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Nuclear Localization of Herpesvirus Proteins: Potential Role for the Cellular Framework

MARGARET P. QUINLAN AND DAVID M. KNIPE*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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Two herpes simplex virus proteins, the major capsid protein and the major DNA binding protein, are specifically localized to the nucleus of infected cells. We have found that the major proportion of these proteins is associated with the detergent-insoluble matrix or cytoskeletal framework of the infected cell from the time of their synthesis until they have matured to their final binding site in the cell nucleus. These results suggest that these two proteins may interact with or bind to the cellular cytoskeleton during or soon after their synthesis and throughout transport into the cell nucleus. In addition, the DNA binding protein remains associated with the nuclear skeleton at times when it is bound to viral DNA. Thus, viral DNA may also be attached to the nuclear framework. We have demonstrated that the DNA binding protein and the capsid protein exchange from the cytoplasmic framework to the nuclear framework, suggesting the direct movement of the proteins from one structure to the other. Inhibition of viral DNA replication enhanced the binding of the DNA binding protein to the cytoskeleton and increased the rate of exchange from the cytoplasmic framework to the nuclear framework, suggesting a functional relationship between these events. Inhibition of viral DNA replication resulted in decreased synthesis and transport of the capsid protein. We have been unable to detect any artificial binding of these proteins to the cytoskeleton when solubilized viral proteins were mixed with a cytoskeletal fraction or a cell monolayer. This suggested that the attachment of these proteins to the cytoskeleton repesents the actual state of these proteins within the cell.

Specific pathways and mechanisms have evolved to ensure that cellular proteins are processed and arrive at the proper destination within or outside of the cell. A major internal compartment of eucaryotic cells is the cell nucleus, and thus specific mechanisms could exist for the nuclear localization of proteins. However, unlike the situation with membrane-bound or secreted proteins (6), very little is known about the mechanism of localization of proteins to the cell nucleus (7). As a model system for the study of the association of proteins with the cell nucleus, we have been studying two proteins encoded by herpes simplex virus (HSV) (19). The two proteins are the major DNA binding protein (ICP8) and the major capsid protein (ICP5). The major DNA binding protein is a beta or delayed early protein with a molecular weight of approximately 130,000 (2, 27, 29, 34). It binds to single-stranded DNA more tightly and efficiently than to double-stranded DNA (19, 29) and can melt a polydeoxyadenylate-polydeoxythymidylate homopolymer (26). Mutants whose lesions map in or near the ICP8 gene (10) or which encode an altered DNA binding protein (26) are defective for viral DNA replication. Therefore, the protein is believed to be essential for viral DNA replication. It is not a major virion component. In contrast, the major capsid protein has a molecular weight of approximately 150,000 and is expressed as a late protein (16). It is assembled in the nucleus into the viral capsid. Both proteins are synthesized in the cytoplasm and accumulate in the infected-cell nucleus (12, 25).

A series of stages in the association of these two proteins has been defined (19). ICP8, for example, is found in the cytoplasm immediately after synthesis but quickly associates with the crude nuclear fraction. At early times after this nuclear association, detergent treatment will remove ICP8 from the crude nuclear fraction, suggesting that the protein is attached to the outside of the nucleus. It then chases into a nucleus-associated, detergent-resistant form and later to a nuclear DNase-sensitive form. The latter association is believed to be with replicating viral DNA because inhibition of viral DNA replication prevents the DNase-sensitive stage. These results suggested that there is a sequence of binding sites for ICP8 during nuclear trans-

port. More important was the observation that the protein may bind to structural elements of the nucleus (19; Quinlan and Knipe, unpublished data). In this paper we report experiments which were designed to determine when ICP8 and ICP5 interact with structural elements of the host cell during their maturation to the cell nucleus. We have used techniques involving gentle detergent extractions of cells to define biochemically the detergent-soluble and -insoluble matrix fractions (8, 9, 11, 14, 21). We have found that after detergent extraction of infected cells ICP8 and ICP5 remain with the insoluble cytoskeletal framework from their time of synthesis until they are bound to structural elements of the cell nucleus. This suggests that these proteins may be bound to the structural framework of the cell during nuclear localization.

MATERIALS AND METHODS

Cells and viruses. HSV type 1 strain mP (15) was grown and titrated as described previously (19). All experimental infections were performed with Vero cells.

Labeling of cells. Uninfected Vero cell monolayer cultures were labeled for 14 to 16 h with 6 μCi of [^{35}S]methionine (New England Nuclear) per ml of minimal essential medium containing one-half the normal level of methionine. To label viral proteins, infected cells were labeled at 4 h postinfection for the specified time intervals with 12 μCi of [^{35}S]methionine per ml of methionine-free minimal essential medium supplemented with 1.0% dialyzed calf serum. In some experiments the cultures were then subjected to chase conditions with 1.0 mM methionine. Incorporation of [^{35}S]methionine into host cell or viral proteins in each subcellular fraction was quantified by trichloroacetic acid precipitation of portions of each fraction.

