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Asynchronous Expression of the Immediate-Early Protein of Herpesvirus Saimiri in Populations of Productively Infected Cells

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SUMMARY

The time course of virus replication in cultures of permissive cells infected with high multiplicities of herpesvirus saimiri (HVS), a gammaherpesvirus, is protracted relative to the replication of herpes simplex virus (HSV), an alphaherpesvirus, under similar conditions. The basis for this difference was investigated by quantitative immunofluorescence microscopy exploiting monoclonal antibodies specific to the HVS 52000 mol. wt. immediate-early polypeptide (IE 52K) and to delayed-early (DE 51K, DE 110K) and late (130K and capsid proteins) gene products to measure the timing of gene expression in individual cells of infected cultures. The timing of the transitions from IE to DE and from DE to late protein synthesis occurred at proportionately different intervals in the growth cycle of HVS, relative to that of HSV. In particular, the DE to late transition occurred relatively later in HVS infections. However, asynchrony in the events leading to the expression of the first class of virus proteins (IE 52K) was the main source of the extended course of HVS replication in populations of infected cells. This asynchrony was not modified significantly by infection at different stages of the host cell-cycle and was reduced, but not overcome, by very high applied multiplicities of infection. Double-antibody staining revealed a positive correlation between the accumulation of high concentrations of parental virus particles at perinuclear sites and early detection of HVS IE 52K gene expression. Both of these events remained sensitive to a microtubule poison (colcemid) for many hours after infection with HVS, whereas the rapid and synchronous expression of the IE 175K protein (ICP4) in HSV-1-infected cells was insensitive to post-infection exposures to this drug. We conclude that significant differences in early stages of virus entry and intracellular processing which precede immediate-early gene expression are largely responsible for differences between the replicative cycles of these representatives of gamma- and alphaherpesviruses in cultures of permissive cells.

INTRODUCTION

Herpesviruses have been divided into three subgroups, alpha-, beta- and gammaherpesviruses, based on differences in their biological properties and some aspects of their molecular biology (Honess & Watson, 1977; Roizman, 1982; Honess, 1984). Typical alphaherpesviruses, such as herpes simplex virus type 1 (HSV-1), have relatively broad in vitro host ranges and short cycles of virus replication in permissively infected cells. Typical betaherpesviruses, such as human cytomegalovirus, and gammaherpesviruses, such as herpesvirus saimiri (HVS), have more restricted host ranges and protracted cycles of virus growth in vitro.

As part of an investigation into the biological and molecular properties of HVS, we have previously described a number of characteristics of the replicative cycle of this virus in cultures of permissively infected cells. As with other herpesviruses, the synthesis of HVS-specified polypeptides proceeds in three main phases. The synthesis of a single major virus-specific

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immediate-early (IE) gene product (a nuclear phosphoprotein with an apparent mol. wt. of 52000, IE 52K) is followed by the synthesis of delayed-early (DE) proteins (e.g. 51K and 110K non-structural DNA-binding proteins and virus-specific thymidine kinase and DNA polymerase activities) and finally by the synthesis of progeny virus DNA and proteins of the third phase, the late gene products (e.g. 160K, 130K and 150K structural proteins: Randall et al., 1983, 1984a, b; Honess et al., 1982; O'Hare & Honess, 1983a, b; Blair & Honess, 1983). However, the growth cycle of the virus and the synthesis of independently regulated virus proteins is relatively protracted in populations of productively infected cells (e.g. Randall et al., 1983; see also Fig. 3 of present paper).

We wished to discover the basis for this extended course of HVS replication for two main reasons: firstly, to place the differences between the growth of representatives of 'slowly' and 'rapidly' cytopathic herpesviruses on a more objective basis and, secondly, to determine to what extent the growth of HVS could be manipulated to enable further studies of the regulation of virus gene expression. Methods to study these processes should be capable of making the critical distinction between a delayed or protracted time course which prevails uniformly throughout a population of infected cells, and a protracted course which is the product of heterogeneity or asynchrony in the timing of events in individual cells of the population. Clearly, measurements of the average properties of infected cultures cannot make this distinction. We have recently isolated and characterized a series of monoclonal antibodies directed against HVS-specified proteins, including IE 52K (Randall et al., 1984a) as well as delayed-early (e.g. 51K and 110K non-structural DNA-binding proteins) and late proteins (e.g. 130K and virus capsid proteins; Randall et al., 1984b). A number of these antibodies gave characteristic and specific patterns of intracellular staining when used to examine infected cells by fluorescence microscopy (e.g. Randall et al., 1984a, b). Moreover, we have also observed that antibodies to virus capsid components could be used to monitor the fate of parental virus particles as well as the de novo synthesis of progeny capsid components in cells infected at high multiplicities (see Results). These reagents therefore provided the means to examine the timing of each of the major phases of the HVS growth cycle in individual cells from an infected culture. In this paper we present results using such antibodies and a DNA-binding fluorochrome to study the cellular basis for the protracted cycle of HVS growth, relative to that of HSV-1, by quantitative three-colour fluorescence microscopy.

