Improvement in Nuclear Entry and Transgene Expression of Baculoviruses by Disintegration of Microtubules in Human Hepatocytes

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***Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), a potent virus for mammalian cell gene delivery, possesses an ability to transduce mammalian cells without viral replication. We examined the role of the cellular cytoskeleton in the cytoplasmic trafficking of viral particles toward the nucleus in human hepatic cells. Microscopic studies showed that capsids were found in the nucleus after either viral inoculation or cytoplasmic microinjection of nucleocapsids. The presence of microtubule (MT) depolymerizing agents caused the amount of nuclear capsids to increase. Overexpression of p50/dynamitin, an inhibitor of dynein-dependent endocytic trafficking from peripheral endosomes along MTs toward late endosomes, did not significantly affect the amount of nuclear accumulation of nucleocapsids in the inoculated cells, suggesting that viral nucleocapsids are released into the cytosol during the early stages of the endocytic pathway. Moreover, studies with recombinant viruses containing the nuclear-targeted expression β -galactosidase gene (β -gal) showed a markedly increased level in the cellular expression of β -galactosidase in the presence of MT-disintegrating drugs. The maximal increase in expression at 10 h postinoculation was observed in the presence of 80 μ M nocodazole or 10 μ M vinblastine. Together, these data suggest that the intact MTs constitute a barrier to baculovirus transport toward the nucleus.**

In recent years, a large number of vectors based on various viruses have been developed as gene transfer vehicles for use in gene therapy applications (21, 40, 44). However, there remain a number of unresolved problems connected to the availability of viral vectors in humans, including the preexisting immunity to most viral vectors of animal origin, as well as the limited ability of vectors to package large DNA inserts. One approach to solving these problems is the development of recombinant viruses of nonhuman origin as vectors for therapeutic gene transfer. Progress in the development of insect baculovirus-derived vectors provides a potential alternative to gene transfer into mammalian cells.

Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) is a large enveloped baculovirus that replicates in insect cells. The viral envelope encloses a 25-by-260-nm cigar-shaped nucleocapsid that contains a 134-kb double-stranded DNA genome. Recombinant baculoviruses have been used in the production of numerous recombinant proteins in insect cells (22, 35). Moreover, their ability to transduce mammalian cells without viral replication and without a cytopathic effect upon infected cells makes AcMNPV a potential nonhuman viral DNA vector for use in gene therapy (2, 11, 22, 25, 29). The efficiency of baculovirus-mediated gene delivery and expression in the recipient cell depends on the entry process and the strength of the promoter used to control the transcription

of the foreign gene. Previous studies have demonstrated that baculoviruses are able to deliver transgenes to various hepatic and nonhepatic mammalian cell types (3, 37, 41). Moreover, it has been shown that AcMNPV enters human hepatic cells in preference to other mammalian cells (8, 19).

The mechanism of entry for baculoviruses have mostly been studied in insect cells. In these cells extracellular budded baculoviruses are internalized by receptor-mediated endocytosis (9, 33, 46, 49). The viral envelope protein gp64 is responsible for the acid-induced membrane fusion and endosomal escape of nucleocapsids into the cytosol (10, 30), where they induce the formation of thick transient actin bundles at one end of the nucleocapsid. During transport through the cytosol toward the nucleus, the viral nucleocapsids exploit the polymerization ability of actin (13, 24). Apparently intact nucleocapsids have been seen inside the nucleoplasm of the insect cell (17). Although the mechanism and strategies by which baculovirus enters mammalian cells have not yet been well characterized, a study using several mammalian cell types has indicated that, after entering the cell via endocytosis, viruses are released from endosomes into the cytoplasm by an acid-induced fusion event. The viruses are then transported through the cytosol to the nucleus, most likely using actin-mediated transport (3, 23, 45).

