Synthesis, Posttranslational Modifications, and Nuclear Transport of Polyomavirus Major Capsid Protein VP1†

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Polyomavirus major capsid protein VP1 synthesis was studied in infected primary baby mouse kidney cells. A standard curve of VP1 protein was used to quantitate VP1 in the cytoplasm and nucleus of infected cells during the time course of infection. Polyomavirus VP1 continued to be accumulated in the cytoplasm of the cells until 27 h postinfection, at which time the synthesis of VP1 leveled off. VP1 continued to accumulate in the nucleus of the infected cells throughout the course of infection. The presence of the six isospecies, A to F, of polyomavirus VP1 was also studied to determine the relative quantity of each species during the time course of infection. All six species were found in the cytoplasm and nucleus of infected cells at various times postinfection. However, the relative quantity of each species was different at early as compared with later times of infection. In addition, phosphorylated VP1 was found in isolated polyribosomes of infected cells, suggesting that phosphorylation of VP1 is a cotranslational modification. Examination of the effect of macromolecular synthesis on the transport of VP1 into the nucleus of infected baby mouse kidney cells as well as the rate of its nuclear accumulation during and after protein synthesis inhibition revealed that the continual transport and accumulation of VP1 in the nucleus required protein synthesis.

Lytic infection of mouse kidney cells by polyomavirus is a multistep process, requiring 30 to 36 h for a complete lytic cycle. Many of these steps, including virion binding (4, 9, 27-29), entry (15, 17, 27), nuclear uncoating (41), structural protein synthesis (31), and assembly (12, 45), have been studied and characterized. Polyomavirus assembly occurs in the nucleus, as demonstrated by the isolation of stable assembly intermediates (consisting of DNA and capsomere subunits) from infected-cell nuclei (12, 45). In order for the assembly process to occur, the three structural proteins VP1, VP2, and VP3 of polyomavirus must be transported from their site of synthesis in the cytoplasm into the nucleus of the infected cell.

Previously our laboratory produced monospecific antisera to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-derived polyoma virion polypeptides (31). These antisera were used to detect, by immunofluorescence, cytoplasmic synthesis of the polyomavirus structural proteins and their subsequent transport to the nucleus. However, the immunofluorescence technique used in these studies lacked the ability to quantitate the polypeptides being synthesized in the cytoplasm and transported to the nucleus. Recently, Stamatou et al., using recombinant vaccinia virus containing the polyomavirus structural protein genes VP1, VP2, and VP3, demonstrated the synthesis as well as the time course of polyomavirus protein transport to the nucleus in CV1 cells. It was also shown that VP1 promotes nuclear localization of VP2 and VP3 (40). However, this interesting work did not deal with the mechanism(s) of protein transport or with the modifications of the structural proteins. We report here the quantitation of polyomavirus VP1 synthesis in the cytoplasm and its subsequent accumulation in the nucleus of infected baby mouse kidney cells (MKC).

Our laboratory as well as others has evidence that the VP1 capsid protein of polyomavirus is phosphorylated (1-3, 12,

34, 35), and recently VP1 has also been shown to be modified by sulfation (26). These (and possibly other) modifications are thought to give rise to the six species of VP1 separable by isoelectric focusing. It is unknown whether the VP1 modifications occur in the cytoplasm or the nucleus of the infected cell during viral maturation. We have conducted experiments to localize the subcellular site of VP1 isospecies as well as their relative abundance during lytic infection of MKC. Experiments have also been performed to demonstrate that phosphorylation of VP1 occurs in the cytoplasm at the ribosome level.

Many investigators have recently studied the transport of several viral and normal cellular proteins into cell nuclei (8, 10, 11, 19-21, 24, 36). Even though these reports indicate a requirement for inherent structural moieties within the transported proteins' primary structure, little information is available regarding other events (factors) involved in the transport process. We have examined the requirement of protein synthesis for the transport of polyomavirus VP1 during lytic infection of MKC. In this report we provide evidence that the continual transport and accumulation of polyomavirus VP1 is linked to continual protein synthesis in the cell.

