Intranuclear Localization of Herpes Simplex Virus Immediate-Early and Delayed-Early Proteins: Evidence that ICP 4 Is Associated with Progeny Virus DNA

By R. E. RANDALL and N. DINWOODIE

Department of Biochemistry and Microbiology, University of St. Andrews, St. Andrews, Fife KY16 9AL and Division of Virology, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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SUMMARY

The localization of ICP 4, ICP 8, DNA polymerase and alkaline exonuclease within herpes simplex virus type 1 (HSV)-infected cells has been examined by immunofluorescence using specific antibodies to these proteins. Cells were simultaneously counterstained with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI) to reveal the intranuclear distribution of DNA. These studies showed that in the absence of virus DNA replication ICP 4, ICP 8 and DNA polymerase were diffusely distributed throughout the nucleus but during virus DNA replication these proteins accumulated at specific foci within the nucleus. Initially these foci were near the nuclear membrane but with continuing virus DNA replication they increased in size until the whole of the nucleus became affected. The increase in size of these foci was coincident with a redistribution of nuclear DNA and margination of chromatin at the nuclear membrane, as revealed by DAPI staining. The number of foci initially present in an infected cell was dependent on the multiplicity of infection. The distribution of ICP 4, ICP 8 and DNA polymerase within the nucleus was altered by treating the cells with DNase. The majority of alkaline exonuclease was diffusely distributed throughout the nucleus during virus DNA replication and did not localize at specific foci within the nucleus. Autoradiographic examination of the incorporation of [3H]thymidine in cells infected with HSV showed that viral DNA replication occurred in restricted areas within the nucleus that were similar, in terms of number, location and size, to the foci where ICP 4, ICP 8 and DNA polymerase accumulated. Furthermore, in cells blocked in mitosis following infection with HSV, ICP 4, ICP 8 and DNA polymerase, but not alkaline exonuclease, localized in areas outside the condensed chromatin structures. DAPI staining revealed the presence of DNA in these areas and, as such structures were never seen when uninfected cells had entered mitosis, it is suggested that this extrachromosomal DNA is of viral origin. These studies therefore suggest that ICP 4 is associated with progeny virus DNA and that while its intranuclear localization is initially at non-viral sites, as DNA replication proceeds so ICP 4 is recruited into areas of virus DNA transcription and replication.

INTRODUCTION

After infection of cells with herpes simplex virus type 1 (HSV) there are complex biochemical and morphological changes within infected cells which culminate in the production of new virus particles. The expression of virus genes in productively infected cells proceeds in three main phases, α- or immediate-early (IE), β- or delayed-early (DE) and γ- or late (Honess & Roizman, 1974, 1975). Only one of the five immediate-early proteins, ICP 4 or IE 175, has been shown to be unconditionally required throughout the infection cycle to act as a cofactor in the transcription of both β- and γ-genes (e.g. Honess & Roizman, 1975; Preston, 1979; Dixon & Schaffer, 1980; Watson & Clements, 1980; Sandri-Goldin et al., 1983; Dennis & Smiley, 1984).
The protein products of β-genes include virus-specific enzymes involved in virus DNA replication, e.g. DNA polymerase, a major DNA-binding protein (ICP 8, Honess & Roizman, 1973; or ICSP 11,12, Purifoy & Powell, 1976), alkaline exonuclease, thymidine kinase and ribonucleotide reductase (e.g. Weissbach et al., 1973; Purifoy & Powell, 1976; Powell & Purifoy, 1977; Hoffman & Cheng, 1978; Banks et al., 1983; Kit & Dubbs, 1963; Dutia, 1983). Normal rates of late or γ-protein synthesis are dependent on the successful replication of virus DNA (Powell et al., 1975; Honess & Watson, 1977; Holland et al., 1980).

