Enhancement of Polyhedrin Nuclear Localization during Baculovirus Infection

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Polyhedrin is the major component of the nuclear viral occlusions produced during replication of the baculovirus *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV). Since viral occlusions are responsible for the horizontal transmission of AcMNPV in nature, the biosynthesis, localization, and assembly of polyhedrin are important events in the viral replication cycle. We recently defined the sequence requirements for nuclear localization and assembly of polyhedrin. In this study, we examined the localization of polyhedrin at different times of infection. The results showed that nuclear localization of polyhedrin becomes more efficient as the occlusion phase of infection progresses. Several different factors were identified that might contribute to this overall effect, including a higher rate of polyhedrin nuclear localization and a higher rate of polyhedrin biosynthesis. We also examined the biosynthesis and processing of polyhedrin in cells infected with an AcMNPV few polyhedra (FP) mutant, which produces smaller numbers of viral occlusions that contain few or no virions. Compared with wild type, the FP mutant produced polyhedrin more slowly and localized it to the nucleus less efficiently at the beginning of the occlusion phase of infection (24 h postinfection). This supported the idea that the efficiency of polyhedrin nuclear localization is tightly coupled to its rate of biosynthesis. It also revealed that expression of the viral 25K gene, which is inactivated in the FP mutant, is directly or indirectly associated with an enhancement of polyhedrin biosynthesis and nuclear localization at the beginning of the occlusion phase of infection. This enhancement effect appears to be necessary to ensure the normal assembly of viral occlusions.

Autographa californica multicapsid nuclear polyhedrosis virus (AcMNPV) is a member of the family *Baculoviridae*, a large group of DNA-containing viruses that infect insects and other invertebrates (28). AcMNPV is most widely known for its practical applications as an expression vector (reviewed in references 24, 25, and 29) and biological insecticide (5, 30). However, AcMNPV also serves as a good tool for studying protein biosynthesis and processing in insect cells. A common approach is to construct a recombinant that can express a well-characterized foreign protein, use it to infect cultured insect cells, and analyze the biosynthesis of that model protein in those cells (reviewed in reference 18). Alternatively, wild-type AcMNPV can be used to study the biosynthesis and processing of native viral proteins during the course of infection. Besides providing new information on protein biosynthesis pathways in lepidopteran insect cells, both of these approaches can provide information on the AcMNPV replication cycle and the virus-host interaction, which have some interesting and unique features relative to other animal virus systems.

One of the unique features of AcMNPV is that it has a biphasic replication cycle which results in the production of two distinct forms of viral progeny (reviewed in reference 6). Extracellular progeny are produced starting at about 12 h postinfection, when viral nucleocapsids migrate from the nucleus to the plasma membrane and bud from the surface of the infected cell. These budded virus particles disseminate the infection from the primary site in the midgut to numerous other tissues throughout the insect. Viral occlusions are produced later, starting at about 24 h postinfection, when enveloped viral nucleocapsids are embedded within a

paracrystalline matrix inside the nucleus. These large, refractile particles are released into the environment when the infected insect dies and can remain infectious under harsh conditions for a long time. Viral occlusions are responsible for the horizontal transmission of AcMNPV infection, which occurs when a susceptible insect ingests food that is contaminated with these particles.

There is an inverse relationship between the production of budded and occluded virus during AcMNPV infection, suggesting that there is a tangible switch from the production of one form of progeny to the other during the viral replication cycle (46). This idea is supported by the observation that nuclear polyhedrosis virus few polyhedra (FP) mutants, which produce fewer viral occlusions than wild type, also produce more budded virus than wild type (10, 14, 20, 34). The nature of the molecular event(s) that might simultaneously down-regulate budded virus production and up-regulate occluded virus production is unknown. However, one tantalizing hint is that many FP mutants lack a functional 25K gene product (3, 11), and insertional inactivation of this gene will produce a virus with an FP phenotype (4).

The most abundant protein in viral occlusions is polyhedrin, a slightly acidic protein with a molecular weight of about 29,000 (41; reviewed in references 36 and 44). The gene encoding the AcMNPV polyhedrin protein has been cloned and sequenced (15). The polyhedrin gene contains a strong transcriptional promoter, which accounts, at least in part, for the ability of the virus to produce large amounts of polyhedrin in infected cells. Theoretically, newly synthesized polyhedrin is small enough to diffuse into the nucleus through the nuclear pore complexes (reviewed in references 12, 13, and 39). However, it has been shown that polyhedrin contains a nuclear localization signal, which includes the sequence KRKK, located from amino acids 32 to 35 in the

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polypeptide chain (16). Based on recent work in other systems, it is likely that the polyhedrin nuclear localization signal is recognized by a cytoplasmic receptor that plays a functional role in the nuclear localization process (1, 2, 22, 23, 31, 38, 49; reviewed in references 12 and 39). Once it gets inside the nucleus, polyhedrin somehow assembles into the paracrystalline matrix that surrounds enveloped viral nucleocapsids, resulting in the production of viral occlusions, as described above. It has been shown that polyhedrin requires the domain between amino acids 19 and 110 to assemble into occlusion-like particles that can be detected by light microscopy (16). It also is known that the occlusions produced by FP mutants generally contain few, if any nucleocapsids, indicating that these particles were assembled by an abnormal process (10, 34, 35). However, little else is known about the supramolecular assembly of polyhedrin and the production of viral occlusions in the nuclei of AcMNPV-infected insect cells.