To better understand the translocation process of baculovirus nucleocapsids in mammalian cells, we studied whether the intracellular transport of nucleocapsids toward the nucleus is affected by the microtubule (MT) network. We inoculated cells in the presence or absence of MT-affecting drugs and monitored nuclear import of nucleocapsids. In addition, we exam-

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ined the contribution of the dynein and/or dynactin motor to cellular trafficking and nuclear import of capsids by overexpressing the p50/dynamin. Moreover, we analyzed the transgene expression in cells inoculated with a virus, LacZ virus, expressing β -galactosidase (β -Gal) when the cells were treated with MT-depolymerizing agents. These studies provide new insights into the mechanism of baculovirus entry into mammalian cells and may also have implications for the optimal use of baculovirus vectors in gene therapy.

MATERIALS AND METHODS

Cells and viruses. Human hepatoma cells (HepG2) used in the experiments were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Gibco-BRL, Paisley, United Kingdom). *Spodoptera frugiperda* Sf9 cells (CRL 1711; American Type Culture Collection, Manassas, Va.), cultured at 27°C in HyQ SFX-Insect medium (HyClone, Logan, Utah), were used to propagate the Bacmid-derived AcMNPV E2 strain (27) virus, as well as vp39EGFP baculovirus, which displays the enhanced green fluorescent protein (EGFP) and the LacZ expression cassette virus. Production of vp39EGFP and LacZ constructs was described in detail previously (2, 23). To localize intracellular nucleocapsids by confocal microscopy, cells were immunolabeled with the anti-EGFP antibody and the anti-lamin A/C monoclonal antibody (MAb) or with the anti-vp39 capsid protein MAb. The relatively weak EGFP signal of the vp39EGFP capsids was amplified by using anti-EGFP antibody labeling. The double-labeling experiment with VP39 and anti-GFP showed that usage of anti-GFP antibody, together with GFP virus, did not cause any changes to the intracellular localization of the virus. To prepare concentrated batches of the viruses, cells were inoculated with wild-type or recombinant viruses at a multiplicity of infection (MOI) of 0.1. At 4 days postinfection the viruses were collected from the medium as described earlier (2). For the microinjection experiments vp39EGFP virions were stripped of their envelopes by gently shaking them in a lysis buffer (500 mM NaCl, 20 mM Tris [pH 7.4], 0.5% Triton X-100, 1 mM EDTA) for 30 min on ice (34). Nucleocapsids were layered onto a sucrose gradient and collected as a UV light-scattering zone (L. Gilbert, personal communication). Prior injection the capsids were resuspended in the microinjection buffer (10 mM Tris-HCl, 120 mM KCl [pH 7.4]). Capsid concentration was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Antibodies and chemicals. A mouse MAb to the AcMNPV vp39 capsid protein was a generous gift from Loy Volkman (University of California, Berkeley). A rabbit antibody to EGFP was obtained from Molecular Probes (Eugene, Oreg.), and a mouse anti-nuclear lamins A/C MAb was obtained from Novocastra Laboratories, Ltd. (Newcastle upon Tyne, United Kingdom). MTs were visualized by using a mouse anti- α -tubulin MAb (Amersham, Little Chalfont, Buckinghamshire, United Kingdom), and actins were visualized by using a rabbit antibody to actin (Sigma, St. Louis, Mo.). A mouse anti- β -Gal MAb was obtained from Biotest (Saco, Maine) and a mouse anti-myc MAb from the American Type Culture Collection (9E10). In the double-labeling studies, Alexa-546- or Alexa-488-conjugated anti-mouse antibodies and Alexa-488- or Alexa-546-conjugated anti-rabbit antibodies from Molecular Probes were used. Nanogold-conjugated polyclonal goat anti-mouse immunoglobulin G (IgG) was purchased from Nano-Probes (Yaphank, N.Y.).

Nocodazole, latrunculin A, and cytochalasin D were purchased from Sigma, and taxol (paclitaxel) and jasplakinolide were from Molecular Probes. Nanogold and HQ-silver enhancement reagents were obtained from Nano-Probes. Epon LX-112 was obtained from Ladd Research Industries (Williston, Vt.).

