Ultrastructural Localization of the Herpes Simplex Virus Major
DNA-binding Protein in the Nucleus of Infected Cells

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SUMMARY

Using immunocytochemical procedures in conjunction with electron microscopy we have examined the distribution of the major DNA-binding protein (DBP) of herpes simplex virus (HSV) in infected nuclei. In embedded specimens, DBP was preferentially associated with fibrillar material of electron-translucent viral inclusions, and to a lesser extent with peripheral (marginated) host chromatin. The latter association was sensitive to a non-ionic detergent ('Photo tiC). In chromatin spread by the Miller technique, DBP was found to be a component of the 10 nm 'thick filaments' previously described in HSV infection.

INTRODUCTION

During the course of infection, herpes simplex virus (HSV) induces the synthesis of several DNA-binding proteins (DBPs; Bayliss et al., 1975; Powell & Purifoy, 1976; Wilcox et al., 1980). The most abundant of these is a virus-encoded polypeptide with an estimated molecular weight (M,) of about 130000 (Bayliss et al., 1975; Powell & Purifoy, 1976; Purifoy & Powell, 1976; Leinbach & Casto, 1983). It goes by several names, depending on which laboratory is describing it: ICP8 (Honess & Roizman, 1973; Morse et al., 1978; Wilcox et al., 1980; Conley et al., 1981) and Vmw 130 (Hay & Hay, 1981) for HSV type 1 (HSV-1) protein and ICSP 11, 12 for its HSV-2 counterpart (Purifoy & Powell, 1976). The HSV type 1 and 2 major DBPs exhibit strong serological cross-reactivity (Powell et al., 1981; Yeo et al., 1981), and in the present study we have used a serum raised against the type 2 protein (Powell et al., 1981) to examine, by a combination of electron microscopy and immunocytochemical techniques, the association of the major DBP with various structures in HSV type 1-infected nuclei.

Previous electron microscopic studies of HSV-infected nuclei have revealed a certain number of characteristic structures. In thin sections, one sees nuclear modifications principally related to the margination of host chromatin, a partial disruption of the nucleoli, the formation of electron-dense 'dense bodies', and the appearance of electron-translucent regions containing virus nucleocapsids in various degrees of completion (Darlington & James, 1966; Schwartz & Roizman, 1969; Dupuy-Coin et al., 1978; Puvion-Dutilleul et al., 1982). In chromatin samples prepared by the spreading technique introduced by Miller & Beaty (1969), the most notable structures are filaments about 10 nm in thickness associated with double-stranded DNA molecules devoid of nucleosomes (Müller et al., 1980; Puvion-Dutilleul et al., 1982). These 'thick filaments' recall morphologically similar structures present during adenovirus replication which consist of single-stranded viral DNA complexed with the adenovirus 72K DBP (Miller & Hodge, 1975; Kedinger et al., 1978; Matsuguchi et al., 1979; Beyer et al., 1981; Puvion-Dutilleul et al., 1984).

Here we report that the HSV-1 major DBP is present mainly in the electron-translucent regions of thin sections, and is associated with the 'thick filaments' observed in Miller spreads.
Cells and viruses. RS 537 rabbit fibroblasts were grown and infected at 37 °C with HSV-1 (strain A44 or strain KOS) as previously described (Puvion-Dutilleul et al., 1982). All observations were performed on cultures at 7 h post-infection. No differences were observed between infections initiated by A44 or KOS.

Antisera. Rabbit anti-ICSP 11, 12 (Powell et al., 1981) was generously provided by K. L. Powell (Leeds, U.K.). This serum gives a single band at 130K in PAGe of labelled polypeptides immunoprecipitated from HSV-2-infected cells (Powell et al., 1981) or HSV-1-infected cells (strain KOS, Leinbach & Casto, 1983; and A44, F. Puvion-Dutilleul & M. Laither, unpublished observations). Control antisera was obtained from normal rabbits.

