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A Mutant Baculovirus with a Temperature-Sensitive IE-1 Transregulatory Protein

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We have mapped the mutation responsible for the temperature-sensitive (*ts*) phenotype of *ts*B821, a mutant of the baculovirus *Autographa californica* nuclear polyhedrosis virus (H. H. Lee and L. K. Miller, J. Virol. 31:240–252, 1979), to a single nucleotide which changes alanine 432 of the multifunctional regulatory protein IE-1 to a valine. Mapping was done with a combination of marker rescue and transient expression assays, hybrid gene construction by overlap PCR gene splicing, and nucleotide sequence analysis. Cells infected with *ts*B821 at high multiplicities of infection showed a spectrum of responses from severe cytopathic effects, including apoptosis, to a lack of obvious signs of infection. Protein synthesis in *ts*B821-infected cells at the restrictive temperature appeared similar to uninfected cell protein synthesis, but viral DNA replication and budded virus production were observed, albeit in a delayed manner. The dependence of early and late promoter activity on the wild-type IE-1 gene, *ie-1*, was observed in transient expression assays. However, the dependence of early promoter activity on *ie-1* was strongest in the absence of other viral genes. Thus, other viral genes appear to be able to compensate, at least in part, for the lack, or low levels, of *ie-1* in transient expression assays using early promoters. The mutant should prove useful in further defining the function(s) of IE-1.

A number of baculovirus regulatory genes have been identified on the basis of their ability to transactivate reporter plasmids in transient expression assays (2, 14, 17, 27, 33, 34, 40, 41). Expression from many viral promoters is strongly, if not absolutely, dependent on the multifunctional regulatory gene *ie-1* in these assays (16, 31, 34).

The *ie-1* product, IE-1, not only acts as a strong transactivator of some promoters but also downregulates other promoters (4, 23) and can stimulate expression through *cis*-acting sequences known as homologous regions (*hr*1 through *hr*5) (15). The homologous regions are interspersed in the *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) genome (8, 13) and appear to function both as transcriptional enhancers (15) and as origins for DNA replication (20, 35) in transient assays. Gel shift assays indicate that the 67-kDa IE-1 is involved in protein/DNA complex formation with *hr*5 (12, 21). The abilities of IE-1 to participate in *hr*5/protein complex formation and to transactivate promoters are governed, in part, by the C-terminal portion of the polypeptide (21), which is more highly conserved than the N terminus (19, 40).

Transcripts of *ie-1* initiate from two different locations which ɔ rise to a set of spliced and unspliced transcripts (5, 22). spliced transcripts originate from a promoter immediately tream of the *ie-1* open reading frame (ORF) and encode 1 (16). These RNAs are detectable within 1 h postinfection .), accumulate during infection, and are most abundant late nfection (23). Spliced *ie-1* RNAs encode IE-0 and originate m a promoter upstream of an exon (exon 0) located 4 kbp from the *ie-1* ORF (5, 22). The product of these spliced RNAs is IE-0, which is the same as IE-1 except for an additional 54 amino acids at the N terminus. Steady-state levels of RNA which specifically encode *ie-0* are maximum at 2 to 3 h p.i. (5, 22), although additional spliced RNAs also originate from the *ie-0* promoter region late in infection (22). In transient expression assays, the *ie-1* promoter is stimulated by *ie-1*, *ie-0*, and another early gene, *ie-2* (formerly known as *ie-n*) (3), while the *ie-0* promoter is downregulated by *ie-1* (23). IE-1 and IE-0 have distinct transregulatory properties in transient expression assays (23).

IE-1-dependent promoters belong to the early as well as late classes of promoters, although late promoter dependence on IE-1 may be a secondary result of dependence on the presence of early gene products. Expression from some promoters, often referred to as immediate-early promoters and including the promoters of *ie-1*, *ie-0*, and *ie-2*, is less dependent on or even negatively regulated by *ie-1* in transient assays (4, 14, 23). The delayed-early class of promoters appear to depend on *ie-1* only in transient assays; a requirement for prior viral protein synthesis is not observed during viral infection (9, 10, 37). However, IE-1 is clearly the key regulatory protein in Ac*M*NPV gene expression in transient assays, and it is important to understand its normal role during virus infection.

Previous studies of a temperature-sensitive (*ts*) Ac*M*NPV mutant, *ts*B821 (26), suggest that this mutant is blocked at a very early stage of virus infection although DNA replication and budded virus formation are only delayed (26, 29). We have confirmed this phenotype and have mapped the mutation responsible for the *ts* phenotype to a single nucleotide change in *ie-1* which alters an amino acid in the C-terminal third of IE-1 (and IE-0). Thus, IE-1 is necessary for timely early gene expression and DNA replication. At high multiplicities of infection (MOI), infected cells displayed a variety of responses ranging from lack of cytopathic effects to apoptosis and full infection. The *ts*B821 mutant should prove useful in further analyses of IE-1 function.

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MATERIALS AND METHODS

Cells and viruses. Separate cultures of the *Spodoptera frugiperda* (fall armyworm) IPLB-SF-21 (SF-21) cell line (44) were adapted and maintained at either 23 or 33°C in TC100 medium supplemented with 10% fetal bovine serum and 0.26% tryptose broth as described previously (32). The *ts*B821 mutant of Ac*M*NPV was derived previously (26) by 5-bromodeoxyuridine mutagenesis of the wild-type (wt) virus, Ac*M*NPV L-1 (25). The *ts* virus was propagated and titered at the permissive temperature, 23°C, and phenotypically characterized at the restrictive temperature, 33°C. A p35 mutant of AcMNPV, vAcAnh (6), was propagated and titered at 27°C in TN-368, a cell line derived from *Trichoplusia ni* (44), before use as a control in the experiments described.

Measurements of rate of budded virus release and DNA synthesis. SF-21 cells (2×10^6 cells per 60-mm-diameter dish) adapted to either 23 or 33°C were infected with virus at an MOI of 20 PFU per cell. After 1 h of adsorption, the inocula were removed and the cell monolayers were washed twice with 2 ml of phosphate-buffered saline (PBS; pH 6.2) (26) and then incubated at either 23 or 33°C in 4 ml of complete TC100. To determine the rate of release of budded virus, aliquots of extracellular fluid were removed at specified times p.i. and stored at 4°C before titering on SF-21 cells maintained at 23°C. To determine the rate of viral DNA synthesis, cells were harvested at specified times p.i. and counted with a hemocytometer. The cells were pelleted in a microcentrifuge and resuspended in distilled water (2×10^5 cells per ml), and the suspension was stored at −20°C until all samples were collected. A portion of the cells (2×10^4 or 1×10^4) in 100 μl of distilled water was placed in 1.5-ml microcentrifuge tubes, 81 μl of hot, supersaturated sodium iodide was added to each tube, and the mixture was boiled at 100°C for 10 min. (Supersaturated sodium iodide was prepared by dissolving 100 g of sodium iodide in 40 ml of hot distilled water; the solution was stored in the dark and redissolved prior to use by boiling.) Denatured DNA samples were removed individually from the boiling water and applied immediately to a nylon membrane in a slot blot apparatus connected to a vacuum to ensure rapid (less than 30 s) passage through the membrane. The membranes, containing bound denatured DNA, were washed briefly with 70% ethanol to remove excess sodium iodide and then washed briefly with water. Membranes were prehybridized at 65°C in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)-Denhardt's hybridization buffer (38) for 15 min and then hybridized overnight at 65°C with nick-translated Ac*M*NPV DNA. The blots were washed twice in 2× SSC–0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min per wash. Bound radioactivity was detected by autoradiography.

