JOURNAL OF VIROLOGY, Feb. 1987, p. 516–525
0022-538X/87/020516-10$02.00/0

Vol. 61, No. 2

Expression of Polyomavirus Virion Proteins by a Vaccinia Virus Vector: Association of VP1 and VP2 with the Nuclear Framework

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Received 24 March 1986/Accepted 27 October 1986

The polyomavirus proteins VP1, VP2, and VP3 move from their cytoplasmic site of synthesis into the nucleus, where virus assembly occurs. To identify cellular or viral components which might control this process, we determined the distribution of VP1, VP2, and VP3 in a soluble fraction, a cytoplasmic cytoskeleton fraction, and a nuclear framework fraction of infected cells. All three proteins were detected in a detergent-extractable form immediately after their synthesis in polyomavirus-infected cells. Approximately 50, 25, and 40% of pulse-labeled VP1, VP2, and VP3, respectively, associated with the skeletal framework of the nucleus within 10 min after their synthesis. The remaining portion of each labeled protein failed to accumulate on the nuclear framework during a 40-min chase and was degraded. When expressed separately by recombinant vaccinia viruses, VP1 and VP2, but not VP3, accumulated on the nuclear framework. This association was not dependent on other polyomavirus proteins or viral DNA. The amount of total VP1 and VP2 which was bound to the nuclear framework approximated 45 and 20%, respectively. Indirect immunofluorescence demonstrated an exclusive nuclear localization of VP1 in situ. In coinfection experiments, a greater percentage of total VP2 and VP3 was bound to the nuclear framework of cells which cosynthesized VP1. These results indicate that although VP1 and VP2 can bind independently to the insoluble nuclear framework, the association of VP3 with this nuclear structure is promoted by the presence of VP1.

The complex organization of a eucaryotic cell requires a reproducible means of directing specific proteins to their appropriate intracellular location. Signal sequences commit secretory, membrane, and mitochondrial proteins to a pathway leading to their ultimate destination (5, 47, 49). Nuclear proteins also are directed to their proper location by specific amino acid sequences (8, 14–16, 22, 26, 30). For proteins larger than the dimensions of the nuclear pore complex (9 to 10.5 nm in diameter) (41, 44, 56), a signal sequence appears to facilitate transit across the nuclear membrane by promoting an interaction with the pore complex (18). Other sequences within large nuclear proteins specify binding to precise intranuclear sites (13, 16, 43). Proteins which are smaller than the exclusion limit of the nuclear pore can diffuse across the nuclear membrane at a rate inversely related to their size (7, 42). These small proteins can be retained in the nucleus by binding to a nondiffusible nuclear component (17).

The cytoskeletal framework of virus-infected cells has been shown to support the orderly intracellular movement of viral components (2, 3, 12, 32, 45, 59). Evidence from several laboratories (2, 3, 45) suggests that the cytoskeleton may function as conduit for transport of viral proteins into the nucleus. In these studies, it was demonstrated that herpesvirus- and simian virus 40 (SV40)-encoded proteins migrate directly from the cytoplasmic cytoskeleton to the nuclear matrix, the site of virus maturation. This finding is supported by electron micrographs showing that intermediate filaments, which are concentrated in the perinuclear area (reviewed in reference 31), are contiguous with the nuclear matrix (11).

The papovaviruses SV40 and polyomavirus replicate in the nuclei of infected cells (reviewed in reference 54). During the period of virion assembly, the viral proteins VP1, VP2, and VP3 move from their site of synthesis in the cytoplasm into the nucleus (28, 36), where they associate with and encapsidate viral DNA. Owing to their small size, the minor virion proteins VP2 (35 kilodaltons [kDa]) and VP3 (23 kDa) could diffuse into the nucleus and be retained by binding to viral components or to the nuclear matrix. The larger size of VP1 (45 kDa), along with its tendency to form oligomers, suggests that entry into the nucleus may occur by a facilitated transport mechanism. It has been proposed that the SV40 structural proteins may enter the nucleus in the form of a complex (27, 32). All three proteins have been found associated with the nuclear matrix (2, 9). It is not clear, though, whether they are bound individually, in complex with each other or with the nonstructural viral proteins (such as large T antigen), or with viral DNA.

In an initial approach to defining the mechanism(s) whereby polyomavirus virion proteins enter and accumulate in the nucleus, we determined the kinetics of association of VP1, VP2, and VP3 with the nuclear framework of polyomavirus-infected cells. The rapid rate of association of each protein with the nuclear framework suggested the possibility that VP1, VP2, and VP3 accumulate in the nucleus in a coordinated manner. By expressing VP1, VP2, and VP3 separately in recombinant vaccinia viruses, we were able to analyze the capacity of each protein to associate independently with the nuclear structural framework. We show in this report that only VP1 and VP2 have the capacity to bind to the nuclear framework in the absence of other polyomavirus proteins or viral DNA. Furthermore, VP1 can

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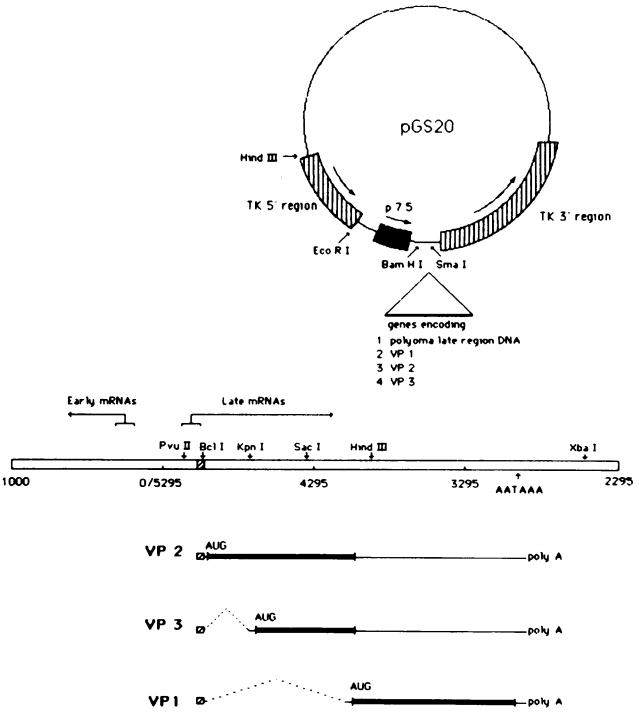

FIG. 1. Organization of the late region of the polyomavirus genome encoding VP1, VP2, and VP3 and of the vaccinia virus insertion vector pGS20. Restriction sites used to excise the coding sequences for each protein are shown in the figure and discussed in Materials and Methods. The unique mRNA for each viral protein, which is derived from the same primary transcript in polyomavirus-infected cells, is aligned with the corresponding sequence of the viral genome. The mRNAs for VP3 and VP1 are formed by a splicing reaction (elevated dashed line) which adjoins the 3' end of the leader sequence (stippled box) to a region proximal to the respective translation initiation condon AUG. Several tandem repeats of the leader sequence appear at the 5' terminus of each mRNA (55). The protein-coding sequence on each mRNA is highlighted.

promote the nuclear localization of VP3 and, to a lesser extent, of VP2.

MATERIALS AND METHODS

Growth of cells and viruses. 3T6 cells, a continuous cell line derived from mouse embryo fibroblasts, (obtained from K. Takemoto, National Institutes of Health, Bethesda, Md.) and CV-1 African green monkey kidney cells were grown at 37°C as monolayers in Dulbecco modified minimal essential medium (Flow Laboratories) supplemented with 5 and 10% fetal bovine serum (M.A. Bioproducts), respectively. Conditions for the growth of HeLa cells and human 143 tk^- cells have been described previously (51). The Lp-D clone of polyomavirus isolated by Vogt and Dulbecco (57) from the LID-1 strain was propagated in 3T6 cells at a low multiplicity of infection and used as stock virus and as a source of DNA for the experiments described in this report. The WR strain of vaccinia virus and the recombinant viruses were grown in HeLa cells and purified from cytoplasmic extracts by sucrose gradient centrifugation (25).

