Genetic Analysis of a Baculovirus, *Autographa californica* Nuclear Polyhedrosis Virus

I. Isolation of Temperature-Sensitive Mutants and Assortment into Complementation Groups

MARTHA BROWN, ALLAN M. CRAWFORD, AND PETER FAULKNER*

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada

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Temperature-sensitive (*ts*) mutants were isolated from the baculovirus *Autographa californica* (alfalfa looper) MNPV, grown in *Spodoptera frugiperda* (fall armyworm) cells in the presence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Of 567 plaque isolates screened, 27 were temperature sensitive (*ts*), representing a mutation frequency of 4.8%. Ten *ts* mutants were studied in detail: six failed to yield nonoccluded virus at 33°C (*NOV* mutants), whereas the other four produced nonoccluded virus but were restricted in formation of polyhedra at 33°C (*Poly* mutants). One of the six *NOV* mutants failed to synthesize viral DNA. Reversion and leak frequencies were determined, and the mutants were assorted into complementation groups based on the yield of polyhedrin synthesis in cells coinfecting with pairs of mutants at 33°C, as measured by radioimmunoassay. For *NOV* mutants, complementation indexes were also based on virus yield and were consistent with those based on polyhedrin synthesis. Nine mutants were assorted into five complementation groups. One mutant remained unclassified.

Studies over the past decade have identified the genome of baculoviruses as covalently closed circular double-stranded DNA (1, 28, 30, 31) with molecular weight of 60×10^6 to 85×10^6 (4, 5, 13, 18). However, there are no reports on gene organization within the genome or on gene interaction in mixedly infected cells. Such studies are of importance in view of a growing use of baculoviruses in biological control programs directed against insect pests (34). *Autographa californica* (alfalfa looper) NPV is being considered for registration as a pesticide in the United States, and because it is pathogenic to several lepidopteran genera (36) and is easily grown in cell culture (22, 35) it was chosen as a suitable candidate for genetic studies. The use of temperature-sensitive (*ts*) mutants for the genetic analysis of several vertebrate viruses is well documented (7, 11, 29, 38). With the development of a plaque assay for nuclear polyhedrosis viruses using a solid overlay (3), it was possible to clone mutants and begin a genetic analysis of a baculovirus. This paper describes the isolation of 27 *ts* mutants of *A. californica* NPV and the assortment of 10 of these into five complementation groups.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* (fall armyworm) cells, IPLB-SF21 (37), were used between

passage levels 207 and 331 as host cells. They were routinely grown in BML-TC/10 medium containing 10% fetal calf serum (10) at 33°C and were incubated at either 33 or 25°C after infection. Doubling times were 29 and 20 h at 25 and 33°C, respectively.

The virus used to initiate the genetic studies was a plaque-purified MP strain of *A. californica* MNPV (14, 26). This was further plaque-purified three times at 33°C using a solid agarose overlay (3). The final plaque isolate was designated wild type (wt) and was grown at 33°C to passage 3 as a working stock using an input multiplicity of infection (MOI) of 0.1.

Mutagenesis. Cells were infected with wt virus at an MOI of 5 and incubated in the presence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG, 3.0 µg/ml) at 25°C. Culture fluids were harvested 38 h postinfection (p.i.) and clarified by centrifugation at $1,200 \times g$ for 30 min. Supernatants were dialyzed overnight against phosphate-buffered saline (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.3) containing bovine serum albumin (2 g/liter) with one change of buffer to remove the mutagen. Each sample was filtered through a 0.22-µm Millex filter (Millipore Corp.) and stored at 4°C.

Virus titration. Nonoccluded virus (*NOV*; i.e., virus not occluded in inclusion bodies) was assayed by an endpoint dilution technique described previously (2) using *S. frugiperda* cells.

Isolation of *ts* mutants. Virus that had replicated in the presence of NTG was plated on *S. frugiperda* monolayers at 25°C (3). Four separate mutagenized stocks were used. Well-isolated plaques were picked 5 days p.i. using sterile capillary pipettes, and each

plaque isolate was expelled into a vial containing 1 ml of BML-TC/10 (10). Samples were left overnight at 4°C, then were screened as follows. Microtest plates (Falcon Plastics) were seeded with 1,500 cells per well in 5- μ l aliquots, and undiluted 5- μ l aliquots of each plaque isolate were added to duplicate wells on separate plates. One plate was incubated at permissive temperature (25°C) and examined 5 days p.i. using an inverted microscope; the other was incubated at non-permissive temperature (33°C) and examined 4 days p.i. Plaque isolates that gave rise to a typical MP cytopathic effect (CPE; more than 10 polyhedra per cell [14, 20, 26]) at 25°C but not in the corresponding wells at 33°C were considered presumptive *ts* mutants. Culture fluid containing NOV was harvested, using a micropipette, from the appropriate wells in the plate that had been incubated at 25°C. Isolates from each well were diluted in 1 ml of BML-TC/10 and screened again in the same manner. Those isolates that remained *ts* with regard to CPE were plaque-purified three times at 25°C. Isolates were screened for temperature sensitivity in Microtest plates after each plaque passage to check that they had not reverted to wt phenotype. Mutants were grown to passage 3 at 25°C using an input MOI = 0.01. Virus preparations were used in experiments at either the second or third passage.