**METHODS**

**Cells and viruses.** Vero cells (Flow Laboratories), owl monkey kidney cells (OMK-210, provided by Professor M. A. Epstein, Medical School, University of Bristol, Bristol, U.K.) and marmoset embryo fibroblasts (MEF; recently established from an embryo of the common marmoset Callithrix jacchus) were grown at 37 °C as monolayers on 25 cm² or 75 cm² tissue culture flasks or on rotating Winchester bottles, in Dulbecco's modification of Eagle's tissue culture medium containing 10% newborn calf serum. HSV-1 strain HFEM/STH2 was grown in Vero cells. HVS strain 11 or its attenuated derivative (HVS-IIAtt; Schaffer et al., 1975) were used for most experiments illustrated in this paper. Some comparative studies used HVS strains KM744 (isolated by Professor K. McCarthy, The Medical School, Liverpool, U.K.), SMBI (isolated by Professor F. Deinhardt's group in Chicago) and HOT. HVS was propagated at low multiplicities of infection (0.01 to 0.5 p.f.u./cell) with virus stocks of 1 x 10⁵ to 8 x 10⁶/ml being obtained from infected OMK or Vero cultures (2.5 to 20 p.f.u./cell) and of 6 x 10⁷ to 2 x 10⁸/ml from infected MEF cultures (15 to 50 p.f.u./cell). All titres quoted in this paper were estimated by adsorbing 1 ml of diluted virus stock on monolayers in 25 cm² plates for 2 h at 37 °C and then overlaying the monolayers with culture medium supplemented with 2% calf serum and containing 0.5% (w/v) CM-cellulose. Under these conditions the specific infectivities of virus stocks were 50 to 300 particles/p.f.u. No reproducible difference was observed between the efficiency of plaque formation by HVS-11 on OMK, Vero or MEF monolayers, although plaques were somewhat larger on MEF monolayers than on Vero or OMK monolayers. However, the estimated titre of a given virus stock could be changed by altering the assay conditions, for example a 20- to 30-fold lower plaquing efficiency was obtained by adsorbing 1 ml of diluted virus stock on monolayers formed in 24-well Linbro plates than in 25 cm² plates. In addition, high concentrations of calf serum (> 10%) in the medium used to dilute the virus stock significantly reduced the estimated titre of virus (see Results).

**Optimizing conditions for high-multiplicity infections of culture cells with HVS.** The results presented in this paper have some practical implications for optimizing infections of OMK and Vero cell cultures with HVS. Thus, the
degree of asynchrony in infections with HVS was minimized by inoculating subconfluent monolayers of OMK, Vero or MEF cells which had been in culture for > 20 h since their last exposure to trypsin (to regenerate trypsin-sensitive receptors) with high multiplicities of virus suspended in medium which did not contain fresh calf serum. In addition, the synthesis of late proteins was selectively reduced in cultures infected with HVS-11 if the medium became acidic (less than pH 7.0, not shown). It was therefore important to ensure that the pH of the culture medium remained on the alkaline side of neutrality (pH 7.4) for the course of the infection. The pH of the culture medium was monitored (colorimetrically with phenol red as indicator) and all results given in this paper are from infections where the medium pH remained between 7.2 and 7.8 using standard medium with bicarbonate buffer.

Monoclonal antibodies. The isolation and characterization of monoclonal antibodies to HVS proteins have been reported in detail elsewhere (Randall et al., 1984b). The monoclonal antibodies used for the experiments reported in this paper had specificities to the major IE 52K protein [SB(52K)], major DE DNA-binding proteins of 51K and 110K [DI(51K), DC(110K) respectively] and to late structural proteins [DE(130K), DH(capsid)]. A monoclonal antibody (58S) that reacts with the IE 175K protein of HSV (ICP4) was kindly provided by Dr M. Zweig. The isolation and characterization of this antibody was described by Showalter et al. (1981).

Immunofluorescence. OMK or Vero cells were grown as monolayers on coverslips with 2 × 10⁵ cells seeded into each well of 24-well Linbro plates. Monolayers were infected 18 to 24 h after plating with either HVS or HSV by rocking 0.5 to 1 ml of virus inoculum over the monolayer for 2 to 4 h at 37 °C, after which time the inoculum was removed and the cells were re-incubated in growth medium (see text). At appropriate times after infection, coverslips were removed and the monolayers were fixed with 5% formaldehyde, 2% sucrose in phosphate-buffered saline (PBS) for 10 min at 20 °C. The monolayers were then washed three times with PBS containing 1% calf serum, permeabilized by treatment with 1% Triton X-100, 10% sucrose, 1% calf serum in PBS for 5 min at 20 °C, and washed three times with PBS containing 1% calf serum before being reacted with monoclonal antibodies. For direct immunofluorescence, monolayers were reacted with purified fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody for 1 h at 20 °C, washed once with 1% Triton X-100, 10% sucrose, 1% calf serum in PBS and four times with PBS containing 1% calf serum. For indirect immunofluorescence, monolayers were reacted with monoclonal antibody (as ascitic fluid diluted 1/100 in PBS with 1% calf serum and filtered through a 0.22 μm Millipore filter) for 1 h at 20 °C. The monolayers were washed four times with PBS containing 1% calf serum and incubated with rhodamine-conjugated goat anti-mouse immunoglobulin (Nordic, Maidenhead, U.K.) for 1 h at 20 °C. The monolayers were washed once with PBS containing 1% Triton X-100, 10% sucrose and 1% calf serum and four times with PBS containing 1% calf serum. Cell nuclei were stained by adding a fluorescent DNA-binding dye [4,6-diamidino-2-phenylindole (DAPI), 20 μg/ml; Russell et al., 1975] to the monoclonal antibodies. In experiments in which both direct and indirect immunofluorescence were used to stain different virus proteins in the same cells, the procedure for indirect immunofluorescence was carried out first and then the procedure for direct immunofluorescence. Cells were examined for immunofluorescence using a Zeiss photomicroscope with ×10 or ×25 objectives. The excitation wavelengths used for FITC were between 450 and 490 nm (excitation filter BP450/490) and for rhodamine between 510 and 546 nm (excitation filter BP546/10); DAPI was excited by u.v. light (excitation filter G365). After excitation FITC emitted green light (barrier filter LP520-560), rhodamine emitted red light (barrier filter LP590) and DAPI emitted blue light (barrier filter LP418). Photographs were taken on Ilford HPS film with exposure times of 30 to 120 s for FITC and rhodamine and 0.1 to 1 s for DAPI. It should be emphasized here that great care must be taken when simultaneously staining with DAPI and FITC. Exposure of DAPI to u.v. light for longer than 5 to 10 s results in a breakdown of the fluorochrome so that it emits green light when excited with wavelengths of 450 to 490 nm. Thus, when examining cells stained with both FITC and DAPI, photographs were first taken of FITC fluorescence and then of DAPI fluorescence.