Extraction procedures. Cells were washed with phosphate-buffered saline at the temperature indicated for the extraction. Cells were extracted for 2.5 min at 4.0°C with buffer A {10 mM PIPES [piperazine-*N*, *N*'-bis(2-ethanesulfonic acid)], pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride [PMSF], 100 kallikrein units of aprotinin per ml, 1% Triton X-100} by the procedure of Cervera et al. (9) and Boss et al. (8). Extraction of cells with buffer B (buffer A plus 1 mM $CaCl_2$) was also performed by the method of Cervera et al. (9). Extraction of cells with buffer C [100 mM PIPES, pH 6.8, 1 mM $MgSO_4$, 2 mM ethylene glycol-bis(β-aminoethylether)-*N*, *N*-tetra-acetic acid, pH 6.9, 2 M glycerol, 0.1% Nonidet P-40, 1 mM PMSF, 100 kallikrein units of aprotinin per ml] was performed at 22°C by the procedure of Duerr et al. (11). Extraction of cells with buffer D (100 mM PIPES, pH 6.9, 0.5 mM $MgCl_2$, 4 M glycerol, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 100 kallikrein units of aprotinin per ml) was performed at 37°C by the procedure of Heuser and Kirschner (14).

The insoluble fraction from extraction with buffer A was divided into the cytoplasmic and nuclear components by the procedure of Lenk and Penman (21) as modified by Boss et al. (8). We minimized proteolysis in the resulting fractions by immediate precipitation of proteins from the soluble and cytoplasmic fractions with 9 volumes of acetone. The acetone precipitates were dissolved in 60 mM Tris-hydrochloride (pH 7.6)–2% sodium dodecyl sulfate–0.5% β-mercaptoethanol–1 mM PMSF and reprecipitated with 2.5 volumes of ethanol. The ethanol precipitates and the insoluble framework were dissolved in gel sample buffer (60 mM Tris-hydrochloride, pH 7.6, 2% sodium dodecyl sulfate, 20% glycerol, 0.002% bromophenol blue, 0.5% β-mercaptoethanol, 1 mM PMSF) and subjected to electrophoresis on 9.25% polyacrylamide gels as described previously (19). Autoradiography and quantitation of specific protein bands were performed as described previously (19).

RNA extractions. The detergent-insoluble matrices were first treated with DNase I at 20 μg/ml for 15 min at 0 to 4°C to lower the viscosity. The soluble and insoluble fractions were brought to 0.2 M NaCl–10 mM EDTA–1% sodium dodecyl sulfate. Two phenol-chloroform-isoamyl alcohol extractions and two chloroform-isoamyl alcohol extractions were performed. The nucleic acids were recovered by ethanol precipitation and resuspended in 10 mM Tris-hydrochloride (pH 7.6)–10 mM EDTA.

Sucrose gradient velocity centrifugation. Sucrose gradients (15 to 30%) were made in 10 mM Tris-hydrochloride (pH 7.6)–100 mM NaCl–1 mM PMSF for RNA analysis or 10 mM PIPES (pH 6.8)–100 mM KCl–2.5 mM $MgCl_2$–1 mM PMSF for insoluble matrix analysis. After the layering of the samples, the gradients were subjected to centrifugation for 17 h at 30,000 × *g* and 4°C in a Beckman SW41 rotor. Fractions of 0.5 or 1.0 ml were collected. For detection of RNA, the absorbance at 260 nm was measured in a Gilford 250 spectrophotometer. The areas of the peaks resulting from 28S rRNA or 4 to 5S RNA were derived by planimetry. For analysis of the proteins in the gradient fractions from the cytoskeleton, sodium acetate was added to 0.2 M and the proteins were recovered by ethanol precipitation. The ethanol precipitates were dissolved in gel sample buffer, and the samples were subjected to gel electrophoresis.

RESULTS

Fractionation of HSV-infected cells. We have previously reported that two HSV proteins, the major DNA binding protein and the major capsid protein, are localized to the cell nucleus and appear to interact with structural elements of the nucleus (19). To determine when these proteins become associated with structural elements of the cell, we performed a series of experiments in which we fractionated infected cells by several extraction procedures designed to preserve the structural elements of cells in a detergent-insoluble fraction (8, 9, 11, 14, 21). The soluble components of the cell are recovered in the detergent-released fraction. The insoluble fraction contains many of the cytoplasmic and nuclear fibrous and structural elements and has been referred to as the "cytoskeletal framework" of the cell. Monolayer cultures of Vero cells infected with HSV-1 strain mP virus were labeled with [^{35}S]methionine for 15 min at 4 h postinfection

TABLE 1. Distribution of host and viral proteins by different extraction procedures

Extraction[a]	% Insoluble				
	Total host cell proteins		Total viral proteins	ICP5	ICP8
	Mock infected	Infected			
Buffer A, 4°C	52	51	42	95	85
Buffer B, 4°C	48	45	42	89	64
Buffer C, 22°C	10	11	24	72	68
Buffer D, 37°C	10	18	18	66	63
Scraped cells, buffer A, 4°C	23	ND[b]	17	35	39

