulkner (1975) also reported that there was no direct correlation between cell doubling time and virus production. In this study, the growth rate of Se301 cells was found to be lower than that of the parent Se3FH cell line. It is noteworthy that the doubling time of Se3FH cell line has changed from 30 h to 17 h during about 100 serial passages in 1.5 years; however, its susceptibility to SeNPV has not changed. Further characterization of the *S. exigua* cell lines is required to determine whether there is any correlation between their biological characteristics and the level of virus production.

The growth kinetic study showed that a plaque purified virus isolate replicated more rapidly, at the early stage of virus infection, than

the wild isolate. It was also found that the level of PIB production could be increased by employing a plaque purified isolate. Plaque purification of field isolates of AcNPV showed that each isolate consisted of several clones or substrains having slightly different restriction enzyme digestion profiles and usually one particular profile was predominant (Lee and Miller, 1978; Smith and Summers, 1978). Knell and Summers (1981) reported that, of the 13 plaque purified isolates from a single wild isolate of *S. frugiperda* NPV, five distinct variants were obtained. Thus, the wild isolate SeNPV-IW used in this study is likely a mixture of variants and the genetic heterogeneity in the wild isolate may influence the level of virus production to some extent.

The promoters encoding the polyhedrin or p10 proteins of AcNPV are currently used in baculovirus expression vector systems for production of prokaryotic and eukaryotic proteins (Smith *et al.*, 1983; Granados and Federici, 1986; Summers and Smith, 1987; Miller, 1988; Maeda, 1989). Expressed products of foreign genes in insect cells undergo post-translational modifications, such as glycosylation, phosphorylation, and secretion and they are biologically active. AcNPV has a broad host range infecting over 25 insect species and can replicate in many cell lines derived from six families of Lepidoptera (Granados and Hashimoto, 1989; Adams and McClintock, 1991). In particular, IPLB-SF 21 cell line and its clonal isolate SF9 from *S. frugiperda* are extensively used for studies on the expression of foreign genes (Vaughn *et al*, 1977). Hink *et al.* (1991), in their comparative studies on the expression of ß-garactosidase using recombinant AcNPV in 23 insect cell lines, demonstrated that SF9 cells had the highest yield of ß-garactosidase among cell lines used. In the present study, however, the total amounts of ß-garactosidase produced in Se301 cell line was on a high level through postinoculation and was approximately 7 times greater than in

SF9 cell line 72 h p.i. This indicates that Se301 cell line is more susceptible to recombinant AcNPV than SF9 cell line and considered as a candidate for production of recombinant proteins.

CHAPTER V

In Vivo and *In Vitro* Characterization of Several Isolates of *Spodoptera exigua* Nuclear Polyhedrosis Virus

The infection of nuclear polyhedrosis virus (NPV) in *Spodoptera exigua* was first reported by Steinhaus (1949) in California. Later, several *S. exigua* (SeNPV) isolates have been collected from California (Hunter and Hall, 1968; Smith and Summers, 1978; Gelernter and Federici, 1986a). SeNPV isolates collected from different geographical regions have also been reported (Vlak *et al*, 1982; Caballero *et al.*, 1992; Kondo *et al.*, 1994). Most SeNPV isolates are highly infectious for *S. exigua* larvae and possess similar biological activity and biochemical characteristics. Also, the nucleotide sequence and regulation of SeNPV gene expression, in particular the polyhedrin and p10 genes, have been well studied (van Strien *et al.*, 1992; Zuidema *et al.*, 1993). Polyhedrin genes are highly conserved among baculoviruses and the SeNPV polyhedrin gene was most closely related to that of *S. frugiperda* NPV, differing only five amino acids (Vlak and Rohrmann, 1985; Gonzales *et al.*, 1989; van Strien *et al.*, 1992).

The restriction endonuclease (REN) analysis of baculovirus DNA has been used to distinguish and characterize closely related genotypic variants and species (Tanada and Kaya, 1993). Studies on genetic heterogeneity of wild isolates of NPVs have often revealed that a single isolate is a mixture of genotypically different variants (Lee and Miller, 1978; Smith and Summers, 1978; Knell and Summers, 1981; Cherry and Summers, 1985; Maeda *et al.*, 1990). The closely related genotypic variants have been identified in a collection of geographic isolates of SeNPV using REN analysis (Gelernter and Federici, 1986a, 1990; Smits

and Vlak, 1988b) and there was no significant difference in their biological activity determined by LD_{50} value on *S. exigua* larvae (Caballero *et al.*, 1992).

In this Chapter, comparative studies of five wild isolates of SeNPV collected in Japan and Thailand were done by analysing their REN profiles of virus DNA, level of virus production *in vitro* and biological activity to *S. exigua* larvae. Also, the DNAs from several plaque-purified isolates were analyzed by REN fragment pattern to examine the genetic heterogeneity within a single wild isolate of SeNPV.

MATERIALS AND METHODS

Cells and Viruses

The cloned cell line, Se301, derived from a continuous Se3FH cell line of *S. exigua* was cultured at 27°C in IPL-41 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 25-cm^2 tissue culture flasks (Costar, 3055).

Origin and source of the five SeNPV wild isolates used are listed in Table 6. *Autographa californica* NPV(AcNPV) strain E2 was obtained from Dr. Y. Hashimoto (Kyoto Institute of Technology, Kyoto, Japan). Viruses were propagated by infecting the Se301 cells in logarithmic phase as described in Chapter III.

