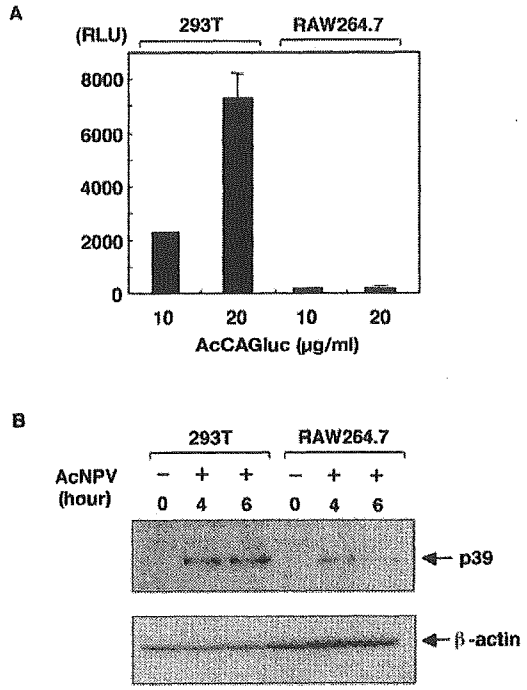

FIG. 8. AcNPV penetrates macrophages through the phagocytic pathway. (A) 293T and RAW264.7 cells (10^6 cells/well) were inoculated with a recombinant baculovirus possessing the luciferase gene under the control of the CAG promoter, AcCAGluc (49) (10 and 20 μg/ml). Cells were harvested 24 h after infection, and relative luciferase activities were determined. (B) 293T and RAW264.7 cells (10^6 cells/well) were inoculated with AcCAGluc (40 μg/ml), washed extensively after 1 h of adsorption, and harvested after 4 or 6 h of incubation. The presence of the p39 capsid protein in cells inoculated with AcNPV was determined by immunoblotting with an anti-p39 monoclonal antibody.

and *N*-acetyl-glucosamine modifications but no detectable galactose or terminal sialic acid residues (29). The mannose receptor (MR) recognizes a range of carbohydrates present on the surfaces and cell walls of microorganisms. MR is primarily expressed on macrophages and DCs and is involved in MR-mediated endocytosis and phagocytosis. In addition, MR plays a key role in host defense and the induction of innate immunity (8). Therefore, it is tempting to speculate that gp64 interacts with MR through its mannose modifications in macrophages and DCs of mice inoculated with AcNPV. However, our data contradict such a model; instead, we show that it is AcNPV DNA, not the gp64 glycoprotein, that induces immune system activation in a MyD88/TLR9-dependent manner.

Recently, it was shown that plasmacytoid DCs (pDCs) naturally produce IFN-α in response to viruses (30). HSV-1 and -2, whose genomes contain abundant CpG motifs, are able to induce the production of IFN-α in pDCs. The HSV-induced production of IFN-α in pDCs derived from MyD88- and TLR9-deficient mice was completely eliminated (33, 40). The recognition of the HSV genome by TLR9 was shown to be mediated by an endocytic pathway that can be inhibited by chloroquine or bafilomycin A1. In this study, we demonstrated that AcNPV induces proinflammatory cytokines through a MyD88/TLR9-dependent signaling pathway, whereas signaling molecules other than MyD88 may participate in IFN-α production in response to AcNPV. Recently, MyD88-independent TLR signaling events involving TIR domain-containing adaptor inducing IFN-β (TRIF) were described (59). Therefore, it is possible that the TRIF pathway is one means by which AcNPV induces MyD88-independent IFN production. However, future studies are needed to clarify the precise mechanisms of this induction.

While UV irradiation of AcNPV abolishes its ability to stimulate an immune response, the addition of liposomes is able to restore this activity. UV-inactivated HSV is capable of inducing the production of IFN-α in pDCs (40), indicating that viral replication is not required for the HSV-induced immune response. In contrast, UV irradiation of AcNPV abolishes immune stimulation in macrophages, while internalization of the inactivated virus by liposomes restores the activity. These results, in conjunction with our data for AcNPVΔ64, indicate that the AcNPV-induced production of cytokines in immunocompetent cells requires a fusion process mediated by gp64 that leads to internalization of the viral genome into the cells.

Recently, several viral envelope glycoproteins were shown to induce immune system activation through TLRs (10, 22, 34, 47). However, gp64 does not directly participate in a TLR-mediated immune response. TLR family members are expressed differentially at very low levels on the surfaces of different immune cells and appear to respond to different stimuli (43). A recent study indicated that LPS and CpG-rich DNA activate TLRs in distinct cellular compartments (3). Internalization and endosomal maturation are required for CpG-rich DNA to activate TLR9, but not for LPS to activate TLR4 on the plasma membrane. We showed here that the inhibition of endosomal maturation by a treatment with chloroquine abolishes the immune system activation of AcNPV in a dose-dependent manner. These results imply that immune system activation by AcNPV through TLR9 requires membrane fusion via gp64 as well as the liberation of the viral genome into cytoplasmic vesicles expressing TLR9.

Interestingly, Lund et al. demonstrated that the TLR7-mediated immune recognition of single-stranded RNAs from vesicular stomatitis virus and influenza virus requires endosomal acidification (41). The recognition of HSV-1 and HSV-2 viral DNAs through a TLR9/MyD88-dependent pathway in pDCs also requires endosomal acidification (40). These data indicate that TLR7 and TLR9 expressed in the endosomal or lysosomal compartments of immunocompetent cells recognize the viral genome entering the cell through receptor-mediated endocytosis or phagocytosis, leading to the secretion of inflammatory cytokines and IFNs. However, the precise mechanisms by which viral genomes translocate to TLR-expressing compartments are still unknown.

Since the first report on the immunostimulatory potential of bacterial DNA, which found that the main immunogenic fraction of mycobacterial lysates consists of genomic DNA (55, 56), substantial progress has been made towards understanding the immunostimulatory potency of CpG-rich DNA motifs, which are more common in bacteria than in vertebrates. For instance, TLR9 was shown to be responsible in vivo for immune system stimulation by oligodeoxynucleotides containing unmethylated CpG motifs (24). Like bacteria, AcNPV contains a significant

number of potentially bioactive CpG motifs. Interestingly, the frequency of CpG motifs in HSV DNA, which has been shown to be involved in the induction of angiogenesis in stromal keratitis (61), was similar to that in *E. coli* DNA. In contrast, the frequency of CpG motifs in the genome of an insect poxvirus was much lower than that for AcNPV (Table 1).

In conclusion, we have demonstrated that AcNPV has the ability to induce innate immune system activation through a MyD88/TLR9-dependent pathway. The molecular mechanisms of viral uptake, intracellular processing, and the induction of potent antiviral activity in immune cells require further investigation. However, the strong immune response induced by AcNPV makes it a promising candidate for a novel, adjuvant-containing vaccine vehicle against infectious diseases. In particular, our findings raise the possibility that AcNPV may be harnessed therapeutically to induce a host immune response against various infectious diseases caused by pathogens invading the respiratory tract.

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Ligand-Directed Gene Targeting to Mammalian Cells by Pseudotype Baculoviruses†

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The baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) can infect a variety of mammalian cells, as well as insect cells, facilitating its use as a viral vector for gene delivery into mammalian cells. Glycoprotein gp64, a major component of the budded AcMNPV envelope, is involved in viral entry into cells by receptor-mediated endocytosis and subsequent membrane fusion. We examined the potential production of pseudotype baculovirus particles transiently carrying ligands of interest in place of gp64 as a method of ligand-directed gene delivery into target cells. During amplification of a gp64-null pseudotype baculovirus carrying a green fluorescent protein gene in gp64-expressing insect cells, however, we observed the high-frequency appearance of a replication-competent virus incorporating the gp64 gene into the viral genome. To avoid generation of replication-competent revertants, we prepared pseudotype baculoviruses by transfection with recombinant bacmids without further amplification in the gp64-expressing cells. We constructed gp64-null recombinant bacmids carrying cDNAs encoding either vesicular stomatitis virus G protein (VSVG) or measles virus receptors (CD46 or SLAM). The VSVG pseudotype baculovirus efficiently transduced a reporter gene into a variety of mammalian cell lines, while CD46 and SLAM pseudotype baculoviruses allowed ligand-receptor-directed reporter gene transduction into target cells expressing measles virus envelope glycoproteins. Gene transduction mediated by the pseudotype baculoviruses could be inhibited by pretreatment with specific antibodies. These results indicate the possible application of pseudotype baculoviruses in ligand-directed gene delivery into target cells.

The baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is an insect virus possessing a 134-kb double-stranded circular DNA genome (3). Due to the strong polyhedrin and p10 promoters, baculovirus is commonly used as a tool for the large-scale production of recombinant protein in insect cells (32, 38). Baculovirus is also capable of entering into a variety of mammalian cells to facilitate the expression of foreign genes under the control of the mammalian promoters without replication of the viral genome (8, 21, 61). Therefore, baculovirus is a useful viral vector, not only for the abundant expression of foreign genes in insect cells, but also for efficient gene delivery to mammalian cells (29). AcMNPV has a number of unique beneficial properties as a viral vector, including a large capacity for foreign gene incorporation, easy manipulation, and replication competence in insect cells combined with incompetence in mammalian cells. Therefore, the possibility of generating replication-competent revertants expressing baculoviral gene products, which can often lead to harmful immune responses against mammalian cells, is significantly lower than for other viral vectors presently in use. Furthermore, studies of host responses to baculovirus infection in vivo revealed that AcMNPV can stimulate interferon production in mammalian cell lines, conferring protection from lethal encephalomyocarditis virus infections in mice (18). Intranasal inoculation with AcMNPV also induces a strong innate immune response, protecting mice from lethal challenges of influenza A or B virus (1). The precise mechanism of protective immune response induction by AcMNPV, however, remains unclear.

Recently, several groups have reported enhanced gene transfer in a variety of cell lines infected with recombinant baculoviruses expressing either foreign viral envelope proteins, such as vesicular stomatitis virus envelope G protein (VSVG), or excess amounts of the endogenous envelope glycoprotein, gp64, on the virion surface (4, 65, 66). Although modification of the virion surface enhances the efficiency of gene transduction into a variety of cell lines, the utility of recombinant baculoviruses in cell-type-specific gene transduction is still unsatisfactory. Ojala et al. demonstrated that, while baculoviruses bearing either a single chain antibody fragment specific for carcinoembryonic antigen or a synthetic immunoglobulin G (IgG) binding domain derived from protein A could specifically bind target cells, cell type-specific gene transduction was unsuccessful (44, 45). Although gp64-null pseudotype baculoviruses expressing a foreign viral envelope protein, such as VSVG or fusion envelope glycoproteins from other baculoviruses, exhibited high infectivity to insect cells, their capacity for gene transduction into mammalian cells has yet to be explored (33, 34). The inefficiency of present gene transfer vectors in gaining entry into cells needing treatment can be problematic, as many therapeutic genes may be deleterious if delivered to bystander cells. Therefore, the development of a ligand-directed gene delivery vector capable of distinguishing between

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† This study is dedicated to the memory of Ikuko Yanase

target and nontarget tissue is essential for both the safety and efficacy of gene therapy.

In this study, we examined the stability of a generated gp64-null pseudotype baculovirus possessing the green fluorescent protein (GFP) gene during passages in insect cells stably expressing the gp64 protein. Replication-competent revertant viruses emerged with high frequency during passage in the cell line, incorporating the gp64 gene into the revertants' viral genomes. To overcome the emergence of revertant viruses during passage, we generated recombinant bacmids lacking the gp64 gene and carrying a ligand of interest and a reporter gene under the control of the polyhedrin and the CAG promoters, respectively. Pseudotype baculoviruses generated from these bacmids exhibited specific ligand-directed gene delivery into target cells. These pseudotype baculovirus vectors may be useful in future clinical gene targeting.

MATERIALS AND METHODS

Cells. *Spodoptera frugiperda* (Sf9) cells were grown in TC-100 medium (Sigma, St. Louis, Mo.) supplemented with 0.26% tryptose phosphate broth (Difco, Detroit, Mich.) and 10% (vol/vol) fetal bovine serum (FBS) (Sigma) (66). To establish a cell line constitutively expressing gp64, Sf9 cells were transfected with pAFgp64 (see below) and pIB/V5-His (Invitrogen, Carlsbad, Calif.) using UniFector reagent (B-Bridge, Sunnyvale, Calif.). Thirty-six hours after transfection, Sf9 cells were selected in TC-100 medium containing blasticidin (50 μg/ml; Invitrogen). Resistant cells were stained with anti-gp64 antibodies (AcV1) (kindly provided by P. Faulkner) (22); positive cells were sorted using a FACSCalibur (Becton Dickinson, Franklin Lakes, N.J.) to establish a cell line, Sfgp64, stably expressing gp64 at the cell surface. The human embryonic kidney cell line 293T and the hamster kidney cell line BHK, purchased from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 2 mM L-glutamine, penicillin (50 IU/ml), streptomycin (50 μg/ml), and 10% FBS (66).