Reporter plasmids. Plasmids pETCAThr5, pCAPCAT, pETCAT, pCAPCAT-hr5, phcIE1, phcIE0, and phcPE38 were used as reporter plasmids in transient expression assays. Plasmids pETCAThr5 (34), pCAPCAT (43), phcIE1, phcIE0, and phcPE38 (30) were described previously. pETCAT is the same as pETCAThr5 (34) but lacks the *hr*5 sequences. To construct pCAPCAT-hr5 (pCAPCAT lacking the *hr*5 sequences), pCAPCAT was digested with *Nde*I and *Sfi*I, blunt ended, and ligated to remove a 0.5-kbp fragment containing *hr*5. Plasmids pETCAT and pETCAThr5 have the chloramphenicol acetyltransferase gene (*cat*) under the control of an Ac*M*NPV early gene (*etl*) promoter (9). Plasmids pCAPCAT-hr5 and pCAPCAT have *cat* under the control of a late Ac*M*NPV gene (*vp39*) promoter (42). Plasmids phcIE1, phcIE0, and phcPE38 contain *hr*5, and *cat* under the control of the Ac*M*NPV early gene promoters *ie-1*, *ie-0*, and *pe-38*, respectively (30).

Other plasmids. Plasmids pH_3G, $ptsH_3G$, pH_3F, pPstN, pPE-38, pBSIE1HC (also known as pIE1/HC), and pBStsIE1HC contain genomic fragments of Ac*M*NPV or *ts*B821 (in the case of $ptsH_3G$ and pBStsIE1HC) within the region from approximately 91 to 3.4 map units (m.u.). Plasmids pH_3G, pH_3F, pPSTN, pPE-38, containing Ac*M*NPV *Hin*dIII-G, *Hin*dIII-F, *Pst*I-N, and a *Bgl*II-*Eco*RI fragment (98.4 to 100.0 m.u.), respectively, have all been described earlier (34). Plasmid $ptsH_3G$ was constructed by digesting *ts*B821 DNA with *Hin*dIII, cloning the resulting genomic fragments into pBluescript (Stratagene, La Jolla, Calif.), and isolating a clone containing the *Hin*dIII G fragment (91.0 to 96.9 m.u.). Plasmid pBSIE1HC contains *ie-1* within a 3.1-kbp *Cla*I-*Hin*dIII fragment of Ac*M*NPV (94.7 to 96.9 m.u.) cloned into the *Hin*dIII and *Cla*I sites of pBluescript. Plasmid pBStsIE1HC was constructed by subcloning the 3.1-kbp *Cla*I-*Hin*dIII fragment of $ptsH_3G$ into the *Cla*I and *Hin*dIII sites of pBluescript.

Ac*M*NPV clone library. A library containing overlapping clones that comprise the entire Ac*M*NPV genome has been previously described (34) and was used in the initial mapping of the *ts*B821 mutation by marker rescue.

DNA cotransfections and transient expression assays. Monolayers of SF-21 cells (2×10^6/60-mm-diameter dish) preadapted for growth at 33°C were transfected with DNA by liposome-mediated transfection using Lipofectin (GIBCO BRL). The cells were maintained at 33°C for 12 h for cotransfections with the reporter plasmids phcIE1, phcIE0, and phcPE38, 12 or 24 h for cotransfections with pETCAT and pETCAThr5, and 48 h for cotransfections with pCAPCAT-hr5 and pCAPCAT. Each cotransfection mixture contained 2 μg of reporter plasmid DNA and approximately 1 μg of viral PCR product or test plasmid DNA. CAT assays (11) were performed with 20% of the cell lysates.

Metabolic labeling of SF-21 cells. Monolayers of SF-21 cells were infected (at 23 and 33°C) with either wt Ac*M*NPV or *ts*B821 virus at an MOI of 20 PFU per cell. After 1 h, inocula were replaced with complete TC100. Two hours before the appropriate time point, the cells were suspended in complete TC100, placed in sterile tubes, centrifuged, and provided incomplete TC100 lacking cysteine and methionine. One hour before the time point, the cells were centrifuged, pulse-labeled by replacing the medium with fresh cysteine- and methionine-free medium containing 25 μCi of [^{35}S]Trans-label, a mixture of radioactive cysteine and methionine (ICN Biochemicals), and incubated at 23 or 33°C for 1 h. At the time point, the cells were centrifuged, the medium was removed, and the cells were washed twice with 0.5 ml of PBS (pH 6.2). The cells were centrifuged, and the cell pellet was stored at −80°C. Lysis buffer (50 μl) was added to the cell pellet, and the lysates were boiled for 5 min in a SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (2% SDS, 62.5 mM Tris-HCl [pH 6.7], 15% glycerol, 100 mM dithiothreitol). Labeled proteins were analyzed by SDS-PAGE, and the radioisotope distribution was detected by fluorography (1).

Analysis of the size of DNA from infected SF-21 cells. Monolayers of SF-21 cells (2×10^6 cells per 60-mm-diameter dish) were infected with either Ac*M*NPV, *ts*B821, or vAcAnh at an MOI of 20 PFU per cell. After 1 h, inocula were replaced with complete TC100. At selected times during infection at 33°C, medium was removed and the cells were lysed in 0.4 M Tris (pH 8.0)–0.1 M EDTA–1% SDS–200 μg of proteinase K per ml. The lysates were incubated at room temperature for 24 h, phenol extracted, and ethanol precipitated. Half of each sample was loaded on a 1.2% agarose gel and separated by electrophoresis overnight. The gel was then treated with 20 μg

TABLE 1. Marker rescue of *ts*B821

Viral DNA	DNA clone[a]	Rescue[b]
*ts*B821	IE15	+++++
*ts*B821	pPE-38	−
*ts*B821	pH_3F	−
*ts*B821	pPstN	−
*ts*B821	pH_3G	++
*ts*B821	pBSIE1HC	+

[a] Except for IE15, the clones of the overlapping Ac*M*NPV clone library (34) were negative for rescue.

[b] SF-21 cells were cotransfected with *ts*B821 DNA and the lambda clone IE15 or smaller plasmid DNAs within this region. Rescue was scored by the presence of plaques at 33°C by 7 days after cotransfection of the DNAs. The results are a compilation of results of eight different experiments. A plus sign indicates that occlusion bodies, reflecting successful marker rescue, were observed. The number of plus signs indicates the relative level of rescue observed. A minus sign indicates that no occlusion bodies were observed; the clone was negative for rescue.