Immunofluorescence. Monolayers were washed with Ca2+- and Mg2+-free phosphate-buffered saline (PBS), fixed at −20 °C with methanol-acetone (3/7) and stored at −20 °C until required. The presence of virus-specific antigens was determined using the indirect immunofluorescence method as described previously (Puvion-Dutilleul et al., 1984) employing the antisera to ICSP 11, 12 (1/10 in PBS) followed by rhodamine (TRITC)-conjugated goat anti-rabbit IgG serum (1/10 in PBS) (Institut Pasteur Production, Paris, France). Normal rabbit serum and uninfected cell monolayers were used as controls.

Immunocytochemistry with embedded material. Immunoperoxidase labelling was performed both on non-loosened material (Puvion-Dutilleul et al., 1984) and on partially loosened material (Puvion-Dutilleul & Puvion, 1980). Immunogold labelling was performed on material embedded in Lowicryl K4M (Carlemalm et al., 1980; Roth et al., 1981).

For immunocytochemistry performed on non-loosened material, plastic coverslip cultures were fixed with formaldehyde and permeabilized by a Triton X-100 treatment before the immunoperoxidase labelling as previously described (Puvion-Dutilleul et al., 1984). Samples were then embedded in situ with Epox and thin sections were observed without additional staining. As a control, immunoperoxidase labelling was also performed on monolayers fixed as for fluorescence with cold methanol-acetone.

For immunocytochemistry on loosened material, reactions were performed either on pellets of cells or on coverslip cultures as described elsewhere (Puvion-Dutilleul et al., 1982) and immunoperoxidase labelling was performed as above for non-loosened material.

Non-specific labelling was estimated by incubating the material with labelled immunoconjugate either directly after bovine serum albumin (BSA) treatment (5% in PBS) or after incubation in normal rabbit serum (1/10 in PBS).

Immunogold labelling was performed on thin sections of cells fixed with 4% formaldehyde in 0.1 M-phosphate buffer for 1 h at 4 °C and embedded in Lowicryl K4M (Carlemalm et al., 1980; Roth et al., 1981). Thin sections were floated on antiserum (1/10 in PBS) and then on goat-rabbit IgG conjugated to colloidal gold particles, 5 nm in diameter (1/50 in PBS) (Janssen Pharmaceutica, Beerse, Belgium) as previously described (Puvion-Dutilleul et al., 1984). For controls, immune serum was replaced with normal serum.

Immunocytochemistry on spread preparations. Nucleoproteins of infected cells were visualized either by direct spreading of cells or after fractionation of subcellular components. For direct spreading, cells were lysed according to a slight modification (Puvion-Dutilleul et al., 1977b) of the original technique of Miller (Miller & Beatty, 1969). The lysed material was centrifuged onto carbon-coated grids (copper or gold), rinsed in Photo flo (Kodak-Path6, Chalon, France), and air-dried. In subfractionation experiments, cells were collected by scraping in a medium containing 0.5% Cemulsol NP 6 (Rhône-Poulenc, Paris, France) as previously described (Puvion-Dutilleul et al., 1977a). They were then disrupted by 10 or 20 strokes in a Dounce homogenizer fitted with a tight pestle. After centrifugation, nuclei were resuspended and spread as above. No differences were observed when whole cells or isolated nuclei were examined, confirming the intranuclear origin of the 'thick filament'.

For routine observations, copper grids were either stained with 2% uranyl acetate or 1% ethanolic phosphotungstic acid (PTA), or were rotary-shadowed with platinum or tungsten. For immunocytochemistry, material spread on gold grids was stored at −20 °C until used. The immunological procedures were as described previously for the detection of 72K antigenic sites in adenovirus-infected cells (Puvion-Dutilleul et al., 1984). Anti-ICSP 11, 12 or normal serum, and peroxidase-labelled anti-rabbit IgG were diluted tenfold in PBS whereas goat anti-rabbit IgG conjugated to colloidal gold particles was diluted 50-fold. Preparations were observed either without additional staining (immunoperoxidase labelling) or after uranyl acetate staining (immunogold labelling).