Kinetics of Virus Growth and Plaque Assay

Se301 cells in exponential phase were infected with virus at a MOI of 0.5 PFU per cell in multiwell culture plate (Falcon, 3014) containing 0.24 x 10^6 cells/well in IPL-41 medium supplemented with 10% FBS. Following the 1 h virus adsorption period, the cells were washed, added with fresh medium, and incubated at 27°C. At various time after inoculation, the culture supernatant containing the infectious extracellular viruses (ECVs) was removed, centrifuged at 3,000 rpm for 10 min, and stored at -80°C prior to plaque assay. To determine polyhedral inclusion body (PIB) concentration, 1% (final concentration) SDS was added to infected cultures and incubated at 37°C for 30 min.

Plaques were produced on a confluent monolayer of Se301 cells which had been seeded into multiwell culture plate at a density of 0.25 ×

TABLE 6

***Spodoptera exigua* nuclear polyhedrosis virus wild isolates used**

Virus isolate	Geographic origin	Year isolated	Source
IW	Nagasaki, Japan	1990	K. Yokomizo[a]
KW	Kagoshima, Japan	1991	K. Kusigemati[b]
SW	Kagoshima, Japan	1991	K. Tsuda[c]
OW	Oita, Japan	1992	K. Tsuda
TW	Kanchanaburi,Thailand	1991	K. Tsuda

[a] Nagasaki Agricultural and Forestry Experiment Station, Nagasaki, Japan.
[b] Faculty of Agriculture, Kagoshima University, Kagoshima, Japan.
[c] Fukuoka Agricultural Research Center, Fukuoka, Japan.

10^6 cells/well, as described in Chapter III. Serial 10-fold-diluted virus suspension in IPL-41 medium were inoculated to cells and allowed to adsorb to cells for 60 min at 27°C. The cells were then overlaid with 0.3 ml/well of 0.75% (w/v) Seaplaque agarose in IPL-41 medium containing 10% FBS at 36°C. The number of plaques were counted 6 days after incubation at 27°C without staining.

Virus DNA Extraction

ECVs from virus infected Se301 cells 3 to 4 days p.i. were used as the source of virus DNA for restriction endonuclease analysis. The supernatant was collected from the infected cell culture by centrifugation at 3,000 rpm for 20 min. Virus was then pelleted by centrifugation at 20,000 rpm for 40 min (SW 28 rotor), and the pellets were suspended in TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) at 4°C overnight. The virus suspension was incubated with 1% (final concentration) SDS for 10 min at 37°C. Subsequently, proteinase K (Merck) was added at a concentration of 1 mg/ml and incubated for 30 min. The virus DNA was extracted with an equal volume of TE buffer-saturated phenol, phenol-chloroform (1 : 1, v/v) and chloroform. The extracted DNA solution was stored at 4°C until use.

Digestion of DNA with Restriction Endonucleases and Gel Electrophoresis

The virus DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, and *Sal*I restriction endonucleases (Nippon Gene Co., Ltd.). DNA (two μg) was incubated with 10-20 units of enzyme at 37°C for 60min. Reaction was stopped by addition of 0.1 vol of 0.1 M EDTA (pH 8.0),

40% glycerol and 0.25% (w/v) bromophenol blue. Horizontal slab gels (5 × 125 × 200 mm) containing 0.7% agarose (Agarose I, Dojindo Laboratories) and 0.5 µg/ml ethidium bromide dissolved in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) were used for electrophoresis. A constant current of 30 mA was applied across the gel for 24 h at room temperature, in TAE buffer containing 0.5 µg/ml ethidium bromide. The molecular sizes of the virus DNA were determined on slab gels by comparison with the fragments of *Hin*dIII digested lambda phage DNA.

Insect Rearing

The cultures of *S. exigua* were maintained on an artificial diet at 25°C as described in Chapter I.

Dosage-Mortality Studies

PIBs from SeNPV infected Se301 cells were ingested with an artificial diet to early 4th-instar *S. exigua* larvae. The virus infected larvae were homogenized in 1 M NaCl, and the suspension was filtrated through two layers of Kimwipe. PIBs were pelleted by centrifugation at 3,000 rpm for 10 min and then incubated with 50 mM Tris containing 0.5 M urea and 0.1% SDS at 37°C for 5 min. After washing with distilled water, PIBs were purified on 45-55% (w/w) sucrose density gradient centrifugation at 3,000 rpm for 30 min. PIBs were washed two times with distilled water, and stored at 4°C.

Second-instar larvae of *S. exigua* were used to determine the biological activity of SeNPV isolates. Larvae were starved for 5-6 h and then fed on 20 mg artificial diet surface-contaminated with PIBs at ap-

propriate concentrations. Dosages used were 1, 4, 16, 64, and 256 PIBs per larva. Larvae were individually placed into wells of multi-well tissue culture plate (Costar, 3524) and incubated at 27°C in constant darkness. The mortality was recorded for 10 days after inoculation and the LD_{50} value was calculated by the probit analysis method (Finney, 1978).

RESULTS

Restriction Endonuclease Analysis of SeNPV Wild Isolates

The DNAs from SeNPV wild isolates were exposed to six restriction endonucleases in order to characterize the virus genome. The *Eco*RI and *Pst*I REN profiles of five wild isolates and one cloned plaque-purified isolate are shown in Fig. 21. These SeNPV isolates produced 22-23 fragments after DNA digestion with *Eco*RI. The five wild isolates of SeNPV had the similar overall patterns with minor differences. The *Eco*RI profiles of SeNPV-KW, SW, OW, and TW were identical, but distinct from that of SeNPV-IW in some fragments. In addition, the profile of SeNPV-I1, plaque-purified isolate from SeNPV-IW, lacked the submolar fragment of 9.6 kbp that was present in all SeNPV wild isolates. The *Eco*RI REN profiles of SeNPV isolates showed distinctive differences from that of AcNPV.