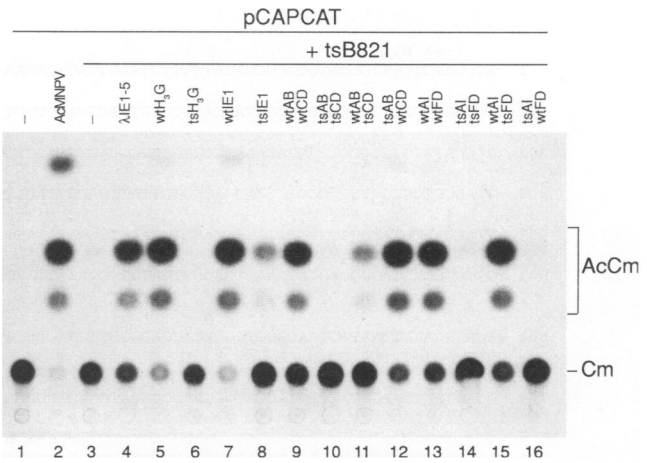


FIG. 1. Mapping the *ts* mutation in *ts*B821 in transient expression assays. Monolayers of SF-21 cells were cotransfected with the reporter plasmid pCAPCAT alone (lane 1), pCAPCAT plus Ac*M*NPV DNA (lane 2), pCAPCAT plus *ts*B821 DNA (lane 3), pCAPCAT plus *ts*B821 DNA and lambda clone IE15 (lane 4), pCAPCAT plus *ts*B821 and subclones of IE15 (lanes 5 and 7), pCAPCAT plus *ts*B821 DNA and clones derived from *ts*B821 DNA (lanes 6 and 8), or pCAPCAT plus *ts*B821 DNA and SOE PCR products (lanes 9 through 16). Lanes designated wtIE-1 and tsIE-1 contain subclones pBSIE1HC and pBStsIE1HC, respectively. AB, CD, AI, and FD indicate the primers used for the first set of SOE PCRs during hybrid gene construction; ts or wt preceding the primer name indicates whether the primer was used to synthesize wt or *ts* portions of *ie-1* DNA. Transfected cells were maintained at 33°C, and CAT activity was determined by enzyme assays at 48 h. Acetylated (AcCm) and unacetylated (Cm) [^{14}C]chloramphenicol were separated by thin-layer chromatography and visualized by autoradiography.

of RNase A per ml in electrophoretic running buffer (containing ethidium bromide [1 μg/ml]) at room temperature before visualization with UV light.

Sequencing of the *ie-1* genes from Ac*M*NPV and *ts*B821. Plasmids pBSIE1HC and pBStsIE1HC were used for sequencing the *Cla*I-*Hin*dIII fragments (94.7 to 96.9 m.u.) from Ac*M*NPV and *ts*B821, respectively. Both DNA strands were sequenced by the dideoxyribonucleotide chain termination method (39), using primers of 17 or 18 nucleotides in length, and a DNA sequencing kit (United States Biochemical, Cleveland, Ohio).

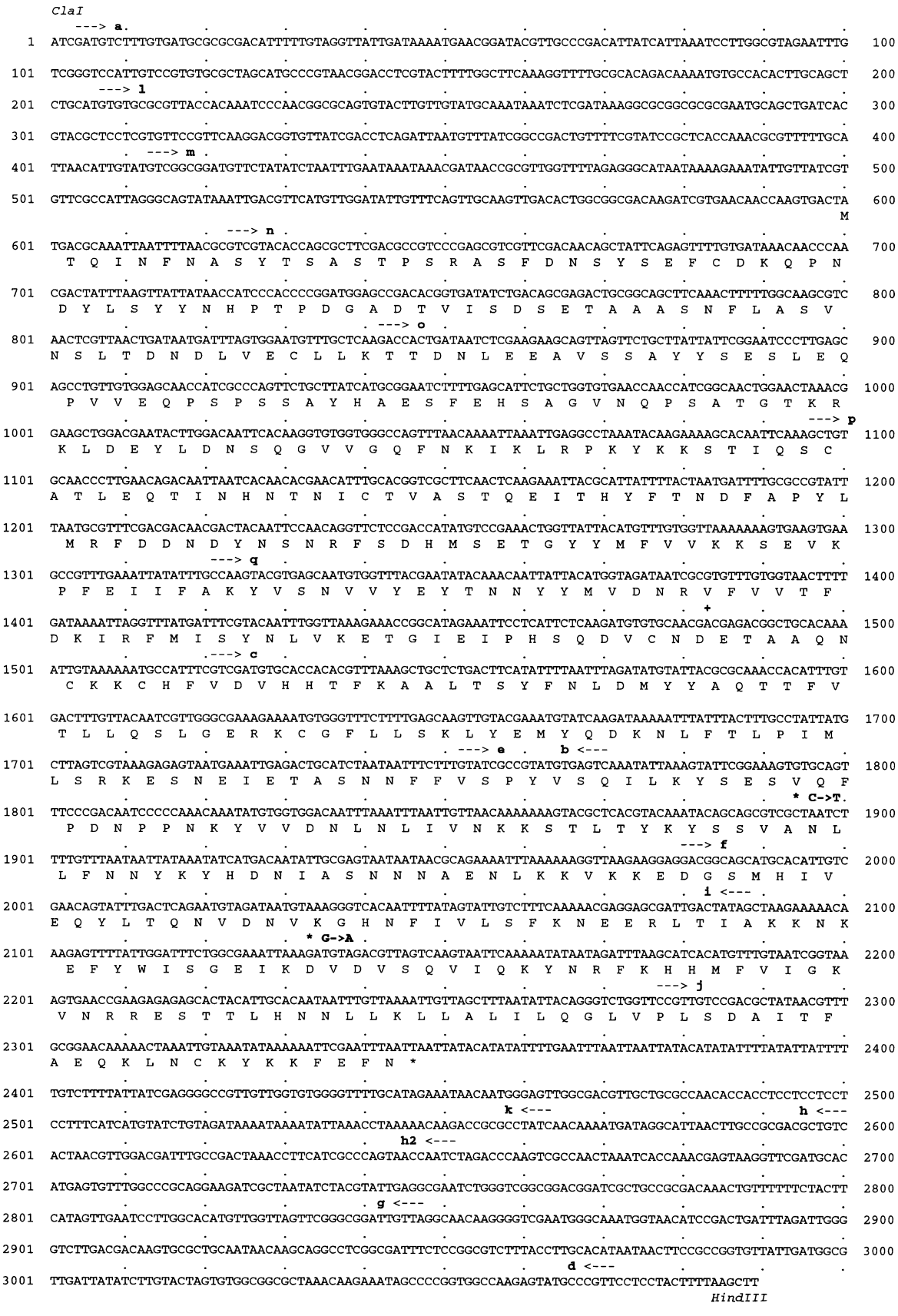
Construction of hybrid genes. Hybrid *ie-1*s composed of portions of both wt and *ts*B821 alleles were constructed by the gene splicing by overlap extension (SOE) method (18), using either pBSIE1HC and pBStsIE1HC or Ac*M*NPV and *ts*B821 DNAs.

RESULTS

Mapping of the *ts*B821 mutation. To map the *ts*B821 mutation, *ts*B821 DNA was cotransfected into SF-21 cells with each of an overlapping set of DNA clones, described previously (34), which collectively comprise the entire Ac*M*NPV DNA genome. Rescue of the mutant to a *wt* phenotype was scored by observing an increase in the number of plaques at the restrictive temperature (28). Only one clone, IE15, comprising the region from approximately 90 to 3 m.u., rescued *ts*B821. To locate the mutation more precisely, subclones of IE15 were used in further marker rescue experiments (Table 1). The smallest clone which provided rescue was pBSIE1HC (ca. 94.7 to 96.9 m.u.), comprising *ie-1*. Marker rescue using smaller clones proved difficult; the number of plaques observed was dependent on the size of the DNA used for rescue, probably because of reduction in the length of DNA available for homologous recombination. We have observed similar effects of fragment size on marker rescue with other *ts* mutants (unpublished data).

