MOLECULAR AND CELLULAR BIOLOGY, July 1986, p. 2371–2381
0270-7306/86/072371-11$02.00/0

Vol. 6, No. 7

A Mutant Herpesvirus Protein Leads to a Block in Nuclear Localization of Other Viral Proteins

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Received 16 December 1985/Accepted 23 March 1986

The herpes simplex virus mutants KOS1.1 *ts*756 and HFEM *ts*LB2 express temperature-sensitive ICP4 proteins that are not localized properly to the cell nucleus at the nonpermissive temperature. In these infected cells at the nonpermissive temperature, nuclear localization of at least two other viral proteins, ICP0 and ICP8, is impaired. Replacement of the mutated sequences in the ICP4 gene of *ts*LB2 restored proper nuclear localization of all of the proteins. The ICP0 and ICP8 proteins expressed in cells transfected with their individual genes were localized to the cell nucleus. Therefore, in infected cells, the mutant ICP4 gene product appears to be the primary defect which leads to the block in nuclear localization of the other proteins. One viral protein, ICP27, was not inhibited for nuclear localization in these cells. These data indicate that there are at least two pathways for nuclear localization of HSV proteins, one of which is inhibited by the mutant ICP4 protein. The mutant ICP4 protein may define a probe for one of the pathways of nuclear localization of proteins.

Little is known about the interactions between proteins and other cytoplasmic macromolecules during localization of the proteins into the cell nucleus. Some proteins are localized to the nucleus as a multimeric complex. These include the cellular protein nucleoplasmin (5), the adenovirus hexon protein (15, 43), the simian virus 40 large-T antigen (22), and the polyomavirus VP1 protein (R. Garcea, unpublished data). The adenovirus hexon forms a trimer in the cytoplasm, and this assembly requires the adenovirus 100,000-molecular-weight protein, which is not present in the final trimer or assembled capsid (10, 27, 37, 46). Assembly of the trimer and further conformational changes are required for nuclear localization of the hexon protein (18, 23). It is not known how formation of the complexes or the conformational changes might facilitate nuclear localization. Proteins that shuttle in and out of the nucleus have been identified (12), but it has not been determined whether these or other molecules serve as carrier molecules for proteins entering the nucleus. Some proteins destined for the nucleus seem to interact with the cytoskeleton (2, 33), but specific components of the cytoskeleton that function in the transport of proteins to the nucleus have not been identified.

The proteins encoded by herpes simplex virus (HSV) provide a system in which interactions between multiple proteins destined for the nucleus can be studied. Transcription, replication, and encapsidation of the viral genome occur in the nucleus of the infected cell, and many viral proteins are localized to the cell nucleus to participate in or regulate these events. HSV proteins are expressed in a series of coordinately regulated groups (reviewed in reference 36). Immediately after infection, the immediate early or α proteins are expressed. These gene products promote expression of the delayed early, or β, gene products and the late, or γ, gene products. The αICP4 gene product is required for optimal expression of β and γ gene products in infected (6, 19, 29, 44) or transfected cells (7, 9, 26, 35). The αICP0 gene product stimulates β gene expression in cotransfected cells (7, 9, 26, 35). The αICP27 gene product plays a role in regulating γ gene expression (38). The β gene products are involved in the replication of the viral genome which promotes the expression of the γ gene products. One of the β gene products, ICP8, promotes viral DNA replication (4, 28) and inhibits viral transcription (11).

In cells infected at the nonpermissive temperature with viruses encoding a temperature-sensitive (*ts*) αICP4 gene product, very low levels of expression of β and γ gene products are observed (6, 19, 29, 44). In transfected cells, either the αICP4 or αICP0 gene products can stimulate the expression of a β gene, but the two gene products can behave synergistically during the stimulation of expression of a β gene (7, 9, 35). It was suggested that, in cells infected with the ICP4 *ts* mutants, the mutant ICP4 molecules might be dominant over and inhibit the activity of the wild-type ICP0 (9, 26, 35). To investigate the interactions between ICP4 and ICP0, we examined the nuclear localization of ICP4 and ICP0 in cells infected with different viruses. We report here that the mutant ICP4 molecules can inhibit the nuclear localization of both ICP0 and another viral proein, ICP8.

MATERIALS AND METHODS

Cells and viruses. All infections and transfections were performed with Vero cells. The virus stocks were prepared and titrated as described previously (21). The original references and sources of the viruses are as follows: KOS1.1 wild-type (*ts*$^+$ [16]) and KOS1.1 *ts*756 (16) viruses were provided by M. Levine, University of Michigan; HFEM *ts*$^+$ (45) and HFEM *ts*LB2 (14) viruses were provided by I. Halliburton, University of Leeds, Great Britain; KOS *ts*$^+$ (42), KOS *ts*B2 (39), and KOS *ts*B21 (39) viruses were provided by P. Schaffer, Dana-Farber Cancer Institute, Boston, Mass. The *ts*$^+$ virus LG17 was obtained by marker rescue of *ts*LB2 by recombination between the *ts*LB2

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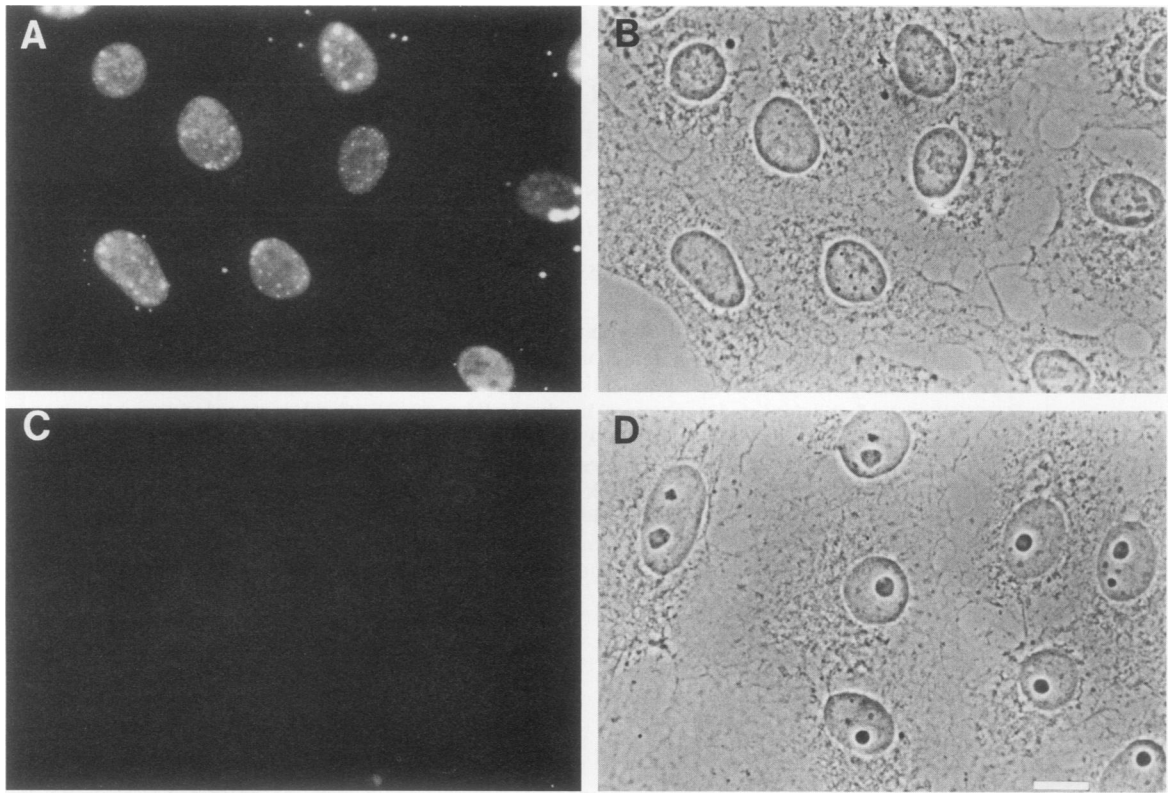