As well as the biochemical changes involved in the synthesis and replication of virus proteins and nucleic acids there are a number of morphological changes that take place within a cell productively infected with HSV. The earliest changes are observed in the nucleus, and these include margination of host chromatin, disaggregation of the nucleolus, and the appearance of electron-translucent viral inclusions (e.g. Love & Wildy, 1963; Schwartz & Roizman, 1969; Dargan & Subak-Sharpe, 1983; Puvion-Dutilleul et al., 1982). There have also been a number of studies on the localization of virus proteins within infected cells. For example the intranuclear localization of alkaline exonuclease has been examined (Banks et al., 1985) and it has been shown that ICP 4 and ICP 8 localize in specific areas within the infected nuclei, the distribution of ICP 4 and ICP 8 being influenced by virus DNA replication (Cabral et al., 1980; Quinlan et al., 1984). ICP 8 has been shown to localize in areas of virus DNA replication and to form complexes with virus DNA (Lee & Knipe, 1983; Puvion-Dutilleul et al., 1985). Furthermore, Vaughan et al. (1984) presented evidence that ICP 8, DNA polymerase, alkaline exonuclease and possibly ICP 4 may be associated in a complex. Uptake of [3H]thymidine by HSV-infected cells has shown that virus DNA synthesis is restricted to well-defined sites within the nucleus which increase in size as the infection proceeds (Roizman, 1969; Rixon et al., 1983).

In this paper we show that ICP 4, ICP 8 and DNA polymerase co-localize in specific areas within the nucleus and provide further evidence that these are areas of virus DNA replication. Such studies suggest that ICP 4 is associated with progeny virus DNA and that ICP 4 is recruited from non-viral sites within the nucleus into areas of virus DNA replication and transcription.

**METHODS**

**Cells and viruses.** Vero cells (Flow Laboratories) were grown as monolayers in 75 cm² tissue culture flasks or in rotating Winchester bottles, in Dulbecco's modification of Earle's tissue culture medium containing 10% newborn calf serum. The wild-type HSV-1 strain HFEM/STH2 (see Honess et al., 1984) and ts mutants in ICP 4 (tsD of HSV-1 strain MP17; Brown et al., 1973) and in DNA polymerase (tsD9 of HSV-1 strain KOS; Schaffer et al., 1973) were grown and titrated under appropriate conditions in Vero cells.

**Antibodies.** Monoclonal antibodies that reacted with ICP 4 and alkaline exonuclease were kindly provided by Dr M. Zweig (58 S; Showalter et al., 1981) and Dr K. Powell (Q1; Banks et al., 1983). In addition, Dr K. Powell also very kindly provided rabbit polyclonal antisera to the major DNA-binding protein ICP 8 (ICSP 11,12; Purifoy & Powell, 1976) and to DNA polymerase. The antisera to DNA polymerase had antibodies to both a 150000 mol. wt. protein and to ICSP 34,35 (Powell & Purifoy, 1977; K. Powell, personal communication).

**Immunofluorescence.** 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 25 h after plating with HSV by rocking 0.5 to 1 ml of virus inoculum over the monolayers for 1 h at 37 °C, after which time the inoculum was removed and the cells were re-incubated in growth medium. Infection of Vero cells with tsD and tsD9 were performed in the same manner but at the appropriate temperatures (see text). At various times after infection (see text), coverslips were removed and the monolayers 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 with 0.5% Nonidet P40, 10% sucrose, 1% calf serum in PBS for 5 min at 20 °C, and washed three times with PBS containing 1% calf serum. The monolayers were reacted for 1 h at 20 °C, with monoclonal antibodies (as ascitic fluid diluted 1/100 in PBS with 1% calf serum and filtered through a 0.22 μm Millipore filter) or polyclonal antisera (diluted 1/100 in PBS with 1% calf serum) that had been pre-absorbed with uninfected cell antigens (3 ml of diluted antisera was rocked over a fixed and permeabilized Vero cell monolayer in a 75 cm² tissue culture flask, at 37 °C for 2 h and filtered through a 0.22 μm Millipore filter). The monolayers were washed four times with PBS containing 1% calf serum and incubated, for 1 h at 20 °C, with rhodamine-conjugated goat anti-mouse immunoglobulin or rhodamine-conjugated goat anti-rabbit immunoglobulin (diluted 1/100 in PBS with 1% calf serum and pre-absorbed with uninfected cell antigen as described above; Nordic Immunological Laboratories, Maidenhead, U.K.). The monolayers were washed once with PBS containing 0.5% Nonidet P40, 10% sucrose and
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1% calf serum and four times with PBS containing 1% calf serum. Total nuclear DNA was stained by adding a fluorescent DNA-binding dye [4,6-diamidino-2-phenylindole (DAPI), 20 μg/ml; Russell et al., 1975] to either the goat anti-mouse or anti-rabbit immunoglobulin. Cells were examined for immunofluorescence using a Zeiss photomicroscope with ×25 or ×40 objectives. The excitation wavelengths for rhodamine were between 510 and 546 nm (excitation filter BP 546/10); DAPI was excited by u.v. light (excitation filter G365). After excitation, rhodamine emitted red light (barrier filter LP590) and DAPI emitted blue light (barrier filter LP418). Photographs were taken on Ilford HP5 film.