RIA procedure. The amount of polyhedrin in infected cells was measured by radioimmunoassay (RIA) using the direct sandwich method as previously described (8).

Preparation of antiserum for RIA. *A. californica* MNPV polyhedra were grown, purified, and dissolved in 0.1 M sodium carbonate as described (8). Polyhedrin was purified from dissolved polyhedra by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% acrylamide preparative slab gel (3 mm by 14 cm by 10 cm). The polyhedrin band, identified by staining a small strip of the gel with Coomassie blue, was cut out, and the polyhedrin was recovered by diffusion into 10 ml of phosphate-buffered saline. The purified polyhedrin in a 1-ml volume (300 μ g/ml) was emulsified in an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). A 6-month-old New Zealand white rabbit was injected subcutaneously at four sites (0.2 ml per site) along its back. Similar injections were given 2 weeks later with *A. californica* NPV polyhedrin (300 μ g/ml of phosphate-buffered saline) emulsified in an equal volume of Freund incomplete adjuvant. A third and fourth subcutaneous injection series was given 1 and 3 weeks after the second injection, using antigen without adjuvant. After a test bleed 10 days after the final injection, the rabbits were exsanguinated.

When tested by gel double diffusion (8), the precipitin endpoint of the antiserum was 1:16 against 15 μ g of purified *A. californica* NPV polyhedrin antigen. The serum was stored in an equal volume of glycerol at -20°C.

Immunoglobulin specific for polyhedrin was obtained by using an immunoadsorption procedure and labeled with ¹²⁵I using the chloramine T method as previously described (8).

Complementation analysis. Plastic tissue culture plates (Corning, 35-mm) were seeded with 2×10^6 *S.*

frugiperda cells and infected at total input MOI = 12 with single mutants or with mixtures of two mutants in equal proportion. In each case, the inoculum volume was 0.1 ml. Plates were placed in a humid container on a rocker platform for 1 h at room temperature for virus adsorption. Inoculum was aspirated off, and the plates were washed three times with 1 ml of BML-TC/10 lacking fetal calf serum. Fresh BML-TC/10 containing 10% fetal calf serum was added to the plates, and they were incubated in humid containers at 33°C. Cultures were examined 5 days p.i. Cells were scraped into the culture fluid using a rubber scraper that had been dipped in alcohol and flamed, and the contents of each plate were transferred to a sterile tube. Suspensions were clarified by centrifugation at $1,200 \times g$ for 30 min, and the supernatants were decanted into sterile tubes and stored at 4°C for assay of NOV. Pellets were stored frozen for determination of polyhedrin content by RIA.

For each pair of mutants, a complementation index (CI) was calculated as follows: CI = (amount of polyhedrin in cells coinfecting with mutants A and B)/(amount of polyhedrin in cells infected with A + amount of polyhedrin in cells infected with B), where A and B are different *ts* mutants.

For pairs of NOV mutants, it was possible to calculate CI based on release of NOV, as follows: CI = [(virus yield from A + B) titrated at 25°C]/[(virus yield from A + virus yield from B) titrated at 25°C], where A and B represent different *ts* mutants.

DNA-DNA hybridization. Pure *A. californica* NPV DNA, used to prepare the cellulose nitrate filters (25-mm diameter, Schleicher and Schuell type B6), was obtained by phenol extraction of virions purified from *A. californica* NPV polyhedra (23).

