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NOTES

Ultrastructural Characterization of an Early, Nonstructural Polypeptide of Herpes Simplex Virus Type 1

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An immunoperoxidase procedure was employed to study the expression of a large-molecular-weight, virus-induced polypeptide (VP175; molecular weight, 175,000) at the light and electron microscopic levels in Vero cells infected with herpes simplex virus type 1 or with tsB2, a DNA-negative, temperature-sensitive mutant of herpes simplex virus type 1. In cells infected with herpes simplex virus type 1 and in cells infected with tsB2 at the permissive temperature (34°C), VP175 was found within the nucleus. The protein was detected as early as 2 h postinfection and, by 3 h postinfection, was generally distributed in a marginated pattern contiguous with, and extending from, the inner lamella of the nuclear membrane. At 6 h postinfection, protein accumulations were dispersed throughout the nucleus, and, by 9 h postinfection, these accumulations tended to be localized in a marginated pattern near the nuclear membrane. It was also noted that, at 9 h postinfection, under permissive conditions, VP175 was not found in association with nucleocapsids or enveloped particles. In contrast, in cells infected with tsB2 at the nonpermissive temperature (39°C) and harvested at 6 or 9 h postinfection, accumulations of VP175 were identified not only within the nucleus, but also within the cytoplasm in the form of annular or globular aggregates. These aggregates consisted of a granular matrix and were not bound by membranes.

Viral protein 175 (VP175) (5), or infected-cell protein 4 (ICP4) (11), is a nonstructural polypeptide which is synthesized early in herpes simplex virus type 1 (HSV-1)-infected cells. This polypeptide has an average molecular weight of 175,000, has been grouped in the α-kinetic class of HSV-1 proteins (11), undergoes post-translational modification resulting in an increase in molecular weight (5, 10), and is phosphorylated (20). The use of monospecific antiserum to VP175 has demonstrated that it is found primarily in the nucleus of cells infected with wild-type (WT) virus (5). Additional studies have demonstrated that VP175 is overproduced in cells infected either with certain temperature-sensitive mutants (5, 27) or with HSV-1 stocks containing a high proportion of defective virus (10, 17).

In an effort to clarify further the role of this protein in virus replication, this laboratory employed immunoperoxidase staining at the light and electron microscopic levels to determine the distribution of VP175 in Vero cells infected with HSV-1 or with tsB2, a DNA-negative, temperature-sensitive mutant of this virus (21). At the nonpermissive temperature, this mutant is characterized by the overproduction of VP175 (5) and the failure to induce the synthesis of detectable amounts of viral DNA (23) or recognizable virions (24).

African green monkey (Vero) cells in 16-ounce (ca. 0.473-liter) prescription bottles, in 100-mm petri plates, or on cover slips in 60-mm petri plates were grown and maintained in Eagle medium in humidified CO_2 (5%) incubators (Wedco, Inc., Silver Spring, Md.) with temperature variations of ±2°C, as previously described (3). Permissive and nonpermissive temperatures were 34 and 39°C, respectively. The preparation of virus stocks (1, 6, 24) and the performance of infectivity assays (8) have been reported previously.

Subconfluent monolayers were infected 24 h after seeding with cell-free WT HSV-1 (strain KOS) or tsB2 at a multiplicity of infection of 0.5 and 10 PFU/cell for cover slip and petri plate cultures, respectively. Duplicate cultures were mock infected with 0.05 M Tris buffer, pH 7.6.

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Infected cultures were incubated for 1 h at 37°C to allow for virus absorption, medium containing 5% fetal bovine serum was added, and monolayers were maintained at 34 or 39°C.

VP175 was isolated from virus-infected cells by sequential sodium dodecyl sulfate-preparative and cylindrical polyacrylamide gel electrophoresis, and antiserum to the purified polypeptide was prepared in rabbits as previously reported (5).

Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G was prepared by immunization of goats with rabbit immunoglobulin G purified DEAE-cellulose chromatography (9), precipitation of goat globulins with Na_2SO_4 (14), filtration of globulins through Sephadex G-150 superfine, and conjugation of the eluted immunoglobulin G to horseradish peroxidase by a periodation technique (18). The conjugate was evaluated by radial immunodiffusion and by immunoelectrophoresis followed by treatment with 0.05% 3,3'-diaminobenzidine tetrahydrochloride–0.1% H_2O_2 to demonstrate peroxidatic activity.