We therefore developed a second method to map the mutation in *ts*B821 that was based on a previous observation that *cat* expression from pCAPCAT, a reporter plasmid carrying *cat* under the control of the late *vp39* promoter, is dependent on *ie-1* function in transient expression assays (34). When pCAPCAT was cotransfected with *ts*B821 DNA at 33°C, no *cat* expression was observed (Fig. 1, lane 3), whereas extensive CAT activity was found upon cotransfection with wt Ac*M*NPV DNA (lane 2). When *ts*B821 DNA was cotransfected with the IE15 clone, *cat* expression from pCAPCAT was observed (lane 4); none of the other clones of the overlapping library were able to rescue *cat* expression in this assay. When cells were cotransfected with *ts*B821, transient expression rescue was also observed with plasmids pH_3G (91.0 to 96.9 m.u.; lane 5) and pBSIE1HC (94.7 to 96.9 m.u.; indicated as wtIE1; lane 7). Rescue was not observed if the equivalent regions of *ts*B821 ($ptsH_3G$ and pBStsIE1HC) were cotransfected with *ts*B821 DNA (lanes 6 and 8, respectively). Thus, the results of this assay were identical to those obtained by using conventional marker rescue by plaque assay, and the *ts* mutation of *ts*B821 was localized to the *Cla*I-*Hin*dIII fragment from 94.7 to 96.9 m.u. This fragment includes the entire ORF of *ie-1* as well as 599 bp upstream of the translation start site and 740 bp downstream of the translation stop codon.

To further localize the mutation, we constructed hybrids of wt *ie-1* (wtIE1) and *ts*B821 *ie-1* (tsIE1) by using SOE, a PCR-based gene splicing technique (18). Four primers were used for the construction of the first set of hybrid genes. Primers a and b were used to amplify 1,770 bp containing the N-terminal half of *ie-1* (see Fig. 2 for positions of the primers), and primers c and d were used to amplify 1,550 bp containing the C-terminal half. The PCR products from primers a and b and primers c and d overlapped by 149 bases. The N-terminal half of the wt *ie-1* was designated wtAB, while the C-terminal wt half was designated wtCD. Likewise, the two halves of the *ts*B821 region were designated *ts*AB and *ts*CD. The PCR products were combined as follows: wtAB/wtCD, *ts*AB/*ts*CD, wtAB/*ts*CD, and *ts*AB/wtCD. Primers a and d were then added to each of the PCR product combinations, and a further PCR was carried out to produce a wt gene, wtAB/wtCD, a *ts* gene,


ClaI
---> a.
---> l
---> m .
---> n .
M
---> o .
---> p
---> q .
---> c .
---> e .
b <---
* C->T.
---> f .
i <---
* G->A
---> j .
k <---
h <---
h2 <---
g <---
d <---
HindIII

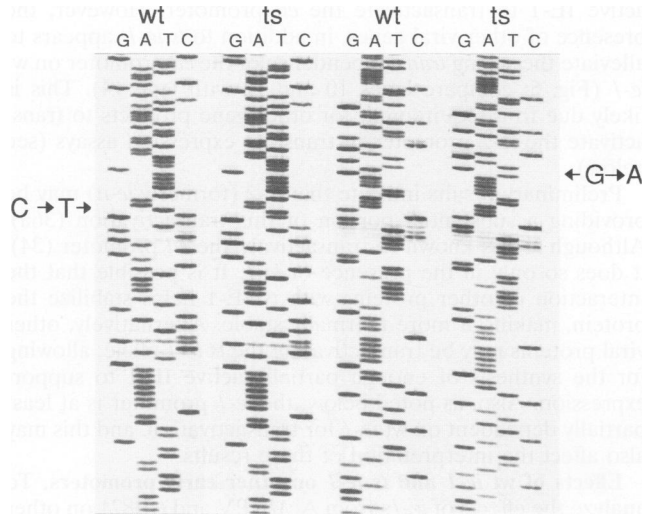


FIG. 3. DNA sequence localization of mutations in *ie-1* of *ts*B821. An autoradiogram of a gel showing a portion of the sequence of wt *ie-1* and *ts ie-1* obtained by using primer e or f (Fig. 2). Arrows point to sequence differences: a C-to-T change at nucleotide 1894 and a G-to-A change at nucleotide 2133. Each lane represents a sequencing reaction; the dideoxynucleotides used are indicated above the lanes.

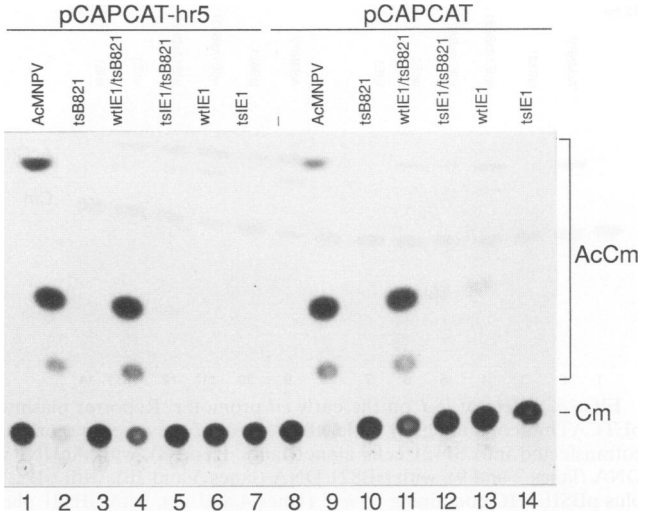


FIG. 4. Effect of *hr5* on transactivation of a late Ac*M*NPV promoter in transient expression assays. Reporter plasmids pCAPCAT and pCAPCAT-hr5 (with and without *hr5* sequences, respectively) and *cat* under the control of the *vp39* promoter were cotransfected into SF-21 cells. Cells were transfected with the reporter plasmids alone (lanes 1 and 8), with Ac*M*NPV DNA (lanes 2 and 9), with *ts*B821 DNA (lanes 3 and 10), with *ts*B821 plus either pBSIE1HC (lanes 4 and 11) or pBStsIE1HC (lanes 5 and 12), and with pBSIE1HC (lanes 6 and 13) or pBStsIE1HC (lanes 7 and 14) in the absence of viral DNA. Cells were maintained at 33°C, and CAT activity in transfected cells was determined by enzyme assays at 48 h. Acetylated (AcCm) and unacetylated (Cm) [^{14}C]chloramphenicol were separated by thin-layer chromatography and visualized by autoradiography.

*ts*AB/*ts*CD, and two hybrid genes, wtAB/*ts*CD and *ts*AB/wtCD. The latter PCR products were then cotransfected with *ts*B821 DNA and pCAPCAT in transient assays (Fig. 1, lanes 9 through 12). Results of these assays showed that the hybrid *ts*AB/wtCD gene, but not the *ts*AB/*ts*CD or wtAB/*ts*CD gene, rescued *ts*B821. Thus, the *ts* mutation was localized in the C-terminal half of *ie-1* (Fig. 1; compare lanes 11 and 12).

Sequencing the *ie-1* region of Ac*M*NPV and *ts*B821. The entire *Cla*I-to-*Hin*dIII fragments containing *ie-1* of both wt Ac*M*NPV and *ts*B821 were sequenced by using primers a to q (Fig. 2). Our sequence of wt *ie-1* was virtually identical to the sequence published by Chisholm and Henner (5); only a single nucleotide difference was observed at position 1483 from G to A (GGC to GAC), changing glycine 295 to aspartic acid. This difference was also observed in the *ts ie-1* sequence. Two differences between wt *ie-1* and *ts ie-1* were observed (Fig. 2, asterisks; Fig. 3). The first was a C-to-T change at nucleotide 1894, and the second was a G-to-A change at nucleotide 2133 (Fig. 3); both changes affect the IE-1 amino acid sequence.