As an initial step in the determination of a time course for synthesis and nuclear accumulation of polyomavirus VP1, we sought to establish a quantitative assay to detect and determine nanogram amounts of VP1. This was accomplished by using a VP1 standard curve produced by application of increasing quantities of CsCl-purified virions to SDS-PAGE and subsequent Western immunoblotting. The blots were then probed with anti-VP1 immunoglobulin G (IgG) (rabbit) and ¹²⁵I-labeled protein A. Scanning densitometry of the resulting autoradiogram allowed the production of the VP1 standard curve of increasing VP1 protein quantity versus A₄₄₀, which was linear in the range of 5 to 500 ng of VP1 protein (Fig. 1, inset).

To determine the time course of synthesis and nuclear accumulation of VP1 protein, infected MKC were harvested and fractionated into nuclear and cytoplasmic fractions at 3-h intervals between 15 and 36 h postinfection (PI). Immu-

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FIG. 1. Quantitation of cytoplasmic and nuclear VP1 in infected MKC. MKC grown on 100-mm dishes were infected at a multiplicity of infection of 150, predetermined to infect 100% of the cells as assayed by the immunofluorescence technique described previously (31). The infected monolayers were lysed at 3-h intervals between 15 and 36 h PI in 1 ml of lysis buffer (0.5% Nonidet P-40 in 0.1 M Tris [pH 7.9], 0.1 M NaCl, and 10 μ g of aprotinin, phenylmethylsulfonyl fluoride, TPCK, TLCK, and leupeptin per ml [as protease inhibitors]) at 4°C for 20 min with constant rocking. The lysates were then centrifuged at $2,000 \times g$ for 5 min to pellet the nuclei. The supernatant (cytoplasmic fraction) was stored, and the pellet (nuclear fraction) was washed twice with lysis buffer. The nuclei were resuspended in lysis buffer and disrupted by sonication, and both the nuclear and cytoplasmic fractions were brought to 2% SDS and 100 mM dithiothreitol, boiled for 2 min, and acetone precipitated (5:1, acetone-sample) at -20°C overnight. Precipitated samples were resuspended in 10 mM Tris (pH 7.4) buffer containing 0.1% SDS, and equal amounts of protein from each preparation were then treated with sample buffer, applied to 15% SDS-PAGE gels, and blotted onto nitrocellulose. The VP1 band was identified by using anti-VP1 IgG and 125 I-labeled protein A. The quantity of VP1 in each fraction was determined by interpolation from the VP1 standard curve (inset, described in text) produced simultaneously with the time course experiment. Solid bars, Nuclear VP1; open bars, cytoplasmic VP1.

noblotting of equal protein quantities of each time-point sample was performed simultaneously with a VP1 standard curve in order to circumvent 125 I decay calculations. The quantity of VP1 protein in each time-point preparation was then interpolated from the standard curve. Figure 1 demonstrates that small quantities of cytoplasmic VP1 were detected as early as 15 h PI and continued to accumulate until 27 h PI. VP1 accumulation in the nucleus displayed a continuous pattern initially as early as 15 h PI, and the accumulation was maintained throughout the experiment. This pattern of continuous nuclear accumulation was expected, since polyomavirus assembly occurs in the nucleus and requires a constant supply of structural protein transport for the assembly process. Based on calculations from our results, at 15 h PI there was approximately 0.37 pg of VP1 protein per cell, compared with 2.38 pg/cell at 36 h PI, a 6.43-fold increase in total VP1 protein per cell. In the nucleus of infected cells, there was a rise from 0.052 to 0.77

pg of VP1 per cell from 15 to 36 h PI, a 15-fold increase in the amount of VP1 protein present in the nucleus.

