On the cellular localization of the components of the herpes simplex virus type 1 helicase–primase complex and the viral origin-binding protein

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We constructed recombinant viruses based on the herpes simplex virus type 1 mutant tsK which individually were able to express the products of four viral DNA replication genes (UL5, UL8, UL9 and UL52) in the absence of any of the other proteins required for viral DNA synthesis. These viruses were used in immunofluorescence experiments to investigate the cellular localization of the four replication proteins expressed. The results demonstrated that all three components of the viral helicase–primase complex (UL5, UL8 and UL52 proteins) must be co-expressed to allow their efficient localization to the nucleus. Since the UL5 and UL52 proteins together form a complex which is enzymatically indistinguishable from a complex formed from all three proteins, a possible role of the UL8 protein may be in facilitating nuclear uptake. The UL9 protein (origin-binding protein) efficiently entered the cell nucleus when expressed alone. Both UL9 protein and the tripartite helicase–primase complex exhibited patterns of fluorescence which resembled the ‘pre-replicative sites’ described previously.

Introduction

Herpes simplex virus type 1 (HSV-1) genes UL5, UL8, UL9, UL29, UL30, UL42 and UL52 encode a set of seven proteins which have essential roles in viral DNA replication. Each of these genes identifies a complementation group, the members of which exhibit defects in viral DNA synthesis (reviewed by Challberg & Kelly, 1989; Weller, 1991). Moreover, in a transient assay in transfected tissue culture cells the seven genes are both necessary and sufficient for viral origin-dependent DNA replication (Wu et al., 1988; Heilbronn & zur Hausen, 1989).

Genes UL30 and UL42 encode the catalytic and accessory subunits of the viral DNA polymerase (Gallo et al., 1989; Hernandez & Lehman, 1990; Gottlieb et al., 1990), an ssDNA-binding protein is specified by the UL29 gene (Weller et al., 1983; Quinn & McGeoch, 1985), and the product of the UL9 gene binds to specific DNA sequences within the viral origins of replication (Olivo et al., 1988; Weir et al., 1989). The proteins encoded by genes UL5, UL8 and UL52 form a complex in HSV-1-infected cells which exhibits DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities (Crute et al., 1988, 1989).

The UL5, UL8 and UL52 proteins have been expressed by recombinant baculoviruses and shown to assemble into a functional complex in triply infected insect cells (Dodson et al., 1989; Calder & Stow, 1990). Although no enzymatic activity has been attributed to any of the individual subunits of the helicase–primase complex, it has been shown that a sub-assembly of the UL5 and UL52 proteins formed in doubly infected insect cells exhibits each of the activities associated with the UL5–UL8–UL52 complex (Calder & Stow, 1990; Dodson & Lehman, 1991). Therefore, despite its well documented involvement in viral DNA synthesis (Wu et al., 1988; Carmichael and Weller, 1989), no role can yet be assigned to the UL8 protein.

We have previously constructed four recombinant viruses, tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52, in which additional copies of the UL5, UL8, UL9 or UL52 gene are inserted into the genome of the HSV-1 temperature-sensitive (ts) mutant tsK under the control of the immediate early (IE) gene 3 promoter (Weir et al., 1989). At the non-permissive temperature (NPT) tsK accumulates IE polypeptides but fails to induce the expression of early and late genes (Preston, 1979; Watson & Clements, 1980). Under these conditions the recombinant viruses overexpress the product of the inserted gene, but do not synthesize any of the other DNA replication proteins because these remain under their normal early gene control. In this paper we report the use of the recombinant viruses to investigate whether, in the absence of other DNA replication proteins, the UL5, UL8, UL9 and UL52 proteins localize
to the nucleus of mammalian tissue culture cells. The results suggest that the UL8 protein may play a role in facilitating efficient entry of the helicase–primase complex into the nucleus.

