Herpes simplex virus type 1 origin-dependent DNA replication in insect cells using recombinant baculoviruses

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The minimal set of seven herpes simplex virus type 1 (HSV-1) genes required for viral origin-dependent DNA synthesis was previously identified using a transient replication assay in a mammalian cell line permissive for HSV-1 growth. We have constructed recombinant baculoviruses which efficiently express the products of each of these seven genes in infected Spodoptera frugiperda (Sf) insect cells. When Sf cells were transfected with a plasmid containing a functional HSV-1 origin of replication, and subsequently superinfected with a mixture of these seven viruses, the input plasmid was amplified. This amplification exhibited properties characteristic of genuine HSV-1 DNA replication: all seven HSV-1 replication gene products were required, replicated DNA was detected as concatemers, and mutated origins were impaired to similar extents in insect cells and cells permissive for HSV-1 replication. These results demonstrate that the HSV-1 proteins expressed in Sf cells are fully competent for viral DNA synthesis, and indicate that any host function essential in mammalian cells must also be present in the infected insect cells. This system also provides a convenient method by which mutated replication proteins can be screened for function and produced in amounts sufficient for biochemical studies. Using this approach we show that the ability of the UL9 protein to bind to the viral origins of replication is not sufficient for it to facilitate DNA synthesis.

Introduction

The herpes simplex virus type 1 (HSV-1) genome is a linear double-stranded DNA of approximately 152 kbp (McGeoch et al., 1988a). Viral origins of DNA replication are specified by two related sequences. One (oriL) lies close to the centre of the long unique (UL) region, while the other (oriR) is present, in two copies, in the inverted repeats TR5 and IR8 (Spaete & Frenkel, 1982; Mocarski & Roizman, 1982; Stow & McMonagle, 1983; Weller et al., 1985). To date, no cell-free system capable of carrying out faithful origin-dependent HSV-1 DNA replication has been described. However, using a transient transfection assay in a cell line permissive for HSV-1 growth, Wu et al. (1988a) identified a set of seven HSV-1 genes which together encode all the viral proteins required for origin-dependent DNA synthesis. This finding has stimulated many recent investigations of the functions of the encoded proteins, and roles in replication have now been assigned to all seven products (reviewed by Challberg & Kelly, 1989; Weller, 1991). The viral DNA polymerase is a heterodimer consisting of catalytic and accessory subunits encoded by genes UL30 and UL42, respectively (Powell & Purifoy, 1977; Vaughan et al., 1985; Gallo et al., 1989; Crute & Lehman, 1989; Gottlieb et al., 1990). An ssDNA-binding protein is encoded by gene UL29 (Weller et al., 1983; Quinn & McGeoch, 1985; Olivo et al., 1989), and a sequence-specific origin-binding protein by gene UL9 (Olivo et al., 1988; Weir et al., 1989). The remaining three proteins, encoded by genes UL5, UL8 and UL52, form a complex which exhibits DNA helicase and DNA primase activities (Dodson et al., 1989; Crute et al., 1989; Calder & Stow, 1990). It is not known whether additional functions essential for HSV-1 DNA synthesis are provided by the host cell.

Recent studies of the biochemical properties of the HSV-1 replication proteins have been greatly facilitated by the use of expression vectors, in particular the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV). Indeed, six of the replication proteins have already been expressed in a functional form in Spodoptera frugiperda (Sf) insect cells infected with appropriate (AcNPV) recombinants (Olivo et al., 1988; Dodson et al., 1989; Gottlieb et al., 1990; Marcy et al., 1990; Calder & Stow, 1990). Because many future efforts are aimed at utilizing these heterologously expressed proteins in cell-free systems, it is important that they should be able to perform all the replicative functions normally carried out by their counterparts during a lytic infection of permissive cells, and not merely those activities for which convenient assays are presently available. The experiments presented in this paper were therefore designed to determine whether HSV-1 origin-
dependent DNA synthesis could be reconstituted on Sf cells expressing all seven HSV-1 DNA replication proteins. The results indicate that such synthesis can occur and that it exhibits the important features of HSV-1 DNA replication in permissive mammalian cells.