To obtain the ³H-labeled DNA for hybridization, 25-cm² plastic tissue culture flasks (Corning) containing 10^6 infected cells (MOI = 10) and incubated at 33°C were pulsed for either 2 or 3 h with BML-TC/10 medium without fetal calf serum but containing either 20 or 50 μ Ci of [³H]thymidine (Amersham) per ml. After the pulse, the medium was removed, and 2 ml of 0.5% sodium lauryl sarcosine in 0.01 M Tris buffer (pH 7.6) containing 0.001 M EDTA was added to lyse the cells. After incubation of the lysate for 5 min at 60°C, it was extracted twice with redistilled phenol saturated with Tris buffer as above. The aqueous phase was placed in a cellulose nitrate test tube (Beckman, 0.5 by 2 inches [ca. 1.3 by 5 cm]) and sonicated in a Virsonic Cell Disruptor (model no. 16-850) using the small probe at maximum power for five 30-s periods. The sonicated aqueous phase was diluted 1:10 in 2 $\times$ SSC (8.8 g of trisodium citrate and 17.5 g of sodium chloride in 1 liter of distilled water), and 1-ml aliquots were used for each DNA-DNA hybridization.

The DNA-DNA hybridization was carried out exactly as described by Green et al. (12). The amount of ³H-labeled DNA added to the filter was determined by 10% trichloroacetic acid precipitation (30 min at 4°C) of a 1-ml aliquot of diluted extract. The precipitate was collected on a 24-mm-diameter glass fiber disk (Whatman GF/A), washed three times with 5% trichloroacetic acid and once with absolute ethanol, then dried and counted. Glass fiber disks and cellulose nitrate filters were counted in a nonaqueous scintillant

(4 g of PPO [2,5-diphenyloxazole] + 0.15 g of POPOP [1,4-bis-(5-phenyloxazolyl)-benzene] per liter of toluene) using a liquid scintillation spectrometer.

RESULTS

NTG inactivation. NTG inhibited the replication of NPV at a concentration of 2.5 $\mu\text{g/ml}$ (Fig. 1A). The experiment was repeated using a range of NTG concentrations from 1 to 5 $\mu\text{g/ml}$, and at 3 $\mu\text{g/ml}$ NTG inhibited virus replication by 95% (Fig. 1B). Based on these results, an NTG concentration of 3 $\mu\text{g/ml}$ was chosen for the induction of mutants, since it was reasoned that a lower NTG concentration would induce

fewer mutants and a higher NTG concentration may induce mutants with multiple lesions.

Growth characteristics of *ts* mutants. Virus progeny replicated in the presence of NTG (3 $\mu\text{g/ml}$) were plated at 25°C. A total of 567 well-isolated plaques were picked from virus progeny replicated in the presence of NTG and plated at 25°C. Of these, 27 were temperature sensitive. This represented a mutation frequency of 4.8%. The first 10 *ts* mutants available were studied in detail and their growth characteristics are shown in Table 1. Mutants 1, 7, 8, 9, 10, and 12 appeared to be defective in production of NOV as well as polyhedra at 33°C and are referred to as *NOV* mutants. Alternatively, mutants 2, 3, 4, and 11 were restricted in production of polyhedra but did produce NOV at 33°C and thus were designated *Poly* mutants.

Reversion and leak frequencies are shown for each mutant in Table 1. Reversion frequencies were sufficiently low to indicate that the mutants were stable. It was not possible to measure leak frequencies of *Poly* mutants by using the assay methods employed in this study because *Poly* mutants produced considerable amounts of NOV at the nonpermissive temperature.

Yield of polyhedrin at permissive and nonpermissive temperatures. The yield of polyhedrin, the principal matrix protein of the polyhedron (32), can be measured by using RIA (8, 17, 24). In this study, it was first necessary to demonstrate that the RIA procedure was specific for polyhedrin and did not detect other viral proteins. As shown in Fig. 2, NOV concentrated from culture fluid by centrifugation did not bind ^{125}I -labeled anti-polyhedrin antiserum, indicating that polyhedrin and NOV proteins did not cross-react in this assay. Comparative yields of polyhedrin in mutant-infected cells at permissive and nonpermissive temperatures are given in Table 2. At nonpermissive temperature, mutant-infected cells synthesized 3 to 9% the amount of polyhedrin synthesized by cells infected with the same mutant at permissive temperature. In wt-infected cells, the yield of polyhedrin at nonpermissive temperature was 47% of the yield at permissive temperature. Polyhedrin yield in mutant-infected cells may also be expressed as a percentage of the yield in wt-infected cells at the same temperature. Thus, cells infected with *ts* mutants synthesized 56 to 127% and 4 to 22% the amount of polyhedrin synthesized by wt-infected cells at the permissive and nonpermissive temperatures, respectively.