Drug treatments. To test changes in the organization of the MT network or actin filaments for the intracellular trafficking of virions, cells were incubated either in a medium containing 60 μ M nocodazole, 10 μ M vinblastine, 2 μ M taxol, or 4 μ M cytochalasin D, 23 μ M latrunculin A, 10 μ M jasplakinolide 30 min prior to vp39EGFP or wild-type virus inoculation at an MOI of 100. The drug was then maintained for 8 to 10 h until fixation in methanol (6 min, -20°C). Each experiment was conducted three times, and at least 100 cells were examined. Intracellular nucleocapsids were localized by confocal microscopy and immunolabeling with the anti-EGFP antibody and the anti-lamin A/C MAb. In control studies, cells were stained with an anti-tubulin MAb or an anti-actin antibody to confirm the effect of the drug on the MT or the actin cytoskeleton.

Dynamin overexpression experiments. For transfections we used plasmid encoding the myc-tagged dynamin (p50) subunit of the dynactin complex under the control of the cytomegalovirus promoter (a generous gift from R. Vallee,

University of Massachusetts, Worcester, Mass.). Cloning of p50/dynamin plasmid was described previously (12). The HepG2 cells, plated on 13-mm round coverslips 24 h before transfection, were transfected (Fugene 6; Roche, Indianapolis, Ind.) with Qiagen-purified (Santa Clarita, Calif.) plasmid DNAs (4 μ g/3-cm dish) and inoculated 48 h later with vp39EGFP, LacZ, or wild-type virus at an MOI of 100. Cells were incubated for 8 h at 37°C in the presence or absence of 60 μ M nocodazole or 20 μ M vinblastine before fixation in 20°C methanol for 6 min and immunostaining with the anti-myc MAb and the anti-EGFP antibody.

Electron microscopy (EM). HepG2 cells on 35-mm-diameter plastic culture dishes were grown to ~80% confluency. For the infection assays, cells were inoculated at an MOI of 600 and incubated for 6 h prior to fixation. Some of the cells were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), followed by washing with buffer. They were then postfixed in 1% OsO₄ with 50 mM K₃Fe(CN)₆ in cacodylate buffer and dehydrated in ethanol series, followed by embedding in Epon LX-112. After polymerization, capsules were warmed up to 100°C and removed carefully, and sections parallel to the bottom were cut with an ultramicrotome (Ultracut 8008; Reichert-Jung, Iowa City, Iowa) set to 50 nm, picked up on a copper grid, stained with 2% uranyl acetate and lead citrate, and examined by using a JEOL JEM-1200EX transmission electron microscope at 60 kV.

Pre-embedding was done as previously described (43). Briefly, cells were fixed with PLP fixative (32) for 2 h at room temperature (20 to 23°C) and permeabilized with buffer A (0.01% saponin and 0.1% bovine serum albumin in 0.1 M phosphate buffer [pH 7.4]). In addition, some samples were treated with 0.05% Triton X-100 in buffer A to ensure that the nuclear membrane was permeabilized. Immunolabeling was done by using monoclonal anti-vp39 followed by 1.4-nm gold particle-conjugated goat anti-rabbit IgG. Nano-gold was silver-enhanced for 9 min by using the HQ-silver enhancement reagents and gold-toned with 0.05% gold chloride (7). Cells were postfixed with 1% osmium tetroxide for 1 h at room temperature and dehydrated with a graded series of ethanol. Plastic capsules filled with Epon LX-112 were placed upside down on top of cells. After polymerization capsules were treated as described above.

Microinjection of capsids. Microinjection into HepG2 cells was performed by using a semiautomatic system comprising a Transjector 5246 and Micromanipulator 5171 (Eppendorf, Hamburg, Germany) on an inverted microscope. Cells for injections were grown to 80% confluency on microgrid coverslips (grid size, 175 nm; Eppendorf). Concentrated vp39EGFP nucleocapsids at 2 to 4 mg/ml were injected into the cytoplasm of cells in the absence or presence of 60 μ M nocodazole. After 6 h of incubation, cells were fixed with 4% paraformaldehyde (20 min at room temperature) and then incubated with phosphate-buffered saline containing 0.1% Triton X-100, 1% bovine serum albumin, and 0.01% sodium azide for 20 min at room temperature prior to immunolabeling. Cells were stained either with the anti-EGFP and anti-lamin A/C antibodies or with the anti-vp39 MAb, mounted in Prolong antifade mounting medium (Molecular Probes), and subjected to laser scanning microscopy by using Zeiss LSM 510 inverted microscope.

