Characterization of an *Autographa californica* multiple nucleopolyhedrovirus dual mutant: ORF82 is required for budded virus production, and a point mutation in LEF-8 alters late and abolishes very late transcription

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A temperature-sensitive (ts) *Autographa californica* multiple nucleopolyhedrovirus dual mutant, ts42, was generated that displayed tiny-plaque and polyhedral inclusion body (PIB)-defective phenotypes at 33 °C. The mutation responsible for the tiny-plaque phenotype was mapped to orf82, which was characterized as a late gene. Its product was not studied. The mutation responsible for the PIB-defective phenotype was mapped to a highly conserved region of lef-8, which encodes the largest subunit of the viral RNA polymerase. These mutations did not cause a global defect in viral DNA replication or a defect in the shutoff of host protein synthesis. However, the mutation in orf82 caused a dramatic defect in the production of progeny budded virus (BV) but did not decrease the infectivity of those BVs that were released. Hence, ORF82 is required for BV production. The mutation in lef-8 affected a conserved residue that is part of a highly conserved region of LEF-8. This mutation abolished very late transcription whilst altering the transcript size and level of transcription of two late genes.

**INTRODUCTION**

Baculoviruses are DNA viruses that exist in two forms: occluded virus (OV), which spreads the infection between hosts, and budded virus (BV), which disseminates the infection within the host until a systemic infection is reached (Keddie *et al.*, 1989). OVs are enclosed within protective structures called polyhedral inclusion bodies (PIBs), where polyhedrin protein is the major constituent (Rohrmann, 1986). Viral gene expression is regulated mainly at the transcription level (Friesen & Miller, 1986; Huh & Weaver, 1990a, b; Kelly, 1982) and is divided into three temporal phases: early, late and very late (Blissard & Rohrmann, 1990; O’Reilly *et al.*, 1994). Proteins expressed in each phase are required for the next phase, with an increasing global level of expression. These three phases dictate, respectively: (i) reprogramming of the host cell to suppress apoptosis, and deviation of the host machinery towards viral gene transcription and viral genome replication; (ii) BV production; and (iii) OV production.

*Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) diverts the host transcription and translation machineries to produce early gene products encoded by the viral DNA. The viral RNA polymerase is synthesized during this early phase (Guarino *et al.*, 1998). Early gene products are essential to the viral DNA replication process (Erlandson *et al.*, 1985; Gordon & Carstens, 1984; Lu & Miller, 1995), which is initiated at 6 h post-infection (p.i.). Late transcription begins at the onset of viral DNA replication and peaks between 12 and 24 h p.i. Viral DNA synthesis is required for late and, consequently, very late transcription (Lu & Miller, 1995; Passarelli & Miller, 1993; Rice & Miller, 1986). At 12 h p.i., BVs start budding from infected cells, and by 24 h p.i., BV production diminishes and OVs begin to be occluded within PIBs (Blissard & Rohrmann, 1990). Very late transcription occurs in a strong burst that begins at 18 h p.i., and is allowed by the concomitant shutoff of host protein synthesis. There are only two very late genes, *polh* (encoding polyhedrin) and *p10*, which are involved in PIB formation.

Early promoters resemble class II promoters from the host and are recognized by the host RNA polymerase II. In contrast, late and very late promoters, which are recognized...
by the virus-encoded RNA polymerase (Beniya et al., 1996; Fuchs et al., 1983; Glocker et al., 1993; Grula et al., 1981; Guarino et al., 1998; Huh & Weaver, 1990a, b; Xu et al., 1995; Yang et al., 1991), are very similar to one another and quite distinct from host class II promoters. Indeed, both late and very late promoters contain one or several conserved and essential (A/T/G)TAAG motifs where transcription is initiated (Blissard & Rohrmann, 1990; Lu & Miller, 1997; O’Reilly et al., 1994). AATAAA motifs are found in the 3’ region of virtually all late and very late genes (Westwood et al., 1993), and AATAAA polyadenylation signals have been reported to determine the polyadenylation site of transcripts (Friesen, 2007; Hodgson et al., 2007; Lin et al., 2001; Rankin et al., 1986; van Oers et al., 1999). Nevertheless, an in vitro study suggested that transcription terminates at T-rich sequences and transcripts are then polyadenylated in an AATAAA-independent manner (Jin & Guarino, 2000). However, this report could not eliminate a role for the well-conserved AATAAA motifs in vivo, and suggested that they might act as positioning agents for termination. In fact, a previous study has shown that the 3’ processing of baculoviral transcripts requires signals in addition to the AATAAA motif (Westwood et al., 1993). In eukaryotes, this process requires a GT- and/or T-rich sequence downstream of an AATAAA polyadenylation signal (Gil & Proudfoot, 1987; McDevitt et al., 1986).