The purpose of this study was to extend our previous studies on nuclear localization and assembly of AcMNPV polyhedrin by examining its localization at various times during the occlusion phase of infection. We found that the efficiency and rate of polyhedrin nuclear localization increase as the occlusion phase progresses. This correlated with an increased rate of polyhedrin biosynthesis. At the beginning of the occlusion phase of infection, an FP mutant lacking a functional 25K gene (4) expressed polyhedrin at lower rates and localized it to the nucleus less efficiently. These results suggested that the 25K gene directly or indirectly enhances the biosynthesis and nuclear localization of polyhedrin at the beginning of the occlusion phase, which appears to be necessary to ensure the normal production of viral occlusions during AcMNPV infection.

MATERIALS AND METHODS

Cells and viruses. The Sf9 subclone of IPLB-Sf21-AE (43) was maintained as a Spinner culture in TNM-FH medium (42) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Hazelton Research Products, Inc., Lenexa, Kans.), 1.25 μ g of amphotericin B (Sigma Chemical Co., St. Louis, Mo.) per ml, 25 μ g of gentamycin per ml (Sigma), and 0.1% (wt/vol) pluronic F68 (BASF Wyandotte Corp., Parsippany, N.J.) (32). Radiolabeling medium was methionine-free Grace's medium (42) supplemented with these same concentrations of heat-inactivated fetal bovine serum and antibiotics. Chase medium was TNM-FH supplemented with five times the normal concentration of nonradioactive amino acids, 10 μ g of cycloheximide per ml, and serum and antibiotics, as described above. Sf9 cells were routinely maintained at 28°C at densities between 0.5×10^6 and 2.5×10^6 cells per ml. The E2 strain of wild-type AcMNPV (41), the FP- β gal recombinant (4), VL720 (26), and all the other recombinant baculoviruses used in this study (18) have been described previously. Briefly, FP- β gal is a recombinant in which the viral 25K gene has been disrupted by the insertion of a *lacZ* gene. VL720 encodes nonfused β -galactosidase (GAL), GUS encodes nonfused β -glucuronidase, and all the other recombinants encode polyhedrin-GAL or polyhedrin-GUS fusions in place of polyhedrin. The names of the latter recombinants indicate which polyhedrin amino acids and reporter protein are included in the fusion protein and, in each case, the polyhedrin fragment is N terminal. All baculoviruses were propagated and titrated by plaque assay in Sf9 cells, as described by Summers and Smith (42), except a second agarose overlay containing 0.1% (wt/vol) neutral red

was added at 6 days postinfection to enhance the detection of plaques at 7 days postinfection.

Quantitative biochemical fractionation procedures. Sf9 cells were seeded at a density of 10^6 cells per well in six-well plates, infected at the appropriate multiplicity of infection, and radiolabeled at various times postinfection for 4 h with 100 μ Ci of 35 S-Translabel (ICN Radiochemicals, Irvine, Calif.) per ml of radiolabeling medium. After labeling, the cells were separated into cytosolic and nuclear fractions by using either a detergent-based or a detergent-free fractionation procedure.

The detergent-based procedure has been described previously (16). Briefly, the cells were treated for 10 min on ice with TBN buffer (10 mM Tris [pH 6.5], 140 mM NaCl, 3 mM MgCl₂, 0.5% [vol/vol] Nonidet P-40, 500 Kallikrein inactivating units of aprotinin [Boehringer Mannheim Corp., Indianapolis, Ind.] per ml), scraped into the buffer with a rubber policeman, and centrifuged for 5 min in a microcentrifuge. The supernatant was taken as the cytosolic fraction, and NaOH was added to a final concentration of 0.1 N. The nuclear pellet was extracted with an equal volume of radioimmunoprecipitation assay RIPA buffer (50 mM Tris [pH 8.0], 100 mM NaCl, 3 mM MgCl₂, 1% [vol/vol] Nonidet P-40, 500 Kallikrein inactivating units of aprotinin per ml), NaOH was added to a final concentration of 0.1 N, and the extract was forced through a 25-gauge needle to shear the high-molecular-weight DNA. The extract was clarified for 10 min in a microcentrifuge, and the supernatant was taken as the nuclear fraction.