**Methods**

**Cells and viruses.** Baby hamster kidney 21 clone 13 (BHK) cells (Macpherson & Stoker, 1962) were grown in Eagle's medium supplemented with 10% tryptose phosphate broth and 10% newborn calf serum. The viruses used were the HSV-1 ts mutant tsK (Preston, 1979) and recombinant viruses tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52 (Weir et al., 1989). These viruses contain additional copies of early genes UL5, UL8, UL9 and UL52 inserted under the control of the IE gene 3 promoter within the thymidine kinase (TK) gene of tsK, and were constructed using an approach similar to that described by Hummel et al. (1986). Briefly, plasmid p23, a gift from Dr C. Preston, contains a 361 bp fragment (nucleotides 131759 to 131399; McGeoch et al., 1989a) specifying the IE gene 3 promoter, 5' end and upstream regulatory sequences inserted at nucleotide 47358 within the TK gene coding region of the HSV-1 BamHI fragment. The inserted fragment is oriented so that transcription from the IE gene 3 promoter is in the same direction as that of the TK gene, and therefore the TK gene mRNA processing signals are potentially available for any transcript expressed from the IE gene 3 promoter. Plasmid p23 contains a unique XhoI site immediately downstream of the IE3 promoter, into which were inserted, in the correct orientation, DNA fragments containing the complete UL5, UL8, UL9 and UL52 open reading frames (ORFs). The inserted fragments correspond to nucleotides 15166 to 12128, 20492 to 17855, 23542 to 20670 and 108969 to 112514 respectively. The recombinant plasmids were cotransfected into BHK cells with intact tsK DNA and incubated at the permissive temperature (31 °C) TK virus progeny containing additional copies of the UL5, UL8, UL9 and UL52 genes inserted into the TK locus were enriched by growth in the presence of 100 μg/ml bromodeoxycytidine and purified. The structures of the recombinant virus genomes were confirmed by restriction endonuclease digestion and Southern blotting using appropriate probes. Stocks of the parental virus, tsK, and the resulting recombinant viruses, tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52, were propagated in BHK cells at 31 °C.

**Preparation and analysis of [35S]methionine-labelled polypeptides.** BHK cells in the 15 mm diameter wells of multi-well plates were mock-infected or infected with 10 p.f.u./cell of tsK or the recombinant viruses and incubated at the NPT, 38.5 °C for 6 h. The medium was then replaced with 200 μl PBS containing 15 μCi [35S]methionine (Amersham; sp. act. > 800 Ci/mmol). Incubation was continued for a further 2-5 h at 38.5 °C. At the end of the labelling period the supernatant was removed and the cultures were lysed and harvested as described by Marsden et al. (1978). Samples were analysed by electrophoresis through an SDS–polyacrylamide gel containing 9% acrylamide cross-linked with 1:40 (w/w) N,N'-methylene-bis-acrylamide. Gel fixation, drying and autoradiography were as previously described (Marsden et al., 1978).

**Immunofluorescence.** Sparse monolayers of BHK cells (4 x 10^5 cells) in 35 mm plastic Petri dishes were infected with 10^7 p.f.u./dish of tsK or each recombinant virus as indicated, and incubated at 38.5 °C. The monolayers were then washed with 1:1 methanol:acetone mixture. The monolayers were washed further with PBS and allowed to become almost dry. A 50 μl drop of a 1:30 dilution (in PBS containing 1% foetal calf serum) of the primary antibody was applied to a marked region in the middle of the dish. The primary antisera were prepared in rabbits against synthetic decapetides from the C termini of the UL5, UL8, UL9 and UL52 proteins (Olivo et al., 1989), and were a kind gift from Dr M. D. Challberg. After 1 h at room temperature, the monolayers were washed four times with PBS and reacted similarly with a 1:80 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma). The cells were washed once with 10% sucrose, 0.5% NP40 in PBS and four times with PBS, and examined using a Leitz u.v. fluorescence microscope. Photographs were taken on Tri-X Pan 400 film with 25 or 40 x objective lenses. Exposure times and printing conditions were kept constant for all the samples within any one experiment.

**Results**

**Polypeptide synthesis in cells infected with tsK recombinant viruses**

Recombinant viruses in which additional copies of the HSV-1 replication genes UL5, UL8, UL9 and UL52 had been individually inserted under IE promoter control into the tsK genome were generated as described in Methods. At the NPT tsK overexpresses IE genes, but, with the exception of Vmw136, the product of 'pseudo-IE' gene UL39 (Wymer et al., 1989), does not synthesize any of the remaining early or late viral proteins (Preston, 1979). Since UL5, UL8, UL9 and UL52 are all early genes (Olivo et al., 1989), the copies inserted under IE promoter control, but not the normally regulated resident copies, should be expressed at the NPT.