RESULTS

Immunofluorescence

Indirect fluorescence of 7 h infected cells with anti-ICSP 11, 12 serum gave intense nuclear labelling with weaker fluorescence in cytoplasmic regions (Fig. 1). The nuclear fluorescence was patchy in some cells (Fig. 1a) and diffuse in others (Fig. 1b). The labelling patterns...
Intranuclear HSV major DNA-binding protein

Fig. 1. Indirect immunofluorescence of HSV-1 (strain A44)-infected cells using anti-ICSP 11, 12 serum. (a) Irregular fluorescent patches are located in the interior of the nuclei. (b) Diffuse nuclear labelling. Magnification × 200.

corresponded to those observed by several workers (Powell et al., 1981; Yeo et al., 1981; Ben Ze'ev et al., 1983; Littler et al., 1983). No fluorescence was observed with uninfected cells, or with normal rabbit serum and infected cells (not shown).

Immunocytochemistry

Embedded material

In order to carry out initial observations resembling those used for immunofluorescence, we embedded cells fixed in situ with methanol–acetone (Methods). The distribution of immunoperoxidase label in the nuclei of infected cells (Fig. 2a) closely paralleled that found by immunofluorescence, with the majority of electron-dense deposits being restricted to centrally located inclusions known to contain viral material (Darlington & James, 1966; Schwartz & Roizman, 1969; Dupuy-Coin et al., 1978; Puvion-Dutilleul et al., 1982). Occasionally, label was also observed in the peripheral regions of condensed chromatin (Fig. 2a), but never covering the entirety of these regions.

Greater morphological detail was obtained in nuclei fixed with formaldehyde followed by permeabilization with non-ionic detergent (Fig. 2b). Here, the peroxidase label is seen to be generally distributed over the clear viral inclusions, occasionally in peripheral chromatin and not in association with recognizable structures such as nucleoli, 'dense bodies' or viral nucleocapsids (Fig. 2b). With both formaldehyde-fixed and methanol–acetone-fixed preparations, no labelling was observed with uninfected cells, or with infected cells treated with normal rabbit serum (Fig. 3a, b).

An alternative method of treating cells prior to embedding is a 'mild loosening' procedure in the presence of formaldehyde (Puvion-Dutilleul & Puvion, 1980). This treatment which employs Photo flo, a commercial preparation containing Triton X-100, resulted in the elimination of all labelling in the peripheral host chromatin; otherwise, the labelling pattern did not differ from that obtained with the two previously described treatments (Fig. 3c, and Fig. 4a, b).

Under certain experimental conditions it is possible to detect antigens directly at the surface of thin sections (Dunker & Brown, 1979; Cabral et al., 1980; Garaud et al., 1980). The advantage of such a technique resides in the suppression of the permeabilization step and thus a minimum of disruption of nuclear structure. Our initial attempts with Epon embedding were unsuccessful, although the use of this material had proven successful in detecting the adenovirus 72K DBP (Puvion-Dutilleul et al., 1984). We therefore turned to Lowicryl K4M, which involves a milder treatment, to expose surface antigen (Bendayan, 1983; see Methods). With this embedding
Fig. 2. Immunoperoxidase labelling, using anti-ICSP 11, 12 serum, of HSV-1-infected cells fixed with cold methanol-acetone (a) or Triton X-100 (b). Irregular patches of electron-dense deposits are present in the large centrally located region (CR) of the nuclei. Host chromatin (HC) shows weak and irregular labelling. The capsid (arrow) and the dense body (triangle) are unlabelled. Bar markers represent (a) 1 μm and (b) 0.5 μm.
Fig. 3. Immunoperoxidase labelling of HSV-1-infected cells using normal rabbit serum. (a) Cold methanol–acetone fixation, (b) Triton X-100 treatment, and (c) mild loosening procedure. Indicated structures are: the large centrally located region (CR), peripheral host chromatin (HC), dense bodies (triangles) and viral capsids (arrow). Bar markers represent (a, b) 1 μm and (c) 0.5 μm.
Fig. 4. Immunoperoxidase labelling of loosened infected nuclei using anti-ICSP 11, 12 serum. Electron-dense deposits are restricted to the centrally located region (CR) of the nuclei. Host chromatin (HC), nucleocapsids (arrows) and dense bodies (triangles) are unlabelled. Bar markers represent 1 μm.
medium and immunogold staining (Fig. 5a, b), we obtained labelling patterns similar to those described above for immunoperoxidase labelling. In order to quantify the distribution of immunogold label in various cellular regions, we performed grain counts on infected and uninfected preparations. In uninfected cells treated with anti-ICSP 11, 12 serum (Fig. 6) or infected cells treated with normal serum, grains were evenly distributed over nucleus and cytoplasm at a density of 15 ± 2 per μm². For infected cells treated with anti-ICSP 11, 12 serum, the grain density over virus-specific inclusions was typically 80 ± 14 per μm², while in the region of the margined host chromatin the density was 38 ± 15 per μm². The same level of labelling was observed in the cytoplasm with the exception of mitochondria, which were labelled at background level (about 15 per μm²).