Complementation analysis. Mutants whose defects are in different genes should complement each other in mixed infection. A qualitative assessment of complementation was often

FIG. 1. Inactivation of *A. californica* MNPV by NTG. Cells (3×10^6 per 25-cm² flask) were infected with wt virus at input MOI = 5 and incubated in the presence of BML-TC/10 containing NTG at the concentrations shown above. Culture fluids were harvested 38 h p.i., clarified by centrifugation at $1,200 \times g$ for 30 min, and dialyzed overnight against phosphate-buffered saline containing bovine serum albumin (2 g/liter) with one change of buffer. Samples were filtered through 0.22- μm Millex filters and titrated for NOV by an endpoint dilution method (2). A and B represent different experiments. Titer of progeny virus grown in absence of NTG was 2.8×10^7 infectious units per ml in experiment A and 3.3×10^7 infectious units per ml in experiment B.

TABLE 1. Growth characteristics of *A. californica* MNPV temperature-sensitive mutants^a

Designation	Mutant	33°C/25°C yield ^b	Reversion frequency ^c	Leak frequency ^d
	wt	$(8.0 \pm 4.3) \times 10^{-2}$		
NOV	<i>ts1</i>	$(5.5 \pm 1.9) \times 10^{-5}$	$<(3.06 \pm 1.1) \times 10^{-5}$	$(4.3 \pm 1.2) \times 10^{-4}$
Poly	<i>ts2</i>	$(1.4 \pm 0.5) \times 10^{-2}$	$(7.2 \pm 5.8) \times 10^{-4}$	NC ^e
Poly	<i>ts3</i>	$(1.6 \pm 0.3) \times 10^{-2}$	$(2.6 \pm 0.2) \times 10^{-4}$	NC
Poly	<i>ts4</i>	$(8.2 \pm 0.1) \times 10^{-3}$	$(7.8 \pm 0.1) \times 10^{-4}$	NC
NOV	<i>ts7</i>	$(4.0 \pm 2.3) \times 10^{-5}$	$<(2.1 \pm 0.8) \times 10^{-5}$	$(8.5 \pm 3.3) \times 10^{-4}$
NOV	<i>ts8</i>	$(6.5 \pm 2.7) \times 10^{-6}$	$<(4.5 \pm 2.0) \times 10^{-5}$	$(8.3 \pm 5.5) \times 10^{-4}$
NOV	<i>ts9</i>	$(7.4 \pm 4.8) \times 10^{-5}$	$<(2.1 \pm 0.8) \times 10^{-5}$	$(6.9 \pm 2.8) \times 10^{-4}$
NOV	<i>ts10</i>	$(2.8 \pm 1.6) \times 10^{-5}$	$<(2.1 \pm 0.8) \times 10^{-5}$	$(1.1 \pm 0.3) \times 10^{-4}$
Poly	<i>ts11</i>	$(3.8 \pm 0.6) \times 10^{-3}$	$(4.4 \pm 0.5) \times 10^{-4}$	NC
NOV	<i>ts12</i>	$(1.3 \pm 1.0) \times 10^{-4}$	$<(2.1 \pm 0.8) \times 10^{-5}$	$(3.3 \pm 1.3) \times 10^{-3}$

^a Plastic tissue culture plates (35-mm) were seeded with 2×10^6 cells, and the cells were infected at input MOI = 12. Culture fluids were harvested 5 days p.i. and clarified by centrifugation at $1,200 \times g$ for 30 min, and NOV were titrated by an endpoint dilution method (2). Each value is the mean result of two or more experiments $\pm$ standard error.

^b Yield at 33°C as ratio of yield at 25°C, both titrated at 25°C.

^c Reversion frequency = (mutant yield at 33°C, titrated at 33°C)/(wt yield at 33°C, titrated at 33°C). For Poly mutants, titer at 33°C is titer that gave rise to wt, not mutant, phenotype at 33°C.

^d Leak frequency = (mutant yield at 33°C, titrated at 25°C – mutant yield at 33°C, titrated at 33°C)/(wt yield at 33°C, titrated at 25°C).

^e NC, Not calculated.

FIG. 2. Direct sandwich solid-phase RIA using a purified polyhedra preparation dissolved with 0.1 M sodium carbonate (○) and NOV concentrated from culture fluid by centrifugation (□). Procedure used was described previously (8). Percent binding refers to the amount of ^{125}I -labeled anti-polyhedrin antibody bound per well as a percentage of amount added to each well of a disposable Microtiter plate.

possible after observing cultures that had been mixedly infected with mutants and incubated at 33°C. When complementation occurred, cells showing typical MP morphology could be seen (Fig. 3). The yield of polyhedrin in cultures coinfecting with pairs of mutants was determined by RIA and compared to the yield of polyhedrin in cultures infected with single mutants. CIs were calculated as described in Materials and Methods and are listed in Table 3. CIs greater than or equal to 1.5 with respect to polyhedrin synthesis were considered to indicate complementation between the two mutants examined.

