Isolation and characterization of a temperature-sensitive mutant of Bombyx mori nucleopolyhedrovirus for a putative RNA polymerase gene

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Temperature-sensitive (ts) mutants of Bombyx mori nucleopolyhedrovirus (BmNPV), which exhibited no polyhedra formation at the non-permissive temperature of 33 °C, were produced using the base analogue 5-bromodeoxyuridine. A unique ts mutant, designated ts-S1, was characterized in terms of gene mutation and virulence in cultured cells and silkworm larvae. Mutant-infected BmN4 cells at 33 °C showed normal viral DNA synthesis but defective budded virus production and polyhedrin synthesis, suggesting the absence of late and very late gene expression. Silkworm larvae were injected with ts-S1 and reared at 33.5 °C. At 7 days post-injection, none of the larvae exhibited nucleopolyhedrosis but some possessed viral DNA, detected by PCR using virus-specific primers. Continued rearing of the larvae at a permissive temperature of 25 °C showed that, while most developed into normal adults, some developed nucleopolyhedrosis, indicating that the former larvae had aborted the virus infection during the course of rearing at 33.5 °C. No viral DNA was detected in the adults. Marker rescue tests to identify the lesion involved in the ts phenotype of ts-S1, and nucleotide sequencing of the identified genome region, showed a single nucleotide mutation of a putative RNA polymerase gene, late expression factor-8 (lef-8). These results indicate that lef-8 is essential for BmNPV replication in vitro and in vivo.

Introduction

Nucleopolyhedroviruses (NPV), belonging to the family Baculoviridae, have a covalently closed circular and double-stranded DNA. Viral gene expression is regulated in cascade fashion during virus replication and divided into three temporal phases: early, late and very late (Blissard & Rohrmann, 1990). Early and late gene expression is differentiated by the onset of viral DNA replication (Gordon & Carstens, 1984). Early genes are transcribed by the host RNA polymerase without any viral terms of gene mutation and virulence in cultured cells and silkworm larvae. Mutant-infected BmN4 cells at 33 °C showed normal viral DNA synthesis but defective budded virus production and polyhedrin synthesis, suggesting the absence of late and very late gene expression. Silkworm larvae were injected with ts-S1 and reared at 33.5 °C. At 7 days post-injection, none of the larvae exhibited nucleopolyhedrosis but some possessed viral DNA, detected by PCR using virus-specific primers. Continued rearing of the larvae at a permissive temperature of 25 °C showed that, while most developed into normal adults, some developed nucleopolyhedrosis, indicating that the former larvae had aborted the virus infection during the course of rearing at 33.5 °C. No viral DNA was detected in the adults. Marker rescue tests to identify the lesion involved in the ts phenotype of ts-S1, and nucleotide sequencing of the identified genome region, showed a single nucleotide mutation of a putative RNA polymerase gene, late expression factor-8 (lef-8). These results indicate that lef-8 is essential for BmNPV replication in vitro and in vivo.

Mutants and transient expression assays of a regulatory gene have been carried out. In Autographa california NPV (AcMNPV), various genes which regulate viral DNA replication and late gene expression have been identified. Eighteen genes, ie-1, ie-2, dnapol, p143, late expression factors (lef)-1 to -11, p47, p39 and p35, are required for viral late gene expression, and 6 of these 18 genes, ie-1, lef-1, lef-2, lef-3, p143 and p35, are required for viral DNA replication in Spodoptera frugiperda (Sf21) cells (Todd et al., 1995; Lu & Miller, 1995). In analyses of the ts mutants, a mutation of p143 in ts8 was identified as the cause of defective viral DNA replication in late and very late gene expression (Lu & Carstens, 1991). Mutations of p47 in ts317 and lef-4 in ts538 were shown to cause a defect in budded virus (BV) production and polyhedra formation (Carstens et al., 1993, 1994), while mutation in ie-1 of tsB821 caused a defect in plaque formation (Ribeiro et al., 1994). These results were consistent with those obtained by transient expression assay, in that these genes were shown to be essential for virus replication.

Analysis of Bombyx mori NPV (BmNPV) nucleotide sequence data has indicated that 18 homologues of AcMNPV lef genes are encoded in the genome. Deletion analysis of the
individual genes showed that four genes, ie-2, p35, p39 and lef-7, are not required for virus replication in BmN cells (Gomi et al., 1997).