Synchronization of cells at the G1–S boundary of the cell cycle. Vero cells (2 × 10⁵) were seeded into each well of 24-well Linbro plates in growth medium supplemented with 10% calf serum. After 6 h at 37 °C, the culture medium was replaced with growth medium containing 1 μg/ml aphidicolin and supplemented with 10% calf serum. The aphidicolin block of cellular DNA synthesis was removed after 18 h by washing the monolayers four times with growth medium containing 10% calf serum. Passage of cells through the cell cycle was monitored by three methods. (i) The incorporation of [³H]thymidine into TCA-precipitable material was used as a measure of cellular DNA synthesis. At 2-hourly intervals for 26 h, duplicate monolayers were incubated with growth medium containing 10 μCi/ml [³H]thymidine (48 Ci/mol, Amersham) for 15 min. Cultures were then washed three times with ice-cold PBS, lysed in 1% SDS, sonicated and aliquots (10⁵ cells) air-dried onto glass fibre discs (Whatman GF/C, 2.5 cm) which were washed sequentially with 10% (w/v) TCA, 5% (w/v) TCA and 100% ethanol and dried for the estimation of TCA-precipitable radioactivity. (ii) The percentage of cells passing through the cell cycle was determined by estimating the percentages of cells that had entered mitosis at various times after their release from an aphidicolin block. Colcemid was added to the growth medium (0.6 μg/ml) of a series of cultures immediately after their release from an aphidicolin block. Duplicate cultures were taken at 2 h intervals for 26 h, the cell nuclei stained with DAPI (see Immunofluorescence) and the percentage of cells blocked in mitosis was estimated by scoring at least 1000 nuclei. (iii) The degree of synchronization was estimated by
examining the percentage of cells that were in mitosis at a given time. Duplicate cultures were taken every 2 h for 26 h after their release from the aphidicolin block, stained with DAPI and the percentage of cells in mitosis estimated by counts of the number of mitotic figures in 1000 nuclei.

Analysis of virus protein synthesis in the TPA-treated and in untreated cultures of HVS-infected cells. Replicate monolayers of OMK or Vero cells in 25 cm² tissue culture flasks (2 × 10⁶ cells/flask) were mock-infected or infected with 40 p.f.u./cell of HVS strain 11 in a volume of 1.0 ml of medium and incubated for an adsorption period of 2 h at 37 °C. Inocula were then decanted and replaced with 5 ml of growth medium supplemented with 10% calf serum or with this medium containing, in addition, 20 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA). TPA-treated and untreated cultures of infected and uninfected cells were labelled with L-[³⁵S]methionine (5 μCi/ml, >500 Ci/mmol; Amersham) in a modified growth medium containing one-tenth of the normal concentration of methionine and with 20 ng/ml TPA or in the absence of this compound. At the end of the labelling interval (see text), cultures were removed, washed with ice-cold PBS, lysed in gel electrophoresis sample buffer (0.05 M-Tris-HCl pH 7.0, 2% SDS, 5% 2-mercaptoethanol, 5% glycerol) and after heating (80 °C, 10 min) the dissociated polypeptides were separated by SDS-gel electrophoresis. After electrophoresis, gels containing the separated polypeptides were fixed, stained and dried, and the labelled polypeptides visualized by autoradiography of the dried gel films. Virus-specific polypeptides were annotated as described in detail elsewhere Randall et al., 1983, 1984a, b; Blair & Honess, 1984).

RESULTS

Description of asynchrony

In order to identify the rate-limiting step in the growth of HVS two series of experiments were performed. In the first, we examined the multiplicity dependence of infectious centre formation. Provided that the conditions of high-multiplicity infection and of the plaque titration were identical (see Methods), the proportion of infectious centres (P) did not differ significantly from that predicted from the applied multiplicities of infection, m (i.e. P = 1 – e⁻ᵐ), and essentially all cells in a culture gave rise to infectious centres when m > 5.