A purified viral RNA polymerase was found to transcribe from late and very late promoters with equal efficiencies (Guarino et al., 1998), and additional factors dictated appropriate temporal expression levels (McLachlin & Miller, 1994; Passarelli & Guarino, 2007; Todd et al., 1996; Xu et al., 1995; Yang & Miller, 1998, 1999). The AcMNPV RNA polymerase is composed of four subunits: LEF-8, LEF-4, LEF-9 and P47 (Guarino et al., 1998). Each subunit is indispensable to late and very late transcription, but their specific roles remain unclear. LEF-8 is the largest subunit, and all baculoviral LEF-8 homologues contain a 13 aa motif near their C terminus that is also conserved near the C terminus of the largest subunits of DNA-directed RNA polymerases from animals, plants and bacteria (Passarelli et al., 1994). This motif is thought to be part of the catalytic site of these RNA polymerases (Schultz et al., 1993).

In this study, we generated a temperature-sensitive (ts) AcMNPV mutant with point mutations in two genes, orf82 and lef-8, which are well conserved among baculoviruses (Rohrmann, 2011), suggesting their importance in the virus replication cycle. The mutations in orf82 and lef-8 illuminated the functions of their products, including the role of an important region of LEF-8 in late and very late transcription.

RESULTS

Mapping the mutations responsible for the tiny-plaque and PIB-defective phenotypes

The ts mutant ts42 was generated by treating wild-type (WT) AcMNPV-infected cells with nitrosoguanidine. Cells infected with ts42 formed tiny plaques exclusively and produced no PIBs at 33 °C. We mapped the mutation(s) responsible for these phenotypes by marker-rescue analysis with five overlapping cosmids representing the entire AcMNPV genome and then with plasmids containing smaller fragments.

We found that cosmid 1 [33.8-66 map units (m.u.)], HindIII-A (37.3-53.4 m.u.), EcoRI-C (42.8-52.6 m.u.) and SacI-L (50.2-51.6 m.u.) rescued the tiny-plaque phenotype in ts42-infected cells. This generated SR1 (single-rescued 1), which produced WT-sized plaques but retained the PIB-defective phenotype. This showed that at least two mutations were responsible for the two ts phenotypes. Sequencing the entire SacI-L region from WT, ts42 and SR1 revealed that the tiny-plaque phenotype formed by ts42-infected cells at 33 °C was due to a single G→A mutation of nt 67701, changing cysteine (TGC) to tyrosine (TAC) in codon 73 of orf82. Next, we found that cosmids 9 (19.91-47.04 m.u.), PstI-E (29.81-37.12 m.u.) and PN5 (29.81-33.83 m.u.) rescued the PIB-defective phenotype in SR1-infected cells. This generated DR2 (double-rescued 2), which displayed WT phenotypes for plaque formation and PIB generation. Sequencing the entire PN5 region from WT, SR1 and DR2 revealed that the PIB-defective phenotype in ts42- and SR1-infected cells at 33 °C was due to a single T→C mutation at nt 41562, changing leucine (TTG) to serine (TCC) in codon 531 of lef-8. These mutations did not affect the ORFs of the adjacent genes (Fig. 1), nor did they alter their TAAG regulatory motifs (data not shown).

The tiny-plaque and PIB-defective phenotypes are not the consequences of a defect in viral DNA replication

Late and very late transcription depend on viral DNA replication. It was therefore possible that the tiny-plaque and PIB-defective phenotypes observed at 33 °C resulted from an indirect effect of defective viral DNA replication. To test this possibility, we performed a viral DNA replication assay at various times p.i. at 25 and 33 °C (Fig. 2). At either temperature, ts42 could replicate its DNA in infected cells to WT levels, albeit with a short delay of up to 6 h observed at 33 °C. However, viral DNA accumulated in ts42-infected cells at 33 °C at least as rapidly as in WT-infected cells at 25 °C. Hence, the tiny-plaque and PIB-defective phenotypes were not caused by a defect in viral DNA replication. The higher abundance of viral DNA at a given time point observed at 33 °C was probably due to faster cell metabolism at the elevated temperature. This phenomenon was also observed at the protein and RNA levels (see below), and has been reported by others (Partington et al., 1990).