Methods

Isolation of recombinant baculoviruses. Recombinant viruses AcUL5, AcUL8 and AcUL52, containing the UL5, UL8 and UL52 genes, respectively, have been described previously (Calder & Stow, 1990). The recombinant virus AcUL9 (containing the UL9 gene) was similarly constructed except that a fragment of HSV-1 DNA containing the UL9 gene (nucleotides 20670 to 23542; McGeoch et al., 1988a) was first cloned as an XhoI fragment using linker oligonucleotides, and subsequently excised, filled-in and ligated into the filled-in BamHI site of pAcYM1 (Matsuura et al., 1987). Recombinant AcUL9CT is identical to AcUL9 except that an internal in-frame deletion (nucleotides 21655 to 23226) was introduced between BamHI and BsrYI sites within the UL9 coding region of the pAcYM1 derivative. This virus specifies a UL9 protein consisting of the N-terminal 10 and C-terminal 317 amino acids of the intact protein (835 amino acids).

A fragment containing the UL42 gene (nucleotides 92885 to 95299) was cloned using BamHI linkers into the recombinant vector pAcYM1. The UL29 and UL50 gene fragments (nucleotides 57748 to 62173 and 62759 to 67261, respectively) were cloned using XbaI linkers into a derivative of pAcYM1 in which the BamHI cloning site had been converted to an XbaI site. The HSV-1 genes in the three plasmids described above were introduced into the AcNPV genome by recombination with viral DNA which had been linearized downstream of the polyhedrin gene promoter, as described by Kitts et al. (1990). Appropriate linear molecules were produced by cleaving DNA of the parental virus AcRP23lacZ (Possee & Howard, 1987) at the unique Bsu36I site within the inserted lacZ gene. In each instance greater than 20% of the progeny of cotransfected cells was recombinant virus containing an inserted HSV-1 gene. Recombinants AcUL29, AcUL30 and AcUL42, containing the UL29, UL30 and UL42 genes, respectively, were obtained by plaque purification.

Sf cells (strain IPLB-SF-21: Kitts et al., 1990) were maintained in TC100 medium (Life Technologies) containing 5% (v/v) foetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Preparation and titration of virus stocks were carried out as described by Brown & Faulkner (1977) and Matsuura et al. (1987).

Assay for HSV-1 ori₅ activity in Sf cells. Plasmid pST19 contains a 100 bp fragment specifying a functional HSV-1 ori₅ origin replication origin inserted into the vector pTZ19U. The derivatives pST19ΔII, pST19ΔIII and pST19ΔIIII contain 11 bp deletions inactivating UL9 binding sites I and II or removing a closely related sequence, motif III, respectively. Plasmid pST19pmI contains a point mutation which inactivates binding site I. These plasmids have been described previously (Weir & Stow, 1990).

Monolayers of Sf cells in 35 mm Petri dishes (1.4 × 10⁶ cells/dish) were exposed to 0.4 μg plasmid DNA using a liposome-mediated transfection procedure. Liposomes were prepared from dimethyl dioctadecylammonium bromide (DDAB) and dioleoyl phosphatidyl ethanolamine (DOPE) as described by Felgner et al. (1987) and Rose et al. (1991). A solution of 10 mg DDAB and 10 mg DOPE in 10 ml chloroform was evaporated to dryness under vacuum, the lipids were resuspended in 10 ml sterile H₂O and sonicated to clarity using a Dawe Soniprobe at approximately 100 W.

Optimem 1 medium (Life Technologies) was mixed with 265 μl concentrated HCl per 100 ml to bring the pH to approximately 5.8 prior to use. A mix of 1 ml Optimem 1, 15 μl liposomes and 0.4 μg plasmid DNA was kept for 10 min at room temperature and then added to a monolayer of Sf cells which had been washed once with Optimem 1 and then drained. The cells were incubated 4 h at 28 °C, the Optimem mix was removed and 200 μl of appropriately diluted virus was added for 1 h at room temperature. Finally, the inoculum was replaced with 2 ml Sf growth medium and incubation continued at 28 °C. For multiple infection an m.o.i. of 5 p.f.u./cell of each of the seven recombinant viruses was used. Control infections with parental viruses employed an m.o.i. of 35 p.f.u./cell.