For light microscopy, cover slip cultures were harvested at 2, 3, 6, 8, and 9 h postinfection (p.i.), washed with Tris, air dried, and fixed in acetone for 5 min at room temperature. All sera were absorbed with rabbit liver powder and with uninfected Vero cells (approximately 10^8 cells per ml of serum) for 1 h at 37°C and 12 h at 4°C, followed by centrifugation at 100,000 × *g* for 20 min in a Spinco 40 rotor. After rehydration in phosphate-buffered saline (PBS; 0.13 M NaCl, 0.02 M sodium phosphate buffer [pH 7.3], 5×10^{-3} M $MgCl_2$, 1×10^{-4} M $CaCl_2$, 5×10^{-3} M KCl), the cultures were incubated with rabbit anti-VP175 antiserum (1:2) or normal rabbit serum (1:2), washed three times with PBS, and treated with goat anti-rabbit immunoglobulin G-horseradish peroxidase for 30 min. After a brief wash in Tris buffer, cultures were treated in the dark for 10 min with 0.1% 3,3'-diaminobenzidine tetrahydrochloride–0.01% H_2O_2 in the same buffer. After a brief rinse in distilled water and dehydration in a graded series of ethanol, cover slips were mounted in Permount.

For immunoelectron microscopy, cultures in 100-mm petri plates were harvested at 3, 6, and 9 h p.i. Briefly, cells were washed in situ with cold (4°C) PBS, scraped into this buffer, pelleted at 1,200 rpm at 4°C, and fixed with periodate-lysine-paraformaldehyde solution (16) for 3 h at 4°C. After washing for 12 h in three changes of cold 0.05 M sodium phosphate buffer (pH 7.3) containing 4.5% sucrose and 0.05 M lysine, the cells were dehydrated in a graded series of ethanol and embedded in Epon 812. Thin sections on nickel grids were etched with 5% H_2O_2 for 5 min, incubated for 2 to 5 min with normal goat serum (1:30 in PBS), treated with rabbit anti-VP175 (1:4) or normal rabbit serum (1:4), washed in a jet of PBS, and floated on 15 μl of goat anti-rabbit immunoglobulin G-horseradish peroxidase (1:10). After washing with Tris, the grids were immersed in 0.02% 3,3'-diaminobenzidine tetrahydrochloride–0.05% H_2O_2 in Tris for 2 to 3 min with continuous stirring, rinsed in distilled water, and treated with 2% aqueous osmium tetroxide for 1 h. Grids were examined without heavy metal staining in a Hitachi HU11B electron microscope operating at an accelerating voltage of 75 kV.

For standard electron microscopy, cultures in 100-mm plates were harvested at 6 h p.i. as described for immunoelectron microscopy. Pellets were fixed in cold 2.5% glutaraldehyde in Sorensen buffer (pH 7.2), washed in buffer, post-fixed with osmium tetroxide, dehydrated in ethanol, and embedded in Epon-Araldite as previously described (3). Thin sections were examined in the electron microscope after staining with uranyl acetate and lead citrate.

Light microscopic observations of virus-infected cells stained by the immunoperoxidase technique for the demonstration of VP175 were essentially in accord with those noted previously employing immunofluorescence staining (5). In cells infected with WT virus at 34 or 39°C, staining for VP175 was localized primarily within the nucleus. At 3 h p.i., brown clusters were noted at or near the center of the nucleus. At 6 h p.i., the intranuclear deposits were present in a dispersed punctate pattern or in large, crescent-shaped inclusions (Fig. 1A). In approximately 30% of the cells examined, the nucleus was sharply defined by immunostaining (Fig. 1A, arrow). By 9 h p.i., intranuclear brownish granules were prominently displayed in a marginated pattern in approximately 75% of the cells examined.