Detergent-free fractionations were done by using the hexylene glycol method described by Wray and coworkers (48). Radiolabeled cells were washed twice with ice-cold HPN buffer (0.05 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.8], 0.5 M hexylene glycol, 1.0 mM CaCl₂), resuspended in the same buffer, and homogenized in a Dounce homogenizer with a tight-fitting pestle (Wheaton Scientific, Millville, N.J.) until over 95% of the cells were broken, as determined by phase-contrast microscopy. The homogenate was centrifuged at $2,000 \times g$ for 3 min at 4°C to pellet the nuclei, and the supernatant was harvested as the cytosolic fraction. The pellet was washed once with HPN buffer, resuspended in 20% (wt/vol) sucrose in HPN buffer, and layered onto a 50% (wt/vol) sucrose cushion prepared in the same buffer. The cushion was centrifuged at 24,000 rpm for 1 h at 4°C in an SW28 swinging-bucket rotor (Beckman Instruments, Inc., Fullerton, Calif.). The nuclear pellet was recovered, washed once with HPN buffer, and extracted with RIPA buffer plus 0.1 N NaOH as described above. Total protein was recovered from the cytosolic fraction by acetone precipitation and centrifugation.

Equivalent aliquots of the cytosolic and nuclear fractions were boiled for 3 min in protein sample buffer and analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21). The gels were stained with Coomassie blue, destained, dried, and autoradiographed. The bands of interest were excised from the gels, the gel slices were solubilized, and radioactivity was measured in a Beckman model LS6000-IC liquid scintillation counter as described previously (16). Total 35 S incorporation, which reflected the biosynthetic rate for the protein of interest, was taken as the sum of the counts per minute detected in the nuclear and cytosolic populations, and the percentage of nuclear protein was calculated on the basis of that value.

Kinetics studies. The rates of nuclear protein localization in baculovirus-infected Sf9 cells were measured by pulse-

chase analysis, as described previously (17). Briefly, infected cells were starved in radiolabeling medium from 22 to 24 or 46 to 48 h postinfection and then pulse-labeled for 5 min with 500 μ Ci of 35 S-Translabel per ml of the same medium. After pulse-labeling, the cells were diluted 1:10 into chase medium, pelleted, and resuspended in fresh chase medium at a density of 10^6 cells per ml. A 1.0-ml sample was taken immediately (time zero) and at various times after further incubation on a rocker apparatus at 28°C. The samples were immediately fractionated by the detergent-based procedure, and samples from each fraction were analyzed by quantitative SDS-PAGE as described above.

RESULTS

Efficiency of polyhedrin nuclear localization during baculovirus infection. As part of a prior study on nuclear localization of polyhedrin, we constructed a large number of recombinant baculoviruses encoding different polyhedrin-GAL and polyhedrin-GUS fusion proteins (16). The distribution of these fusion proteins in infected Sf9 cells was determined by using a detergent-based biochemical fractionation procedure. Based upon the results of that analysis, which are summarized in Table 1, we concluded that detergent-stable nuclear localization of polyhedrin requires the domain between amino acids 30 and 110. We also found that a slightly larger domain, located between amino acids 19 and 110, is required for the assembly of polyhedrin into viral occlusions or occlusion-like particles. Subsequently, further experiments were done to examine the localization of polyhedrin at different times during the occlusion phase of infection. These experiments involved quantitative biochemical fractionation of Sf9 cells infected with wild-type AcMNPV or recombinants that encode nonfused controls or the different fusion proteins. Infected cells were pulse-labeled from 20 to 24 h postinfection to examine the behavior of the newly synthesized proteins at the beginning of the occlusion phase or at 44 to 48 h postinfection to examine their behavior later in infection. At the end of the labeling period, cytosolic and nuclear fractions were prepared and resolved by SDS-PAGE, and then the proteins of interest were excised from the gels and quantitated as described in Materials and Methods. The results showed that the efficiency of polyhedrin nuclear localization, defined throughout this study as the percentage of newly synthesized polyhedrin found in the nuclear fraction, was higher at the later time of infection (Table 1). Similar results were obtained for all the nuclear fusion proteins, while there was little or no change in the distribution of the cytosolic fusion proteins.