*Pst*I digestion of DNA from SeNPV wild isolates yielded 12-14 fragments with total molecular sizes ranging from 105.6 to 122.6 kbp (Fig. 21, Table 7). Three wild isolates, SeNPV-KW, SW, and TW, had similar *Pst*I patterns with a total molecular size of 105.6 kbp. However, these profiles was distinct from those of SeNPV-IW and OW. *Pst*I profiles of these two isolates were nearly the same, although the fragment of 16.7 kb was not present in SeNPV-OW. The DNA REN pattern of plaque-purified SeNPV-I1 isolate was the same as that of the parental isolate SeNPV-IW, except for the absence of the 14.4 and 11.3 kbp submolar fragments. The *Pst*I profiles of SeNPV and AcNPV DNA also different from each other.

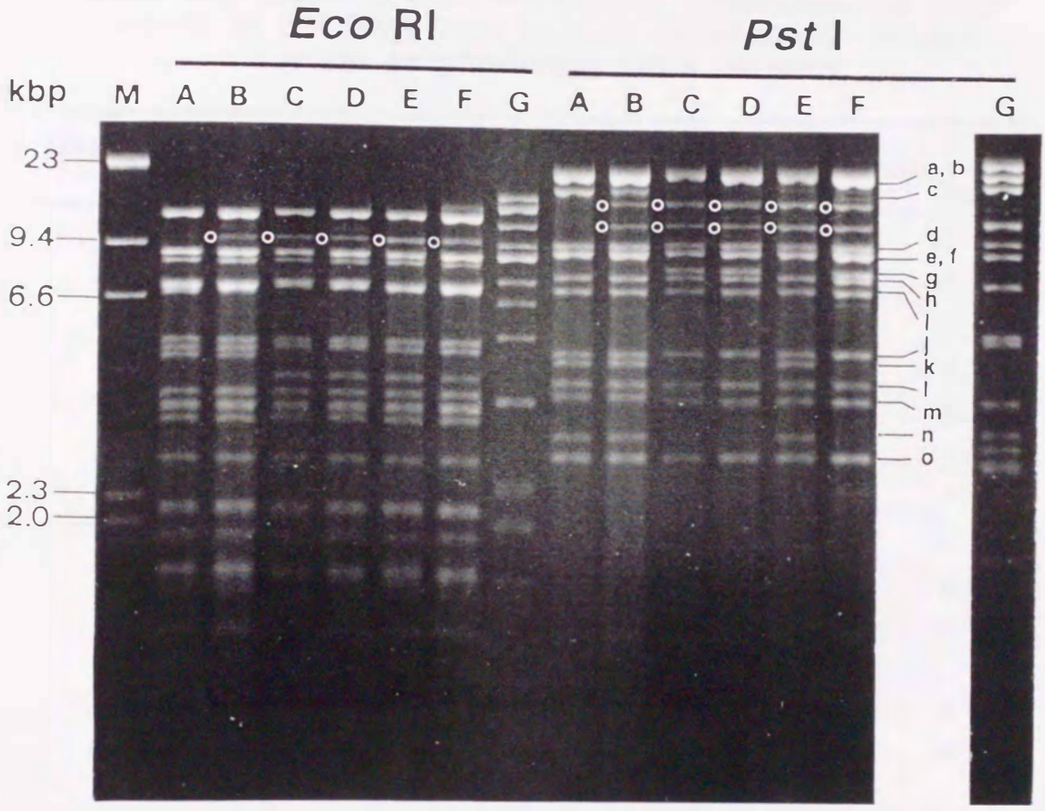

Fig. 21. Restriction endonuclease profiles of the DNA from *Spodoptera exigua* nuclear polyhedrosis virus (SeNPV) isolates after digestion with *Eco*RI or *Pst*I. (A) SeNPV-I1, plaque-purified isolate from SeNPV-IW. (B) SeNPV-IW. (C) SeNPV-KW. (D) SeNPV-SW. (E) SeNPV-OW. (F) SeNPV-TW. (G) *Autographa californica* NPV. Sizes (kbp) of restriction fragments of lambda phage DNA digested with *Hin*dIII (M) are shown to the left. Submolar fragments determined by density analysis are indicated by open circles to the left of the band. For the location of the viruses, see Table 6.

TABLE 7

Molecular sizes of *Pst*I restriction endonuclease fragments of the DNA from various *Spodoptera exigua* nuclear polyhedrosis virus isolates

Fragment	Molecular size[a] (kilobase pairs)					
	I1	IW	KW	SW	OW	TW
a	21.6	21.6	21.6	21.6	21.6	21.6
b	21.6	21.6	21.6	21.6	21.6	21.6
c	16.7	16.7				
d	9.2	9.2	9.2	9.2	9.2	9.2
e	8.4	8.4	8.4	8.4	8.4	8.4
f	8.4	8.4	8.4	8.4	8.4	8.4
g			7.4	7.4		7.4
h	7.2	7.2	7.2	7.2	7.2	7.2
i	6.4	6.4	6.4	6.4	6.4	6.4
j	4.6	4.6	4.6	4.6	4.6	4.6
k	4.4	4.4			4.4	
l	4.0	4.0	4.0	4.0	4.0	4.0
m	3.8	3.8	3.8	3.8	3.8	3.8
n	3.3	3.3			3.3	
o	3.0	3.0	3.0	3.0	3.0	3.0
Total	122.6	122.6	105.6	105.6	105.9	105.6

[a] Estimation of the molecular size of each *Pst*I fragment was made by comparing with standard curves obtained from lambda phage DNA *Hin*dIII fragments used as size makers. Submolar fragments were removed for the estimation.