Preparation of recombinant DNA. Polyomavirus DNA was extracted from infected 3T6 cells by the Hirt procedure (23). Sequences encoding the three virion proteins and the late primary transcript were excised from the viral genome as indicated below and in Fig. 1. The numbering system of Soeda et al. (52) was used to describe the polyomavirus genome. For VP1, the *Sac*I fragment containing the VP1 gene was digested with BAL 31 to remove upstream, noncoding ATGs and then was digested with *Xba*I. Results from M13 dideoxy sequencing (39, 48) confirmed that the VP1-encoding fragment, spanning nucleotides (nt) 4123 to 2522, contained only 46 nt upstream of the VP1 translation initiation ATG. For VP2, the *Bcl*I (nt 5022)-*Xba*I (nt 2522) fragment contained 21 nt 5' to the VP2 initiation codon. For VP3, viral DNA was cut with *Kpn*I (nt 4693) and *Xba*I (nt 2522) to generate a fragment extending 38 nt 5' of the VP3 initiation ATG. For the late primary transcript, the *Pvu*II (nt 5134)-*Xba*I (nt 2522) fragment encompassed sequences for the leader and the three virion proteins and retained 128 nt upstream from the VP2 initiation ATG. The distance from the translation initiation ATG to the 5' restriction site in each fragment contained no additional ATGs. In addition, all fragments extended past the eucaryotic polyadenylation signal (AATAAA) and terminated at the same *Xba*I site.

Purified DNA fragments encoding the virion proteins were subcloned into the *Sma*I-*Xba*I sites (VP1, VP2, and late primary transcript) or the *Kpn*I-*Xba*I sites (VP3) of pUC18 with T4 DNA ligase. Following amplification of the recombinant plasmids in transformed *Escherichia coli*, fragments containing polyomavirus sequences were excised by restriction at the *Xba*I site and another site dependent on the insert. Restriction of the VP1-coding sequence with *Sst*I-*Xba*I added 11 nt of the pUC18 polylinker to the ATG-proximal sequences of the original insert. Likewise, restriction of the VP2-pUC18 and late primary transcript-pUC18 constructs with *Eco*RI-*Xba*I added 18 nt of pUC18 to each. Excision of the VP3-containing fragment with *Kpn*I-*Xba*I generated no additional pUC18 sequences for that insert. Each fragment was ligated into the *Sma*I site of the vaccinia virus insertion vector pGS20 (33) (Fig. 1). After all DNA manipulations, the 5' terminus of each mRNA was expected to contain 35 nt of vaccinia virus sequences from the 7.5-kDa gene transcription start site to the *Sma*I insertion site and a variable amount of pUC18 (0 to 18 nt) and polyomavirus DNA (21 to 128 nt).

DNA restriction and modification enzymes (International Biotechnologies and Bethesda Research Laboratories, Inc.) were used as recommended by the suppliers. DNA fragments were resolved on 1.0% agarose gels, and bands from restriction digests were isolated by being electroblotted onto Schleicher & Schuell NA-45 membranes. Pretreatment of the membranes and elution of bound DNA were as suggested by the manufacturer. Transformation of competent *E. coli* HB101 with each of the constructs, selection of colonies containing the proper recombinant plasmids by the miniscreen alkaline lysis procedure, and chloramphenicol amplification and purification of the plasmid DNAs were done by established methods (4, 35).

Selection and analysis of recombinant viruses. Vaccinia virus recombinants containing genes for polyomavirus virion proteins were isolated from CV-1 cells, which had been infected with wild-type vaccinia virus and subsequently transfected with the insertion vectors, by procedures which have been described in detail previously (33, 50, 51). To verify the presence and orientation of polyomavirus genes in the recombinants, viral DNA was extracted and digested with *Hind*III, and fragments, separated by eletrophoresis through agarose gels, were transferred to nitrocellulose membranes (53) and detected by hybridization (58) to a nick-translated probe of the VP1 gene. Recombinant viruses

expressing the respective polyomavirus genes were referred to as follows: VP1-Vac, VP2-Vac, VP3-Vac, and LTr-Vac.

Fractionation of infected cells. To determine the subcellular distribution of viral proteins, we modified a procedure used by others (2, 11, 19, 32, 45) to fractionate cells. Monolayers of 3T6 and CV-1 cells were grown to subconfluency in 35-mm tissue culture dishes and were infected, respectively, with polyomavirus or recombinant vaccinia viruses at a multiplicity of 30. When indicated, cells were infected with the recombinant virus expressing VP2 and VP3 and then coinfected with the appropriate virus 2 h later. The monolayer of infected cells was washed twice with phosphate-buffered saline (PBS) (pH 7.4) and overlaid with 0.25 ml of a solution containing 10 mM Tris hydrochloride (pH 7.4), 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% Triton X-100, 1.2 mM phenylmethylsulfonyl fluoride, and 100 Kallikrein U of aprotinin per ml. After a 5-min incubation on ice, this solution, containing soluble proteins, was removed. The extracted cell monolayer was washed with an additional 0.15 m of fresh solution, which was combined with the original extraction buffer. To separate the cytoplasmic cytoskeletal fraction from the nuclear skeletal framework, the subcellular components still attached to the plate were scraped into 0.40 ml of a buffer containing 10 mM Tris hydrochloride (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 1% Tween 40, 0.5% deoxycholate, 1.2 mM phenylmethylsulfonyl fluoride, and 100 Kallikrein U of aprotinin per ml, disrupted in a tight-fitting Dounce homogenizer, and centrifuged for 6 min at 1,000 × *g*. The solubilized cytoplasmic cytoskeleton in the supernatant was removed, and the insoluble nuclear framework was resuspended in 0.40 ml of the same buffer. When examined by light microscopy, these detergent-extracted nuclei appeared to be free of contaminating cytoplasmic material. An equal volume of 2× solubilization buffer (29) was added to the three fractions, and samples were boiled for 7 min.

Labeling of proteins in infected cells. Cells infected by polyomavirus or recombinant vaccinia viruses were incubated for 30 and 90 min, respectively, with methionine-free Dulbecco modified Eagle medium supplemented with 5% dialyzed fetal bovine serum. Proteins in polyomavirus-infected cells were labeled for 5 min at 37°C with 150 μCi of [^{35}S]methionine (New England Nuclear Corp.; 1,109 Ci/mmol) per ml; labeling in vaccinia virus-infected cells was carried out for 6 min with 300 μCi of [^{35}S]methionine per ml. When indicated, cells were incubated for an additional period in Dulbecco modified Eagle medium containing 10× unlabeled methionine.

Indirect immunofluorescence. Monolayers of CV-1 cells were grown to subconfluency on tissue culture dishes and infected at a multiplicity of 1 with VP1-Vac or wt vaccinia virus. At 6 h postinfection, cells were washed three times with PBS, fixed for 10 min at 4°C with 3% formaldehyde (in PBS), and then permeabilized in a solution of 3% formaldehyde containing 0.5% Triton X-100. Cells were incubated for 1 h at 23°C with a 1:75 dilution (in PBS containing 1% bovine serum albumin and 10% calf serum) of a polyclonal goat antibody to VP1 (36) (kindly provided by Richard Consigli) which had been preabsorbed to a monolayer of wt vaccinia virus-infected CV-1 cells fixed as described above. Cells were then incubated sequentially with rabbit anti-goat (1:150 dilution) and fluoresceinated goat anti-rabbit (1:200 dilution) antibodies (Cooper Biomedical, Inc.) (both diluted in PBS–1% bovine serum albumin–5% calf serum). Cells were washed several times with PBS between each incubation. They were then counterstained with Evans blue (0.4 mg/ml), mounted in 90% glycerol, and viewed with a Nikon phase-epifluorescence microscope.