Infected cells were harvested 50 h post infection (p.i.) and total cellular DNA was prepared as previously described (Stow et al., 1983). Samples of DNA corresponding to the yield from 1 × 10⁸ cells were cleaved with EcoRI plus DpnI and the resulting fragments separated by electrophoresis through a 1% agarose gel. The fragments were transferred to a Hybond-N membrane (Amersham) and hybridized to pTZ19U DNA which had been 3²P-labelled in vitro by nick translation.

Purification of UL9 proteins and assay for DNA binding and DNA helicase. High-salt nuclear extracts were prepared from approximately 3 × 10⁸ Sf cells infected with AcUL9 or AcUL9CT as described by Calder & Stow (1990) except that KCl replaced NaCl in buffer C. The extracts were diluted to a final KCl concentration of 150 mM with buffer A (Weir et al., 1989) and passed through a 0.5 ml bed volume DNA sequence-affinity column. The column matrix, prepared as described by Wu et al. (1988b), consisted of a double-stranded synthetic oligonucleotide containing the sequence of UL9 binding site I covalently linked to CNBr-activated Sepharose 4B. UL9 and UL9CT proteins were eluted at a 0.2 to 0.9 M-KCl gradient in buffer B. Both proteins eluted at approximately 0.5 M-KCl, and were estimated, by SDS-PAGE, to be at least 80% pure. Gel retardation and DNA helicase assays were performed on the peak fractions as described by Weir et al. (1989) and Calder & Stow (1990). The labelled probe fragment in the gel retardation assay contained a functional UL9 binding site I (Weir & Stow, 1990), and the helicase substrate consisted of a 45 base oligonucleotide annealed to M13mp18 single-stranded DNA so as to leave a 3′ unannealed tail of 23 bases (Calder & Stow, 1990). This substrate did not contain a specific UL9 binding site.

Results

Expression of HSV-1 replication proteins by recombinant baculoviruses

Sf cells were mock-infected or infected with 20 p.f.u./cell of wild-type (wt) AcNPV, AcRP23lacZ, AcUL5, AcUL8, AcUL9, AcUL9CT, AcUL29, AcUL30, AcUL42 or AcUL52. After incubation at 28 °C for 50 h the cells were washed twice with Tris-buffered saline solution and total cellular proteins were prepared by direct lysis in sample buffer (Marsden et al., 1978). Each lane of a 9% polyacrylamide gel was loaded with material recovered from 1 × 10⁶ cells, and after electrophoresis the gel was stained with Coomassie blue. Fig. 1 shows the proteins visualized. With the exception of the intact UL9 protein, which was synthesized in smaller amounts, the HSV-1 polypeptides were all expressed at high levels (comparable to or greater than polyhedrin protein by wt...
Expression of HSV-1 DNA replication genes

Fig. 1. Polypeptides synthesized by recombinant baculoviruses. Proteins synthesized in mock-infected Sf cells (M) or cells infected with AcUL5 (5), AcUL8 (8), AcUL9 (9), AcUL29 (29), AcUL30 (30), AcUL42 (42), AcUL52 (52), wt AcNPV (wt), AcRP23lacZ (Z) or AcUL9CT (9C) were separated by electrophoresis through a 9% polyacrylamide gel and detected by staining with Coomassie blue. The products of the inserted genes, and of polyhedrin protein synthesized in wt AcNPV-infected cells, are indicated by open circles.

AcNPV and ß-galactosidase by AcRP23lacZ). The products of the seven inserted HSV-1 genes exhibit gel mobilities close to those predicted from DNA sequence analysis (McGeoch et al., 1988a, b), and in a variety of experiments have been shown to comigrate with the corresponding products of wt HSV-1-infected baby hamster kidney cells (data not shown). Minor bands visible in Fig. 1 and specific to individual recombinant viruses (e.g. AcUL29, AcUL30 and AcUL52) probably represent degraded forms of the over-expressed proteins.