The patterns of immunostaining observed in cells infected with WT virus at 34°C were also noted at these times in tsB2-infected cells incubated at the permissive temperature (i.e., 34°C). However, in cells infected with the mutant and incubated at the nonpermissive temperature (i.e., 39°C), a significant difference in the distribution of staining for VP175 was noted. Although brownish deposits were found within the nucleus in a pattern similar to that noted in WT virus-infected cells at 3 h p.i., at 6 h p.i. immunostaining was present within both the nucleus and the cytoplasm. The cytoplasmic staining was seen in the form of large granules which were concentrated in the perinuclear region. This perinuclear arrangement was particularly pronounced at 9 h p.i. (Fig. 2A). In addition,

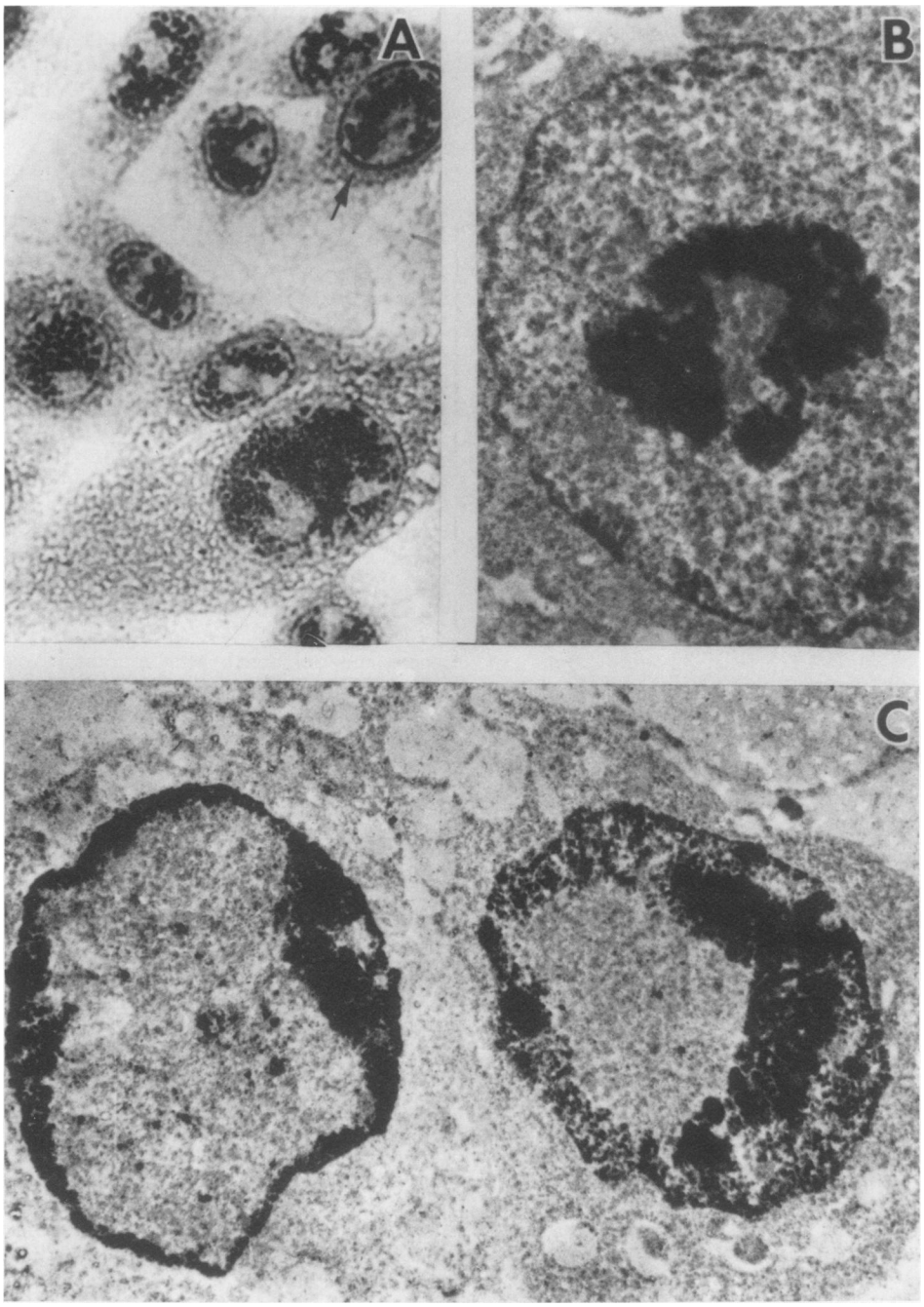

FIG. 1. *Vero cells infected with HSV-1 (strain KOS) at 39°C and processed by the indirect immunoperoxidase technique. (A) Light microscopy of cells processed at 6 h p.i. The protein is either dispersed in the nucleus or found in a reniform arrangement at the nuclear periphery (arrow). Note that some of the staining for VP175 tended to be localized in a marginated pattern within the nucleus. ×800. (B and C) Immunoelectron microscopy. (B) Nucleus of infected cell at 6 h p.i. Electron-dense staining is distributed along the periphery of a body resembling a nucleolus. ×10,800. (C) At 9 h p.i., accumulations of VP175 were generally in a marginated pattern within the nucleus. ×6,100.*