The results of a more complete time course experiment, which included some intervening time points, are shown in Fig. 1 and 2. Again, pulse-labeling was used to assess the fates of proteins synthesized during 4-h periods at different times of infection, starting at the beginning of the occlusion phase. The cells were fractionated at the end of the labeling period, and the proteins of interest were quantitated as described above. Each panel in Fig. 1 shows the time course for one protein of interest, from 20 to 48 h postinfection, in the middle eight lanes of the gel. Extracts that lacked the protein of interest were run in the outside lanes as controls. This makes it easy to identify each protein of interest by comparing the profiles in the middle lanes with those in the control lanes. The results showed that the proportion of newly synthesized polyhedrin found in the nuclear fraction increased steadily with increasing time of infection (Fig. 1A). Similar results were obtained for a nuclear fusion protein

TABLE 1. Distribution of polyhedrin fusion proteins at different times of infection^a

Virus	Expected location ^b	% total radio activity at:	
		24 h	48 h
Polyhedrin	N	46	96
GAL	C	2	1
AcP1-11 GAL	C	2	1
AcP1-22 GAL	C	4	3
AcP1-25 GAL	C	1	6
AcP1-28 GAL	C	1	1
AcP1-31 GAL	C	3	1
AcP1-57 GAL	C	2	2
AcP1-110 GAL	N	49	96
GUS	C	3	6
AcP1-22 GUS	C	6	1
AcP1-25 GUS	C	4	1
AcP1-31 GUS	C	3	1
AcP1-57 GUS	C	2	2
AcP1-110 GUS	N	16	88
AcP15-31 GAL	C	1	1
AcP15-35 GAL	C	1	1
AcP15-57 GAL	C	2	1
AcP15-110 GAL	N	8	67
AcP19-31 GAL	C	2	1
AcP19-35 GAL	C	3	2
AcP19-57 GAL	C	2	1
AcP19-110 GAL	N	2	60
AcP30-35 GAL	C	2	1
AcP30-57 GAL	C	4	1
AcP30-110 GAL	N	5	8

^a Sf9 cells were infected with recombinant baculoviruses encoding the indicated proteins, radiolabeled from 20 to 24 or 44 to 48 h postinfection, and cytosolic and nuclear fractions were prepared by the detergent-based method. Fractions were resolved by SDS-PAGE; then protein bands were excised, solubilized, and counted in a liquid scintillation counter. The results are presented as the percentage of the total radioactivity found in the nuclear fraction. The names of the various recombinants and the fusion proteins they express are defined in Materials and Methods.

^b Based on biochemical fractionation data from reference 16. C, cytosolic; N, nuclear.

(AcP1-110 GAL; Fig. 1D), while nonfused GAL (Fig. 1B) and a cytosolic fusion protein (AcP1-28 GAL; Fig. 1C) remained predominantly cytosolic throughout infection. These results are presented quantitatively in Fig. 2.

The results of these experiments suggested that nuclear localization of polyhedrin becomes more efficient as the occlusion phase of AcMNPV infection progresses. However, this revealed nothing about the possible mechanism(s) responsible for this effect. One possibility was that polyhedrin is actually transported into the nucleus more quickly at later times of infection. A second possibility was that polyhedrin is retained more tightly inside the nucleus at later times of infection. Considering our experimental approach, which involved detergent-based biochemical fractionation, and the fact that polyhedrin assembles into paracrystalline occlusions inside the nucleus, the second possibility seemed more likely. The detergent-based fractionation procedure provides nuclei with little or no cytosolic contamination (7, 33), but it also can extract some nucleoplasmic proteins into the cytosolic fraction (40). Thus, if some newly synthesized polyhedrin was loosely associated with the nucleoplasm at

FIG. 1. Efficiency of nuclear localization at different times of infection. Sf9 cells were infected with AcMNPV (A), VL720 (GAL; B), AcP1-28 GAL (C), or AcP1-110 GAL (D) at a multiplicity of 5 PFU per cell. The infected cells were radiolabeled for 4 h, ending at the time points indicated above each pair of lanes. Then cytosolic (C) and nuclear (N) fractions were prepared by using the detergent-based procedure, and samples were resolved on 12% acrylamide gels, as described in Material and Methods. The middle eight lanes in each panel show the time course for one protein of interest, from 20 to 48 h postinfection. The outside pairs of lanes show control extracts that lack the protein of interest. The positions of the proteins of interest can easily be identified by comparing the profiles in the middle lanes with those in the control lanes. The positions of polyhedrin (P) and GAL (G) or the polyhedrin-GAL fusion proteins (G) are marked on the left-hand side of each panel. The molecular weight standards are identified by their molecular weights ($\times 10^3$) on the right-hand side of each panel.

earlier times of infection, it could be extracted into the cytosol during the fractionation procedure. If polyhedrin assembles faster or more efficiently later in infection, there might not be a nucleoplasmic pool of polyhedrin. Instead, most of the newly synthesized polyhedrin would be sequestered into detergent-resistant occlusions, and a higher percentage would remain in the nuclei during the fractionation procedure.