FIG. 1. Comparison of the 58S (anti-ICP4) antibody reaction with infected and uninfected cells. Vero cells were infected with HSV-1 KOS1.1 ts^+ virus or mock infected at 37°C. At 4 h postinfection, the cells were fixed, permeabilized, and incubated with 58S monoclonal antibody and rhodamine-conjugated goat anti-mouse immunoglobulin antibody. (A) Immunofluorescence micrograph, infected cells; (B) phase-contrast micrograph of the field shown in panel A; (C) immunofluorescence micrograph, mock-infected cells; (D) phase-contrast micrograph of the field shown in panel C.

genome and the HSV-2 *Xba*I-I DNA fragment arising from the S component (20). Dixon and Schaffer (6) reported that *ts*LB2 could be rescued by sequences in the *Bam*HI-Y DNA fragment. We isolated the ts^+ virus LB2*Rs*1 by marker rescue of *ts*LB2 by recombination between the *ts*LB2 genome and the cloned HSV-1 KOS1.1 *Bam*HI-Y DNA fragment (generously provided by P. J. Godowski). LB2*Rs*1 was isolated by three cycles of plaque purification at 39.5°C under agarose overlay (unpublished data).

Plasmids and transient expression. The plasmid pSG1-ES1 (35), which contains the complete ICP0 gene, was introduced into Vero cells by the calcium phosphate procedure described previously for transfection (35). Plasmid pBR325 DNA was used as the carrier DNA.

Indirect immunofluorescence. Cells were grown on 12-mm circular glass cover slips in glass shell vials (2.1 by 7 cm) closed with rubber stoppers. The cultures were infected with the indicated viruses at a multiplicity of infection of 20 in 0.2 ml of phosphate-buffered saline (pH 7.6)–0.1% glucose–1% heat-inactivated calf serum. After a 1-h adsorption period, the virus inoculum was removed, and 1 ml of medium 199–1% heat-inactivated calf serum–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.6) was added to each culture. The vials were then placed in a circulating water bath maintained at the appropriate temperature.

The fixation and staining procedures described previously (32) were used for this work. Microscopy was performed with a Zeiss standard microscope equipped for phase-contrast and fluorescence microscopy with a Neofluar 63× objective. Within each figure, micrographs with similar exposure periods were chosen so that relative intensities can be compared to determine the relative amounts of the proteins in the various compartments of the cell.

The H1083 (anti-ICP0) and H1113 (anti-ICP27) monoclonal antibodies (1) were kindly provided by L. Pereira, and the 58S (anti-ICP4) and 39S (anti-ICP8) monoclonal antibodies (40) were kindly provided by M. Zweig.

RESULTS

Localization of ICP4 and ICP0 in cells infected with *ts*756. To examine the interactions of ICP4 and ICP0, we determined the cellular localization of ICP4 and ICP0 in cells infected with the ICP4 mutant KOS1.1 *ts*756 (16). This mutant is classified in complementation group 1-2 (40), a group of viruses that encode defective ICP4 proteins. The lesion in this virus maps in or near the ICP4 gene (D. Knipe and J. Smith, manuscript in preparation). At the nonpermissive temperature, this virus expresses almost exclusively α gene products (11; unpublished data).

We examined the localization of ICP4 in infected cells by indirect immunofluorescence with the 58S monoclonal antibody (41). This antibody immunoprecipitates only ICP4 from infected cell extracts (41; C. Lee and D. Knipe, unpublished data). Using this antibody for indirect immunofluorescence, we observed nuclear fluorescence in cells infected with KOS1.1 ts^+ virus but little staining in mock-infected cells