Treatment of cells with DNase before immunofluorescence. Monolayers of Vero cells, grown on coverslips, were infected with HSV as described above. Five h post-infection the monolayers were fixed with 0.5% paraformaldehyde in PBS for 45 min at 20°C. The monolayers were washed three times with PBS and permeabilized with 0.5% Nonidet P40, 10% sucrose, 1% calf serum in PBS for 5 min at 20°C. The monolayers were then washed three times with PBS and once with nuclease buffer (0.85% NaCl, 10 mM-Tris–HCl pH 7.0, 5 mM-MgCl2, 1 mM-CaCl2) and reacted for 1 min with 50 μg/ml of DNase I (Sigma). The monolayers were washed once with PBS and treated with 5% formaldehyde for 10 min at 20 °C to fix the internal cellular proteins. Finally, the monolayers were washed three times with PBS with 1% calf serum before being stained with specific antibodies for immunofluorescence as described earlier.

Autoradiography of [3H]thymidine incorporation into DNA. Monolayers of Vero cells, grown on coverslips, were infected with 5 to 10 p.f.u./cell of HSV strain HFEM/STH2, in the presence or absence of aphidicolin (0.5 μg/ml). At 4 h post-infection the culture medium was removed and replaced with growth medium containing 10 μCi/ml [3H]thymidine (48 Ci/mmol, Amersham) and re-incubated at 37°C for 1 h. The monolayers were fixed, permeabilized and washed as described for treatment of cells for immunofluorescence (see above) before either being stained with specific antibodies for immunofluorescence, or being examined for the incorporation of [3H]thymidine into DNA by autoradiography. For autoradiography the monolayers were coated with a thin layer of photographic emulsion (Ilford nuclear research emulsion, K5) and exposed for 8 to 12 weeks before photographic development. The monolayers were then treated with DAPI (20 μg/ml) in PBS for 1 h and washed four times with PBS. The autoradiographs were examined by light microscopy and the DAPI staining by fluorescence microscopy using a Zeiss photomicroscope. Pictures were taken on Ilford HP5 film.

RESULTS

Intranuclear localization of ICP 4, ICP 8, DNA polymerase and alkaline exonuclease

