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Import of simian virus 40 virions through nuclear pore complexes

(DNA tumor virus/nuclear transport)

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ABSTRACT **How the DNA tumor virus, simian virus 40, reaches the nucleus is unknown. In this report we have tested the affinity of simian virus 40 toward the nucleus by microinjecting virion particles into the cytoplasm under conditions in which cell-surface-mediated viral infection was blocked. Subcellular localization of viral structural proteins Vp1, Vp2, and Vp3, large tumor antigen, and virion particles was followed immunocytochemically and ultrastructurally. Both virion particles and viral structural proteins localized in the nucleus within 1–2 hr after cytoplasmic injection and subsequently expressed large tumor antigen, which was detected in the nucleus as early as 3 hr after cytoplasmic injection. Vp1 and large tumor antigen nuclear accumulation, as well as virion nuclear entry, were blocked by wheat germ agglutinin and an anti-nucleoporin monoclonal antibody, mAb 414. Virion particles were visualized in the vicinity of nuclear pores and in the cytoplasm with this agent. We conclude that virion particles are karyophilic and enter through nuclear pores. This study suggests that virion structural proteins facilitate virion import into the nucleus and viral gene expression.**

An explanation of how infectious animal viruses reach their virion reproductive site upon cell entry is central to the understanding of virion morphogenesis and the prevention of infectious disease. Papovaviruses adsorb to the cell surface via receptors, where they are internalized in endocytic vesicles; these virion-containing vesicles are reported to be transported across the cytoplasm to the nucleus, the reproductive site of the virion (1–9). The surface of the nonenveloped virus is assumed to contain information necessary for targeting the infectious particles to the reproductive site. During normal cell-surface-mediated viral infection, some intracellular virion particles have been found in association with the nuclear fraction as early as 15 min–2 hr of infection, and dissociation of virions into viral structural proteins Vp1, Vp2, and Vp3 and viral DNA occurs there 4–6 hr after infection (2–4). This result indicates that a fraction of the infectious particles reaches the nucleus shortly after infection, and uncoating follows thereafter. This relatively short nuclear transit and disassembly time contrasts with the long time seen for commencement of viral gene expression and that seen for intracytoplasmic trafficking of the particles. (*i*) A long latent period occurs before onset of viral gene expression after viral infection (1, 10). (*ii*) Most virion particles have been found in many cytoplasmic compartments, including endoplasmic reticulum, lysosomes, and mitochondria for long periods after infection, whereas only a few particles have been seen in the nucleus, both in the nucleoplasm and the intracisternal space of the nuclear envelope (2–4, 5–7). In fact, the latter observation has led to a proposal that the infectious virions enter the nucleus through fusion of the outer nuclear membrane with endocytotic vacuoles containing virion particles (2–4, 7). These observations, however, did not show whether cytologically visualized virion particles are those capable of gene expression and propagation of the infectious cycle.

Reproduction of simian virus 40 (SV40), a member of the papovavirus family, occurs in the nucleus of African green monkey kidney cells (1). We have mapped the nuclear transport signal (NTS) of Vp2/3 to residues 316–324 of Vp2 and 198–206 of Vp3, GPNKKKRKL (in one-letter code) (11), which is within a Vp2/3 nuclear localization signal (NLS) (12). The distinction between an NTS and an NLS is as follows: an NTS can target nonnuclear proteins to the nucleus, whereas an NLS can localize the signal-harboring proteins to the nucleus by undefined means without any NTS activity. The amino-terminal 8 residues of SV40 Vp1 contain an NLS (13) but do not promote entry of nonnuclear proteins into the nucleus (14) and, hence, appear insufficient as an NTS. These signals presumably target recently synthesized viral proteins from the cytoplasm to the nucleus for virion assembly. Because a virion particle is composed of these three structural proteins, these signals may also be involved in initially routing infectious virion particles to the reproductive site. In this study we have used microinjection to introduce virion particles directly into the cell cytoplasm to test whether virions, infectious particles, are effectively targeted to the nucleus, and if so, whether they can initiate viral gene expression.