Construction of plasmids. We constructed two expression plasmids, pAF-MCS1 and pAF-MCS2, harboring the A3 actin promoter, a multiple cloning site, and the polyadenylation signal derived from the *Bombyx mori* fibroin H-chain gene, for the subcloning of ligand molecules. First, the promoter and polyadenylation signal were excised from pA3Fb-Luc, kindly provided by H. Bando (Hokkaido University, Sapporo, Japan), and inserted into pUC18. To generate pAFgp64, the gp64 gene was excised from pFBgp64 (see below) by digestion with SalI and HindIII. This fragment was then inserted into the SalI-HindIII site of pAF-MCS1. Recombinant baculoviruses were constructed using the transfer vector pFASTBAC1 (Invitrogen). To measure the expression of foreign genes in mammalian cells, the firefly luciferase gene under the control of the CAG promoter (43) was subcloned into pFASTBAC1. To construct the transfer vector pFB-CAluc, the CAG-luciferase cassette was excised from pCAGluc (61) by digestion with SalI, extension with Klenow enzyme, and redigestion with BamHI and inserted into the SnaBI-BamHI site of pFASTBAC1.

pUCgp64locus was generated by cloning the EcoRI-SmaI fragment from AcMNPV genomic DNA (corresponding to 107,325 to 112,041 nt) (3) into the EcoRI-SmaI site of pUC18. To generate pUCgp64, a fragment encoding the gp64 gene was excised from pUCgp64locus by digestion with SpeI and BglII and then cloned into the XbaI-BamHI site of pUC18. The gp64 gene was excised from pUCgp64 by digestion with SalI and KpnI and inserted into the SalI-KpnI site of pFASTBAC1. The resulting plasmid was designated pFBgp64. To generate pFBgp64CAluc, the cassette including the polyhedrin promoter and the gp64 gene was excised from pFBgp64 by digestion with SnaBI and KpnI and cloned into pFBCAluc, which was digested with SalI, extended with Klenow enzyme, and redigested with KpnI. The VSVG gene fragment was excised from pCAG-VSVG (64) by digestion with EcoRI and cloned into the EcoRI site of pFASTBAC1 to create pFBVSVG. pFBGFP was constructed by excision of the GFP gene from pAcVSVG-CAGFP (65) by digestion with EcoRI and subsequent insertion into the EcoRI site of pFASTBAC1. To generate pFBVSVGCAluc and pFBGFPCAluc, the DNA fragment encoding the polyhedrin promoter and either the VSVG or GFP gene was excised from pFBVSVG or pFBGFP, respectively, by digestion with SnaBI and XhoI and cloned into pFBCAluc, which was digested with SalI, extended with Klenow enzyme, and redigested with XhoI.

cDNAs encoding human CD46 and signaling lymphocyte activation molecule (SLAM; also known as CDw150) were amplified from the genomic DNAs of CHO/CD46 (kindly provided by T. Seya) (25) and CHO.SLAM (kindly provided by Y. Yanagi) (67) cells, respectively, by PCR. The CD46-Fw (1st) (5′-TTT CCTCCGGAGAAATAACAGC-3′) and CD46-Rv (1st) (5′-CTAAGCCAC AGTTGCACTCATG-3′) primers were used to amplify CD46 cDNA, and the SLAM-Fw (1st) (5′-TGACACGAAGCTTGCTTCTG-3′) and SLAM-Rv (1st) (5′-GTCGACCTTTGTTGGTCTCTGGTG-3′) primers were used to amplify SLAM cDNA. These PCR products were used as templates for a second PCR with the primers CD46-Fw-*Hind*III (5′-CCCCAAGCTTCCGCGCCGCG CATGGG-3′) and CD46-Rv-*Sal*I (5′-TTTTGTCGACTCAGCCTCTCTGCTC TGCTG-3′) to amplify CD46 cDNA and SLAM-Fw-*Hind*III (5′-CCCCAAGC TTCCTCATTGGCTGATGGATC-3′) and SLAM-Rv-*Sal*I (5′-AAAAGTCGA CTCAGCTCTCTGGAAGTGTCA-3′) to amplify SLAM cDNA. The amplified CD46 and SLAM cDNAs were digested with HindIII and SalI and then cloned into the HindIII-SalI sites of pAF-MCS2 to create pAFCD46 and pAFSLAM, respectively. The CD46 and SLAM cDNAs were excised from pAFCD46 and pAFSLAM, respectively, by digestion with HindIII, extension with Klenow enzyme, and redigestion with XbaI and cloned into pFASTBAC1. To generate pFBCD46CAluc and pFBSLAMCAluc, the DNA fragments encoding the polyhedrin promoter and either the CD46 gene or the SLAM gene were excised from pFBCD46 or pFBSLAM, respectively, by digestion with SnaBI and PvuI and cloned into pFBCAluc. A mutant SLAM gene, SLAMcyto7, possessing a truncated cytoplasmic domain of 7 amino acids, was generated by PCR with the primers SLAM-Fw-SmaI (5′-CCCCCCGGGCCTCATTGGCTGATGGATC-3′) and SLAM-7aa-stop-Rv-*Sal*I (5′-GGGGGGTCGACTCAGTTCGTTTT ACCTCTTCTTCTCAAC-3′). This PCR product was digested with SmaI and SalI and then cloned into the SmaI-SalI sites of pAF-MCS1 to create pAFSLAMcyto7. To construct pFBSLAMcyto7CAluc, the SLAMcyto7 gene was excised from pAFSLAMcyto7 and substituted for the full-length SLAM gene of pFBSLAMCAluc. All plasmids containing PCR-derived sequences were confirmed by sequence analyses. For infection with pseudotype baculoviruses bearing CD46 or SLAM, we transfected target cells with expression plasmids encoding either the hemagglutinin and fusion proteins of the Edmonston strain (EdH and EdF) or those of the Ichinose strain (IcH and IcF) measles viruses. The pCA-EdH, pCA-EdF, pCA-IcH, and pCA-IcF plasmids were kindly provided by K. Takeuchi (63).

Construction of pseudotype baculoviruses. The gp64 gene of the AcMNPV-bacmid (bMON14272; Invitrogen) was replaced with the chloramphenicol acetyltransferase (CAT) gene as described previously with slight modifications (5, 33). Briefly, the CAT gene was amplified by PCR with the Chl-Fw-*Spe*I (5′-GGAC TAGTCCGAATAAATACCTGTGACGG-3′) and Chl-Rv-*Bgl*II (5′-GAAG ATCTCGTCAATTATTACCTCCACGG-3′) primers using the pBT plasmid (Stratagene, La Jolla, Calif.) as a template. Following digestion with SpeI and BglII, the amplified CAT gene replaced the gp64 gene of pUCgp64locus to create p64locus/cat. To construct a gp64-null AcMNPV-bacmid, bMONΔ64/cat, the p64locus/cat plasmid was linearized by digestion with NdeI and cotransfected with bMON14272 into Sf9 cells. Forty-eight hours posttransfection, the cells were washed with cold phosphate-buffered saline and lysed in proteinase K buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate [SDS]). DNA was purified from cell lysates by phenol-chloroform extraction and then transformed into *Escherichia coli* DH10B competent cells (Invitrogen) by electroporation using a Gene Pulser (Bio-Rad, Hercules, Calif.). Resistant colonies were selected in kanamycin and chloramphenicol. Disruption of the gp64 gene was confirmed by PCR in a bMON14272-transformed colony that was resistant to kanamycin and chloramphenicol (Fig. 1A). To generate DH10BacΔ64/cat, we cotransfected bMONΔ64/cat and the helper plasmid pMON7124 into DH10B cells. To construct recombinant bacmids, DH10BacΔ64/cat was transformed with transfer vectors and selected according to the manufacturer's instructions. To separate recombinant bacmids from the pMON7124 helper plasmid, miniprep bacmid DNA was transformed into DH10B cells by electroporation. To generate pseudotype baculoviruses, bacmids lacking the gp64 gene and possessing both an exogenous ligand gene and the luciferase gene under the polyhedrin and CAG promoters, respectively, were transfected into Sf9 cells. Fifteen micrograms of the bacmid DNA was used to transfect 5×10^6 Sf9 cells in a 10-cm-diameter dish by using 30 μl of UniFector reagent (B-Bridge). Four days after transfection, 500 ml of culture supernatants (50 dishes) was harvested. The resulting pseudotype baculoviruses, AcΔ64/gp64/CAluc, AcΔ64/VSVG/CAluc, AcΔ64/CD46/CAluc, AcΔ64/SLAM/CAluc, and AcΔ64/SLAMcyto7/CAluc, were concentrated ~2,000 times by ultracentrifugation as described previously (66). The number of virus particles was determined from the signal intensity by Western blotting for the capsid protein vp39. Although both AcΔ64/gp64/CAluc and AcΔ64/VSVG/CAluc infected and repli-

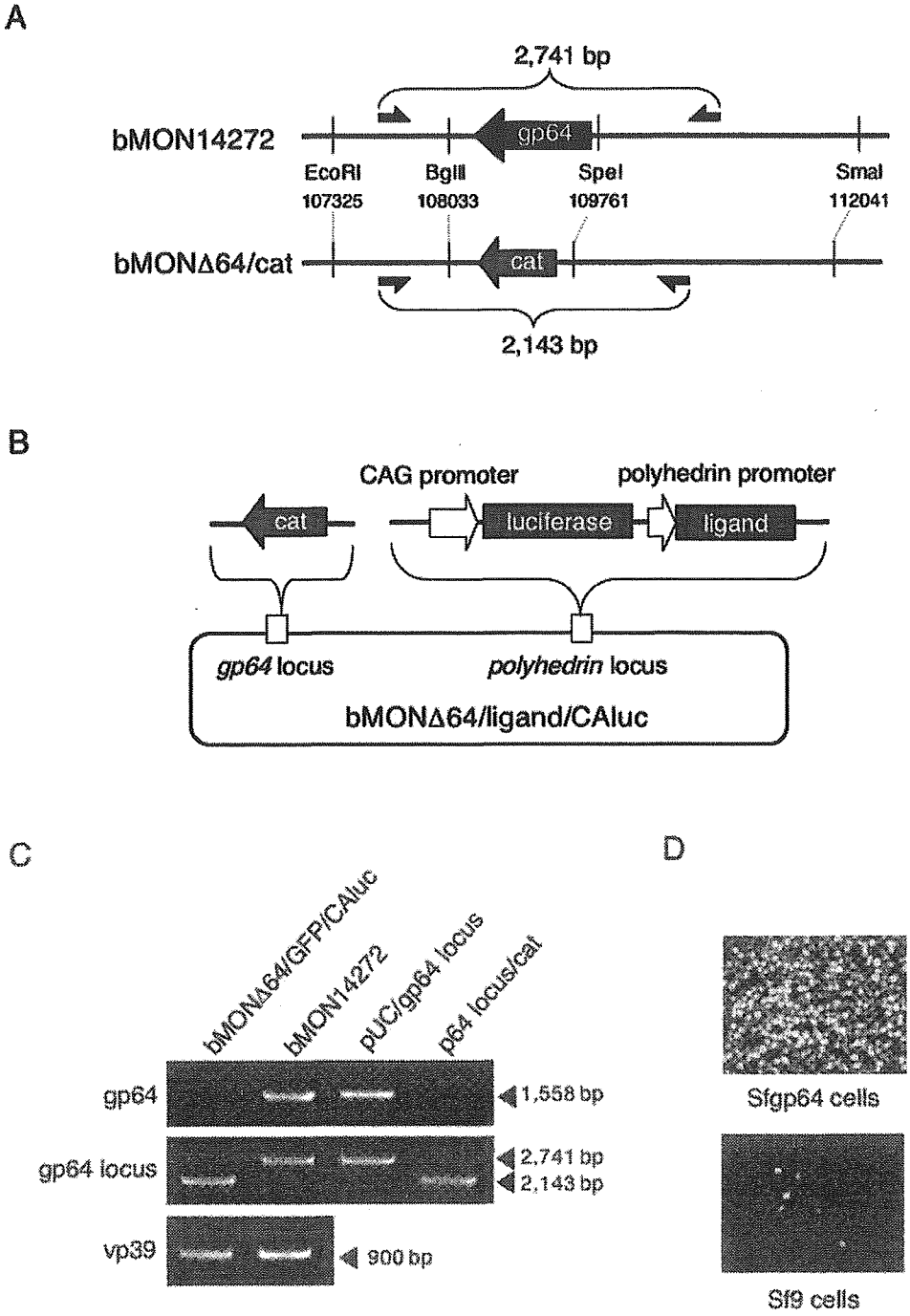

FIG. 1. (A) Schematic representations of the gp64 loci of the AcMNPV (bMON14272) and gp64-null AcMNPV (bMONΔ64/cat) bacmids. The gp64 gene (BglII/SpeI fragment corresponding to 108,033 to 109,761 nt) (3) of bMON14272 was replaced with the CAT gene by homologous recombination. The arrows indicate the locations of the PCR primers within the gp64 loci. (B) Construction of the recombinant bacmid bMONΔ64/ligand/CAluc. The gp64 gene in bMON14272 was replaced with the CAT gene. The desired ligand and luciferase genes were inserted under the control of the polyhedrin and CAG promoters, respectively, within the polyhedrin locus. (C) The bacmids bMONΔ64/ligand/CAluc and bMON14272 and plasmids containing the gp64 locus, pUC/gp64locus, and p64locus/cat (the gp64 locus with the CAT gene replacement) were amplified by PCR using primers specific for gp64, the gp64 locus, and vp39, a nucleocapsid protein of AcMNPV used as an internal control. Primers for gp64 and vp39 amplified fragments of 1,558 and 900 bp, respectively. The gp64 locus primers generated 2,741- and 2,143-bp fragments corresponding to the wild-type gp64 locus and the mutant locus with the CAT gene replacement shown in panel A, respectively. (D) Sfgp64 and Sf9 cells were transfected with bMONΔ64/GFP/CAluc. GFP expression was examined by fluorescence microscopy 4 days posttransfection.

cated in Sf9 cells, it was not possible to determine the infectivity in the cases of the pseudotype baculoviruses possessing ligands incapable of entering into insect cells. To standardize the viral titer, we determined the amount of viral capsid protein vp39 by semiquantitative Western blot analysis. The infectious titer determined by plaque assay in Sf9 cells correlated well with the intensity of the vp39 signal obtained by Western blotting for both AcΔ64/gp64/CAluc and AcΔ64/VSVG/CAluc (data not shown). Pseudotype baculovirus titers are expressed as relative infectious units (RIU) in this study. To confirm the absence of gp64 in the bacmids, we synthesized oligonucleotide primers specific for the gp64 gene, the gp64 locus, and the vp39 gene as follows: for the gp64 gene, gp64-Fw (Bgl) (5′-AAAGATCTACCATGGTAAGCGCTATTGTTT-3′) and gp64-Rv (Sal) (5′-TTGTCGACTTAATATTGTCTATTACGGTTT-3′); for the gp64locus, gp64locus-Fw (5′-GCACGGATTGGGGAGAGGACGGATTTT-3′) and gp64locus-Rv (5′-AGCTCGTTATTCAAGTGTCCCGCGTAC-3′); and for vp39, vp39-Fw (5′-ATATGGCGCTAGTGCCCGTGGGTATGG-3′) and vp39-Rv (5′-GACGGCTATTCCTCCACCTGCTGCCTG-3′). PCR amplification was performed using *Taq* DNA polymerase (Invitrogen) according to the manufacturer's protocol.