The distribution of DNA within the nuclei of cells is dramatically altered after infection with HSV and can be visualized by staining the cells with DAPI. The nucleolus became indistinct and large dark patches appeared within the DAPI staining, and the nucleus took on a very distinctive 'glazed' appearance (Fig. 1). Infected nuclei had this 'glazed' appearance regardless of whether the cells had been fixed before staining, demonstrating that this appearance was not an artefact of the staining procedure (data not shown). The redistribution of host cell DNA, as visualized by DAPI staining, was presumably the well-documented feature of margination of host chromatin in infected nuclei. The localization of ICP 4, ICP 8, HSV DNA polymerase and HSV alkaline exonuclease within cells was examined by immunofluorescence staining of infected cells with antibodies specific for these proteins. It can be seen from Fig. 1 that ICP 4, ICP 8 and DNA polymerase localize in discrete foci, whilst alkaline exonuclease had a more diffuse distribution within infected nuclei. The distinctive foci of ICP 4, ICP 8 and DNA polymerase accumulation corresponded to the dark areas within DAPI-stained nuclei (Fig. 1). The accumulation of ICP 4, ICP 8 and DNA polymerase in these discrete foci within infected nuclei was dependent on the time after infection, multiplicity of infection and virus DNA replication. Fig. 2 shows the intranuclear localization of ICP 4 and ICP 8 in Vero cells at various times after infection with 5 to 10 p.f.u./cell of HSV. At early times after infection (2 h) the majority of ICP 4 and ICP 8 was diffusely distributed throughout the nucleus. However, even at these early times after infection small foci of accumulation of ICP 4 and ICP 8 could be visualized. As the infection proceeded the foci expanded, coalesced and eventually occupied the entire nucleus (Fig. 2). At the same time as there was an increase in the amount of ICP 4 and ICP 8 in these foci so there was a decrease in the amount of ICP 4 and ICP 8 visualized as being diffusely distributed throughout the nucleus (Fig. 2).

At high multiplicities of infection (10 p.f.u./cell) the earliest times at which cells became positive for ICP 4 fluorescence was between 1.5 and 2 h post-infection. At low multiplicities the latest time at which cells first became positive for ICP 4 fluorescence was between 4 and 5 h
Fig. 1. Monolayers of Vero cells were left uninfected (a) or infected (b) with 5 p.f.u./cell of HSV and incubated at 37°C for 5 h. Monolayers were fixed, and stained with specific antibodies using indirect immunofluorescence to show the localization of ICP 4, ICP 8, DNA polymerase (pol.) and alkaline exonuclease (alk. exo.) within infected cells (left). Total nuclear DNA was visualized by counterstaining the cells with DAPI (right).

post-infection (data not shown; Randall et al., 1985). The number of foci in which ICP 4 and ICP 8 accumulated per nucleus was dependent on the multiplicity of infection (Fig. 3 and 4). Thus, it could be demonstrated that even by 2 h post-infection there were a large number of individual foci per nucleus in cells infected with HSV at 25 p.f.u./cell but not by 5 h post-infection in cells infected with 0.25 p.f.u./cell (data not shown). The number of foci per nucleus at later times in infection, when the foci were larger, was also multiplicity dependent (Fig. 4). Furthermore, if cells were infected at a very low multiplicity (0.01 p.f.u./cell) then during the first round of replication no more than one to two foci were seen per infected nucleus. However, as the infection proceeded to surrounding cells so the number of foci per nucleus dramatically
Fig. 2. Photographs showing changes in the intranuclear localization of (a) ICP 4 and (b) ICP 8 with time after infection of Vero cells with HSV. Monolayers were infected with 5 to 10 p.f.u./cell of HSV and incubated at 37 °C. At 2 h, 3 h, 4 h and 5 h post-infection, monolayers were fixed and stained with specific antibodies to ICP 4 and ICP 8 using indirect immunofluorescence.
increased, presumably because these cells were being infected at high multiplicity (Fig. 3). At early times of infection the foci of accumulation of ICP 4 and ICP 8 appeared near the nuclear membrane and it was only as they increased in size that the inner part of the nucleus became affected (Fig. 2 and 3). The areas of these foci completely correlated with the dark areas of DAPI staining within infected nuclei. As the size of these foci increased so did the size of the dark areas of DAPI staining (Fig. 3 and 4).

The distribution of ICP 4 in cells in which virus DNA replication had or had not taken place was examined by infecting cells in the presence or absence of phosphonoacetic acid (PAA), which selectively inhibits HSV DNA polymerase activity. In the absence of virus DNA replication ICP 4 remained evenly distributed throughout the nucleus and did not accumulate in discrete foci (Fig. 4). Correspondingly, the appearance and distribution of DNA in infected nuclei in which virus DNA replication had or had not taken place was clearly different, as visualized by DAPI staining (Fig. 4). Thus, at high multiplicities of infection in the presence of PAA, whilst the nucleolus became indistinct and the distribution of DAPI within the nucleus was different from that of uninfected cells (compare Fig. 1a with panel f, Fig. 4), there were no dark areas within the DAPI staining as seen during normal replicative infection with HSV. The difference in the appearance of the nuclei between uninfected cells and cells infected with HSV in the presence of PAA was multiplicity-dependent. Thus, at low multiplicities of infection there was little difference in the pattern of DAPI staining between infected and uninfected cells (data not shown).