Expression of β -Gal. A lacZ expression cassette containing the cytomegalovirus immediate-early promoter and a gene encoding a nuclear-targeted β -Gal (β -gal) was cloned into the baculovirus genome as previously described by Airenne et al. (2). The intracellular β -Gal expression of virus-inoculated cells was detected by staining with anti- β -Gal MAb. The β -Gal activity of cells inoculated with LacZ virus was determined by using the luminescent β -Gal detection assay (BD Biosciences Clontech, Palo Alto, Calif.). Cell lysates and detection steps were performed as described in the manufacturer's protocol. To determine the effect of drug concentration on viral β -Gal activity, cells were incubated for 30 min before inoculation (at an MOI of 100, 350, or 500) in medium supplemented with either nocodazole at 40 to 200 μ M, vinblastine at 10 to 50 μ M, or taxol at 1 to 8 μ M and were maintained in these drugs thereafter for 10 h prior to analysis. To study the timing of the β -Gal expression, cells were inoculated at an MOI of 100 or 500 and then analyzed at various times between 4 and 72 h thereafter. To further examine the effects of MT-affecting drugs on β -Gal expression, cells were inoculated at an MOI of 100 and incubated for 10 h in the presence of 60 μ M nocodazole, 20 μ M vinblastine, or 2 μ M taxol and analyzed as described above.

RESULTS

Effect of cytoskeleton on intracellular trafficking of capsids.

It has been shown previously that actin filaments are essential for the cytoplasmic transport of baculovirus nucleocapsids during entry into insect and mammalian cells (13, 45). We further

FIG. 1. Intracellular localization of viral nucleocapsids in the presence of drugs affecting the MT or actin cytoskeleton. (A) Cells inoculated in the presence or absence of nocodazole, vinblastine, or taxol. (B) Cells inoculated in the presence of cytochalasin D, latrunculin, or jasplakinolide. Cells were incubated for 8 h after inoculation and then fixed and stained for nucleocapsids with rabbit polyclonal anti-EGFP IgG, followed by Alexa-488 conjugated goat anti-rabbit IgG (green). The nuclear membrane was visualized by using a mouse anti-lamin MAb, followed by Alexa-546 conjugated goat anti-mouse IgG (red).

examined the role of actin and MTs in the intracellular trafficking of nucleocapsids. In order to monitor the intracellular traffic, nucleocapsids were labeled either with the MAb against the major capsid protein vp39 or with the antibody against the EGFP of the vp39EGFP capsid fusion protein (Fig. 1A). Anti-EGFP labeling was used to amplify the relatively weak EGFP signal given by the vp39EGFP capsids. Control studies in which cells were fixed 8 h after inoculation and stained both for vp39 and EGFP confirmed that anti-vp39 MAb and EGFP IgG staining showed similar staining patterns. In nontreated control cells 8 h after inoculation, ca. 90% of cells showed some nuclear localization of nucleocapsids, although the majority of the nucleocapsids remained scattered throughout the cytoplasm. Nucleocapsids appeared as small aggregations within the nucleus (Fig. 1A). In cells treated with MT-affecting drugs the percentage of cells showing the nuclear localization of capsid aggregates was not significantly higher than the values seen in untreated cells. However, the amount of nuclear aggregates per cell, indicating the presence of viral nucleocapsids, was markedly higher in these cells (Fig. 1A). In contrast, in cells treated with actin-affecting drugs nucleocapsids remained in the cytoplasm and accumulated in the cell periphery (Fig. 1B). In control studies, cells treated either with nocoda-

zole or cytochalasin D showed extensive disruption of MTs or actins.