In this study, we isolated BmNPV ts mutants defective in polyhedra formation at the non-permissive temperature of 33 °C. One of four ts mutants obtained, designated ts-S1, was further characterized. In an established silkworm cell line, BmN4, the ts-S1 mutant was defective in BV production and polyhedra formation, but not in viral DNA synthesis, at 33 °C; this was similar to the characteristics of AcMNPV mutants ts317 and ts538. Marker rescue tests and nucleotide sequence analysis of ts-S1 revealed a single nucleotide mutation in the lef-8 gene, which was different from ts317 and ts538 mutations.

We examined the virulence of ts-S1 in silkworm larvae by injection of BV at 33.5 °C and found abortive infection of the larvae, indicating that lef-8 is an essential gene for virus replication not only in vitro but also in vivo. Temperature-sensitive characteristics of ts-S1 in vitro are discussed in terms of virulence in larvae reared at the non-permissive temperature.

Methods

Cultured cells, virus and silkworm larvae. An established silkworm cell line, BmN4 (Maeda, 1989), was maintained at 27 °C using Grace’s insect culture medium (GIBCO BRL), supplemented with 10% heat-inactivated foetal bovine serum, 0.26% tryptose and 0.035% sodium bicarbonate. A wild-type (wt) virus, BmNPV D1 (wtBmNPV) (Hashimoto et al., 1994), was propagated on BmN4 cells and BV titre was determined by measuring TCID50 at 27 °C. Commercial silkworm eggs, Kinshu × Showa or Shunrei × Shogetsu (Kanebo), were incubated for several days at 25 °C in high humidity. After hatching, silkworm larvae were reared on an artificial diet (Silkmate 2S, Nosan-Kogyo) at 25 °C. Newly eclosited fourth instar larvae were used for virus bioassay.

Mutagenesis and mutant screening. BmN4 cells (2 × 106) were infected with wtBmNPV at an m.o.i. of 1.5 TCID50 per cell and incubated at 25 °C in the presence of 5-bromodeoxyuridine (BrDU) at 10, 30 and 40 μg/ml. Culture fluids were harvested at 5 days post-infection (p.i.) and excess BrDU was removed by dialysis in buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 7H2O, 1.4 mM KH2PO4, pH 7.2, plus 0.5%, w/v, BSA). Mutated viruses were separated once by plaque purification and the purified virus suspension was propagated in BmN4 cells in duplicate on 96-well plates. Each plate was incubated at 25 or 33 °C. When polyhedra formation was observed in the cells incubated at 25 °C, supernatants were recovered from wells containing mutants, as characterized by the absence of polyhedra formation, at 33 °C. Further purification of the selected ts mutants by plaque formation was carried out twice.

Measurement of BV production in ts mutant-infected BmN4 cells at 25 °C and 33 °C. BmN4 cells (2 × 106) were infected with the virus at an m.o.i. of 30 TCID50 per cell and incubated at 25 and 33 °C. Aliquots of 104 cells were harvested at 0, 6, 12, 24, 36, 48 and 72 h p.i. and aliquots of 103 cells lysed with a sample buffer (5% β-mercaptoethanol, 2% SDS, 125 mM Tris–HCl pH 6.8, 10% glycerol, 1% bromophenol blue) and boiled at 100 °C for 5 min. Polypeptides were separated in a 12% acrylamide gel and detected by Coomassie brilliant blue (CB) staining (Asubel et al., 1994b).

Marker rescue. A cosmid library containing overlapping sets of clones of the wtBmNPV genome (Shikata et al., 1998) was used in the mapping of the ts mutation by a marker rescue experiment. Caesium chloride-purified cosmids DNA (2 μg) and ts mutant DNA (1 μg) were cotransfected into BmN4 cells by the calcium phosphate precipitation method. Culture medium was recovered after cell culture at 25 °C for 5 days. To confirm the rescue of rescued virus, the medium was inoculated into BmN4 cells and the cells fixed in 1:5% agarose. Positive rescue was determined by plaque formation at 33 °C. To narrow the region of the virus genome relating to rescue, a further marker rescue was carried out using DNA from the HindIII plasmid library, HindIII-K, -P, -D, -O and -S (Hashimoto et al., 1994), and the sub-clones of HindIII-O, such as pD1H3O (30.2–31.4 m.u.) and pDH1HSO (31.4–32.0 m.u.).