Analysis of proteins by gel electrophoresis and immunoblotting. The conditions for preparation of 12 % sodium dodecyl sulfate (SDS)-polyacrylamide slab gels and running buffer and solubilization of samples were those described by Laemmli (29). To detect polyomavirus proteins immunologically, proteins were transferred to Schleicher & Scheull BA85 nitrocellulose membranes (10) in buffer containing 25 mM Trizma base, 192 mM glycine, and 20% methanol. Membranes were blocked in a solution containing 3% bovine serum albumin and 0.1% Tween 20 in PBS, which was adjusted to pH 7.4. The membranes were incubated for 4 h at 23°C with a 1:300 dilution of a goat polyclonal antibody to the SDS-denatured polyomavirus virion proteins (36) and then with a rabbit anti-goat immunoglobulin G conjugated to horseradish peroxidase (Dakopatts). After membranes were washed for 30 min in PBS containing 0.05% Nonidet P-40, antibody-antigen complexes were detected by exposure to a solution of 50 mM Tris (pH 7.6)–0.5 mg of diaminobenzidine (Sigma Chemical Co.) per ml–0.02% H_2O_2. Alternatively, ^{125}I-labeled protein A, (30 mCi/mg; Amersham Corp.) was used in place of the secondary rabbit antibody. The amount of each polyomavirus virion protein was quantitated by densitometric tracing of autoradiographs and by excising the individual protein bands from the nitrocellulose membranes and determining the associated counts per minute of ^{125}I-labeled protein A. Similar relative values were obtained by both methods. The numbers shown in Table 1 represent an average of data obtained by both procedures from each of three separate experiments.

RESULTS

Polyomavirus virion proteins bind to the insoluble nuclear framework. Previous studies of polyomavirus and SV40 assembly used immunofluorescence to demonstrate the nuclear accumulation of the viral proteins VP1, VP2, and VP3 (28, 36). To identify structural components of the cell which may have a role in localizing polyomavirus virion proteins to the nucleus, we used an in situ cell fractionation procedure successfully used by others (2, 3, 12, 32, 45, 59) to separate soluble cytoplasmic and nucleoplasmic proteins and the cytoplasmic cytoskeleton from an insoluble nuclear framework. When purified in this manner, the nuclear framework is composed primarily of nuclear matrix and chromatin (3, 11, 32, 45). Light-microscopic analysis demonstrated that these nuclei had been cleanly separated from the extensive network of cytoplasmic filaments (data not shown). We determined the steady-state distribution of VP1, VP2, and VP3 in these three fractions of polyomavirus-infected cells at a stage in the lytic cycle (26 h postinfection) following initiation of viral DNA replication when these virion proteins were briskly synthesized but when only small amounts of mature virus had been assembled. Although more than 75% of total protein from a monolayer of infected cells was extracted into the soluble fraction, as determined by Coomassie blue staining (Fig. 2A, lane S), the major capsid protein VP1 remained associated predominantly with the nuclear framework (lane N). It was necessary to use immunological means to detect VP2 and VP3 (Fig. 2B) because they are synthesized in small amounts and comigrate with some cellular proteins. As was the case with VP1 (Fig. 2A, lane N), most VP3 was found associated with structural elements of the nucleus. However, unlike VP1 and VP3, VP2 was present in nearly equal amounts in both soluble and nuclear framework fractions.

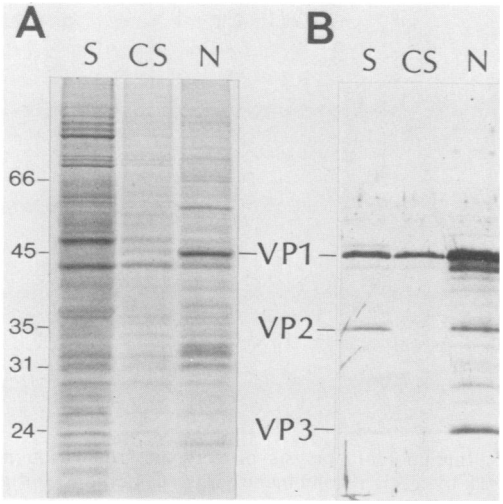

FIG. 2. Distribution of cellular and viral proteins in the soluble (lanes S), cytoplasmic cytoskeleton (lanes CS) and nuclear framework (lanes N) fractions of polyomavirus-infected mouse 3T6 cells. Conditions for the growth, infection, and fractionation (at 26 h postinfection) of cells are described in Materials and Methods. Proteins in a sample representing 2.5% of each subcellular fraction were separated by SDS-PAGE and visualized by being stained with Coomassie blue (A). Alternatively, separated proteins in 0.8% of each fraction were transferred to nitrocellulose membranes, and the virion proteins were detected by an immunoblot (B). As is explained in the text, panel B does not represent accurately the subcellular distribution of VP1. Detection of VP1 in the S and CS fractions and of VP2 and VP3 in the S or N fractions or both by the immunoblot is attributable to the greater sensitivity of the immunoassay than the Coomassie blue staining. Comigration of a small amount of VP1 with actin in lane N may explain why a band of 42 kDa reacts with antibody raised to the polyomavirus virion proteins. The migration of the molecular mass markers run in an adjacent lane is indicated (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; pepsin, 35 kDa; carbonic anhydrase, 31 kDa; and trypsinogen, 24 kDa).

When analyzing an amount of material which enabled us to detect the small amounts of VP2 and VP3 by the immunoblot, we found that abundant proteins such as VP1 (in the nuclear framework fraction) saturated the binding sites on the nitrocellulose membrane before being transferred completely (unpublished results). Consequently, in Fig. 2B, the amount of VP1 in the soluble fraction and attached to the cytoplasmic cytoskeleton was artificially exaggerated in relation to the amount of VP1 bound to the nuclear framework. The Coomassie blue-stained gel in Fig. 2A represents much more accurately the subcellular distribution of VP1.

VP1, VP2, and VP3 associate with the nuclear framework at similar rates. To determine the kinetics with which newly synthesized VP1, VP2, and VP3 associate with the nuclear framework, we performed pulse-chase experiments. We labeled proteins with [^{35}S]methionine for 5 min at 26 h postinfection and fractionated cells after chase periods of increasing duration. The same percentage of total protein from each fraction was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3), and the subcellular distribution of each virion protein was quantified by excising each viral protein from the gel and determining the amount of associated ^{35}S (Fig. 4). Immediately after the pulse, more than 80% of each labeled virion protein was found in the soluble fraction (Fig. 3 and 4). The amount of ^{35}S-labeled VP1 associated with the nuclear framework rose threefold within a 5-min chase and reached a maximum within 10 min.