MATERIALS AND METHODS

Cell lines, conditions for cell culture, and preparation of rabbit anti-Vp1 and anti-Vp3 sera have been described (15), as have the procedures for immunofluorescence microscopy and for the use of wheat germ agglutinin (WGA) and Con A (11). Guinea pig anti-Vp1 was prepared as described (15). SV40 was purified from infected cell lysates as described (16). The purified virus was centrifuged to equilibrium in CsCl/TD buffer (0.14 M NaCl/5 mM KCl/0.7 mM Na_2HPO_4/2.5 mM Tris·HCl, pH 7.5), and the virus band at 1.34 g/cc was collected and stored in the CsCl/TD buffer at −20°C. Concentration of virus was extrapolated from virion protein mass visualized by Coomassie blue staining after $NaDodSO_4$/PAGE of virion samples compared with known amounts of protein marker(s), as well as from spectrophotometric measurements of the sample at 260 and 280 nm (17). Purified virus preparations contained 1×10^{12} and 1×10^{14} physical particles per ml (corresponding to 1×10^{10} and 1×10^{12} plaque-forming units per ml, assuming 100 physical particles comprise 1 plaque-forming unit). The number of virion particles cytoplasmically microinjected was, unless otherwise noted, from 5×10^2 to 5×10^3 particles for the immunofluorescent studies and from 1×10^4 to 5×10^4 particles for EM studies. Purified monoclonal antibody (mAb) 414 (18) was

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Abbreviations: NLS, nuclear localization signal; NTS, nuclear transport signal; CGP–BSA, colloidal gold particle–bovine serum albumin; WGA, wheat germ agglutinin; mAb, monoclonal antibody; hpmj, hours post-microinjection; hpmf, hours post-microinfection.

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obtained from David Goldfarb (University of Rochester, Rochester, NY) and was coinjected with virus at 12.5 mg/ml, as was an anti-*Escherichia coli* β-galactosidase mAb, anti-β-gal (Promega).

Microinjection of cells was done as described (11). The microinjection experiments were done, except for those used for EM, with 0.04 ml of virus neutralizing antibody (calf anti-SV40, Flow Laboratories) per ml of the medium. For EM thin sectioning, 5-nm colloidal gold particles (CGP) coupled to bovine serum albumin (BSA) (1×10^{14} particles per ml, Ted Pella, Redding, CA) were included in the microinjection sample. Microinjected cells were marked as an islet by scraping off adjacent and surrounding nonmicroinjected cells with a glass capillary before incubation. A microinfection procedure was developed in which the capillaries were brought close to, but did not touch, the cell surface. The microinfection was done either with or without neutralizing anti-SV40 antibody and cells were cultured in the absence or presence of the antibody, respectively.

For EM, microinjected cell islets were fixed, ethanol-dehydrated, and processed for EM thin section as described (19). Sections were viewed on a Philips 420 electron microscope at 120 KeV. For visualization of virion particles or 5-nm CGP–BSA, droplets containing samples were applied to the parlodion-coated grids, rinsed with water, ethanol-dehydrated, and viewed by EM with or without uranyl acetate treatment, respectively.