[a] Unless otherwise noted, the extractions were performed on cell monolayers. See text for compositions of extraction buffers.
[b] ND, Not determined.

and fractionated by the different protocols described in Materials and Methods. The amounts of the DNA binding protein (ICP8) and the capsid protein (ICP5) in each fraction were determined (Table 1). With all protocols for extraction of a cell monolayer, the majority of the ICP5 (60 to 95%) and ICP8 (65 to 85%) proteins was found in the detergent-insoluble fraction, although 50 to 90% of the total incorporated radioactivity was extracted in the soluble fraction (Table 1). We also observed that the amount of ICP5 and ICP8 remaining with the detergent-insoluble fraction varied from 60 to 90% when the same extraction procedure was used in repetitive trials. Thus, these two viral proteins appeared to be enriched in the insoluble fraction.

These extraction procedures yielded comparable results for the fractionation of infected or uninfected cells in that similar distributions of incorporated radioactivity were observed in subcellular fractions from cells labeled with [^{35}S]methionine and then infected or mock infected (Table 1). Also, the distribution of host cell protein species was identical in subcellular fractions from infected and mock-infected cells (Fig. 1). Thus, the structure of infected cells had not been altered from that of control cells by 4 h postinfection as assayed by these fractionation procedures.

Characterization of a specific fractionation protocol. We chose one of the procedures described to further analyze the maturation of viral proteins. We observed that extraction of infected or uninfected cells by the method of Cervera et al. (9) led to a detergent-insoluble matrix consisting of a nuclear structure surrounded by a diffuse cytoplasmic skeleton as seen by phase microscopy (not shown). To better characterize this fractionation protocol, we examined the distri-

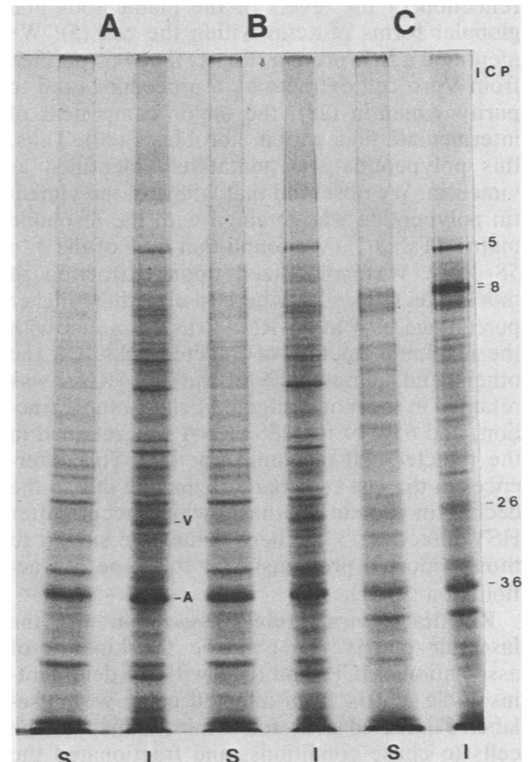

FIG. 1. Distribution of host cell and viral polypeptides in subcellular fractions of infected and mock-infected cells. Extraction was with buffer A at 0 to 4°C for 3 min. Shown is the autoradiogram of the gel in which the proteins from each fraction were subjected to electrophoresis. (A) Host cell polypeptides from mock-infected cells, 4 h post-mock infection; (B) host cell polypeptides from infected cells, 4 h postinfection; (C) viral polypeptides, 4 h postinfection. S, Soluble fraction; I, detergent-insoluble fraction. The conditions for labeling host cell or viral proteins are described in the text. A, Actin; V, vimentin.

TABLE 2. Characterization of subcellular fractions[a]

Marker	% of total in each fraction			
	Mock infected		Infected	
	Soluble	Insoluble	Soluble	Insoluble
Actin	49	51	55	45
Vimentin	2	98	2	98
4–5S RNA	58	42	73	27
28S rRNA	12	88	34	66

[a] Subcellular fractions from extraction of a monolayer with buffer A at 4°C.

bution of several cellular biochemical markers by the fractionation of infected cells and mock-infected cells. We observed that the actin protein was approximately equally distributed in the soluble and insoluble fractions of both sets of cells (Table 2; Fig. 1). This distribution is consistent with that observed previously (9) and is a reflection of the levels of the filamentous and globular forms of actin within the cell (5). We identified a 58K protein (Fig. 1) that was purified from Vero cell extracts by a procedure used to purify vimentin (28), the major component of intermediate filaments in fibroblasts (20). Thus, this polypeptide was tentatively identified as vimentin. We observed that >98% of the vimentin polypeptide was retained with the insoluble matrix (Table 2). We found that 60% of the 4 to 5S RNA was solubilized upon extraction of mock-infected cells, whereas a slightly higher percentage of 4 to 5S RNA was extracted with the insoluble fraction of infected cells. On the other hand, almost 90% of the 28S rRNA was retained in the mock-infected-cell insoluble fraction, and 65% of the 28S rRNA was retained in the infected-cell insoluble fraction. The differences in the last two markers may be due to the decline in protein synthesis which occurs after HSV infection (33). These values are similar to those reported previously for this type of fractionation (9, 21).