In the second series of experiments we exploited monoclonal antibodies directed against representative IE, DE and late proteins to analyse the causes of the protracted time course of virus replication. Results from one such experiment with cultures of OMK cells infected with HVS strain 11 and reacted with antibody to the IE 52K protein of HVS are shown in Fig. 1. Expression of the earliest known virus gene product was asynchronous. In this experiment 5 to 10% of cells expressed high levels of the IE protein at 12 h and this proportion increased to reach 50 to 70% by 36 h both in the presence and absence of inhibitors of virus (phosphonoacetic acid, PAA) or cellular (aphidicolin, APH; O'Hare & Honess, 1983a) DNA synthesis. Similar measurements of the expression of DE (51K and 110K DNA-binding proteins) and late (130K polypeptide and capsid proteins) gene products showed that there was little delay between the expression of IE and DE proteins (2 to 4 h) but a significantly longer delay between the detection of newly synthesized IE and late proteins (see Table 1 and Fig. 6 and accompanying text). The major rate-limiting step in the replication of HVS was therefore located at some stage between adsorption of virus to susceptible cells and the expression of the IE 52K protein. Similar experiments with other lines of susceptible cells (OMK-637, Vero, marmoset embryo kidney and marmoset embryo fibroblasts) infected with HVS strain 11 and with subclones of strain 11 and with other strains of virus in OMK-210 cells (e.g. KM744, HOT, SMB1) gave qualitatively similar results to those documented here for HVS strain 11 in OMK-210 and Vero cells.

Comparisons of the multiplicity dependence of IE gene expression in cultures infected with HVS and HSV

HSV-1 undergoes a normal productive cycle of virus growth in cultures of Vero and OMK-210 cells and a monoclonal antibody to the major IE 175K protein (ICP4) of HSV was made available to us through the generosity of Dr M. Zweig (Showalter et al., 1981). We therefore made a direct quantitative comparison of the multiplicity dependence of detectable IE gene expression relative to infectious centre formation in cultures of Vero cells infected with HVS and with HSV. The results (Fig. 2) showed that under comparable conditions of infection a similar multiplicity dependence of infectious centre formation (points to the right of curves in both panels) was observed with the two viruses, but the multiplicity dependence of the timing of IE
Untreated

+PAA

+APH

Fig. 1. Photographs illustrating the asynchronous expression of the HVS 52K IE protein in populations of cells infected at high multiplicities. Monolayers of OMK-210 cells in 24-well Linbro trays were infected with 20 p.f.u./cell of HVS-11Att and incubated at 37 °C for 12, 24 and 36 h in the absence of inhibitors (untreated) or in the presence of 300 µg/ml phosphonoacetic acid (PAA) or 1 µg/ml aphidicolin (APH). Monolayers were fixed and reacted with monoclonal antibody to the IE 52K protein and stained with rhodamine-conjugated anti-immunoglobulin. The proportion of cells showing the nuclear staining characteristic of the IE protein was estimated by counting the total nuclei per field in samples counterstained with DAPI (not shown, see text).

Asynchronous expression of HVS IE 52K in populations of TPA-treated cells and in infections of synchronized cells

It has been suggested that the growth cycle of HVS is advanced in TPA-treated relative to untreated cultures of OMK cells (Modrow & Wolf, 1983). We therefore examined the effects of TPA treatment on the time course of protein synthesis both by an analysis of labelled polypeptides separated from infected cultures by SDS-gel electrophoresis (Fig. 3) and by quantitative fluorescence microscopy using antibodies to IE 52K and to DE (51K) and late (capsid) proteins (Table 1). We found no differences in the distribution of times at which cells became positive for IE, DE or late proteins in comparison with untreated cultures of infected cells (Table 1) and these cultures did not differ in the times at which maximum rates of virus
protein synthesis were first observed (Fig. 3). Treatment with TPA did however have some effects on the morphology of infected and uninfected cultures. Thus, all treated cultures showed a degree of cell rounding and then resumed grossly normal appearance by 6 h after treatment. However, in the experiment shown treated infected cultures then began to show a typical virus-induced c.p.e. (gross cell rounding) at significantly earlier times than their untreated infected counterparts such that by 14 h treated cultures displayed a 20 to 30% c.p.e. whereas not until 24 h did untreated infected cultures exhibit a 10 to 30% c.p.e. The results shown are from infected cultures of Vero cells; we have obtained similar results with infected cultures of OMK cells.

In randomly growing cultures an effect of the host cell-cycle could clearly contribute to
Fig. 3. Effects of TPA treatment on the time course of protein synthesis in HVS-infected cells. Autoradiogram of labelled polypeptides separated by SDS-gel electrophoresis from lysates of uninfected Vero cells (lane 1) and Vero cells infected with 40 p.f.u./cell of HVS and labelled with \([^{35}S]\)methionine for the intervals shown (h) during infections in the presence of 20 ng/ml TPA (lanes 6 to 9) or in the absence of the drug (lanes 2 to 5). Selected virus-specific polypeptides are annotated with their apparent mol. wt. \(\times 10^{-3}\), as described elsewhere (Randall et al., 1983; Blair & Honess, 1983).