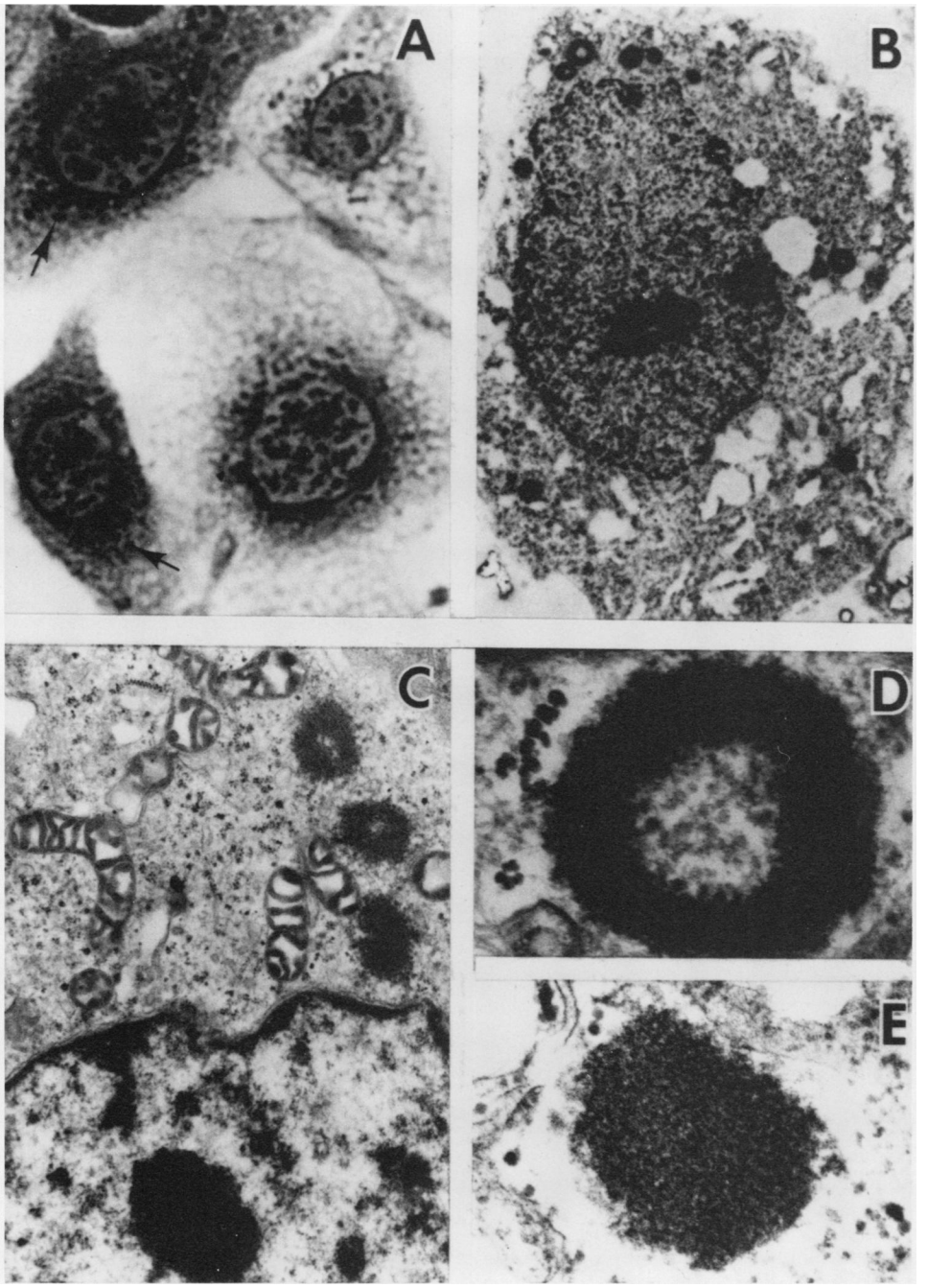

FIG. 2. *Vero cells infected with tsB2 at 39°C, the nonpermissive temperature. (A) Light microscopy of cells processed at 9 h p.i. Staining for VP175 is dispersed in the nucleus and concentrated in the perinuclear area of the cytoplasm (arrow). ×800. (B) Immunoelectron microscopy of cell processed at 6 h p.i. Staining for VP175 is dispersed in the nucleus. Staining also is localized at the periphery of a structure which may represent the nucleolus. Positive-staining annular and globular aggregates are dispersed in the cytoplasm. ×5,760. (C, D, and E) Standard electron microscopy. (C) Three protein clusters of confirmation similar to that seen using immunoperoxidase are present in the cytoplasm. ×17,000. (C and D) High-resolution electron microscopy of globular and annular structures seen in the tsB2-infected cells at the nonpermissive temperature. These accumulations are composed of a granular matrix and are not bounded by membranes. ×57,000.*

staining was distributed randomly throughout the nucleus at both time periods.

Immunostaining was not seen in virus-infected cells incubated with normal rabbit serum rather than with monospecific antiserum to VP175. Staining also was absent in mock-infected cells treated with preimmune sera or with antisera to VP175.

Immunoperoxidase staining at the ultrastructural level confirmed the patterns for VP175 in HSV-1- and in tsB2-infected cells seen by light microscopy. At 3 h p.i., electron-dense deposits were seen within the nucleus of cells infected with WT virus at 34 or 39°C. In addition, staining was denoted along the outer periphery of an intranuclear body resembling the nucleolus. At 6 h p.i., immunostaining was present throughout the nucleus in a fine granular pattern and also adjoined the inner lamella of the nuclear membrane. Staining was observed along the periphery of nucleolus-like bodies in a pattern similar to that noted at 3 h p.i. (Fig. 1B). By 9 h p.i., staining for VP175 was distributed in a marginated pattern within the nucleus. This arrangement was especially pronounced in WT virus-infected cells maintained at 39°C (Fig. 1C). Immunostaining for VP175 was not associated with either nucleocapsids or enveloped particles.

The staining patterns observed in cells infected with tsB2 at 34°C did not differ significantly from those described for cells infected with WT virus at this temperature.