Stability of pseudotype baculoviruses during passage in Sfgp64 cells. Culture supernatants from Sfgp64 cells transfected with recombinant bacmids were harvested 4 days after transfection. After serial passage in Sfgp64 cells for 4 days, each Sfgp64 cell supernatant was inoculated into Sf9 cells. The culture supernatants were further inoculated into Sf9 cells to examine the generation of replication-competent revertants during the replication in Sfgp64 cells. The presence of replication-competent virus in the culture supernatants was assessed by the appearance of cytopathic effect and GFP expression in Sf9 cells. GFP expression in insect cells was observed by fluorescence microscopy (UFX-II; Nikon, Tokyo, Japan). The generation of replication-competent viruses incorporating gp64 was examined by PCR using the viral DNA as a template. The supernatants of Sfgp64 cells were concentrated by centrifugation at 18,000 × *g* for 45 min at 4°C. Viral DNA, purified from replication-competent revertants by phenol-chloroform extraction, was examined by Southern blot analysis. DNA was digested with BglII or PstI, separated by electrophoresis on a 0.6% agarose gel, and transferred to a Hybond N+ nylon membrane (Amersham Biosciences, Piscataway, N.J.). PCR primers [gp64-Fw (Bgl) and gp64-Rv (Sal) for the gp64 gene or vp39-Fw and vp39-Rv for the vp39 gene] were used to amplify the target fragments for use as hybridization probes. PCR products were purified and labeled using the ECL direct nucleic acid labeling and detection system (Amersham Biosciences) according to the manufacturer's instructions. Fragments containing the gp64 or vp39 gene were visualized using image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Incorporation of ligands into pseudotype particles. To examine the expression of ligand proteins in insect cells or the incorporation of the ligands into pseudotype particles, cell lysates or purified baculoviruses were separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto Hybond-P polyvinylidene difluoride membranes (Amersham Bioscience). After being blocked in phosphate-buffered saline containing 5% skim milk and 0.05% Tween 20 (Sigma), the membranes were incubated at room temperature for 1 h with a rabbit polyclonal anti-CD46 antibody (H-294; 1:200) (Santa Cruz, Santa Cruz, Calif.) or one of the following mouse monoclonal antibodies: anti-gp64 (AcV5; 1:1,000) (kindly provided by P. Faulkner) (22), anti-VSVG (P5D4; 1:2,000) (Sigma), anti-SLAM (123317; 1:200) (R&D systems, Minneapolis, Minn.), or anti-vp39 (236; 1:2,000) (kindly provided by G. F. Rohrmann) (51). The membranes were then incubated in horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies at room temperature for 1 h. Immunoreactive bands were visualized using enhanced-chemiluminescence Super Signal West Femto substrate (Pierce, Rockford, Ill.) (47).

Reporter gene expression by pseudotype baculoviruses. AcΔ64/gp64/CAluc and AcΔ64/VSVG/CAluc baculoviruses were inoculated into 3.0×10^4 293T and BHK cells. Twenty-four hours after infection, the cells were lysed in Bright-Glo luciferase substrate (Promega, Madison, Wis.) according to the manufacturer's instructions. Relative light units were measured using a luminometer (AB-2200; ATTO Co. Ltd., Tokyo, Japan). To demonstrate ligand-directed gene targeting by AcΔ64/CD46/CAluc, AcΔ64/SLAMCAluc, and AcΔ64/SLAMcyto7/CAluc baculoviruses, 3.0×10^4 BHK cells were cotransfected with either pCA-EdF and pCA-EdH or pCA-IcF and pCA-IcH and then infected with 5.0×10^6 RIU of pseudotype baculoviruses at 24 h posttransfection. Luciferase expression was determined after a 24-h incubation.

Inhibition of gene transduction by specific antibodies against ligands. To examine ligand-directed gene transduction by pseudotype baculoviruses, we examined the neutralization of gene transduction by antibodies specific for the ligands presented by the pseudotypes. The appropriate dilutions of anti-gp64 (AcV1), anti-VSVG (I1) (kindly provided by M. A. Whitt) (30), anti-CD46 (M75) (Seikagaku Co. Ltd., Tokyo, Japan), or anti-SLAM (IPO-3) (Biodesign International, Saco, Maine) antibodies were preincubated with each virus (10^6 RIU) at 37°C for 60 min and then inoculated into the appropriate target cells. After incubation at 37°C for 24 h, we determined the neutralization by the included antibodies from the reduction of luciferase expression.

Entry of pseudotype baculovirus into target cells. BHK cells expressing hemagglutinin and fusion proteins derived from the Edmonston strain of measles virus were preincubated with either ammonium chloride (2, 10, or 50 mM) (Wako Pure Chemical Industries, Osaka, Japan) or chloroquine (20, 100, or 500 μM) (Sigma) for 1 h. The cells were then inoculated with 1.0×10^6 RIU of AcΔ64/CD46/CAluc, AcΔ64/gp64/CAluc, or AcΔ64/VSVG/CAluc in the presence of the above-mentioned reagents. The effects of ammonium choloride and chloroquine on gene transduction by pseudotype baculoviruses were determined by the changes in luciferase expression.

Electron microscopy. Viral particles purified by ultracentrifugation as described above were put onto carbon-coated copper 400-mesh electron microscopy grids for 15 min. After being washed in water, the grids were negatively stained with 1% (wt/vol) uranyl acetate and examined using a Hitachi (Tokyo, Japan) H-7100 electron microscope at 75 kV. For immunoelectron microscopy, virus particles put onto grids were incubated with murine monoclonal antibodies specific for VSVG (11) or CD46 (E4.3) (Santa Cruz) and then treated with a gold particle-conjugated anti-mouse IgG antibody (British Biocell International, Ltd., Cardiff, United Kingdom). Samples were stained and observed as described above.

RESULTS

Construction of recombinant AcMNPV lacking the gp64 gene. The gp64 gene of the AcMNPV bacmid, bMON14272, was replaced with the CAT gene by homologous recombination in Sf9 cells using a modification of the methods reported by Bideshi and Federici (5) and Lung et al. (33) (Fig. 1A). We cotransfected bMON14272 and a linearized p64locus/cat plasmid bearing the CAT gene in place of the gp64 gene into Sf9 cells. DNA, extracted from the cells 48 h after transfection, was then transformed into competent DH10B cells. The disruption of the gp64 gene in colonies selected with kanamycin and chloramphenicol was confirmed by PCR (data not shown). We also constructed a recombinant bacmid, bMONΔ64/GFP/CAluc, which contained the insertion of the GFP gene under the control of the polyhedrin promoter and the luciferase gene under the control of the CAG promoter into the polyhedrin locus of the gp64-null bacmid (Fig. 1B). Disruption of gp64 in bMONΔ64/GFP/CAluc was confirmed by PCR using a series of specific primers (Fig. 1C). PCR with primers specific for the vp39 gene, used as an internal control for the AcMNPV bacmid, amplified a 900-bp product from both the bMONΔ64/GFP/CAluc and parent bMON14272 bacmids. The gp64 gene (1,558 bp) was amplified from bMON14272 and pUC/64locus, but not from bMONΔ64/GFP/CAluc and p64locus/cat. The 2,741- and 2,143-bp fragments corresponding to the wild-type and mutant gp64 genes, respectively, were amplified using gp64 locus-specific primers. The wild-type gene was amplified from bMON14272 and pUC/gp64locus, while the mutant gene was amplified from bMONΔ64/GFP/CAluc and p64locus/cat (Fig. 1A and C). These data indicate that the gp64 gene was replaced with the cat gene in bMONΔ64/GFP/CAluc. Previous studies demonstrated that gp64-null AcMNPV could propagate in $Sf9^{OP64\text{-}6}$ or $Sf9^{Op1D}$ cell lines constitutively expressing the gp64 protein of *Orgyia pseudotsugata* NPV (OpNPV) but not in untransfected Sf9 cells (40, 49). We then established a cell line, Sfgp64, constitutively expressing the gp64 gene derived from AcMNPV. The pAFgp64 plasmid, carrying the gp64 gene of AcMNPV without any flanking sequence, was used to

avoid homologous recombination between the viral genome and the plasmid. To examine the replication competency of gp64-null AcMNPV (AcΔ64/GFP/CAluc), Sfgp64 and Sf9 cells were transfected with bMONΔ64/GFP/CAluc. We assessed the propagation of infectious virus by measuring GFP expression by fluorescence microscopy. Forty-eight hours posttransfection, ~10% of the Sfgp64 and Sf9 cells were GFP positive (data not shown). While Sfgp64 cells exhibited the spread of infection 96 h posttransfection, Sf9 cells did not (Fig. 1D). These data indicate that AcΔ64/GFP/CAluc can replicate only in Sfgp64 cells, not in Sf9 cells.

Appearance of revertants incorporating the gp64 gene during replication in Sfgp64 cells. To determine the stability of AcΔ64/GFP/CAluc during replication in Sfgp64 cells, we serially passaged AcΔ64/GFP/CAluc in Sfgp64 cells. Culture supernatants of Sfgp64 cells collected 4 days after transfection with bMONΔ64/GFP/CAluc (passage 1) were inoculated into Sfgp64 cells. The supernatants were further passaged in Sfgp64 cells for 4 days. To examine the appearance of replication-competent viruses, the culture supernatants from each passage were inoculated into Sf9 cells. At 4 days postinfection, we examined GFP expression in Sf9 cells by fluorescence microscopy (Fig. 2A). The expression of GFP was observed in Sf9 cells inoculated with Sfgp64 culture supernatants, irrespective of the passage history. As gp64-negative AcΔ64/GFP/CAluc baculovirus only transiently carries gp64, progeny viruses produced in Sf9 cells should not be infectious. The supernatants of Sf9 cells inoculated with supernatants recovered after >3 passages (passages 3, 4, and 5) with Sfgp64 cells exhibited infectivity to Sf9 cells, suggesting the generation of replication-competent revertants incorporating the gp64 gene into the viral genome. To confirm the incorporation of gp64 into the viral genome, virus particles were purified from the supernatants of each Sfgp64 passage. The presence of the gp64 gene within the viral genome was determined by PCR. We detected the gp64 gene in viruses obtained from the culture supernatants of passages 3, 4, and 5 but not in those from the first and second passages (Fig. 2B). Furthermore, PCR amplification of viral DNA with the gp64 locus-specific primers revealed that a 2,143-bp fragment, corresponding to the mutant form, was detected in the genome of AcΔ64/GFP/CAluc, while a 2,741-bp fragment, corresponding to the wild-type form, was amplified from Ac14272, irrespective of the number of passages. These results confirmed that the emergence of replication-competent virus during the passage in Sfgp64 cells is not due to the contamination of the parental virus, Ac14272. The recombinant virus incorporated the gp64 gene into the AcΔ64/GFP/CAluc genome during propagation in Sfgp64 cells.