The finding that ICP 4 and ICP 8 accumulation within the nucleus was dependent on the replication of virus DNA was confirmed by the use of ts mutants. Two ts mutants were used in these studies; tsD has a defect at the non-permissive temperature in the switch-on of delayed early proteins (Marsden et al., 1976) and tsD9 has defective DNA polymerase activity at non-permissive temperatures (Purifoy & Powell, 1977). In cells infected with tsD at non-permissive
Fig. 4. Photographs showing that the localization of ICP 4 within infected nuclei is dependent on the m.o.i. and on virus DNA replication. Monolayers of Vero cells were infected with either 25 p.f.u./cell (a, b, e, f) or 1 p.f.u./cell (c, d) in the presence (e, f) or absence (a to d) of PAA (300 μg/ml). Monolayers that had been infected with 25 p.f.u./cell in the presence of PAA were fixed at 8 h post-infection, and those infected with 25 p.f.u./cell or 1 p.f.u./cell in the absence of PAA were fixed at 4 h and 6 h post-infection respectively. The monolayers were then stained with antibody specific to ICP 4 (a, c, e) and with DAPI (b, d, f). Mitotic cells are illustrated by an arrow and the letter M.

temperatures ICP 4 did not localize within the nucleus but was found in the cytoplasm (Fig. 5); at 35 °C whereas most ICP 4 was still located within the cytoplasm that ICP 4 which was present within the nucleus was located in discrete foci (data not shown). Interestingly, a certain amount of ICP 8 was made at the non-permissive temperature, most of which was diffusely distributed throughout the nucleus (Fig. 5). In cells infected with tsD9 at the non-permissive temperature ICP 4 was diffusely distributed throughout the nucleus (Fig. 5) but at permissive temperatures ICP 4 accumulated in discrete foci. At the non-permissive temperature ICP 8 was localized in small, distinct areas spaced regularly throughout the nucleus (Fig. 5), while at permissive
temperatures, ICP 8 localized in large discrete foci randomly distributed within the nucleus. The number of small areas of accumulation of ICP 8 in the absence of virus DNA replication was not multiplicity-dependent and we could find no correlation between these areas of accumulation of ICP 8 and the foci in which ICP 8 accumulated during virus DNA replication.

Evidence that ICP 4, ICP 8 and DNA polymerase locate in areas of virus DNA replication

The intranuclear localization of virus DNA synthesis was visualized by light microscopic examination of autoradiographs of [3H]thymidine incorporation in infected cells. In order to decrease the amount of [3H]thymidine being incorporated into host cell DNA by DNA polymerase α, the cells were infected with HSV strain HFEM/STH2 in the presence or absence of 0.5 μg/ml of aphidicolin. The DNA polymerase activity of this strain is largely resistant to this concentration of aphidicolin, while uninfected cell DNA polymerase α is sensitive (Honess et al., 1984). The nuclear incorporation of [3H]thymidine into DNA was markedly different between infected and uninfected cells. In uninfected cells [3H]thymidine was found either at the periphery of the nucleus in a ring-like fashion or was found uniformly distributed throughout the nucleus (Fig. 6c and d). In infected cells the distribution of incorporated label was in discrete areas which increased in size with time after infection (Fig. 6a and b). These areas were identical in distribution and size to the discrete foci of accumulation of ICP 4, ICP 8 and HSV DNA polymerase. It should be noted that in these experiments the autoradiographic image of [3H]thymidine incorporation interfered with DAPI staining. However, the DAPI staining pattern has been used to demonstrate the localization of the autoradiographic images within a nucleus (Fig. 6). The results presented here on the incorporation of [3H]thymidine into HSV type 1-infected cells were thus similar to those presented by Rixon et al. (1983) for HSV type 2-
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Fig. 6. Monolayers of Vero cells were infected with 5 to 10 p.f.u./cell of HSV (a, b) or mock-infected (c, d) in the presence (b, d) or absence (a, c) of aphidicolin (0-5 μg/ml). Cells were labelled with [3H]thymidine (10 μCi/ml) for 30 min, from 4 h post-infection (a, c) or 8 h post-infection (b, d) before being fixed. The monolayers were coated with Ilford K5 emulsion which was developed after 8 weeks exposure (autoradiographs, left). After development of the autoradiographs the cells were counterstained with DAPI (right). It should be noted that the incorporation of [3H]thymidine into DNA of uninfected cells which resulted in a ring-like distribution of label (d) was not unique to cells treated with aphidicolin but was also observed in control uninfected cells.