Polyomavirus VP1 protein is known to be modified by phosphorylation and sulfation (1-3, 12, 26, 34, 35). These and possibly other modifications are believed to give rise to the six different species (A to F) of VP1 identified by isoelectric focusing (2, 3, 12). With the knowledge of VP1 synthesis over time and nuclear accumulation, it was also our goal to determine the subcellular site for these modifications. Using the presence of VP1 isospecies as an index of VP1 modification, we subjected samples from our time course experiment to tube isoelectric focusing. Western blotting and scanning densitometry of these cytoplasmic and nuclear preparations demonstrated that early in infection (18 h PI), all six isospecies of VP1 were present in both the cytoplasm and nucleus of infected MKC (Fig. 2). The middle (27 h PI) and late (36 h PI) time points of infection also demonstrated the presence of all six VP1 isospecies. Even though all six species of VP1 were present in the cytoplasm and nucleus of the cells at the different times examined during the time course of infection, the ratio of the species present at each time differed markedly. Table 1 shows the relative quantity of each VP1 species present in the nuclear and cytoplasmic preparations from the different time points assayed. At 18 h PI, VP1 isospecies A, the most basic species of VP1, was present at a much higher ratio (30% of total VP1) than in purified virions (12%). However, at 27 and 36 h PI, this species was present at a ratio (16 and 11%, respectively) resembling that found in purified virions. The three phosphorylated species of VP1, D, E, and F, were present in approximately equal proportions (16 to 18% of total VP1) at 18 h PI, but at 27 and 36 h PI, approximately two- to threefold more D was present than either E or F (30% vs. 12 and 9%, respectively). Species C was the only species that was present at a constant ratio (20% of total VP1, resembling purified virions) throughout the time course examined. It is likely that the relative quantities of these species (and possibly their modifications) are in a state of dynamic flux during virion maturation.

The presence of all six VP1 isospecies in the cytoplasm and the nucleus of the infected cells suggested that modification of VP1 is a cytoplasmic event. In order to investigate this, phosphorylation of VP1 was studied in the cytoplasm of the infected cells at the ribosome level. At 24 h PI, infected MKC were pulse labeled with 32 P_i for 1 h. Immunoprecipitation of isolated polyribosomes from these cells revealed the presence of 32 P-labeled VP1 (Fig. 3A, lane 3). This indicated that VP1 phosphorylation is in fact a cotranslational process. As expected, immunoprecipitation of the nuclear (lane A1) and cytoplasmic (lane A2) fractions from the pulse labeling experiments displayed 32 P-labeled VP1 as well. The identity of the enriched 32 P-labeled 33-kilodalton (kDa) band observed in the polyribosome preparation (lane A3) is not known. It is likely that this band represents either an immature nascent VP1 protein within the polyribosomes or a degradation product of VP1 protein caused by the polyribosome isolation procedure. This band does not represent either of the minor capsid proteins of polyomavirus, VP2 or VP3, since these samples were immunoprecipitated with IgG specific for the VP1 protein. Figure 3B demonstrates the results obtained when the same nuclear (lane B1), cytoplasmic (lane B2), and polyribosome (lane B3) preparations were subjected to SDS-PAGE and Western blotting. As expected, VP1 was detected in all three preparations by this method. The same 33-kDa band was also reactive with anti-VP1 IgG in the polyribosome preparation (lane B3) on a

FIG. 2. Isospecies of VP1 identified in nuclear and cytoplasmic fractions of infected MKC. Nuclear and cytoplasmic fractions from samples taken at 18, 27, and 36 h PI in 10 mM Tris (pH 7.4)–0.1% SDS buffer were treated with 9.5 M urea, 5% mercaptoethanol, and 1% ampholines (pH range 3 to 10; LKB) and subjected to isoelectric focusing by the method of O'Farrell as described previously (2, 3, 33, 34). Resulting tube gels were sliced (2-mm thick) and blotted onto nitrocellulose paper with an ABN transblotter. The nitrocellulose sheets were treated as described in the legend to Fig. 1, and the resulting autoradiogram was scanned at 440 nm. In order to quantitate the ratio of each VP1 isospecies, the area under each peak was calculated, and the sum of the six species was taken as 100% VP1. Each species was then represented as a fraction of this total. The nuclear and cytoplasmic species were compared with identified species of VP1 found in purified virions (panel 7). The right vertical axis is the pH values as represented by the pH gradient line crossing the peaks. Panels 1 to 3 are cytoplasmic and panels 4 to 6 are nuclear fractions from 18, 27, and 36 h PI, respectively.

Western blot. It is therefore unlikely that this band represents a cellular polypeptide which was associated with the VP1 protein in the polyribosomes and co-immunoprecipitated with the anti-VP1 IgG.