Plasmid DNA can be integrated into multiple sites within the viral genome by nonhomologous recombination upon cotransfection of plasmid DNA with the baculovirus genome in Sf9 cells (71). To determine if gp64 genes integrated into the baculovirus genome by nonhomologous recombination during propagation in Sfgp64 cells, we analyzed the DNAs of three independent revertant viruses by PCR and Southern blot analyses. Viral DNA was extracted from these revertant viruses and analyzed by PCR as described above (Fig. 3A). We detected the gp64 gene in all revertant viruses and bMON14272 but not in the parental bacmid, bMONΔ64/GFP/CAluc. The gp64 locus primers amplified the mutant 2,143-bp fragment from all revertant viruses and the parental bMONΔ64/GFP/CAluc bacmid, not the 2,741-bp wild-type fragment that could be amplified from bMON14272. These results confirmed that the three independent revertant viruses, instead of deriving from contaminating wild-type virus, had incorporated the gp64 gene into their genomes exogenously. DNA from the revertants was digested with BglII or PstI, which do not digest sequences within the gp64 or vp39 genes, and hybridized to gp64- or vp39-specific probes (Fig. 3B). If the gp64 gene integrated into the viral genome by nonhomologous recombination, the digested fragments containing the gp64 gene would be of different sizes. Following digestion with BglII, the DNA fragments containing the gp64 gene in the revertants differed in size from each other (Fig. 3B, lanes 3 to 5). When digested with PstI, the sizes of the fragments containing the gp64 gene were similar in revertant clones 2 and 3 (Fig. 3B, lanes 9 to 10), indicating that the gp64 gene may have integrated into nearby sites in the viral genomes of clones 2 and 3. The fragment containing the gp64 gene in revertant clone 1 following digestion with either BglII or PstI was similar to that seen in bMON14272 (Fig. 3B lanes 3 and 8). These results, however, were not due to contamination with bMON14272, as the PCR analysis demonstrated that the gp64 locus of revertant clone 1 was of the mutant type (Fig. 3A). These data suggested that the gp64 gene integrated into the virus genomes of the revertants by nonhomologous recombination. As an internal control, the vp39 gene was detected in fragments of the predicted sizes (31,975 bp when digested with BglII and 29,009 bp when digested with PstI) in all viruses. To determine the sites of integration of the gp64 gene in the genomes of the revertants, we tried to sequence from within the gp64 gene out into the baculovirus genome by using an internal gp64 primer. In revertant 2, the sequences including the actin promoter and the gp64 gene were detected upstream of the polyhedrin promoter, where no homologous sequence was observed. In revertants 1 and 3, however, sequence analyses by the internal primer obtained only sequences of pAFgp64 and could not reach the integration site, due to a large insertion of the plasmid sequence (data not shown).

Characterization of pseudotype baculovirus carrying VSVG. Previous studies demonstrated that the gp64 protein plays a critical role in infection of various mammalian cells, as well as insect cells (66). To determine if the pseudotype baculoviruses bearing foreign viral envelope proteins in place of gp64 can infect and express foreign genes within mammalian cells, we constructed a gp64-null pseudotype virus, AcΔ64/VSVG/CAluc, by the transfection of bMONΔ64/VSVG/CAluc, which encodes the VSVG gene under the control of the polyhedrin promoter and the luciferase gene under the control of the CAG promoter, into Sf9 cells (Fig. 1B). As a control, we also generated AcΔ64/gp64/CAluc, in which the gp64 gene under the control of the polyhedrin promoter replaced the VSVG gene in the above-mentioned virus. Sf9 cells were transfected with appropriate bacmids and incubated for 4 days. The pseudotype baculoviruses in the culture supernatants were concentrated and purified by ultracentrifugation (10^8 to 10^9 RIU/ml). To examine the expression and incorporation of the glycoproteins into virions, we transfected these bacmid constructs into Sf9 cells. The cell lysates and the purified virus particles were examined by Western blot analysis (Fig. 4A). VSVG and gp64

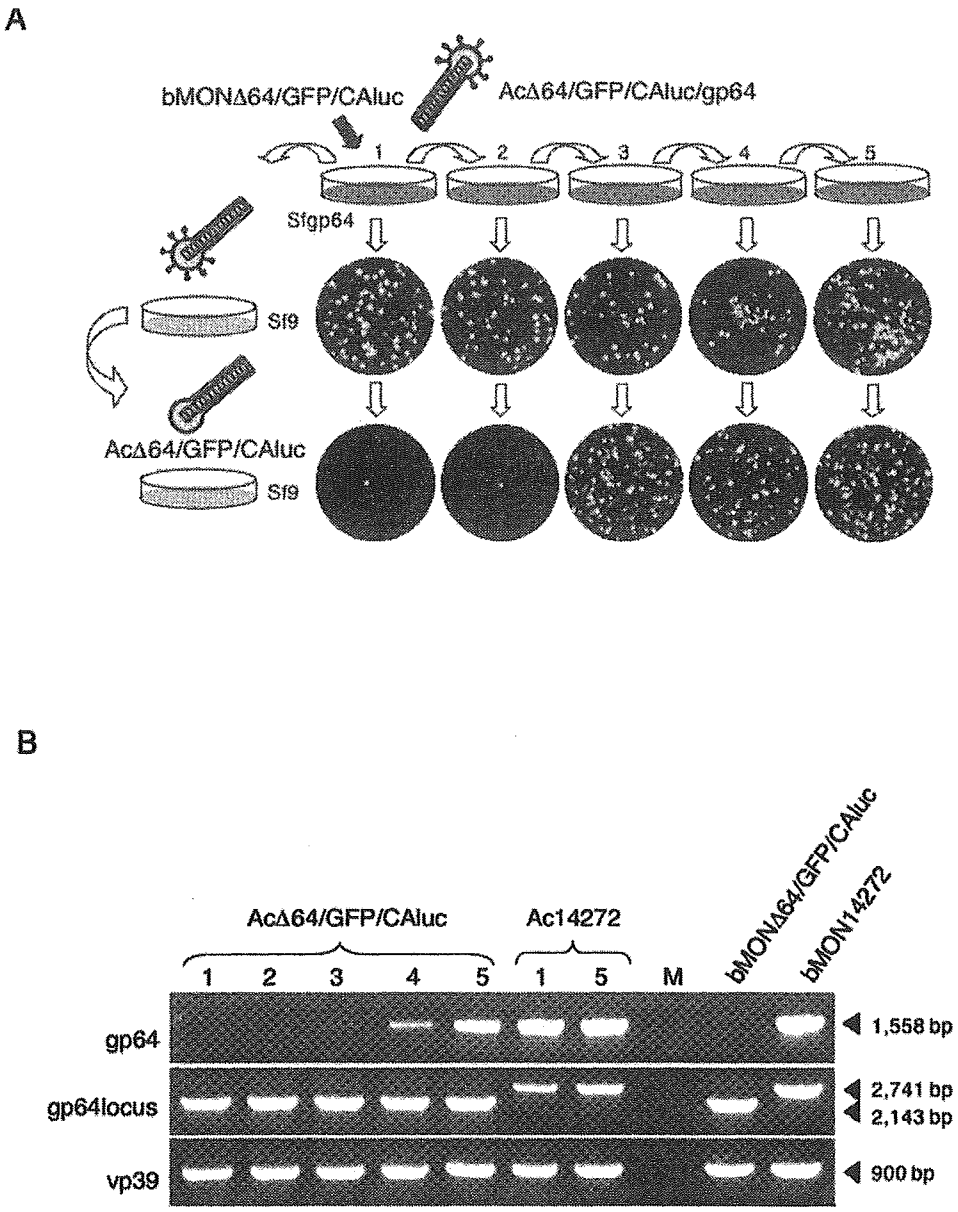

FIG. 2. Appearance of replication-competent viruses incorporating the gp64 gene during passage in Sfgp64 cells. (A) Sfgp64 cells were transfected with bMONΔ64/GFP/CAluc. Culture supernatants were harvested 4 days after transfection and then serially passaged in Sfgp64 cells at 4-day intervals. Each culture supernatant from Sfgp64 cells was passaged two more times in Sf9 cells to detect the appearance of replication-competent viruses. GFP expression in Sf9 cells was examined by fluorescence microscopy 4 days after infection. (B) PCR analysis of purified virus particles from the supernatant of each Sfgp64 cell passage. The gp64 gene was detectable in particles obtained from the third or later passages. The numbers above the lanes represent the passage numbers. The bMONΔ64/GFP/CAluc and bMON14272 bacmids and Ac14272, generated from bMON14272 and passaged in Sfgp64 cells, were used as controls. M is the culture supernatant of uninfected Sfgp64 cells concentrated under the same conditions as the virus particles. The primers amplified fragments as detailed in the legend to Fig. 1.

were expressed in the cells transfected with the appropriate bacmids. The proteins were also detected in the purified AcΔ64/VSVG/CAluc and AcΔ64/gp64/CAluc viruses, respectively, but not in AcΔ64/GFP/CAluc.

To assess the efficacy of mammalian cell gene transduction by the pseudotype baculoviruses, 293T and BHK cells were inoculated with various amounts of pseudotype viruses (Fig. 4B). Similar levels of reporter gene expression were observed in a dose-dependent manner in both cell lines following infection with AcΔ64/gp64/CAluc and AcΔ64/VSVG/CAluc. AcΔ64/GFP/CAluc, however, was unable to infect either cell line. To confirm the role of gp64- or VSVG-mediated gene transduction into mammalian cells by the pseudotype baculoviruses, we attempted to neutralize 293T cell infection using

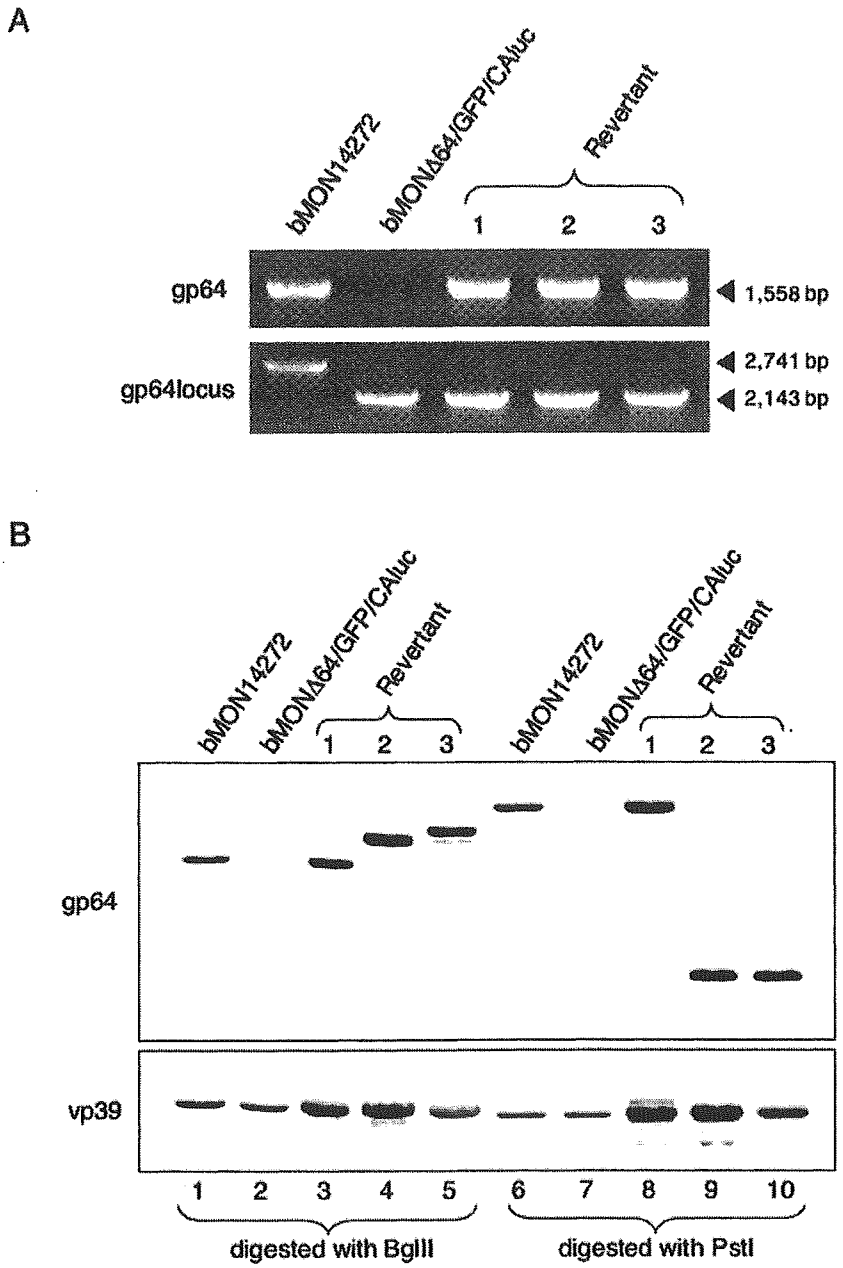

FIG. 3. Incorporation of the gp64 gene into gp64-null baculovirus genomes by nonhomologous recombination. (A) PCR analysis of three independent revertant viruses. In each revertant virus, the gp64 gene and gp64 locus primer pairs produced 1,558- and 2,143-bp fragments, respectively, indicating the presence of the mutant gp64 locus. (B) Southern blot analysis of revertant viruses. Viral DNA was digested with BglII or PstI, separated, and hybridized to gp64- or vp39-specific probes. Fragments containing the gp64 gene were detectable in all of the revertant viruses, but the fragment sizes differed. The vp39 gene, used as an internal control, was detectable in all revertant DNAs and bacmids. The numbers above the lanes represent the revertant clones. The bMONΔ64/GFP/CAluc and bMON14272 bacmids were used as controls.

specific monoclonal antibodies against gp64 and VSVG. Luciferase expression in 293T cells infected with either AcΔ64/gp64/CAluc or AcΔ64/VSVG/CAluc was specifically inhibited by antibodies against gp64 or VSVG, respectively (Fig. 4C). These results indicate that reporter gene expression in mammalian cells inoculated with pseudotype baculoviruses relies on the interactions mediated by the ligand proteins on the viral particles.