Effect of overexpression of dynamitin on intracellular trafficking of capsids. Dynamitin overexpression is able to induce a decrease in MT-, dynein-, and dynactin-dependent endosome trafficking. To study the effect of an excess of dynamitin on the baculovirus infection, the cellular localization capsids were monitored in cells transfected with dynamitin expression vector. In cells overexpressing the dynamitin, the majority of the nucleocapsids were accumulated at the cell periphery. However, the level of expression of dynamitin varied in different cells, and in cells that showed lower expression of dynamitin the capsids did not show a localization pattern different from that of control cells, suggesting that the distribution of capsids at the periphery may need excessive dynamitin expression. Interestingly, dynamitin overexpression did not significantly affect the nuclear import of capsids in inoculated cells (Fig. 2A). Furthermore, when the dynamitin-overexpressing cells were inoculated in the presence of drugs affecting the MT cytoskeleton, the intracellular localization or amount of nuclear nucleocapsids was not affected (Fig. 2B). Overexpression of dynamitin affected the intracellular localization of nucleocapsids but not the nuclear accumulation of capsids, suggesting

FIG. 2. Effect of myc-dynamin overexpression on the intracellular localization of viral nucleocapsids. Immunofluorescence microscopy of cells transfected with dynamitin and 36 h later inoculated with virus (A) or inoculated with virus and incubated for 10 h in the presence of nocodazole (B). Cells were fixed with methanol and double-labeled for dynamitin (myc) (red) and viral capsids (green).

that capsids once released into the cytoplasm are able to reach the nucleus without the assistance of the dynactin complex. Taken together, the microscopy studies showed that, in general, the overexpression of dynamitin caused the accumulation of nucleocapsids at the cell peripheral vesicles, whereas it did not significantly affect the nuclear import of capsids.

Intracellular localization of capsids. Due to the large size of AcMNPV capsids, they can also readily be visualized with EM without immunolabeling (Fig. 3A). In the immunolabeling EM analysis viral nucleocapsids were labeled with an MAb against the vp39 capsid protein, followed by Nanogold-conjugated secondary antibody and silver enhancement. By using this method, intracellular capsid antigen was visualized as small, intensely labeled grainy spots (Fig. 3B and C). At 6 h postinoculation, the most of the nucleocapsids were visibly enveloped inside cytoplasmic vesicular structures, with only a small proportion of nucleocapsids visible free in the cytosol (Fig. 3A). At the same time some nonenveloped nucleocapsids had reached the nucleus, and they appeared to be adjacent to the nuclear membrane (Fig. 3B). Moreover, capsid antigen associated with seemingly intact nucleocapsids were also seen in the nucleus. Whole nucleocapsids and their cross-sections were visible (Fig. 3C). The slight degeneration of the fine structure of cells was due to the Triton X-100 treatment used to permeabilize the nuclear membrane to allow antigen labeling inside the nucleus.

Nucleus-directed transport of nucleocapsids. In order to determine whether nucleocapsids need endocytic modifications for transport through the cytosol toward the nucleus, nucleocapsids were injected directly into the cytosol of cells. Localization of nucleocapsids was monitored in cells labeled with the antibody against the EGFP of the vp39EGFP capsid fusion protein. Nucleocapsids were distributed throughout the

cytoplasm immediately after the microinjection. At 6 h postinjection injected capsids were found in both the cytoplasm and nucleoplasm (Fig. 4). In some of the images, capsids appeared to be associated with the nuclear membrane or they were detected as intranuclear fine punctate structures (Fig. 4A). Moreover, in some cells capsids were seen in large amounts in the nucleus (Fig. 4B). Control studies in which cells were injected with vp39EGFP capsids and stained for vp39 confirmed that anti-vp39 MAb and EGFP IgG staining showed similar staining patterns.

Intracellular expression of β -Gal. To study the efficiency of baculovirus-mediated *lacZ* gene delivery into human hepatocytes, HepG2 cells were inoculated with LacZ virus and examined in the presence or absence of drugs affecting MTs. The immunolabeling experiments showed β -Gal label accumulation in the nucleus of inoculated cells after 12 h of incubation at 37°C (Fig. 5A). The kinetics of the β -Gal expression was examined by inoculating cells with the LacZ virus at an MOI of 150 and then determining the proportions of β -Gal-expressing cells at various time points. A significant increase in cellular β -Gal expression was observed after 12 h, and the maximum percentage of the β -Gal-expressing cells was reached at 24 h when 20% of cells showed visible amounts of β -Gal in the nucleus (Fig. 5B).