On this basis, the 10 *ts* mutants were sorted into five complementation groups (Table 4).

Values shown in parentheses in Table 3 are CIs based on release of NOV and support the assignment of mutants to the complementation groups shown in Table 4. The CIs based on NOV yield from cells coinfecting with *ts1* and *ts12* and with *ts9* and *ts12* were low in repeated experiments but appeared to indicate complementation, in contrast to the CIs based on polyhedrin synthesis for the same mutants. To determine whether the low CIs in this case indicated delayed complementation, the experiment was re-

peated, and the NOV was harvested after one replication cycle, 36 h p.i. The CIs calculated for cultures coinfecting with *ts1* and *ts12* and with

TABLE 2. Comparative yields of polyhedrin in infected cells at permissive and nonpermissive temperatures^a

Mutant	Ratio of polyhedrin yields		
	33°C/25°C ^b	Mutant/wt at 33°C	Mutant/wt at 25°C
wt	0.47 ± 0.13	1.0	1.0
<i>ts1</i>	0.09 ± 0.05	0.10 ± 0.04	0.89 ± 0.09
<i>ts2</i>	0.04 ± 0.02	0.12 ± 0.04	1.00 ± 0.25
<i>ts3</i>	0.09 ± 0.02	0.22 ± 0.04	0.76 ± 0.12
<i>ts4</i>	0.05 ± 0.01	0.09 ± 0.03	0.56 ± 0.08
<i>ts7</i>	0.04 ± 0.01	0.05 ± 0.02	0.96 ± 0.17
<i>ts8</i>	0.03 ± 0.01	0.04 ± 0.02	0.85 ± 0.24
<i>ts9</i>	0.03 ± 0.01	0.05 ± 0.01	0.86 ± 0.14
<i>ts10</i>	0.05 ± 0.02	0.10 ± 0.03	1.27 ± 0.30
<i>ts11</i>	0.03 ± 0.01	0.06 ± 0.02	0.85 ± 0.23
<i>ts12</i>	-0.06 ± 0.02	0.12 ± 0.03	1.17 ± 0.26

^a Cells (2×10^6 per 35-mm plate) were infected at input MOI = 12. Polyhedrin was measured 5 days p.i. by RIA as described in the text.

^b Polyhedrin yield (micrograms per 2×10^6 cells) from infected cells at 33°C as ratio of polyhedrin yield in cells infected with the same virus at 25°C.

ts9 and *ts12* were 1.9 and 2.3, respectively. Thus, in group A, *ts12* complemented *ts1* and *ts9* at a low level for the production of NOV but not polyhedrin; however, *ts1* and *ts9* did not complement each other for the synthesis of either NOV or polyhedrin. Mutant *ts3* clearly complemented only one mutant, *ts8*, and thus is included in four complementation groups, A, B, C, and E.

DNA synthesis of *ts* mutants. The quantity of *A. californica* NPV bound to cellulose nitrate filters (3 µg) was found to be at least twice that required to hybridize ³H-labeled viral DNA present in samples of wt-infected cell extracts used for DNA-DNA hybridization (Table 5). Mock-infected and *ts8*-infected cells showed levels of ³H-labeled DNA hybridization equivalent to that found on DNA-free filters, indicating the absence of virus-specific DNA synthesis (Table 5). All the remaining NOV *ts* mutants showed virus-specific DNA synthesis. The pattern of DNA synthesis in *ts8* was further examined by pulsing with [³H]thymidine at 3-h intervals for the first 21 h after infection and comparing the virus-specific DNA hybridization of the pulses with that obtained from pulses of wt-infected cells (Fig. 4).

Synthesis of total DNA was markedly in-

FIG. 3. Complementation between *ts* mutants. Cells were infected at total input MOI of 12 with *ts3* or *ts8* alone or with a mixture of *ts3* and *ts8* in equal proportions. Cultures were incubated at 33°C and photographed 5 days p.i. using an inverted microscope. (a) Cells infected with *ts3*; (b) cells coinfecting with *ts3* and *ts8*; (c) cells infected with *ts8*. Bar represents 50 µm.