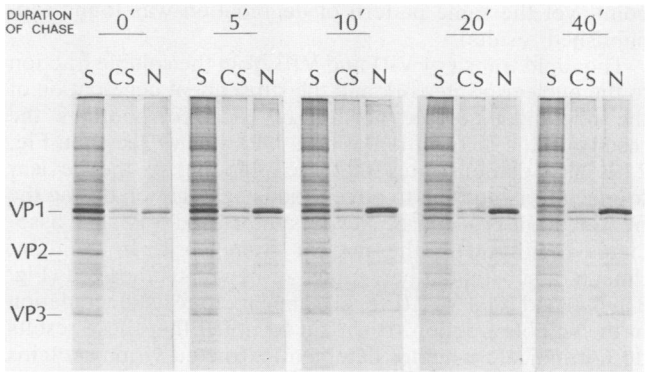

FIG. 3. Subcellular distribution of ^{35}S-labeled VP1, VP2, and VP3 after a 5-min pulse-label and chases of increasing duration (min). Conditions for infection and fractionation of cells and for labeling (at 26 h postinfection) and electrophoresis of proteins were as described in Materials and Methods. Aliquots representing 2.5% of the soluble (lanes S), cytoplasmic cytoskeleton (lanes CS), and nuclear framework (lanes N) fractions were analyzed by SDS-PAGE. Dried gels were exposed for autoradiography to Kodak BB-5 film for 20 h. Proteins from purified polyomavirus were used as markers and run in an adjacent lane.

Approximately 50% of labeled VP1 failed to associate with the nuclear framework. This portion of VP1 was degraded during the ensuing 40-min chase. We found no diminution in the amount of labeled VP1 bound to the nuclear framework after a 90-min chase (unpublished results), suggesting that nuclear-framework-bound VP1 was not degraded.

Newly synthesized VP2 and VP3 also associated with the nuclear framework rapidly. Maximal amounts of each pulse-labeled protein were bound to the nuclear framework within 10 min (Fig. 3 and 4). As was the case with soluble VP1, soluble VP3 was degraded rapidly. In contrast, soluble VP2 appeared to be more resistant to proteolysis during the chase period (Fig. 4). That the protein degradation was not an artifact of the fractionation procedure was seen in separate experiments in which unfractionated cells were solubilized immediately with SDS-PAGE sample buffer at each chase

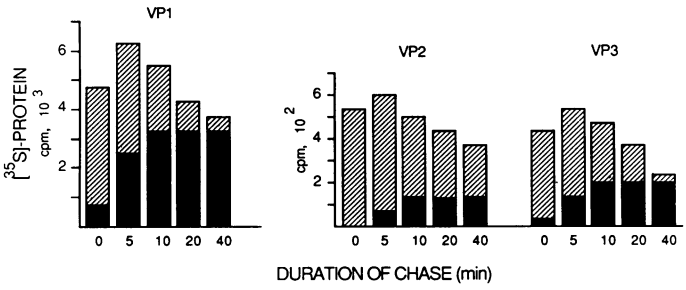

FIG. 4. Kinetics of association of pulse-labeled VP1, VP2, and VP3 with the nuclear framework of polyomavirus-infected cells. The amount of each ^{35}S-labeled virion protein in the subcellular fractions described in the experiment shown in Fig. 3 was quantitated by excising the separated proteins from the gel and determining the ^{35}S content in each. The full height of each column represents the intracellular amount of labeled VP1, VP2, and VP3 at the various chase points. Symbols: ■, amount of protein bound to the nuclear framework; ▨, amount of labeled protein in the soluble and cytoplasmic cytoskeletal fractions. The data shown represents the average of two determinations from each of three separate experiments.

point, yet the same pattern of degradation was found (unpublished results).

The rapid transit of VP1 and VP3 from the soluble fraction to the nuclear framework and the subsequent degradation of the remaining soluble portion of each corroborates the results on the distribution of total VP1 and VP3 seen in Fig. 2. It also demonstrates that their binding to the nuclear framework is not due to adventitious aggregation during the fractionation procedure. Newly synthesized VP2 also associates rapidly with the nuclear framework. In contrast, though, a substantial percentage of newly synthesized (Fig. 3 and 4) and total VP2 (Fig. 2B) appears to resist degradation in the soluble fraction. It should be noted that these results do not indicate whether detergent-extracted virion proteins had actually entered the nucleus, since nucleoplasmic proteins are removed in the soluble fraction. The exclusive nuclear localization of each protein is suggested, though, by indirect immunofluorescence analyses (28, 36). The rapid rate at which the three proteins bind to the nuclear framework may be due to (i) similar rates of transit across the nuclear membrane, (ii) transport into the nucleus in a complex, (iii) equivalent affinity for binding sites on the nuclear framework, or (iv) coordinated interactions with viral chromatin or subviral assembly intermediates or both.

Recombinant vaccinia viruses express polyomavirus virion proteins separately. To determine the capacity of VP1, VP2, and VP3 to associate independently with a nuclear framework which was devoid of other polyomavirus proteins and viral DNA, we expressed each protein separately in recombinant vaccinia viruses. The sites in the late region of the polyomavirus genome which were used to excise coding sequences for the three polyomavirus virion proteins are described in the legend to Fig. 1 and in Materials and Methods. A fourth recombinant containing a vaccinia virus promoter adjacent to the entire late region of the polyomavirus genome also was constructed. If VP1, VP2, and VP3 were expressed by this recombinant, as occurs in polyomavirus-infected cells, we would be able to determine whether one of the proteins influenced the subcellular distribution of the others. Presumably, synthesis of VP3 and VP1 would require either splicing of the primary transcript or transcriptional or translational initiation at internal sites (Fig. 1). Following the construction and selection of recombinants as described in Materials and Methods, the predicted genome organization of each virus was confirmed by restriction mapping (data not shown).

Each recombinant virus directed the synthesis of the correct polyomavirus protein as seen in Fig. 5 where total protein from cells infected by each recombinant was separated by SDS-PAGE and analyzed by an immunoblot using antibody to SDS-denatured VP1 (45 kDa), VP2 (35 kDa), and VP3 (23 kDa). In addition to the full-length virion protein, trace amounts of breakdown products of VP1 (29 and 40 kDa) (1) and of VP2 (26 kDa) were evident in cells synthesizing each protein. It is noteworthy that only VP2 was detected in cells infected by the recombinant containing polyomavirus DNA encoding the late primary transcript. The absence of VP1 and VP3 suggests that the appropriate spliced transcripts were not formed in appreciable amounts. It should also be noted that despite being under the transcriptional control of the same vaccinia virus promoter for the gene encoding the 7.5-kDa protein, the three polyomavirus gene products were expressed at different levels. When measured at 6 h postinfection, the levels of VP1 and VP2 were more than 20- and ca. 5-fold higher, respectively, than that of VP3. The relatively weak expression of VP3 was not due to its release or secretion from the infected cell into the media, nor could higher amounts of VP3 be detected in cells infected at multiplicities greater than 30 (data not shown). The levels of polyomavirus-specific mRNA in cells infected by each recombinant were equivalent when measured by Northern blots (unpublished results) as expected, since the same vaccinia virus promoter was used in each case. This finding suggests that the difference in expression may lie at the translational level.