RESULTS

Blockade of Cell-Surface-Mediated Viral Infection. Microinjection experiments were done under constant air pressure in which infectious particles continuously leak out from a glass capillary tip. It was, therefore, imperative to first demonstrate that cell-surface-mediated viral infection was effectively blocked and that the biological activities presented here originated solely from the cytoplasmically microinjected particles. We established a microinfection procedure in which the capillary was brought to the surface of a permissive host (TC7) cell without penetrating the cytoplasmic membrane, and infectious particles were delivered to the cell surface. Cells growing along a diamond knife-etched line on glass coverslips were infected with virion particles in medium either containing virion neutralizing antiserum (Fig. 1 *A–C*) or lacking the antiserum (Fig. 1 *D–F*). Virion particles could infect cells, and both an early gene product, large tumor antigen (Fig. 1*E*), and a late gene product, Vp1 (Fig. 1*F*), were found in the proper cell compartments without antiserum. In contrast, with the neutralizing reagent, cell-surface-mediated viral infection was effectively blocked for as long as 24 hr after microinfection, as judged by lack of both gene products (Fig. 1 *B* and *C*). Consequently, the cytoplasmic microinjection experiments presented here were done, except for the EM study, in the presence of the neutralizing antiserum to block cell-surface-mediated infection.

SV40 Particles Are Karyophilic. When virion particles (Fig. 2*A* shows the composition of their structural proteins) were directly introduced into the cytoplasm of TC7 cells together with nonnuclear rhodamine-labeled BSA (11), the major coat protein Vp1 accumulated in the nucleus within 2 hr of microinjection [hours post-microinjection (hpmj)] (Fig. 2*B*), whereas the rhodamine–BSA remained in the cytoplasm of the same cells (Fig. 2*C*). Nuclear Vp1 accumulation was seen as early as 1 hpmj and appeared complete by 2 hpmj (Fig. 3 *A*, *C*, *E*, and *G*). At 0 hpmj, virion-associated Vp1 could be seen entirely in the cytoplasm of microinjected cells (data not shown). The accumulation could be visualized for up to 6 hpmj and then became undetectable by 6–12 hpmj (J.C., unpublished results).

Nuclear Vp1 accumulation could result from dissociation of the virion particles in the cytoplasm and then entry of the structural proteins. Alternatively, the accumulation could reflect the nuclear entry of intact virion particles. We attempted to distinguish between these alternatives by asking whether minor structural proteins Vp2/3 accompany Vp1 nuclear accumulation or whether virion particles can be visualized in the nucleus. Presumably because of low molar content of Vp2/3 relative to that of Vp1 in virion particles, Vp2/3 was not detected in cells injected with 5×10^2 to 5×10^3 particles (data not shown). When cells were microinjected with a high concentration of the virion particles, Vp2/3