Additional screening with REN profiles by *Bam*HI, *Hind*III, *Kpn*I and *Sal*I revealed that all SeNPV wild isolates had identical or quite similar patterns (data not shown).

*Eco*RI *Analysis among SeNPV Clones*

16 SeNPV clones from SeNPV-IW wild isolate by plaque purification method on *S. exigua* cells were successfully obtained. Of the 16 plaque-purified isolates, 14 isolates (SeNPV-I1 to I14) were many polyhedra (MP) mutants and 2 isolates (SeNPV-IF1 and IF2) were few polyhedra (FP) mutants. Digestion of the plaque-purified virus DNAs with *Eco*RI resulted in considerable similarity to wild isolate (Fig. 22). Seven distinct patterns were observed among plaque-purified SeNPV isolates, when six fragments (a, b, c, d, e, and f) were employed for characterization of each isolate. In eight isolates (SeNPV-I1, I5, I6, I7, I8, I12, IF1, and IF2), the DNA REN patterns were similar, in which four fragments (a, b, d, and f) were missing. All of the six fragments were missing in SeNPV-I2, I3, and I4 that showed identical patterns one another. The *Eco*RI patterns of other 5 isolates (SeNPV-I9, I10, I11, I13, and I14) were different one another in some fragments.

Characteristics of Virus Growth in vitro

Virus growth curves of five wild isolates and one plaque-purified isolate of SeNPV in Se301 cells are shown in Fig. 23. The Se301 cells showed significant cytopathic changes and had a high level production of PIBs after inoculation of each SeNPV isolate. All six SeNPV isolates replicated well and formed plaques clearly on the monolayer of Se301 cells. The virus growth kinetics of six isolates revealed typical SeNPV

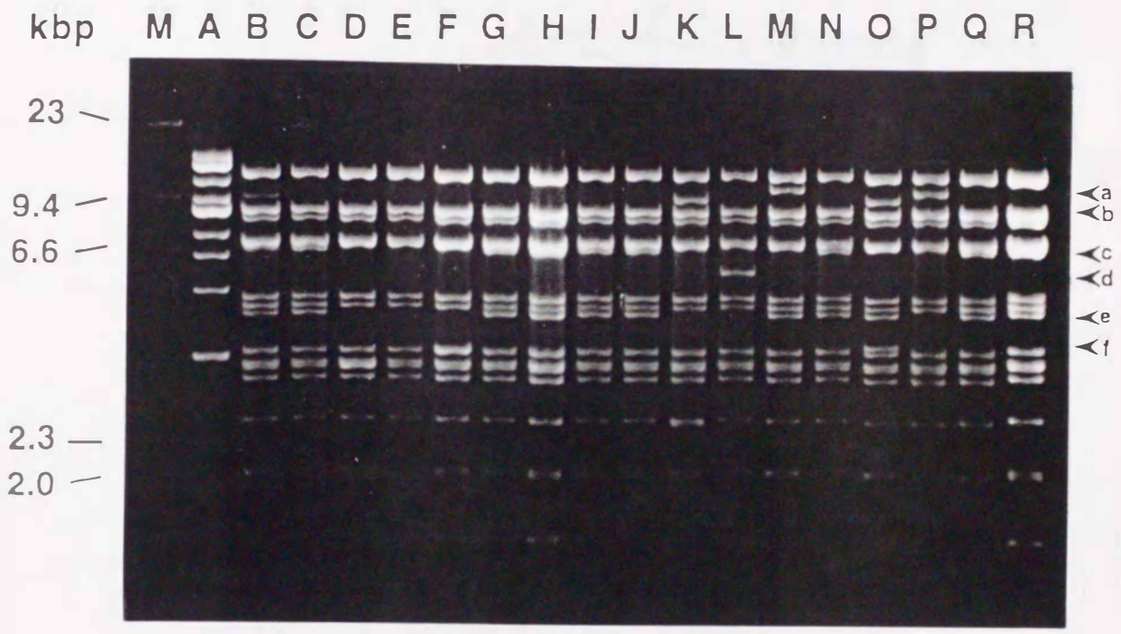

Fig. 22. *Eco*RI fragmentation profiles of the DNA of plaque-purified isolates from *Spodoptera exigua* nuclear polyhedrosis virus Isahaya wild isolate, SeNPV-IW. (A) *Autographa californica* NPV. (B) SeNPV-IW. (C to P) SeNPV-I1 to I14, plaque-purified isolates from SeNPV-IW, respectively. (Q and R) SeNPV-IF1 and -IF2, FP mutants from SeNPV-IW, respectively.