Ligand-directed gene targeting by pseudotype baculovirus. To demonstrate the ligand-directed gene transduction of target cells by pseudotype baculoviruses, we constructed pseudotype viruses bearing CD46 or SLAM in place of the gp64

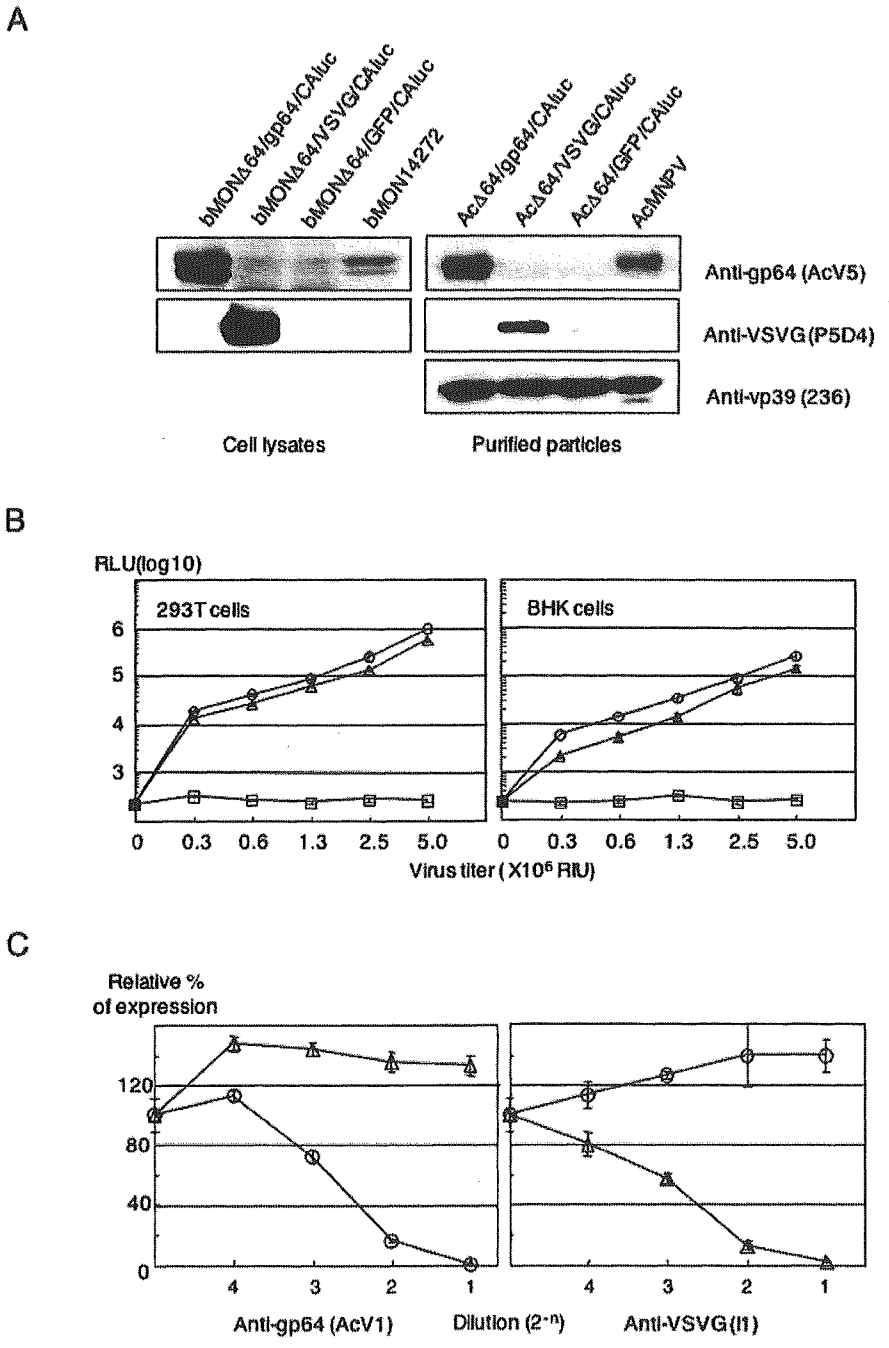

FIG. 4. Characterization of pseudotype baculoviruses bearing VSVG. (A) VSVG and gp64 expression in Sf9 cells transfected with the bMONΔ64/gp64/CAluc, bMONΔ64/VSVG/CAluc, bMONΔ64/GFP/CAluc, or bMON14272 bacmid were examined by Western blot analysis using monoclonal antibodies specific for VSVG (P5D4) and gp64 (AcV5) (left). The incorporation of gp64 and VSVG into pseudotype particles, AcΔ64/gp64/CAluc, AcΔ64/VSVG/CAluc, AcΔ64/GFP/CAluc, or AcMNPV, was examined by Western blot analysis using monoclonal antibodies specific for gp64, VSVG, and vp39 (236) (right). (B) Gene transduction into mammalian cells by pseudotype baculoviruses. 293T or BHK cells (3×10^4) were inoculated with various amounts of AcΔ64/gp64/CAluc, AcΔ64/VSVG/CAluc, or AcΔ64/GFP/CAluc. The pseudotype titers are expressed as RIU. Luciferase expression was determined 24 h after infection. The results shown are the means of three independent assays, while the error bars represent the standard deviations. RLU, relative light units. (C) Neutralization of gene transduction into mammalian cells by pseudotype baculoviruses by antibodies specific for the particle ligands. AcΔ64/gp64/CAluc or AcΔ64/VSVG/CAluc (10^6 RIU) was preincubated with the indicated dilutions of monoclonal antibodies specific for gp64 (AcV1) or VSVG (11), respectively, for 60 min at 37°C. Residual activity, determined as luciferase expression in 293T cells 24 h postinfection, is expressed as the relative percentages of expression. The results shown are the means of three independent assays, with the error bars representing the standard deviations.

protein. The receptor usage of measles virus has been well characterized; while laboratory strains of measles virus, such as the Edmonston strain, can use either CD46 or SLAM as receptors, wild-type strains, such as the Ichinose strain, can only use SLAM for entry (15, 24, 42, 48, 67). Expression of these receptor molecules in Sf9 cells transfected with the bMONΔ64/CD46/CAluc or bMONΔ64/SLAM/CAluc bacmid (Fig. 1B) and subsequent incorporation of the receptors into progeny particles (AcΔ64/CD46/CAluc and AcΔ64/SLAM/CAluc, respectively) were confirmed by Western blotting (Fig. 5A). CD46 was detected in cells transfected with bMONΔ64/CD46/CAluc and in the purified particles of AcΔ64/CD46/CAluc, whereas SLAM was detected in cells transfected with the bacmid but not in the particles of AcΔ64/SLAM/CAluc.

The gp64, CD46, and SLAM proteins are all type I membrane proteins. SLAM has a 77-amino-acid cytoplasmic domain (23), while gp64 and CD46 have only 7- and 33-amino-acid tails, respectively (49, 60). Therefore, we speculated that SLAM may be only inefficiently incorporated into baculovirus particles, due to its large cytoplasmic domain. To examine the effect of the cytoplasmic domain length on incorporation into baculovirus particles, we constructed a mutant SLAM molecule, SLAMcyto7, with a deletion in the C-terminal cytoplasmic domain that preserves only the seven membrane-proximal amino acids. Western blot analysis revealed that SLAMcyto7 was efficiently expressed in Sf9 cells transfected with bMONΔ64/SLAMcyto7/CAluc and subsequently incorporated into AcΔ64/SLAMcyto7/CAluc particles at levels similar to those seen for CD46 inclusion into AcΔ64/CD46/CAluc (Fig. 5A).

To determine the efficiency of ligand-directed gene delivery, BHK cells were cotransfected with expression plasmids encoding the measles virus H and F glycoproteins of the Edmonston (EdH and EdF) or Ichinose (IcH and IcF) strain. These cells were inoculated with pseudotype baculoviruses (Fig. 5B). AcΔ64/CD46/CAluc exhibited gene delivery specifically to cells expressing EdH and EdF, but not IcH and IcF. Although the efficiency of gene transduction was 10 times lower than that seen with AcΔ64/CD46/CAluc, AcΔ64/SLAMcyto7/CAluc could also deliver a reporter gene to cells expressing the Edmonston and Ichinose strain glycoproteins but not to control cells. While AcΔ64/gp64/CAluc and AcΔ64/VSVG/CAluc could effectively deliver a reporter gene to all of the cells examined, AcΔ64/SLAM/CAluc was ineffective against all of the cell lines tested, likely due to the lack of SLAM incorporation into the virions.

To confirm ligand-directed gene delivery by AcΔ64/CD46/CAluc and AcΔ64/SLAMcyto7/CAluc to cells expressing appropriate measles virus glycoproteins, we tested the neutralization of gene transduction by specific monoclonal antibodies against CD46 and SLAM (Fig. 5C). Gene transduction of target cells by either AcΔ64/CD46/CAluc or AcΔ64/SLAMcyto7/CAluc, but not by AcΔ64/gp64/CAluc, could be inhibited in a dose-dependent manner by anti-CD46 and anti-SLAM monoclonal antibodies, respectively. These results indicate that pseudotype baculoviruses can deliver foreign genes to target cells in a ligand-directed manner.

Entry pathway of the pseudotype baculoviruses. Virus entry occurs either by the direct fusion of viral envelope proteins with the host plasma membrane at neutral pH, as seen for measles virus, or following receptor-mediated endocytosis, as seen for AcMNPV and VSV, in which envelope glycoproteins undergo conformational changes into a fusion-competent state, leading to fusion between viral and host membranes at low pH within endosomes (6, 36, 69). Ammonium chloride and chloroquine, which inhibit endosomal acidification, have been used as entry inhibitors for viruses that penetrate cells through receptor-mediated endocytosis (7). To examine the entry pathways used by the pseudotype baculoviruses, we examined the infectivity of AcΔ64/gp64/CAluc, AcΔ64/VSVG/CAluc, and AcΔ64/CD46/CAluc to BHK cells expressing EdH and EdF in the presence or absence of ammonium chloride or chloroquine (Fig. 6). Although these compounds inhibited gene transduction of BHK cells inoculated with AcΔ64/gp64/CAluc or AcΔ64/VSVG/CAluc in a dose-dependent manner, gene transduction by AcΔ64/CD46/CAluc was not inhibited. In contrast, ammonium chloride treatment enhanced gene expression following AcΔ64/CD46/CAluc infection. These results indicate that the pseudotype baculoviruses utilize entry pathways conferred by the nature of the ligand protein replacing gp64.

Morphology of pseudotype baculovirus. To address any alterations in pseudotype baculovirus morphology, we examined the AcMNPV, AcΔ64/VSVG/CAluc, and AcΔ64/CD46/CAluc virus particles by transmission electron microscopy (Fig. 7A to C). All of the pseudotype baculoviruses exhibited rod shapes and similar sizes, indistinguishable from the wild-type baculovirus. To examine the incorporation of exogenous ligands into the virion, purified AcΔ64/VSVG/CAluc and AcΔ64/CD46/CAluc particles were examined by immunoelectron microscopy using specific monoclonal antibodies against VSVG and CD46 (Fig. 7E and F). In both pseudotype viruses, gold particles were detected on the virion surface from the stalk to the head domains, indicating that VSVG and CD46 were incorporated into the AcΔ64/VSVG/CAluc and AcΔ64/CD46/CAluc virus particles, respectively.

DISCUSSION

Baculovirus is a useful tool for gene delivery to mammalian cells due to the large capacity of the virus to incorporate foreign genes, the wide host range, and the lack of replication in mammalian cells, providing minimal toxicity (29, 53, 55, 61, 68). The gp64 envelope glycoprotein, involved in attachment to both insect and mammalian cells, is required for low-pH-triggered membrane fusion following endocytosis during virus entry (6, 8, 12, 14, 20, 21, 31, 40, 41, 66, 69, 70). We have previously demonstrated that the interaction of gp64 with cell surface phospholipids may be important in baculovirus infection of mammalian cells (66). As the recombinant baculoviruses used for gene delivery to mammalian cells in this system retained the gp64 envelope glycoprotein, it was difficult to deliver foreign genes to specific target cells.