The polypeptides synthesized at 38.5 °C in mock-infected BHK cells, or cells infected with tsK or the four recombinant viruses tsK/UL5, tsK/UL8, tsK/UL9 or tsK/UL52 were labelled and analysed as described in Methods. Fig. 1 demonstrates that in addition to the major viral polypeptides detected in tsK-infected cells (IE proteins Vmw175, Vmw110, Vmw68 and Vmw63 plus Vmw136), the recombinant viruses each produce a single novel species. Several observations confirmed these novel proteins to be the products of the inserted UL5, UL8, UL9 and UL52 ORFs. First, their apparent M, was approximated to the values of 98710, 79921, 94246 and 114416 predicted for the UL5, UL8, UL9 and UL52 proteins, respectively, from DNA sequence data (McGeoch et al., 1988b). Second, the proteins comigrated on gels with the products expressed from the same ORFs in baculovirus vectors (unpublished results). Third, they reacted with antisera raised against peptides predicted from the DNA sequence of the ORFs (immunofluorescence data presented in this paper, and unpublished results of immunoprecipitation experiments).
Fig. 1. Polypeptide synthesis in cells infected with the recombinant viruses. Polypeptides synthesized at the NPT in mock-infected (lane MI) BHK cells or cells infected with \( \text{tsK} \) (lane K), \( \text{tsK/UL5} \) (lane 5), \( \text{tsK/UL8} \) (lane 8), \( \text{tsK/UL9} \) (lane 9), \( \text{tsK/UL52} \) (lane 52) were labelled and analysed as described in Methods. The \( \text{tsK} \)-induced polypeptides (\( \text{Vmw175}, \text{Vmw136}, \text{Vmw110}, \text{Vmw68} \) and \( \text{Vmw63} \)) are indicated. Novel polypeptides synthesized by the recombinant viruses are marked with arrowheads.

**Immunofluorescence studies on the UL5, UL8 and UL52 proteins synthesized by recombinant viruses**

The ability of the \( \text{tsK} \) recombinant viruses to synthesize the UL5, UL8, UL9 and UL52 proteins at the NPT allowed examination of their cellular localization in a cell line permissive for HSV-1 replication when expressed in isolation from the other DNA replication proteins. The UL5, UL8 and UL52 proteins exist as a complex in HSV-1-infected cells, so we also performed experiments in which these proteins were expressed either individually or in all possible combinations, and examined the cellular location of the expressed proteins by immunofluorescence.

The results of single infections are shown in Fig. 2. Replicate sets of sparse monolayers of BHK cells were infected at the NPT with \( \text{tsK} \), \( \text{tsK/UL5} \), \( \text{tsK/UL8} \) or \( \text{tsK/UL52} \) and subsequently processed as described in Methods. One set was reacted with anti-UL5 antibody (a to d), a second set with anti-UL8 antibody (e to h) and a third with anti-UL52 antibody (i to l). Following incubation with FITC-conjugated goat anti-rabbit IgG the cells were examined by u. v. fluorescence microscopy. The anti-UL5 antibody gave weak background staining with the \( \text{tsK} \)-, \( \text{tsK/UL8} \)- and \( \text{tsK/UL52} \)-infected cells (a, c and d), but a much brighter cytoplasmic fluorescence was apparent in cells infected with recombinant \( \text{tsK/UL5} \) (b). Similarly, the anti-UL52 antibody reacted specifically with cells infected with \( \text{tsK/UL52} \), revealing bright cytoplasmic fluorescence (l). The pattern observed was more discrete than in (b), and distinct, predominantly perinuclear foci were apparent in the majority of cells. The result was less clear-cut with the anti-UL8 antibody, but nevertheless a slightly increased intensity of fluorescence was apparent in the \( \text{tsK/UL8} \) infection (g). The specificity of the reaction between the anti-UL8 antibody and \( \text{tsK/UL8} \)-infected cells is also demonstrated in Fig. 3 (f and g).

These results indicate that the anti-UL5, anti-UL8 and anti-UL52 antibodies are each capable of specifically detecting the corresponding gene product in cells infected with the \( \text{tsK} \) recombinant viruses. Furthermore, they demonstrate that the UL5, UL8 and UL52 proteins, when expressed individually in the absence of any other HSV-1 DNA replication protein, do not localize to the cell nucleus, the site of viral DNA synthesis (Rixon et al., 1983) and the location of these proteins during the normal course of HSV-1 infection (Olivo et al., 1989). In additional experiments, which serve as controls for the mixed infections described below, it was found that a two- or threefold increase in the virus inoculum did not significantly affect either the pattern or intensity of fluorescent staining (data not shown).