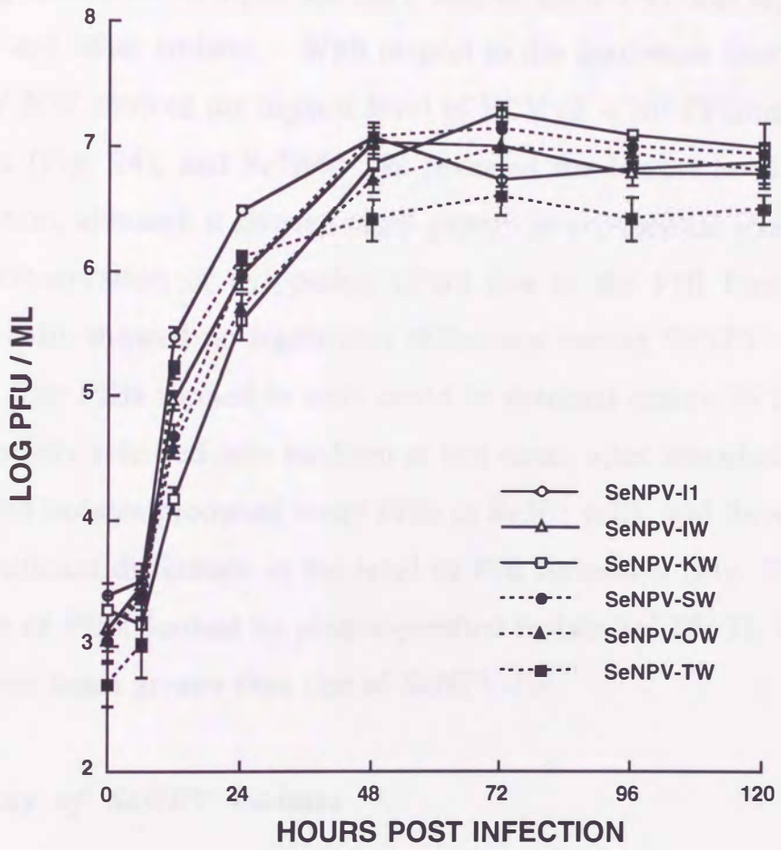

FIG. 23. Replication of five wild isolates and a plaque-purified isolate of *Spodoptera exigua* nuclear polyhedrosis virus in the Se301 cell line of *S. exigua*. Cells were infected with the virus at a MOI of 0.5 PFU per cell. Data represent means ± SE of a typical experiment (n = 5 / sample).

growth patterns with high level of progeny virus production; the ECVs appeared as early as 6 h p.i., and the infectivity reached a maximum titer of about 10^7 PFU/ml 48 to 72 h p.i. There was a small difference in the level of ECVs production among isolates. In exponential phase ranging from 6 to 48 h p.i., the ECV titer of SeNPV-I1 was higher than that of any other isolates. With respect to the maximum titer of ECV, SeNPV-KW showed the highest level of ECV (2×10^7 PFU/ml) among isolates (Fig. 24), and SeNPV-TW revealed the lowest level of ECV production, although it showed rapid growth in exponential phase.

Observation of cytopathic effect due to the PIB formation in Se301 cells showed no significant difference among SeNPV wild isolates. The PIBs formed in cells could be detected before 24 h p.i. and were mostly released into medium at late times after inoculation. All five wild isolates produced many PIBs in Se301 cells, and there was little significant difference in the level of PIB formation (Fig. 25). The number of PIBs formed by plaque-purified isolate SeNPV-I1, however, was three times greater than that of SeNPV-IW.

Bioassay of SeNPV Isolates

To examine the virulence of the six SeNPV isolates against *S. exigua* larvae, LD_{50} values were determined by oral inoculation of PIBs into 2nd-instar larvae of *S. exigua*. All isolates killed the larvae within 5-7 days with the symptoms typical of NPV infection: the hemolymph turned milky and larval integuments became fragile. The infected larvae produced a large number of PIBs with no differences in the size and shape. Table 8 shows the statistics of the regression lines and the values of LC_{50} and LD_{50}. SeNPV-KW was most virulent to *S. exigua* larvae with the LD_{50} value of 3.0 PIBs per larva. Significant difference was

not observed in LD_{50} values between plaque-purified isolate SeNPV-I1 and wild isolate SeNPV-IW.

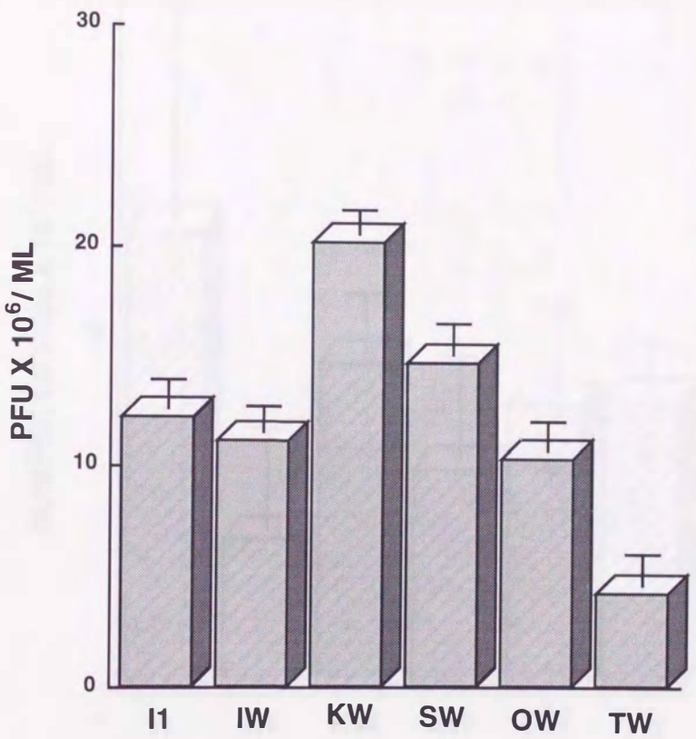

FIG. 24. Maximum titers of extracellular virus of five wild isolates and a plaque-purified isolate of *Spodoptera exigua* nuclear polyhedrosis virus in the Se301 cell line of *S. exigua.* Data represent means ± SE of a typical experiment (n = 5 / sample).