DNA sequencing and computer analysis of the sequence data. The plasmid which showed positive in the marker rescue test, pDH1HSO, and the ts-S1 DNA clone in the equivalent region of wtBmNPV were sequenced by the chain-termination method with a Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham). Plasmid clones pD1H3DL (27.9–30.2 m.u.) and pDH1HSO, which were derived from Xhol digests of the HindIII-D and HindIII-O fragment, respectively, were used for sequencing templates. DNA sequence data (291–320 m.u.) were analysed by GENETYX-WIN (Software Development) and registered in the DDBJ database as AB009987.

Bioassay. The inoculum of the ts mutant was prepared at 104 TCID50/ml, and 10 μl inoculum was subcutaneously injected into the leg of the eighth segment of newly eclosed fourth instar larvae. Thirty larvae were injected with the ts mutant and reared individually in a 150 mm dish (Falcon 1058) at 33.5 °C. This rearing temperature was defined as a non-permissive temperature for in vivo experiments, since it is generally accepted that the breathing of silkworm larvae slightly reduces body temperature. After 7 days, the rearing temperature was reduced to 25 °C and larvae were continuously reared until they died or developed into adults. Dead larvae were microscopically examined for the presence of polyhedra indicative of nucleopolyhedrosis.

Detection of viral DNA in ts mutant-infected larvae. Twenty of the fourth instar larvae were injected with the ts mutant at 104 TCID50 per larva and reared individually at 33.5 °C for 7 days. Ten larvae were collected and stored at 20 °C; the remaining larvae were reared at 25 °C. Larvae which died of nucleopolyhedrosis or physiological damage during breeding were collected and stored at 20 °C. Adults were also stored at 20 °C prior to DNA extraction. Insects were ground to fine powder in liquid nitrogen and treated with proteinase K and SDS. DNA infected with the virus at an m.o.i. of 30 TCID50 per cell and incubated at 25 and 33 °C. Aliquots of 104 cells were harvested at 0, 6, 12, 24, 36, 48 and 72 h p.i. and lysed with 200 μl 0.5 M NaOH at 100 °C. After adding 50 μl 0.5 M EDTA, samples were dot-blotted onto a nylon membrane (Hybond-N+, Amersham). Hybridization was carried out by a standard hybridization procedure (Asubel et al., 1994a) with [32P]ATP random primer-labelled probe of HindIII- and Xhol-labelled BmNPV DNA. Hybridization signals were detected by autoradiography.

SDS-PAGE analysis of viral DNA synthesis in ts mutant-infected BmN4 cells. BmN4 cells (105) were infected with the virus at an m.o.i. of 30 TCID50 per cell and incubated at 25 or 33 °C. Cells were collected at 72 h p.i. and aliquots of 103 cells lysed with a sample buffer (5% β-mercaptoethanol, 2% SDS, 125 mM Tris–HCl pH 6.8, 10% glycerol, 1% bromophenol blue) and boiled at 100 °C for 5 min. Polypeptides were separated in a 12% acrylamide gel and detected by Coomassie brilliant blue (CB) staining (Asubel et al., 1994b).

Putative gene sequences and nucleotide sequence data (29 ± 10 m.u.) determined by Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham). Plasmid clones pD1H3DL (27.9–30.2 m.u.) and pDH1HSO (31.4–32.0 m.u.) were sequenced by the chain-termination method with a Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham). Plasmid clones pD1H3DL (27.9–30.2 m.u.) and pDH1HSO, which were derived from Xhol digests of the HindIII-D and HindIII-O fragment, respectively, were used for sequencing templates. DNA sequence data (291–320 m.u.) were analysed by GENETYX-WIN (Software Development) and registered in the DDBJ database as AB009987.
was expected as a PCR product. PCR products were separated in 0.7% agarose gel and transferred onto a nylon membrane (Hybond-N+, Amersham). Hybridization was carried out with pD1H3OL probe, using a Renaissance random primer biotin dUTP labelling kit (NEN).

Isolation of ts mutants

To isolate ts mutants defective in polyhedra formation at the non-permissive temperature of 33 °C, wtBmNPV was amplified on BmN4 cells in the presence of 10, 30 and 40 µg/ml BrdU at 25 °C. A total of 875 plaques, all of which contained polyhedra in the nucleus at 25 °C, were isolated and screened for temperature sensitivity at 33 °C. Further plaque purification was carried out and four ts mutants were cloned. In this study, we characterized mutant ts-S1, isolated from a virus fluid treated with 40 µg/ml BrdU. The temperature-sensitive phenotype of the ts-S1 was stable during the course of consecutive propagation.