Kinetics of viral protein association with the insoluble matrix. To examine the kinetics of association of ICP5 and ICP8 with the detergent-insoluble matrix from infected cells, we pulse-labeled infected cells for 5 min, subjected the cells to chase conditions, and fractionated the cells at different times. Immediately after the 5-min pulse-label, the two proteins were associated with the insoluble cytoskeleton framework (Fig. 2). The association of these two proteins with the cytoskeleton continued through 120 min of chase. We have shown previously that by 120 min of chase the majority of ICP8 is associated with viral DNA in the cell nucleus (19). Therefore, ICP8 is associated with the insoluble matrix throughout the period of time during which it is being localized to the nucleus. These and the previous results also imply that viral DNA with attached ICP8 is bound to the nuclear framework. These results suggest that ICP8 and ICP5 may, in fact, be bound to structural elements of the cell during nuclear localization.

Exchange from the cytoplasmic to nuclear cytoskeleton. Lenk et al. (22) and Boss et al. (8) have further separated the detergent-insoluble cytoskeleton into the nuclear and cytoplasmic components by Dounce homogenization in the presence of detergents. We used this protocol to further define the location of ICP8 on the detergent-insoluble cytoskeleton. We compared the protein profiles of infected cell nuclei isolated by mechanical homogenization of cells with that of the nuclear detergent-insoluble matrix, and we found the proteins of the latter to be a subset of the proteins in the former (Fig. 3). We therefore believed that the nuclear insoluble skeleton was a valid measure of association with the cell nucleus. We observed that, after a 5-min pulse-label, most of the ICP8 was associated with the cytoplasmic insoluble cytoskeleton (Fig. 4A). ICP8 then chased from the cytoplasmic skeleton to the nuclear skeleton without passing through a detectable soluble intermediate phase. This supported the contention that ICP8 interacts with the cytoskeleton throughout its localization to the nucleus.

Enhancement of nuclear transport of ICP8. To establish a functional correlation between the cytoskeleton and nuclear transport of herpesviral proteins, we examined conditions which alter the nuclear transport of these viral proteins. We examined the subcellular distribution of ICP8 and ICP5 under infection conditions in which viral DNA replication is blocked. This situation enhanced the rate and extent of nuclear transport of ICP8 but blocked the nuclear transport of ICP5 (19). In the presence of phosphonoacetate, which effectively blocks HSV DNA replication, the association of ICP8 with the cytoskeleton was greater than in control cells (Fig. 4B). Virtually no ICP8 was detected in the soluble fraction from cells incubated with phosphonoacetate; approximately 10% of ICP8 was always detected in the soluble fraction from control cells (Fig. 4A). In addition, the rate of exchange from the cytoplasmic to the nuclear framework was increased in the presence of phosphonoacetate (Fig. 4A and B). Thus, inhibition of viral replication enhanced association of ICP8 with the cytoskeleton and increased the rate of nuclear cytoskeletal exchange and the rate of nuclear transport of ICP8 (19), suggesting a correlation between these events. On the other hand, ICP5 was attached to the cytoskeleton in the absence of viral DNA replication but associated with the nucleus very slowly (Fig. 5). From these results, it is clear that binding to the