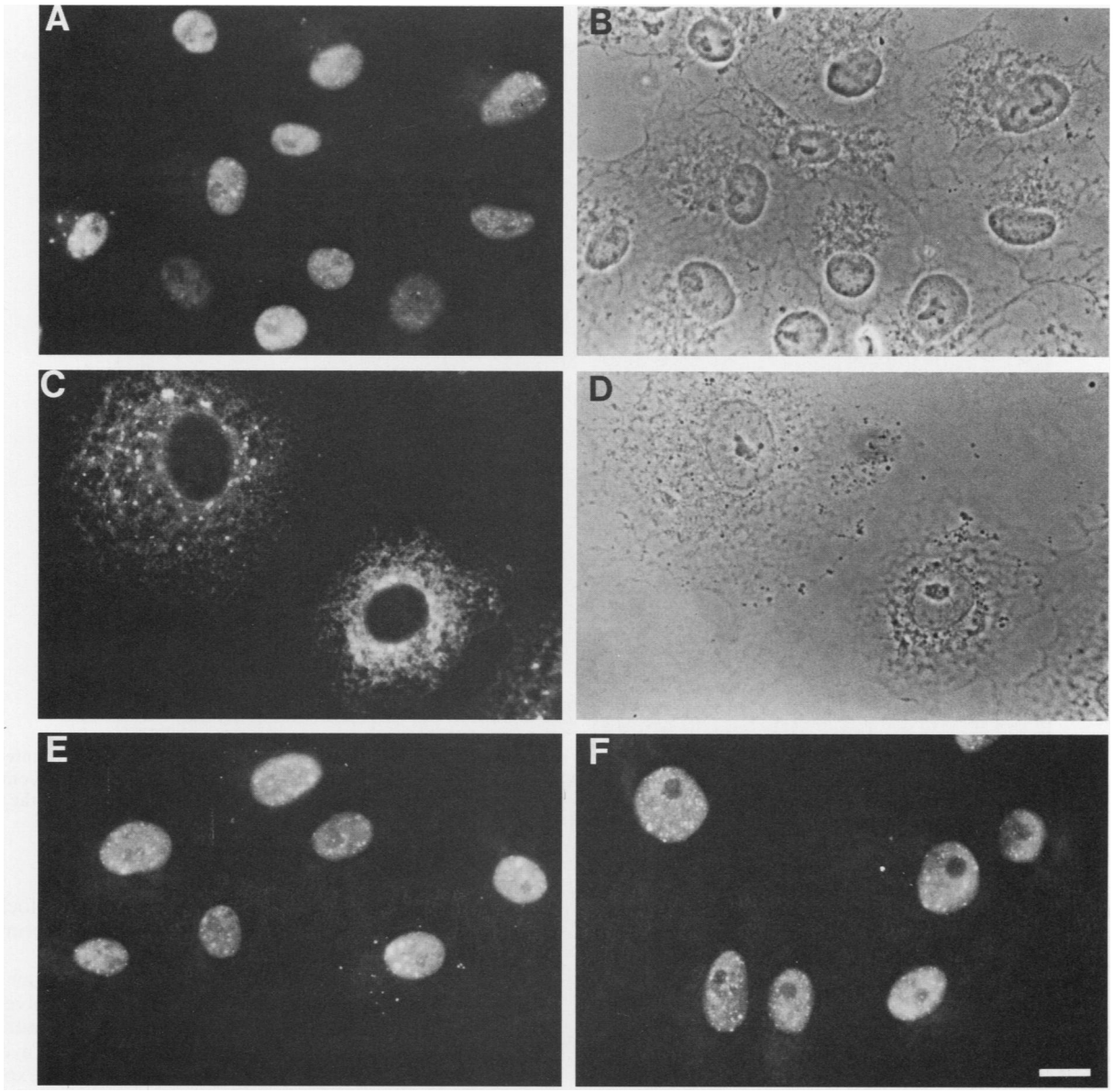


FIG. 2. Localization of ICP4 in cells infected with KOS1.1 ts^+ or *ts*756 virus. Cells were infected with KOS1.1 ts^+ or *ts*756 virus at 33 or 39.7°C. At 4 h postinfection, the cells were processed as described in the legend to Fig. 1. (A) Immunofluorescence micrograph, ts^+ infection at 39.7°C; (B) phase-contrast micrograph of the field shown in panel A; (C) immunofluorescence micrograph, *ts*756 infection at 39.7°C; (D) phase-contrast micrograph of the field shown in panel C; (E) immunofluorescence micrograph, ts^+ virus infection at 33°C; (F) immunofluorescence micrograph, *ts*756 virus infection at 33°C.

(Fig. 1). In cells infected with KOS1.1 ts^+ virus, ICP4 was localized to the nucleus at either the nonpermissive temperature (39.7°C; Fig. 2A) or the permissive temperature (33°C; Fig. 2E). In cells infected with KOS1.1 *ts*756 at 33°C, ICP4 was localized to the nucleus (Fig. 2F). However, at 39.7°C, ICP4 was distributed in the cytoplasm in large granules and a fibrous network (Fig. 2C). Therefore, the nuclear localization of the ts mutant ICP4 was temperature sensitive.

To examine the localization of ICP0, we performed indirect immunofluorescence by using the monoclonal antibody H1083 (1). This antibody reacts with ICP0 on Western blots (1), and it reacts with cells transfected with the cloned fragment of the HSV genome containing the ICP0 gene (see below). The antibody does not react with cells transfected with a plasmid containing a deleted ICP0 gene (unpublished data). We observed nuclear staining in cells infected with KOS1.1 ts^+ virus (Fig. 3A) but only a weak perinuclear staining in mock infected cells (Fig. 3C). ICP0 was also localized to the cell nucleus in cells infected with KOS1.1 ts^+ virus at 33 or 39.7°C (Fig. 4A and E). ICP0 was localized to the nucleus in cells infected with *ts*756 at 33°C (Fig. 4F), but it was localized in large cytoplasmic granules in cells infected with the mutant virus at 39.7°C (Fig. 4C). These granules were frequently perinuclear, but they were sometimes scattered throughout the cytoplasm in linear arrays (Fig. 4C). The fluorescent-staining granules coincided with dense granules visible by phase microscopy (Fig. 4D).

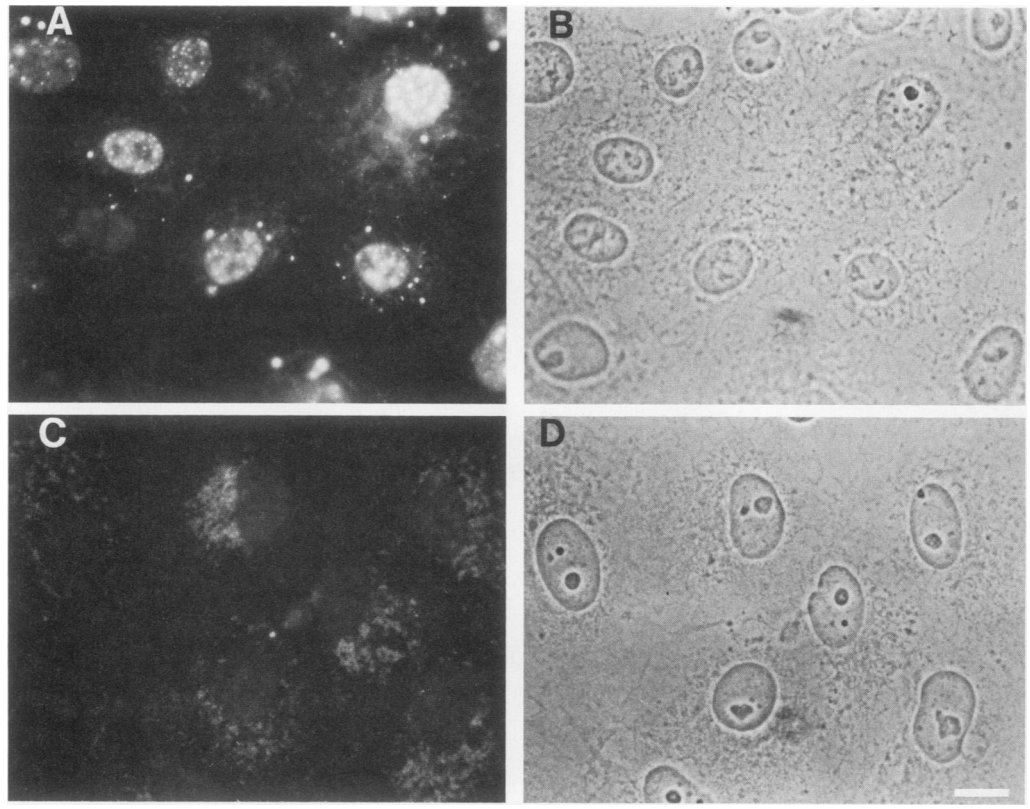


FIG. 3. Comparison of the H1083 (anti-ICP0) monoclonal antibody reaction with infected and uninfected cells. Vero cells were infected or mock infected and processed as described in the legend to Fig. 1. (A) Immunofluorescence micrograph, infected cells; (B) phase-contrast micrograph of the field shown in panel A; (C) immunofluorescence micrograph, mock-infected cells; (D) phase-contrast micrograph of the field shown in panel C.