In cells infected with tsB2 at 39°C and harvested at 3 h p.i., staining was seen within the nucleus in a pattern similar to that noted in WT virus-infected cells examined at the same period. However, in agreement with light microscopic observations, staining deposits were seen in both the nucleus and the cytoplasm at 6 and 9 h p.i. (Fig. 2B). The cytoplasmic deposits were globular or ring shaped, ranged in diameter up to 0.5 μm, and were usually localized in the perinuclear region. They were neither encompassed by membranes nor found in association with smooth or rough endoplasmic reticulum or other cytoplasmic organelles. In addition, immunostaining in a marginated pattern, similar to that seen in the nuclei of WT virus-infected cells, was absent. Controls similar to those utilized in the light microscopic study were employed at the ultrastructural level.

Standard electron microscopy was undertaken to determine whether tsB2-infected cells maintained at 39°C contained aberrant virus structures or expressed virus-induced cellular alterations which could be related to the phenotype of VP175 overproduction at 39°C. Cells were infected with HSV-1 or with tsB2 at 34 or 39°C and were examined at 3, 6, and 9 h p.i. Cytoplasmic aggregates of annular or globular confirmation (Fig. 2C, D, and E), similar to those noted in the immunoelectron microscopic studies, were observed in cells infected with tsB2 at 39°C and harvested at 6 and 9 h p.i. The aggregates were composed of a granular matrix and were not bound by membranes. In addition, the accumulations were not associated with cytoplasmic membranes or organelles. These aggregates were never seen in tsB2-infected cells maintained at 34°C or in WT virus-infected cells.

It has been suggested that VP175 functions within the nucleus of infected cells because this protein is transported into that organelle after its synthesis in the cytoplasm. Indeed, VP175 has been shown to bind to DNA and may have a DNA-related function. Powell and Purifoy (21), using DNA-cellulose chromatography, were able to isolate 17 infected-cell-specific polypeptides (ICP) of HSV-1 and HSV-2. Included among the HSV-1 polypeptides which bound reversibly to either native or denatured salmon sperm DNA was ICP172-178, or VP175. This protein was classified as a weakly binding protein on the basis of the molarity of salt required for elution from denatured DNA-cellulose.