It was possible to test these ideas by using a detergent-free biochemical fractionation procedure to compare the intracellular distribution of polyhedrin at 24 and 48 h postinfection. If the difference in polyhedrin nuclear localization observed at 24 and 48 h postinfection reflected a difference in its retention inside the nucleus during the detergent-based fractionation procedure, as proposed, then nuclei prepared in the absence of detergent should contain an equal percentage of polyhedrin at both times of infection. However, the results of the detergent-free fractionation experiment showed, again, that the percentage of polyhedrin in the nuclear fraction was higher at 48 h postinfection (Fig. 3). Thus, essentially identical results were obtained whether the cells were fractionated in the presence or absence of deter-

FIG. 2. Quantitative efficiency of nuclear localization at different times of infection. Proteins of interest were excised from the gels shown in Fig. 1, the gel slices were solubilized, and radioactivity was quantitated in a liquid scintillation counter, as described in Materials and Methods. The percentage of total radioactivity found in the nuclear fraction was plotted against time of infection. Symbols: ●, polyhedrin; ○, GAL; ■, AcP1-28 GAL; □, AcP1-110 GAL.

FIG. 3. Distribution of polyhedrin by detergent-free biochemical fractionation. Sf9 cells were infected with AcMNPV or AcP1-28 GAL at a multiplicity of 5 PFU per cell, radiolabeled from 20 to 24 or 44 to 48 h postinfection, and fractionated in the absence of detergent, as described in Materials and Methods. Samples were resolved by SDS-PAGE, and the amounts of radiolabeled polyhedrin or the polyhedrin-GAL fusion protein in each fraction were quantitated as described in the legend to Fig. 2. The results obtained by detergent-free fractionation (Det-) are compared with the results obtained by using the detergent-based (Det+) fractionation procedure (data from Table 1).

gent. These results indicate that the higher percentage of polyhedrin found in the nuclear fraction at later times of infection does not result from a higher rate or efficiency of polyhedrin assembly, which could increase nuclear retention as described above. There must be other factors that contribute to this effect, and further experiments were designed to elucidate these factors, as described below.

Permeability of nucleus during baculovirus infection. The results of the controls for the experiment shown in Fig. 3 revealed a rather surprising factor that could contribute to a higher efficiency of polyhedrin nuclear localization at later times of infection. AcP1-28 GAL is a cytosolic fusion protein which is only about 1% nuclear in detergent-based biochemical fractionation experiments at any time of infection (Table 1 and Fig. 1 and 2). By contrast, AcP1-28 GAL was about 10% and 40% nuclear in detergent-free fractionation experiments done at 24 and 48 h postinfection, respectively (Fig. 3). This could simply indicate that the nuclei prepared by the detergent-free fractionation procedure were significantly contaminated with cytosolic proteins. However, the nuclei appeared to be free of cytoplasm by phase-contrast microscopy (data not shown), they were subsequently purified through a sucrose cushion, and there was no reason to expect a higher percentage of cytosolic contamination later in infection. A more likely explanation for these results is that the nuclear envelope becomes more permeable at later times of baculovirus infection. This would explain how a nonnuclear protein could enter the nucleus and remain associated with the nuclear fraction in the absence of detergent. The inability of this protein to specifically associate with an intranuclear compartment would explain why it is completely extracted into the cytosolic fraction when the cells are fractionated in the presence of detergent. A virus-induced effect on the nuclear envelope might be more pronounced later in infection, which would explain why there is a higher percentage of this nonnuclear protein in the nuclear fraction at 48 h postinfection. Based on these interpretations, we tentatively propose that an increase in the

FIG. 4. Kinetics of polyhedrin nuclear localization at different times of infection. Sf9 cells were infected with AcMNPV (○, ●) or AcP1-28 GAL (□, ■) at a multiplicity of 5 PFU per cell. Then pulse-chase analysis was done at 24 h (●, ■) or 48 h (○, □) postinfection, as described in Materials and Methods. Samples were resolved by SDS-PAGE, and the amounts of radiolabeled polyhedrin or the polyhedrin-GAL fusion protein in each fraction were quantitated as described in the legend to Fig. 2.

permeability of the nuclear envelope might be one factor that contributes to the observed increase in the efficiency of polyhedrin nuclear localization at later times of infection.

Rate of polyhedrin nuclear localization during baculovirus infection. A more obvious factor that would influence the efficiency of polyhedrin nuclear localization is a change in its rate of nuclear localization. This possibility was addressed by performing pulse-chase analyses at 24 and 48 h postinfection, as described in Materials and Methods. The results clearly showed that polyhedrin was localized to the nucleus at a much faster rate at the later time of infection (Fig. 4). The half-time for nuclear localization was about 2 h at 24 h postinfection, compared with only about 10 min at 48 h postinfection. Cytosolic controls remained predominantly cytosolic throughout these pulse-chase experiments, which utilized the detergent-based procedure for subcellular fractionation.