Therefore, nuclear localization of ICP0 was also temperature sensitive in cells infected with *ts*756.

Localization of ICP4 and ICP0 with other ICP4 mutants. To determine whether the defect in ICP0 localization was a general property of ICP4 mutants, we examined cells infected with other ICP4 ts mutants. Nuclear localization of ICP4 was temperature sensitive in cells infected with HFEM *ts*LB2 (Fig. 5C and F), KOS *ts*B2, and KOS *ts*B22 (S. Rice and D. Knipe, unpublished data). The nuclear localization of ICP0 was also defective in cells infected with these mutant viruses. In cells infected with *ts*LB2 at 39.7°C, ICP0 accumulated in large perinuclear granules (Fig. 5D) similar to those in cells infected with *ts*756 at the nonpermissive temperature. Similar results were observed with *ts*B2 and *ts*B22, but nuclear localization of ICP4 and ICP0 was normal in cells infected with HSV-1 17 *ts*K (S. Rice and D. Knipe, unpublished data). Thus, this phenotype was observed for nearly all of the ICP4 *ts* mutants.

To confirm that the primary defect in localization was caused by the mutant ICP4 proteins, we examined ICP4 and ICP0 localization in cells infected with ts^+ rescued derivatives of *ts*LB2. The recombinant LG17, derived by marker rescue of *ts*LB2 with a wild-type HSV-2 DNA fragment from the S component (20), was used to infect cells. In these cells, nuclear localization of ICP4 (Fig. 5E) and ICP0 (Fig. 5F) was normal at 33 or 39.7°C (Fig. 5E and F). Similar results were obtained with the virus LB2*Rs*1, a ts^+ virus obtained by marker rescue of *ts*LB2 with a cloned HSV-1 DNA fragment (data not shown). Therefore, replacement of the mutated ICP4 gene sequences was sufficient to reverse the block in ICP0 localization, and ICP4 appeared to be the primary cause of the block in nuclear localization.

Nuclear localization of ICP8. To determine whether the nuclear localization of other HSV gene products involved in gene regulation was affected, we examined the nuclear localization of ICP8, the β DNA-binding protein. In cells infected with *ts*LB2 at 39.7°C, there was low-level expression of ICP8, the β DNA-binding protein. Some cells were observed to express substantial levels of ICP8, but it was blocked from localizing into the nucleus (Fig. 6A), in contrast to the nuclear location usually observed for this protein in infected or transfected cells (31, 33, 34). Therefore, at least one protein besides ICP0 was blocked for nuclear transport in these mutant-infected cells.

Nuclear localization of ICP27. To determine whether other α proteins were blocked for nuclear transport, we examined the localization of ICP27 using the H1113 monoclonal antibody (1) for indirect immunofluorescence. This antibody reacts wtih ICP27 on Western blots (1). In cells infected with KOS1.1 ts^+ virus at 37°C, nuclear fluorescence was observed (Fig. 7A), but little staining was observed in mock-infected cells (Fig. 7C). In contrast to ICP0, ICP27 was localized normally to the nucleus in cells infected with KOS1.1 ts^+ or *ts*756 viruses at 33 or 39.7°C (Fig. 8). These data indicate that nuclear localization of at least one viral protein was apparently normal in these mutant-infected cells.