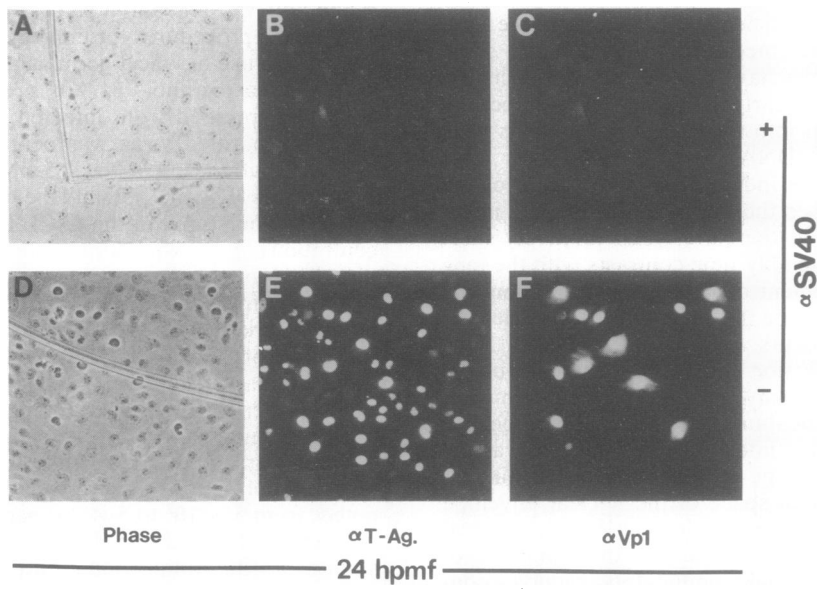

FIG. 1. SV40 microinfection. TC7 cells were microinfected with SV40 in the presence (+, *A*) or absence (−, *D*) of anti-SV40 neutralizing antibodies (αSV40). Cells were cultured for 24 hr (24 hpmf), at which time they were fixed and doubly stained with 1:60 dilution of hamster anti-large tumor antigen (αT-Ag, *B* and *E*) and 1:25 dilution of rabbit anti-Vp1 (αVp1, *C* and *F*) sera, followed by 1:25 dilution of rhodamine- or fluorescein-labeled secondary antibodies (Organon Teknika–Cappel), respectively. *B* and *C* or *E* and *F* represent the same field of cells viewed under phase in *A* or *D*, respectively. Lines in *A* and *D* are diamond knife-etched marks along which microinfection occurred. (×50.)

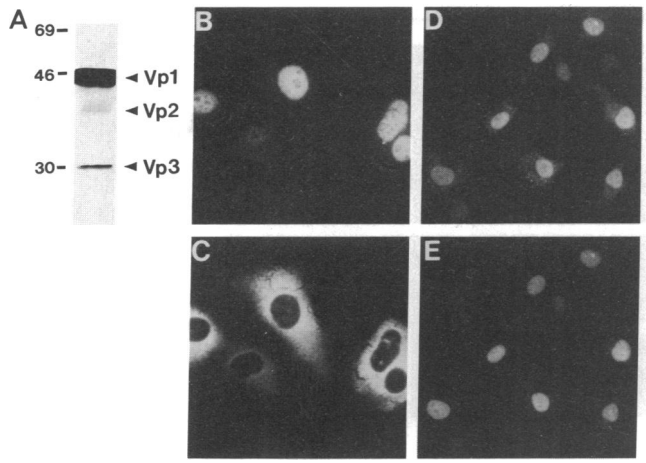

FIG. 2. Composition of SV40 proteins and cytoplasmic virion microinjection. (*A*) Autoradiogram of [^{35}S]methionine-labeled virion proteins separated on a $NaDodSO_4$/15% polyacrylamide gel subjected to fluorography. The labeled virions were prepared from ^{35}S-labeled cell lysates as described (20). Three bands corresponding to viral capsid proteins Vp1, Vp2, and Vp3 are indicated by an arrowhead at right, whereas positions of the molecular-mass standards carbonic anhydrase (30 kDa), ovalbumin (46 kDa), and BSA (69 kDa) are indicated at left. (*B* and *C*) The same field of cells cytoplasmically microinjected with SV40 virions and rhodamine-labeled BSA (2 mg/ml, Organon Teknika–Cappel). Cells were cultured for 2 hr, then fixed, and stained with rabbit anti-Vp1 serum followed by fluorescein-labeled secondary antibodies in *B*. Rhodamine-labeled BSA is shown in *C*. (×130.) (*D* and *E*) The same field of cells cytoplasmically microinjected with 1×10^4 to 5×10^4 virion particles per cell. After 2-hr culture, cells were fixed, treated with 1:10 dilution each of guinea pig anti-Vp1 and rabbit anti-Vp3, followed by 1:25 dilution each of rhodamine- and fluorescein-labeled secondary antibodies, respectively. *D* shows anti-Vp1 staining, whereas *E* shows anti-Vp3 staining. (×100.)

colocalized to the nucleus by 2 hpmj (Fig. 2*E*) just as did most of the Vp1 (Fig. 2*D*).