TABLE 3. Complementation analysis of *A. californica* MNPV temperature-sensitive mutants^a

Mutant	<i>ts1</i>	<i>ts2</i>	<i>ts3</i>	<i>ts4</i>	<i>ts7</i>	<i>ts8</i>	<i>ts9</i>	<i>ts10</i>	<i>ts11</i>	<i>ts12</i>
<i>ts1</i>	—	1.24 ± 0.13	1.23 ± 0.43	1.46 ± 0.26	1.55 ± 0.45 (167 ± 106)	8.90 ± 1.20 (15,000)	0.44 ± 0.09 (0.7)	3.87 ± 2.64 (484 ± 316)	1.20 ± 0.55	0.52 ± 0.13 (8.0 ± 6.5)
<i>ts2</i>		—	0.85 ± 0.10	2.20 ± 0.65	1.41 ± 0.72	9.00 ± 4.00	1.66 ± 0.56	1.43 ± 0.37	2.02 ± 1.02	1.19 ± 0.19
<i>ts3</i>			—	1.36 ± 0.27	0.98 ± 0.13	1.95 ± 0.20	0.96 ± 0.39	0.85 ± 0.26	1.06 ± 0.30	1.03 ± 0.24
<i>ts4</i>				—	1.44 ± 0.15	4.10 ± 1.10	4.70 ± 2.80	3.90 ± 2.70	6.00 ± 4.60	1.36 ± 0.16
<i>ts7</i>					—	6.39 ± 1.30 (6,600)	2.98 ± 0.50 (257 ± 168)	1.67 ± 0.26 (270)	2.71 ± 0.80	2.06 ± 0.83 (430)
<i>ts8</i>						—	4.50 ± 0.69 (3,600)	2.73 ± 0.57 (15,000)	3.73 ± 0.38	5.15 ± 0.46 (1,400)
<i>ts9</i>							—	2.18 ± 0.39 (437)	0.64 ± 0.18	0.73 ± 0.14 (2.2 ± 0.1)
<i>ts10</i>								—	2.56 ± 1.19	1.82 ± 0.84 (79 ± 43)
<i>ts11</i>									—	0.82 ± 0.32
<i>ts12</i>										—

^a Cells (2×10^6 cells per 35-mm plate) were infected at total input MOI = 12 with single mutants or with pairs of mutants in equal proportions. Cultures were incubated at 33°C for 5 days, and then cells were scraped into the culture fluid and transferred to sterile tubes. After centrifugation at $1,200 \times g$ for 30 min, supernatants were decanted and stored at 4°C for assay of NOV (2). Pellets were stored frozen for RIA (8). CI was calculated as described in the text. Numbers shown are mean values ± standard error of CIs determined from two or more experiments. Numbers in parentheses refer to the CI based on NOV yield. For mutant crosses in which complementation was clearly positive or negative, NOV was titrated in one experiment; for those crosses that gave ambiguous results, NOV was titrated in two or more experiments.

TABLE 4. Assortment of *A. californica* MNPV *ts* mutants into complementation groups^a

Complementation group	Mutants
A	1, 2, <u>3</u> , 9, 11, 12
B	4, <u>3</u>
C	7, <u>3</u>
D	8
E	10, <u>3</u>

^a According to the data shown in Table 3, pairs of mutants for which the CI, based on polyhedrin yield, was less than or equal to 1.5 were placed in the same complementation group; those with a CI greater than or equal to 1.5 were sorted into different complementation groups. Using this criterion, *ts3* is underlined because it fits into more than one complementation group.

hibited by 9 h p.i. in cells infected with wt and *ts8* at 33°C (Fig. 4). During the same period, little virus-specific DNA synthesis was detected. This result confirms that the rate of cellular DNA synthesis decreases after infection (20a). Later in infection, virus-specific DNA synthesis increased in cells infected with wt virus, but the rise did not occur with *ts8* infection (Fig. 4).

DISCUSSION

Each *ts* mutant that was isolated either failed to induce a CPE or induced an altered CPE at 33°C. A variety of CPE was observed, ranging from an FP (few polyhedra) response (14, 26), through a granular appearance, to the absence of inclusions in swollen cells. Accordingly, the mutants were grouped into two principal classes: (i) those that produced high yields of NOV while

TABLE 5. DNA synthesis in *A. californica* NPV NOV temperature-sensitive mutants

Virus type	DNA bound to filter (μg)	Total [³ H]-DNA added ^a (cpm)	[³ H]DNA hybridized (cpm ± SD ^b)	Percent hybridized
wt	0.0	11,480 ± 736	441	4
wt	0.003	11,480 ± 736	433 ± 8	4
wt	0.03	11,480 ± 736	708 ± 37	6
wt	0.3	11,480 ± 736	2,524 ± 100	22
wt	1.5	11,480 ± 736	6,147 ± 78	54
wt	3.0	11,480 ± 736	6,082 ± 109	53
Mock-infected cells	3.0	13,821	243 ± 4	2
<i>ts1</i>	3.0	20,421	19,670 ± 197	96
<i>ts7</i>	3.0	18,225	10,677 ± 1,716	59
<i>ts8</i>	3.0	1,491	205 ± 4	13
<i>ts9</i>	3.0	13,531	12,588 ± 412	93
<i>ts10</i>	3.0	6,818	3,771 ± 471	55
<i>ts12</i>	3.0	25,397	19,918 ± 5,751	78

^a ³H-labeled DNA was extracted, as described in the text, from infected or mock-infected cells at 33°C pulsed with 50 μCi of [³H]thymidine per ml for 2 h between 12 and 14 h p.i.