Reversal of the block after temperature shift. We examined

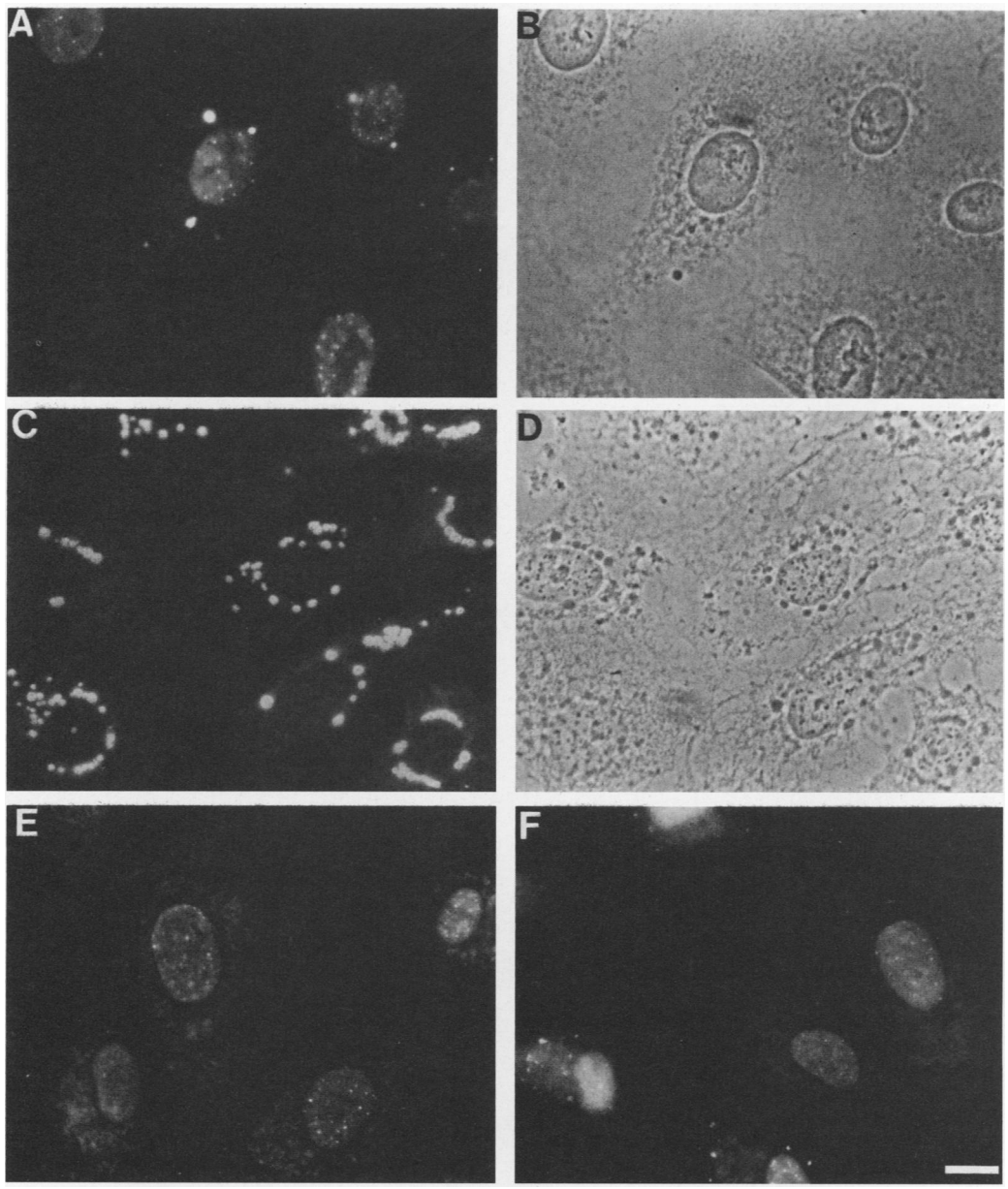


FIG. 4. Localization of ICP0 in cells infected with KOS1.1 ts^+ or *ts*756 virus. Cells were infected with ts^+ or *ts*756 virus at 33 or 39.7°C. At 4 h postinfection, the cells were fixed, permeabilized, and incubated with H1083 antibody and rhodamine conjugate. (A) Immunofluorescence micrograph, ts^+ infection at 39.7°C; (B) phase-contrast micrograph of the field shown in panel A; (C) immunofluorescence micrograph, *ts*756 infection at 39.7°C; (D) phase-contrast micrograph of the field shown in panel C; (E) immunofluorescence micrograph, ts^+ infection at 33°C; (F) immunofluorescence micrograph, *ts*756 infection at 33°C.

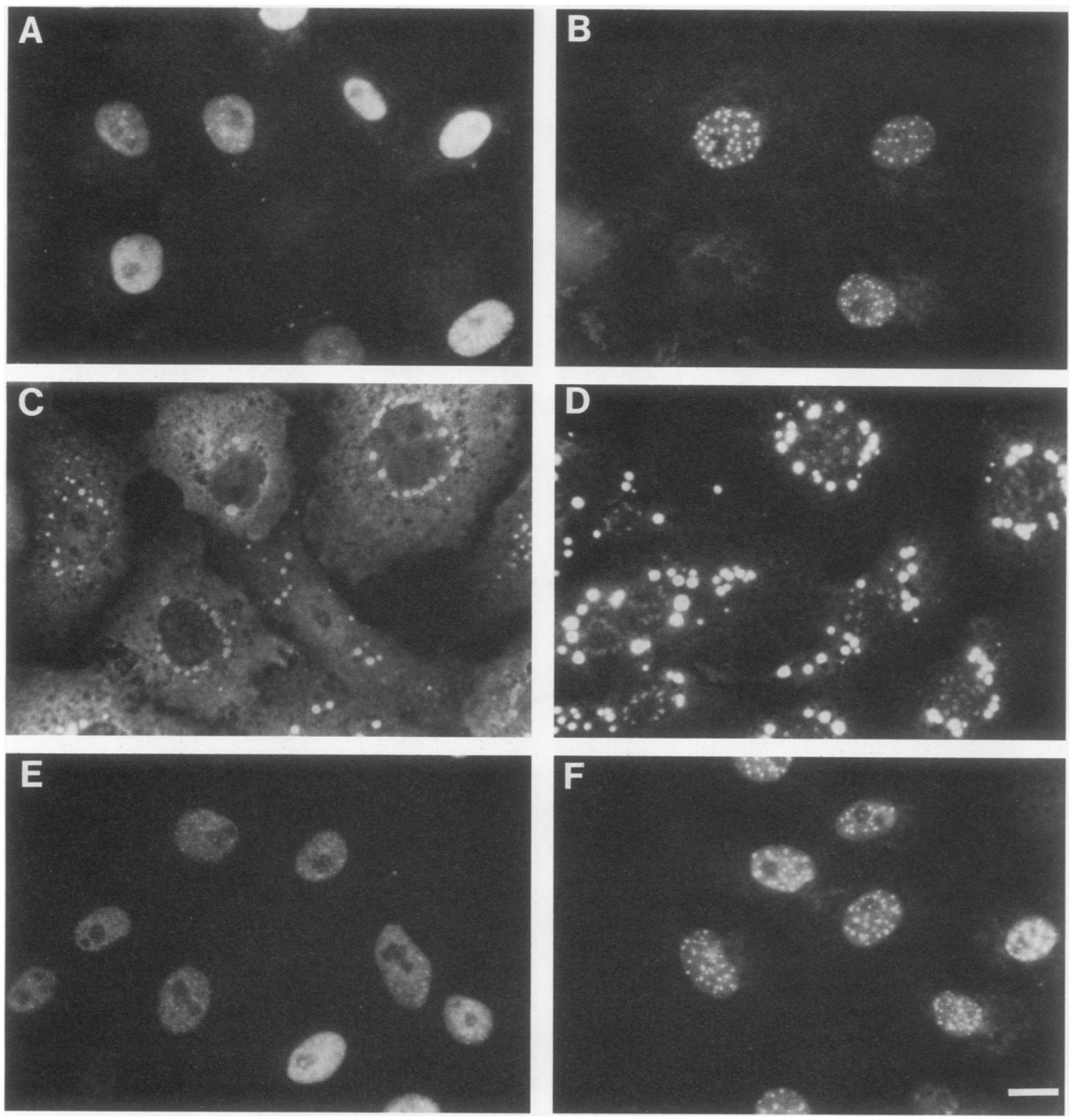


FIG. 5. Localization of ICP4 and ICP0 in cells infected with HFEM ts^+, HFEM *ts*LB2, or LG17 at 39.7°C. The immunofluorescence micrographs shown are the result of staining with 58S (anti-ICP4) or H1083 (anti-ICP0) antibodies. (A) ICP4 distribution, ts^+ infection; (B) ICP0 distribution, ts^+ infection; (C) ICP4 distribution, *ts*LB2 infection; (D) ICP0 distribution, *ts*LB2 infection; (E) ICP4 distribution, LG17 infection; (F) ICP0 distribution, LG17 infection.