The seven HSV-1 replication proteins expressed in Sf cells activate replication of a plasmid containing HSV-1 ori₅

To determine whether the seven HSV-1 replication proteins over-expressed by the recombinant baculoviruses were competent for HSV DNA synthesis, Sf cells were transfected with plasmid pST19, which contains a functional copy of the ori₅ replication origin, and subsequently superinfected with a mixture of viruses expressing the seven proteins. DNA was prepared from the cells at various times p.i. and examined for the presence of replicated (DpnI-resistant) plasmid sequences. An m.o.i. of 5 p.f.u./cell of each virus was used for superinfection. Theoretically, 95% of the cell population should have received all seven viruses (Wardlaw, 1985).

The results are shown in Fig. 2. A band corresponding to replicated input plasmid DNA, which comigrated with EcoRI-cleaved pST19 DNA, was first detectable at 29 h p.i. and increased in amount until 70 h p.i. This band was not detected in the DNA from cells harvested

Fig. 2. Time course of plasmid pST19 amplification. Sf cells were transfected with plasmid pST19 and either mock-infected (MI) or infected with a mix of AcNPV recombinants. DNA prepared from cells harvested at the indicated times (h p.i.) was analysed following cleavage with EcoRI plus DpnI. The fragments were separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized to ³²P-labelled pTZ19U DNA. An autoradiograph of the washed filter is shown. The position of DpnI-resistant EcoRI-cleaved molecules is indicated by an arrowhead. The smaller fragments detected are DpnI cleavage products of unreplicated input plasmid DNA. The marker (M) is EcoRI-cleaved pST19 DNA. Longer autoradiographic exposure more clearly revealed the presence of small amounts of replicated pST19 DNA in the 29 h p.i. sample (not shown).
Fig. 3. Gene requirements for plasmid amplification. Sf cells were transfected with pST19 DNA and either mock-infected (MI) or infected with AcRP23lacZ (Z), the mix of AcNPV recombinants specifying all seven HSV-1 replication proteins (7R) or similar mixes from which one recombinant had in turn been omitted (−5, −8, etc. indicate mixes from which AcUL5, AcUL8, etc. had been omitted). DNA was prepared 50 h p.i. and analysed as described in the legend to Fig. 2. The markers (M) are 3.8 and 2.8 kbp, the smaller being 100 bp shorter than linearized pST19DNA. The position of replicated pST19 monomers is indicated by an arrowhead.

Fig. 4. Replication of plasmids containing mutations within oriS. Sf cells were transfected with the vector pTZ19U (v), parental plasmid pST19 (ST and Co), pST19delI (d1), pST19delII (d2), pST19delIII (d3) or pST19pmI (pm1) and either mock-infected (Co) or infected with the mix of AcNPV recombinants specifying all seven HSV-1 replication proteins. DNA was prepared 50 h p.i. and analysed as described in the legend to Fig. 2. The marker (M) is EcoRI-cleaved pST19 DNA and the expected position of replicated plasmid DNA molecules is indicated by an arrowhead.

DNA and the inserted lacZ gene of the virus. This fragment was not detected when wt AcNPV was used for control infections (e.g. see Fig. 8). A fragment corresponding to replicated pST19 molecules was not detected in mock-infected cells or in cells infected with AcRP23lacZ, but was present in large amounts in cells coinfected with viruses expressing all seven HSV-1 replication proteins. Omission of any one of the seven viruses resulted in a very low level of pST19 replication which was detectable only upon longer exposure of the autoradiograph. This result indicates that the plasmid replication observed in infected Sf cells resembles that seen in transfected Vero cells (Wu et al., 1988a) in that the presence of all seven HSV-1 replication proteins is required for efficient DNA synthesis.