Furthermore, in order to confirm whether our result showing the increased amount of nuclear nucleocapsids in cells treated with drugs was consistent with the detectable amount of β -Gal activity, we analyzed the β -Gal production of the LacZ virus-inoculated hepatocyte cells. To quantify the changes in β -Gal activity in the presence of various concentrations of drugs affecting MTs, cells were inoculated at an MOI of 350 in the presence of nocodazole, vinblastine, or taxol at concentrations of 1, 2.5, or 40 μ M or higher and maintained in

FIG. 3. EM localization of baculovirus nucleocapsids in inoculated cells. Cells were inoculated with virus at an MOI of 600 and then incubated for 6 h. (A) Accumulation of enveloped virus particles within cytoplasmic vesicles. (B) Immunolabeled viral nucleocapsid interacting with the outer nuclear membrane (arrow) and (C) three nucleocapsids within the nucleus. Cross-sections of two nuclear virus particles were visible (arrowheads). Cytoplasm (Cy) and Nucleus (Nu) are marked in the figure. Scale bars, 200 nm.

these drugs thereafter for 10 h before the analysis. β -Gal activity reached a maximum when the cells were treated in a medium containing 80 μ M nocodazole, 10 μ M vinblastine, or 4 μ M taxol. The difference in β -Gal activity compared to that in cells inoculated in the absence of drugs was significant in the presence of nocodazole or vinblastine but was only slightly increased in taxol-treated cells (Fig. 6).

To examine the effect of virus concentration on the amount of β -Gal expression, cells were inoculated with LacZ virus either at an MOI of 100 or 500, and the activity was determined at various times. In general, β -Gal activity increased, along

with an increase in an MOI. A slight increase in activity was observed between 10 and 30 h postinoculation, a significant increase was detected after 40 h and maximum values were reached by 48 h for both MOIs. At 48 h the β -Gal activity was approximately twofold higher with an MOI of 500 than with an MOI of 100 (Fig. 7A). The results shown in the Fig. 1 suggest that the nuclear import of baculovirus capsids is restricted by the intact MT network. Similarly, we observed that, upon the disintegration of MTs with depolymerizing drugs, the β -Gal activity of the LacZ inoculated cells was markedly increased at 10 h after the inoculation. The β -Gal activities of cells treated

FIG. 4. Localization of capsids after microinjection into cytoplasm. Cells were injected with capsids, incubated for 6 h at 37°C, fixed, and then stained for nucleocapsids with rabbit anti-EGFP IgG followed by Alexa-488 conjugated goat anti-rabbit IgG (green). Capsid localization panels in close proximity to the nuclear membrane (A) and in the nucleus (B) are shown. The nuclear membrane was visualized by using a mouse anti-lamin MAb, followed by Alexa-546-conjugated goat anti-mouse IgG (red).

with nocodazole or vinblastine were at least sixfold higher than they were in untreated cells (Fig. 7B).

DISCUSSION

The entry of baculovirus into host cells is a complex process. The virus needs to enter the cell and penetrate the cytosol, after which the viral DNA-protein complex has to traverse through the cytoplasm and enter the nucleus. Baculovirus is known to enter mammalian cells via endocytosis, followed by acid-induced release to the cytoplasm and nuclear import of nucleocapsids (23, 45). However, little is known about specific intracellular mechanisms used by the incoming baculovirus to transport the genome and other components through the cytoplasm into the nucleus. The purpose of the present study was to further clarify the mechanism and factors affecting the cytoplasmic transport of nucleocapsids toward the nucleus of the mammalian cell.