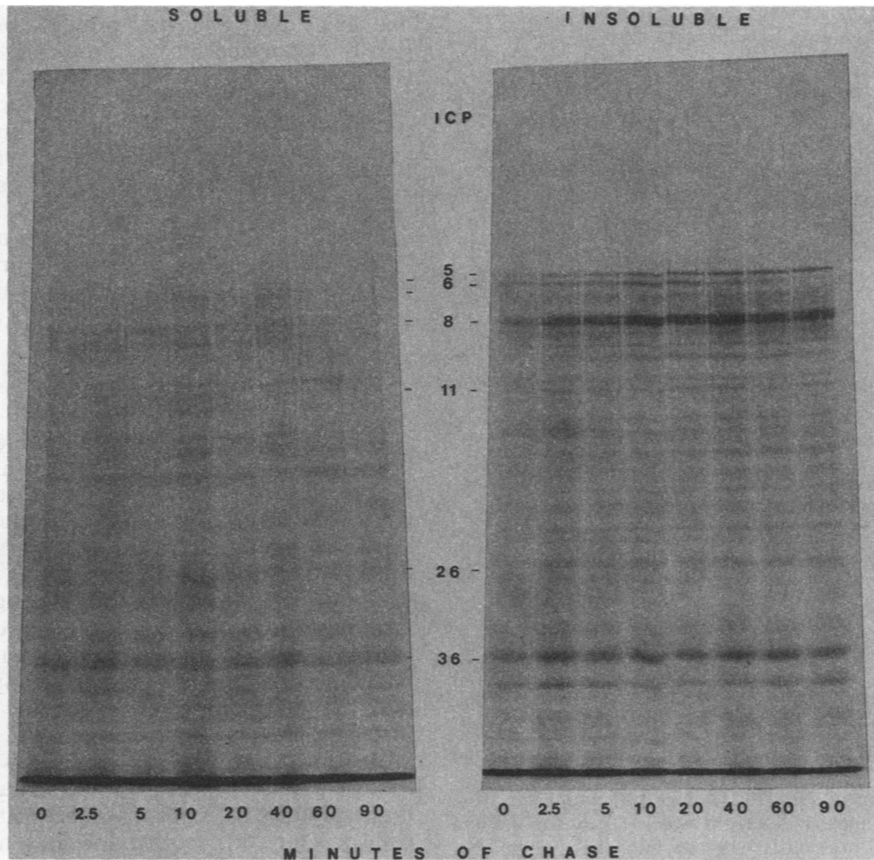

FIG. 2. Distribution of viral polypeptides in subcellular fractions from infected cells subjected to chase conditions for different periods of time. Infected cultures (4 h postinfection) were labeled for 5 min before the start of the chase period. At the times of the chase period indicated, the cells were fractionated as described in the legend to Fig. 1. Shown is the autoradiogram of the gel in which the proteins were subjected to electrophoresis.

cytoskeleton is not sufficient for transport of ICP5 to the nucleus.

Specificity of attachment of ICP8 and ICP5. We performed a series of experiments to examine the specificity of the association of ICP8 and ICP5 with the detergent-insoluble matrix. First, we designed a series of reconstitution trials in which labeled soluble fractions either were mixed with unlabeled insoluble fractions or were used to extract a new monolayer of cells. Alternatively, labeled cytoplasmic extracts were prepared, detergent was added, and the mixture was added to an insoluble matrix or cell monolayer. When the matrix fractions were isolated, they generally contained 10 to 15% of the labeled proteins from the supernatant (Table 3). Therefore, ICP5 and ICP8 did not nonspecifically associate with the cytoskeleton fraction to a significant extent under these conditions. Second, we studied the form of ICP8 and its solubility by centrifugation of the detergent-insoluble matrix fraction through sucrose gradients. We scraped the insoluble matrix material from the culture dish and layered it on a sucrose gradient prepard in extraction buffer. After centrifugation, ICP8 was largely at the top of the gradient, sedimenting in 4 to 10S structures (Fig. 6). Some ICP8 did sediment to the bottom of the gradient, but much of this was eliminated by removal of the nuclear cytoskeleton before centrifugation (not shown). This experiment shows that the association of ICP8 with the cytoplasmic cytoskeleton or the cytoskeleton itself was disrupted by removal from the culture dish. Therefore, ICP8 did not appear to be aggregated into large insoluble complexes with itself or other cytoplasmic components.

Consistent with the idea that the cytoskeleton or the linkage of ICP8 is very sensitive to mechanical forces was an experiment shown in Table 1. When we scraped cells from the culture dish and then fractionated them, we observed

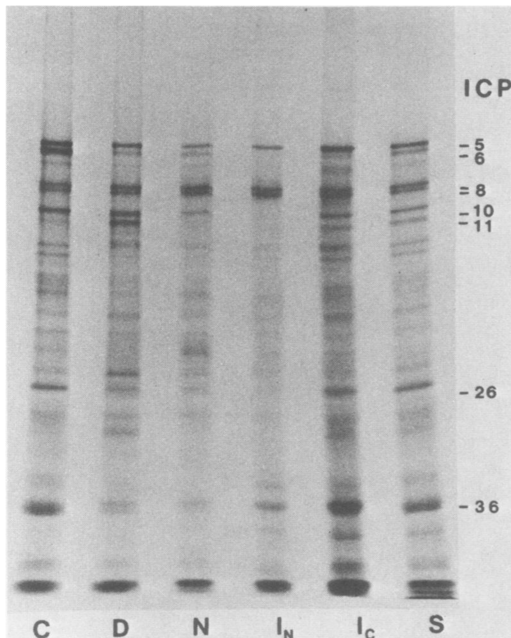

FIG. 3. Comparison of the distribution of viral polypeptides in subcellular fractions derived by mechanical homogenization or gentle detergent extraction of infected cells. Infected cultures were labeled for 30 min at 4 h postinfection with [^{35}S]methionine and fractionated into the nuclear (N), cytoplasmic (C), or crude nuclear detergent wash (D) by the method described previously (19) or into the soluble (S), nuclear insoluble (I_N), or cytoplasmic insoluble (I_C) fraction by the protocol described in the text.

that many host and viral proteins were more readily solubilized by this protocol. ICP8 was also more extensively solubilized by this protocol. Either the increased physical shear forces or the altered cell structure in the suspended cells led to an altered distribution upon fractionation. These results all argue that ICP8 does not precipitate into a large insoluble mass or adhere nonspecifically to cellular structures under our extraction conditions. Instead, the protein appears to be specifically attached to the detergent-insoluble fraction of the infected cell.