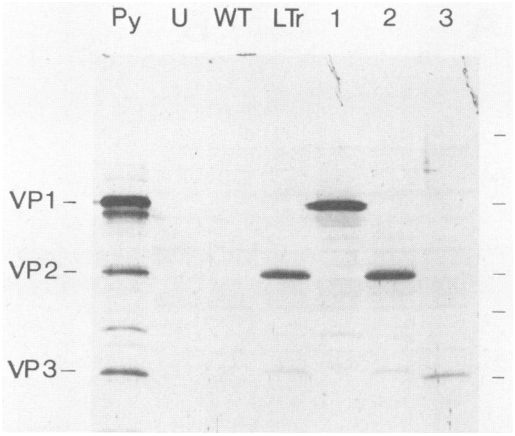

FIG. 5. Immunoblot analysis of polyomavirus virion proteins synthesized by each recombinant virus. Conditions for growth, infection, and fractionation (at 6 h postinfection) of CV-1 monkey kidney cells are described in Materials and Methods. Proteins in an aliquot representing 4% of total material from uninfected cells (lane U), cells infected by wild-type vaccinia virus (lane WT) and by recombinants containing the genes for the late primary transcript (lane LTr), VP1 (lane 1) and VP2 (lane 2) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected with antibody to the polyomavirus proteins. Proteins in 6% of total material were sampled from cells infected by the VP3 recombinant (lane 3). Viral proteins from polyomavirus-infected mouse cells (lane Py) were used as markers. Molecular mass markers used in Fig. 2 were run in an adjacent lane, transferred to the nitrocellulose membrane, and detected by staining with amido black. Their position of migration is indicated by the dashes on the right side of the figure. The high level of background was due to the long (1 min) exposure to diaminobenzidine which was needed to detect the small amount of VP3 synthesized.

VP1 is transported to the nucleus of cells infected by VP1-Vac. The intracellular distribution of VP1 in cells infected by the VP1-Vac recombinant was determined in situ by indirect immunofluorescence. For this analysis, it was necessary to infect cells under conditions such that adequate amounts of polyomavirus proteins were synthesized, yet cell morphology was not drastically altered, nor was the distinction between nucleus and cytoplasm compromised by the vaccinia virus infection. These criteria were satisfied only in cells infected by the VP1-Vac recombinant, perhaps because of the higher level of expression of this gene. VP1 was detected exclusively in the nucleus (Fig. 6A and B), using a polyclonal goat antibody to VP1 (36). Only faint staining was detected in cells infected by wt vaccinia virus (Fig. 6C and D) and in cells infected by VP1-Vac and then reacted with nonimmune goat serum as primary antibody (data not shown). The exclusive nuclear localization of VP1 when expressed by the VP1-Vac recombinant virus was similar to results obtained from cells infected by wt polyomavirus (compared with data in reference 36).

Proteins bind normally to the nuclear framework in vaccinia

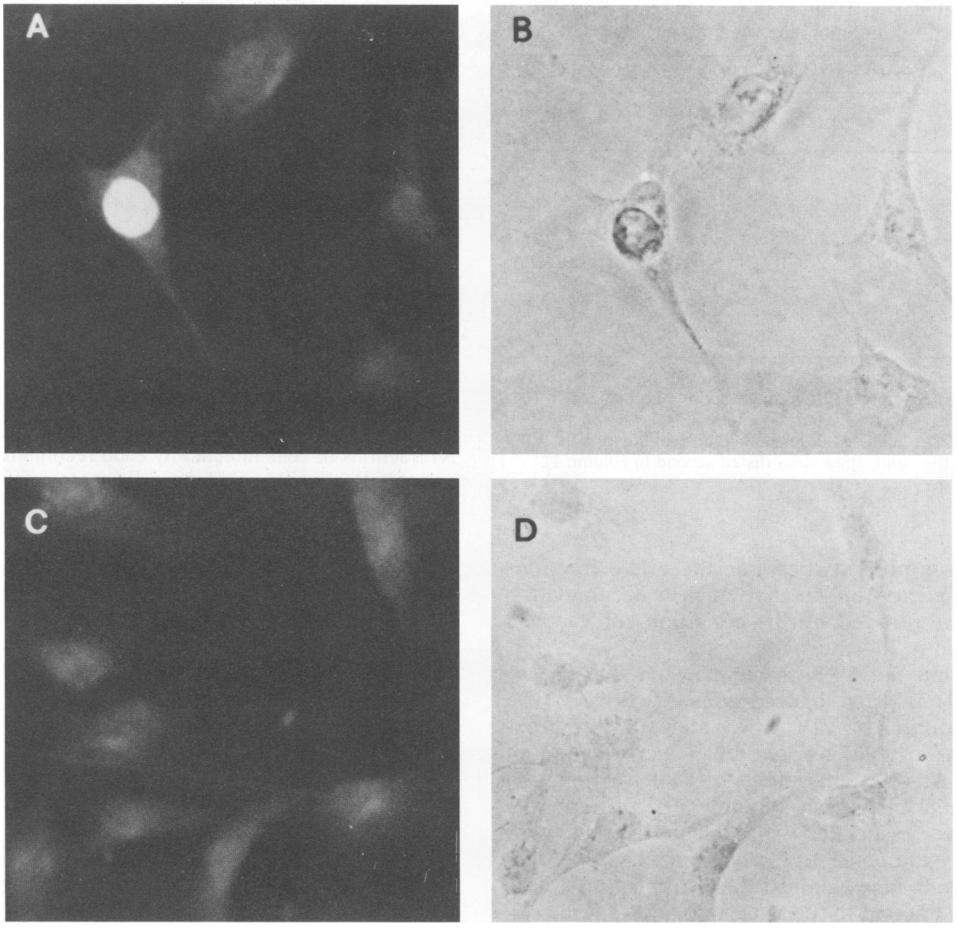

FIG. 6. Intracellular localization of VP1 determined by immunofluorescence microscopy of cells infected by VP1-Vac. Conditions for infection of cells and for immunostaining are described in Materials and Methods. (A and B) Cells infected by VP1-Vac. (C and D) Cells infected by wt vaccinia virus. Phase-contrast photographs (B and D) are shown for the respective immunofluorescence photographs (A and C). Magnification, ×200.

virus-infected cells. The finding that VP1 is transported to the nuclei of cells infected by VP1-Vac is consistent with other reports which show that exogenous viral proteins expressed by recombinant vaccinia viruses are correctly processed, glycosylated, and transported to the cell surface (34, 46, 51). Nevertheless, since nuclear transport and accumulation may be independent events (14), we explored the possibility that vaccinia virus-induced alterations in cell morphology and gene expression disturb the normal association of nuclear proteins with the nuclear framework.

In an initial control experiment, we determined the effect of vaccinia virus infection on the nuclear accumulation of polyomavirus virion proteins synthesized during the normal course of a polyomavirus infection. Cells infected with polyomavirus were superinfected at 18 h postinfection with vaccinia virus at multiplicities of 5, 15, and 30, the proteins were pulsed with [^{35}S]methionine 6 h later, and cells were fractionated after a chase with cold methionine as described in Materials and Methods. The protein content of each fraction was determined by SDS-PAGE as described in the legend to Fig. 3. Despite undergoing the changes in morphology characteristic of vaccinia virus infection, cells continued to synthesize the polyomavirus virion proteins at a level sufficient to be distinguished from vaccinia virus-encoded proteins by densitometry of autoradiographs. Under these conditions of infection, VP1, VP2, and VP3 associated with the nuclear framework with kinetics similar to those observed in cells infected by polyomavirus alone (data not shown).