A series of plasmids carrying mutated copies of HSV-1 oriS, which had previously been examined for activity in permissive tissue culture cells superinfected with wt HSV-1 (Weir & Stow, 1990), were tested for their ability to replicate in the presence of the seven baculovirus-expressed replication proteins. The results are presented in Fig. 4. No replication of input plasmid was detected in mock-infected cells which had received the unmutated

43 or 70 h after mock infection. Inspection of the ethidium bromide-stained gel revealed that the DNAs of the superinfecting viruses were replicated much earlier than pST19 DNA, the maximum yield of AcNPV DNA having been achieved by 29 h p.i. These results are consistent with the expected time course of AcNPV DNA synthesis and with replication of pST19 DNA being driven by products expressed from the late polyhedrin gene promoter rather than by baculovirus DNA replication proteins.

The requirements for specific HSV-1 gene products for amplification of pST19 were investigated. Replicate monolayers of Sf cells were transfected with pST19 DNA and either mock-infected, infected with the parental virus AcRP23lacZ or with separate mixtures containing either all seven recombinants expressing HSV-1 replication proteins, or mixtures from which one virus in turn had been omitted. The analysis of DNA from these cells is shown in Fig. 3. The slowly migrating fragment detected in the cells infected with AcRP23lacZ results from similarity between lacZ sequences of the probe DNA and the inserted lacZ gene of the virus. This fragment was not detected when wt AcNPV was used for control infections (e.g. see Fig. 8). A fragment corresponding to replicated pST19 molecules was not detected in mock-infected cells or in cells infected with AcRP23lacZ, but was present in large amounts in cells coinfected with viruses expressing all seven HSV-1 replication proteins. Omission of any one of the seven viruses resulted in a very low level of pST19 replication which was detectable only upon longer exposure of the autoradiograph. This result indicates that the plasmid replication observed in infected Sf cells resembles that seen in transfected Vero cells (Wu et al., 1988a) in that the presence of all seven HSV-1 replication proteins is required for efficient DNA synthesis.

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Expression of HSV-1 DNA replication genes

of a related 11 bp sequence (motif III; plasmid pST19delIII) resulted in a less severe impairment. These results are qualitatively very similar to those obtained with the same plasmids in baby hamster kidney cells (Weir & Stow, 1990), and indicate that the presence of a functional HSV-1 oriS is necessary for the plasmid amplification mediated by HSV-1 replication proteins in Sf cells.

Structure of the products of replication of pST19 DNA

A sample of the DNA recovered from Sf cells transfected with pST19 DNA and superinfected with a mix of the seven recombinants expressing HSV-1 DNA replication proteins was cleaved to completion with DpnI and then treated for various times with EcoRI. The products were analysed following electrophoresis through an agarose gel. Fig. 5 shows that the products of replication are high Mr molecules which upon complete digestion with EcoRI yield fragments which comigrate with linearized molecules of the input plasmid. Partial digestion with EcoRI resulted in the production of a ladder of DNA fragments consistent with the presence of pST19 multimers. Replication of plasmid pST19 in the mixedly-infected Sf cells thus appears to proceed by a mechanism which closely resembles the situation previously reported for oriS-containing plasmids replicated in permissive cells in the presence of wt HSV-1 as helper (Stow, 1982).

Effect of input m.o.i. on pST19 replication

Sf cells were transfected with plasmid pST19 and superinfected with recombinant baculovirus mixes corresponding to various input multiplicities of each of the seven viruses. The replicated plasmid molecules were analysed as described above and the results are presented in Fig. 6. Replication of pST19 remained detectable even when each virus was present at an m.o.i. of only 0-16 p.f.u./cell, suggesting that at least a proportion of the cell population was expressing all seven replication proteins. Theoretical considerations (Wardlaw, 1985) indicate that at input multiplicities of 11, 5, 1-6, 0-5, 0-16 and 0-05 p.f.u./cell of each virus the percentage of cells receiving all seven viruses should be 99, 95, 20, 0-15, 1-5 \times 10^{-4} and 6-8 \times 10^{-8} respectively. It is apparent that the level of replication observed does not decrease as rapidly as would be predicted from these values. The most probable explanation for this is that titration of the virus stocks on monolayers of Sf cells underestimates the number of virus particles present which are capable of expressing the various replication proteins.
Fig. 6. Effect of input multiplicity on plasmid amplification. Sf cells were transfected with pST19 and either mock-infected (MI) or infected with mixes of the AcNPV recombinants expressing all seven HSV-1 DNA replication proteins in which each virus was present at the indicated m.o.i. (p.f.u./cell). DNA was prepared at 50 h p.i. and analysed as described in the legend to Fig. 2. The marker (M) is pST19 cleaved with EcoRI and the position of replicated input plasmid DNA is indicated by an arrowhead.