Spread material

As mentioned in the Introduction, a distinctive structure found in Miller spreads of HSV-1-infected nuclei is the ‘thick filament’ (Müller et al., 1980; Puvion-Dutilleul et al., 1982). Thick filaments are thickened regions (about 10 nm in width) of double-stranded DNA molecules (about 2 nm in width) ranging in length from less than 1 μm to slightly more than 10 μm (Fig. 7a). Immunoperoxidase staining of spread preparations with anti-ICSP 11, 12 serum resulted in the labelling of DNA-associated filamentous structures varying in width from 50 nm to 120 nm, the lengths of which fell in the range expected for thick filaments (Fig. 7b). The gross morphology of these structures as compared to control preparations (Fig. 7c), as well as their grouped distribution on the grid, indicated that they correspond to heavily labelled thick filaments.

Immunogold staining permitted a somewhat better appreciation of the morphology of labelled filaments (Fig. 8a, b). Here, the (unshadowed) filament width varied generally from 20 nm to 60 nm, presumably because of thickening by reaction with antibody, while DNA fibres, although not always easy to visualize (Fig. 8b), were about 2 to 3 nm wide. Particle counts confirmed visual impressions concerning the association of gold label with the thick filaments; in any region > 80% of the gold particles were not farther than 5 nm from the filament. No labelling of filaments was observed with either stain when normal rabbit serum was employed (Fig. 7c, 9). Nor was label found in association with other recognizable structures, such as viral capsids (Fig. 10a), transcription complexes (Fig. 10b) and nucleosomal DNA (Fig. 10c, d).

DISCUSSION

The present results permit a preliminary description of the manner in which the major HSV DBP is distributed in the nuclei of infected cells. Using thin sections of Lowicryl-embedded material, which, of the several fixation techniques used in this study, probably provides the truest representation of nuclear structure, we found the greatest concentration of immunolabel associated with fibrillar material in the clear central region (Fig. 5). A quantitative estimate of grain counts in this region gave approx. 65 grains per μm² after correction for non-specific background (approx. 15 grains per μm² using non-immune serum). It is in this region that viral DNA is replicated (Okada et al., 1974; our unpublished observations on high resolution autoradiographs), and so the presence of DBP is consistent with genetic (Powell et al., 1981; Knipe & Spang, 1982; Littler et al., 1983; Weller et al., 1983), and biochemical (Powell et al., 1981) studies demonstrating the requirement for DBP in HSV DNA replication. Three recognizable structures other than fibrillar material in this region, the nucleolus, virus capsids, and ‘dense bodies’, were not labelled by anti-DBP serum (≤ 15 grains per μm² uncorrected, Fig. 2, 4, 5).

