Isolation, Complementation, and Initial Characterization of Temperature-Sensitive Mutants of the Baculovirus *Autographa californica* Nuclear Polyhedrosis Virus†

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Sixteen temperature-sensitive mutants of *Autographa californica* nuclear polyhedrosis virus were isolated. Several interesting phenotypes were observed. A large proportion of the mutants were unable to form polyhedral occlusion bodies (polyhedra) at the nonpermissive temperature (32.5°C). At 32.5°C, one mutant formed plaques in which the cells lacked polyhedra. Another mutant type was defective in the production of progeny extracellular nonoccluded virus and produced a "plaque" consisting of only a single cell containing polyhedra at 32.5°C. One mutant was defective in plaque formation, progeny nonoccluded virus formation, and polyhedra formation at 32.5°C. Several mutants produced nonoccluded virus but failed to produce plaques or polyhedra at 32.5°C. Other phenotypes were also distinguished. Complementation analyses, performed by either measuring the increase in extracellular nonoccluded virus formation or by observing polyhedra formation in mixed infections at 32.5°C, indicated the presence of 15 complementation groups. A high frequency of recombination was observed. Four of the mutants were found to be host dependent in their temperature sensitivity for polyhedra formation.

Nuclear polyhedrosis viruses (NPVs), members of the family Baculoviridae, possess enveloped, rod-shaped nucleocapsids containing a double-stranded, circular, supercoiled DNA genome of approximately 85 megadaltons (1, 3, 13, 20). Viral DNA replication and nucleocapsid formation occur in the nuclei of invertebrate host cells. Progeny nucleocapsids can acquire envelopes by budding through cellular membranes and can be released from infected cells in this form (5, 12). Alternatively, the nucleocapsids can acquire membrane envelopes by an envelopment process within the nucleus (4, 12, 19). Enveloped nucleocapsids in the nucleus may be occluded within crystalline protein matrices known as polyhedral occlusion bodies or, simply, polyhedra. Polyhedra are highly refractive and quite large (1 to 15 µm in diameter), thus being readily visible by light microscopy (18).

The complex development of NPVs, including the formation of both occluded viruses and extracellular nonoccluded viruses (NOVs), is an important aspect of the NPV infection process (4, 5). Occluded viruses effect the horizontal transmission of NPV infection from host organism to host organism; the crystalline protein matrix provides protection to the virions in the

† Idaho Agricultural Experiment Station research paper no. 7952.

environment and enroute to the midgut via the alimentary canal. NOVs are responsible for the spread of infection from cell to cell via the invertebrate hemolymph in vivo or in cell culture media (2, 5, 6, 25). The formation of NOVs and the formation of polyhedra are apparently under temporal control, with the appearance of extracellular NOVs preceding polyhedra formation (26). A genetic analysis of NPVs could provide considerable insight into the control of virus development and the process of baculovirus infection generally.

Our laboratory has undertaken an extensive molecular genetic analysis of an NPV, *Autographa californica* NPV (AcNPV). AcNPV is the most extensively studied baculovirus in terms of its DNA genome (13), structural proteins (23), and cell culture replication properties (2, 26). We have previously demonstrated the ability to clone AcNPV by the isolation of a variety of genotypic variants distinguished by restriction endonuclease analysis (11). We now report the successful isolation of AcNPV temperature-sensitive (ts) mutants, many of which are defective in occluded virus or NOV formation or both. This work is expected to provide a basis for (i) further understanding the control of occluded virus development, (ii) further understanding the mechanism of baculovirus infection, which

is of considerable importance in relationship to the use of these viruses as biological pesticides (22), and (iii) the development of a genetic map which will be of importance in exploring AcNPV as a potential vector for recombinant DNA research in invertebrates.

MATERIALS AND METHODS

Virus and cells. The parent virus used in this work was the L1 variant of AcNPV, which was cloned and described previously (11). Virus used for inoculating cell cultures was NOV derived from cell culture media (11). Long-term serial passage in cell culture was minimized by replaquing or passage through insect larvae approximately every sixth passage. A *Spodoptera frugiperda* cell line (IPLB-SF-21) was used for propagation and plaque assay of NOV (11). Cells were routinely maintained in TC-100 medium at 27°C and passed every 3 days. For experiments requiring large numbers of cells, the cells were cultured in 200- to 300-ml suspension cultures at room temperature. Cell growth was exponential, and growth could be maintained by removing a portion of the cells for experimental use and replenishing the remaining cell suspension culture with TC-100 medium.

In host range studies, a cell line of *Trichoplusia ni*, TN-368, was employed (7). This cell line was adapted for growth in TC-100 medium and was provided by D. L. Knudson, Yale Arbovirus Research Unit.

Mutagens. 5-Bromodeoxyuridine (BUdR) was purchased from P. L. Biochemicals, Inc. A stock BUdR solution was prepared by dissolving 1 mg of BUdR in 10 ml of distilled water. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was purchased from Aldrich Chemical Co. A stock NTG solution was prepared by dissolving 1 mg of NTG in 10 ml of distilled water.

Plaque assay. Plaque assays were performed as previously described (11), with the following modification. Petri dishes were seeded with 2×10^6 cells per dish and allowed to attach for 2 h before virus inoculation.

Mutagenesis of AcNPV by NTG. A mutagenesis procedure described for herpesvirus using NTG (17) was modified for AcNPV mutagenesis. Approximately 2×10^6 cells growing exponentially in suspension culture were seeded in tissue culture dishes (60 by 10 mm) and incubated for 2 h at 27°C for attachment. The medium was removed, and the cells were infected with wild-type virus, AcNPV L1, at a multiplicity of infection (MOI) of 5 PFU/cell. After 1 h of adsorption at room temperature, the monolayers were washed twice with TC-100 medium, and the dishes were incubated with 5 ml of medium for 10 h at 27°C. At 10 h postinfection, the stock solution of NTG was diluted so that the dishes contained 1, 2, 5, or 8 μg of NTG per ml of medium. The dishes were incubated at 23.5°C for 2 h, and then the NTG-containing medium was replaced with fresh NTG-free TC-100 medium. The dishes were incubated for 20 h at 23.5°C before collection of extracellular NOV. The infected culture media were centrifuged at $2,000 \times g$ for 20 min, and the virus-containing supernatants were titrated by plaque assay at 23.5°C and stored at 4°C as mutagenized virus stocks.