BV production of BmN4 cells infected with ts-S1 at 25 °C and 33 °C

Mutant ts-S1 formed polyhedra in infected BmN4 cells at 25 °C, but not at 33 °C. For determination of BV production, BmN4 cells infected with wtBmNPV or ts-S1 at an m.o.i. of 30 TCID₅₀ per cell were cultured at 25 or 33 °C. Culture media were collected up to 72 h p.i., and BV titres determined by the end-point dilution method (Fig. 1a). At 25 °C, BV production of ts-S1 increased linearly up to 48 h p.i. and then levelled off to approximately 10⁶ TCID₅₀/ml. The growth curve of ts-S1 was similar to that of wtBmNPV at 25 °C. Although the BV titre of wtBmNPV at 33 °C was reduced to one-tenth of that at 25 °C, BV titre of ts-S1 at 33 °C remained at basal level over the incubation period. These results suggest that the defective BV production of ts-S1 at 33 °C is solely due to the temperature-sensitive mutation.

Viral DNA in ts-S1-infected BmN4 cells

To determine the accumulation of viral DNA in mutant-infected BmN4 cells at 25 and 33 °C, BmN4 cells were infected with ts-S1 and wtBmNPV, harvested at various time-points up to 72 h p.i. and lysed in an alkaline solution. DNA from the lysed cells was dot-blotted and hybridized with labelled BmNPV DNA digests (Fig. 1b). In wtBmNPV-infected BmN4 cells at 25 °C, viral DNA accumulated up to 24 h p.i., after which it decreased slightly and then remained constant up to 72 h p.i. A similar dot-blot pattern, but with slightly less viral DNA and a lower maximum DNA concentration at 24 and 36 h p.i., was detected in wtBmNPV-infected BmN4 cells at 33 °C. The dot-blot pattern of ts-S1-infected BmN4 cells showed significant amounts of viral DNA, which were comparable to those of wtBmNPV-infected BmN4 cells. At 25 °C, ts-S1 DNA accumulated up to 24 h p.i., reached a steady-state at 36 h p.i. and levelled off at 48 h p.i. At 33 °C, a similar pattern of dot-blot, with a shift to an earlier time phase, was observed. These results show that the temperature-sensitive mutation of ts-S1 did not affect viral DNA synthesis.

Protein profiles in wtBmNPV- or ts-S1-infected BmN4 cells

Under microscopic observation, ts-S1 was defective in polyhedra formation at 33 °C. To detect the non-crystallized polyhedrin polypeptides, ts-S1-infected BmN4 cells were analysed by SDS–PAGE. The ts-S1- or wtBmNPV-infected cells were collected at 72 h p.i. and lysed. Protein samples were separated on a 12% SDS–polyacrylamide gel and subjected to CBB staining (Fig. 1c). The polypeptide patterns of cells infected with wtBmNPV at 25 and 33 °C, and with ts-S1 at 25 °C, were very similar to each other. However, at 33 °C, the polypeptide pattern of cells infected with ts-S1 showed no polyhedrin polypeptide, being very similar to that of mock-infected cells at 33 °C. These results demonstrate a substantial reduction in polyhedrin production at the non-permissive temperature, together with an absence of host protein synthesis inhibition.