Rate of polyhedrin biosynthesis during baculovirus infection. The rate of polyhedrin nuclear localization might be increased by increasing its rate of biosynthesis. This could raise the cytoplasmic concentration of polyhedrin, thereby increasing the chance of a productive interaction between a polyhedrin molecule and a putative nuclear localization signal receptor in the cytoplasm and/or the nuclear pore complex, and the net result would be a higher rate of nuclear localization. This possibility was addressed by the experiment shown in Fig. 5, in which the overall rates of polyhedrin biosynthesis are shown as a function of time of infection. This graph demonstrated that the rate of polyhedrin biosynthesis increased steadily with increasing time of infection. This correlated directly with the higher rate (Fig. 4) and efficiency (Fig. 1 and 2) of polyhedrin nuclear localization at later times of infection. Similar results were observed for the nuclear fusion protein AcP1-110 GAL (Fig. 1, 2, and 5).

The rate of polyhedrin biosynthesis at 24 h postinfection depends strictly on the multiplicity of AcMNPV infection. As shown in Fig. 6A, there was a fourfold increase in the rate of polyhedrin biosynthesis when the multiplicity of infection was increased from 0.5 to 10 PFU per cell. This was accompanied by a threefold increase in the percentage of polyhedrin found in the nuclear fraction, as shown in Fig.

FIG. 5. Kinetics of polyhedrin biosynthesis at different times of infection. The graph shows the total radioactivity incorporated into polyhedrin or AcP110 GAL at various times of infection, from data obtained in the experiment shown in Fig. 1 and 2.

6B. Thus, the results of this experiment support the idea that there is a direct correlation between the rate of polyhedrin biosynthesis and the efficiency of polyhedrin nuclear localization, as both can be manipulated by changing the multiplicity of infection.

Together, the results of these experiments suggest that there are at least three different factors that could contribute to the higher efficiency of polyhedrin nuclear localization at later times of baculovirus infection: (i) a higher rate of polyhedrin biosynthesis; (ii) a higher rate of polyhedrin nuclear localization; and, perhaps, (iii) an increase in the permeability of the nuclear envelope.

Biosynthesis and nuclear localization of polyhedrin by an FP mutant. The ability to produce large numbers of nuclear occlusions during the very late phase of infection is a definitive characteristic of the nuclear polyhedrosis viruses. Since polyhedrin is the major component of these particles, it must be required in large amounts in the nucleus, and perhaps this explains why it is synthesized and localized faster and more efficiently as the viral infection progresses through the occlusion phase. AcMNPV FP mutants produce relatively smaller numbers of nuclear occlusions that generally contain few or no viral nucleocapsids (14, 35). We hypothesized that this phenotype might result from the inability of an FP mutant to synthesize and localize polyhedrin as fast and efficiently as wild-type AcMNPV.

This hypothesis was tested by comparing the biosynthesis and nuclear localization of polyhedrin in cells infected with wild-type AcMNPV or FP- β gal, an FP mutant that was produced by insertional inactivation of the AcMNPV 25K gene (4). The results of this analysis showed that polyhedrin was synthesized about twice as fast from 20 to 24 h postinfection in cells infected with the wild-type virus (Fig. 7 and 8). Furthermore, the efficiency of polyhedrin nuclear localization during this period was about twice as high in wild-type virus-infected cells as in cells infected with the FP mutant. At 48 h postinfection, the rates of polyhedrin biosynthesis in cells infected with either virus were similar, as were the efficiencies of nuclear localization. We were unable to compare the rates of polyhedrin nuclear localization in wild-type- and FP mutant-infected cells at 24 h postinfection, because the mutant synthesized polyhedrin too slowly for sufficient ³⁵S incorporation during the short labeling time required for the pulse-chase experiment (data not shown).

FIG. 6. Polyhedrin biosynthesis and nuclear localization at different multiplicities of infection. Sf9 cells were infected with AcMNPV at the indicated multiplicities of infection, radiolabeled from 20 to 24 or 44 to 48 h postinfection, and fractionated by the detergent-based procedure. Samples were resolved by SDS-PAGE, and the amounts of radiolabeled polyhedrin in each fraction were quantitated as described in the legend to Fig. 2. (A) Kinetics of polyhedrin synthesis as total ³⁵S incorporation at different multiplicities of infection. (B) Efficiency of polyhedrin nuclear localization as the percentage of total radioactivity found in the nuclear fraction at different multiplicities of infection.

These results support the idea that the efficiency of polyhedrin nuclear localization is tightly coupled to its rate of biosynthesis. They also suggest that expression of the viral 25K gene is directly or indirectly associated with an enhancement of polyhedrin biosynthesis and nuclear localization at the beginning of the occlusion phase of infection. This function appears to be critical for the normal assembly of viral occlusions.