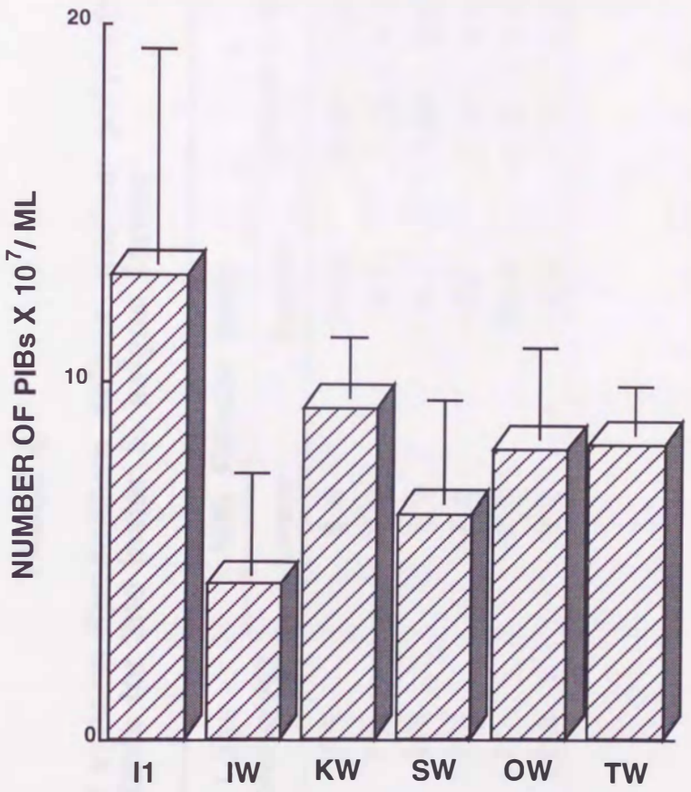

FIG. 25. Polyhedral Inclusion body (PIB) formation by five wild isolates and a plaque-purified isolate of *Spodoptera exigua* nuclear polyhedrosis virus in the Se301 cell line of *S. exigua*. Data represent means ± SE of a typical experiment (n = 3 / sample).

TABLE 8

LC_{50} and LD_{50} values of various *Spodoptera exigua* nuclear polyhedrosis virus isolates for 2nd instar *S. exigua* larvae

SeNPV isolate	LC_{50} (PIBs/ml)	LD_{50} (PIBs/larva)	95% Fiducial limits		Slope ± SE	Intercept
			Lower	Upper		
I1	9.5×10^3	9.5	8.0	10.9	0.87 ± 0.39	4.15
IW	7.1×10^3	7.1	5.6	8.6	1.53 ± 0.12	3.70
KW	3.0×10^3	3.0	1.9	4.1	3.85 ± 0.88	3.14
SW	15.6×10^3	15.6	14.2	17.0	1.20 ± 0.14	3.57
OW	22.1×10^3	22.1	20.7	23.4	1.13 ± 0.18	3.48
TW	12.3×10^3	12.3	9.2	15.4	1.37 ± 0.06	3.51

DISCUSSION

In recent studies, REN analysis of baculovirus DNAs has been a useful technique for identification of closely related virus isolates (Smith and Summers, 1978; Miller and Dawes, 1978a,b; Vlak and Gröner, 1980; Kislev and Edelman, 1982). The REN fragment patterns of DNAs from wild isolates of *S. exigua* NPV indicate that these isolates are closely related but slightly different one another. The REN profiles of SeNPV wild isolates used in this study do not resemble that of AcNPV and it is concluded that these profiles are typical of SeNPV. There have been several reports on the isolation of SeNPV from California, Egypt, Netherlands, Spain, Thailand, and Japan (Gelernter and Federici, 1990; Vlak *et al.*, 1982; Caballero *et al.*, 1992; Kondo *et al.*, 1994). The beet armyworm is an immigrant pest, coming from its homelands in subtropics, Egypt, Algeria, and Tunisia, to nothern directions, Italy, Spain, and eventually to North Europe. There it does not form persistent populations, but with these migrations NPV isolates are redistributed in temporary invaded areas. Therefore, it is interesting to compare SeNPV isolates in different areas and look for diversity of introduced and selected isolates. Vlak *et al.*, (1982) showed that SeNPV isolates from Egypt, Netherlands, and California are genetically distinct. However, it also appeared, from the results of REN analysis, that the isolates from California, Thailand, and Spain are closely related, and the California isolate, SeNPV-US, was proposed to consider as the type strain of SeNPV (Caballero *et al.*, 1992). The present results clearly show that the SeNPV isolates used in the present study are closely related to the SeNPV-US reported by Caballero *et al.* (1992), although minor differences are observed. Diversity of SeNPV isolates may offer isolates suitable for introduction or colonization in areas with adapted

and compatible isolates in local sedentary population. These studies will provide the information toward a better understanding of the diversity of SeNPV isolates in East and South-East Asia.