Mutagenesis of AcNPV by BUdR. The cells were seeded and infected with AcNPV L1 as described above for NTG mutagenesis. After a 1-h adsorption, the infected cells were incubated in 5 ml of TC-100 medium containing BUdR at final concentrations of 1, 2, 4, 6, 10, 20, and 50 μg/ml of medium. After incubation at 23.5°C for 48 h, the culture media were centrifuged at $2,000 \times g$ for 20 min. The supernatants were titrated and then stored at 4°C as mutagenized virus stocks.

Isolation of ts mutants. Mutagenized virus stocks were diluted appropriately for plaque assay. The infected monolayers, overlaid with media containing 0.5% agarose, were incubated at 23.5°C for 4 to 5 days and stained with neutral red. Well-isolated plaques were randomly picked from plates containing fewer than 10 plaques and transferred into 0.5 ml of TC-100 basal medium. Each of the plaques was used to inoculate two sets of tissue culture dishes. The inoculated monolayers were overlaid with 0.5% agarose medium and incubated at 23.5 or 32.5°C and screened for cytopathic effects at 23.5 and 32.5°C after 4 to 5 days. Viruses indicating temperature sensitivity were replaqued and developed into virus stocks at the permissive temperature. Each of the ts virus stocks was titrated again at 23.5 and 32.5°C to confirm the temperature sensitivity.

Rate of NOV release at 23.5 and 32.5°C. Duplicate dishes (100 by 20 mm) of *S. frugiperda* cells containing 4×10^6 cells were each infected with virus at an MOI of 5 PFU/cell. After a 1-h adsorption at room temperature, the cells were washed twice with medium and then incubated in 15 ml of TC-100 medium. One dish of a duplicate set was incubated at 23.5°C, and the other was incubated at 32.5°C. At scheduled times, 0.5 ml of culture medium containing NOVs was removed and stored at 4°C until all samples were collected, and then the samples were titrated at 23.5°C.

Adsorption test of ts mutants of AcNPV. *S. frugiperda* cells (2×10^6 cells per dish) were infected with approximately 100 PFU of virus in 0.1 ml of TC-100 medium. Before infection, the cells and virus solutions were prewarmed to either 23.5 or 32.5°C. One dish of a duplicate set was incubated at 23.5°C for a 1-h adsorption, and the second dish was incubated at 32.5°C for a 1-h adsorption. After the 1-h adsorption period, the plates were washed twice with media prewarmed at the appropriate temperatures. The plates were overlaid with 0.5% agarose medium (11) and incubated at 23.5°C for 5 days. Cells were stained with neutral red (11), and the number of plaques per dish was counted.

Complementation tests of ts mutants of AcNPV. *S. frugiperda* cells (1.5×10^6 cells per dish [60 by 15 mm]) were infected with pairs of ts mutants (double infections) or with each of the mutants separately (single infections). The MOI was 10 PFU/cell in single infections and 5 PFU/cell for each of the two mutants in double infections.

Adsorption was carried out at room temperature for 1 h with tilting every 15 min. The cells were washed with TC-100 medium, and 4 ml of the medium was added. The infected cells were incubated at 32.5°C. At 48 h postinfection, cells were suspended in the me-

dium, and the percentage of cells containing polyhedral occlusion bodies was determined under a light microscope. In the cases where complementation analysis was performed by determining the increase in NOV formation, the medium was collected by centrifugation, and the yield of NOV was determined by plaque assay at 23.5°C. Plaque assay at 32.5°C provided information concerning recombination.

Electron microscopic studies of cells infected with ts-B1074 and wild-type virus. Duplicate sets of *S. frugiperda* cells (2 × 10^6 cells per dish [60 by 15 mm]) were infected with ts-B1074 and wild-type virus at an MOI of 5 PFU/cell and incubated at 23.5 and 32.5°C for 3 days. The infected cells were harvested by centrifugation at 2,000 × *g* for 15 min. The pellets were fixed in 5% glutaraldehyde solution for 1 h and then in 2% osmium tetroxide for 1 h according to the method of Harrap (4), except that the two fixatives were dissolved in phosphate buffer, pH 6.2 (11). The fixed samples were embedded in agar and dehydrated through a series of increasing alcohol concentrations. The dehydrated samples were embedded in Epon and sectioned. The sections were stained in 2% (wt/vol) uranyl acetate in a solution of acetic acid and lead citrate (16) and visualized with a Zeiss 10 electron microscope at an accelerating voltage of 80 kV.

Temperature shift experiments. *S. frugiperda* cells (2 × 10^6 cells per dish [60 by 15 mm]) were infected with each ts mutant at an MOI of 5 PFU/cell. After a 1-h adsorption, 5 ml of TC-100 medium was added to each, and the infected cells were incubated at 23.5 and 32.5°C for 12 h. At 12 h postinfection, the infected cells at the low temperature were shifted from the low to the high temperature and vice versa. Cytopathic changes, particularly polyhedral occlusion body formation in the infected cells, were observed by light microscopy at 18, 20, 22, 24, 26, 28, 30, 32, 40, 48, and 60 h postinfection. Controls at 23.5 and 32.5°C without temperature shift were also performed.

Host range test of ts mutants on TN-368 cells. Duplicate dishes of *S. frugiperda* cells and of TN-368 cells (2 × 10^6 cells per dish [60 by 15 mm]) were infected with each of the ts mutants at an MOI of 5 PFU/cell. After a 1-h adsorption, 5 ml of TC-100 medium was added to each dish, and the dishes were incubated at 23.5 or 32.5°C for 3 days. The extent of

the cytopathic effects, particularly polyhedral occlusion body formation, was periodically checked by light microscopy.

RESULTS

Mutagenesis of AcNPV. The effects of increasing concentrations of the mutagens BUdR and NTG on the production of extracellular NOVs and the proportion of ts mutants observed are presented in Table 1. In the case of BUdR mutagenesis, the dramatic drop in NOV titer between 20 and 50 μg of BUdR per ml paralleled a decrease in cell health. There was no general increase in the proportion of ts mutants observed with increased BUdR treatment under the conditions employed. In the case of NTG mutagenesis, there was a trend of an increasing proportion of ts mutants with increasing NTG concentrations.

A total of 2,111 plaque isolates were screened for their ability to multiply at 23.5 and 32.5°C. Sixteen isolates were found to be temperature sensitive for one or more characteristics of normal (wild-type) virus growth at 32.5°C. Of the 16 ts mutants, 11 were derived from the BUdR-treated virus stocks, and 5 were derived from the NTG-treated stocks. The mutants are listed in Table 1 relative to mutagen conditions and screening results.