The presence of the origin-binding domain is not sufficient for the UL9 protein to function in viral DNA synthesis

We previously demonstrated that sequence-specific origin-binding activity resided in the C-terminal 317 amino acids of the UL9 protein (Weir et al., 1989). A deleted form of the UL9 protein (UL9CT) consisting of this domain linked to the N-terminal 10 amino acids was therefore expressed by a recombinant baculovirus (AcUL9CT; Fig. 1). The intact UL9 and UL9CT polypeptides were purified from infected Sf cells by sequence-specific DNA affinity chromatography and examined for their origin-binding and DNA helicase activities. The results are shown in Fig. 7. As previously demonstrated, both the intact and truncated proteins were able to bind to a labelled oligonucleotide containing the binding site I recognition sequence and cause its retardation upon electrophoresis through a polyacrylamide gel (Fig. 7a). Intact UL9 protein also contains an intrinsic DNA helicase activity, as demonstrated by displacement of an annealed oligonucleotide from a single-stranded circular DNA molecule (Bruckner et al., 1991). Incubation of a substrate of this type with the intact UL9 protein, but not the C-terminal fragment, resulted in displacement of the labelled 45 base oligonucleotide (Fig. 7b). This result indicates that the region of the UL9 protein between amino acids 11 and 518 contains sequences essential for helicase activity, and is consistent with the presence within this region of a series of amino acid motifs characteristic of many helicases (Gorbalenya et al., 1989).

To determine whether the C-terminal domain of the UL9 protein is sufficient for HSV-1 DNA replication, experiments were performed in which AcUL9CT was substituted for AcUL9 in the mix of seven recombinant baculoviruses used to infect Sf cells transfected with plasmid pST19. Fig. 8 shows that efficient replication occurred only when the intact UL9 protein was present. Moreover, when AcUL9CT was added at 5 p.f.u./cell together with the mix of seven viruses required for efficient plasmid replication, a marked reduction in plasmid amplification occurred. These data therefore indicate that origin-binding activity is not sufficient to allow the UL9 protein to function in viral DNA synthesis, and suggest that the presence of the C-terminal
Expression of HSV-1 DNA replication genes

Fig. 8. Replicative ability of the UL9 and UL9CT proteins. Sf cells were transfected with pST19 and infected as follows: wt, 35 p.f.u./cell of wt AcNPV; all7, 5 p.f.u./cell of each of the seven recombinants expressing HSV-1 DNA replication proteins; -UL9, a similar mix from which AcUL9 had been omitted. AcUL9CT (5 p.f.u./cell) was also present (+) or absent (-). DNA was prepared 50 h p.i. and analysed as described in the legend to Fig. 2. The marker is EcoRI-cleaved pST19 DNA and the expected position of replicated plasmid DNA molecules is indicated by an arrowhead.