To investigate whether co-expression of the UL5, UL8 and UL52 proteins affected cellular localization, cells were infected with a mixture of constructs and examined similarly by immunofluorescence microscopy. The results are shown in Fig. 3. UL5 protein was predominantly cytoplasmic when expressed alone or in combination with UL8 protein (b and c). In the presence of UL8 protein a slightly increased proportion appeared to enter the nucleus (d), but only in the presence of both UL8 and UL52 proteins was efficient localization to the nucleus detected (e). Similarly, co-expression of all three proteins was necessary for efficient nuclear localization of the UL8 and UL52 proteins (j and o). The UL8 protein, however, appeared to exhibit a small increase in nuclear uptake in the presence of UL52 alone, and the UL52 protein exhibited a similar response in the presence of either UL5 or UL8 (i, m and n). These results indicate mutual dependence between the UL5, UL8 and UL52 proteins for fully efficient nuclear localization, suggesting that they may be transported into the nucleus as a complex. In cells co-expressing UL52 and either UL5 or...
UL8 there appeared to be a small enhancement of nuclear uptake of both proteins, although this may reflect the presence of protein on the surface of rather than inside the nucleus. The observation is nevertheless consistent with some form of interaction between these pairs of polypeptides in doubly infected cells.

**Cellular localization of the UL9 polypeptide**

Recombinant virus tsK/UL9 was used similarly to determine the cellular localization of the UL9 polypeptide when expressed in the absence of other HSV-1 DNA replication proteins. Sparse monolayers of BHK cells were infected at the NPT with tsK, tsK/UL9 or a combination of tsK/UL5, tsK/UL8 and tsK/UL52. Duplicate plates were stained with either anti-UL9 antibody or anti-UL52 antibody and examined by immunofluorescence. The results are shown in Fig. 4. The anti-UL9 antibody reacted efficiently with only the tsK/UL9-infected cells (b), demonstrating the specificity of the antibody for the UL9 polypeptide, and revealed specific localization of the UL9 protein within the nucleus. The cells exhibited a speckled staining pattern with distinct foci which was very similar to the pattern exhibited by UL52 protein in triply infected cells (f). This result indicates that the UL9 protein, in contrast to the UL5, UL8 and UL52 proteins expressed individually, efficiently enters the nucleus in the absence of any other viral DNA replication protein.

**Discussion**

We have used ts HSV-1 recombinant viruses to study the localization of four viral DNA replication proteins, UL5, UL8, UL9 and UL52, in a cell type permissive for virus growth. The parental virus, tsK, is very tightly blocked at the IE stage of infection (Preston, 1979; Watson &
Fig. 3. Immunofluorescence microscopy of cells expressing various combinations of the UL5, UL8 and UL52 proteins. Cells were infected with 10^7 p.f.u./dish of each of the following viruses and processed as described in Methods: tsK (a, f and k); tsK/UL5 (b); tsK/UL8 (g); tsK/UL52 (l); tsK/UL5 plus tsK/UL8 (c and h); tsK/UL5 plus tsK/UL52 (d and m); tsK/UL8 plus tsK/UL52 (i and n); tsK/UL5 plus tsK/UL8 plus tsK/UL52 (e, j and o). The antibodies used were anti-UL5 (a to e), anti-UL8 (f to j) and anti-UL52 (k to o). The cells were photographed using a 25× objective lens. The bar marker represents 50 μm.

Fig. 4. Immunofluorescence microscopy of BHK cells expressing the UL9 protein. Cells were infected as described in the legend to Fig. 3 with tsK (a and d), tsK/UL9 (b and e) or tsK/UL5 plus tsK/UL8 plus tsK/UL52 (c and f). The antibodies used were anti-UL9 (a to c) or anti-UL52 (d to f). The cells were photographed using a 40× objective lens. The bar marker represents 30 μm.
Clements, 1980), and the recombinants differ from it at the NPT only in that they also express the products of the inserted genes. The observation that HSV-1 IE proteins are dispensable for viral DNA synthesis in transfected cells (Heilbronn & zur Hausen, 1989) further suggests that the proteins expressed by tsK at the NPT are unlikely to have an important role in determining the localization of the viral DNA replication proteins. Our results show that of the UL5, UL8, UL9 and UL52 proteins, only UL9 expressed individually is able to enter the cell nucleus. Therefore, nuclear uptake of the UL9 protein appears to be independent of any of the other components of the viral DNA replication machinery, and it is quite likely that the UL9 polypeptide contains a specific nuclear localization sequence (reviewed by Silver, 1991).