The mutation in lef-8 abolishes polyhedrin production

To determine whether the defect in PIB formation was due to insufficient levels of polyhedrin protein, we pulse-labelled
proteins in cells infected at 27 and 33 °C at various times p.i. and separated them by SDS-PAGE (Fig. 3). At both temperatures, WT- and DR2-infected cells showed similar patterns of protein synthesis, including the burst of polyhedrin protein synthesis. At 27 °C, ts42- and SR1-infected cells also showed a burst of polyhedrin synthesis, although this was delayed by up to 24 h compared with WT. Hence, the mutation in lef-8 has an effect on the timing of polyhedrin expression at the permissive temperature. In addition, at 27 °C, ts42-infected cells produced less polyhedrin than SR1-infected cells, which in turn produced less than the WT-infected cells. This showed that, even at the permissive temperature, both mutations had an effect on polyhedrin production. At 33 °C, ts42- and SR1-infected cells did not show any polyhedrin expression, clearly establishing that the PIB-defective phenotype observed at 33 °C was the result of a defect in polyhedrin expression. Moreover, the global shutoff of host gene expression appeared to occur normally in cells infected by any of the four viral strains at either temperature, as the spectrum of proteins synthesized was significantly different and more discrete than that in mock-infected cells as early as 24 h p.i.

Levels of infectious BVs released are dramatically decreased by the mutation in orf82 and mildly decreased by the mutation in lef-8

The abnormally small plaques observed at 33 °C when cells were infected with ts42 could have been due to a defect in BV production or in the infectivity of the released BVs. To investigate this, we first performed an infectious BV release assay at 27 and 33 °C for 48 h, in which the progeny BVs in the supernatants were titrated by plaque assay (Fig. 4a). At 33 °C, DR2-infected cells produced WT levels of infectious BVs. However, ts42-infected cells released only 4% of the number of BVs released by WT-infected cells at that temperature. As the mutation causing the tiny-plaque phenotype is rescued in SR1, we expected SR1-infected cells to produce WT levels of infectious BVs at both temperatures. However, these cells released only 56% infectious BVs compared with WT levels at 33 °C. Thus, the mutation in lef-8, which remains in SR1, depressed the release of infectious BVs (to 56%), and addition of the mutation in orf82 further decreased it dramatically (to 4%). We argue below that the mutation in lef-8 may achieve its mild effect indirectly by affecting late gene expression. At 27 °C, cells infected with DR2 and SR1 produced titres of infectious BVs that were not significantly different from the titres produced by WT-infected cells, but this temperature was not fully permissive for cells infected with ts42, as they released significantly fewer infectious BVs (45%) than WT-infected cells. It should be noted that cells infected with ts42 still produced BVs at the restrictive temperature, although only at very low levels (4%), suggesting that the mutation in orf82 is leaky.
The decrease in the levels of infectious BVs is due to a defect in BV production and not to reduced infectivity

The infectious BV release assay can measure only the number of progeny BVs that can form plaques. Hence, if non-infectious BVs were also released, they would not be accounted for by this assay. Therefore, we conducted a DNA slot-blot analysis to determine the amount of viral DNA present in the same supernatants that were titrated previously in the infectious BV release assay (Fig. 4a). The quantity of viral DNA should be directly proportional to the number of BVs, whether infectious or not. Hence, we disrupted the BVs from these supernatants, applied the extracts to a slot blot and hybridized the blot with a total viral DNA probe obtained from disrupted WT virions (Fig. 4b). Quantification of the DNA slot blot by phosphorimaging (Fig. 4b) revealed that the relative percentages of DNA present in the supernatants were very similar to the relative percentages of infectious BVs titrated with the infectious BV release assay (Fig. 4a). At 27 °C, the supernatant from ts42-infected cells contained 4 and 5 % of WT levels of infectious BVs and viral DNA, respectively. Therefore, the tiny-plaque phenotype observed following infection with ts42 was not due to weaker infectivity but resulted from a defect in BV production. Moreover, at both temperatures, the supernatants from DR2-infected cells contained at least WT levels of viral DNA. Significantly less viral DNA (44 %) was present in the supernatant from cells infected with SR1 at 33 °C, supporting the conclusion that the mutation in lef-8 moderately depressed BV production. At 27 °C, the supernatant from ts42-infected cells contained significantly less viral DNA (41 %) than that of cells infected with WT, reinforcing the notion that 27 °C is not fully permissive for the mutation in orf82.