In a separate analysis, we fractionated cells infected by the VP1-Vac recombinant virus, after proteins had been pulse-labeled and chased for various periods, to determine whether we could (i) observe the movement of proteins from the soluble fraction to the nuclear framework and (ii) identify several proteins which were enriched in the different fractions. ^{35}S-labeled VP1 was found mainly in the soluble fraction immediately after its synthesis (Fig. 7). Approximately 40% of labeled VP1 migrated from the soluble fraction to the nuclear framework within a 15-min chase. In addition, a vaccinia virus protein of 33 kDa, possibly a vaccinia virus-specified nuclear protein identified by others (24), was found predominantly attached to the nuclear framework directly following its synthesis. The majority of vaccinia virus proteins (such as 27-, 39- and 66-kDa proteins) were found only in the soluble fraction, while another set of vaccinia virus proteins was distributed in all three cell fractions. Thus, the movement of VP1 from the soluble fraction to the nuclear framework and the ordered partition-

TABLE 1. Subcellular distribution of polyomavirus proteins synthesized by recombinant vaccinia viruses

Protein(s) expressed	% of VP1, VP2, or VP3 in subcellular fractions[a]		
	Soluble	Cytoplasmic cytoskeleton	Nuclear framework
VP1	47	8	45
VP1[b]	46	10	44
VP2	78	3	19
VP2 + VP1	67 (40)[c]	5 (23)	28 (37)
VP2 + VP3	84 (ND)[d]	2 (ND)	14 (ND)
VP3	99	0	1
VP3 + VP1	76 (35)	3 (19)	21 (46)
VP3 + VP2	97 (80)	1 (2)	2 (18)

[a] Quantitation of proteins was as described in Materials and Methods. The total amount of each protein from all three fractions was normalized to 100.
[b] Cells infected by VP1-Vac at 1/10 normal multiplicity.
[c] Numbers in parentheses indicate the subcellular distribution of VP1, VP2, or VP3 synthesized by the coinfecting virus (listed second in column 1).
[d] ND, Not determined.

ing of vaccinia virus proteins between these two fractions suggest that the capacity for selective nuclear accumulation of proteins is not destroyed by the conditions of vaccinia virus infection used in this study.

VP1 and VP2, but not VP3, accumulate on the nuclear framework of cells infected by recombinant viruses. The intracellular distribution of VP1, VP2, and VP3, now expressed separately in cells whose nuclei were devoid of polyomavirus DNA, was determined by cell fractionation and immunoblot analysis. Approximately 50% of total VP1 and 20% of VP2 were associated with the nuclear framework of cells infected by the respective recombinant virus (VP1-Vac, VP2-Vac; Fig. 8, lane N, and Table 1). In contrast, less than 5% of VP3 had bound to the nuclear framework in cells infected under the same conditions by VP3-Vac (Fig. 8 and Table 1). The association of VP1 with the nuclear framework was not dependent on its intracellular concentration or on

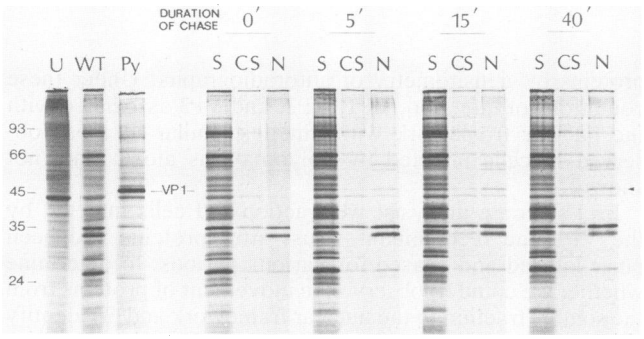

FIG. 7. Subcellular distribution of ^{35}S-labeled VP1 and vaccinia virus-specific proteins synthesized in cells infected by the VP1-Vac recombinant after a 6-min pulse-label and chases (minutes) of increasing duration. Conditions for growth, infection, and fractionation of cells (at 4.5 h postinfection), as well as for labeling and electrophoresis of proteins, are described in Materials and Methods. Aliquots representing 2.5% of the soluble (lane S), cytoplasmic cytoskeleton (lane CS), and nuclear framework (lane N) fractions were analyzed by SDS-PAGE. Pulse-labeled proteins in a whole lysate of uninfected (lane U) and wt vaccinia virus-infected (WT) (at 4.5 h postinfection) CV-1 cells, as well as from polyomavirus-infected (lane Py) mouse cells are presented for comparison. Molecular mass markers which were run in an adjacent lane, are identical to those used in Fig. 2 with the addition of phosphorylase *b* (93 kDa).

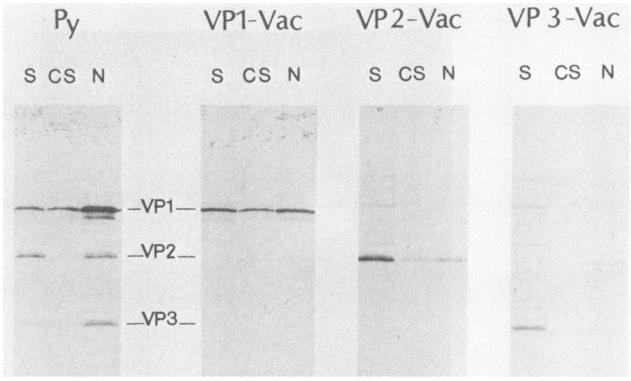

FIG. 8. Distribution of VP1, VP2, and VP3 in soluble (lanes S), cytoplasmic cytoskeleton (lanes CS), and nuclear framework (lanes N) fractions of cells infected by each recombinant virus. Monolayers of CV-1 monkey kidney cells were infected with VP1-Vac, VP2-Vac, or VP3-Vac and were fractionated 6 h postinfection as described in Materials and Methods. Proteins in an amount representing 6% of each fraction were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with antibody to the polyoma virion proteins. Proteins in the VP1-Vac panel were exposed to diaminobenzidine for 20 s; exposure of the VP2-Vac and VP3-Vac lanes continued for 1 min. The distribution of viral proteins in polyomavirus-infected cells (Py) is shown for comparison.

the background level of vaccinia virus macromolecular synthesis. In cells infected at a low multiplicity with VP1-Vac, such that the amount of VP1 was comparable to that of VP3 made by VP3-Vac, VP1 still associated efficiently with the nuclear framework (Table 1).

As was observed in polyomavirus-infected cells (Fig. 3 and 4), only a portion of newly synthesized VP1 and VP2 which was expressed by the vaccinia virus recombinants accumulated on the nuclear framework (Fig. 7 and 8). In contrast, the fate of soluble VP1 was different in cells infected by VP1-Vac than in polyomavirus-infected cells. The high levels of soluble VP1 detected by the Western blot (Fig. 8, VP1-Vac) and by the pulse-chase experiment (Fig. 7) may be attributed to less active degradation of VP1 (Fig. 7) than was detected in polyomavirus-infected cells and does not necessarily indicate less efficient binding to the nuclear framework.

These results indicate that VP1 and, to a lesser extent VP2, are able to associate with the nuclear framework independently of other polyomavirus proteins or viral DNA. In contrast, VP3 expressed by VP3-Vac was unable to accumulate on either the cytoplasmic cytoskeleton or the nuclear framework. These observations raised the possibility that VP1 influences the association of VP3 with the nuclear framework.

VP1 promotes nuclear localization of VP2 and VP3. To determine whether the subcellular distribution of VP2 and VP3 could be altered in the presence of VP1, cells infected by the VP2-Vac and VP3-Vac recombinants were coinfected by the VP1-Vac virus. Thus, we constructed an artificial condition in which VP1 and VP2 or VP1 and VP3 were synthesized concurrently in the same cell. Under these conditions, the percentage of total VP2 and VP3 which was bound to the nuclear framework was found in repeated experiments to be greater than when these proteins were synthesized alone (Table 1). The increase in nuclear framework-bound VP3 from 1 to 21% appeared to be significant. The subcellular distribution of both VP2 and VP3 was not altered when these two proteins were coexpressed.

These data suggest that VP1 can help localize VP2 and VP3 to the nuclear framework. For VP2, the presence of VP1 appears to provide an additional pathway for localizing VP2 to the nucleus. It is not clear whether VP1 affects the transport of these proteins across the nuclear membrane or whether nuclear VP1 provides a binding site for VP2 and VP3, which may enter the nucleus independently. VP1 may even influence the nuclear localization of VP2 and VP3 by a yet unknown process.