Nuclear translocation of various cellular and viral proteins has been studied (8, 10, 11, 19–21, 24, 36). In order to assess the effect of cellular macromolecular synthesis on VP1 nuclear transport, experiments were performed to study this process in the presence of the protein synthesis inhibitor cycloheximide. Preliminary experiments indicated that 100 μ g of cycloheximide per ml, when incubated with cells for 1 h, resulted in 97% inhibition of protein synthesis in infected MKC as assayed by [35 S]methionine incorporation. This quantity of cycloheximide was nontoxic to the cells, as evident from full recovery of protein synthesis upon removal of the cycloheximide. A time course experiment in which

infected MKC were fractionated into cytoplasmic and nuclear fractions at various intervals after the addition of cycloheximide revealed the effect of protein synthesis inhibition on the accumulation of VP1 in the nucleus. The synthesis of VP1 in the cytoplasm (Fig. 4A) was immediately halted after the addition of cycloheximide compared with untreated infected cells. The nuclear fractions (Fig. 4B) from these same time intervals demonstrated a different pattern of VP1 accumulation. VP1 continued to be transported and accumulated in the infected-cell nuclei for a short period (30 to 45 min) after the addition of cycloheximide but was halted thereafter. In order to substantiate this finding, experiments were also conducted in which MKC were labeled with [35 S]methionine from 15 to 27 h PI, at which point the medium was removed and replaced with fresh medium containing cycloheximide (100 μ g/ml). MKC were then

TABLE 1. VP1 species in the nucleus and cytoplasm of infected MKC

Fraction	VP1 species	% of total VP1 present ^a			
		Time PI (h)			Standard VP1
		18	27	36	
Cytoplasmic	A	30.0	15.8	10.4	11.9
	B	5.3	22.2	18.4	14.7
	C	19.6	20.0	19.2	22.7
	D	17.8	15.8	27.9	33.4
	E	17.2	15.4	13.2	9.4
	F	10.1	10.8	10.9	7.9
Nuclear	A	22.2	6.7	13.5	11.9
	B	8.2	25.6	17.1	14.7
	C	19.0	18.8	18.2	22.7
	D	17.9	27.2	30.0	33.4
	E	16.3	11.8	12.2	9.4
	F	16.4	9.9	8.9	7.9

^a The ratio of each VP1 species was quantitated by calculation of the area under each peak in Fig. 2, and the summation of all six species at each time interval was taken as 100% VP1. The ratio of each species is presented as a fraction of 100% total VP1.

fractionated at various intervals, and the quantity of ³⁵S-labeled VP1 in the cytoplasm and nucleus was determined. In infected, untreated cells, radiolabeled VP1 accumulated continually in the nucleus (Fig. 5B), with a concomitant decrease in the cytoplasmic pool of radiolabeled VP1 (Fig. 5A). In infected cells treated with cycloheximide, VP1 accumulation in the nucleus (Fig. 5B) was halted 30 to 45 min after the addition of cycloheximide. These findings suggest a requirement for protein synthesis for continuous transport and accumulation of polyomavirus VP1 in infected-cell nuclei.

In order to determine whether the pool of VP1 protein remaining in the cytoplasm during protein synthesis inhibition is capable of nuclear transport upon removal of cycloheximide, infected MKC were labeled from 15 to 24 h PI with [³⁵S]methionine in methionine-free medium. After the addition of cycloheximide for 1 h (from 24 to 25 h PI), the cycloheximide was removed and fresh medium was placed on the cells. At various intervals following the removal of cycloheximide, the cells were fractionated into cytoplasmic and nuclear fractions and the quantity of radioactive VP1 in each sample was determined. Figure 6 shows that the VP1 remaining in the cytoplasm during cycloheximide treatment was fully capable of nuclear translocation within 30 min after removal of the protein synthesis inhibitor. As expected, the rise in the quantity of labeled VP1 in the nuclei of these cells was accompanied by a concomitant decrease in the quantity of labeled VP1 in the cytoplasmic pool.