Characteristics of ts mutants. Wild-type AcNPV forms plaques 3 to 4 mm in diameter at 23.5°C; cells within these plaques contain polyhedral occlusion bodies. At 32.5°C, wild-type virus produces plaques 2 to 3 mm in diameter with polyhedral occlusion bodies. Differences in the ts mutants at 23.5 and 32.5°C were observed by plaque assay. The ts mutants could be generally divided into four groups by their characteristics in plaque assays at the nonpermissive temperature (Table 2).

(i) Single-cell infections. Two mutants, ts-

TABLE 1. *Characteristics of mutagenesis of AcNPV*

Mutagen	Concn (μg/ml)	No. of PFU/ml	No. of plaques screened	No. of ts mutants found	Mutants
BUdR	0	5 × 10^7	0		
	1.0		280	1	ts-B113
	2.0	2 × 10^7	100	0	
	6.0	4 × 10^6	100	1	ts-B1148
	10	4 × 10^6	100	2	ts-B1074, -B1075
	20	1.5 × 10^6	370	5	ts-B15, -B821, -B837, -B918, -B951
	50	4 × 10^3	265	2	ts-B97, -B305
NTG	0	1 × 10^7	0		
	1	6 × 10^6	154	0	
	2	4 × 10^6	262	1	ts-N706
	5	7 × 10^5	402	2	ts-N356, -N482
	8	6 × 10^5	178	2	ts-N332, -N1054

TABLE 2. *Properties of ts mutants of AcNPV in plaque assay*

Mutant group	Virus	23.5°C		32.5°C	
		PFU/ml	Plaque size (mm)	PFU/ml	Plaque size
	Wild type	8.9×10^7	3–4	8.6×10^7	2–3 mm
I	ts-N1054	2.0×10^7	1.0	1.3×10^6	1 cell
	ts-B1074	3.0×10^7	1.0	2.0×10^7	1 cell
II	ts-B1148	1.0×10^8	2–3	1.4×10^7	1–3 cells
III	ts-B821	3.9×10^7	1.0	2.8×10^4	0.5 mm
	ts-B837	3.6×10^7	2.0	5.0×10^6	0.5 mm[a]
	ts-B918	7.0×10^7	2.0	3.7×10^4	0.2 mm
	ts-B951	2.4×10^7	2–3	5.9×10^5	1.0 mm
	ts-B1075	2.4×10^8	2–3	2.1×10^6	0.4 mm
IVa	ts-B15	3.7×10^7	0.5	1×10^{4b}	
	ts-B305	2.7×10^7	1.0	1×10^{4b}	
	ts-N332	3.5×10^7	1.0	1×10^{4b}	
	ts-N356	6.5×10^7	1.0	1×10^{4b}	
	ts-N706	3.0×10^7	2.0	1×10^{4b}	
IVb	ts-B97	4.2×10^7	1.0	1×10^{4c}	
	ts-B113	2.4×10^7	1.0	1×10^{4c}	
	ts-N482	2.3×10^7	0.5	1×10^{4c}	

[a] At 32.5°C, ts-B837 formed plaques without polyhedral occlusion bodies.

[b] At 32.5°C, group IVa mutants caused cytopathic effects at low dilutions; no polyhedral occlusion bodies were observed.

[c] At 32.5°C, group IVb mutants caused cytopathic effects at low dilutions; a small proportion of affected cells contained polyhedral occlusion bodies.

N1054 and -B1074, infected only a single cell at 32.5°C. Single-cell infections could be observed under the light microscope due to polyhedral occlusion body formation. There was no indication of spread of infection to neighboring cells at the nonpermissive temperature. At 23.5°C, the mutants formed multiple-cell infections similar to those of wild-type virus, with plaque sizes approximately 1.0 mm in diameter. The number of PFU per milliliter observed at 23.5°C for ts-B1074 was similar to the number of single-cell infections observed at 32.5°C (Table 2). Experiments to further understand the nature of these mutants are discussed below.

(ii) **Few-cell infection.** Mutant ts-B1148 formed minute centers of infection at the non-permissive temperature, whereas at 23.5°C the mutant formed normal plaques. The infectious centers formed at 32.5°C usually consisted of only 1 to 3 cells containing polyhedral occlusion bodies. The ability of mutant ts-B1148 to achieve a spread of infection under plaque assay conditions was apparently partially impaired at 32.5°C.

(iii) **Reduced size and number of plaques.** Four ts mutants, ts-B821, -B837, -B918, and -B1075, formed plaques which were reduced in size and were from 1/4 to 1/10 the diameter of wild-type plaques at the nonpermissive temper-

ature (Table 2). ts-B837 formed small plaques which lacked polyhedral occlusion bodies at 32.5°C. The other three mutants formed small plaques with polyhedral occlusion bodies at 32.5°C. At 23.5°C, all four mutants formed plaques of normal size containing polyhedral occlusion bodies. Some variation in plaque size occurred with both wild-type and mutant clones. The mutant ts-B837 was unique in its formation of plaques lacking polyhedral occlusion bodies at the high temperature. The ability to observe distinct plaques of ts-B837 at high temperatures also indicates that the plaque system utilized in this work (11) does not rely on polyhedra production for plaque visualization. The mutants ts-B821, -B918, and -B1075 showed a 2 to 3 log reduction in the number of plaques, whereas ts-B951 showed only a 40-fold reduction in plaque number at the nonpermissive temperature of 32.5°C (Table 2). The possibility that these mutants might have a high reversion rate was tested by passing the mutants several times in cell culture at 23.5°C. No alteration in the ratio of plaques at 23.5°C to plaques at 32.5°C was observed on passage.

(iv) **No plaque formation.** The ts mutants which completely lacked the ability to form plaques at the nonpermissive temperature of 32.5°C were ts-B15, -B97, -B113, -B305, -N332,

-N356, -N482, and -N706 (Table 2). This group of ts mutants could be divided into two subgroups according to the tendency to form polyhedral occlusion bodies at 32.5°C: (a) the complete absence of polyhedral occlusion bodies in infected cells, and (b) the presence of polyhedral occlusion bodies in some of the infected cells at the nonpermissive temperature of 32.5°C.