Table 1. *Asynchronous expression of HVS immediate-early (IE 52K), delayed-early (DE 51K) and late (capsid) proteins in untreated infected Vero cell cultures and in infected cultures treated with TPA*

<table>
<thead>
<tr>
<th>Time (h) p.i.*</th>
<th>Untreated</th>
<th>TPA-treated (20 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-52K</td>
<td>Anti-51K</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>&lt;0·01</td>
</tr>
<tr>
<td>12</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>18</td>
<td>49</td>
<td>38</td>
</tr>
<tr>
<td>24</td>
<td>77</td>
<td>65</td>
</tr>
</tbody>
</table>

* Post-infection.
† General nuclear staining (progeny capsid proteins).

asynchrony of HVS IE gene expression. An experiment to examine the influence of the cell-cycle on the events leading to the expression of the HVS IE 52K protein is summarized in Fig. 4. Aphidicolin-mediated synchronization of uninfected cultures was highly effective; two peaks of
Fig. 4. Expression of the HVS IE 52K protein in synchronized cultures infected at different stages of the cell-cycle. Cultures of Vero cells, grown on coverslips in 24-well Linbro trays, were synchronized at the G1–S boundary by inhibiting cellular DNA synthesis by incubation for 18 h at 37 °C in medium containing 1 µg aphidicolin per ml. Aphidicolin was removed (0 h) and four cultures were infected with 40 p.f.u./cell of HVS at 2-hourly intervals to 26 h. Two of each of these cultures were removed at 10 h and two at 18 h after infection and the percentage of cells expressing the HVS IE 52K protein was measured by immunofluorescence microscopy [(b); results from samples taken at 10 h (■) and 18 h (○) after infection are each plotted at the time of inoculation relative to the removal of aphidicolin shown on the abscissa]. At each of the 2 h intervals after the removal of aphidicolin, the relative rates of cellular DNA synthesis were measured by incorporation of [3H]thymidine into TCA-precipitable material in uninfected cultures [(a) left-hand ordinate (■); incorporation during 15 min labelling intervals with medium containing 10 µCi [3H]thymidine per ml] and the percentages of cells undergoing mitosis at each 2 h interval were estimated by fluorescence microscopy of cultures stained with DAPI [(a) right-hand ordinate (○)]. In addition, the cumulative percentages of uninfected cells which had entered mitosis at times after the removal of aphidicolin were estimated by fluorescence microscopy of DAPI-stained cultures which were treated with 0.6 µg colcemid per ml of medium from 0 h [(a) right-hand ordinate (▲)]. Both infection and subsequent incubations were in medium containing 10% fresh calf serum in order to permit unrestricted growth of cell populations.
Table 2. Correlation between nuclear accumulation of parental capsid proteins and expression of the virus IE 52K protein measured by three-colour fluorescence microscopy of infected cultures stained with anti-IE 52K (fluorescein), anti-capsid (rhodamine) and DAPI

<table>
<thead>
<tr>
<th>Relative virus dilution*</th>
<th>Time p.i.†</th>
<th>% Cells positive</th>
<th>(IE + capsid)/capsid, × 100§</th>
<th>(IE + capsid)/IE, × 100</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>47</td>
<td>88</td>
<td>54</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1/3</td>
<td>4</td>
<td>87</td>
<td>91</td>
<td>97</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1/9</td>
<td>4</td>
<td>10</td>
<td>30</td>
<td>28</td>
<td>100</td>
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<tr>
<td></td>
<td>8</td>
<td>59</td>
<td>70</td>
<td>81</td>
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<tr>
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<td>59</td>
<td>70</td>
<td>81</td>
<td>95</td>
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</table>

* Virus inocula and conditions of infection were as described in the legends to Fig. 2 and 6, i.e. undiluted virus was at 10⁸ p.f.u./ml.
† Post-infection.
‡ All reactions were perinuclear, i.e. measurements of parental capsid proteins; see Fig. 5 and 6 and text.
§ The percentage of nuclei positive for capsid fluorescence that were also positive for IE 52K.
|| The percentage of nuclei positive for IE 52K that were also positive for capsid fluorescence.

cellular DNA synthesis (S phase; Fig. 4a) were separated by 18 h with at least 70% of cells entering the intervening mitosis between 8 and 14 h after the removal of aphidicolin and with 15% of cells in mitosis at 10 h after the removal of the drug. Thus, the cycle time was approximately 18 h and at least 70% of cells were passing through the cycle with a good degree of synchrony. However, infection at intervals throughout the cell-cycle had no significant, reproducible, effect on the asynchronous expression of the IE 52K protein and at 18 h after infection (equivalent to one cell-cycle) only 50 to 60% of cells were positive for IE 52K (Fig. 4b). We conclude that the major cause of asynchrony in the expression of the IE 52K protein does not vary solely as a function of the cell-cycle.