The rate of newly synthesized VP1 was also assessed following the removal of the cycloheximide. At 27 h PI, infected cells were radioactively labeled with [³⁵S]methionine following the removal of cycloheximide from the infected cells. As evident in Fig. 7A, newly synthesized VP1 was detected in the cytoplasm of the cells approximately 30 min after the removal of the inhibitor. A longer lag period was observed before radioactive VP1 could be detected in the nuclei of these cells (Fig. 7B). This longer lag period may be due to the presence of a large pool of presynthesized unlabeled VP1 (awaiting transport to the nucleus) which dilutes the newly synthesized radioactive VP1 in the cytoplasm of the cell.

In this report we describe the construction of a standard curve used for the specific quantitation of nanogram quanti-

FIG. 3. Identification of phosphorylated VP1 associated with isolated polyribosomes from infected MKC. Infected MKC were maintained in phosphate-free Eagle medium for 2 h prior to radioactive labeling. At 24 h PI, cells grown on four 100-mm dishes were pulsed for 1 h with 500 μ Ci of ³²P_i per ml in phosphate-free Eagle medium. Polyribosomes were prepared from the cells by the method of Schimke (37). Briefly, the cells were incubated with polysome buffer (25 mM Tris [pH 7.6], 25 mM NaCl, 5 mM MgCl₂, 0.14 M sucrose, and 500 μ g of sodium heparin per ml) and disrupted with a Dounce homogenizer, and the homogenate was centrifuged for 5 min at 5,000 $\times$ g to pellet the nuclei. The nuclear pellet was disrupted by sonication and immediately frozen to be processed later. A portion of the nucleus-free cellular lysate was further fractionated by centrifugation at 100,000 $\times$ g, and the supernatant was used as the cytoplasmic fraction. The remainder of the nucleus-free lysate was then centrifuged for 10 min at 27,000 $\times$ g, and the supernatant was layered over a discontinuous sucrose gradient containing 2 ml of 2.5 M sucrose and 4 ml of 1 M sucrose (both prepared in polysome buffer containing 100 μ g of sodium heparin per ml). The preparations were then centrifuged for 110 minutes at 180,000 $\times$ g in a Beckman SW41 rotor, and the polysome band at the 2.5 M sucrose interface was collected. The nuclear, cytoplasmic, and polysome preparations were dialyzed against 20 mM MOPS (morpholinepropanesulfonic acid) buffer, pH 7.0, containing 150 mM NaCl and either immunoprecipitated with rabbit anti-VP1 IgG and Pansorbin *Staphylococcus aureus* cells (Calbiochem) or subjected to SDS-PAGE and blotted onto nitrocellulose. The immunoprecipitates were resuspended in sample buffer and subjected to SDS-PAGE and autoradiography. The blots were probed with the same anti-VP1 IgG preparation used for the immunoprecipitation, and the reactive bands were visualized by incubation with horseradish peroxidase-conjugated protein A (Bio-Rad Laboratories) and developed with 4-chloro-1-naphthol and H₂O₂ based on the recommendations of the manufacturer. (A) Immunoprecipitated samples; (B) Western blots. Lanes 1, nuclear; lanes 2, cytoplasmic; lanes 3, polyribosome. Size standards (in kilodaltons) are indicated between the two panels, and the polyomavirus structural proteins are shown on both sides.

ties of polyomavirus VP1 protein in infected-cell nuclei and cytoplasm. The standard curve produced by Western blotting was sensitive and linear in the concentration range of 5 to 500 ng of VP1. Figure 1 shows a time course experiment of VP1 synthesis in which this standard curve was used for VP1 quantitation. We demonstrated the presence of nanogram quantities of polyomavirus VP1 in both the cytoplasm

FIG. 4. Effect of protein synthesis inhibition on VP1 cytoplasmic synthesis and nuclear transport. Infected MKC monolayers were treated with cycloheximide (100 μ g/ml) at 24 h PI. At indicated intervals following cycloheximide addition, cells were lysed, and nuclear (B) and cytoplasmic (A) fractions were assayed for VP1 quantity with a standard VP1 curve (inset, panel A) as described in the text and Fig. 1 legend. Cycloheximide-treated cells (●) were compared with infected cells not treated with cycloheximide (□).