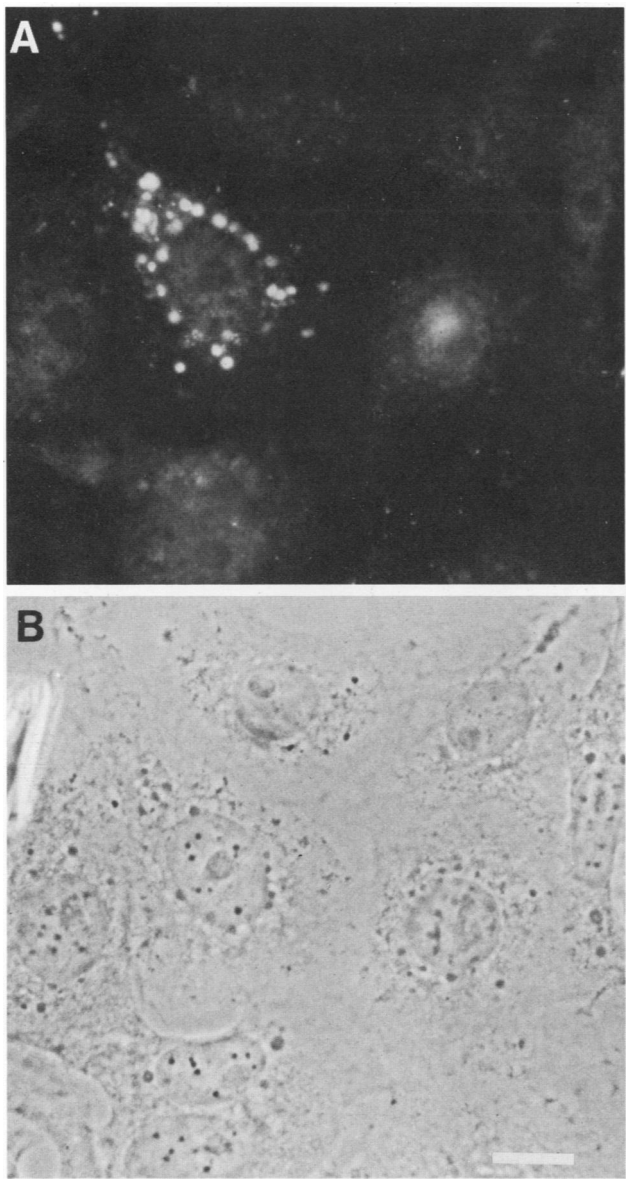


FIG. 6. Localization of ICP8 in cells infected with *ts*LB2. Cells were infected with *ts*LB2 at 39.7°C. At 4 h postinfection the cells were fixed, permeabilized, and incubated with the 39S monoclonal antibody and rhodamine conjugate. (A) Immunofluorescence micrograph; (B) phase-contrast micrograph of the field shown in panel A.

the distribution of ICP4 and ICP0 after temperature shift-down of infected cells to determine whether the block in nuclear localization was reversible. At 4 h post-infection, cycloheximide was added to cultures of cells infected with *ts*756 at 33 or 39.7°C, and some of the cultures were shifted from 39.7 to 33°C. ICP4 accumulated specifically in the nucleus within 2 h after the shift-down (Fig. 9B and C), but the distribution of ICP0 did not change significantly after the temperature shift (Fig. 9E and F). The difference in the behavior of these two proteins following shift-down may indicate that there is not a specific complex formed between the two proteins in the cytoplasm. Instead, the mutant ICP4 protein may block a pathway for nuclear transport of certain viral proteins.

Localization of ICP0 in transfected cells. To determine whether ICP0, independent of ICP4, is capable of localization to the nucleus in cells, we transfected cells with the plasmid pSG1-ES1, which contains the ICP0 gene but not the ICP4 gene (35). ICP0 was detected by immunofluorescence in a significant number of the transfected cells, and it was localized to the cell nucleus (Fig. 10A). ICP0 was expressed at high levels in these cells, and the nuclei contained large intranuclear granules that coincided with the ICP0 in the nucleus (Fig. 10A and B). Therefore, ICP0 can localize to the cell nucleus in cells where no ICP4 is present and in which ICP0 is expressed to high levels.

DISCUSSION

The nuclear localization of several viral proteins was inhibited in cells infected with certain HSV mutants encoding a temperature-sensitive ICP4 gene product. One other situation has been reported in which a mutant protein exhibited *trans*-dominant effects on nuclear localization. The simian virus 40 *cT* mutation causes a dominant defect in large-T antigen localization to the nucleus, but transport of other viral proteins is not affected (22). This is most likely the result of the coaggregation of mutant and wild-type T antigen (8, 13, 31). The wild-type ICP4 is believed to form a homodimer in infected cells (24), and the mutant ICP4 defect for nuclear localization is dominant over the wild-type phenotype (data not shown). However, ICP0 and ICP8 transport is also defective in these infected cells. ICP4 and ICP0 may form a functional complex in infected cells because their stimulation of β gene expression is synergistic. ICP0 and ICP8 apparently do not require ICP4 for nuclear localization, because they can localize to the nucleus in cells transfected with their individual genes. The mutant ICP4 could complex with ICP0 and prevent its normal transport. The inhibition is somewhat broader, in that ICP8 transport is also blocked in these cells. There is no evidence for a complex between ICP4 and ICP8, but this remains a possibility. Because the mutant phenotype is dominant, the inhibition of nuclear localization would appear to be the result of some activity of the mutant protein. One possibility is that the mutant ICP4 molecule binds to a transport molecule or part of the cytoplasmic structure and prevents their normal function in nuclear localization of HSV proteins. We are currently investigating whether the mutant ICP4 protein can inhibit the localization of nuclear proteins encoded by cells or other viruses. A second possibility is that mutant ICP4 inhibits nuclear localization of viral proteins by binding to those proteins with which the wild-type protein normally interacts in the nucleus.

The defect in localization is probably not due simply to the overexpression of α proteins in the mutant-infected cells. In cells infected with HSV 17 *ts*K at the nonpermissive temperature, α proteins are overproduced (29), but ICP4 and ICP0 are transported into the nucleus normally (D. Knipe and J. Smith, unpublished data). Thus, it appears that overexpression of α proteins is not sufficient to block localization of ICP0. The defect in localization or another property of the mutant ICP4 appears to be responsible for the dominant phenotype.

The fibrous staining pattern of ICP4 in the cytoplasm suggests that it may be bound to a part of the cytoskeleton or other cytoplasmic structure. Because the block in transport is readily reversed upon temperature shift-down of the infected cells, the mutant ICP4 is blocked either in the normal transport pathway or close to it. It will be of interest