DISCUSSION

The nuclear polyhedrosis viruses are unique in their ability to produce two different forms of progeny during replication. One form is produced relatively early, beginning at about 12 h postinfection, when viral nucleocapsids migrate from the nucleus to the plasma membrane and bud from the surface of the infected cell. The other form appears later, when enveloped nucleocapsids are embedded within a paracrystalline matrix inside the nucleus. This results in the production of multiple viral occlusions, which are large, highly stable

FIG. 7. Polyhedrin expression and nuclear localization by an FP mutant. Sf9 cells were infected with AcMNPV (WT) or FP- β gal (FP) at a multiplicity of 5 PFU per cell. The cells were labeled from 20 to 24 or 44 to 48 h postinfection and split into cytosolic (C) and nuclear (N) fractions by the detergent-based procedure, and samples were resolved by SDS-PAGE. The position of polyhedrin (Poly) is indicated on the left-hand side, and the sizes ($\times 10^3$) of the molecular weight standards (lane M) are indicated on the right-hand side of the photograph.

particles responsible for the horizontal transmission of virus infection. The matrix structure in these particles is composed of a single virus-encoded protein called polyhedrin. Thus, the biosynthesis, nuclear localization, and assembly of large amounts of polyhedrin are critical for the production of viral occlusions.

One factor that helps to ensure an adequate supply of polyhedrin is the presence of a strong promoter in the polyhedrin gene. This promoter induces the transcription of a large pool of mRNA, which can be used to translate large amounts of polyhedrin in the cell cytoplasm. Once synthesized, polyhedrin is probably small enough to simply diffuse into the nucleus through the nuclear pore complexes (reviewed in references 12, 13, and 39). However, this would be a relatively inefficient mechanism, which might explain why polyhedrin has a nuclear localization signal (16). This signal probably facilitates the nuclear localization of polyhedrin by allowing it to participate in a more efficient receptor-mediated transport mechanism.

FIG. 8. Quantitation of polyhedrin biosynthesis and nuclear localization in AcMNPV and an FP mutant. The polyhedrin bands were excised from the gel shown in Fig. 7, and radioactivity was quantitated as described in Materials and Methods. The bars show the rates of polyhedrin biosynthesis as total ^{35}S incorporation, and the numbers above the bars show the percentage of polyhedrin found in the nuclear fraction.

ated transport mechanism. This idea is consistent with the fact that there are nuclear localization signals in other small nuclear proteins, which appear to be localized by a receptor-mediated process (8).

Polyhedrin biosynthesis begins at about 20 h postinfection, which marks the beginning of the occlusion phase of AcMNPV infection. Viral occlusions can sometimes be seen by 24 h, and large numbers of occlusions are obvious by 36 to 48 h postinfection. Thus, as the occlusion phase progresses, there must be a sharp increase in the amount of polyhedrin needed in the nucleus. On the basis of this idea, we decided to compare the efficiencies of polyhedrin nuclear localization at different times of AcMNPV infection.

The results of quantitative biochemical fractionation experiments showed that the percentage of newly synthesized polyhedrin in the nuclear fraction increased steadily from 20 to 48 h postinfection. Initially, we thought that these results might reflect an increase in the efficiency of polyhedrin assembly, because the fractionation procedure could extract nucleoplasmic proteins and polyhedrin ultimately assembles into insoluble particles inside the nucleus. However, when this hypothesis was tested by using a detergent-free fractionation procedure, it turned out to be incorrect. Instead, there appeared to be a true increase in the nuclear targeting efficiency of polyhedrin at later times of AcMNPV infection. Further experiments were designed to identify specific factors that might contribute to this overall effect.

One possible factor revealed by these experiments was an increase in the permeability of the nuclear envelope at later times of infection. This interpretation must be considered cautiously, as it is based on the finding that a nonnuclear protein was found in nuclei prepared in the absence of detergent, and an alternative interpretation is that the nuclear fraction was simply contaminated. However, there are several reasons to believe that this was not true. Phase-contrast microscopy showed that the nuclei were free of cytoplasm, and the nuclei were subsequently purified through a sucrose cushion. Furthermore, a higher percentage of the nonnuclear marker was found in the nuclear fraction at later times of infection. This is hard to explain on the basis of cytoplasmic contamination, but it could easily be explained by a virus-induced effect on nuclear permeability, which would be more pronounced later in infection. Finally, other workers have detected GAL in the nuclei of recombinant AcMNPV-infected Sf9 cells by immunoelectron microscopy (43a). Thus, our data are consistent with the interpretation that AcMNPV induces a change in the permeability of the nucleus during infection. Clearly, further and more direct studies are needed to investigate this possibility. Meanwhile, it is interesting to consider how this change might occur. It could be caused by breakdown of the nuclear envelope during AcMNPV infection, a possibility which is supported by some ultrastructural evidence (27, 47). Alternatively, AcMNPV might induce qualitative or quantitative changes in the nuclear pore complexes. This possibility is supported by studies which indicate that the transport capabilities of the nuclear envelope can fluctuate when cells are exposed to growth factors or hormones (37), when they are transformed (19), or in response to changes in cell cycle or state of cellular differentiation (9).