and the nucleus of infected cells as early as 15 h PI. VP1 continued to accumulate in the cytoplasm of infected MKC through 27 h PI, at which point the quantity of VP1 in the cytoplasm plateaued. This may be due to a heightened demand for VP1 protein in the nucleus after 27 h for the assembly process. We therefore expected to observe continual transport of newly synthesized structural proteins (e.g., VP1) into the nucleus of infected cells. VP1 protein quantitation in the nucleus of infected MKC between 15 and 36 h PI demonstrated a continuous pattern of accumulation in the nucleus. It is also interesting that VP1 protein was detectable in the nucleus of the infected cells as early as in the cytoplasm of these cells. This indicates that the transport of VP1 occurred concurrently with its synthesis and did not require the build-up of large cytoplasmic pools of synthesized VP1 prior to transport.

Polyomavirus is one of the finest examples of genetic economy in nature. With enough genetic information to code for only six proteins (three early tumor antigens and three

FIG. 5. Fate of presynthesized radiolabeled VP1 during protein synthesis inhibition. Infected MKC were radioactively labeled with 25 μ Ci of [³⁵S]methionine per ml from 15 to 27 h PI. At 27 h PI, medium on the cells was removed and replaced with fresh Eagle medium containing cycloheximide (100 μ g/ml) and methionine (4 mg/ml). At indicated intervals following cycloheximide addition, cells were lysed, and equal amounts of protein from the nuclear (B) and cytoplasmic (A) fractions were subjected to SDS-PAGE. The VP1 bands from stained gels were excised, and the amount of radioactivity in each band was determined by scintillation counting. Cycloheximide-treated cells (●) were compared with infected cells not treated with cycloheximide (□).

late structural proteins), polyomavirus is able to expand its functional capabilities by utilizing post-translationally modified versions of its structural proteins (e.g., VP1) for various required functions (e.g., cellular attachment and hemagglutination) (2, 3). It is important to note that the enzymes responsible for these modifications are a property of the host cell (25). Polyomavirus VP1 has been demonstrated to separate into six distinct species, A (most basic) through F (most acidic), based on their isoelectric points (3). Various posttranslational modifications (e.g., phosphorylation and sulfation) are thought to be responsible for the different isoelectric points observed with these species. Figure 2 shows that all six species of VP1 were present in the cytoplasm of infected cells when assayed by isoelectric focusing. Since VP1 is synthesized in the cytoplasm of the cell and later transported to the nucleus for assembly, the presence of modified species of VP1 in the cytoplasm

FIG. 6. Fate of presynthesized VP1 upon reversal of protein synthesis inhibition. Infected MKC monolayers were labeled from 15 to 24 h PI with 25 μ Ci of [35 S]methionine per ml. At 24 h PI, cycloheximide (100 μ g/ml) was added to the cells for 1 h and then removed, and fresh medium was added to the cells. Cells were then lysed at indicated time intervals. Equal amounts of protein from each cytoplasmic (●) and nuclear (□) fraction were subjected to SDS-PAGE, the VP1 band was excised, and the amount of radioactivity was determined by scintillation counting.

suggested that modification of VP1 occurred in the cytoplasm before its translocation into the nucleus of the infected cells.

Essential to understanding the temporal relationship between synthesis, posttranslational modification, and nuclear transport of polyomavirus VP1 in infected cells is the determination of not only the intracellular site but also the time

course of the occurrence of these modifications. Quantitation of the relative amounts of each VP1 isospecies revealed differences as to their presence at various times during infection. Even though at 18 h PI all six species of VP1 were present in both the cytoplasm and nucleus of infected cells, species A, the most basic species of VP1, was the predominant species present. VP1 isospecies A has previously been shown to be associated with the viral minichromosome and is thought to replace histone H1 early in the assembly process (3, 45). It is therefore possible that an abundant supply of isospecies A is required early in infection for proper condensation of the viral minichromosome prior to the assembly of capsomere subunits about the DNA. Later in infection, at 27 and 36 h PI, the relative quantity of each species resembled the profile observed when purified virions are subjected to the same conditions of isofocusing. This result is expected, since at later times in infection the infected cell has accumulated a large number of mature virions. Pulse-labeling experiments with 32 P_i demonstrated the presence of phosphorylated VP1 protein in polyribosome preparations from infected MKC. These findings reflect the cotranslational nature of this modification.