Mapping of the mutation in ts-S1 and nucleotide sequence of the mutated gene

To locate the mutation in ts-S1, marker rescue tests were carried out using a cosmid library covering the entire wtBmNPV genome (Fig. 2). Rescue of the mutation to a wt phenotype was scored by observing plaque formation at 33 °C. The cosmid clones pWB60 and pWBE5, comprising regions 96–8–32.5 m.u. via 0 m.u. and 7.3–42.7 m.u., respectively, rescued ts-S1 mutation. Other cosmid clones, such as pWBE8, pWBE25 and pWBE1, failed to rescue the mutation. The mutation was located within an overlapped region of cosmids clones pWB60 and pWBE5 (i.e., 19.0–32.5 m.u.). Further marker rescue tests were carried out using plasmid clones of HindIII-D, -K, -O, -P and -S of wtBmNPV DNA, which covered the region defined above. The HindIII-O fragment (30.2–32.0 m.u.) rescued ts-S1 mutation. The HindIII-O fragment was restricted with Xhol and the digests were sub-cloned to pD1H3OL (30.2–31.4 m.u.) and pD1H3OS (31.4–32.0 m.u.). Finally, pD1H3OL was identified as the smallest plasmid clone competent to rescue the ts-S1 phenotype. The nucleotide sequence of the inserted viral DNA in pD1H3OL showed high sequence identity to that of AcMNPV lef-8 gene. The corresponding region of ts-S1 DNA was cloned into
Fig. 1. Phenotypic characterization of ts-S1. (a) Time-course of BV production in BmN4 cells infected with ts-S1 or wtBmNPV at an m.o.i. of 30 TCID₅₀ per cell and incubated at 25 or 33 °C. Culture medium (50 µl) was harvested at the time-points indicated and BV titre determined by the end-point dilution method. Solid lines indicate culture at 25 °C and dotted lines at 33 °C. ○, ts-S1; □, wtBmNPV. (b) Viral DNA accumulation in ts-S1- or wtBmNPV-infected BmN4 cells at 25 or 33 °C. BmN4 cells were infected and incubated as in (a). Cells (10⁴) harvested at the time-points indicated were lysed with 0.5 M NaOH at 100 °C. Samples containing DNA were applied onto a nylon membrane. Hybridization was carried out with random primer-labelled wtBmNPV DNA digests as a probe. Standard amounts of viral DNA are indicated on the left (lane C). (c) Protein profiles of ts-S1- or wtBmNPV-infected BmN4 cells at 25 or 33 °C. BmN4 cells were infected and incubated as in (a). Cells harvested at 72 h p.i. were lysed in SDS–sample buffer at 100 °C. Proteins were separated on 12% SDS–polyacrylamide gel and stained with CBB. The sizes of molecular mass markers are indicated on the right. Black arrowhead indicates the polyhedrin.

Fig. 2. Marker rescue and identification of a point mutation relating to a temperature-sensitive phenotype of ts-S1. The HindIII restriction map of wtBmNPV DNA is shown at the top. DNA fragments of wtBmNPV cloned in cosmid or plasmid are shown as thick white bars in the middle section. Black bars show viral DNA fragments competent to rescue the ts-S1 phenotype. In the bottom section, the large open arrow indicates the BmNPV lef-8 gene ORF and the details of the single nucleotide mutation are interpreted.

a plasmid, designated pS1H3OL, and sequenced. There was a single nucleotide alteration from cytosine in pD1H3OL to thymine in pS1H3OL (Fig. 2). To determine the nucleotide sequence of the complete open reading frame (ORF) of the wtBmNPV lef-8, upstream and downstream of region 302–314 m.u. was sequenced (AB009987). The wtBmNPV lef-8
ORF consisted of 2634 bp with a predicted protein of 877 amino acids.

**Comparison of amino acid sequences between BmNPV Lef-8 and other baculovirus Lef-8 proteins**

The putative RNA polymerase gene *lef-8* has been identified in five baculoviruses: AcMNPV L1 (Passarelli et al., 1994), AcMNPV C6 (Ayres et al., 1994), BmNPV T3 (GenBank accession number L33180), Orgyia pseudotsugata NPV (OpMNPV) (Ahrens et al., 1997) and Helicoverpa zea NPV (HzSNPV) (Le et al., 1997). Comparing the nucleotide sequence of the *lef-8* obtained in this study with those previously reported, we found amino acid sequence identities of Lef-8 between BmNPV D1 and other baculoviruses as follows: 99-4% BmNPV T3; 97-4% AcMNPV L1; 97-4% AcMNPV C6; 69-0% OpMNPV; and 61-4% HzSNPV. The mutated nucleotide cytosine at position 1625 of the ts-S1 *lef-8* gene corresponded to a single amino acid change from alanine to valine at position 542 of the Lef-8 polypeptide. Amino acid residues of the aligned Lef-8 polypeptides of the five baculoviruses were all alanine, which was identical to that of wtBmNPV (Fig. 2). The amino acid residue involved in thermosensitivity is conserved among the known baculovirus Lef-8 proteins, but is not conserved in other RNA polymerases (data not shown); it is out of the polymerase conserved region GXKX4HGQ/NKG (Passarelli et al., 1994).