Discussion

The knowledge that only seven HSV-1-encoded proteins are required for replication of the viral genome (Wu et al., 1988a; Heilbronn & zur Hausen, 1989), and the relatively low abundance of most of these in virus-infected cells, has stimulated the use of expression systems to facilitate purification of amounts sufficient for biochemical studies. A major hope is that these reagents will allow the development of a cell-free system for HSV-1 origin-dependent DNA synthesis. One of the most successful systems employed has been expression under the control of the late polyhedrin promoter of the insect baculovirus, AcNPV. Successful expression of enzymatically active HSV-1 helicase–primase (UL5, UL8 and UL52 proteins), DNA polymerase catalytic and accessory subunits (UL30 and UL42 proteins), and origin-binding protein (UL9 protein) have been reported (Olivo et al., 1988; Dodson et al., 1989; Gottlieb et al., 1990; Marcy et al., 1990; Calder & Stow, 1990). Although the baculovirus-expressed proteins exhibit the same enzyme activities as the corresponding proteins purified from HSV-1-infected cells, it is difficult to be certain that they have the potential to carry out all the functions necessary for replication of a double-stranded DNA molecule containing a viral replication origin. It can be envisaged that certain activities (e.g. the ability to interact with other components of a replication complex) might be lacking due to differences in post-translational processing in infected Sf cells, or to the introduction of mutations during the various cloning procedures. In the case of the UL8 protein [which is not required for the enzymatic activities of the helicase–primase complex (Calder & Stow, 1990; Dodson & Lehman, 1991)] and the UL29 protein (major ssDNA-binding protein), the current in vitro assays probably assess only a limited aspect of the in vivo function.

This paper reports the efficient expression of all seven HSV-1 replication proteins in recombinant baculoviruses, and the demonstration that they are competent for replication of a plasmid containing the viral ori5. HSV-1 origin-dependent DNA replication was achieved in transfected Sf cells by superinfection with a mixture of recombinants capable of expressing the seven replication proteins. The observed synthesis closely resembled viral origin-dependent replication in permissive mammalian cells since (i) the same seven HSV-1-encoded replicative proteins were both necessary and sufficient, (ii) mutated copies of ori5 were impaired to similar extents in the two cells types and (iii) the products of replication were high Mr concatemers. These results indicate that baculovirus proteins do not efficiently substitute for any of the HSV-1 DNA replication proteins and additionally imply that if mammalian cell factors are necessary for HSV-1 DNA replication their functions must also be present in the infected insect cells (specified by either the Sf cells or baculovirus vector). Preparations containing the seven baculovirus-expressed proteins, perhaps supplemented with mammalian or Sf cell extracts, should therefore provide an appropriate starting point for attempts to develop a cell-free system for origin-dependent HSV-1 DNA synthesis.

Three of the recombinant viruses used in this study (AcUL29, AcUL30 and AcUL42) were generated using a method (Kitts et al., 1990) which reproducibly yields a high proportion of recombinant viruses. In contrast to our previous serial enrichment approach (Calder & Stow, 1990), purified recombinant viruses were obtained rapidly and only small numbers of plaques needed to be analysed. This method of isolating recombinants should greatly simplify the construction of a library of viruses containing specific mutations within an inserted gene.
The development of the Sf cell replication assay enables mutated proteins to be screened for replicative ability and produced in amounts sufficient for biochemical assay. As an example of this approach, experiments with a virus expressing a deleted version of the UL9 protein (Fig. 7 and 8) have demonstrated that specific origin-binding activity of the UL9 protein is not sufficient to allow DNA replication. The importance of sequences in the N-terminal two-thirds of the protein for DNA synthesis is consistent with the mapping of mutations conferring temperature sensitivity (to tsS) and host-range for growth (to hr156) within this region of the protein (E. C. Stow, unpublished results; Weller, 1991). In accordance with the presence of characteristic helicase motifs within the portion of the UL9 protein missing from UL9CT (Gorbalenya et al., 1989), the truncated polypeptide did not exhibit detectable helicase activity. It will be of interest to determine whether the HSV-1 mutants described above are also defective in UL9 helicase activity. Although not being competent for viral DNA replication, the UL9CT protein nevertheless exerted an inhibitory effect on synthesis mediated by the full complement of replication proteins. A possible explanation for this phenomenon is that the C-terminal fragment competes with the intact protein for binding orfs, although further experiments are necessary to verify this hypothesis.

Expression of the HSV-1 replication proteins by the recombinant viruses was in each instance under the control of the efficient polyhedrin promoter, and it is quite probable that the relative amounts synthesized are far from optimal. Although only limited variation in these amounts is likely to be possible with the present viruses, the use of different promoters might allow greater modulation and an increased efficiency of plasmid replication. This might prove a particular asset in studies of interactions between the replication proteins and of intermediates generated during plasmid amplification.

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References


Expression of HSV-1 DNA replication genes


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