infected cells. The results on the incorporation of [3H]thymidine into uninfected cells were of interest in that the proportion of cells showing incorporation at the periphery of the nucleus compared to the number of cells showing incorporation throughout the nucleus was higher in cultures treated with aphidicolin than in control cultures (Fig. 6). We think that the most likely explanation for these results is that aphidicolin reduced the overall rate of DNA replication to a
Fig. 7. Monolayers of Vero cells were infected with 5 to 10 p.f.u./cell of HSV. At 5 h post-infection the cells were fixed with paraformaldehyde, permeabilized, and were (right) or were not (left) treated with 50 μg/ml of DNase I for 1 min (see Methods). The intranuclear distribution of ICP 4, ICP 8 and DNA polymerase (pol.) in cells treated or not treated with DNase was visualized by staining the cells with specific antibodies to these proteins using indirect immunofluorescence (b). The total nuclear DNA content of the cells was visualized by DAPI staining (a). Closed and open arrows indicate the same cells stained with DAPI or with antibody to ICP 8.

level which increased the chance of visualizing areas of maximal DNA replication under the conditions used.

These results suggested that ICP 4 and ICP 8 and HSV DNA polymerase located in areas of virus DNA replication and that these proteins may have been associated with progeny virus
DNA. This supposition was supported by the finding that the localization of these proteins within the nucleus was disrupted by treatment of the cells with DNase I. Fig. 7 shows the localization of ICP 4, ICP 8 and DNA polymerase both before and after treatment of HSV-infected cells with 50 μg/ml of DNase I for 1 min. It can be seen from the DAPI staining that this treatment altered the distribution of DNA within the nuclei. The location of ICP 4, ICP 8 and DNA polymerase was also drastically altered by such treatment. No longer were these proteins located within discrete foci but they were dispersed throughout the nucleus. ICP 4 and DNA polymerase were diffusely distributed throughout the nucleus after DNase treatment but in agreement with the results of Quinlan et al. (1984) ICP 8 was relocated in small discrete foci evenly distributed throughout the nucleus (Fig. 7). Although it was impossible to quantify the amount of these proteins within infected nuclei by immunofluorescence, there was a decrease in the overall intensity of fluorescence, which was particularly marked with anti-ICP 4 fluorescence (Fig. 7).

Localization of HSV proteins in cells that had been blocked in mitosis

Several reports have shown that HSV infection can both abort and prevent mitosis (Stoker & Newton, 1959; Vantsis & Wildy, 1962) and these observations were confirmed in this study (Fig. 8 and 9). The localization of ICP 4, ICP 8, DNA polymerase and alkaline exonuclease in cells that had been blocked in mitosis by infection with HSV was striking. Fig. 8 and 9 clearly demonstrate that ICP 4, ICP 8 and DNA polymerase did not associate with host cell chromatin but accumulated in specific regions outside the areas of chromatin condensation. Interestingly, there was evidence of DNA within these areas as revealed by DAPI staining (Fig. 8 and 9). The DNA excluded from host cell chromatin structures, as revealed by DAPI staining, was never seen in uninfected cells undergoing mitosis (data not shown) and was therefore presumably of viral origin.