The REN analysis of baculovirus DNAs from wild isolates has revealed the presence of submolar fragments (Miller and Dawes, 1978a, b; Smith and Summers, 1978; Knell and Summers, 1981; Gettig and McCarthy, 1982). These results have suggested that the wild isolate is a mixture of two or more closely related genotypic mutants. For instance, a wild isolate of AcNPV had submolar fragments and subsequent plaque purification of the isolate resulted in the isolation of several different mutants (Smith and Summers, 1978). Maeda *et al.* (1990) also reported that a *Spodoptera littura* NPV wild isolate was a mixture of several different NPVs including AcNPV and *Spodoptera littoralis* NPV. Recently, several investigators (Gelernter and Federici, 1990; Caballero *et al*, 1992) have demonstrated the presence of the submolar fragments in SeNPV wild isolates. In the present study, 16 plaque-purified isolates from SeNPV-IW could be grouped into seven distinct genotypic mutants and two phenotypic mutants. This is the first report showing that the SeNPV wild isolate is a mixture of genotypic and phenotypic mutants. In Chapter IV, it was observed that a plaque-purified SeNPV-I1 isolate consistently produced more ECVs and PIBs than SeNPV-IW wild isolate in cultured cells. These findings suggest that the genetic heterogeneity influences the level of virus production. These genotypic mutants may be generated from a single origin genotype by a deletion and/or an insertion of extra restriction site of virus DNA. However, the origin and significance of these mutants are not fully understood. The preferental selection of the mutants may occur during the distribution of virus, or when the virus is ingested by differ-

ent insect hosts. The appearance of such mutants might play an important role in the diversity and the evolution of baculoviruses.

The numerous serial passage of NPVs in cultured cells causes the production of FP mutants (MacKinnon *et al.*, 1974; Burand and Summers, 1982; Kumar and Miller, 1987; Granados *et al.*, 1994). The occurrence of FP mutants is considered to be the results of the insertion of transposon-like elements (Miller and Miller, 1982; Blissard and Rohrmann, 1990) or host DNA (Fraser *et al.*, 1983) into the virus genome. In the present study, the FP mutants were isolated from SeNPV wild isolate after one passage through *S. exigua* larve and one passage in cultued *S. exigua* cells, suggesting SeNPV wild isolate containing FP mutants. Further works are needed to determine whether FP mutants occur in nature and are relatively stable.

In this study, it was shown that SeNPV wild isolates were genetically closely related one another with small differences in REN profiles of virus DNA, as well as in virus production level *in vitro* and in virulence against *S. exigua* larvae. Gelernter and Federici (1990) demonstrated that a high degree of uniformity was observed among SeNPV isolates in Californian populations and virus epizootics were caused by a single virus or its closely related variants. In addition, there was no occurrence of apparent variation in the field populations of Californian SeNPV over a 16-year period (Gelernter and Federici, 1986a). It is probable that a significant genetic change in the SeNPV populations has not occurred. This may reflect the narrow host range of SeNPV both *in vivo* and *in vitro*. Further studies of the generation of genotypic variants will provide insights into the determination of host specificity of baculoviruses.

SUMMARY

Five cell lines, designated Se3FH, Se4FH, Se5FH, Se6FHA, and Se6FHB, were established from minced neonate larvae of the beet armyworm, *Spodoptera exigua.* There are two cell types in each cell line, spindle-shaped and spherical cells. These newly established *S. exigua* cell lines were heteroploidy and polyploidy, and had a population doubling time raging from 19 h to 37 h. All cell lines supported the replication of *S. exigua* nuclear polyhedrosis virus (SeNPV), with infection ratios of 41-73% in Se3FH, Se5FH, Se6FHB and < 2% in Se4FH and Se6FHA.

SeNPV was examined for replication in 20 continuous cell lines from eight lepidopteran species; *Spodoptera frugiperda, Spodoptera littoralis, Spodoptera litura, Spodoptera exigua, Pseudaletia separata, Mamestra brassicae, Plutella xylostella,* and *Bombyx mori.* Of these, only five homologous cell lines, established from *S. exigua*, were permissive for infection with the virus. The growth kinetics of SeNPV in *S. exigua* Se3FH cells showed that extracellular viruses (ECVs) were released from infected cells 6 h p.i. and reached a maximal titer 72 h p.i. The number of polyhedral inclusion bodies (PIBs) reached a maximum of 10^7 PIBs/ml 96 h p.i.

The SeNPV and *Autographa californica* NPV (AcNPV) produced plaques in a newly established cell line of *S. exigua.* Plaques were composed of infected cells containing many PIBs and were visible without any staining procedure. Dose-response assays showed direct correlation between the number of plaques and the inoculum size. Growth kinetic studies of the two viruses, using the plaque assay system devel-

oped, revealed that the release of ECVs began 6 h p.i. and the titer reached the stationary period 48 h p.i. The sensitivity of the plaque assay system was 100 times greater for the heterologous AcNPV than for the homologous SeNPV.

A cloned cell line, designated Se301 derived from a continuous Se3FH cell line of *S. exigua,* showed ten times greater sensitivity when tested with the plaque assay to a SeNPV as compared to the parent cell line. Nearly 100% of Se301 cells were infected with the plaque purified isolate SeNPV-I1 or the wild isolate SeNPV-IW. Plaques were first detected 3 to 4 days in Se301s faster than the Se3FH cell line. The size of plaques formed in Se301 cell line was markedly larger than that of the parental cell line. At 12 h p.i., the production of the ECVs in the Se301 cell line infected with either SeNPV-I1 or SeNPV-IW was 20 - 40 times greater than that in Se3FH cell line. In contrast, Se301 cells infected with SeNPV-I1 or SeNPV-IW produced PIBs at lower levels compared to Se3FH cells. The isolate SeNPV-I1 consistently produced more ECVs and PIBs, in both Se301 and Se3FH cell lines, than the isolate SeNPV-IW.