For EM observations cells were microinjected with 5-nm CGP–BSA together with a high concentration of virion particles. In thin sections, the former served as electron-dense markers to distinguish microinjected cells from noninjected cells. An example of the CGP–BSA in a thin section as well as free CGP–BSA is shown in Fig. 4 *E* and *D*, respectively. In thin sections, electron-dense particles matched in size to that of virion particles (Fig. 4*A*) were mostly found in the

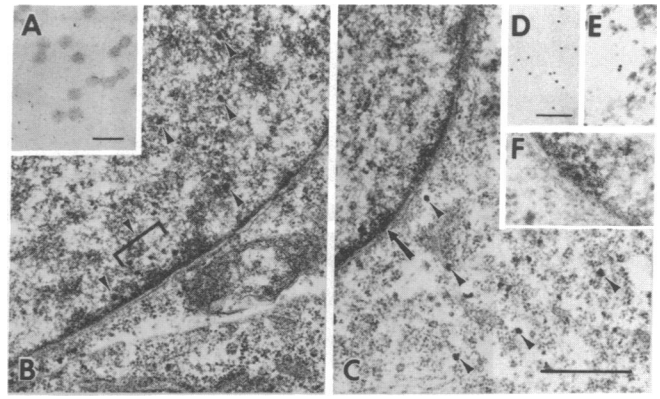

FIG. 4. Virion particles in microinjected cells. (*A* and *D*) Photographs of SV40 (*A*) and 5-nm CGP–BSA (*D*) directly visualized by particle adsorption to EM-supporting membrane. (*B* and *C*) Photographs of comicroinjected cell with virion–CGP–BSA (*B*) and virion–mAb 414–CGP–BSA (*C*). (*E*) Photograph of 5-nm CGP–BSA in cytoplasmic area. (*F*) Enlarged photograph of area of nuclear pore marked by arrow in *C*. Arrowheads, virion particles. Distribution of virion particles was determined by counting particles in 4 μm^2 of two adjacent areas, one in the nucleus and the other in the cytoplasm, both of which were separated by a double nuclear membrane. Results were expressed as means of the number of the particles per μm^2 and SDs, derived from 10 cells. [Bars: *A*, 0.1 μm (magnification the same for *F*); *D*, 0.05 μm (magnification the same for *E*); *C*, 0.5 μm (magnification the same for *B*).]

nucleus (1.7 ± 0.7 particles per μm^2) but were scarce in the cytoplasm (0.2 ± 0.4 particles per μm^2) at 1.5 hpmj (Fig. 4*B*). These electron-dense particles were not present in noninjected cells (data not shown). Such experiments show that SV40 particles are karyophilic and enter the nucleus shortly after their introduction into the cytoplasm by microinjection.

Virion Nuclear Entry Is Blocked by WGA and mAb 414. Both WGA and mAb 414 bind to the nucleoporin p62 (18) at the central transporter apparatus of the nuclear pore complex (21) and block entry of signal-mediated nuclear proteins (22–24). Con A, on the other hand, binds to gp210, which spans the lumen of the nuclear envelope (25–28) and does not inhibit transport (22, 23). We tested the effect of these reagents on nuclear Vp1 accumulation by cytoplasmic comicroinjection of virion particles with each reagent or a control antibody against β-galactosidase. Nuclear Vp1 was seen in cells coinjected with either Con A (Fig. 5*E*) or control antibody (Fig. 5*F*) and was comparable in intensity to the