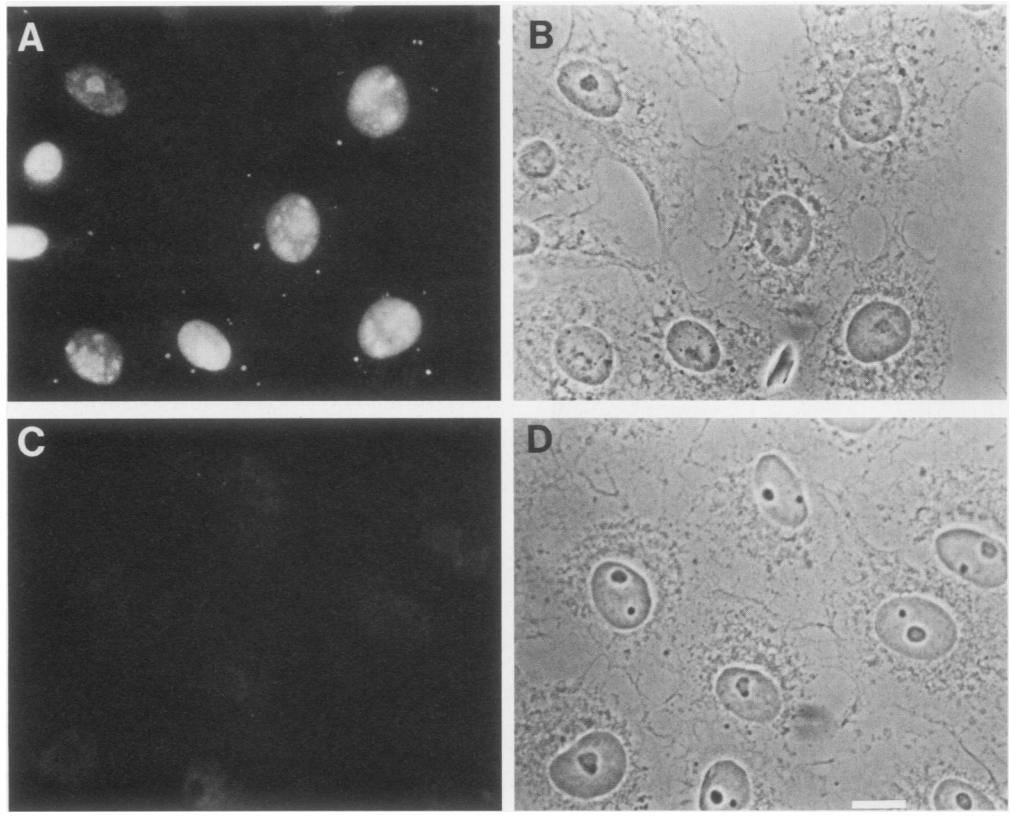


FIG. 7. Comparison of the H1113 (anti-ICP27) antibody reaction with infected and mock-infected cells. Cells were infected with KOS1.1 ts^+ virus or mock infected, fixed, and permeabilized as described in the legend to Fig. 1. They were then reacted with H1113 antibody and rhodamine conjugate. (A) Immunofluorescence micrograph, infected cells; (B) phase-contrast micrograph of the field shown in panel A; (C) immunofluorescence micrograph, mock-infected cells; (D) phase-contrast micrograph of the field shown in panel C.

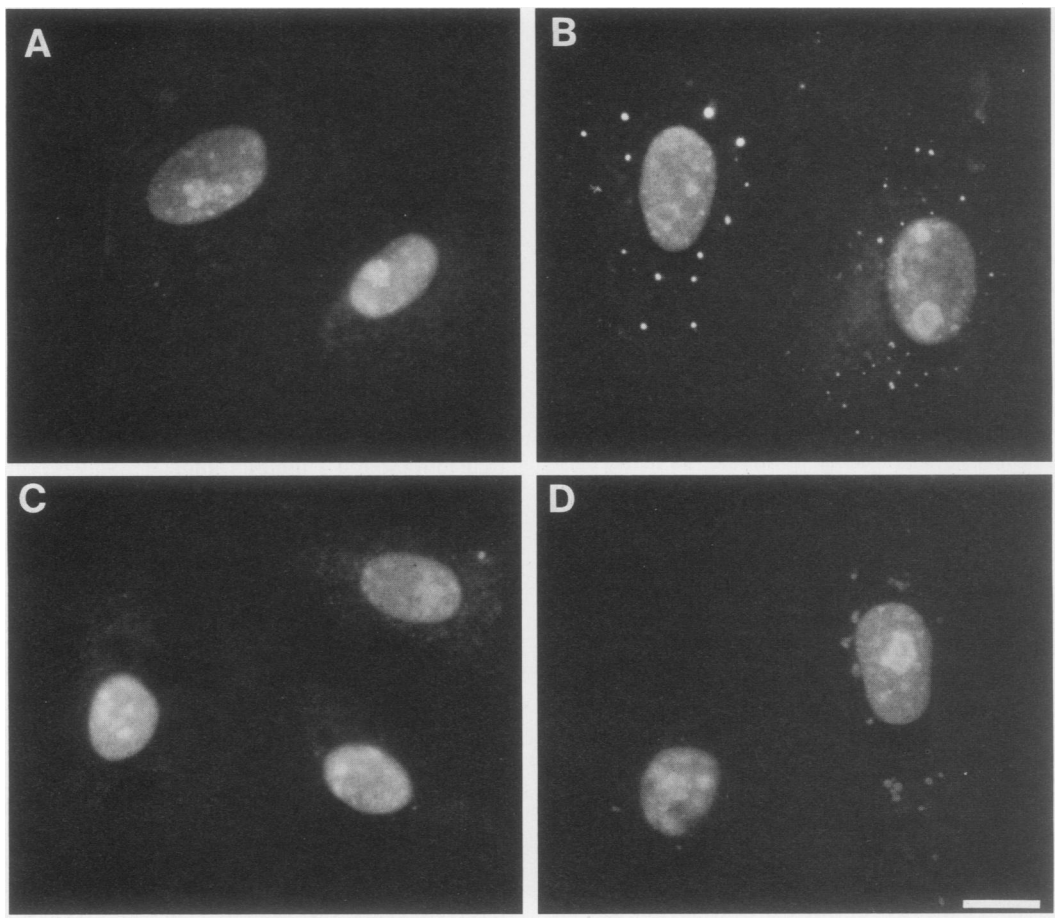


FIG. 8. Localization of ICP27 in cells infected with KOS1.1 ts^+ or *ts*756 virus. The immunofluorescence micrographs shown were the result of staining with H1113 antibody and rhodamine conjugate. (A) ts^+ virus infection at 33°C; (B) ts^+ virus infection at 39.7°C; (C) *ts*756 infection at 33°C; (D) *ts*756 infection at 39.7°C.