To confirm that ts42 is defective in BV production, we pulse-labelled proteins in cells infected for 48 h at 27 and 33 °C using m.o.i. ranging from 0.5 to 20 and subjected them to SDS-PAGE (Fig. 5). At both temperatures, WT-infected cells showed similar bursts of polyhedrin protein synthesis and host protein synthesis shutdown at all the m.o.i., including the lowest of 0.5. At 27 °C, ts42-infected cells still showed the burst of polyhedrin synthesis at all the m.o.i. but, as seen in Fig. 3, this burst was not as pronounced as in WT-infected cells. Also, there were globally more non-polyhedrin proteins and less polyhedrin synthesized at the lower m.o.i. (0.5 and 1) than at higher ones (5, 10 and 20). This phenomenon was another reflection of the fact that 27 °C is not fully permissive for ts42. At 33 °C, cells infected with ts42 using the lowest m.o.i. (0.5 and 1) expressed proteins with a pattern and intensity typically observed in mock-infected cells. However, at the same temperature, cells infected with ts42 using the highest m.o.i. (5, 10 and 20) displayed dramatically decreased levels of global protein synthesis, as observed in WT-infected cells, but without the...
burst of polyhedrin synthesis. This result indicated that, at
33 °C, the spread of infection from cell to cell was impaired,
and that this defect was essentially masked at the highest
m.o.i., which permitted most cells to be synchronously
infected. This m.o.i. dependence was relatively weak in

ts42-infected cells at 27 °C and was not observed in WT-infected
 cells at either temperature. These findings are consistent
with the notion that 
ts42 is defective in BV production.

orf82 is a late gene

A Northern blot with RNA collected at various times p.i.
and probed with an orf82 probe showed the orf82 transcript
to be about 1.3 kb (Fig. 6a). This transcript was too short
to be a transcript of the adjacent orf81 or orf83 genes (data
not shown). The temporal expression analysis of orf82
revealed that the steady-state levels of its transcript started
to accumulate at 6 h p.i., peaked between 12 and 24 h p.i.
and then greatly decreased up to 48 h p.i. Moreover, the 5’
region of orf82 contains two ATAAG sequences, which are
typical transcription start sites of strong late promoters.
Thus, orf82 is a late gene generating a 1.3 kb transcript.

The mutation in lef-8 alters the sizes and
increases the steady-state levels of two late
transcripts

To determine whether the modest decrease in BV pro-
duction caused by the mutation in lef-8 was due to
an alteration in late gene expression, we performed
Northern blot analysis of the total RNA collected at 48 h
p.i. from cells infected at 27 and 33 °C, probing with orf82
and vp39 (encoding the major capsid protein) probes (Fig.
6b). The steady-state levels of the orf82 and vp39 transcripts
reached at least WT levels in cells infected with each of the
viral strains at either temperature. Interestingly, in ts42-
and SR1-infected cells at 33 °C: (i) these levels were
higher than in cells infected with WT and DR2; (ii) an
orf82 transcript that was 200 nt larger than the WT orf82
An AcMNPV with mutations in orf82 and lef-8

The mutation in lef-8 abolishes very late transcription

Because the mutation in lef-8 affected the virus-encoded RNA polymerase, we conducted Northern blot analysis to determine the influence of this mutation on the steady-state levels of the very late transcripts. For this purpose, we employed the same Northern blot used in the study of the orf82 and vp39 transcripts, and probed it with polh and p10 probes (Fig. 6b). The steady-state levels of the polh and p10 transcripts reached WT levels in DR2-infected cells at both temperatures. In cells infected with ts42 and SR1 at 27 °C, the steady-state levels of polh transcripts appeared to be normal, whilst the levels of p10 transcripts were reduced. Most importantly, ts42- and SR1-infected cells at 33 °C showed negligible levels of the polh and p10 transcripts. Hence, the mutation in lef-8 caused a loss of very late transcription at 33 °C.