**Virus entry and IE gene expression**

During the course of experiments to characterize the monoclonal antibodies we observed that antibodies to capsid components gave two patterns of staining with cultures of cells infected at high multiplicities. The first type of fluorescence was observed at high multiplicities, was unaffected in cultures infected in the presence of cycloheximide (50 µg/ml), and was characterized by early punctate fluorescence at perinuclear sites (see e.g. Fig. 5). It is clear that this pattern of fluorescence results from reactions with parental capsid proteins from the virus inoculum. The second type of fluorescence was only observed at late times after infection, it was observed in cultures infected at high or low multiplicities, its appearance was prevented by cycloheximide and it was sensitive to PAA. This pattern of fluorescence was characterized by a reaction throughout the volume of the nucleus (Fig. 6, compare n and q with b and e) and appeared to reflect the accumulation of newly synthesized capsid proteins. We have used these distinctive patterns of reactivity to correlate variations in the perinuclear accumulation of virus from the inoculum with the expression of the IE 52K protein (Fig. 5 and 6 and Table 2).

The time course of accumulation of parental virus at perinuclear sites of cells infected with high multiplicities is illustrated in Fig. 5, together with the reactions of the same cells with antibody to the IE 52K protein. The majority of anti-capsid fluorescence was at the cell surface at 2 h. There was then a progressive increase in perinuclear fluorescence and a decrease in surface fluorescence, so that by 8 h the majority of cells were positive for perinuclear reactions (Fig. 5, Table 2). In addition to the relatively protracted course of virus transfer to the nucleus the cell population was heterogeneous with respect to the times at which perinuclear fluorescence was first observed and a small fraction of cells appeared to exclude the majority of the infecting virus (see legend to Fig. 5, and compare Fig. 6b and c). The proportion of cells which appeared to exclude virus was too small for the effect to make a significant contribution to asynchrony but differences in the rate and amount of virus accumulating at perinuclear sites...
Fig. 5. Photographs illustrating the time course of nuclear accumulation of parental virus and correlation with IE 52K protein expression. Replicate monolayers of Vero cells grown on coverslips in 24-well Linbro trays were infected with 500 p.f.u./cell of HVS and incubated at 37 °C either in growth medium with no additions or with growth medium containing 50 μg cycloheximide (CX) per ml. Duplicate coverslips from each set of cultures were removed at 2, 4, 6 and 8 h after the addition of the inoculum and each coverslip was stained for three-colour fluorescence microscopy to detect virus capsid proteins, virus IE 52K protein and nuclei in each cell (anti-capsid reactivity was detected by indirect immunofluorescence with rhodamine-conjugated sheep anti-mouse immunoglobulin followed by the addition of anti-IE 52K conjugated with fluorescein and staining with DAPI to reveal nuclear DNA). The row of three panels for each time point are each photographs of a single field of cells illuminated to detect the three reagents. Filled arrows indicate single cells in samples at 4, 6 and 8 h which are stained with all three reagents. The short open arrow (in the photograph of the anti-capsid reaction at 4 h) indicates a cell showing surface fluorescence and no perinuclear fluorescence at a time when the majority of cells only have perinuclear reactions.
Asynchrony in gammaherpesvirus replication

<table>
<thead>
<tr>
<th>Relative virus dilution</th>
<th>Anti-52K</th>
<th>Anti-capsid</th>
<th>DAPI</th>
</tr>
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<tr>
<td>8 h</td>
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<td><img src="b" alt="Image" /></td>
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<td><img src="q" alt="Image" /></td>
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</tr>
<tr>
<td>8 h</td>
<td><img src="s" alt="Image" /></td>
<td><img src="t" alt="Image" /></td>
<td><img src="u" alt="Image" /></td>
</tr>
<tr>
<td>1/27</td>
<td><img src="v" alt="Image" /></td>
<td><img src="w" alt="Image" /></td>
<td><img src="x" alt="Image" /></td>
</tr>
<tr>
<td>12 h</td>
<td><img src="y" alt="Image" /></td>
<td><img src="z" alt="Image" /></td>
<td><img src="aa" alt="Image" /></td>
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**Fig. 6.** Photographs illustrating the effects of the applied multiplicity of infection on the fraction of cells that are positive in immunofluorescence reactions with anti-52K and for reactions against parental (perinuclear fluorescence at 8 h) and progeny (general nuclear fluorescence, e.g. at 12 h) capsid proteins (anti-capsid: see text and Fig. 5). Conditions of infection were as given in the legend to Fig. 2 and samples were stained for three-colour fluorescence microscopy as described for Fig. 5. Times shown are h post-infection. Each set of three panels (anti-52K, anti-capsid, DAPI) illustrates a single field of cells; single cells stained with all three reagents are indicated by arrows.

were correlated with IE 52K expression. Thus, the early perinuclear accumulation of high concentrations of parental capsids was correlated with early expression of IE 52K protein (Fig. 5 and 6, and Table 2: compare overall % IE-positive with the % of cells positive for perinuclear fluorescence which are IE-positive). It was also clear, however, that at no time was the correlation perfect. Thus, although a cell with high concentration of parental virus at a perinuclear site early in infection could be up to sixfold (Table 2) more likely to show early expression of the IE 52K protein, not all cells showing early accumulation of parental virus at the nucleus showed early expression of IE 52K.
Table 3. Effects of calf serum during the period of virus adsorption on the infection of OMK and Vero cells with HVS*

<table>
<thead>
<tr>
<th>Calf serum concentration (%)</th>
<th>OMK</th>
<th>Vero</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-52K</td>
<td>Anti-capsid</td>
</tr>
<tr>
<td>1</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>25</td>
<td>0·3</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

* Replicate monolayers of OMK and Vero cells (2 × 10^5 cells/well in 24-well Linbro plates) were inoculated with 500 p.f.u./cell of HVS in a volume of 1·0 ml of culture medium containing 1, 5 or 25% of fresh newborn calf serum and incubated at 37 °C for 2 h. Inocula were then removed and cultures re-incubated with growth medium containing 10% calf serum until 8 h, at which time cultures were removed and processed for immunofluorescence measurements of the percentage of cells reacting with anti-(IE 52K) and anti-capsid (parental perinuclear reaction) antibodies.