DISCUSSION

We have shown that two HSV proteins, destined for the infected cell nucleus, are associated with the detergent-insoluble cytoskeletal framework immediately after and possibly during their synthesis. Their association with the structural framework is maintained throughout transport to the nucleus and even upon binding at their final sites in the nucleus. We could detect no soluble phase for these proteins during their maturation to the cell nucleus, implying that they do not simply diffuse into the nucleus. Instead, there may be a specific route for these proteins involving the cellular framework. The lack of a soluble phase for the maturation of these proteins is similar to the maturational pathways for other proteins localized to specific sites in the cell. For example, viral membrane proteins (17) and cellular secreted and mem-

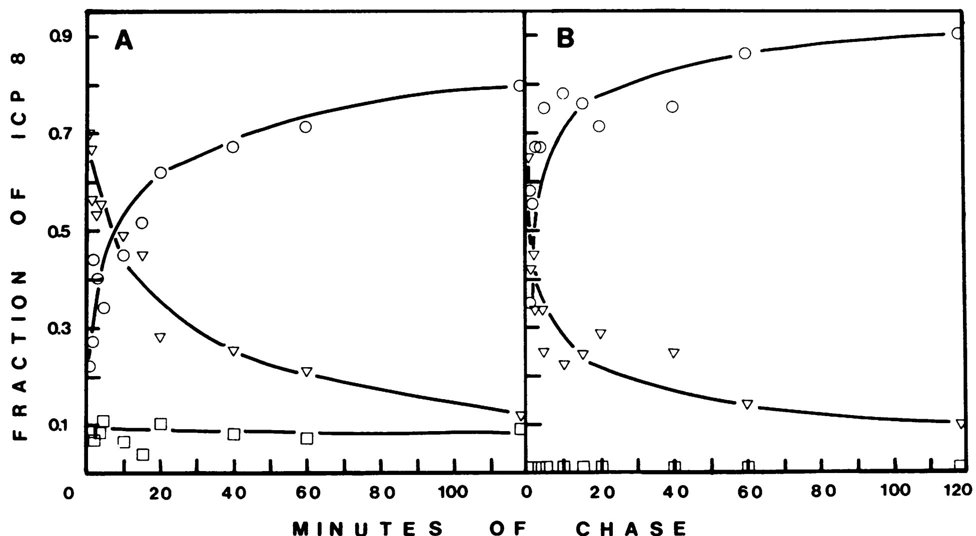

FIG. 4. Kinetics of association of ICP8 with the soluble, cytoplasmic insoluble, and nuclear insoluble fractions of infected cells in the absence (A) or presence (B) of 400 μg of phosphonoacetate per ml of medium. Infected cultures were labeled and subjected to chase conditions as in the legend to Fig. 2. At the chase times indicated, the cells were fractionated into soluble (□), cytoplasmic insoluble (▽), and nuclear insoluble (○) fractions. The amount of ICP8 in each fraction was then quantified as described in the text.

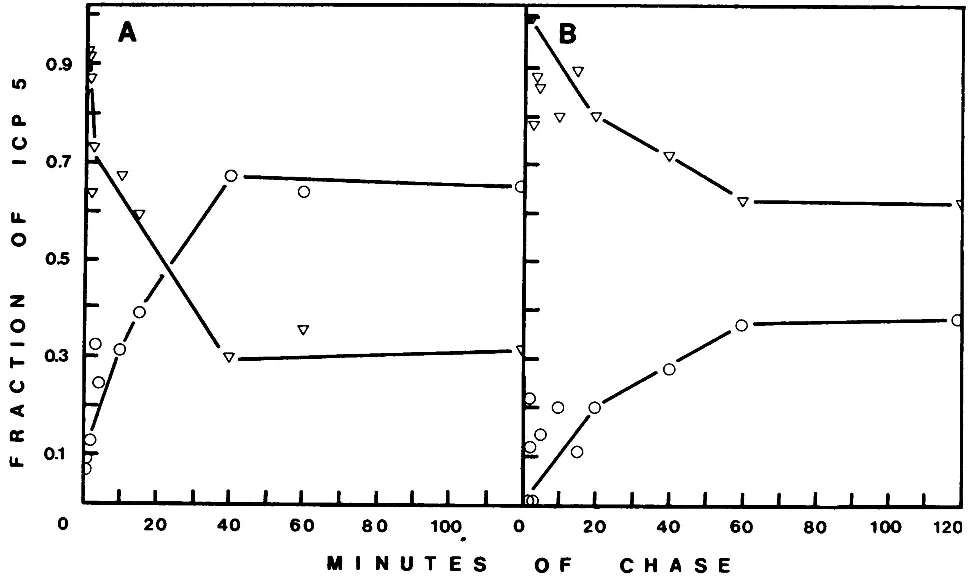

FIG. 5. Kinetics of association of ICP5 with subcellular fractions. The analysis was identical to that used for Fig. 4. ICP5 was examined in cells infected in the absence (A) or presence (B) of phosphonoacetate. ICP5 was not detected in the soluble fraction. Symbols: ▽, cytoplasmic insoluble fraction; ○, nuclear insoluble fraction.

brane proteins (6) are never found in a soluble form within cells. Instead, they are immediately inserted into the membrane of the endoplasmic reticulum (31). In addition, cytoskeletal proteins may attach to the cytoskeleton immediately upon synthesis and migrate away from their site of synthesis (13).