To determine whether both or only one of these mutations was involved in the *ts* phenotype, a second set of SOE hybrid genes was constructed. Primers f and i (Fig. 2) were synthesized and used in constructing hybrid *ie-1*s containing neither, both, or either mutation. Primers a and i and primers f and d were used in the first SOE PCRs to produce four separate fragments: wtAI (2,088 bp), wtFD (1,091 bp), *ts*AI (2,088 bp), and *ts*FD (1,091 bp); the N- and C-terminal fragments overlap by 108 bases. These fragments were then mixed (wtAI/wtFD, *ts*AI/*ts*FD, wtAI/*ts*FD, and *ts*AI/wtFD) and subjected to a second set of SOE PCRs in the presence of primers a and d. The resulting genes, wtAI/wtFD, *ts*AI/*ts*FD, wtAI/*ts*FD, and *ts*AI/wtFD, were cotransfected with *ts*B821 and pCAPCAT, and CAT activity was assayed (Fig. 1, lanes 13 through 16). Lanes with *ie-1* containing *ts*AI portions (Fig. 1, lanes 14 and 16) exhibit a *ts* phenotype, whereas wtAI/*ts*FD shows a wt phenotype. Thus, only the C-to-T change at position 1894 (within the AI region) is responsible for the *ts* phenotype of *ts*B821. This C-to-T transition is predicted to change an alanine codon, GCT, to a valine codon, GTT, within the *ie-1* ORF.

Effects of *hr5* on *ts* IE-1-mediated expression from pCAPCAT. Because IE-1 is known to interact with *hr5*/protein complexes in vitro, we were interested in determining whether the *cis*-linked *hr5* in pCAPCAT influenced *cat* expression. We therefore compared expression from pCAPCAT and pCAPCAT-hr5, which lacks *hr5*. There is little, if any, effect of a *cis*-linked *hr5* on the ability of wt viral DNA or *ts*B821 in combination with wt *ie-1* to transactivate the *vp39* promoter of pCAPCAT (Fig. 4; compare lanes 2 to 9 and 4 to 11). As expected for this late promoter, wt or *ts ie-1* alone had no transactivating effect on pCAPCAT in this assay. The *ts*B821 DNA alone (lanes 3 and 10) or in combination with the *ts ie-1* (lanes 5 and 12) was unable to transactivate the *vp39* promoter at 48 h (we have not studied later times posttransfection).

FIG. 2. Nucleotide sequence and deduced amino acid sequence differences of a 3.2-kb *Cla*I-*Hin*dIII fragment containing *ie-1* of *ts*B821 compared with the sequences of wt Ac*M*NPV (L-1). The inserts of pBSIE1HC and pBStsIE1HC were sequenced by using 18 primers (a through q). The tails of the arrows are above the first nucleotide of each primer, and the arrowheads show the 5′-to-3′ direction. Six primers (a, b, c, d, f, and i) were also used in SOE PCRs. The two mutations found in the *ts*B821 *ie-1* sequence are indicated by asterisks at position 1894, a C-to-T change, and at position 2133, a G-to-A change. The difference in our wt *ie-1* sequence compared with the sequence of Chisholm and Henner (5) is indicated by a plus sign at nucleotide 1483.

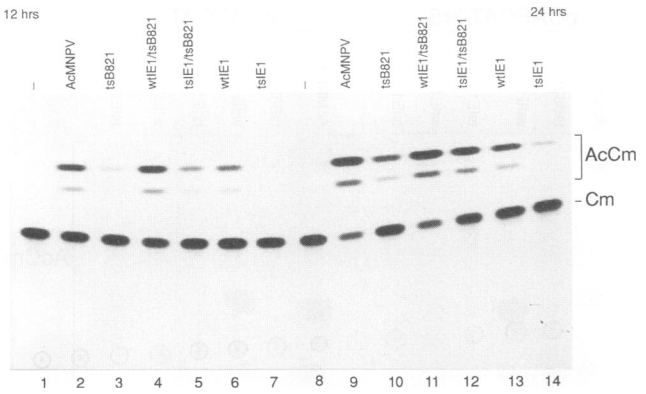


FIG. 5. Effect of *ie-1* on the early *etl* promoter. Reporter plasmid pETCAThr5 containing *cat* under the control of the *etl* promoter was cotransfected into SF-21 cells alone (lanes 1 and 8), with Ac*M*NPV DNA (lanes 2 and 9), with *ts*B821 DNA (lanes 3 and 10), with *ts*B821 plus pBSIE1HC containing wt *ie-1* (lanes 4 and 11), with *ts*B821 plus plasmid pBStsIE1HC containing *ts ie-1* (lanes 5 and 12), or with pBSIE1HC or pBStsIE1HC in the absence of viral DNA (lanes 6, 7, 13, and 14). Cells were maintained at 33°C, and CAT activity was determined by enzyme assays of cell lysates prepared at 12 h (lanes 1 to 7) and 24 h (lanes 8 to 14). The acetylated (AcCm) and unacetylated (Cm) [^{14}C]chloramphenicol were separated by thin-layer chromatography and visualized by autoradiography.

Thus, the *ts ie-1* effect on late gene expression is not being mediated through its interaction with the *hr*5 sequence in pCAPCAT. However, *ts* effects on homologous regions in the viral genome may affect early gene expression which, in turn, would affect late gene expression.

Effects of wt and *ts ie-1* on early *et1* promoter activity in transient expression assays. To determine whether the *ts*B821 *ie-1* exhibited a *ts* phenotype in transactivating a delayed-early gene promoter (one requiring *ie-1* in transient assays), we used the reporter plasmid pETCAThr5, which contains *cat* under the control of the *etl* promoter (9). This promoter was previously shown to be strongly dependent on *ie-1* and further enhanced by *ie-2* (34). At 12 h posttransfection, *cat* expression from the *etl* promoter is strongly dependent on wt *ie-1* (Fig. 5, lanes 2 through 7). When pBStsIE1HC, containing only *ts ie-1*, was cotransfected with the reporter plasmid (Fig. 5, lane 7), no *cat* expression was observed, whereas expression was observed when the equivalent plasmid containing wt *ie-1* was supplied (Fig. 5, lane 6). At 12 h posttransfection, low levels of CAT activity were observed when *ts*B821 genomic DNA was cotransfected with the reporter plasmid in the presence or absence of the *ts ie-1* plasmid (Fig. 5, lanes 3 and 5). Much higher CAT levels were observed with wt Ac*M*NPV DNA and *ts*B821 plus wt *ie-1* (lanes 2 and 4, respectively).

When assayed at 24 h (Fig. 5, lanes 8 through 14), the dependence of the *etl* promoter on wt *ie-1* is still apparent but much less dramatic when other viral genes are supplied with *ts ie-1*. Cotransfection of pETCAThr5 with *ts*B821 DNA alone or in the presence of *ts ie-1* plasmid DNA (Fig. 5, lane 10 or 12) produced significant levels of CAT. Addition of plasmid DNA containing only *ts ie-1* provided very low but detectable levels of CAT (lane 14) which were higher than the level observed with the reporter plasmid alone (lane 8). These results suggest that *ts ie-1* mutation is providing at least a low level of transactivation. Thus, *ts* IE-1 may be leaky. The difference in *cat* expression observed at 12 and 24 h for transfections with *ts*B821 DNA may reflect the time required to make enough active IE-1 to transactivate the *etl* promoter. However, the presence of other viral genes, in addition to *ts ie-1*, appears to alleviate the strong *trans* dependence of the *etl* promoter on wt *ie-1* (Fig. 5; compare lanes 10 and 12 with lane 14). This is likely due to a requirement for other gene products to transactivate the *ie-1* promoter in transient expression assays (see below).