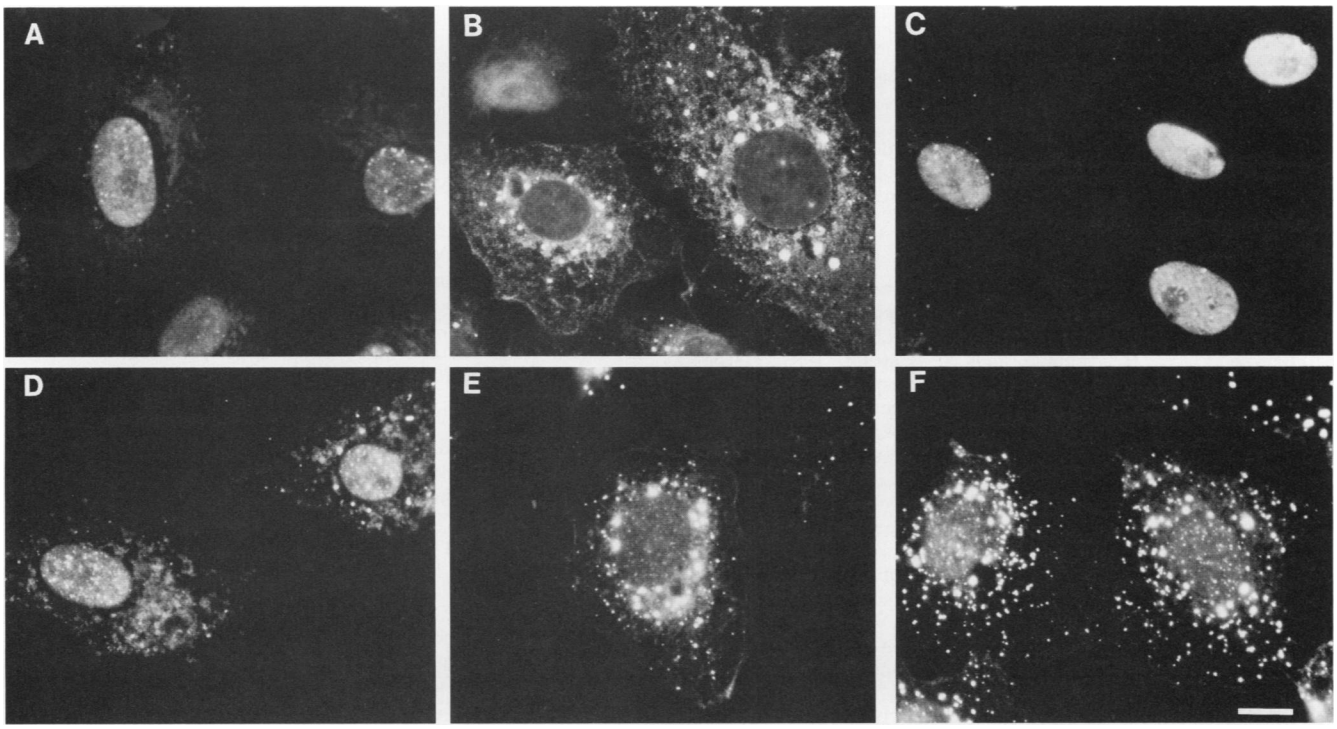
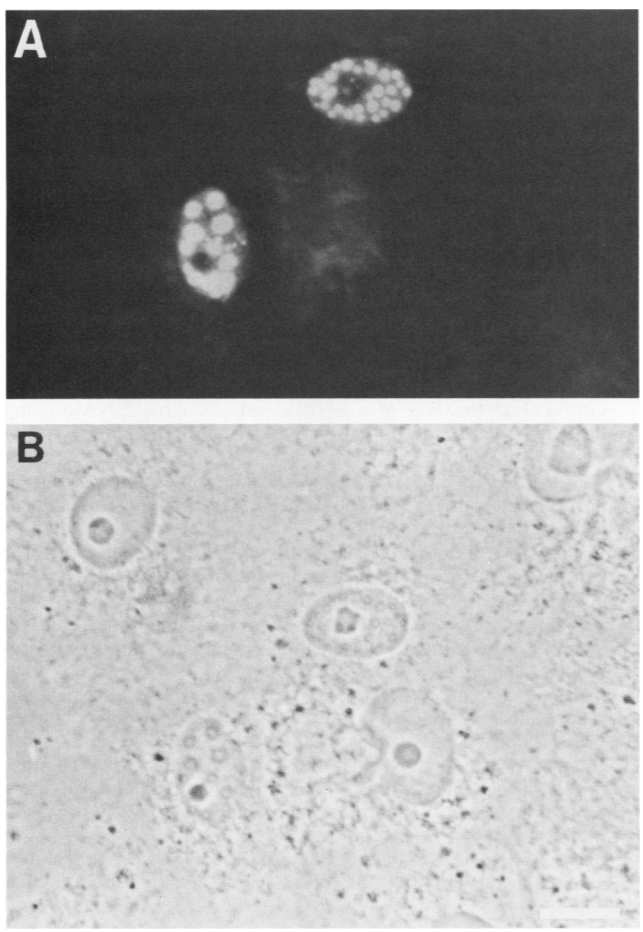


FIG. 9. Distribution of ICP4 and ICP0 after temperature shift-down of *ts*756 virus-infected cells. Replicate cultures infected with *ts*756 were maintained at 33 or 39.5°C for 4 h. At that time cycloheximide (50 μg/ml) was added in all cultures, and some of the cultures were shifted from 39.5 to 33°C. The cells were incubated for 2 h and then were fixed and stained with either 58S or H1083 antibody. (A) ICP4 distribution, 33°C infection; (B) ICP4 distribution, 39.5°C infection; (C) ICP4 distribution after the 39.5 to 33°C temperature shift; (D) ICP0 distribution, 33°C infection; (E) ICP0 distribution, 39.5°C infection; (F) ICP0 distribution after the 39.5 to 33°C temperature shift.



to determine whether there are specific cellular proteins that are bound to the mutant ICP4 molecules in the cytoplasm, because this approach may define cellular molecules that interact with a protein during nuclear transport. In contrast, the block in nuclear transport of ICP0 is not readily reversed upon shift-down. ICP0 appears to be aggregated into the large cytoplasmic granules in a form that cannot reenter the transport pathway. The nature and composition of these granules remain to be determined.

Alternate pathways for nuclear localization of HSV proteins. The ICP27 protein is not blocked for nuclear localization in the mutant-infected cells. This protein either does not interact with ICP4 or utilizes a different mechanism for nuclear localization. In either case, it appears that ICP27 interacts with different molecules from the other HSV proteins during entry into the nucleus. ICP27 has an apparent molecular weight of 58,000 to 63,000 (25, 30); thus, it may be small enough to diffuse through the cytoplasm or the nuclear pores or both (3), while the larger ICP4, ICP0, and ICP8 proteins may need to interact with transport molecules to get to and through the nuclear pores. Further studies are needed to compare the mechanisms of nuclear localization of the different HSV proteins and the amino acid signals (17) needed for their localization.

FIG. 10. Nuclear localization of ICP0 in transfected cells. Subconfluent monolayers of cells were transfected with pSG1-ES1 plasmid containing the ICP0 gene. After 48 h, the cells were fixed and stained with H1083 antibody and rhodamine conjugate. (A) Immunofluorescence micrograph; (B) phase-contrast micrograph of the field shown in panel A.

ACKNOWLEDGMENTS

We thank the members of our laboratory for comments on this manuscript and Lenore Pereira and Marty Zweig for their generous gifts of antibodies.

This research was supported by Public Health Service grant AI 20530 from the National Institute of Allergy and Infectious Diseases. D.M.K. was supported by a Faculty Research Award from the American Cancer Society.

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