^b SD, Standard deviation.

exhibiting a restriction in polyhedra formation (*Poly* mutants), and (ii) those in which NOV and polyhedra production were both restricted (*NOV* mutants). Of these, *ts8* was the only mutant that failed to synthesize viral DNA at the nonpermissive temperature. The inhibition of cellular DNA synthesis by *ts8* is consistent with the observation that cells infected with this mutant at the nonpermissive temperature do not grow (data not shown).

The data in Table 1 show that the yield of wt virus at 33°C was approximately 1 log lower than the yield at 25°C. This consistent finding

FIG. 4. Patterns of virus-specific DNA synthesis in wt- and ts8-infected cells at 33°C. ^3H -labeled DNA from cells pulsed with 20 μCi of [^3H]thymidine per ml for 3-h periods during the first 21 h of infection was hybridized to cellulose nitrate filters containing 3 μg of *A. californica* NPV DNA. The ^3H -labeled DNA added to the filter (unshaded histogram) gives an indication of total DNA synthesis, since the labeling and DNA extraction procedures were identical for all pulses. The ^3H -labeled DNA hybridized to the filter (shaded histogram) was corrected for nonspecific binding to DNA-free filters. Error bars represent the standard deviation of results from two filters.

does not affect interpretation of the results, since the virus yields of NOV mutants at 33°C were several logs lower than the yields at 25°C. A similar decrease in yield of wt virus at nonpermissive temperature has been reported for herpes simplex virus (9).

Synthesis of polyhedrin in cells infected with single mutants at nonpermissive temperature (Table 2) probably reflects leakiness of the mutants.

In this study, assignment to complementation groups depended on observation of an increased yield of polyhedrin in cells coinfecting with pairs of mutants. Several lines of evidence suggest that polyhedrin is a late protein in virus replication. Growth studies (39) and ultrastructural studies of morphogenesis (15, 19, 21) indicate that polyhedra are formed after budding of NOV and enveloping of nucleocapsids in the nucleus. In addition, kinetic experiments involving protein synthesis in infected cells have shown that polyhedrin is synthesized after many virion structural proteins (D. C. Kelly and T. Lescott, Abstr. Fourth Int. Congr. Virol., 1978, w42, p. 562). An immunoperoxidase staining technique has also demonstrated that polyhedrin synthesis in infected cells follows that of other viral proteins (33).

CIs that were considered positive ranged from 1.5 to 9.0 for polyhedrin synthesis and from 2 to 15,000 for NOV yields (Table 3). Similar wide

ranges in CI have been reported in genetic studies with other animal viruses, and it is interesting to speculate on the reason for the variation. Microscopic examination of cultures coinfecting with pairs of mutants indicated that some cells in the population exhibited the morphology induced by single mutants, whereas others produced polyhedra. The latter were those in which complementation had occurred. A high CI was associated with an increased proportion of cells that produced polyhedra rather than an increased production of polyhedra in each cell. According to the Poisson distribution, an input MOI of 12, as used in the experiments, is sufficient to ensure that 99.95% of the cells interact with at least three infectious units of virus, and the probability that the three infecting mutants are the same is small (1/8). Thus the lack of complementation in some cells of the population is not due to the fact that those cells were not infected with both mutants. Cairns (6) proposed that for each virion that penetrates the host cell, there is a constant probability that in a given period of time a virion will begin its replicative cycle. It is possible, therefore, that one mutant of a pair may start replicating and interfere with replication of the other mutant. If so, complementation would occur only in cells in which both mutant virions begin replicating at the same time. Another possibility is that a low CI may indicate intragenic complementation, whereas a high CI may reflect intergenic complementation. A protein composed of identical subunits may be partially functional as a result of intragenic complementation. Mutant subunits with different defects may interact to yield a partially functional protein, whereas subunits that all possess the same defect interact, but yield a nonfunctional product. Alternatively, intragenic complementation could be reflected in the frequency of subunit interaction to yield a completely functional protein. For example, mutant subunits with different defects may interact with lower frequency than wt subunits but with higher frequency than mutant subunits all with the same defect. Intragenic complementation has been shown to occur with deoxypyrimidine kinase mutants of herpes simplex virus (16) and may account for the complementation of *ts1* and *ts12* by *ts9* for the production of NOV but not polyhedrin.