Preliminary results indicate that *ie-2* (formerly *ie-n*) may be providing a substantial portion of this transactivation (36a). Although *ie-2* is known to transactivate the *etl* promoter (34), it does so only in the presence of *ie-1*. It is possible that the interaction of other proteins with *ts* IE-1 helps stabilize the protein, making it more thermally stable. Alternatively, other viral proteins may be transactivating the *ts ie-1* allele, allowing for the synthesis of enough partially active IE-1 to support expression. Also, as noted below, the *ie-1* promoter is at least partially dependent on wt *ie-1* for transactivation, and this may also affect the interpretation of these results.

Effects of wt *ie-1* and *ts ie-1* on other early promoters. To analyze the effects of *ie-1*s from Ac*M*NPV and *ts*B821 on other early promoters, including those of the immediate-early class that are reported to be active in the absence of *ie-1* function in transient expression assays, we cotransfected SF-21 cells with plasmids containing either wt or *ts ie-1* and the reporter plasmids phcIE1, phcIE0, and phcPE38, containing *cat* under the control of Ac*M*NPV *ie-1*, *ie-0*, and *pe-38* promoters, respectively.

Using the reporter phcIE-1, we found that expression from the *ie-1* promoter depended on wt *ie-1* when provided in the absence of other viral genes (Fig. 6, lanes 1, 6, and 7). However, intact *ts*B821 DNA also stimulated expression from phcIE-1 (Fig. 6, lanes 2 and 3). The lack of activity with *ts ie-1* alone was not due to a dominant negative effect because transactivation was also observed when this *ts ie-1*-containing plasmid was cotransfected with *ts*B821 DNA (lane 5). Thus, *ie-1* promoter activity was autotransactivated and may also be activated by other viral genes in the presence of a *ts ie-1* allele; the *ie-1* promoter is effectively a delayed-early promoter when assayed at 12 h in transient expression assays. Autoregulation by *ie-1* was also reported by Kovacs et al. (23), although the strength of the dependence that we observed is notable.

The *ie-0* promoter was also transactivated by wt *ie-1* but not by *ts ie-1* in the absence of other viral genes (Fig. 6A, lanes 8, 13 and 14). The *ie-0* promoter was more dependent on other viral genes in addition to *ie-1* for full expression than the *ie-1* promoter was (compare lanes 4 and 6 with lanes 11 and 13). Ac*M*NPV or *ts*B821 plus wt *ie-1* was able to transactivate *ie-0* strongly (lanes 9 and 11), whereas *ts*B821 DNA alone or *ts ie-1* plus *ts*B821 (lanes 10 and 12) exhibited only low levels of transactivation from phcIE0. Thus, wt *ie-1*, in conjunction with other viral genes (which may be transactivated by *ie-1*), provides full transactivation of the *ie-0* promoter.

Ac*M*NPV but not *ts*B821 DNA was able to transactivate *pe-38*-driven *cat* expression (Fig. 6B, lanes 1 to 3). CAT activity was observed when wt *ie-1* was present either alone (lane 6) or with *ts*B821 DNA (lane 4). The patterns of dependence of *pe-38* on *ie-1* and other viral functions appeared to be quite similar to that observed for the *etl* promoter (compare Fig. 5, lanes 1 through 7, and Fig. 6B).

Thus, for each of these three viral promoters, *ie-1* transactivated, at least to some extent, *cat* expression. A *ts*B821 phenotypic effect was observed for each of the promoters, but genomic DNA provided significant additional transactivating function which alleviated, in part, the *ts* effect. Each of the promoters, however, responded in a different fashion. In the case of the *ie-1* promoter, additional viral factors almost fully

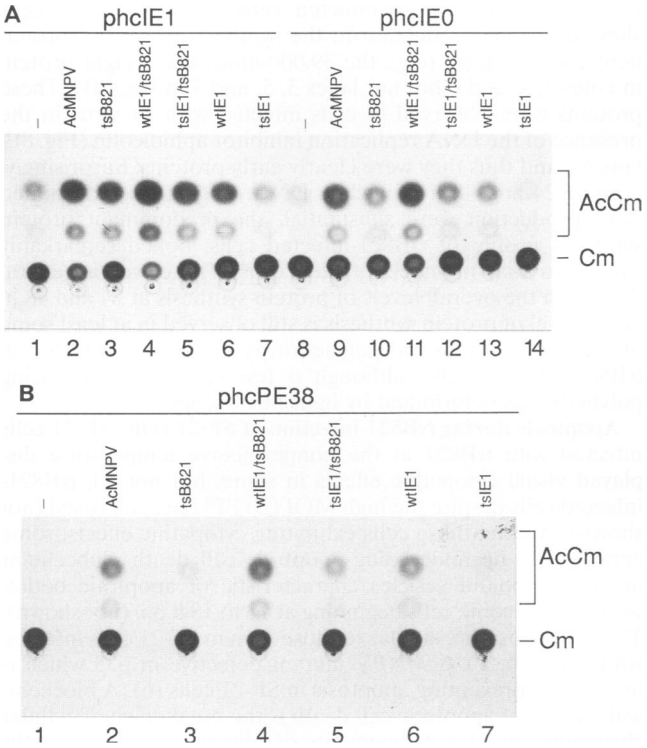


FIG. 6. Effects of wt *ie-1* and *ts ie-1* on *ie-1*, *ie-0*, and *pe-38* promoters in transient expression assays. SF-21 cells were cotransfected with DNA from wt Ac*M*NPV, *ts*B821, pBSIE1HC (wt *ie-1*), and pBStsIE1HC (*ts ie-1*) alone or in combinations indicated above the lanes and with one of the three reporter plasmids phcIE1 and phcIE0 (A) and phcPE38 (B). Cells were maintained at 33°C, and CAT activity in cell lysates was determined by enzyme assays at 12 h, using 1% of the cell lysates. The acetylated (AcCm) and unacetylated (Cm) [^{14}C]chloramphenicol were separated by thin-layer chromatography and visualized by autoradiography.

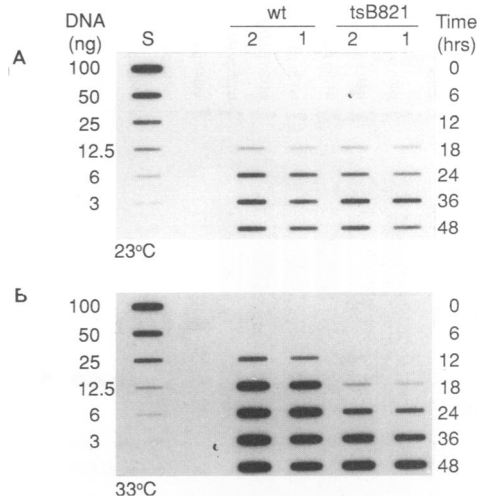


FIG. 7. Kinetics of viral DNA synthesis. SF-21 cells were infected with Ac*M*NPV or *ts*B821 at 23 and 33°C. At the indicated times p.i., the cells were lysed and slot blotted onto nylon membranes. The membranes were hybridized to radiolabeled Ac*M*NPV DNA. Lanes 1 and 2 for wt and *ts*B821 show one- and twofold the amount of infected cell lysate used per slot. Cells were maintained either at 23°C (A) or 33°C (B). Standard amounts of Ac*M*NPV DNA were applied to slots on the left.

compensated for the *ie-1* defect (Fig. 6A; compare lanes 3 and 7). In the case of *ie-0*, the additional factors only partially compensated for the *ts* defect. The *pe-38* promoter showed a strong dependence on wt *ie-1*, and the additional viral factors provided only moderate additional transactivation in this assay.