Recombinant AcNPV carrying ß-galactosidase was used to study the gene expression in *S. exigua* cell lines. The production of ß-galactosidase was first detected 24 h p.i. and the dramatic increase was found between 48 and 72 h p.i. in Se301 and Se3FH cell lines. The total amounts of ß-galactosidase in Se301 cell line 72 h p.i. was approximately 7 times higher than SF9 cell line.

SeNPVs, isolated from five geographically distinct regions of Japan and Thailand, were characterized by their DNA restriction endonuclease (REN) pattern, level of virus production in a continuous cell

line of *S. exigua* and biological activity to *S. exigua* larvae. The REN profiles of *Eco*RI and *Pst*I fragments exhibited the similar overall patterns with minor differences. Digestion of virus DNA from a plaque-purified isolate, SeNPV-I1, yielded 14 fragments with *Pst*I and the estimated genome size was approximately 123 kbp. The SeNPV wild isolate from Kagoshima, SeNPV-KW, showed the highest production level of ECVs in the Se301 cell line of *S. exigua* among five wild isolates, but there was no significant difference in the level of PIB formation. In comparative studies of biological activity using 2nd-instar *S. exigua* larvae, SeNPV-KW had the highest virulence with an LD_{50} value of 3.0 PIBs per larva. When 16 clones, plaque-purified from the Isahaya isolate, SeNPV-IW, were examined for genetic relatedness, seven distinct *Eco*RI patterns were observed, indicating that SeNPV-IW wild isolate consisted a mixture of different genotypes.

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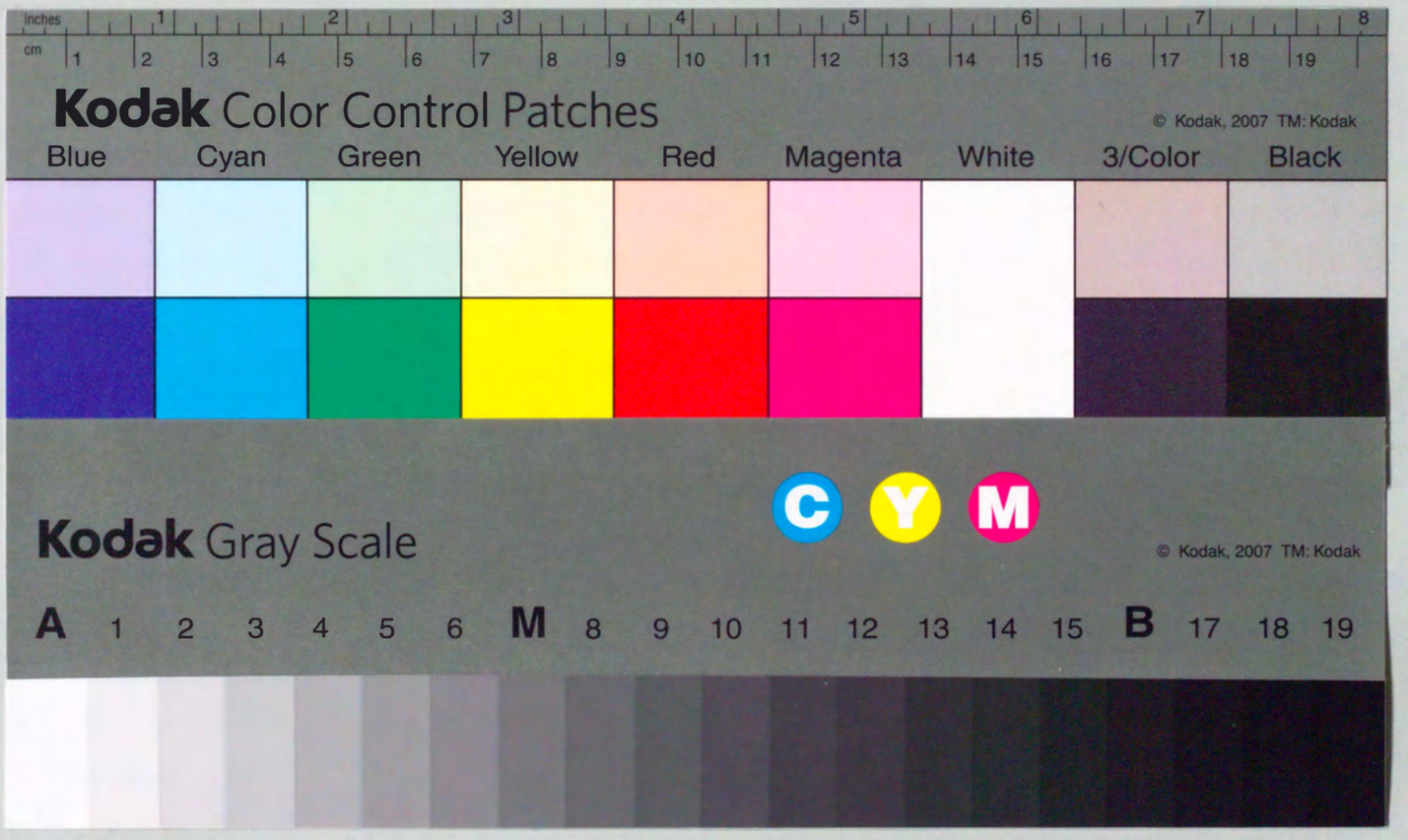
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