Table 4 indicates that the ten *ts* mutants were sorted into five complementation groups, with group A containing six mutants. Although these six mutants were isolated from the same mutagenized stock, they are not clonally related. *ts1*, *ts9*, and *ts12* express a different phenotype at 33°C than *ts2*, *ts3*, and *ts11*. *ts1* and *ts9* both

complement *ts12* to a low degree for NOV production and thus must differ in genotype from *ts12*. Although *ts1* and *ts9* fail to complement each other, recombination studies to be reported later indicate that these mutants recombine with a low but reproducible frequency and hence are not genotypically identical. *ts3* is clearly distinct from *ts2* and *ts11*, since it complements only *ts8*. CIs for pairs involving *ts2* are different than those involving *ts11*, indicating that *ts2* and *ts11* are also distinct mutations.

It is interesting that *ts3* complements only *ts8* and even then to a low degree (CI = 1.95). This might indicate that *ts3* contains multiple defects; however, *ts3* is a *Poly* mutant (i.e., produces NOV but not polyhedra at 33°C), and it seems unlikely that multiple temperature-sensitive mutations would all occur in nonessential genes. Alternatively, *ts3* may be dominant over other mutants when cells are coinfecting. With a virus genome of molecular weight of approximately 60×10^6 to 85×10^6 (4, 5, 13, 18), it is expected that many more complementation groups will be found.

The authors suggest that a uniform system of nomenclature be followed when reporting studies with baculovirus mutants. The one proposed here is based on the nomenclature system described by Schaffer et al. (27) for herpes simplex virus. Each baculovirus mutant was given a name consisting of seven components, outlined as follows:

(i) *MP* or *FP* designates the parental strain of NPV used.

(ii) Lowercase letters describe the properties of the mutant; for example, temperature sensitive (*ts*) or plaque morphology (*pm*).

(iii) A description of the mutant phenotype; for example, *NOV* for those mutants that failed to synthesize NOV and *Poly* for those that failed to produce polyhedra at the nonpermissive temperature.

(iv) A capitalized letter, A to Z, denotes the complementation group to which the mutant has been assigned. A dash (-) indicates that complementation studies have not been done with that mutant, and an asterisk (*) indicates that the mutant failed to complement others and has not been assigned to a complementation group.

(v) A number from 1 upwards is the mutant number. Workers in different laboratories should use different numbers to avoid overlap and confusion. We suggest that the next research group to report the isolation of mutants of *A. californica* NPV number their mutants from 201 to 400, the next group from 401 to 600, and so on.

TABLE 6. Nomenclature of *A. californica* MNPV *ts* mutants^a

Complete designation	Abbreviated designation	Designation used in this report
<i>MP ts (NOV) A1g (SF)</i>	<i>tsA1</i>	<i>ts1</i>
<i>MP ts (Poly) A2g (SF)</i>	<i>tsA2</i>	<i>ts2</i>
<i>MP ts (Poly) *3g (SF)</i>	<i>ts*3</i>	<i>ts3</i>
<i>MP ts (Poly) B4g (SF)</i>	<i>tsB4</i>	<i>ts4</i>
<i>MP ts (NOV) C7g (SF)</i>	<i>tsC7</i>	<i>ts7</i>
<i>MP ts (NOV) D8g (SF)</i>	<i>tsD8</i>	<i>ts8</i>
<i>MP ts (NOV) A9g (SF)</i>	<i>tsA9</i>	<i>ts9</i>
<i>MP ts (NOV) E10g (SF)</i>	<i>tsE10</i>	<i>ts10</i>
<i>MP ts (Poly) A11g (SF)</i>	<i>tsA11</i>	<i>ts11</i>
<i>MP ts (NOV) A12g (SF)</i>	<i>tsA12</i>	<i>ts12</i>

^a The nomenclature system was based on that described by Schaffer et al. (27) for herpes simplex virus. Since *ts3* fitted into more than one complementation group (*), it remained unclassified.

(vi) A lowercase letter indicates the mutagen used; for example, *b* for 5-bromo-2'-deoxyuridine, *g* for NTG, *u* for UV light.

(vii) Uppercase letters designate the cell line in which the phenotype was determined.

According to this system, the 10 *ts* mutants described in this paper have been named as shown in Table 6.

The same techniques that have been used in this study could be applied to select mutants of higher virulence or altered host range, or mutants that give higher yields of polyhedra in cell culture for mass production of polyhedra.

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