Rate of DNA synthesis in *ts*B821-infected cells. A previous study indicated that viral DNA replication is delayed in *ts*B821-infected cells (29). To confirm this observation, cells infected at 23 and 33°C with wt Ac*M*NPV or *ts*B821 were lysed at selected times p.i. DNA from the lysed cells was blotted in slots and hybridized to nick translated Ac*M*NPV DNA. At 23°C, the DNA synthesis patterns of wt and *ts* mutant virus were virtually identical (Fig. 7). At 33°C, *ts*B821 DNA synthesis was delayed by 6 to 12 h. By 48 h, however, *ts*B821 had replicated extensively (Fig. 7). Thus, *ie-1* is required for timely initiation of viral replication as previously reported (29). A delay in accumulation of early gene products necessary for viral DNA replication may be responsible for this effect.

Budded virus release. Although *ts*B821 does not produce plaques at 33°C, it was reported to produce budded virus in a delayed manner. Since these properties seemed inconsistent, we repeated the studies on budded virus production. SF-21 cells were infected with Ac*M*NPV or *ts*B821, and extracellular culture fluid was removed at selected times p.i. and titered at 23°C on SF-21 cells (Table 2). At 23°C, the rate of budded virus release from the wt- and *ts* mutant-infected cells were virtually identical. However, at 33°C, the release of budded virus from the *ts* mutant-infected cells was delayed approximately 12 h, consistent with the observed 6- to 12-h delay in DNA synthesis and the prior study. The final titers of *ts*B821 budded virus were consistently two- to threefold less than those observed for wt Ac*M*NPV at 33°C, but at 23°C, the titers observed for *ts*B821 were usually as high as those of wt Ac*M*NPV (data not shown).

Protein synthesis in cells infected with Ac*M*NPV and *ts*B821. The production of high titers of budded virus by *ts*B821 at late times seemed inconsistent with the previous observation that *ts*B821-infected cells expressed only a limited number of early genes through 24 h p.i. at 33°C (28). To reassess and extend these earlier results, we examined the pattern of protein synthesis in SF-21 cells infected with wt or *ts*B821 virus. At 23°C, the patterns of protein synthesis in wt- and *ts*B821-infected cells were similar, showing normal patterns of expression culminating in polyhedrin product by 48 h p.i. (Fig. 8A). (Lanes 7 and 9 of the *ts* mutant profiles were underloaded and not representative of other patterns obtained for this mutant at this temperature.) The patterns observed at 33°C, however,

TABLE 2. Rate of release of extracellular virus from SF-21 cells infected with wt Ac*M*NPV or *ts*B821

Time (h p.i.)	Virus titer (PFU/ml)			
	23°C		33°C	
	wt	*ts*B821	wt	*ts*B821
0	3×10^3	1×10^3	9×10^3	8×10^3
6	3×10^3	2×10^3	3×10^3	2×10^4
12	1×10^4	2×10^4	1×10^7	9×10^3
18	5×10^6	2×10^6	2×10^8	1×10^4
24	7×10^7	3×10^7	2×10^8	7×10^6
36	7×10^7	5×10^7	2×10^8	7×10^7
48	7×10^7	4×10^7	2×10^8	9×10^7

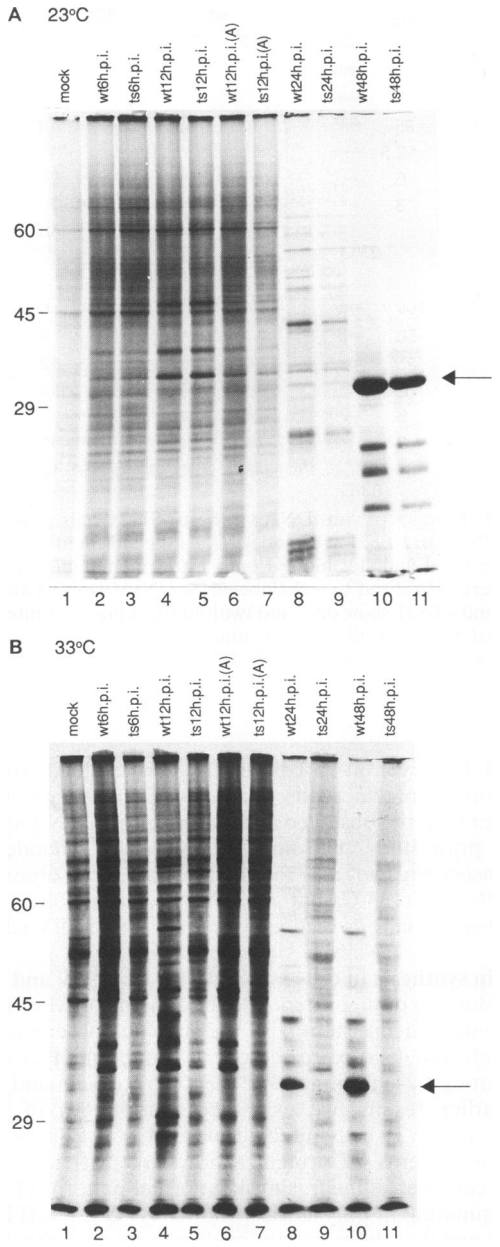


FIG. 8. Kinetics of protein expression in wt Ac*M*NPV- and *ts*B821-infected cells at 23°C (A) and 33°C (B). At selected times after infection, SF-21 cells were pulse-labeled with a mixture of [^{35}S]methionine and cysteine for 1 h before lysis. Times of lysis and virus used (wt or *ts*B821) are indicated above the lanes. The 12-h lanes indicated by (A) contain lysates from cell infected for 12 h in the presence of aphidicolin. Labeled proteins were visualized by fluorography. The sizes (in kilodaltons) of the protein standards are shown on the left. The position of the polyhedrin is indicated by an arrow on the right.

were strikingly different and confirmed previous the finding (29) that *ts*B821 appears to be blocked at an early stage of infection. For example, a prominent early 39,000-molecular-weight protein was observed in the 6-h wt lane and 12-h aphidicolin-treated wt lane but not in the corresponding *ts* lanes. At 12 h, *ts*B821-infected cells still displayed a pattern which was remarkably similar to that of mock-infected cells with the exception of a few new proteins barely visible in the gel. Compared with wt-infected cells, *ts*B821-infected cells showed a strong reduction in the synthesis of several prominent early proteins (e.g., the 39,000-molecular-weight protein in lanes 2, 4, and 6 but not lanes 3, 5, and 7 in Fig. 8B). These proteins were observed in cells infected with wt virus in the presence of the DNA replication inhibitor aphidicolin (Fig. 8B, lane 6), and thus they were clearly early proteins. Surprisingly, even at 24 and 48 h p.i., when DNA replication and budded virus production were substantial, the predominant protein synthesis profile of *ts*B821-infected cells looked remarkably similar to that of mock-infected cells. There was a general decline in the overall levels of protein synthesis at 24 and 48 h. A low level of protein synthesis is still observed in at least some of the cells. Little or no polyhedrin synthesis was detected in *ts*B821-infected cells, although a few cells were producing polyhedra, as determined by light microscopy.