DISCUSSION

The experiments in this report focus on the role of cellular and viral components in directing the nuclear localization of polyomavirus virion proteins following their synthesis. We fractionated polyomavirus-infected cells shortly after onset of exponential replication of virus to follow the kinetics of association of newly synthesized VP1, VP2, and VP3 with the insoluble nuclear framework. More than 80% of each pulse-labeled protein was extracted in a detergent-soluble fraction immediately after its synthesis. After a 10-min chase, approximately 50% of labeled VP1, 25% of VP2, and 40% of VP3 had associated with the nuclear framework. The remaining soluble portion of each protein failed to bind the nuclear framework and was degraded during the extended chase.

It must be stressed that the cell fractionation procedure we used did not enable us to distinguish soluble cytoplasmic proteins from proteins that were free in the nucleus or attached in a detergent-sensitive form to nuclear structures. Thus, the rate of nuclear accumulation for each polyomavirus virion protein may not represent the rate of entry into the nucleus but rather the rate of association in a detergent-resistant form with the nuclear framework. In addition, when purified as described, the nuclear framework comprises chromatin, nuclear matrix and a residual perinuclear skeleton of intermediate filaments (composed of vimentin) (11, 19). These filaments are only a minor portion of the cytoplasmic cytoskeleton and were not sufficiently abundant to be detected when the nuclear framework was observed under a light microscope. Several lines of evidence suggest that the polyomavirus proteins present in the nuclear fraction were not bound to these filaments but rather were bound to intranuclear structures. Electron micrographs have shown the nuclear matrix to be the site of SV40 maturation (2), and pulse-labeled SV40 VP1 and VP3 were found within 15 min of their synthesis in 200S viral assembly intermediates (20). Second, in the only study showing the in situ colocalization of SV40 structural proteins with the cytoplasmic cytoskeleton, the binding was suggested to be to actin, not to vimentin, which is the intermediate filament in our nuclear framework fraction. These findings support our contention that the polyomavirus proteins in the nuclear fraction were indeed intranuclear.

To determine whether VP1, VP2, and VP3 accumulate in the nuclei of polyomavirus-infected cells by binding singularly to the nuclear matrix or by associating with viral proteins or DNA already bound to the nuclear matrix, we constructed recombinant vaccinia viruses to express each polyomavirus virion protein separately. The cytoplasmic site of vaccinia virus replication enabled us to express each protein in a cell whose nucleus was devoid of polyomavirus DNA. Although there have been some reports of the presence of vaccinia virus DNA associated with isolated nuclei from infected cells, in situ hybridization studies with the closely related rabbit poxvirus support an exclusively cytoplasmic localization (38).

When expressed by the respective recombinant virus, VP1 and VP2, but not VP3, were able to bind to the nuclear framework. These results were not an artifact of a vaccinia virus infection, as shown by several lines of evidence. First, the nuclear localization of virion proteins in polyomavirus-infected cells was not impaired following superinfection with vaccinia virus. Second, specific vaccinia virus proteins were enriched either in the soluble fraction or on the nuclear framework of cells infected by each recombinant virus. Third, while the background level of vaccinia virus metabolism in cells infected by each recombinant was identical, the subcellular distribution of VP1, VP2, and VP3 was still different.

Our results from immunofluorescent staining of cells infected by VP1-Vac demonstrate that VP1 is transported efficiently into the nucleus. Only a subset of VP1, though, associated with the nuclear framework. This observation is consistent with our finding that in polyomavirus-infected cells, only 50% of pulse-labeled VP1 became stably bound to the nuclear framework, despite detection of VP1 primarily in the nucleus. Our results are supported by data showing that only a portion of SV40 VP1 accumulates in the nucleus of *Xenopus laevis* oocytes (37), where viral DNA is not replicated but where large T antigen was synthesized and localized exclusively in the nucleus. These findings suggest that the events responsible for the transport of VP1 into the nucleus and its accumulation on the nuclear framework are separable. Alternatively, VP1 may bind to different sites on the nuclear framework with different affinity, and weakly bound VP1 may be solubilized by our detergent extraction. We must be cautious in evaluating these data, since cells were infected for the fractionation studies at a multiplicity 30 times greater than for the immunofluorescent staining. It is possible that the more severe cytopathic effect in multiply infected cells affects the binding of VP1 to the nuclear framework. Despite these possibilities, it is clear that in cells infected by VP1-Vac and wt polyomavirus, only a subset of VP1 reproducibly binds to the nuclear framework. In polyomavirus-infected mouse 3T6 cells, the remaining soluble portion of VP1 was degraded. In monkey CV-1 cells, soluble VP1 remains more stable. It will be of interest to determine the events that commit only a subset of VP1 to bind soon after its synthesis to the nuclear framework.

The simultaneous synthesis of VP1 with VP2 or VP3 in cells coinfected with VP1-Vac and VP2-Vac or VP3-Vac promoted the binding of VP3 and, to a lesser extent, of VP2 to the nuclear framework. However, it is not clear from our results whether VP2 and VP3 are transported to the nucleus in a complex with VP1, as has been suggested to occur in SV40-infected cells (27, 32), or whether VP2 and VP3 enter the nucleus independently and then associate with nuclear VP1. It is possible that VP2 and VP3 localize to the nucleus by several routes and that other factors peculiar to the polyomavirus-infected cell, perhaps other polyomavirus proteins or DNA, also contribute to the nuclear localization of VP1, VP2, and VP3.

It is possible that nuclear framework-bound VP1 provides an organizing center for the maturing polyomavirus particle, a role suggested for the cytoskeleton-bound nucleocapsid protein of vesicular stomatitis virus (12). Expression of VP1 by the VP1-Vac recombinant will enable us (i) to characterize the nuclear component(s) to which VP1 binds, (ii) to determine whether nuclear VP1 alone forms an 8S particle, as observed in SV40-infected cells (40), and (iii) to analyze the nuclear form of VP1 for posttranslational modifications (6, 21) possibly responsible for differences between nuclear

VP1 and VP1 found in the soluble fraction. In addition, by introducing specific mutations in the VP1 gene, we should be able to identify the sequence(s) which governs the nuclear localization of VP1.

ACKNOWLEDGMENTS

This study was supported in part by predoctoral training grant GM07102-10 from the National Institute of General Medical Sciences to N.M.S. and a grant from the Melville A. Hare Basic Research Fund.

N.M.S. is grateful to Peter J. Gomatos for advice and encouragement. We thank Piero Balduzzi and John Frelinger for helpful criticism of the manuscript and Mary Notter for assistance with fluorescence microscopy.