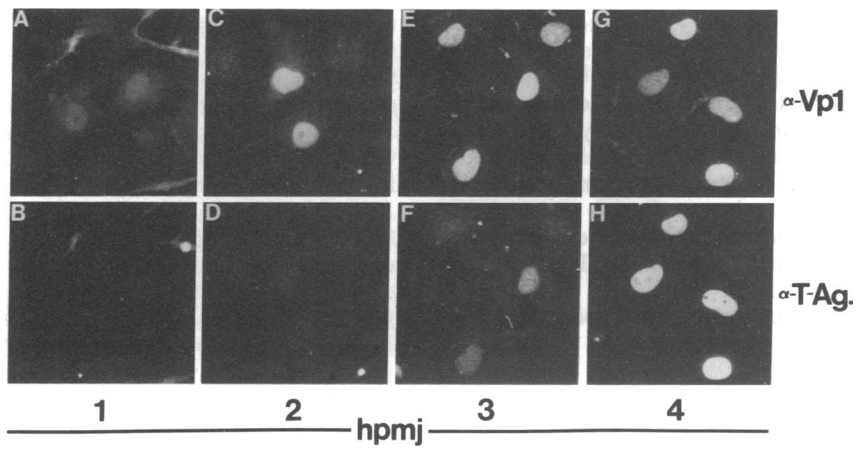

FIG. 3. Time course of nuclear accumulation of Vp1 and large tumor antigen. TC7 cells into which virions had been cytoplasmically introduced were cultured for 1 (*A* and *B*), 2 (*C* and *D*), 3 (*E* and *F*), or 4 (*G* and *H*) hpmj and were fixed and doubly stained with anti-Vp1 (*A*, *C*, *E*, and *G*) and anti-large tumor antigen sera (*B*, *D*, *F*, and *H*), as in Fig. 1. *B*, *D*, *F*, or *H* represents the same field of cells in *A*, *C*, *E*, or *G*, respectively. (×160.)

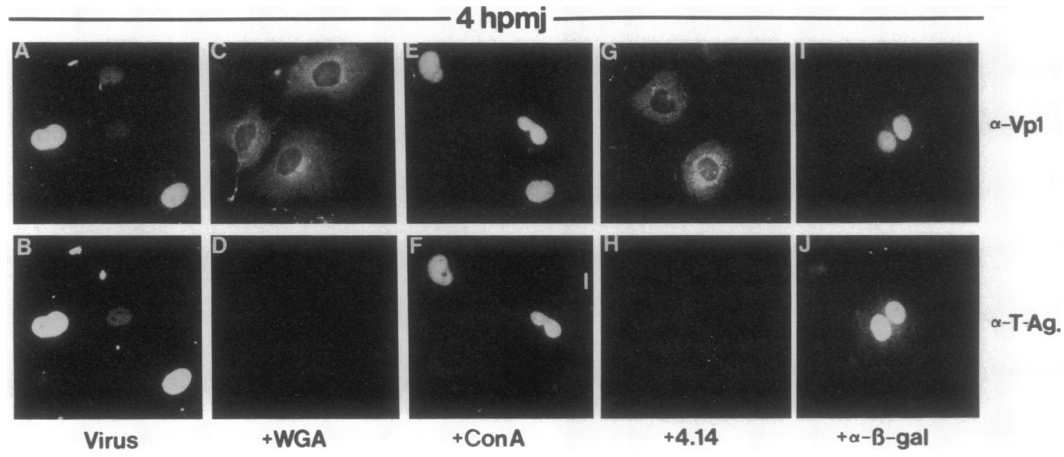

FIG. 5. Effect of various reagents on nuclear accumulation of Vp1 and large tumor antigen. Cells were cytoplasmically microinjected with virus alone (*A* and *B*) or virus plus either WGA (2.5 mg/ml, *C* and *D*), Con A (2.5 mg/ml, *E* and *F*), mAb 414 (12.5 mg/ml, *G* and *H*), and anti-β-galactosidase (anti-β-gal) (12.5 mg/ml, *I* and *J*). Concentration of virion particles injected was the same in all experiments. Cells were fixed at 4 hpmj and doubly stained with anti-Vp1 and anti-large tumor antigen sera, as for Fig. 1. *B*, *D*, *F*, *H*, and *J* represent the same field of cells shown in *A*, *C*, *E*, *G*, and *I*, respectively. (×160.)

control injection (Fig. 5*A*). In contrast, in WGA- or mAb 414-coinjected cells, Vp1 staining in the nucleus was virtually eliminated and was, instead, seen in the cytoplasm (Fig. 5 *C* and *G*). Nuclear accumulation of Vp2/3 was also blocked in cells coinjected with mAb 414, as was Vp1 (data not shown).