Apoptosis during *ts*B821 infection of SF-21 cells. SF-21 cells infected with *ts*B821 at the nonpermissive temperature displayed visual cytopathic effects in some, but not all, *ts*B821-infected cells despite the high MOI (20 PFU per cell) used (not shown). Among those cells exhibiting cytopathic effects, some appeared to be undergoing apoptotic cell death. Subcellular membrane-bound vesicles characteristic of apoptotic bodies were seen in some cells beginning at 12 to 18 h p.i. (not shown). These vesicles were similar to those seen in SF-21 cells infected with vAcAnh, an Ac*M*NPV mutant defective in p35 which is involved in preventing apoptosis in SF-21 cells (6). A biochemical feature of apoptotic cell death is the breakdown of cellular chromatin into DNA fragments of oligonucleosomal lengths (6, 45). To confirm that some cells were undergoing apoptosis during infection with *ts*B821, we determined the size of DNA isolated from infected cells at various times p.i. at 33°C (Fig. 9). By 24 h p.i., fragmentation of DNA into an oligosomal ladder occurred in cells infected with either vAcAnh or *ts*B821 but not wt Ac*M*NPV (Fig. 9). Fragmentation was delayed in *ts*B821-infected cells compared with vAcAnh-infected cells, which showed fragmentation as early as 12 h p.i. (compare lanes 2 to 13). Furthermore, fragmentation of *ts*B821-infected cellular DNA was not as complete as in vAcAnh-infected cells, as judged by the amount of high-molecular-weight DNA remaining at the top of the lanes. As expected, DNA from cells infected with wt Ac*M*NPV remained in high-molecular-weight form for most of the course of infection. By 48 h p.i., some DNA degradation was seen in wt virus-infected cells.

DISCUSSION

We have mapped the mutation responsible for the *ts* phenotype of *ts*B821 to a single nucleotide difference in *ie-1* that changes Ala-432 to valine within a 14-residue stretch which is conserved in IE-1s (19, 40). Another mutation was also found in *ts*B821 *ie-1*; nucleotide 2133 substituted Asp-512 with an asparagine but did not confer a *ts* phenotype on *ie-1*. Because *ts*B821 had a normal phenotype at the permissive temperature, it is unlikely that this mutation or any other second-site mutation had any additional significant effect(s) on the *ts*B821 phenotype.

Consistent with previous results (26), we observed that *ts*B821 DNA replication and budded virus formation were delayed at 33°C by approximately 12 h. High titers of budded virus were produced by 48 h p.i. but were consistently less than one-half that of wt virus. Budded virus production does not appear to correlate with the lack of *ts*B821 plaque formation at 33°C. One explanation is that all time course experiments (i.e., kinetics of budded virus, DNA, and protein synthesis) were performed at relatively high MOIs (5 to 20 PFU per cell) to

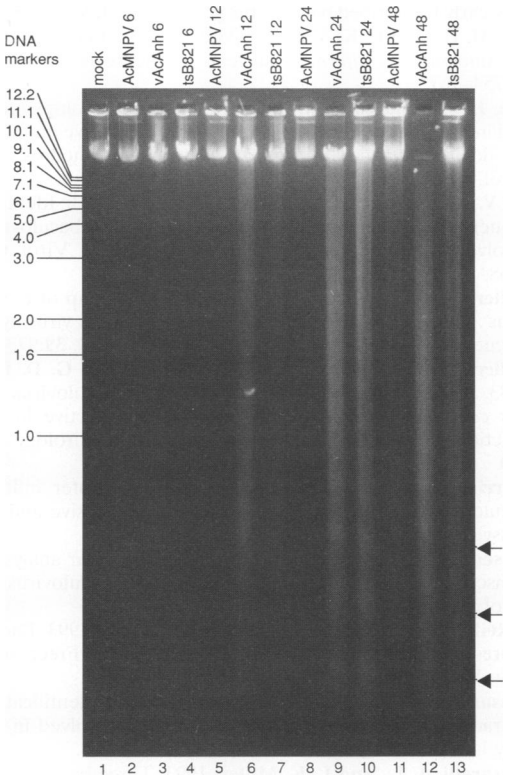


FIG. 9. Apoptosis during infection of SF-21 cells. Monolayers of SF-21 cells (2×10^6 cells per 60-mm-diameter dish) were mock infected or infected with Ac*M*NPV, vAcAnh, or *ts*B821, as indicated above each lane. At specific times p.i., the cells were lysed and the size of the DNA was analyzed by electrophoresis of the lysates in a 1.2% agarose gel. DNA was visualized by ethidium bromide staining. The time (hours) after infection and virus used for infection are indicated above each lane. Positions of the DNA markers are shown on the left in kilobase pairs. Oligonucleosome-size DNA fragments are indicated by arrows on the right.

achieve synchronous infection, whereas plaque assays were performed with dilute virus stocks. If the mutation is leaky and/or if IE-1 enters cells as a component of the virus particle, budded virus formation might occur under high-MOI but not plaque assay conditions.

Protein synthesis profiles of *ts*B821-infected cells were similar to those of mock-infected cells through 12 h p.i., and only minimal evidence of late gene expression was observed even at 48 h p.i., when DNA replication and budded virus release have reached almost wt levels. Thus, the protein profiles may not accurately reflect the status of all cells in the culture. While some *ts*B821-infected cells appeared to be uninfected despite the high MOI, other cells appeared to be fully infected and other cells were undergoing apoptosis. Chromatin degradation data confirmed that apoptosis was occurring in some, but not all, *ts*B821-infected cells. Infected cells undergoing apoptosis shut off all protein synthesis (7), and such cells would not be represented in the protein synthesis patterns. This possibility influences the interpretation of these patterns with regard to *ie-1* influence on the infection process. The varying response of cells to *ts*B821 infection may be due to a variety of factors. For example, the virus may have a greater requirement for IE-1 (or the products of other genes such as *p35* which are transactivated by IE-1) at some points in the cell cycle than at others.

In the absence of other viral genes, transient expression from the early *etl* promoter was strongly dependent on wt *ie-1* function at both 12 h and 24 h. In the presence of other viral genes, dependence of the *etl* promoter on wt *ie-1* function was clearer at 12 h than 24 h. We have not previously found a gene which can replace *ie-1* in transient expression assays using the *etl* promoter (34) and favor the hypothesis that the *ts* IE-1 may be partially active and/or stabilized by interacting with other viral proteins. In the presence of cycloheximide, *etl* is transcribed efficiently during virus infection (9). Preliminary Northern (RNA) blot analyses indicate that *etl* transcription is delayed in *ts*B821-infected cells (36a). Either enough IE-1 is produced in the presence of cycloheximide or a sufficient amount of IE-1 enters infected cells as a component of the virus particle to achieve activation. Our work has not distinguished between these possibilities because incoming IE-1 could be *ts* at 33°C. Thus, *ie-1* is important for timely early gene expression, DNA replication, and productive infection, but there is no definitive evidence for a strict cascade of immediate-early and delayed-early gene transcription. Because the distinction between immediate-early and delayed-early genes is unclear and could be misleading, we have refrained from using these terms except when referring to previously named genes.

The *ie-1* and *ie-0* promoters are transactivated by wt *ie-1* and/or other viral genes, and this influences the interpretation of results with other promoters and could accentuate the leaky behavior of *ts ie-1*. The strong dependence of the *ie-0* promoter on *ie-1* was surprising in view of prior studies, indicating that *ie-1* downregulates this promoter (21). The *ie-0* promoter in phcIE0 consisted of 575 bp (from +36 to −539 relative to the transcriptional start site), similar to that used by Kovacs et al. (21), but was placed in a different context which included *cis*-linked *hr*5 sequences. The effect of context on *ie-0* promoter activity was not tested, but in our analysis, the *ie-0* promoter was transactivated by wt *ie-1* and further transactivated by other viral genes.

Whether the *ts* mutation affects the ability of IE-1 to bind DNA or other proteins has not been determined but will be of future interest. Our study raises many new questions regarding the function of IE-1, but the *ts*B821 mutation will provide a useful tool with which to further define *ie-1* functionally and genetically.

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