(a) Complete absence of polyhedral occlusion bodies in infected cells. Mutants ts-B15, -B305, -N332, -N356, and -N706 formed normal plaques containing polyhedral occlusion bodies at the permissive temperature of 23.5°C but they lacked the ability to form plaques at the nonpermissive temperature of 32.5°C. The group IVa mutants (Table 2) showed temperature sensitivity in the formation of both plaques and polyhedral occlusion bodies. Cytopathic effects without the formation of polyhedral occlusion bodies were observed upon infection of *S. frugiperda* cells by these mutants with MOIs above 0.05 PFU/cell at 32.5°C. The number of cells exhibiting cytopathic effects at the nonpermissive temperature was directly related to the MOI. When MOIs below 0.05 PFU/cell were used, cytopathic effects were not evident.

(b) Presence of some polyhedral occlusion bodies in the infected cells. Mutants ts-B113, -B97, and -B482 failed to form plaques at the nonpermissive temperature, but cytopathic effects were observed at MOIs above 0.05 PFU/cell, with occasional cells producing polyhedral occlusion bodies. Even at very high MOIs (10 PFU/cell), polyhedral occlusion body formation occurred only in a small proportion of infected cells (Table 3). At MOIs below 0.05 PFU/cell cytopathic effects were not evident at 32.5°C. Plaque formation with polyhedral occlusion body formation was normal at 23.5°C.

Temperature dependence of virus adsorption. To determine whether any of the mutants were defective in adsorption at the nonpermissive temperature, similar amounts of virus were allowed to adsorb to cells maintained at 23.5 and 32.5°C. The cells were washed extensively with prewarmed media, and then plaques were developed at 23.5°C. The results in Table 4 indicate that all of the mutants are similar to wild type in their adsorption properties. Wild-type virus showed a slight decrease in adsorption ability at the higher temperature.

Production of infectious extracellular NOV. Each of the mutants was tested for its ability to release NOV into the media of infected *S. frugiperda* cells at 23.5 and 32.5°C. All of the mutants grown at the permissive temperature of 23.5°C released progeny nonoccluded virions into the culture medium in a fashion generally

TABLE 3. *Complementation analysis: percentage of cells containing polyhedral occlusion bodies*

Virus	% of cells containing polyhedral occlusion bodies with:															
	ts-N1054	ts-B1074	ts-B1148	ts-B821	ts-B837	ts-B918	ts-B951	ts-B1075	ts-B15	ts-B305	ts-N332	ts-N356	ts-N706	ts-B97	ts-B113	ts-N482
ts-N1054	8.3	—	99.6	87.6	88.2	98.8	96.6	100	96.9	78.4	99.1	88.4	41.7	90.1	65.0	99.3
ts-B1074		100	—	—	—	—	—	—	—	—	—	—	—	—	—	—
ts-B1148			7.6	85.5	77.7	72.8	99.2	100	100	68.6	100	87.8	48.7	99.1	97.4	99.6
ts-B821				28	68.4	100	54.8	88.9	83.3	93.0	83.7	88.1	100	98.7	97.6	93.1
ts-B837					0	23.9	48.5	87.6	100	58.3	85.4	45.4	61.1	98.9	73.9	99.2
ts-B918						11.5	100	100	99.5	81.3	93.3	75.3	73.4	96.7	58.3	99.7
ts-B951							28.5	92.5	99.8	74.3	63.0	44.2	75.8	98.0	56.5	100
ts-B1075								30	97.7	67.9	97.6	77.7	58.2	93.4	92.2	99.5
ts-B15									0	90.7	98.5	76.4	55.4	38.3	70.6	100
ts-B305										0	60.6	79.1	65.6	89.6	20.5	97.3
ts-N332											0	0	40.3	98.7	56.7	96.6
ts-N356												0	92.8	92.5	73.7	79.5
ts-N706													0	98.8	53.4	51.5
ts-B97														2.0	83.4	99.0
ts-B113															2.3	85.1
ts-N482																12.2

TABLE 4. *Temperature dependence of virus adsorption*

Virus	No. of PFU[a]		Ratio of 32.5°C PFU to 23.5°C PFU
	23.5°C adsorption	32.5°C adsorption	
Wild type	109	71	0.7
ts-N1054	143	73	0.5
ts-B1074	136	71	0.5
ts-B1148	120	85	0.7
ts-B821	108	55	0.5
ts-B837	108	78	0.7
ts-B918	141	115	0.8
ts-B951	94	30	0.3
ts-B1075	117	51	0.4
ts-B15	130	95	0.7
ts-B305	121	42	0.4
ts-N332	91	72	0.8
ts-N356	71	50	0.7
ts-N706	118	80	0.7
ts-B97	80	37	0.5
ts-B113	48	22	0.5
ts-N482	65	45	0.7

[a] The numbers of plaques per plate were determined at 23.5°C after virus adsorption at either 23.5 or 32.5°C.

similar to wild type (Fig. 1). Between 10 and 14 h postinfection, progeny NOV began to appear in the culture media, and NOV release proceeded exponentially until approximately 30 h postinfection at 23.5°C. By 40 h postinfection, maximal levels of NOV were usually achieved, and these levels were then maintained for the duration of the experiments (70 h postinfection). However, some of the mutants continued to release progeny NOV up to 70 h postinfection (Fig. 1). The mutants ts-B918, -B951, and -N332 tended to show delayed release of NOV at 23.5°C (Fig. 1B).

The amount of extracellular virus released at 32.5°C depended on the ts mutant and varied from no release to essentially normal levels (similar to wild type) (Fig. 2). The growth curves of the ts mutants at 32.5°C can be divided into three general categories: (i) those mutants showing essentially normal release, similar to wild type (Fig. 2A and B), (ii) those mutants with impaired ability to release NOVs, approximately 10-fold less than wild type (Fig. 2C), and (iii) those mutants essentially defective in the release of NOVs from infected cells (Fig. 2D).

Mutants ts-B15, -B97, -B113, -B305, -B951, -B821, -B837, -N332, and -N356 released progeny nonoccluded virions from infected cells to a similar extent as wild type at 32.5°C (Fig. 2A and B). The rate of release of the progeny NOVs from infected cells was similar to that observed at 23.5°C, although the maximal level was gen-

erally reached 10 h faster at 32.5°C (by 30 h postinfection) than at 23.5°C. The pattern for NOV release by mutants ts-N482, -B918, -B1075, and -B1148 appeared intermediate between normal (wild type) release and the no-release groups at the nonpermissive temperature of 32.5°C (Fig. 2C). The release of NOV by these mutants at the restrictive temperature was reduced compared with the release at the permissive temperature of 23.5°C and with the release by wild type at 32.5°C. The maximal release of progeny NOVs from the infected cells was approximately 10-fold less than that of the normal release group. The rate of release of progeny NOVs from infected cells by these mutants was also slower. At the permissive temperature of 23.5°C, mutants ts-N706, -B1074, and -N1054 are similar to wild type and release high titers of progeny NOVs. At the nonpermissive temperature of 32.5°C, these three mutants were severely defective in the release of infectious progeny NOV even at an MOI of 5 PFU/cell (Fig. 2D).