The crowded cytoplasm constitutes a diffusion barrier. This barrier is caused by cytoplasmic solutes and macromolecules, along with the lattice-like mesh of MTs, actin, and intermediate filament networks (26, 36). MTs provide distinct polarized tracks through the cell along which a variety of viruses move (39, 42, 50, 51). To define the role of MTs in the nuclear localization of baculoviruses, we tested three drugs that affect the MT cytoskeleton for their effects on the nuclear transport

of baculovirus nucleocapsids during entry into hepatocytes. In the presence of nocodazole (which depolymerizes MTs), vinblastine (which causes MT paracrystal formation), or taxol (which stabilizes MTs), nucleocapsids were able to accomplish their movement into the nucleus. Interestingly, the nuclear localization of nucleocapsids was increased in the presence of nocodazole or vinblastine, suggesting that the MT depolymerization event might improve the nucleus-oriented viral movement (Fig. 1A). This finding is consistent with the report of Volkman and Zaal, who found that MTs were progressively depolymerized during AcMNPV infection in infect cells, and that seemed to be necessary event in the infection (47). The use of three drugs affecting actin—cytochalasin D, latrunculin, and jasplakinolide—resulted in an inhibited nuclear uptake and the peripheral localization of nucleocapsids (Fig. 1B). These experiments confirm previous observations that the role of actin is essential in the cytoplasmic and nuclear transport of nucleocapsids in both insect and mammalian cells (13, 45).

Molecular motors, cytoplasmic dynein, and kinesin are known to mediate organelle movement in opposite directions along MTs. Since the initial discovery of the cytoplasmic dynein complex, it has become apparent that this MT-based motor may require another multisubunit complex, dynactin for most, if not all, cytoplasmic dynein-driven activities (5, 20). An important component of intracellular traffic, the endosomal movement from peripheral early endosomes to late endosomes

FIG. 5. Production of the β -galactosidase in hepatic cells inoculated with LacZ virus. (A) Accumulation of β -Gal in the nucleus of cells (arrows). Inoculated cells were incubated for 12 h prior to fixation and staining for β -Gal with a mouse MAb, followed by Alexa-546-conjugated anti-mouse antibody (red). (B) Kinetics of β -Gal production. Percentages of cells showing detectable amounts of β -Gal. Production at various times between 6 and 24 h after viral inoculation at an MOI of 150.

is driven by the dynein and its activator dynactin (6, 18). Overexpression of the dynactin component dynamitin has been shown to disrupt the dynactin complex and affect the vesicular trafficking in interphase cells (1, 12). The utilization of MTs and dynein during minus-end-directed cytoplasmic motility has been noted with several viruses (15, 28, 39). To elucidate the role of MTs, and specifically dynein motor-dependent endocytic vesicle trafficking during the early steps of baculovirus entry, we blocked the dynein-dependent transport mechanism by overexpressing p50/dynamitin. Our data show that the absence of the dynactin complex in the presence or absence of intact MTs caused the dispersion of entering nucleocapsids to the cell periphery (Fig. 2). Furthermore, it is clear that to accomplish cytoplasmic movement toward and into the nucleus, baculovirus nucleocapsids are not likely to benefit from dynein- or dynactin-mediated movement.

The EM studies presented here confirmed previous studies (16) demonstrating that a majority of the incoming virus particles remained in endosomal vesicle-like structures 6 h after inoculation, and only few of them were found in the cytoplasm (Fig. 3A). Immuno-EM studies showed that, after being re-

FIG. 6. β -Gal expression in the presence or absence of various concentrations of MT-affecting agents. Cells were inoculated with LacZ virus at an MOI of 350, incubated for 10 h before cells were harvested, and assayed for β -Gal activity. The mean values of triplicate samples (A to C) with standard deviations are shown.

leased from vesicles, some of the cytosolic virus particles moved to the nuclear pore, where they were located in close association to the nuclear pore (Fig. 3B). From there, they were transferred through the nuclear pores into the nucleus. The small amount of nucleocapsids showing cytoplasmic or nuclear localization suggest that only a relatively small portion of virus particles entering the cell were released from endosomes and imported into the nucleus. This may reflect the poor endosomal escape of the nucleocapsids or the fast delivery of them into the nucleus, which is followed by immediate capsid

FIG. 7. Baculovirus-mediated expression of β -Gal at various times and in the presence or absence of MT-affecting drugs. Cells were inoculated with LacZ virus prior incubation and assaying for β -Gal activity. (A) Kinetics of β -Gal expression at various times after viral inoculation at an MOI of 100 or 500. (B) β -Gal activity of drug-treated cells 10 h after viral inoculation at an MOI of 100. The mean values of triplicate samples with standard deviations are shown.

uncoating (16). Immunogold-labeled, apparently intact virus particles or their cross-sections were seen inside the nucleoplasm (Fig. 3C). Occasionally, a capsid antigen without visible virus particles was detected inside the nucleus, suggesting that at least partial nucleocapsid disintegration had occurred.