To generate a ligand-directed gene-targeting baculovirus vector, we established a bacmid system to produce recombinant baculoviruses in which the gp64 gene was replaced with other ligand genes of interest. The gp64 protein is required for efficient budding from Sf9 cells; the budding of a mutant virus lacking gp64 was reduced to 2% of that seen for wild-type baculovirus (49). Although AcΔ64/gp64/CAluc, a pseudotype virus in which gp64 was reintroduced into the gp64 deletion mutant, incorporated two to three times as much gp64 protein as seen in wild-type baculovirus (Fig. 4A), the infectious titers

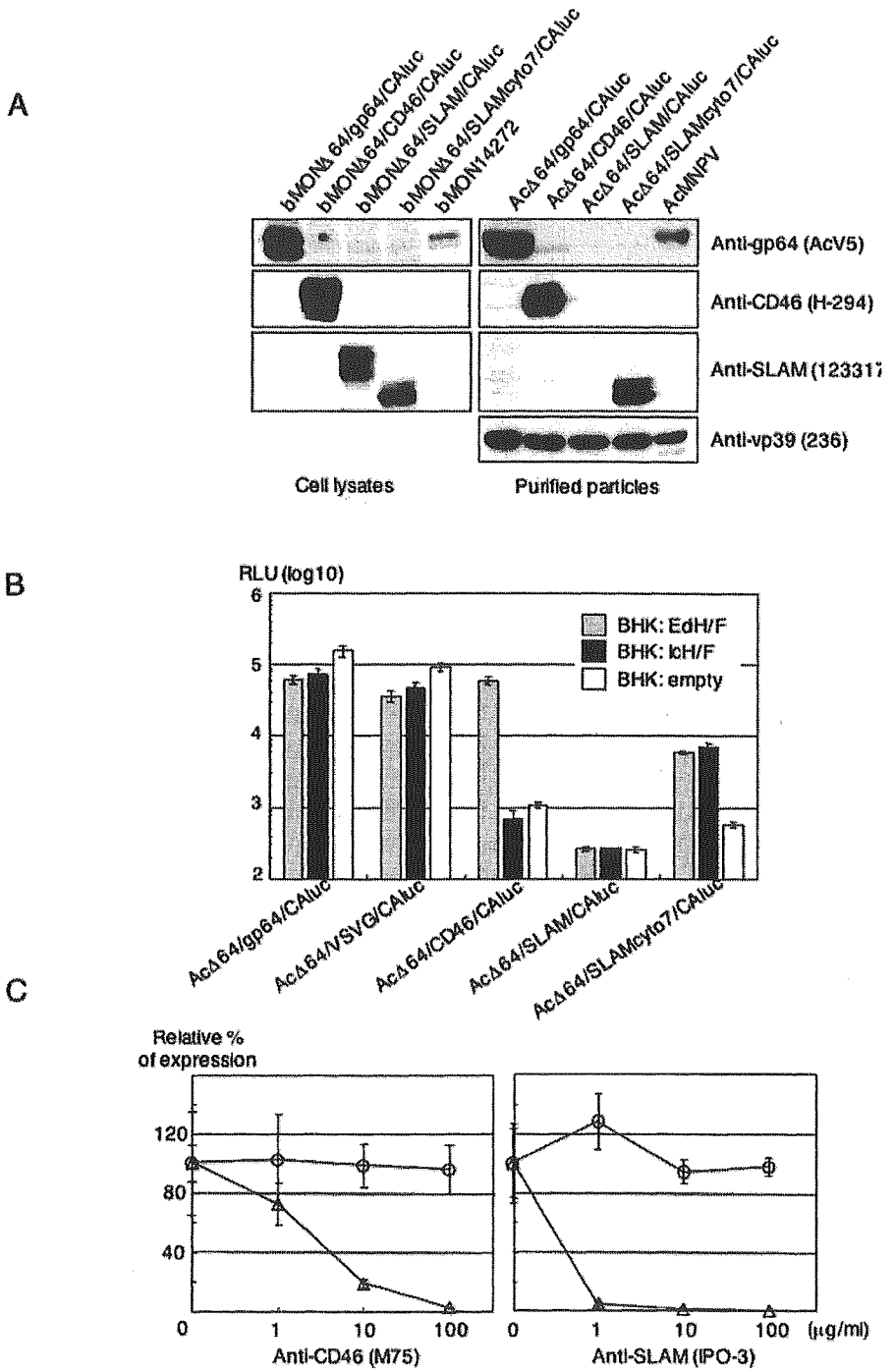

FIG. 5. Ligand-directed gene targeting by pseudotype baculoviruses. (A) The expression of gp64, CD46, SLAM, and SLAMcyto7 in Sf9 cells transfected with the bMONΔ64/gp64/CAluc, bMONΔ64/CD46/CAluc, bMONΔ64/SLAM/CAluc, bMONΔ64/SLAMcyto7/CAluc, or bMON14272 bacmid was examined by Western blot analysis using monoclonal antibodies specific for gp64 (AcV5), CD46 (H-294), and SLAM (123317), as indicated (left). The incorporation of gp64, CD46, SLAM, and SLAMcyto7 into pseudotype particles, AcΔ64/gp64/CAluc, AcΔ64/CD46/CAluc, AcΔ64/SLAM/CAluc, AcΔ64/SLAMcyto7/CAluc, or AcMNPV, was examined by Western blot analysis using monoclonal antibodies specific for gp64, CD46, SLAM, and vp39 (236), as indicated (right). (B) Ligand-directed gene targeting by pseudotype baculoviruses. BHK cells (3×10^4) were co-transfected with expression plasmids encoding measles virus H and F glycoproteins of either the Edmonston (EdH and EdF) or Ichinose (IcH and IcF) strain or with an empty vector and then inoculated with 5×10^6 RIU of AcΔ64/gp64/CAluc, AcΔ64/VSVG/CAluc, AcΔ64/CD46/CAluc, AcΔ64/SLAM/CAluc, or AcΔ64/SLAMcyto7/CAluc 24 h after transfection. Luciferase expression was determined 24 h after infection. The results shown are the means of three independent assays, and the error bars represent the standard deviations. RLU, relative light units. (C) Neutralization of ligand-directed gene targeting by antibodies specific for viral ligands. AcΔ64/gp64/CAluc, AcΔ64/CD46/CAluc, or AcΔ64/SLAMcyto7/CAluc (10^6 RIU) was preincubated with various concentrations of monoclonal antibodies specific for CD46 (M75) or SLAM (IPO-3) for 60 min at 37°C. Residual activity was determined by measurement of luciferase expression in BHK cells expressing the H and F glycoproteins of the Edmonston or Ichinose strain 24 h postinfection. The values are expressed as the relative percentages of expression. The results shown are the means of three independent assays, with the error bars representing the standard deviations.

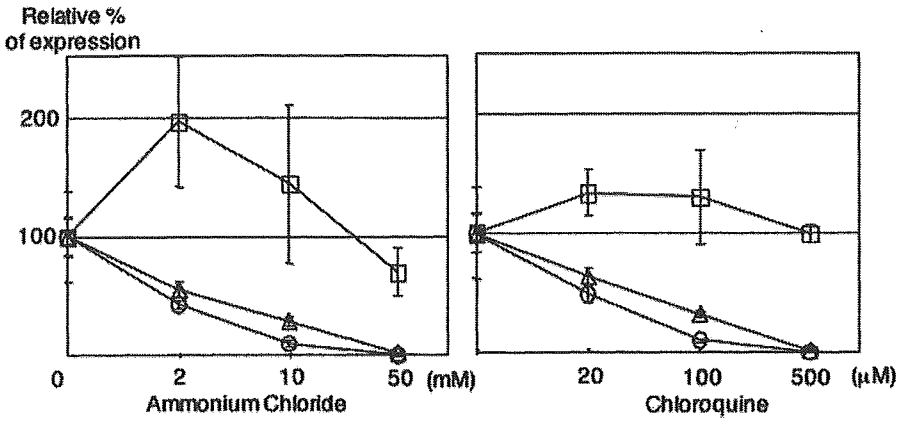

FIG. 6. Effects of lysosomotropic reagents on gene transduction of pseudotype baculoviruses. BHK cells (3×10^4) transfected with expression plasmids encoding the measles virus H and F glycoproteins of the Edmonston strain (EdH and EdF) were pretreated with various concentrations of ammonium chloride or chloroquine for 60 min. The cells were then inoculated with 10^6 RIU of AcΔ64/gp64/CAluc, AcΔ64/VSVG/CAluc, or AcΔ64/CD46/CAluc in the presence of the lysosomotropic reagents. Luciferase expression was determined 24 h postinfection. The results shown are the averages of three independent assays, with the error bars representing the standard deviations.

of the virus, determined by plaque formation in Sf9 cells, were similar. These results suggest that, while the polyhedrin promoter is sufficient to overexpress and incorporate a ligand of interest into the virion, this is not necessarily the best choice to maintain ligand function. The discrepancy between gp64 incorporation and the infectious titer may be attributed to a limited capacity to incorporate functional gp64 into particles and the timing of ligand gene activation. As the polyhedrin promoter is activated in the late stage of infection, baculoviruses budding in the early stage of infection may be unable to incorporate the ligand expressed by the polyhedrin promoter. Use of the immediate-early promoter for ligand expression may improve the efficiency of incorporation into virus particles. In support of this possibility, the infectious titer of a recombinant AcMNPV in which the gp64 gene was replaced with the F gene from *Lymantria dispar* NPV under the control of the polyhedrin promoter was ~60-fold lower than that of a virus with the F gene under the control of the gp64 promoter (33).

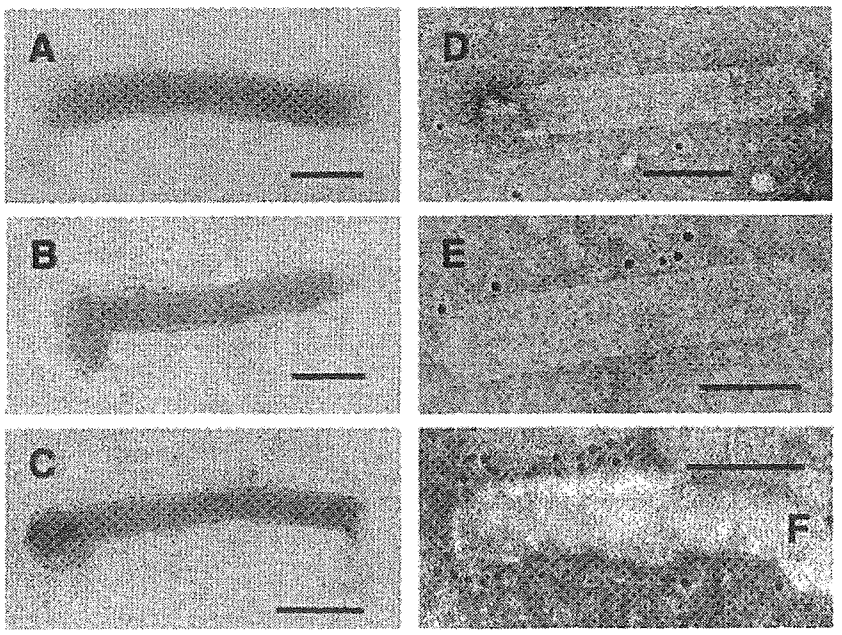

FIG. 7. Electron micrographs of pseudotype baculoviruses. Purified virus particles of wild-type AcMNPV (A), AcΔ64/VSVG/CAluc (B), and AcΔ64/CD46/CAluc (C) were examined by electron microscopy. A typical rod shape was visible in all of the pseudotype baculoviruses. The VSVG or CD46 proteins were observed on the surfaces of AcΔ64/VSVG/CAluc (E) and AcΔ64/CD46/CAluc (F) by immunoelectron microscopy using specific monoclonal antibodies against VSVG and CD46, respectively. AcMNPV treated with the monoclonal antibody against VSVG was used as a control (D). The bars on the panels represent 100 nm.

The replication competency in Sf9 cells of a gp64-null recombinant baculovirus could be rescued by incorporation of the VSVG gene (33, 34). We confirmed that the recombinant baculovirus deleted the gp64 gene and instead incorporated the VSVG gene under the control of the polyhedrin promoter, was replication competent in insect cells, and exhibited a high level of reporter gene transduction into 293T and BHK cells. The compatibility of VSVG in this system may result from similarities of the structural and functional characteristics of VSVG to those of gp64; both proteins are type I membrane glycoproteins, exist in trimeric complexes, and are capable of inducing membrane fusion at low pHs (6, 50, 54). Although the recombinant baculovirus in which gp64 is replaced with VSVG is able to replicate in insect cells and transduce foreign genes into a wide variety of mammalian cells, VSVG was suggested to recognize phosphatidylserine (58) or other ubiquitously expressed molecules other than phosphatidylserine (13) as a receptor(s), making it difficult to confer cell type specificity to gene delivery using VSVG pseudotype baculoviruses.

To establish ligand-directed gene delivery by pseudotype baculoviruses, it is necessary to propagate replication-deficient pseudotype baculoviruses possessing a ligand of interest in Sfgp64, a replication-competent cell line stably expressing gp64. After three rounds of passage of the pseudotype virus in Sfgp64 cells, however, replication-competent revertant viruses that had incorporated the gp64 gene were generated. Sfgp64 cells were established by transfection of Sf9 with a plasmid encoding the gp64 gene lacking any flanking sequences. Southern blot analysis revealed that the gp64 gene was integrated into the genomes of the baculovirus revertants by nonhomologous recombination. Homologous recombination between the AcMNPV genome and either a transfer vector (32, 38), additional AcMNPVs (19), or *B. mori* NPV can occur in insect cells with high frequency (27, 28). In contrast, our data suggest that the revertant viruses were not generated by homologous recombination but by nonhomologous incorporation of the gp64 gene from the Sfgp64 chromosome into the viral genome. Nonhomologous recombination between plasmid DNA and the baculovirus genome was previously reported upon cotransfection into insect cells (71). A gp64-null virus could propagate in $Sf9^{Op1D}$ cell lines constitutively expressing OpNPV gp64 without incorporating the gp64 gene into the viral genome (33, 34). One possible explanation for our result may be the difference between the gp64 genes of OpNPV and AcMNPV. Although the Sfgp64 cell line was established by sorting cells expressing a high level of gp64 on the cell surface, the expression level of gp64 might be lower than that of the $Sf9^{Op1D}$ cell line, and the lower level of expression of gp64 might result in the selective amplification of revertants. To circumvent the high-frequency incorporation of foreign DNA into the baculoviral genome, we attempted the lipofection of recombinant bacmids into Sf9 cells instead of amplification of pseudotype baculoviruses in Sfgp64. Although it is not possible to obtain a high pseudotype virus titer by this method, we can generate pure virus stocks without any contaminating replication-competent baculoviruses incorporating the gp64 gene. To prepare a high-titer stock of a replication-deficient pseudotype baculovirus carrying a foreign ligand, however, it is essential to propagate the pseudotype virus in Sf9 cells expressing gp64 without the transfection of plasmid DNA. Expression of gp64 by RNA transfection, RNA viral vectors, or the RNA replicon system may be able to avoid the incorporation of gp64 DNA into the baculovirus genome.