Electron microscopic analysis of ts-B1074- and wild-type virus-infected cells. Since ts-B1074 was defective in the release of infectious NOV yet produced polyhedral occlusion bodies in single cells at 32.5°C, it was of interest to determine whether nucleocapsids were produced in the nuclei of infected cells at 32.5°C. Cells infected with ts-1074 or wild-type virus at permissive and nonpermissive temperatures were examined by electron microscopy. At 23.5°C, both ts-B1074 and wild-type virus formed polyhedral occlusion bodies containing many virions with multiple nucleocapsids per viral envelope (Fig. 3A). Wild-type virus at the nonpermissive temperature formed polyhedral occlusion bodies, but normal enveloped virions were not present within these occlusion bodies (Fig. 3B). Nucleocapsids were, however, observed in infected nuclei, and some of them appeared to be normally enveloped. ts-B1074 also formed polyhedral occlusion bodies at the nonpermissive temperature, which were devoid of normal enveloped virions (Fig. 3C). ts-B1074 formed nucleocapsid-like structures at 32.5°C, and preliminary steps in nuclear envelopment were observed (Fig. 3D). Because of the rather anomalous behavior of wild-type virus at 32.5°C, more extensive electron microscopic analysis to elucidate the ts-B1074 defect was not pursued.

Effects of temperature on the time of polyhedra formation. Although most of the mutants appeared to be defective in a gene functioning late in the process of NPV infection (since either NOVs or polyhedra were produced by most mutants at 32.5°C), it was of interest to determine whether temperature shift experi-
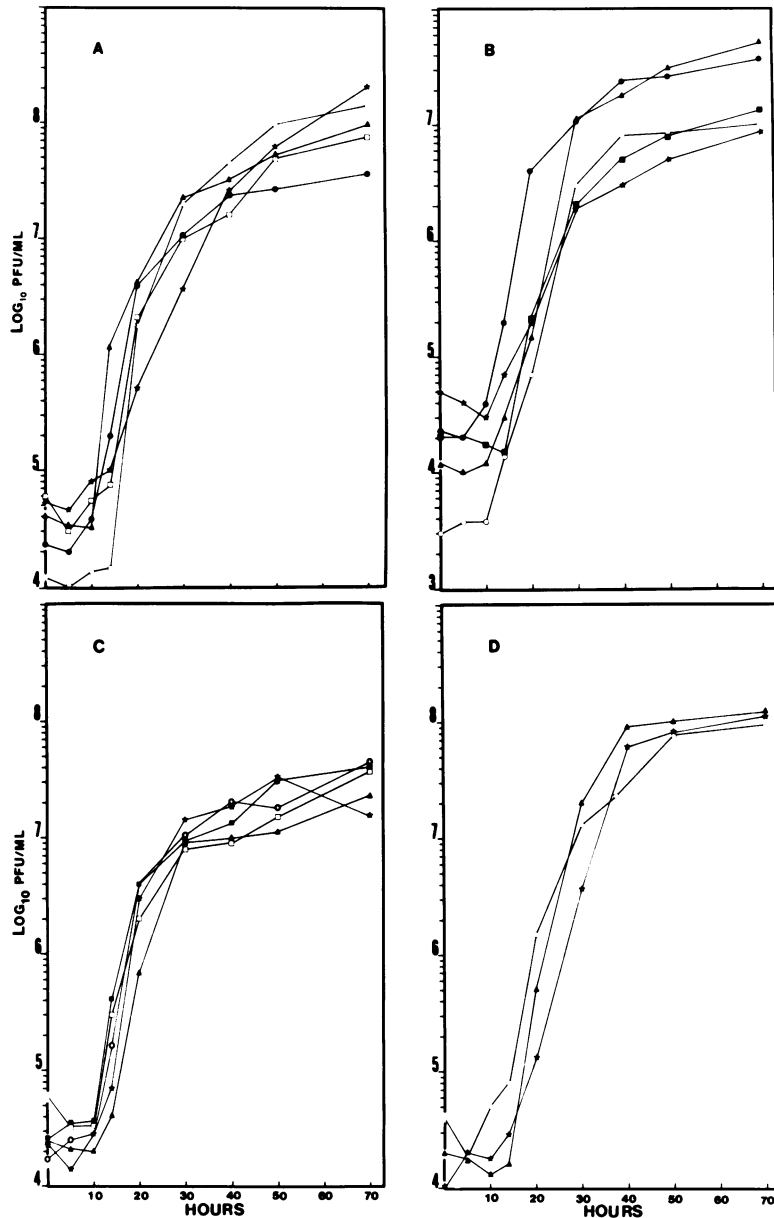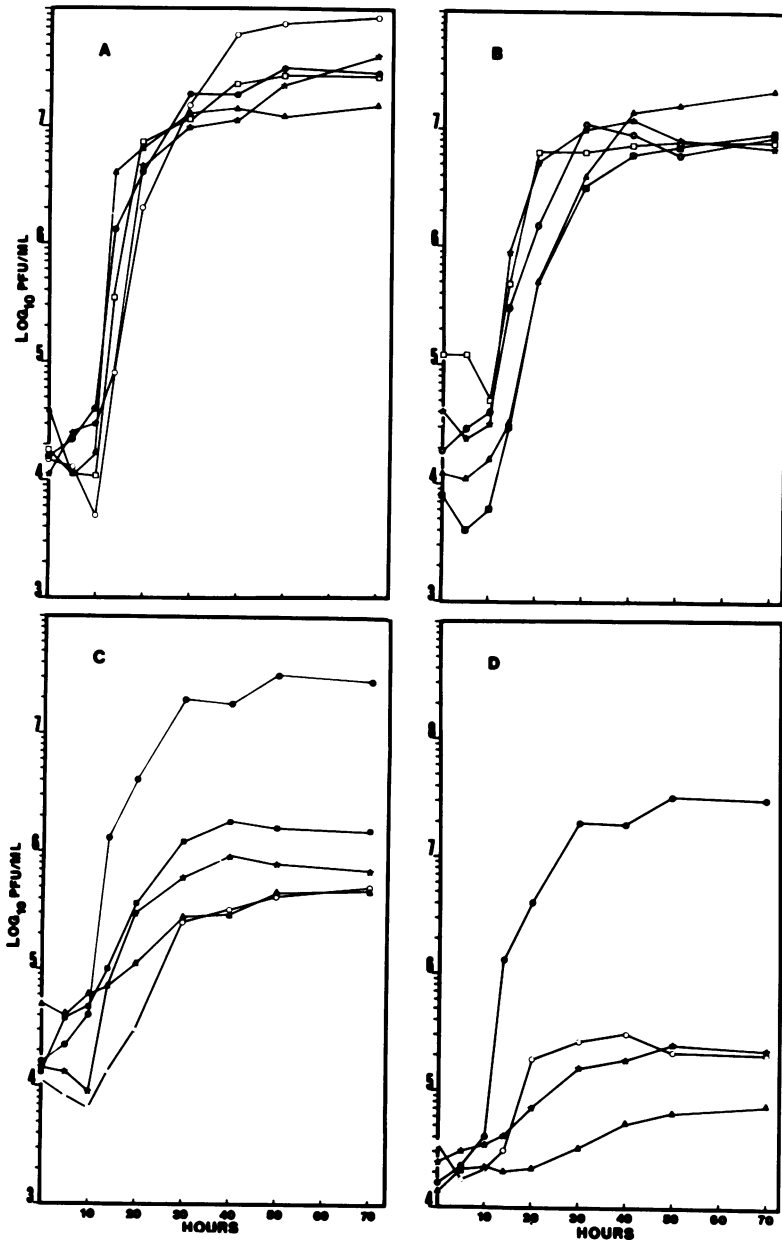
FIG. 1. *Rate of release of infectious extracellular NOV from S. frugiperda cells infected by AcNPV ts mutants at 23.5°C. Wild-type virus (●) profiles are presented in (A) through (D). (A) ts-B113 (★), ts-B305 (○), ts-N356 (▲), ts-B837 (□). (B) ts-N332 (■), ts-B821 (▲), ts-B918 (○), ts-B951 (★). (C) ts-B15 (□), ts-B97 (○), ts-N482 (■), ts-B1075 (★), ts-B1148 (▲). (D) ts-N706 (★), ts-N1054 (○), ts-B1074 (▲).*