DISCUSSION

We have established the importance of two specific codons from two well-conserved baculoviral genes, orf82 and lef-8, in BV production, and in late and very late transcription, respectively. Viral DNA replication and host protein synthesis shutoff occurred normally in cells infected with the baculoviral dual mutant, indicating that viral early genes were globally activated and functioned normally in regulating host transcription.

ORF82 belongs to the TLP20 (Raynes et al., 1994) family of proteins. Alignment of the ORF82 amino acid sequences from 45 baculoviruses revealed that, at position 73, cysteine is not conserved within the TLP20 family (cysteine is present in only seven baculoviruses), and tyrosine is the most commonly found amino acid at this position (in 20 baculoviruses; data not shown). This was surprising, as the mutation we mapped to position 73 of AcMNPV ORF82 caused a change from a cysteine to a tyrosine. A crystallized proteolytic fragment of AcMNPV ORF82 revealed that cysteine 73 is part of a β-strand and appears to play a role in the tertiary structure of the protein (Holden et al., 1996). Interestingly, the adjacent asparagine 74 is conserved in 44 of the 45 baculoviruses. In the context of the AcMNPV ORF82, the change of cysteine 73 to tyrosine causes temperature sensitivity and, as tyrosine is relatively frequent at this position, it is not surprising that this mutation is somewhat leaky.

Several other baculovirus structural proteins are important for BV production, such as GP64 (Oomens & Blissard, 1999), GP41 (Olzewska & Miller, 1997b) and VP1054 (Olzewska & Miller, 1997a). Interestingly, the crystallized proteolytic fragment of ORF82 reveals the topology of a jelly roll that is commonly found in viral coat proteins (Holden et al., 1996). Another study characterized Bombyx mori NPV (BmNPV) orf68, a homologue of AcMNPV orf82, as a late gene encoding a structural protein involved in BV production (Iwanaga et al., 2002). Although the levels of BVs released from cells infected with the BmNPV orf68 deletion mutant used in that study never went below 13% of WT levels during the course of 120 h p.i., and reached 19% at the 48 h p.i. time point that we considered, these and our data point in the same direction. It is therefore likely that AcMNPV ORF82 is also a structural protein.
Our study showed that the _orf82_ transcript is 1.3 kb. The 5′ and 3′ regions of _orf82_ contain several potential transcription start sites and canonical AATAAA polyadenylation signals. The AATAAA sequence at +1078 nt is followed by a T-rich TTTTTATT motif located 24 nt downstream. This closely resembles the TTTATTTT and TTTTTTT _in vitro_ transcription termination sites found 5 and 30 nt downstream of an AATAAA motif, respectively (Jin & Guarino, 2000). Use of the _orf82_ proximal ATAAG transcription start site at −7 nt and the AATAAA polyadenylation signal at +1078 nt followed by the T-rich TTTTTATT motif would give rise to the observed 1.3 kb transcript, assuming that the poly(A) tail is about 185 nt (Fig. 7a). Additionally, the _vp39_ proximal ATAAG at −58 nt and the AATAAA at +1333 nt followed by a T-rich TTGTTT motif 34 nt downstream would generate the detected 1.7 kb transcript, with an approximately 270 nt poly(A) tail (Fig. 7b). These observations agree with the hypothesis that both the polyadenylation signal and the T-rich motif operate in concert for the 3′ processing of late transcripts, whereby AATAAA would specify which downstream and nearby T-rich motif is to be used as polyadenylation site.

Cells infected at 33°C with viral strains carrying the mutation in _lef-8_ altered the transcription of _orf82_ and _vp39_. Indeed, _ts42_- and _SR1_-infected cells synthesized a new _orf82_ transcript that was ~200 nt longer as well as an additional _vp39_ transcript that was ~300 nt longer than their WT counterparts, respectively. Given the distribution of potential polyadenylation signals in the 3′ region of _orf82_ and the size of the WT _orf82_ transcript, the recognition of an alternative AATAAA cannot explain the 200 nt longer mutant _orf82_ transcript. In contrast, the 5′ region of _orf82_ contains three potential transcription start sites, the proximal and middle ones being 163 nt apart, which could account for the new _orf82_ transcript that is 200 nt longer than normal (Fig. 7a). Additionally, the 5′ region of _vp39_ also contains three potential transcription start sites, the proximal and most distal ones being 264 nt apart, which could explain the generation of the additional _vp39_ transcript that is 300 nt longer than normal (Fig. 7b). Our data and observations suggest that, at the restrictive temperature, the mutation in _lef-8_ yields a viral RNA polymerase that has difficulty recognizing the preferred transcription start sites of at least two late genes but that can still transcribe them very efficiently. In fact, the defective viral RNA polymerase yielded steady-state levels for these two late transcripts that were higher than WT levels at 33°C. Because late and very late promoters are recognized by the same core RNA polymerase, and because the defective RNA polymerase cannot transcribe very late genes at 33°C, we propose that it is significantly more