**Bioassay in silkworm larvae by injection with ts-S1**

Fourth instar silkworm larvae were injected with ts-S1 at 10^2 TCID_{50} per larva and reared at 33.5 °C. At 7 days p.i., the rearing temperature was reduced from 33.5 to 25 °C. Table 1 shows the results of the bioassay. At 7 days p.i., no larvae had died. Approximately 30% of larvae infected with ts-S1 eventually died of nucleopolyhedrosis while approximately 10% died of physiological damage due to the high rearing temperature. Most physiological damage was probably due to denaturation of liquid silk in the silk gland, causing cessation of spinning. Reduction in the rearing temperature resulted in development of BmNPV disease and death in 30–37% of the infected larvae, allowing 60% of the larvae to develop into normal adults. In contrast, at the rearing temperature of 25 °C, 100% of larvae injected with ts-S1 died of nucleopolyhedrosis at 4–7 days p.i. These results show that ts-S1 infection becomes abortive at 33.5 °C.

**Detection of viral DNA in silkworm larvae injected with ts-S1**

To examine whether viral DNA was present in larvae infected with ts-S1 and reared continuously at 33.5 °C or at 33.5 °C followed by 25 °C, we carried out PCR and Southern blot hybridization with DNA samples from larvae or adults. PCR and Southern blot hybridization data showed that viral DNA was detected in 3 out of 10 larvae reared at 33.5 °C and collected at 7 days p.i., but not in the remaining 7 larvae (Fig. 3a, b). During further rearing at 25 °C after 7 days 33.5 °C rearing, 2 out of 10 larvae died of nucleopolyhedrosis at 10 days p.i. and 1 larva died of physiological damage. PCR products of the former larvae contained a virus-specific DNA band, but the larva which died of physiological damage showed no such band. The remaining 7 adults failed to show the virus-specific DNA band.

### Table 1. Induction of abortive infection on ts-S1-injected silkworm larvae by rearing at 33.5 °C

<table>
<thead>
<tr>
<th>Injection</th>
<th>No. of larvae tested</th>
<th>Rearing temp.</th>
<th>No. of dead larvae</th>
<th>No. of adults</th>
<th>% Survival</th>
<th>% Death from infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts-S1*</td>
<td>Expt. 1: 30</td>
<td>33.5 °C</td>
<td>2 4 3 (3)*</td>
<td>18</td>
<td>60.0</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>Expt. 2: 30</td>
<td>25 °C</td>
<td>1 3 5 2 (2)*</td>
<td>17</td>
<td>50.7</td>
<td>36.7</td>
</tr>
<tr>
<td>Mock</td>
<td>30</td>
<td></td>
<td>(4)*</td>
<td>26</td>
<td>80.7</td>
<td>0</td>
</tr>
</tbody>
</table>

* Injected into the fourth instar (0 day) larva at an m.o.i. of 1.0×10^2 TCID_{50}.

† Number of larvae that died due to physiological damage such as silk gland denaturation at 33.5 °C.

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**Table 1. Induction of abortive infection on ts-S1-injected silkworm larvae by rearing at 33.5 °C**
Discussion

The characterization of ts mutants of DNA viruses has been employed as a useful approach to elucidating the function of viral genes involved in phenotypes observed at a non-permissive temperature. In AcMNPV, several ts mutants have been isolated and characterized in detail (Brown et al., 1979; Partington et al., 1987; Lee & Miller, 1979; Partington et al., 1990). Several genes crucial for the virus to proceed to a replication phase have been identified, specifically late expression (Carstens et al., 1993, 1994; Ribeiro et al., 1994) and very late expression (McLachlin & Miller, 1994; Fan et al., 1996) genes, and genes controlling DNA replication (Lu & Carstens; 1991), nucleocapsid formation (Olszewski & Miller, 1997b) or egress of nucleocapsids from the nucleus (Olszewski & Miller, 1997b). However, ts mutants of other baculoviruses have not been extensively characterized.