In agreement with the Vp1 immunostaining results, EM confirmed that electron-dense particles were predominantly found in the cytoplasm of mAb 414-coinjected cells (2.6 ± 2.4 particles per μm^2, Fig. 4*C*). Some electron-dense particles (0.3 ± 0.4 particles per μm^2) were also found in transit at the pore (Fig. 4*C*, arrow, and *F*) or just inside the inner nuclear membrane (data not shown).

Large Tumor Antigen Expression Accompanies Virion Nuclear Entry. If the virion particles that are artificially introduced into the cytoplasm and that subsequently enter the nucleus are competent for expression of their own genes, production of an early gene product, large tumor antigen, should accompany the nuclear accumulation of Vp1 or the particles. This hypothesis was tested by indirect, double-immunofluorescent microscopy with anti-Vp1 and anti-large tumor antigen serum after cytoplasmic virion microinjection. Nuclear large tumor antigen staining was not seen by 2 hpmj (Fig. 3 *B* and *D*) but did appear at 3 and 4 hpmj (Fig. 3 *F* and *H*). Thus, the viral genomes were present in the nucleus and were transcribed. The large tumor antigen, which is not a virion structural component, was synthesized and localized to the nucleus within 1–2 hr of the virion nuclear entry. Virion-associated Vp1 nuclear accumulation preceded viral gene-encoded large tumor antigen accumulation by 2 hr (Fig. 3 *A–H*). Furthermore, after cytoplasmic microinjection, large tumor antigen was detected in the nucleus only when Vp1 was found there; both WGA and mAb 414, which prevented virion nuclear entry, also prevented large tumor antigen expression (Fig. 5 *D* and *H*), whereas Con A and anti-β-galactosidase, which did not prevent virion nuclear accumulation, allowed large tumor antigen expression (Fig. 5 *F* and *J*) to the same degree as virions alone (Fig. 5*B*). Thus, cytoplasmically injected virion particles are competent for both nuclear import and viral gene expression.

DISCUSSION

We have established a microinfection assay system in which cell-surface-mediated SV40 infection was efficient but was completely blocked by virus-neutralizing antibody. To assess virion nuclear entry, we then microinjected virion particles directly into the cytoplasm of permissive host cells in the presence of the neutralizing antibody. A significant number of SV40 particles introduced directly into the cytoplasm are karyophilic, enter the nucleus through nuclear pores, and can express an early gene, large tumor antigen, which accumulates in the nucleus of the microinjected cells.

In normal papovavirus infections, virion particles enter the cytoplasm of permissive host cells through the cell surface before they reach the nucleus. The fate of virion particles after infection, as well as the time course of appearance of nuclear large tumor antigen, have been well-documented (1–7, 10, 29, 30). Despite the fact that the estimated number of virion particles injected into the cytoplasm of a cell in our study is equivalent to that used in reported infection studies, this injection study differs from the reported infection studies (2–7, 10, 29) in two major ways: (*i*) the shortened time course for nuclear large tumor antigen accumulation and (*ii*) the site of virion nuclear entry. In our cytoplasmic virion microinjection study, nuclear accumulation of all viral structural proteins took 1–2 hr; most electron-dense virion particles were visualized there at 1.5 hpmj, and large tumor antigen accumulation in the nucleus took only 3–4 hr. Because 1–2 hr are required for virions to enter the nucleus, we estimate that ≈2 hr are necessary for the onset of large tumor antigen gene expression, synthesis, and targeting of the gene product, whereas in viral infection with a multiplicity of infection of ≈5–10 plaque-forming units, nuclear accumulation of large tumor antigen has been noted to occur at 10–12 hr after infection (10, 29). A similar time course for nuclear large tumor antigen accumulation has been seen in our cell-surface-mediated microinfection assay system (J.C., unpublished observation). Thus, introduction of the particles directly into the cytoplasm substantially shortens the duration required for the onset of large tumor antigen expression by ≈7–8 hr. A simple explanation for this difference is difficult. Because the major difference is site of virion delivery, the processes of endocytosis and intracytoplasmic transit could be inefficient or virion particles could be damaged during intracytoplasmic trafficking, resulting in only a small fraction of infectious virion particles reaching the nucleus. Thus, amplification of the viral genome must occur before enough large tumor antigen gene products accumulate for detection.