DISCUSSION

ICP 4 is an essential regulatory protein required as a cofactor in the transcription of β- and γ-genes. It has been shown to bind weakly to DNA (Powell & Purifoy, 1976) but it is not clear whether ICP 4 carries out its regulatory functions in vivo directly by binding to specific sequences on the virus genome, or indirectly via its interactions with other proteins. Results presented in this paper show that at early times during the replicative cycle ICP 4 is diffusely distributed throughout the nucleus but as the infection proceeds so ICP 4 accumulates within restricted foci of the nucleus. The redistribution of ICP 4 within the nucleus is dependent on virus DNA replication. Evidence is provided which strongly suggests that the areas in which ICP 4 accumulates are areas of virus DNA replication. The evidence to suggest this is based on the findings that two other virus proteins which are intimately associated with virus DNA replication, that is the major DNA-binding protein ICP 8 and the HSV-specified DNA polymerase, also accumulate in these intranuclear foci during virus DNA replication. Furthermore, autoradiographic images of [3H]thymidine incorporation into DNA in infected cells show localization in similar areas. Finally, in cells that have been blocked in mitosis, DAPI staining reveals the presence of extrachromosomal DNA that is unique to infected cells precisely where ICP 4, ICP 8 and DNA polymerase accumulate. These foci of accumulation of ICP 4, ICP 8 and DNA polymerase appear initially near the nuclear membrane (Fig. 2), increase in size with time after infection, and the number of foci per nucleus is dependent on the multiplicity of infection. The most likely explanation for these results is that ICP 4 interacts with progeny virus DNA. At early times during an infection ICP 4 is present in excess amounts over the number of virus genomes with which it can interact and is thus located at non-viral sites within the nucleus. As the infection proceeds so ICP 4 is recruited into areas of virus DNA replication either by its direct or indirect interaction with progeny virus genomes. The number of areas in which virus DNA replication occurs is dependent on the number and location of input virus genomes. In the absence of virus DNA replication ICP 4 would also be in excess over input virus genomes and so the majority of ICP 4 would be at non-viral sites within the nucleus. Because the amount of IE
Fig. 8. Photographs showing that ICP 4 localizes in discrete areas outside condensed chromosomes in cells blocked in mitosis following infection with HSV. Monolayers of Vero cells were infected with 1 p.f.u./cell of HSV and incubated at 37 °C for 8 h. Cells were stained with antibody specific for ICP 4 (a) and the DNA content of the cells was visualized by counterstaining with DAPI (b, c). Long (b) and short (c) exposures of DAPI staining are shown. The upper three panels were photographed using a ×25 objective, the lower six panels using a ×40 objective.

Fig. 9. Photographs showing that ICP 8 but not alkaline exonuclease localizes in discrete areas away from condensed chromosomes in cells blocked in mitosis following infection with HSV. Monolayers of Vero cells were infected with 5 p.f.u./cell of HSV and incubated at 37 °C for 10 h. The cells were stained both with (a) rabbit antiserum specific for ICP 8 (in indirect immunofluorescence using rhodamine-conjugated anti-rabbit immunoglobulin) and (b) with a mouse monoclonal antibody to alkaline exonuclease (in indirect immunofluorescence using FITC-conjugated anti-mouse immunoglobulin). (c) The total DNA content of the cell was visualized by counterstaining with DAPI.
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and DE proteins made in the presence or absence of virus DNA replication is similar (Honess & Watson, 1977) then it might be supposed that the major interaction of ICP 4 with progeny virus genomes is involved with late or γ-gene transcription.

The location of ICP 8 and the HSV DNA polymerase within the nucleus is also dependent on virus DNA replication, but this is less surprising as both these proteins are intimately involved in virus DNA replication. Thus, at early times during infection these proteins are also probably in excess over the number of virus genomes, but as the infection proceeds so progeny virus DNA becomes available for replication and so more and more ICP 8 and DNA polymerase become recruited into areas of virus DNA replication. The results presented in this paper on the localization of ICP 8 in the presence or absence of virus DNA replication are in good agreement with those reported by Quinlan et al. (1984). However, in cells where virus DNA replication was proceeding we did not observe an increase in the number of 'globular' foci throughout the infection as was reported by Quinlan et al., only an increase in size of these foci, the number of foci being dependent on the multiplicity of infection.