Previous data have shown that during nuclear transport the baculovirus nucleocapsids are dependent on actin polymerization (13, 45). However, it has not yet been established whether nucleocapsids need endocytic conditions to expose the nuclear localization sequences essential for nuclear import. Here we show that direct microinjection of viral nucleocapsids into the cytoplasm resulted in a nuclear uptake of nucleocapsids (Fig. 4). Our data suggest that, although baculovirus uses endocytic transport to achieve entry into the cell, endocytic modifications of nucleocapsids are not essential for the cytoplasmic trafficking to take place. In addition, the fact that nucleocapsids were able to pass through the nuclear pore complex without endosomal deformation suggests that the nuclear localization sequence mediating the nuclear transport was already exposed on the surface of injected nonenveloped nucleocapsids or became exposed in the cytoplasm. Further work is required to identify the mechanism and interactions involved in the nuclear import of nucleocapsids after their release into the cytoplasm.

When cells were inoculated with LacZ virus, β -Gal was produced and the majority of the β -Gal protein was transported to the nucleus (Fig. 5A). The amount of cells showing nuclear accumulation of β -Gal started to increase after 6 h, reaching the maximum at 24 h after inoculation with LacZ virus (Fig. 5B). In agreement with our microscopic studies (Fig. 1A) demonstrating that the MT network affects the movement and nuclear import of nucleocapsid, we showed that drug-induced loss of MTs caused improved β -Gal gene expression in LacZ virus-inoculated cells. Presumably, viral genomes were delivered to the nucleoplasm in the absence of intact MTs. Moreover, level β -Gal expression was dependent on the concentration of MT-affecting drugs. The maximal amount of β -Gal enzyme activity was observed in the presence of either 80 μ M nocodazole or 10 μ M vinblastine (Fig. 6). On the other hand, the expression of β -Gal correlated both with the amount of inoculated virus and with the amount of time postinoculation. The amount of β -Gal activity was dose dependent and a peak showing maximum activity was observed at 48 h postinoculation (Fig. 7).

Since MTs are involved in the maturation of endosomes to lysosomes, the increase detected in the nuclear transport of capsids and enhanced transgene expression may also be partly due to the improved escape of virus nucleocapsids from the endosomes, leading to decreased degradation of the endocytosed virions (31). Indeed, it is documented that escape from endosomes sets a major barrier to the cytoplasmic entry of incoming substances (4, 38). The fact that half of the internalized baculoviruses have been reported to be degraded by lysosomes in insect cells (14) and that high amount of viruses were detected in the endosomes in the current study further support this idea. Furthermore, Wang and MacDonald reported recently that MT depolymerizing agents dramatically increased transfection of vascular smooth muscle cells, probably by inhibition of the lipoplex transport to lysosomes (48). These authors also suggested activation of transcription via NF- κ B as another possible mechanism for increased transfection. The mechanism by which NF- κ B is able to enhance cytomegalovirus-directed transgene expression would also explain some of our results. The exact impact of each of these separate possible mechanisms on this improved transgene expression remains to be studied further.

In conclusion, our results suggest that endocytic modifications are not essential for the cytoplasmic trafficking or nuclear import of recombinant baculovirus nucleocapsids in mammalian cells. Cytoplasmic movement of baculovirus is not affected by dynein or dynactin complex. In contrast, transport of nucleocapsids seems to be restricted by an intact MT network, whereas the presence of MT-depolymerizing agents such as nocodazole or vinblastine enhance the intracellular movement of the virus toward the nucleus as well as gene expression. MT disruption and reorganization thus provide a simple method with which to enhance baculovirus-mediated gene delivery in mammalian cells.

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