In HSV-1-infected cells the UL5, UL8 and UL52 proteins interact to form the viral helicase–primase complex (Crute et al., 1989), and our results demonstrate that all three proteins of the complex must be present to allow efficient nuclear uptake of any one. The most probable explanation is that the three proteins interact in the cytoplasm to form a complex which presents a signal for nuclear localization that is not recognizable on any of the individual subunits. This could potentially be present on one of the subunits, but masked or in an inappropriate conformation until complex formation occurs. Alternatively, the signal recognized may be distributed over more than one subunit. The electrophoretic mobilities of the UL5, UL8 and UL52 proteins did not differ in singly and triply infected cells (data not shown), suggesting that post-translational polypeptide processing is unlikely to have a determining role in nuclear transport.

Recent experiments in which the UL5, UL8 and UL52 proteins were expressed in various combinations in insect cells using recombinant baculoviruses have demonstrated that the UL5 and UL52 proteins together can form a complex which exhibits all the enzymatic activities associated with the UL5–UL8–UL52 complex found in HSV-1-infected cells (Calder & Stow, 1990; Dodson & Lehman, 1991). Moreover, the UL5–UL8–UL52 and UL5–UL52 complexes purified from infected insect cells are virtually indistinguishable in their enzymatic properties (Dodson & Lehman, 1991). Experiments using the tsK recombinant viruses yielded results very similar to those reported previously for recombinant baculoviruses (Calder & Stow, 1990) in so far as formation of a complex with an associated DNA-dependent ATPase activity occurred in cells co-expressing either the UL5 and UL52 or the UL5, UL8 and UL52 proteins (J. Calder & N. Stow, unpublished observations).

Since the UL8 protein is essential for viral DNA synthesis (Wu et al., 1988; Carmichael & Weller, 1989), these data raise the question of its function. Our immunofluorescence results suggest that an important role of the UL8 protein may be to facilitate entry into the nucleus of the enzymatically active subunits (i.e. the UL5 and UL52 proteins). However, this seems rather unlikely to be its sole function, because nuclear targeting of proteins is achieved readily with signals consisting of short sequences relatively rich in basic amino acids (reviewed by Silver, 1991). The UL8 protein might also interact with other components of the replicative machinery (e.g. the origin-binding protein or the viral DNA polymerase) or modulate the helicase and primase activities on their natural template, the viral genome. It is interesting to note that although Epstein–Barr virus and human cytomegalovirus (human herpesviruses belonging to the gamma- and betaherpesvirus subfamilies, respectively) encode obvious homologues of the HSV-1 UL5 and UL52 proteins, they do not contain ORFs with significant homology to UL8 (McGeoch et al., 1988b; Chee et al., 1990). Whether the UL8 homologue has diverged to such an extent as to be unrecognizable in these viruses, or whether its function is performed by another viral protein (possibly including the UL5 and/or UL52 homologue) or a host factor is not known.

When expressed using the tsK recombinant viruses the UL9 protein and the UL5–UL8–UL52 complex exhibited similar nuclear staining patterns in which protein was detected in numerous small foci (Fig. 4). This pattern resembles the arrangement of 'pre-replicative' sites to which the UL29 product, the major DNA-binding protein, localizes when viral DNA synthesis is blocked by the addition of phosphonoacetic acid (Quinlan et al., 1984). When viral DNA synthesis is permitted, UL29 protein migrates to the larger replication compartments in which viral genome replication occurs (Rixon et al., 1983; Quinlan et al., 1984). Replicating host cell DNA and a number of host cell proteins have also been shown to relocate to the pre-replicative sites or replication compartments following infection with HSV-1 (de Bruyn Kops & Knipe, 1988; Wilcock & Lane, 1991). Moreover, UL29 protein expressed from a transfected plasmid exhibits the 'pre-replicative site' pattern (Quinlan & Knipe, 1985), suggesting that this protein may play an important role in organizing the viral replicative machinery and determining the sites at which viral genome replication will occur (reviewed by Knipe, 1989).

Further experiments are necessary to determine whether the sites to which the UL9 protein and the helicase–primase complex localize are the same, and whether they are functionally equivalent to the pre-replicative sites defined by the presence of UL29 protein. Although recent studies suggest that the presence of functional UL29 protein is important for specific
Localization of the UL42 protein and viral DNA polymerase (Goodrich et al., 1990; Bush et al., 1991), it is possible that other HSV-1 replication proteins may have an intrinsic ability to locate to appropriate sites. It may also be possible to extend the approaches used in this paper to elucidate the minimum requirement of viral proteins for the intranuclear redistribution of replicating host cell DNA and the host proteins described above.

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