Cytoplasmic cytoskeletal framework. The cytoskeletal framework in the cytoplasm has been shown to support the ribosomes and translational apparatus of the cell (22). We observed that the two herpesviral proteins fractionate with the cytoplasmic framework immediately after their synthesis. Thus, they may be bound to the framework during or immediately after their synthesis. It is conceivable that these viral proteins are transiently soluble, and we could not prevent the binding of the proteins by placing the cultures at 0 to 4°C. Further work is needed to determine whether the mRNAs for these two proteins are bound to the cytoskeleton.

The components(s) of the cytoplasmic cytoskeletal framework to which these proteins are bound is presently not known. The microtubules appear not to be solely responsible for the binding because certain extractions (e.g., with buffer B at 4°C) deplete the framework of microtubules but do not remove the majority of the bound viral proteins. Thus, other structural components are involved. There may also be different components of the cytoplasmic framework or more than one type of association utilized at different times during transport into the nucleus. We previously observed the chase of these two proteins from the cytoplasmic fraction to a detergent-sensitive association with the crude nuclear pellet (19). Based on the data

TABLE 3. Reconstruction of mixtures of subcellular fractions

Fraction A[a]	Fraction B[b]	% of protein bound[c]	
		ICP5	ICP8
Control cell monolayer	Soluble fraction	7	6
Control cell monolayer	Cytoplasmic fraction	14	15
Infected cell monolayer	Soluble fraction	10	14
Infected cell monolayer	Cytoplasmic fraction	18	10
Control cell cytoskeleton	Soluble fraction	11	11
Control cell cytoskeleton	Cytoplasmic fraction	9	11
Infected cell cytoskeleton	Soluble fraction	6	7
Infected cell cytoskeleton	Cytoplasmic fraction	12	12

[a] Unlabeled.

[b] Isolated from [^{35}S]methionine-labeled, infected cells.

[c] A and B were mixed, and the soluble and detergent-insoluble fractions were isolated. The amount of ICP5 and ICP8 bound to detergent-insoluble fractions was quantified.