ments, in conjunction with the time of polyhedra appearance, could be used to assess the "early" or "late" nature of the defective gene function. Table 5 summarizes effects of temperature on the time of polyhedra formation in wild-type and mutant virus infections of *S. frugiperda* cell cultures.

In the case of wild-type virus infections, polyhedra formation is 6 h slower at 23.5°C than at 32.5°C. This lag is largely due to an inhibition of polyhedra formation at 23.5°C beginning at approximately 12 h postinfection, a delay in the process of NPV infection at 23.5°C between 0 and 12 h postinfection, or a combination of these

FIG. 2. *Rate of release of infectious extracellular NOV from S. frugiperda cells infected by AcNPV ts mutants at 32.5°C. Wild-type virus (●) profiles are presented in (A) through (D). (A) N332 (★), B305 (○), N356 (▲), B837 (□). (B) B15 (□), B97 (○), B113 (■), B821 (▲), B951 (★). (C) N482 (■), B918 (○), B1075 (★), B1148 (▲). (D) Same as Fig. 1D.*

two factors, as the temperature shift experiments indicate (Table 5).

In the case of the ts mutants, the most obvious feature that can be noted in the temperature shift experiments is the fact that ts-B837, -B15, -B305, -N332, -N356, and -N706 do not form polyhedra even with a 12-h postinfection incubation at 23.5°C, indicating a late function defect in these mutants.

Complementation analysis by observing polyhedral occlusion body formation. Since most of the mutants produced significant quan-
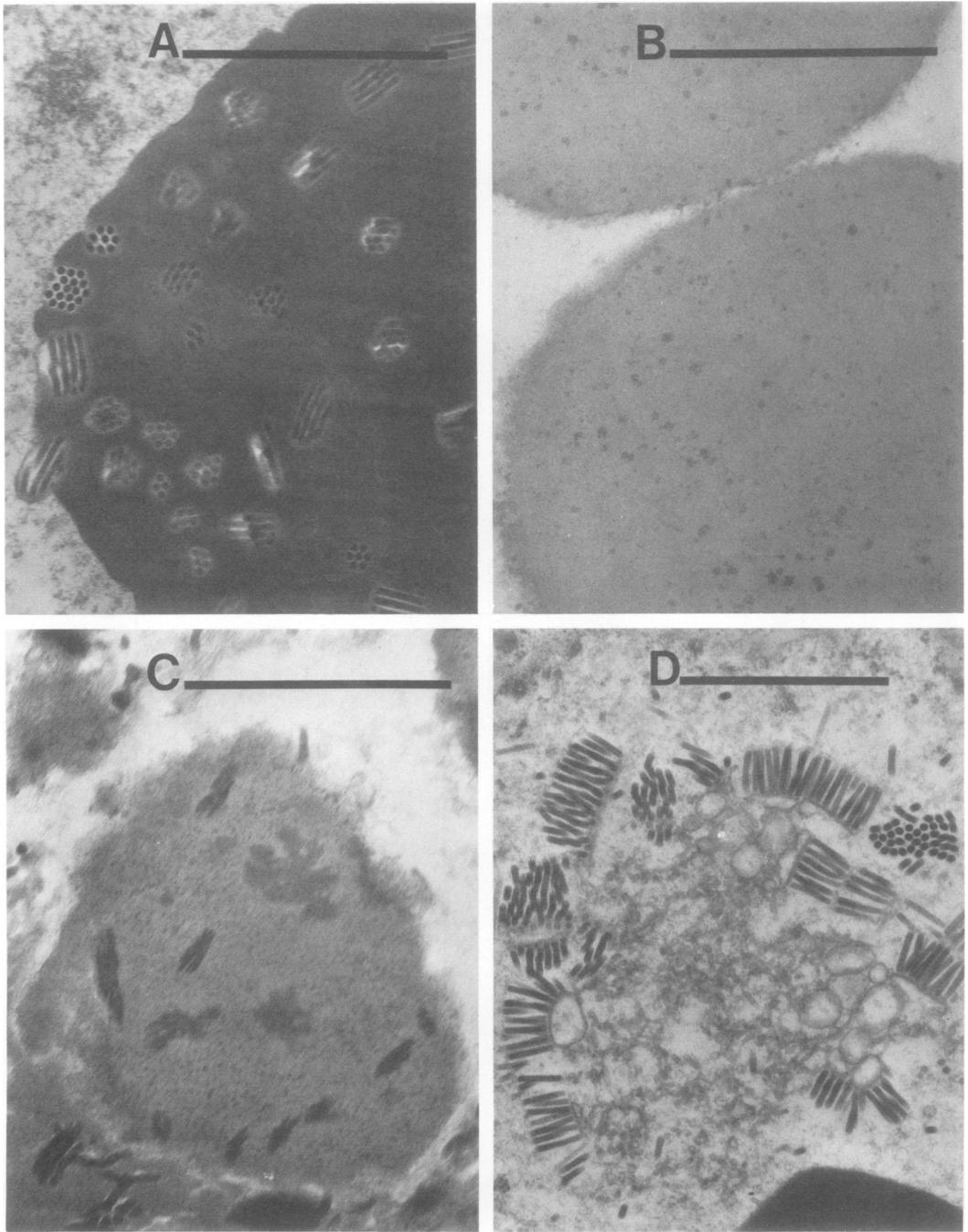
FIG. 3. *Forms of AcNPV observed in infected nuclei of S. frugiperda cells. The bar in each panel represents 1 μm. (A) A wild-type polyhedral occlusion body produced at 23.5°C. (B) Polyhedral occlusion bodies produced at 32.5°C in wild-type virus infections. (C) A polyhedral occlusion body produced at 32.5°C in ts-B1074-infected nuclei. (D) Nucleocapsids and envelope alignment observed in ts-B1074-infected nuclei at 32.5°C.*