Factors affecting virus adsorption and passage to the nucleus

The observation that differences in the rates of virus adsorption and passage to the nucleus appeared to make a contribution to asynchrony led us to re-examine treatments which modified these processes. We found that high concentrations of calf serum during the period of virus adsorption reduced the fraction of virus adsorbed at a given multiplicity and produced corresponding reductions in the expression of the IE 52K protein. An experiment illustrating these effects of calf serum on the nuclear accumulation of parental capsids and IE gene expression is summarized in Table 3. The nuclear accumulation of virus and the expression of IE 52K were inhibited in parallel and infections of OMK cells were more sensitive than were infections of Vero cells (Table 3: 32% of OMK cells infected in the presence of 1% calf serum were expressing IE 52K at 8 h whereas only 0·3% of cells infected in the presence of 25% calf serum were expressing IE 52K at this time). Experiments measuring the effects of calf serum on the removal of infectious virus from an inoculum by monolayers of OMK and Vero cells have confirmed that the interfering effects of calf serum operate specifically to reduce virus adsorption. Variations in the serum concentration in culture medium after the removal of the inoculum do not affect the proportion of cells expressing the IE 52K protein at a given time after infection (results not shown). The interfering components in calf serum are heat-stable (56 °C for 60 min), non-dialysable, and present in many batches of fresh calf serum. They are removed during the growth of uninfected or infected cells and are therefore not present at high concentrations in normal preparations of excreted virus unless these are diluted in medium containing fresh calf serum.

In the course of experiments analysing the effects of the cell-cycle we employed colcemid to measure the fraction of cells that entered mitosis. Results for uninfected cells were presented above (Fig. 4) but treatment of infected cells with colcemid resulted in a marked reduction in IE 52K gene expression. We therefore examined this effect in more detail and included some comparisons of the effects of colcemid on infections with HSV. Cultures of Vero cells were infected with HSV-1 (50 p.f.u./cell) and left untreated or were treated with medium containing 0·6 µg/ml colcemid from 1 h before infection to 3 h after infection. At 3 h post-infection both sets of cultures were removed, stained with antibody to IE 175K (ICP4) and with DAPI and the fraction of cells expressing IE 175K was scored by fluorescence microscopy. In both treated and untreated cultures 88 to 100% of cells were positive for the IE 175K protein at 3 h and we could therefore see no effect of this regime of colcemid treatment on entry and IE gene expression in HSV-1 infected cultures. The results of a similar study on the effects of colcemid on HVS infection of a serum-deprived (non-dividing) culture of Vero cells are summarized in Table 4. Infections with HVS were sensitive to colcemid treatment and, at moderate multiplicities of infection, remained so for many hours after infection (e.g. treatment from 12 h post-infection reduced the percentage of cells positive for IE 52K at 24 and 36 h from 36% and 75% to 10% and
Table 4. Effects of periods of colcemid treatment on the percentage of Vero cells expressing the IE 52K protein at 24 and 36 h after infection with HVS

<table>
<thead>
<tr>
<th>Treatment of infected cultures*</th>
<th>% Cells positive for nuclear immunofluorescence with anti-(IE 52K) at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Untreated</td>
<td>36</td>
</tr>
<tr>
<td>Colcemid-treated</td>
<td></td>
</tr>
<tr>
<td>0–24 h</td>
<td>5</td>
</tr>
<tr>
<td>0–36 h</td>
<td>ND</td>
</tr>
<tr>
<td>0–12 h</td>
<td>19</td>
</tr>
<tr>
<td>12–24 h</td>
<td>10</td>
</tr>
<tr>
<td>12–36 h</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Cultures were grown in medium containing 2% calf serum for 24 h prior to infection and re-incubated with medium containing 2% calf serum after infection with 40 p.f.u./cell of HVS strain 11. At 24 h and 36 h post-infection only 20% and 36% of cells in cultures treated with colcemid (0.6 μg/ml) from 0 h had accumulated due to blocked mitoses.
† ND, Not done.

Asynchrony in the expression of the IE 52K protein correlated with similar reductions in the perinuclear accumulation of parental capsid proteins in treated cells infected at high multiplicities (not shown).