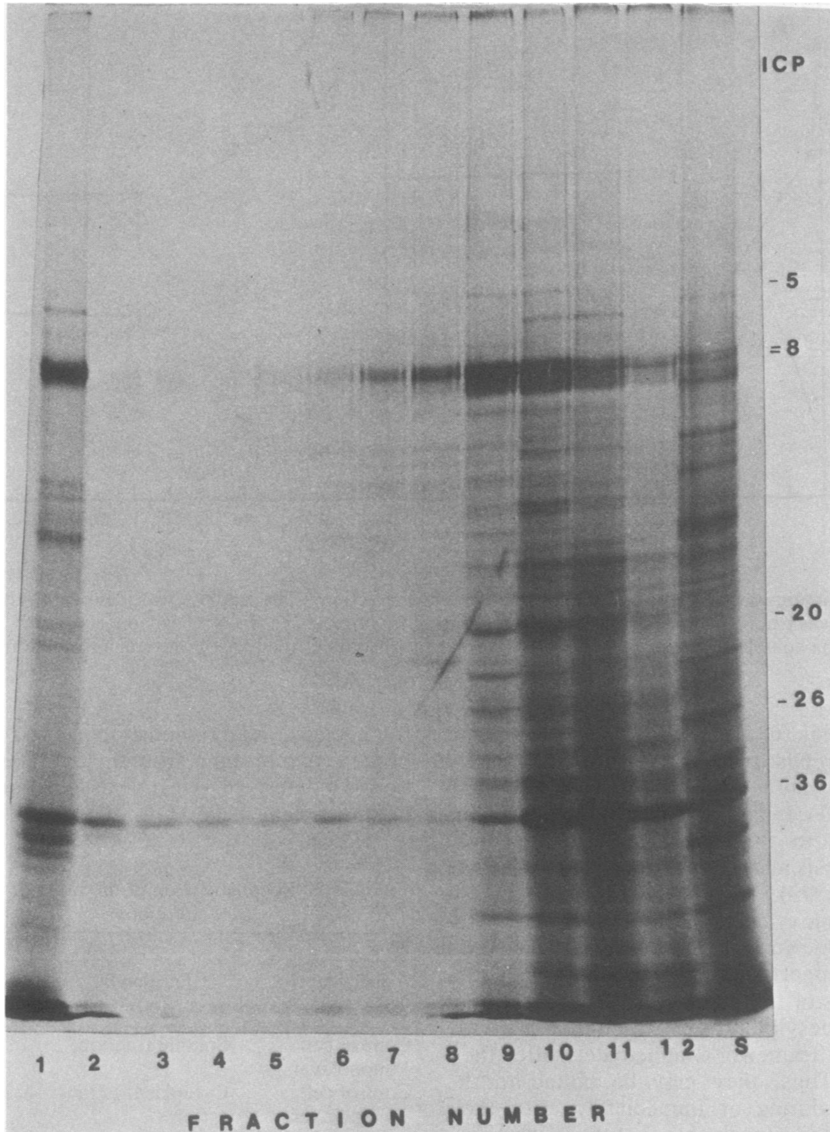

FIG. 6. Sucrose velocity gradient centrifugation of the insoluble matrix from infected cells. Infected cells (4 h postinfection) were labeled for 15 min with [^{35}S]methionine and subjected to chase conditions for 10 min. The detergent-insoluble fraction was isolated, scraped from the culture dish in fresh buffer A, and layered on a 15 to 30% sucrose gradient prepared in buffer A. Centrifugation and recovery of proteins were performed as described in the text. Shown is the autoradiogram of the gel in which the gradient fractions were subjected to electrophoresis. Fraction 1 is the pellet and fractions 2 to 14 are from the bottom to the top of the gradient. In parallel gradients, 28S, 18S, and 4 to 5S RNAs were found in fractions 2 and 3, 5 and 6, and 8 to 10, respectively.

presented in this paper, the detergent-sensitive association could represent the binding of the proteins to cytoskeletal structures attached to the outside of the cell nucleus. For example, intermediate filaments have been shown to be present as a collapsed structure on the surface of isolated nuclei (32).

We have attempted to confirm the association of the DNA binding protein with the cytoplasmic framework by immunofluorescence studies. Because of the rapid transit time to the nucleus (19; Fig. 4), we have been unable to detect sufficient amounts of ICP8 in the cytoplasm by immunofluorescence to be certain of its location. However, it has been possible to detect ICP8 in a fibrous pattern in the cytoplasm of cells infected

with an ICP8 mutant (Quinlan, Chen, and Knipe, unpublished data).

Nuclear cytoskeletal framework. The structural elements of the nucleus or the nuclear matrix (4) are believed to be the site of cellular DNA replication (3). Newly synthesized RNA is also associated with this structure (23, 30). We have shown that the major herpesvirus DNA binding protein associates with structural elements of the cell nucleus in the absence of viral DNA replication but will bind to viral DNA when DNA synthesis resumes (19; Quinlan and Knipe, unpublished data). Here we have shown that ICP5 and ICP8 appear to move directly from the cytoplasmic framework to the nuclear framework without appearing in a soluble phase. Relevant to this are the observations that cytoplasmic and nuclear fibers anchor opposite each other on the nuclear membrane (24) and intermediate filaments interact with the cell nucleus (32). If these fibers attach near the nuclear pores, this could provide a pathway for the transport of these or other proteins to the cell nucleus.

Immunofluorescence studies with a monoclonal antibody specific for ICP8 have shown that in the absence of viral DNA synthesis ICP8 exhibited a distinct pattern of discrete focal sites within the nucleus, including the nuclear membrane (Quinlan et al., unpublished data). When DNA replication takes place, the staining pattern is altered and indicates that the protein is redistributed throughout the nucleus. Thus, in the absence of viral DNA replication, ICP8 may be attached to specific sites within the nuclear framework, including the portion of the nuclear matrix lining the inner side of the nuclear membrane (1). The structural elements of the cell nucleus may serve as a "reservoir" for ICP8 until viral DNA becomes available for binding. Because ICP8 remains associated with the nuclear framework during viral DNA synthesis, but its distribution is altered, there must be more than one type of association for this protein with the nuclear matrix.

Specificity of binding to the cytoskeleton. We have performed several control experiments to ensure that the binding of the two herpesvirus proteins to the cytoskeleton is specific. Mixing of labeled soluble or cytoplasmic extracts with cytoskeletal fractions or cell monolayers did not lead to a significant amount of ICP8 of ICP5 being bound to the insoluble matrix. Therefore, ICP8 and ICP5 do not nonspecifically adhere to the insoluble matrix in the presence of detergent.

ICP8 does not aggregate into large insoluble complexes because centrifugation of cytoskeletal fractions demonstrated ICP8 in 4 to 10S structures. Thus, the linkage of ICP8 to the cytoskeleton or the cytoskeleton itself is very sensitive to physical shear forces. Furthermore, ICP8 is a very solule protein as demonstrated by its high recovery in soluble extracts from cells and ease of chromatography on DNA cellulose columns (19, 29).

We cannot completely exclude the possibility that ICP5 and ICP8, being large proteins, are trapped within the cytoskeletal matrix at the time of detergent lysis of the cell. However, others have shown that the majority of monomeric ribosomes and mature poliovirions are released by detergent extraction of cells (21, 22). Thus, large structures can be solubilized by this extraction procedure. The retention of ICP5 and ICP8 on the insoluble matrix after several different extraction procedures argues that these proteins are indeed associated with structural elements of the cell. Further information about the interaction of these viral proteins with the skeletal framework may come from the study of viral mutants defective in the nuclear localization of these viral proteins.

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