We constructed a pseudotype baculovirus, AcΔ64/CD46/CAluc, bearing human CD46 in place of gp64 on viral particles. CD46 is a multifunctional protein involved in the infection of various microorganisms and the regulation of complement activation (10). CD46, also known as membrane cofactor protein, protects autologous cells from complement attack by serving as a cofactor for factor I-mediated inactivation of C3b and C4b, blocking the complement cascade at C3 activation (2). CD46 also serves as a receptor for human herpes virus 6 (56), group B adenovirus (17, 59, 62), bovine viral diarrhea virus (39), two bacterial strains (*Streptococcus pyogenes* and pathogenic *Neisseria*) (26, 46), and the Edmonston strain of measles virus (15, 42). In this study, we demonstrated that AcΔ64/CD46/CAluc exhibited specific reporter gene transfer to and expression in BHK cells expressing the measles virus H and F glycoproteins of the Edmonston strain but not those expressing the Ichinose strain glycoproteins that require SLAM as a receptor (67). Therefore, a CD46 pseudotype baculovirus bearing a suicide gene may be able to eliminate cells expressing pathogen ligands that utilize CD46 as a receptor. Furthermore, CD46 is frequently overexpressed on cancer cells, possibly serving as a mechanism to overcome lysis by complement (16). In support of the potential utility of this vector, the Edmonston strain of measles virus has a potent and selective oncolytic activity (52). CD46 pseudotype baculovirus may also be applicable for the clearance of tumor cells surviving oncolytic measles virus treatment.

Infections with AcΔ64/gp64/CAluc and AcΔ64/VSVG/CAluc, carrying gp64 and VSVG on the particles, respectively, could be decreased by treatment with ATPase inhibitors, chloroquine, or ammonium chloride, while AcΔ64/CD46/CAluc was resistant to treatment. This finding suggests the possibility of constructing a baculovirus vector capable of both targeting and modulating the viral entry pathway, as seen for VSV (7). This is different from influenza virus vectors, where acidic exposure within endosomes is critical for the dissociation of the matrix protein from the ribonucleocapsid (9, 37). In contrast to AcΔ64/CD46/CAluc, AcΔ64/SLAM/CAluc could not incorporate the full-length SLAM molecule into particles, preventing specific gene transduction. The inability to mediate gene delivery was not due to the absence of an interaction with specific targets, as Sf9 cells expressing SLAM induced membrane fusion with BHK cells expressing the H and F glycoproteins of either the Edmonston or Ichinose strain of measles virus (data not shown). The ligands incorporated efficiently into baculovirus particles, including gp64, VSVG, and CD46, all have relatively short cytoplasmic domains, measuring 7, 29, and 33 amino acids in length, respectively (49, 54, 60). In contrast, SLAM possesses a cytoplasmic tail of 77 amino acids (23). We therefore hypothesized that the length of the ligand cytoplasmic domain may be critical for efficient incorporation into baculovirus particles. AcΔ64/SLAMcyto7/CAluc, possessing a mutant SLAM molecule with the C-terminal 70 amino acids of the cytoplasmic domain deleted, efficiently incorporated the mutant SLAM into particles and exhibited specific gene delivery to BHK cells expressing the H and F glycoproteins of both measles virus strains. Although the mechanism by which SLAM

incorporation into baculovirus particles is enhanced by C-terminal truncation of the cytoplasmic domain is not known, one possibility might be a change in SLAM localization. The gp64 protein localizes to the cell surface but is excluded from lipid raft microdomains (72). As VSVG and CD46 also do not associate with lipid rafts (35, 57), proteins localized to lipid raft microdomains may be excluded during virus assembly and budding. Although the cellular localization of SLAM is not known, further studies will be necessary to clarify the relationship between cell surface localization and incorporation into baculovirus particles and to test the involvement of lipid raft microdomains in this process.

Mangor et al. demonstrated that gp64-null baculoviruses pseudotyped with VSVG were not morphologically distinguishable from budded wild-type AcMNPV particles (34). We confirmed that AcΔ64/VSVG/CAluc, as well as AcΔ64/CD46/CAluc and AcΔ64/GFP/CAluc, exhibited a morphology similar to that of AcMNPV. These results indicate that expression of gp64 is not required for the morphogenesis of a rod-shaped structure for budded AcMNPV particles. Immunogold labeling of AcΔ64/VSVG/CAluc and AcΔ64/CD46/CAluc revealed that the VSVG and CD46 proteins were incorporated into and distributed throughout the whole viral surface. These results are consistent with previous observations that VSVG fusion proteins were distributed throughout the stalk and head domains of baculovirus particles, in contrast to gp64, which was primarily localized in the head domain (11, 44).

In this study, we have demonstrated the capability for ligand-directed gene delivery by pseudotype baculoviruses in vitro. For future in vivo applications of baculovirus vectors for gene targeting to specific organs or virus-infected cells as a method of treatment of inherited or infectious diseases, it is imperative to exhaustively study the transcription of baculoviral genes in mammalian cells for certification of safety. In addition, further studies are needed to establish replication-competent cell lines capable of supporting the propagation of pseudotype viruses without the possibility of replication-competent virus breakthrough by incorporation of gp64 and to optimize the conditions necessary for the efficient incorporation of ligands into recombinant baculovirus particles.

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Nuclear Localization of Japanese Encephalitis Virus Core Protein Enhances Viral Replication†

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Japanese encephalitis virus (JEV) core protein was detected in both the nucleoli and cytoplasm of mammalian and insect cell lines infected with JEV or transfected with the expression plasmid of the core protein. Mutation analysis revealed that Gly^{42} and Pro^{43} in the core protein are essential for the nuclear and nucleolar localization. A mutant M4243 virus in which both Gly^{42} and Pro^{43} were replaced by Ala was recovered by plasmid-based reverse genetics. In C6/36 mosquito cells, the M4243 virus exhibited RNA replication and protein synthesis comparable to wild-type JEV, whereas propagation in Vero cells was impaired. The mutant core protein was detected in the cytoplasm but not in the nucleus of either C6/36 or Vero cell lines infected with the M4243 virus. The impaired propagation of M4243 in mammalian cells was recovered by the expression of wild-type core protein in *trans* but not by that of the mutant core protein. Although M4243 mutant virus exhibited a high level of neurovirulence comparable to wild-type JEV in spite of the approximately 100-fold-lower viral propagation after intracerebral inoculation to 3-week-old mice of strain Jcl:ICR, no virus was recovered from the brain after intraperitoneal inoculation of the mutant. These results indicate that nuclear localization of JEV core protein plays crucial roles not only in the replication in mammalian cells in vitro but also in the pathogenesis of encephalitis induced by JEV in vivo.

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*. Members of the genus *Flavivirus* are predominantly arthropodborne viruses and frequently cause significant morbidity and mortality in mammals and birds (6). JEV is distributed in the south and southeast regions of Asia and kept in a zoonotic transmission cycle between pigs or birds and mosquitoes (6, 50, 57). JEV spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes and causes infection of the central nervous system, with a high mortality rate (6, 57). JEV has a single-stranded positive-strand RNA genome approximately 11 kb in length, which is capped at the 5′ end but lacks modification of the 3′ terminus by polyadenylation (34). The genomic RNA encodes a single large open reading frame, and a polyprotein translated from the genome is cleaved co- and posttranslationally by host and viral proteases to yield three structural proteins, the core, precursor membrane (prM), and envelope (E) proteins, and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (53). Although the core protein has very little amino acid homology to other flaviviruses—for example, the core protein of JEV has only 25% homology to that of tick-borne encephalitis virus (TBEV)—the structural properties, such as the hydrophobicity profile, abundances of basic amino acid residues, and secondary structures, are very similar (11, 20, 36). The flavivirus core proteins commonly contain two hydrophobic sequences in the center and a carboxyl-terminal end, and the carboxyl-terminal hydrophobic region serves as a signal sequence of prM. The signal-anchor sequence is cleaved off by the viral protease NS2B-3, and this cleavage is required for the subsequent liberation of the amino terminus of prM by the host signal peptidase (35, 52, 63). The mature core protein, released from the endoplasmic reticulum (ER) membrane, is believed to bind to the genomic RNA via the basic amino acid clusters at the amino and carboxyl termini and forms nucleocapsids (23). The central hydrophobic region of the core protein may be associated with the ER membrane, and this interaction is believed to facilitate the assembly of nucleocapsid and two membrane proteins, prM and E, and to bud into the ER lumen as virions (39). The removal of the central hydrophobic region of the TBEV core protein increased the production of the subviral particles that consist of (pr)M and E proteins but that lack a core protein and genomic RNA (26, 27).

In addition to their role as structural proteins, core proteins of dengue virus (DEN) and Kunjin virus (KUN) are localized not only in the cytoplasm but also in the nucleus, especially in the nucleoli of several infected cell lines (4, 38, 55, 59, 61). Transport from the cytoplasm to the nucleus occurs through nuclear pore complexes that penetrate the double lipid layers of the nuclear envelope. Small molecules up to 9 nm in diameter (<50 kDa) can freely diffuse through the nuclear pore complexes, while most macromolecules require an active transport process via nuclear import receptor proteins such as impotin-α (37). In general, cargo proteins contain mono- or bi-

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† This study is dedicated to the memory of Ikuko Yanase.

partite cluster sequences of basic amino acids termed nuclear localization signals (NLSs) to bind to nuclear import receptor proteins (5, 21). As flavivirus core proteins are relatively small (approximately 14 kDa), they may diffuse into the nucleus. However, the successful translocation of DEN core protein fused with three copies of green fluorescent protein (GFP) (96 kDa in total) into the nucleus indicates that the DEN core protein is actively translocated into the nucleus by an energy-dependent pathway, and an NLS was assigned to the region of carboxyl-terminal residues from amino acids 85 to 100 (59). Despite the many studies investigating this matter, the biological significance of the nuclear localization of core proteins in the virus replication cycle remains unclear.

In this study, we showed that the JEV core protein is also localized in both the cytoplasm and the nucleus, particularly in the nucleolus, of mammalian and mosquito cell lines and determined that an NLS is present in the core protein. We generated a mutant JEV, replaced the NLS in the core protein with Ala, and confirmed the elimination of the nuclear localization of the mutant core protein in both mammalian and mosquito cells. The characterization of the mutant JEV indicates that the nuclear localization of the core protein plays important roles in the viral replication in mammalian cells and in the pathogenesis of encephalitis in vivo. Finally, we discuss the biological significance of the nuclear localization of the JEV core protein.

MATERIALS AND METHODS

Cells. The mammalian cell lines Vero (African green monkey kidney), 293T (human kidney), BHK (hamster kidney), HeLa (human cervix cancer), HepG2 (human hepatoma), SK-N-SH (human neuroblastoma), and N18 (mouse neuroblastoma) were maintained in Dulbecco's modified Eagle's minimal essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). A mosquito cell line, C6/36 (*Aedes albopictus*), was grown in Eagle's minimal essential medium supplemented with 10% FBS.

Plasmids. The mammalian expression vector pEGFP-C3 was purchased from Clontech (Palo Alto, Calif.). The plasmid pEGFP-JEVC105 was constructed by insertion of cDNA encoding the mature form of the JEV core protein without the C-terminal signal sequence (amino acids 2 to 105 of the AT31 strain) amplified by PCR into pEGFP-C3 as described previously (42). All of the expression vectors coding the enhanced GFP (EGFP)-fused mutant JEV core proteins were constructed based on pEGFP-JEVC105. Briefly, the gene encoding the JEV core protein with amino acids 38 to 44 deleted was amplified by splicing the overlapping extension (16, 17). For alanine scanning in putative NLS regions (amino acids 38 to 44 and 85 to 105), a series of point mutants of the JEV core protein were synthesized by PCR-based mutagenesis (14). All of the mutant genes were cloned into EcoRI and BamHI sites of pEGFP-C3. The plasmid that has a full-length cDNA of the JEV AT31 strain under the control of a T7 promoter was constructed and designated pMWJEATG1 (Z. Zhao, T. Date, Y. Li, T. Kato, M. Miyamoto, K. Yasui, and T. Wakita, submitted for publication). Guanine-to-cytosine and cytosine-to-guanine point mutations were introduced into pMWJEATG1 at nucleotides 220 and 222 of the JEV gene, respectively, by PCR-based mutagenesis to change Gly^{42} and Pro^{43} of the core protein to Ala. The constructed plasmid was designated pMWJEAT/GP4243AA. For the mutant viral replication complementation experiments, the genes coding the C-terminal hemagglutinin (HA)-tagged core proteins derived from pMWJEATG1 and pMWJEAT/GP4243AA were cloned into pCAG-GS vector (43), and the resulting plasmids were designated pCAG-WC-HA and pCAG-MC-HA, respectively.