There was in addition a significant labelling (about 25 grains per μm² above background) of the peripheral nuclear regions (Fig. 2, 5) which are supposed to contain principally condensed host chromatin (Darlington & James, 1966; Schwartz & Roizman, 1969; Dupuy-Coin et al., 1978; Puvion-Dutilleul et al., 1982). After mild loosening of nuclear components in the presence of detergent at low ionic strength, these regions no longer reacted with anti-ICSP 11, 12 serum, although the extent of labelling in the clear central region remained virtually unchanged (Fig. 4).
Fig. 5. Immunogold labelling of Lowicryl-embedded infected cells using anti-ICSP 11, 12 serum. Gold particles are more abundant (see text) over the centrally located region (CR) of the nucleus than over host chromatin (HC) and cytoplasm (Cy). Virions (a, b) and the dense body (b) are not labelled over background. Bar markers represent 0.5 μm.
For legend, see opposite.
Fig. 6. Immunogold labelling, using anti-ICSP 11, 12 serum, of uninfected cells embedded in Lowicryl. A few of the occasional gold particles found in the field are indicated by arrows. Bar marker represents 0.5 μm.
Fig. 7. Contents of infected nuclei spread according to the technique of Miller. (a) Platinum shadow-casting. (b) Immunoperoxidase labelling using anti-ICSP 11, 12 serum; thick filaments are heavily stained (arrows). (c) Control using normal rabbit serum; thick filaments (arrows) are unlabelled. Bar markers represent 1 μm.
Fig. 8. Contents of infected nuclei spread according to the technique of Miller. Immunogold labelling using anti-ICSP 11, 12 serum. Gold particles are preponderantly associated with thick filaments (see text) (arrows). Bar markers represent (a) 1 μm and (b) 0.1 μm.
This indicates that when DBP does associate with peripheral regions, the association is easily disrupted by non-ionic detergent, in contrast to its association with viral chromatin. The same level of labelling, about 25 grains per \( \mu m^2 \), was observed in the cytoplasm (Fig. 5, and additional data not shown), suggesting that the peripheral DBP may be simply undergoing passive diffusion from the cytoplasm, where it is synthesized, to the viral chromatin. Knipe & Spang (1982) have described what appears to be an initial detergent-sensitive step in the transport of the major DBP to viral chromatin and this may be what was observed here.

Perhaps the most important result of this study is the finding that the major DBP is a component of the 10 nm thick filaments present in Miller spreads (Fig. 7b, 8). With HSV-infected cells, in addition to thick filaments this spreading technique reveals nucleosomal and non-nucleosomal (smooth) DNA fibres, virus capsids, and occasional ribonucleoprotein fibrils of transcription complexes (Fig. 10) (Müller et al., 1980; Puvion-Dutilleul et al., 1982). Only the thick filaments were labelled in the presence of ICSP 11, 12 antiserum. The absence of DBP from nucleosomal DNA (i.e. non-viral DNA, see Mouttet et al., 1979; Leinbach & Summers, 1980) (Fig. 10c, d) is consistent with its disappearance from peripheral nuclear regions after detergent treatment.

Until the DNA in thick filaments is characterized it will not be possible to appreciate their role in HSV infection. Because there are no nucleosomes in the vicinity of thick filaments, it has been implicitly assumed that the DNA is viral (Müller et al., 1980; Puvion-Dutilleul et al., 1982), although this has not been directly demonstrated. One would also like to know the strandedness, single or double, of the DNA in thick filaments. The presence of single strands would suggest a role in replication or recombination, while double-stranded DNA would favour a role in the encapsidation process. The morphological resemblance of thick filaments to protein-coated single strands in adenovirus replication (Miller & Hodge, 1975; Kedinger et al., 1978; Matsuguchi et al., 1979; Beyer et al., 1981; Puvion-Dutilleul et al., 1984), or in reconstituted complexes with the T4 gene 32 protein (Müller et al., 1980), could be misleading, since somewhat similar structures (although with considerably greater structural detail) are observed when recA protein of *Escherichia coli* is complexed to double-stranded DNA (Stasiak et al., 1983). It is relevant to the present study that a recent biochemical characterization of chromatin from HSV-infected cells (Leinbach & Casto, 1983) revealed that the majority of DNA associated with the major DBP is double-stranded.

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Fig. 10. Contents of infected nuclei spread according to the technique of Miller. Immunogold labelling using anti-ICSP 11, 12 serum. Occasional gold particles are present over regions containing (a) capsids (arrows), (b) transcription complexes (triangles) and (c, d) nucleosomes (short arrows). The poor contrast of transcription complexes and nucleosomal DNA is typical, due to the protein film deposited during the immunocytochemical steps. Bar markers represent (a to c) 0.5 μm and (d) 0.1 μm.


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