tities of NOV at the nonpermissive temperature but failed to produce polyhedral occlusion bodies, a complementation analysis, based on the formation of polyhedral occlusion bodies in cells infected with pairs of mutants at the nonpermissive temperature, was necessary. The percentage

TABLE 5. *Effects of temperature on the rate of polyhedra formation*

Virus	Time of appearance of polyhedra (h) under the following conditions:			
	23.5°C	32.5°C	Temp shift-up at 12 h post-infection[a]	Temp shift-down at 12 h postinfection[b]
Wild-type	22	16	22	18
ts-N1054	24	24	26	24
ts-B1074	24	16	24	18
ts-B1148	26	22	26	22
ts-B821	26	24	28	24
ts-B837	28	N[c]	N[c]	24
ts-B918	28	22	26	24
ts-B951	28	26	30	24
ts-B1075	24	22	26	22
ts-B15	26	N	N	26
ts-B305	26	N	N	24
ts-N332	26	N	N	26
ts-N356	26	N	N	24
ts-N706	24	N	N	22
ts-B97	24	26	30	22
ts-B113	24	26	30	22
ts-N482	26	22	28	24

[a] The initial incubation temperature was 23.5°C for 12 h postinfection, followed by a shift in temperature to 32.5°C.

[b] The initial incubation temperature was 32.5°C for 12 h postinfection, followed by a shift in temperature to 23.5°C.

[c] N, No polyhedra observed by 60 h postinfection.

of cells with polyhedral occlusion bodies was quantitatively determined for high-multiplicity infections (10 PFU/cell) after 48 h of incubation at 32.5°C (Table 3). The mutants ts-B305, -N332, -N356, -N706, -B15, and -B837 were completely unable to form polyhedral occlusion bodies in single infections at the nonpermissive temperature of 32.5°C. Between 2 and 30% of the cells infected with mutant ts-B97, -B113, -N1054, -B1148, -B1075, -B918, -B482, -B821, or -B951 contained polyhedral occlusion bodies in the single infections (Table 5). In double infections an increase in the percentage of cells containing polyhedral occlusion bodies was observed, with one exception. Mixed infections with ts-B332 and -B356 produced no polyhedra-containing cells at 32.5°C, indicating that these two ts mutants belong to one complementation group. These two mutants were isolated from different mutagenized virus stocks (Table 1) and are distinguishable by slight differences in their NOV release patterns (Fig. 1); they therefore represent two distinct mutants within the same complementation group.

Complementation analysis by NOV formation. Since ts-B1074 infection at 10 PFU/cell resulted in 100% of the cells producing polyhedra (Table 5), complementation analysis for ts-B1074 was carried out by comparing the yield of NOVs in media from single and mixed infections. This complementation was only possible for mutants defective in NOV formation at 32.5°C (Fig. 2D). The 20- to 30-fold increase in PFU per milliliter at 23.5°C with mixed infections (Table 6) indicates that ts-B1074 complements both ts-N706 and ts-N1054.

Observation of recombination between ts mutants. NOVs produced in single and mixed infections with ts-B1074 and -N706 at 32.5°C were titrated at 32.5°C (Table 6). The single-cell infections of ts-B1074 could be easily distinguished from wild-type plaques. NOV from mixed infections contained roughly equal proportions of wild-type recombinants and ts mutants (Table 6), indicating a high recombination frequency. Because of some difficulty in the precise quantitation of the number of single-cell infections per milliliter in the presence of plaques, the recombination rate calculated from these experiments may be high.

Host range observations on ts mutants of AcNPV. The ts properties of the 16 mutants were simultaneously tested on TN-368 and *S. frugiperda* cells to detect host range variations. Although mutants ts-B97, -N332, -N356, and -N706 were unable to form polyhedra in *S. frugiperda* cells at 32.5°C, the formation of polyhedra was detected in TN-368 cells by 48 h postinfection at 32.5°C. The effect of host range on properties of the ts mutants other than polyhedra production remains to be pursued.

DISCUSSION

A variety of interesting phenotypes have been

TABLE 6. *Complementation analysis by NOV formation*

Virus	Virus titer (PFU/ml) at:	
	23.5°C	32.5°C
ts-N706	2.3×10^5	0
ts-B1074	3.7×10^5	(1.4×10^5)[a]
ts-N706 and -B1074	1.6×10^7	8.8×10^5 (5.6×10^5)[a]
ts-N1054	1.2×10^5	NT[b]
ts-B1074	3.7×10^5	NT
ts-N1054 and -B1074	1.2×10^7	NT

[a] Titers in parentheses are reported as single-cell infections per milliliter; at the nonpermissive temperature, ts-B1074 is defective in the spread of infection so that actual plaques are not formed but isolated single-cell infections are readily detected because of polyhedra production.