Antibodies. cDNA encoding the JEV core protein (amino acids 2 to 105) was inserted into pGEX-2TK (Amersham Biosciences, Piscataway, N.J.) and transformed into *Escherichia coli* strain DH5α. The glutathione-*S*-transferase-fused JEV core protein expressed in the bacteria was purified with a column with glutathione Sepharose 4B (Amersham Biosciences) and intradermally injected five times into a Japanese white rabbit purchased from KITAYAMA LABES (Nagano, Japan). The collected antiserum was absorbed with glutathione-*S*-transferase-binding glutathione Sepharose 4B. Anti-JEV monoclonal antibodies (MAb), anti-E 10B4 (E. Konishi, unpublished data) and anti-NS3 34B1 (K. Yasui, unpublished data), were used in immunostaining. Anti-nucleolin MAb, MS-3, and antiactin goat serum were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Rabbit antiserum to PA28 α was purchased from AFFINITI (Exeter, United Kingdom).

Transfection of plasmids. Plasmid vectors were transfected by Superfect (QIAGEN, Tokyo, Japan) for Vero cells or Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.) for 293T, BHK, N18, HeLa, HepG2, and SK-N-SH cells. To examine the intracellular localization of the EGFP or EGFP-fused proteins, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS at 24 h after transfection. After treatment with 1 μg of RNase A (QIAGEN)/ml, the nuclei were stained with 500 μM propidium iodide (Molecular Probes, Eugene, Oreg.). Endogenous nucleolin, a major nucleolar protein (51), was immunostained by an anti-nucleolin monoclonal antibody and Alexa Flour 564-conjugated anti-mouse immunoglobulin G (IgG) antiserum (Molecular Probes). All samples were visualized with a laser scanning confocal microscope (Bio-Rad, Hercules, Calif.).

Generation of JEV from plasmid. The wild-type and mutant (designated M4243) JEVs were generated from plasmids, pMWJEATG1, and pMWJEAT/GP4243AA, respectively, by previous methods (Zhao et al., submitted) with some modifications. Briefly, the plasmid DNAs digested by restriction enzyme KpnI were used as templates for RNA synthesis. Capped full-length JEV RNAs were synthesized in vitro by an mMESSAGE mMACHINE T7 kit (Ambion, Austin, Tex.), purified by precipitation with lithium chloride, and used for electroporation. Trypsinized Vero cells were washed with PBS and resuspended at 10^7 cells/ml in PBS. RNA (10 μg) was mixed with 500 μl of cell suspension and transferred to an electroporation cuvette (Thermo Hybrid, Middlesex, United Kingdom). Cells were then pulsed at 190 V and 950 μF by the use of a Gene Pulser II apparatus (Bio-Rad). Transfected cells were suspended in a culture medium and transferred to 10-cm-diameter culture dishes. After 3 or 4 days of incubation, the culture supernatants were collected as viral solutions. Due to a low viral yield, these viruses were amplified by a single passage in C6/36 cells. Viral infectivities were determined as focus-forming units (FFUs) by an immunostaining focus assay of Vero, C6/36, and 293T cells. Briefly, viruses were serially diluted and inoculated onto cell monolayers. After 1 h of adsorption, the cells were washed with serum free D-MEM three times and cultured in D-MEM containing 5% FBS and 1.25% methylcellulose 4000. At 2 or 3 days later, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Infectious foci were stained with an anti-JEV E monoclonal antibody and visualized with a VECTASTAIN Elite ABC anti-mouse IgG kit with a VIP substrate (Vector Laboratories, Burlingame, Calif.). Vero and C6/36 cells infected with wild-type or M4243 JEV were fixed with cold acetone at 48 h postinoculation and stained with the rabbit anti-JEV core protein antiserum and Alexa Flour 488-conjugated goat anti-rabbit IgG (Molecular Probes) antibody. After treatment with 1 μg of RNase A/ml, nuclei were stained with 500 μM propidium iodide. Samples were examined with a laser scanning confocal microscope.

Subcellular fractionation. At 48 h postinoculation, 2×10^6 Vero cells were fractionalized into cytoplasm and nucleus by using a Nuclear/Cytosol Fractionation kit (BioVision, Mountain View, Calif.) according to the manufacturer's instructions. Finally, 210 μl of the cytoplasmic extracts and 100 μl of the nuclear extracts were recovered and 10 μl of each of the extracts was subjected to electrophoresis on an acrylamide gel. The JEV core protein was detected by Western blotting using the anti-JEV core protein rabbit polyclonal antibody. Endogenous PA28α (3, 42) and nucleolin were detected as controls for the cytoplasmic and nuclear fractions, respectively.

Growth kinetics of mutant JEV in culture cells. Vero or C6/36 cells (2×10^5) in 24-well plates were infected with wild-type or M4243 virus at a multiplicity of infection (MOI) of 5 for 1 h at 4°C, washed three times with a medium to remove unbound viruses, and incubated with a medium supplemented with 5% FBS for a total duration of 30 h. The culture supernatants were used for titration of infectious virus, and cells were used for detection of viral proteins by Western blotting and for detection of negative-strand viral RNA by real-time reverse transcription-PCR (RT-PCR). Total RNAs were extracted from the cells by using an RNeasy Mini kit (QIAGEN) and quantified with a Gene Quant RNA/DNA calculator (Amersham Biosciences). RNA samples (5 μl) were reverse transcribed at 52°C for 30 min with TaqMan reverse transcription reagents (Applied Biosystems, Foster, Calif.) by the use of a negative-strand-specific "tagged" primer corresponding to nucleotides (nt) 9307 to 9332 (5′-GCG TCA TGG TGG CGT ATT TAC CAG AAC TGA TTT AGA AAA TGA A-3′). The tagged sequence, which is underlined, had no correlation to JEV or other flaviviruses. The reverse transcripts were applied to a real-time PCR assay using a TaqMan PCR core reagents kit with sense (5′-GCG TCA TGG TGG CGT

ATT TA-3') and antisense (5'-TGG ACA GCG ATG TTC GTG AA-3') primers corresponding to the tagged sequence and nt 9519 to 9538 of the JEV AT31 strain, respectively. The kinetics of cDNA amplification were monitored with an ABI PRISM 7000 sequence detection system (Applied Biosystems) using a reporter probe corresponding to nt 9363 to 9380 of the JEV AT31 strain (5'-CAC CGC ATG CTC GCC CGA-3') conjugated with 6-carboxyfluorescein at the 5' terminal and 6-carboxy-tetramethylrhodamine at the 3' terminal. As references for the real-time RT-PCR, positive- and negative-strand RNAs were synthesized by in vitro transcription from plasmids containing nt 8907 to 9955 of JEV cDNA inserted in the forward and backward directions under the control of a T7 promoter.

Characterization of viral particles. Vero and C6/36 cells were inoculated with wild-type or M4243 viruses at an MOI of 0.1, and culture fluids harvested after 2 (Vero cells) or 3 (C6/36 cells) days postinoculation were clarified by centrifugation at 6,000 × *g* for 30 min and precipitated with 10% polyethylene glycol (molecular mass, approximately 6,000 kDa). The precipitate was collected by centrifugation at 10,000 × *g* for 45 min and resuspended in TN buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl). The infectious titers of the concentrated viral particles were determined on Vero cells. The hemagglutination (HA) titers were determined at pH 6.6 by the method of Clarke and Casals (9). The viral particles (400 HA units) were applied on 10 to 40% of sucrose gradients and were centrifuged at 147,000 × *g* for 90 min. Fractions collected from the bottom were examined by the HA test.

Complementation of mutant virus replication in mammalian cells. pCAG-WC-HA, pCAG-MC-HA, or pCAG-GS (1 μg) was transfected into 293T cells in a 24-well plate (5×10^4 cells). At 4 h after transfection, the cells were washed three times with a serum-free medium and infected with the wild-type or M4243 JEV at an MOI of 5. At 12, 18, and 24 h after inoculation, the culture supernatants were harvested and infectivity was determined on Vero cells. The infected cells were harvested, and expression levels of the core proteins and replication of viral RNA were determined by Western blotting and real-time RT-PCR, respectively.

Mouse experiments. Female ICR mice of strain Jcl:ICR (3 weeks old) were purchased from CLEA Japan (Osaka, Japan). All mice were kept in pathogen-free environments. Groups of mice (n = 10) were inoculated intracerebrally (ic) with 30 μl of 10-fold-diluted solutions of wild-type or M4243 virus. The virus dilution solution (D-MEM) was administered to 10 mice as a control. The mice were observed for 2 weeks after inoculation to determine survival rates. The value of the 50% lethal dose (LD_{50}) for each virus was determined by the method by Reed and Müench (47). Groups of mice (n = 10 or 11) were inoculated intraperitoneally (ip) with 10^5 FFU (100 μl) of the viruses. The mice were observed for 3 weeks after inoculation to determine survival rates. To examine viral growth in the brain, 100 FFU (ic) or 10^5 FFU (ip) of the viruses were administered to the mice. At 1 to 7 days after inoculation, the mice were euthanized, and the brains were collected. The infectious viral titers in the homogenates of the brains were determined in Vero cells as described above.

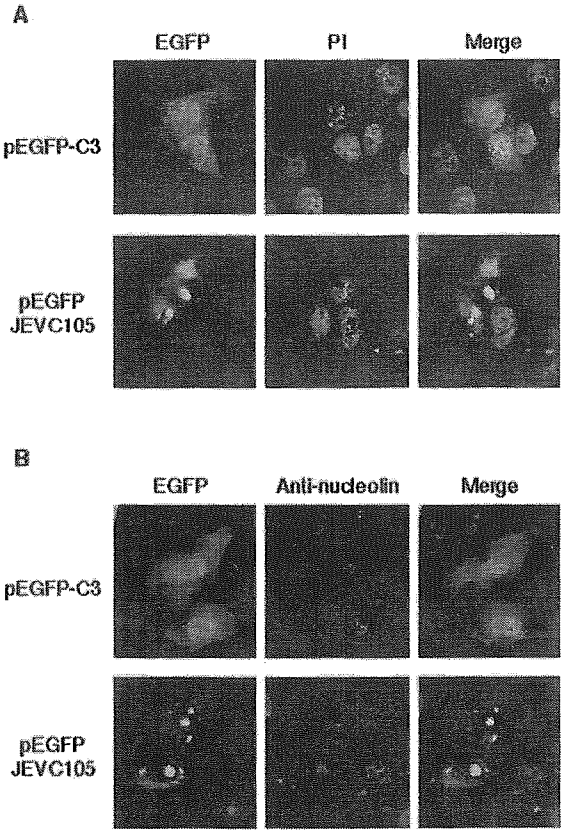

FIG. 1. Intracellular localization of EGFP-fused JEV core protein. Vero cells were transfected with expression plasmids encoding EGFP or EGFP-fused JEV core protein. At 24 h after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. (A) Nuclei were stained with propidium iodide. (B) A representative nucleolar protein, nucleolin, was stained with anti-nucleolin monoclonal antibody. All samples were observed with a confocal microscope.

RESULTS

Determination of amino acids essential for nuclear or nucleolar localization of the JEV core protein. To examine the subcellular localization of the mature JEV core protein without the C-terminal signal sequence in mammalian cells, pEGFP-JEVC105 encoding the EGFP-fused core protein or parental vector, pEGFP-C3, was transfected into Vero cells. EGFP was diffusely distributed in both the cytoplasm and nucleus, while the EGFP-fused core protein exhibited a diffuse distribution in the cytoplasm but granular localization in the nucleus (Fig. 1A). The fusion JEV core protein in the nucleus was colocalized with nucleolin, a major nucleolar component, indicating that the core protein is accumulated at the nucleoli (Fig. 1B). A similar subcellular localization of the fusion core protein was observed in all of the cell lines examined, including neuronal (N18 and SK-N-SH) and nonneuronal (293T, BHK, HeLa, and HepG2) cells (data not shown). Wang et al. (59) reported that the DEN core protein possessed a bipartite NLS in residues 85 to 100 (RKEIGRMLNILNRRKR). A computer program, PSORTII (Institute of Medical Science, Tokyo University [http://psort.ims.u-tokyo.ac.jp/helpwww2.html]), predicted that the JEV core protein also had an NLS at the corresponding region (residues 85 to 101 [KRELGTLIDAVNKRG RK]). To confirm whether the region functions as an NLS, an expression vector for the EGFP-fused mutant core protein in which all of the six basic amino acids (Arg and Lys) that were key amino acids in the NLS motifs were replaced by Ala (AAELGTLIDAVNAAGAA) was transfected into Vero cells. However, these mutations did not affect the nuclear or nucleolar localization of the JEV core protein (data not shown), suggesting that this region of the JEV core protein does not participate as an NLS.

Alternatively, we found another candidate for an NLS in the JEV core protein. The NLS of the core protein of hepatitis C virus (HCV), a member of the same family *Flaviviridae*, has been mapped to the amino acid residues 38 to 43 (54). This domain of the HCV core protein is found to be homologous with flaviviruses, including JEV, St. Louis encephalitis virus, KUN, West Nile virus (WNV), Murray Valley encephalitis virus, and DEN (type 1 to 4) (Fig. 2A). In particular, the two amino acids Gly and Pro are completely conserved among