![Fig. 7. Sequence elements of the _orf82_ and _vp39_ genes. The 5′ and 3′ regions of the _orf82_ (a) and _vp39_ (b) genes are represented. Several TAAG and AATAAA motifs are shown, and the putative transcription initiation, polyadenylation and T-rich sequences are underlined. The distances between the putative transcription start sites and T-rich motifs as well as between the putative alternative transcription start sites are indicated. The calculated lengths of the poly(A) tails to generate the transcripts reported in Fig. 6 are given. The translation initiation and stop codons are shown in bold. The nucleotide numbers from the whole AcMNPV genome are indicated on top of the sequences, whilst numbering with reference to the translation initiation codon is shown below.](image-url)
available to transcribe late genes during the very late phase of infection. Additionally, these increased levels of viral RNA polymerase available for late transcription may disrupt the balance among the factors involved in late transcription. This could result in the recognition of aberrant late transcription initiation sites, leading to the abnormally sized late transcripts observed, although this remains to be tested. Hence, the effect of the mutation in **lef-8** on late transcription may be indirect.

If our assumption about an alternative transcription start site is correct, the abnormally longer **orf82** transcript contains a 5′ untranslated region (UTR) that is 170 nt, whilst that of its WT counterpart is only 7 nt. Computer analyses of RNA secondary structures revealed that the short 5′ UTR of the WT transcript does not appear to be part of any secondary structure, whilst the longer 5′ UTR of the aberrant transcript is predicted to be involved in a total of eight regions of secondary structure, two within itself and six with the coding region (data not shown). This could inhibit scanning to the initiation codon, resulting in less abundant ORF82 protein despite the higher levels of aberrant **orf82** transcript observed. As ORF82 is required for BV production, this could explain the modest yet significant decrease in BV production at the restrictive temperature in the presence of the mutation in **lef-8**.

We mapped the mutation in **lef-8** to codon 531, and this point mutation results in a relatively drastic amino acid change from a leucine to a serine. A study of BmNPV has shown that a change from a leucine to a serine at position 531 of the LEF-8 subunit of the AcMNPV RNA polymerase is part of a highly conserved region, and our studies show that it is critical to very late transcription while having a more subtle effect on late transcription. Further understanding of the role of this important region of LEF-8 may help to elucidate the mechanisms involved in the specific regulation of late and very late transcription.

**Fig. 8.** Result of an amino acid sequence alignment of the 18 aa stretch spanning region 527–544 of LEF-8 from 110 baculoviruses. The amino acids in the LEF-8 proteins of all the baculoviruses were positioned relative to the AcMNPV LEF-8 sequence. Residues with at least 70, 80, 90 and 100 % identity among all 110 baculoviruses are shown. Residues not meeting these criteria are indicated by dashes. Leucine 531 carries the mutation in AcMNPV LEF-8 of **ts42** and SR1, and is indicated by an arrow.

**METHODS**

**Cell culture and viruses.** *Spodoptera frugiperda* IPLB-Sf21 cells were cultured in TC-100 medium (Sigma-Aldrich) supplemented with 10 % FBS. Viruses were derived from the L1 strain of AcMNPV. Infections were conducted at room temperature and, after 1 h, the viral inocula were removed and the cells were further processed.

**Random mutagenesis.** Cells (2×10⁶) were seeded in 6 cm plates and infected with WT virus at an m.o.i. of 10–8 p.f.u. per cell. At 10 h p.i., the cells were incubated in medium containing 8 μg nitrosoguanidine ml⁻¹ for 2 h and then in medium alone for 23 h, both at 25 °C. The supernatants were screened by plaque assay at 33 °C for **ts** mutants, including **ts42**.