DISCUSSION

In this paper we have used monoclonal antibodies directed against IE, DE and late proteins of HVS to analyse the time course of virus growth and gene expression in cultures of productively infected cells. We have shown that population heterogeneity or asynchrony in the timing of events necessary for the synthesis of the earliest known virus gene product (the IE 52K protein) is the major cause of the apparently protracted cycle of HVS replication. In cultures infected with effective multiplicities of 1 to 10 p.f.u./cell, the majority did not express the IE 52K protein until after 10 h, whereas the mean delay between the detection of the IE 52K protein and the detection of DE proteins (e.g. 51K or 110K DNA-binding proteins) was only 2 to 4 h and the delay between the detection of the IE protein and the late proteins (capsid and 130K) was 6 to 12 h. Asynchrony in the expression of the HVS IE 52K protein was reduced, but not abolished, by very high multiplicities of infection and the minimum time observed from inoculation to detection of this protein was only 3 to 4 h relative to 1 to 1.5 h for the IE 175K protein of HSV-1 (e.g. Fig. 2). As a consequence of asynchrony in the events leading to the expression of the IE 52K protein, analyses of the regulation of subsequent events are made considerably more difficult. The minimum observed duration of the complete cycle of HVS replication in a single cell was estimated to be 12 to 20 h compared to average times for whole cultures of from 30 to >48 h. Although the transition from DE to late virus protein synthesis was not the normal cause of the extended cycle of HVS growth, this switch was delayed relative to the IE to DE transition when compared with the timing of the analogous transitions in the growth of HSV (e.g. Honess & Roizman, 1975). There is some evidence that a delayed switch to late gene expression may also be a feature of the growth of at least some betaherpesviruses such as human (Wathen & Stinski, 1982; McDonough & Spector, 1983) and murine (Marks et al., 1983) cytomegaloviruses.

In attempts to manipulate the degree of asynchrony we examined the replication of HVS in cultures of synchronized cells and in cultures treated with the tumour-promoting phorbol ester, TPA. We did not confirm previous claims that TPA treatment shortened the cycle of virus replication (Modrow & Wolf, 1983) and infection of cells at different stages of the cell-cycle had no consistent effects on the asynchronous expression of the IE 52K protein. Because differences in the rates of many cellular processes would be expected to occur throughout the cell-cycle, we were surprised by the latter result. Moreover, the replication of other herpesviruses has been reported to be affected by the host cell-cycle in permissively infected cells (Lawrence, 1971;
Muller & Hudson, 1977), but in these cases there is no evidence that the effect is prior to the expression of virus IE gene products. During the course of the present experiments we also noted a number of variables which significantly affected the efficiency with which a given concentration of HVS initiated an infection. Thus, virus adsorption was reduced by prior trypsinization of OMK cells and also by a component of fresh calf serum and this effect was much more marked in infections of OMK cells than of Vero cells (e.g. Table 3). In order to optimize infections (e.g. Fig. 2) it is therefore necessary to infect subconfluent cultures which have not recently been exposed to trypsin and to eliminate fresh calf serum during the adsorption period.

Measurements of the perinuclear accumulation of parental capsids in HVS infections established a clear correlation between heterogeneity in the transfer of the infecting virus to perinuclear sites and the times at which synthesis of the IE 52K protein could be detected (Fig. 5, 6 and Tables 2, 3). Thus, it appeared that heterogeneity and asynchrony in the transfer of virus from the inoculum to the nucleus was the main cause of the asynchronous synthesis of the IE 52K protein. In addition, measurements on cells infected at high multiplicities gave estimates of an average delay of 2 to 4 h between the detection of perinuclear accumulation of parental virus and the accumulation of detectable levels of the IE 52K protein. We have also noted here that perinuclear accumulation of parental capsid proteins and the de novo expression of IE 52K remained sensitive to the microtubule poison, colcemid, for many hours after inoculation with HVS. This effect clearly requires further investigation, but it would be consistent with significant differences in the rate or route of intracellular transport of HVS compared to HSV in Vero cells, since post-infection exposure to colcemid had no effect on the expression of the IE 175K protein in HSV-1-infected Vero cell cultures.

Virion entry and intracellular translocation are inadequately characterized events in the replication of all herpesviruses and there is very little information on the roles of the many polypeptides which comprise the envelope, tegument and capsid components of the herpesvirion (see e.g. Roizman et al., 1975). However, in preliminary biochemical studies with HSV-1 we have found that although the virus envelope glycoproteins are removed prior to translocation to the nucleus, the major proteins of the virus tegument and capsid are present on the subviral particles that accumulate at perinuclear sites (Wong-Kai-In & R. W. Honess, unpublished results). Moreover, there is independent genetic evidence that proteins of both envelope and tegument have essential roles in virion entry. Thus, mutations in an envelope glycoprotein of HSV-1 (glycoprotein B) affect fusion and the rate of entry (Sarmiento et al., 1979; Bezik et al., 1984), and mutations in a tegument protein (the tsB7 mutation of HSV-1 [STH2]; Batterson et al., 1983) result in the perinuclear accumulation of de-enveloped subviral particles which do not transfer the virus genome to the nucleus. Thus, we may envisage a multistep process of entry and uncoating, with specific interactions between successively exposed virion polypeptides and cellular sites or compartments being the determinants of the steps in virus translocation. It would be surprising if the many differences between the structural proteins of HSV and those of HVS (e.g. Randall & Honess, 1980; Keil et al., 1983; R. W. Honess et al., unpublished data) did not determine significant differences in their entry into different cell types.

REFERENCES


Asynchrony in gammaherpesvirus replication


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