In the present study, immunochemical staining for VP175 demonstrated that, at 9 h p.i., the protein was arranged in an intranuclear marginated pattern in cells infected with WT virus. This pattern of staining parallels the configuration of nuclear chromatin frequently seen in HSV-infected cells (19). In contrast, in cells infected with tsB2 at the nonpermissive temperature, in which VP175 is overproduced (5) and virus DNA synthesis is not detected (1), staining for the protein in a marginated pattern, similar to that noted in cells infected with WT virus, was absent. It has also been observed that VP175 does not assume a marginated intranuclear configuration in the absence of viral DNA synthesis (unpublished data). In HSV-1-infected cells treated with 1-β-D-arabinofuranosylcytosine by immunoflorescence, it was noted that immunofluorescence staining was found in a diffuse pattern throughout the nucleus at all times (3 to 15 h) p.i.

VP175 is one of several α-polypeptides whose functions are required for the synthesis of β-polypeptides and the eventual synthesis of γ-polypeptides (12). The primary lesion in the tsB2 mutant is thought to be in the gene which codes for VP175. The location of the template specifying VP175 on the physical map has been shown to correspond to the location of the α-transcripts (13). Furthermore, the HSV-1 (strain KOS) B cistron, of which tsB2 is a member, has been shown to lie within the c sequences bounding the unique S region of the viral genome (D.

S. Parris, R. A. F. Dixon, and P. A. Schaffer, Virology, in press). Significantly, at least one species of the defective HSV DNA also contains all or part of the B cistron (2) which accounts for the production, but not the accumulation, of VP175 in cells infected with defective virions.

The VP175 polypeptide (ICP4) undergoes significant post-translational modification after its synthesis within HSV-1-infected cells (7, 20). First, with the use of pulse-chase isotopic labeling studies, the polypeptide was detectable after a 10-min pulse as a molecule with a molecular weight of less than 175,000. However, after the chase period, the polypeptide migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a molecule with an apparent molecular weight of 175,000. In contrast, in cells infected with tsB2 and maintained at the nonpermissive temperature, the VP175 polypeptide does not undergo a shift to the higher-molecular-weight polypeptide (7). Instead, one observes the accumulation of the lower-molecular-weight species (7). A second post-translational modification which occurs with this polypeptide is its phosphorylation after synthesis (20). The extent of the phosphorylation of the VP175 in the mutant, however, if indeed phosphorylation occurs at all, is unknown. In any case, despite the change in molecular weight, the limited phosphorylation of the polypeptide in cells infected with tsB2 at the nonpermissive temperature could lead to the production of components exhibiting altered net charge and conformational properties. Because VP175 is a DNA-binding protein, either the absence of phosphorylation or improper phosphorylation could lead to altered DNA-binding properties. Although the exact function of VP175 is speculative, its nuclear localization and DNA-binding properties are reminiscent of those of Epstein-Barr virus-determined nuclear antigen and of T antigen of simian virus 40 and adenovirus, early nuclear antigens which may exert regulatory functions by binding to viral DNA (4, 15).

Previous observations have confirmed that a large number of cistrons of HSV are essential for viral DNA synthesis (22). It is likely that many of the early nonstructural proteins of HSV, such as VP175, function alone or in concert with other proteins in the initiation of DNA and RNA syntheses. Thus, the altered expression of VP175 in cells infected with tsB2 and maintained at the nonpermissive temperature probably contributes to the subsequent failure of viral DNA synthesis and the eventual expression of HSV-specific structural proteins.

An additional observation made in the present study was that in cells infected with the WT virus or with tsB2, immunostaining for VP175 was observed within the nucleus at the periphery of a structure which, upon thin sectioning, resembled the nucleolus. Although the significance of this finding in HSV infection is unknown, a similar pattern of immunochemical staining has been observed in cells infected with parvovirus. Singer (26) described the presence of H1 parvovirus antigens on nucleolar chromatin at the onset of viral protein synthesis. He suggested that the progressive binding of H1 antigens to peripheral nucleolar chromatin was responsible for the early morphological disruption and eventual destruction of the nucleolus. Disaggregation of nucleoli in HSV-infected cells has also been documented (25). The role of VP175 in this process in HSV infection presently is being investigated.

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