**Cosmid library and plasmids.** The cosmid library and *HindIII*-A and *EcoRI*-C plasmids were a gift from Dr G. F. Rohrmann (Oregon State University, OR, USA). The *PstI*-E plasmid was obtained from Dr A. L. Passarelli (Kansas State University, KS, USA). We generated the *SacI*-L and PNS5 plasmids (PNS5 is the 5.37 kb fragment obtained after digesting *PstI*-E with *PstI* and *NotI*).

**Marker rescue and mapping of the mutations.** Cells (1.5×10⁶) were seeded in each well of a six-well plate, infected with **ts42** or SR1 at an m.o.i. of 0.05 and then transfected for 5 h at 33 °C with 1.5 μg cosmid or plasmid using 8 μl Cellfectin (Invitrogen). The transfection mixture was replaced with medium and the cells incubated for 4 days at 33 °C. The supernatants were screened by plaque assay for rescued phenotypes, generating SR1 and DR2 from the picked and amplified plaques.

The *SacI*-L fragments from WT, **ts42** and SR1, and the PNS5 regions from WT, SR1 and DR2 were sequenced to identify the mutations responsible for the tiny-plaque and PIB-defective phenotypes, respectively.

**Viral DNA replication assay.** Cells (2×10⁵) were seeded in each well of a 24-well plate, infected at an m.o.i. of 10, incubated at 25 and 33 °C, and resuspended in 1 ml ddH₂O at each time point. Cell lysis
and DNA denaturation were performed by adding 100 μl cell suspension to 81 μl hot, supersaturated sodium iodide, followed by boiling at 100 °C for 10 min and chilling. The DNA was slot blotted on a Nytran membrane and probed with biotin-labelled total AcMNPV DNA (BioPrime DNA labelling kit; Gibco), followed by hybridization, washing and detection (Southern-Light Chemiluminescent DNA Detection kit; Tropix).

**Pulse labelling of proteins with [35S]methionine.** Cells (1.5 x 10⁷) were seeded in each well of a six-well plate, infected at m.o.i. of 0.5, 1, 5, 10 and 20 and incubated at 27 and 33 °C. At each time point, the cells were cultured for 1.5 h at 27 and 33 °C in starvation medium (SF-900 II SFM without l-methionine or l-cystine; Gibco) containing 27 μCi [35S]methionine in each well, seeding the floating cells back. The cells were washed with PBS, resuspended in equal volumes of TE and gel loading buffer and boiled for 5 min. The proteins were separated by SDS-PAGE (12 % acrylamide), followed by fluorography (Fluoro-Hance; Research Products International).

**DNA slot-blot analysis.** A positively charged nylon membrane was slot blotted with 100 μl virion-disrupted extract, cross-linked and incubated in pre-hybridization buffer [0.01% (w/v) denatured sheared salmon sperm DNA, 20 mM PIPES (pH 6.5), 50 % formamide, 1 % SDS, followed by fluorography (Fluoro-Hance; Research Products International).

**Infectious BV release assay.** Cells (1 x 10⁶) were seeded in the wells of a six-well plate, infected at an m.o.i. of 20 and incubated for 48 h at 27 and 33 °C. The supernatants were titrated by plaque assay. Statistical analysis of the pairwise comparison of the strains within temperature treatments was performed by a two-way ANOVA with a log-transformed count as the response variable.

**Virion disruption.** Virus (8 x 10⁶–1 x 10⁷ p.f.u.) was lysed in 1 ml detergent buffer A [10 mM Tris/Cl (pH 8.3), 50 mM KCl, 0.1 mg gelatine ml⁻¹, 0.45 % NP-40, 0.45 % Tween 20] containing 67 μg proteinase K for 1 h at 60 °C. The proteinase K was inactivated at 95 °C for 10 min and the extracts chilled.

**Northern blot analysis.** Cells (1 x 10⁷) were seeded in 10 cm plates, infected at an m.o.i. of 10 and incubated for 48 h at 27 and 33 °C. Total RNA was prepared (RNAquick-4PCR kit; Ambion) and a 1.2 % agarose/glyoxal gel (NorthernMax-Gly kit; Ambion) was loaded with 6 μg total RNA per well. Following electrophoresis, the RNA was transferred to a positively charged nylon membrane. Probe labelling, pre-hybridization, hybridization, washing and exposure were performed as for the DNA slot-blot analysis.

**REFERENCES**