An understanding of the mechanism by which proteins synthesized in the cytoplasm are specifically sorted to the different cellular compartments is essential for an appreciation of the regulation of functional units within eucaryotic cells. Nuclear entry of larger proteins is a selective mechanism and a property of the amino acid sequence of the protein (10, 18, 32, 38). Recently, the nuclear transport signal of simian virus 40 (SV40) large tumor antigen was identified (19). It comprises a highly basic, lysine-rich stretch of amino acids. It was also demonstrated that a synthetic peptide homologous to the SV40 large T antigen transport signal sequence will transport cross-linked proteins to the nucleus (23, 44). In addition, Wychowski et al. have also demonstrated that the first eight amino-terminal amino acids of SV40 VP1 and VP2/VP3 amino acids 317 to 323 are

FIG. 7. Recovery of VP1 cytoplasmic synthesis and nuclear transport following reversal of protein synthesis inhibition. At 27 h PI, MKC monolayers were treated with cycloheximide (100 μ g/ml) for 1 h. Upon removal of the inhibitor, fresh medium containing 250 μ Ci of [35 S]methionine per ml was added to the cells. At indicated intervals following the removal of the inhibitor, cells were lysed and separated into cytoplasmic (A) and nuclear (B) fractions. Equal amounts of protein from these fractions were then subjected to SDS-PAGE. The VP1 band was excised from stained gels, and the radioactivity was determined by scintillation counting. Cycloheximide-treated (●) cells were compared with infected cells not treated with cycloheximide (□).

involved in the migration of these proteins inside the nucleus (42, 43). It is interesting that the polyomavirus structural protein VP1 (first 12 amino-terminal amino acids) and the last 8 amino acids of VP3 have a similar stretch of basic amino acids with the potential of transporting cytoplasmic proteins to the nucleus.

In order to analyze whether the transport of polyomavirus VP1 is an independent process (requiring only the presence of the signal sequence), we sought to establish the effect of protein synthesis inhibition on the accumulation of newly synthesized VP1 in the nucleus of infected MKC. Figures 4 and 5 suggest that continual accumulation of VP1 protein in the nucleus required protein synthesis. If the transport of VP1 protein was solely dependent on the presence of its putative nuclear transport signal sequence, results exhibiting the continued accumulation of VP1 in the nucleus during protein synthesis inhibition would have been expected. Whether the synthesis of viral or cellular products is required for the transport process is not known at this point. However, our results, together with the work of Stamatos et al. (40), which demonstrates the transport of vaccinia virus-expressed VP1 into the nucleus in the absence of other polyomavirus proteins, extend supportive evidence that cellular constituents may be involved in this process. Recent studies by Gharakhanian et al. have demonstrated that the VP3 protein of SV40, via its carboxyl-terminal 40 amino acids, is involved in interaction with the VP1 structural protein (14). This same region of VP3 has been shown by these authors (13) and others (43) to contain a nuclear localization signal. Gharakhanian et al. suggest that the cluster of positively charged amino acids (similar to the SV40 large T antigen nuclear translocation signal sequence) in this region of VP3 may be involved in electrostatic interactions between SV40 VP1 and VP3. Whether these same interactions occur during polyomavirus structural protein synthesis is not known. However, given the similarities between the two viruses, it is likely that polyomavirus structural proteins VP1, VP2, and VP3 undergo similar protein-protein interactions. It is also likely that polyomavirus VP1 interacts with normal cellular (cytoplasmic or nuclear) proteins through its first 12 amino acids for its nuclear translocation functions. Our results also demonstrated that the pool of VP1 protein remaining in the cytoplasm of the infected cells during protein synthesis inhibition was fully capable of nuclear transport upon removal of cycloheximide (Fig. 6).

With the wealth of accumulated evidence regarding the involvement of the signal sequence in the translocation of proteins into the nucleus, it is our goal to focus our future studies on the examination of VP1 association (interaction) with other viral or cellular proteins in the cytoplasm and nucleus via the signal sequence. These studies may result in the identification of proteins involved in the nuclear transport process in infected cells.

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