These observations offer experimental evidence for virion nuclear entry through nuclear pores and contrast with a nuclear-entry route proposed in normal infection; the latter entry assumes that virions enter the nucleus via a fusion event between the outer nuclear membrane and virion-containing endocytic vesicles that have been transported

across the cytoplasm after cell-surface-mediated infection (2–4, 7). So far we have not found virion particles in the intracisternal space of the nuclear envelope, even though reasonable particle numbers are found in the nucleus. If most virion particles are karyophilic, as suggested here, why have particles been found in a number of cytoplasmic compartments as well as in the intracisternal space of the nuclear envelope (2–4, 7)? One possibility is that the particles may be incorporated into these subcellular compartments but cannot lead to viral gene expression. Indeed, this question has not been addressed. The possibility that an injury by microinjection circumvented normal intracytoplasmic trafficking routes and promoted virion nuclear entry is unlikely because large tumor antigen expression can be prevented by microinjecting mAb 414 cytoplasmically into SV40-microinfected cells (M.Y., unpublished work), indicating the importance of nuclear pores for virus infection.

The use of nuclear pores for virion nuclear entry is supported by the blockade of nuclear Vp1 accumulation by nucleoporin-binding reagents, as well as the blockade of virion particles from the nucleus. These reagents also blocked subsequent large tumor antigen gene expression. The absence of the template viral DNA for transcription as a consequence of the blockade of virion nuclear entry is a reasonable cause for the lack of large tumor antigen gene expression after comicroinjection of virion/mAb 414 or virion/WGA. However, we realize that the reagents could block nuclear entry of auxiliary components required for gene expression.

Although our results clearly demonstrate entry of infectious virion material through the nuclear pore, this observation does not elucidate any mechanism of transit through the nuclear pore. It is possible that the virion particle changes its shape as it goes through a fully extended and open pore or a partial uncoating of the virus occurs in the cytoplasm so that the individual components can enter through the pore. However, the fact that we see electron-dense virion particles within the nucleus supports our idea that intact virions are traversing the pore. NTS-harboring particles up to 26 nm in diameter have been shown to enter the nucleus through nuclear pores (31, 32). Therefore, passage of a virion particle with a diameter of ≈50 nm (1) through a pore must accompany an opening of the channel larger than has been proposed (21, 33). These observations of virion nuclear entry could reflect flexibility of the pore to accommodate incoming particles.

What makes SV40 virion particles karyophilic? As has been shown in both DNA transfection and microinjection experiments, viral DNAs are infectious but appear to require a long latent period for gene expression (29, 34). The experiments reported here, therefore, suggest that viral structural proteins facilitate virion nuclear import as well as viral gene expression. So far, two nuclear targeting signals, Vp1-NLS and Vp2/3-NTS, have been identified in the three structural proteins. Vp1-NLS (13) alone does not promote entry of nonnuclear protein (14); therefore, it is not an NTS and appears to be embedded inside the virion particle (ref. 35; S. Harrison and R. Liddington, personal communication). Vp2/3-NTS, which targets nonnuclear proteins to the nucleus (11), could serve as SV40-NTS for the virion nuclear entry. Although the molar ratio of both Vp2 and Vp3 proteins per particle is less than that of Vp1 (36), this NTS could be exposed at the surface of a virion particle and be accessible to the nuclear pore receptor(s). Unfortunately, the location of Vp2/3 NTS relative to the Vp1 chain in the virus has not been identified. Further structural as well as genetic studies may yield information regarding the geometrical configuration of the signal on the virion surface.

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