[b] NT, Not tested.

observed among the 16 ts mutants (15 complementation groups) isolated after BUdR or NTG mutagenesis of AcNPV-infected *S. frugiperda* cell cultures. One interesting mutant is ts-B1074, which is defective in the production of infectious NOV (Fig. 2D) yet produces polyhedra in infected cells at 32.5°C. The defect in the production of infectious NOV apparently results in the inability of the virus to form actual plaques at 32.5°C. Instead, we observe what we term single-cell infections, where individual cells contain many polyhedra per cell but the surrounding cells have no apparent cytopathic effects. The number of single-cell infections per milliliter with ts-B1074 at 32.5°C is approximately the same as the PFU per milliliter at 23.5°C, and therefore the polyhedra production in scattered cells is not simply a matter of an occasional infected cell producing polyhedra.

The lack of extracellular NOV production in ts-B1074 infections is apparently not due to a defect in nucleocapsid formation since nucleocapsids were observed in ts-B1074-infected cell nuclei at 32.5°C. Since extracellular NOVs derive their membrane envelopes by budding through cellular membranes, a possible explanation of the ts-B1074 defect may be temperature sensitivity of a viral protein inserted in cellular membranes. Further work on this mutant may be of interest from this perspective.

In the course of electron microscopic analysis of ts-B1074 infection at 32.5°C, we noted anomalous behavior of wild-type virus at 32.5°C. Polyhedra produced by wild-type virus at 32.5°C do not contain virions. Polyhedra lacking virions have been observed previously in infections of midgut epithelial cells (5, 21). In our work, this phenomenon is temperature dependent rather than tissue dependent. Care was also taken to avoid long-term serial passage of the virus (9). Nucleocapsid envelopment in the nucleus appears to be a prerequisite for embedding in polyhedra (5, 9). We observed a large proportion of nucleocapsids in the process of envelopment and a few fully enveloped nucleocapsids, so it is not yet clear why empty polyhedra are produced at 32.5°C in wild-type infections.

Another mutant, ts-N1054, was similar in many respects to ts-B1074 but was distinguished by complementation analysis and by its lower ratio of single-cell infections (32.5°C) to PFU (23.5°C). A third mutant, ts-B1148, produced minute centers of infections containing one to three cells per center. The phenotype of ts-B1148 is sufficiently different than those of ts-B1074 and -N1054 to consider it a distinctive phenotype, although the fact that ts-B1148 pro-

duces intermediate levels of extracellular NOV at MOIs of 5 PFU/cell may simply suggest a leaky mutant characteristic.

A surprisingly large proportion of the mutants, 9 of the 16, produced essentially wild-type levels of extracellular NOV at the nonpermissive temperature (Fig. 2), although they were temperature sensitive in some other aspect of virus development, most notably polyhedra formation. If this proportion of mutants is an approximation of target size (the method of mutagenesis may bias the isolation of certain mutant types), the control of polyhedra formation must be quite complex. The crystalline protein matrix of AcNPV polyhedra has one major protein component—a 30,000-dalton protein known as polyhedrin (23). However, the morphogenesis of polyhedra may involve a number of gene products, as electron microscopic analysis of polyhedra morphogenesis suggests (21). Furthermore, there may be intricate temporal control mechanisms operating to regulate polyhedra formation in relationship to extracellular NOV formation (26). Several of the mutants may be useful in further exploring polyhedra development; particularly interesting may be the mutants ts-B837, -B15, -B305, and -N332 (or -N356).

The ts-B837 mutant is unique in that it forms plaques at 32.5°C but cells within the plaques lack polyhedra. The mutants ts-B15, -B305, and -N332 (or -N356 [same complementation group]) are unable to produce either polyhedra or plaques at 32.5°C, although they produce essentially wild-type levels of infectious extracellular virus. There is no apparent defect in adsorption at 32.5°C. These four viruses are also candidates for further exploration of the mechanism of polyhedra formation.

The mutant ts-N706 is unique in that it is defective in plaque formation, polyhedra formation, and progeny NOV formation. Although these properties are suggestive of an early mutant, ts-N706 produces cytopathic effects in infected cells by 8 h postinfection. Furthermore, temperature shift experiments indicate a late defect since incubation at the permissive temperature 12 h postinfection before shift-up prevents polyhedra formation (Table 5).

An interesting feature observed with ts-N706, -N322 (as well as -N356), and -B97 is the host dependency of polyhedra production at 32.5°C. In TN-368 cells at 32.5°C, these four mutants produce significant quantities of polyhedra by 48 h postinfection, but they produce no polyhedra in *S. frugiperda* cells by 4 days postinfection at 32.5°C. There are at least two explanations

for this host dependency of temperature sensitivity. The first possibility is that the three gene products (proteins) affected by the four mutants (complementation analysis indicated that ts-N332 and -N356 affect the same gene product) interact with host proteins in functioning. An alteration in viral protein structure might result in temperature sensitivity in only one of two hosts since the host interactive proteins may vary in their primary, secondary, and/or tertiary structure. Another possibility is that TN-368 cells, but not *S. frugiperda* cells, contain proteins which can substitute for the defective viral proteins. The complexity of control of occlusion body formation cannot be underestimated; there is, for instance, evidence from studies of the baculovirus infection process in insects (5, 21) that occlusion body formation is dependent on the differentiated state of the cell (e.g., midgut versus fat body cells). Host dependency of polyhedra production has been observed for wild-type AcNPV in *Bombyx mori* cell cultures (24). In this instance, polyhedra are produced in only scattered cells of *B. mori* cell cultures, a situation apparently similar to the high temperature response of the group IVb mutants (Table 2) rather than complete absence noted for ts-N706, -N322, and -N356. The *B. mori* restriction to AcNPV is presumably not temperature dependent.

The group IVb mutants (Table 2) do not form plaques at 32.5°C yet produce cytopathic effects which include the occasional appearance of cells containing polyhedral occlusion bodies at 32.5°C. The group IVb mutants are different than the plaque morphology (FP) variants described by Hink and Strauss (8) or Potter and colleagues (15). The group IVb mutants produce a few cells with many polyhedra at 32.5°C. The FP variants produce a few polyhedra in many infected cells and apparently exhibit no temperature sensitivity.

The mutant phenotypes and characterization methods described herein form a basis for continued genetic analysis of the AcNPV. More biochemical work is necessary to further understand the nature of the mutant defects. Several of the mutants may be of considerable interest in exploring the control of baculovirus development. The host-dependent mutants may provide a handle for further understanding the host specificity of NPV infections. Marker rescue experiments (10, 14) are currently underway to correlate a genetic map of the AcNPV genome with the physical restriction endonuclease fragment map.

ACKNOWLEDGMENTS

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