LITERATURE CITED

1. **Anders, D., and R. Consigli.** 1983. Chemical cleavage of polyomavirus major structural protein VP1: identification of cleavage products and evidence that the receptor moiety resides in the carboxy-terminal region. J. Virol. **48**:197–205.
2. **Ben-Ze'ev, A., R. Abulafia, and Y. Aloni.** 1982. SV40 virions and viral RNA metabolism are associated with cellular substructures. EMBO J. **1**:1225–1231.
3. **Ben-Ze'ev, A., R. Abulafia, and S. Bratosin.** 1983. Herpes simplex virus and protein transport are associated with the cytoskeletal framework and nuclear matrix in infected BSC-1 cells. Virology **129**:501–507.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Blobel, G.** 1980. Intracellular protein topogenesis. Proc. Natl. Acad. Sci. USA **77**:1496–1500.
6. **Bolen, J. B., D. G. Anders, J. Trempy, and R. A. Consigli.** 1981. Differences in the subpopulations of the structural proteins of polyoma virions and capsids: biological functions of the multiple VP1 species. J. Virol. **37**:80–91.
7. **Bonner, W. M.** 1975. Protein migration into nuclei. I. Frog oocyte nuclei *in vivo* accumulate microinjected histones, allow entry to small proteins, and exclude large proteins. J. Cell Biol. **64**:421–430.
8. **Bonner, W. M.,** 1975. Protein migration into nuclei. II. Frog oocyte nuclei accumulate a class of microinjected oocyte nuclear proteins and exclude a class of microinjected oocyte cytoplasmic proteins. J. Cell Biol. **64**:431–437.
9. **Buckler-White, A. J., G. W. Humphrey, and V. Pigiet.** 1980. Association of polyoma T antigen and DNA with the nuclear matrix from lytically infected 3T6 cells. Cell **22**:37–46.
10. **Burnette, W. N.** 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
11. **Capco, D. G., K. M. Wan, and S. Penman.** 1982. The nuclear matrix: three dimensional architecture and protein composition. Cell **29**:847–858.
12. **Chatterjee, P. K., M. M. Cervera, and S. Penman.** 1984. Formation of vesicular stomatitis virus nucleocapsid from cytoskeletal framework-bound N protein: possible model for structure assembly. Mol. Cell. Biol. **4**:2231–2234.
13. **Covey, L., Y. Choi, and C. Prives.** 1984. Association of simian virus 40 T antigen with the nuclear matrix of infected and transformed monkey cells. Mol. Cell. Biol. **4**:1384–1392.
14. **Davey, J., N. J. Dimmock, and A. Colman.** 1985. Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in Xenopus oocytes. Cell **40**:667–675.
15. **DeRobertis, E. M., R. F. Longthorne, and J. B. Gurdon.** 1978. Intracellular migration of nuclear proteins in Xenopus oocytes. Nature (London) **272**:254–256.
16. **Dingwall, C., S. V. Sharnick, and R. A. Laskey.** 1982. A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. Cell **30**:449–458.
17. **Einck, L., and M. Bustin.** 1984. Functional histone antibody fragments traverse the nuclear envelope. J. Cell Biol. **98**:205–213.
18. **Feldherr, C., E. Kallenbach, and N. Schultz.** 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. J. Cell Biol. **99**:2216–2222.
19. **Fey, E. G., K. M. Wan, and S. Penman.** 1984. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. J. Cell Biol. **98**:1973–1984.
20. **Garber, E. A., M. M. Seidman, and A. J. Levine.** 1980. Intracellular SV40 nucleoprotein complexes: synthesis to encapsidation. Virology **107**:389–401.
21. **Garcea, R. L., K. Ballmer-Hofer, and T. L. Benjamin.** 1985. Virion assembly defect of polyomavirus *hr-t* mutants: underphosphorylation of major capsid protein VP1 before viral DNA encapsidation. J. Virol. **54**:311–316.
22. **Hall, M. N., L. Hereford, and I. Herskowitz.** 1984. Targeting of *E. coli* β-galactosidase to the nucleus in yeast. Cell **36**:1057–1065.
23. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
24. **Hruby, D. E., D. L. Lynn, and J. R. Kates.** 1980. Identification of a virus-specified protein in the nucleus of vaccinia virus-infected cells. J. Gen. Virol. **47**:293–299.
25. **Joklik, W. K.** 1962. The purification of four strains of poxvirus. Virology **18**:9–18.
26. **Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith.** 1984. Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature (London) **311**:33–38.
27. **Kasamatsu, H., and A. Nehorayan.** 1979. Vp1 affects intracellular localization of Vp3 polypeptide during simian virus 40 infection. Proc. Natl. Acad. Sci. USA **76**:2808–2812.
28. **Kasamatsu, H., and A. Nehorayan.** 1979. Intracellular localization of viral polypeptides during simian virus 40 infection. J. Virol. **32**:648–660.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lanford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37**:801–813.
31. **Lazarides, E.** 1980. Intermediate filaments as mechanical integrators of cellular space. Nature (London) **283**:249–256.
32. **Lin, W., T. Hata, and H. Kasamatsu.** 1984. Subcellular distribution of viral structural proteins during simian virus 40 infection. J. Virol. **50**:363–371.
33. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
34. **Mackett, M., T. Yilma, J. K. Rose, and B. Moss.** 1985. Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. Science **227**:433–435.
35. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **McMillen, J., and R. A. Consigli.** 1977. Immunological reactivity of antisera to sodium dodecyl sulfate-derived polypeptides of polyoma virions. J. Virol. **21**:1113–1120.
37. **Michaeli, T., and C. Prives.** 1985. Regulation of simian virus 40 gene expression in *Xenopus laevis* oocytes. Mol. Cell. Biol. **5**:2019–2028.
38. **Minnigan, H., and R. W. Moyer.** 1985. Intracellular location of rabbit poxvirus nucleic acid within infected cells as determined by in situ hybridization. J. Virol. **55**:634–643.
39. **Norander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–106.
40. **Ozer, H. L., and P. Tegtmeyer.** 1972. Synthesis and assembly of simian virus 40. II. Synthesis of the major capsid protein and its incorporation into viral particles. J. Virol. **9**:52–60.
41. **Paine, P. L.** 1975. Nucleocytoplasmic movement of fluorescent tracers microinjected into living salivary gland cells. J. Cell

Biol. **66**:652–657.
42. **Paine, P. L., L. C. Moore, and S. B. Horowitz.** 1975. Nuclear envelope permeability. Nature (London) **254**:109–114.
43. **Paucha, E., D. Kalderon, R. W. Harvey, and A. E. Smith.** 1986. Simian virus 40 origin DNA-binding domain on large T antigen. J. Virol. **57**:50–64.
44. **Peters, R.** 1984. Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. EMBO J. **3**:1831–1836.
45. **Quinlan, M. P., and D. M. Knipe.** 1983. Nuclear localization of herpesvirus proteins: potential role for the cellular framework. Mol. Cell. Biol. **3**:315–324.
46. **Rice, C. M., C. A. Franke, J. H. Strauss, and D. E. Hruby.** 1985. Expression of Sindbis virus structural proteins via recombinant vaccinia virus: synthesis, processing, and incorporation into mature Sindbis virions. J. Virol. **56**:227–239.
47. **Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik.** 1982. Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. **92**:1–22.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
49. **Schatz, G., and R. A. Butow.** 1983. How are proteins imported into mitochondria? Cell **32**:316–318.
50. **Smith, G. L., and B. Moss.** 1984. Vaccinia virus expression vectors: construction, properties and application. BioTechniques **1984**:306–312.
51. **Smith, G. L., B. R. Murphy, and B. Moss.** 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. USA **80**:7155–7159.
52. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445–453.
53. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
54. **Tooze, J. (ed.).** 1981. DNA tumor viruses: molecular biology of tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
55. **Treisman, R.** 1980. Characterization of polyoma late mRNA leader sequences by molecular cloning and DNA sequence analysis. Nucleic Acids Res. **8**:4867–4888.
56. **Unwin, P. and R. A. Milligan.** 1982. A large particle associated with the perimeter of the nuclear pore complex. J. Cell Biol. **93**:63–75.
57. **Vogt, M., and R. Dulbecco.** 1962. Studies on cells rendered neoplastic by polyoma virus: the problem of the presence of virus-related materials. Virology **16**:41–51.
58. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**:3683–3687.
59. **Weed, H. G., G. Krochmalnic, and S. Penman.** 1985. Poliovirus metabolism and the cytoskeletal framework: detergent extraction and resinless section electron microscopy. J. Virol. **56**: 549–557.