To examine the effect of DNase treatment on the intranuclear distribution of ICP 4, ICP 8 and DNA polymerase we developed a method that fixed cells to the coverslips on which they were grown without 'fixing' the internal cellular proteins as occurs, for example, when formaldehyde is used (data not shown). In the method devised, cells are fixed with paraformaldehyde, which cross-links proteins on the external membranes of the cells but because of its size fails to enter the cell through the plasma membrane and thus fails to react with the internal cellular proteins. The method proved very simple to use and permitted high resolution immunofluorescence to be performed on cells fixed in this manner. By using this method we were able to show that the intranuclear distribution of ICP 4, ICP 8 and DNA polymerase in productively infected cells was altered by DNase treatment. Interestingly, after DNase treatment there was a reduction in the intensity of fluorescence observed with antibody to ICP 4, indicating that there had been a reduction in the amount of ICP 4 within the nucleus; the ICP 4 that remained within the nucleus was diffusely distributed. HSV DNA polymerase also became evenly distributed throughout the nucleus after treatment of the cells with DNase. In contrast and as reported by Quinlan et al. (1984), although ICP 8 was redistributed within the nucleus after DNase treatment some ICP 8 remained located within small foci regularly distributed throughout the nucleus that appeared to be similar to the foci seen when ICP 8 accumulates at late times after infection in the absence of virus DNA replication (Fig. 5). However, we could find no evidence that these sites bore any relationship to the sites of virus DNA replication. Thus, the number of these sites of accumulation of ICP 8 in the absence of virus DNA replication was independent of the multiplicity of infection and showed no similarities to the areas in which [3H]thymidine accumulates during normal virus DNA replication. It may be that the ability of ICP 8 to accumulate in small regular areas in the absence of virus DNA replication is a reflection of a specific property of this protein, such as the ability to self-aggregate, rather than its accumulation at functionally important sites. It should be noted, however, that we also observed at late times in a productive infection the accumulation of ICP 8 into very many small distinct areas (similar to those seen in the absence of virus DNA replication) that were regularly distributed within the discrete 'globular' foci that are characteristic features of cells undergoing DNA replication (data not shown).

Cells infected with HSV undergo a number of well-documented morphological changes, including a redistribution of host cell chromatin within the infected nuclei (see Introduction). Staining infected cells with the DNA-binding fluorochrome DAPI allows these changes to be visualized very easily and rapidly using a fluorescence microscope (e.g. Fig. 1). Thus, intranuclear areas in which ICP 4, ICP 8 and DNA polymerase localize appear darker in cells stained with DAPI. It is likely that the major redistribution of host cell chromatin within infected nuclei is caused by the physical expansion in areas of virus replication. Thus, at low multiplicities of infection, there may be an even distribution of DNA throughout most of the nucleus, apart from areas in which ICP 4, ICP 8 and DNA polymerase have accumulated (see Fig. 3 and 4). These gross changes in the distribution of DNA within the nucleus do not occur in the absence of virus DNA replication. However, at high multiplicities of infection and in the
absence of virus DNA replication we observed changes in the distribution of host cell DNA within the nucleus, without observing margination of the chromatin (Fig. 4). It is unlikely that the effect on host cell DNA seen in the absence of virus DNA replication is caused by any of the IE proteins, as at low multiplicities of infection it is possible to see apparently normal nuclei that are positive for ICP 4 expression. Furthermore, at early times after low multiplicities of infection, cells that are positive for ICP 4 appear to undergo normal mitosis (Fig. 4), while at high multiplicities of infection cells in mitosis appear abnormal even before ICP 4 expression (data not shown). It is therefore likely that virus structural proteins block cells in mitosis and that either input structural proteins or DE proteins alter the structure of the nucleus in the absence of virus DNA replication (Fig. 4f). However it is the replication of virus in particular areas of the nucleus which causes the physical redistribution of host cell DNA and results in margination of the chromatin.

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