Two factors that clearly influenced the efficiency of polyhedrin nuclear localization were its rate of biosynthesis and its rate of nuclear localization. The higher efficiency of polyhedrin nuclear localization observed at later times of infection correlated with higher rates of biosynthesis and nuclear localization. Similarly, the higher efficiency of poly-

hedrin nuclear localization observed at higher multiplicities of infection correlated with a higher rate of polyhedrin biosynthesis. Finally, an FP mutant that synthesized polyhedrin more slowly than wild-type virus at 24 h postinfection also localized it less efficiently and more slowly at that time point. Together, these results strongly suggest that the efficiency of polyhedrin nuclear localization depends on the rate of nuclear localization, which is controlled by the rate of polyhedrin biosynthesis.

One factor that probably contributes to a higher rate of biosynthesis is a growing pool of polyhedrin mRNA in the cytoplasm. As expected from previous studies (reviewed in reference 44), we detected small amounts of cytoplasmic polyhedrin RNA at 12 h postinfection, and the levels increased dramatically with increasing time of infection (unpublished observations). Thus, as the AcMNPV infection progresses, larger amounts of polyhedrin mRNA become available for translation, the rate of polyhedrin protein biosynthesis increases, and the net result is an increased rate and efficiency of polyhedrin nuclear localization. As will be discussed below, a reduction in the rate of polyhedrin biosynthesis and nuclear localization at the beginning of the occlusion phase apparently precludes the normal assembly of viral occlusions.

How are polyhedrin biosynthesis and nuclear localization coupled? The simplest explanation is that a higher rate of polyhedrin biosynthesis increases the cytoplasmic concentration of polyhedrin, which increases its chance of interacting with a nuclear localization signal receptor and/or the nuclear pore complexes. Recent studies in other systems have provided strong evidence that there are cytoplasmic receptors that interact with specific signals in newly synthesized nuclear proteins and play a functional role in nuclear protein localization (1, 2, 22, 23, 31, 38, 49; reviewed in references 12 and 39). Therefore, it is likely that Sf9 cells contain an analogous receptor(s) that recognizes the polyhedrin nuclear localization signal and transports polyhedrin to the nucleus. Another factor that probably contributes to a higher concentration of polyhedrin in the cytoplasm is a reduction in the relative cytoplasmic volume, which results from nuclear swelling at later times of AcMNPV infection (45).

Baculovirus FP mutants produce fewer viral occlusions than wild type, which contain few or no nucleocapsids (10, 14, 20, 34), and inactivation of the viral 25K gene can produce a mutant virus with an FP phenotype (3, 4, 11). We have found that one FP mutant produced polyhedrin more slowly and localized it less efficiently to the nucleus at the beginning of the occlusion phase of infection. This observation provided further evidence that the rate of polyhedrin biosynthesis and efficiency of polyhedrin nuclear localization are tightly coupled, as discussed above. Since this mutant lacks the 25K gene, these data also suggested that expression of this viral gene directly or indirectly enhances the biosynthesis and nuclear localization of polyhedrin. This effect appears to be essential for the normal assembly of viral occlusions during infection. Furthermore, it appears that this enhancement effect occurs only at the beginning of the occlusion phase, as the FP mutant had an essentially wild-type rate of polyhedrin biosynthesis and efficiency of polyhedrin nuclear localization at later times of infection. Perhaps there is a critical point at the beginning of the occlusion phase when a certain concentration of polyhedrin is needed in the nucleus, together with other viral components, to initiate the assembly of viral occlusions. Biosynthesis of polyhedrin might be directly or indirectly enhanced as a

result of 25K gene expression at the beginning of the occlusion phase. Later, this relatively subtle effect might be masked by high-level translation of massive amounts of accumulated mRNA. Further studies on the function of the viral 25K gene product will be necessary to determine how it might affect polyhedrin biosynthesis.

It is interesting to consider that this process might also contribute to the switch from the production of budded virus to the production of viral occlusions during the AcMNPV replication cycle. A higher efficiency of polyhedrin biosynthesis and nuclear localization would up-regulate the production of viral occlusions, and this could simultaneously down-regulate the production of budded virus simply by preventing the movement of viral nucleocapsids out of the nucleus. A virus with a defect in this enhancement process would produce fewer viral occlusions and more budded virus, a classical characteristic of the FP phenotype.

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