In this study, ts-S1 showed phenotypic characteristics of viral DNA replication at 25 and 33 °C similar to those of wtBmNPV. However, polyhedra formation and BV production were completely restricted at 33 °C. SDS-PAGE analysis of BmN4 cells infected with ts-S1 at 33 °C clearly demonstrated restriction of synthesis of polyhedrin and major viral proteins. In contrast, DNA replication of ts-S1 at 33 °C was not significantly restricted as compared with that of ts-S1 at 25 °C and wtBmNPV at 25 and 33 °C. Such phenotypic characteristics are very similar to those of AcMNPV ts mutants ts317 and ts538 (Partington et al., 1990). These mutants are defective in BV production and polyhedra formation, but not in viral DNA synthesis. Rescue tests of these ts mutants showed mutations in p47 and lef-4 genes, respectively, which are indispensable for late gene expression (Carstens et al., 1993, 1994). The lesion of ts-S1 was attributable to one of the lef genes of the baculovirus, lef-8, a gene possessing a temperature-sensitive mutation previously unreported among baculoviruses. Recently, Gomi et al. (1997) showed that BmNPV has 18 gene homologues of AcMNPV: ie-1, ie-2, dnapol, p143, lef-1 to -11, p47, p39 and p35. Deletion analysis of each gene indicated that ie-2, p35, p39 and lef-7 are not essential for virus replication in BmN cells. Their finding that BmNPV lef-8 is necessary for the late phase of virus replication was supported by our phenotypic characterization of ts-S1 replication in vitro at 33 °C.

When ts-S1 was injected into silkworm larvae reared at 33.5 °C for 7 days, no larvae had signs of infection (Table 1), but 3 larvae out of 10 showed the presence of viral DNA in DNA extracts prepared from individual larvae (Fig. 3). In contrast, all 15 larvae injected with ts-S1 and reared at 25 °C died of nucleopolyhedrosis within that period (Table 1). This result indicates that restriction of virus replication at 33.5 °C occurs in vitro, probably in a similar manner observed for in vitro replication of ts-S1 at 33 °C. After the rearing of injected larvae for 7 days at 33.5 °C, a reduction in the rearing temperature to 25 °C resulted in nucleopolyhedrosis in 2 larvae out of 10 (Fig. 3). This fatality rate is similar to the incidence of larvae possessing viral DNA after rearing at

**Fig. 3.** Detection of viral DNA from ts-S1-infected larvae by PCR and Southern blot hybridization. Twenty of the fourth instar larvae were injected with ts-S1 at 10⁷ TCID₅₀ per larva and reared at 33.5 °C. At 7 days p.i., 10 larvae were collected while 10 larvae were continuously reared at 25 °C until they died or emerged. DNA was extracted from the entire body individual insect as described in Methods. PCR products were separated on 7% agarose gel and stained with ethidium bromide (a). In the upper panel of (a), lanes 1–10 are PCR products from the DNA samples of 10 larvae collected at 7 days p.i. at 33.5 °C. Lane M indicates DNA size markers of HindIII digests of λ-phage DNA. – and + indicate PCR products of DNA samples from ts-S1-infected and mock-injected larvae, respectively. In the lower panel of (a), lanes 1–7 are PCR products of DNA samples from adults at 24 days p.i. (33.5 °C rearing for 7 days followed by 25 °C rearing for 17 days). Lanes N1 and N2 are PCR products of DNA samples from larvae with nucleopolyhedrosis that died after rearing for 7 days at 33.5 °C then 3 days at 25 °C. Lane P1 is the PCR product of DNA samples from the larva which died from physiological damage after rearing for 7 days at 33.5 °C then 11 days at 25 °C. Lanes M, + and – are as in (a). Southern blot hybridization of DNA transferred from the agarose gels in (a) was carried out using viral DNA as a probe and the corresponding blots (b) are shown on the right.
33.5 °C for 7 days, suggesting that both the possession of viral DNA and the deaths from nucleopolyhedrosis were due to residual virus replication occurring in these restricted rearing conditions. The contrary explanation is that ts-S1-injected larvae, having once lost viral DNA as a result of restricted conditions of virus replication, would not subsequently die of nucleopolyhedrosis even after transferring to a non-restricted condition (Fig. 3 and Table 1). It can be hypothesized, based on the observations of \textit{in vitro} replication of ts-S1 at 33 °C (Fig. 1), that ts-S1 does not cause a secondary infection of the larvae at 33.5 °C and that the primary infected cells are eliminated by a conventional larval physiological mechanism. According to Wu \textit{et al.} (1992), \textit{Sf}21 cells infected with an AcMNPV ts mutant are probably sensitive to raised temperature. If this were the case for larval cells infected with ts-S1 at 33.5 °C, the infected cells would die as a result of thermal stress and be eliminated from the larvae. However, it cannot be ruled out that some infected cells of the larvae may remain free of serious physiological damage and may continue to propagate along with the viral DNA copies replicated at an undetectable level. Further studies on the fate of infected cells will be an important step towards understanding the \textit{in vivo} mechanism of the elimination and/or association of heterogeneous DNA molecules introduced into the cells.

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References


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