BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 717–722 (1997)
ARTICLE NO. RC977372

Baculoviral Display of the Green Fluorescent Protein and Rubella Virus Envelope Proteins

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Received August 19, 1997

The ability to display heterologous proteins and peptides on the surface of different types of bacteriophage has proven extremely useful in protein structure/function studies. To display such proteins in a eucaryotic environment, we have produced a vector allowing for fusion of proteins to the amino-terminus of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) major envelope glycoprotein, gp64. Such fusion proteins incorporate into the baculoviral virion and display the FLAG epitope tag. We have further produced recombinant baculoviruses displaying the green fluorescent protein (GFP) and the rubella virus envelope proteins, E1 and E2. The incorporation of the GFPgp64, E1gp64, and E2gp64 fusion proteins into the baculovirus particle was demonstrated by western blot analysis of purified budded virus. This is the first report of the display of the GFP protein or the individual rubella virus spike proteins on the surface of an enveloped virus. Such a eucaryotic viral display system may be useful for the display of proteins dependent on glycosylation for activity and for targeting of recombinant baculoviruses to novel host cell types as a gene transfer vehicle.

The ability of various viruses to display foreign proteins and peptides on their surface has been useful in a number of areas in the life sciences, ranging from protein structure studies to gene therapy. Possibly the greatest usage of such technology has been in phage display, where a peptide or protein is fused to the gene III coat protein of the filamentous bacteriophages M13, fd or f1(1, 2). Phage display is an extremely powerful technique due to the direct physical linkage of genotype and phenotype. This linkage enables the selection of mutant proteins with desired characteristics from a library of displayed proteins, as in the selection of mutant forms of growth hormone with increased receptor binding affinity (3). This technology has also found particular application in the generation of antibodies without the need for prior immunization (reviewed in 4). However, phage display does have limitations imposed by the expression host. Since phages are procaryotic viruses, the proteins intended for display must be suitable for secretion from a procaryotic host, and not be dependent on some eucaryotic postranslational modification or folding requirement. Therefore, a eucaryotic viral display system would be useful for the study of such proteins.

The *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been widely used for the expression of heterologous proteins in insect cells (5). As a result the AcNPV virus has become well characterized, including the determination of the complete DNA sequence of the viral genome (6). The virus is a member of the family *Baculoviridae,* a group of large enveloped double-stranded DNA viruses which are pathogens of insects. The form of the virus responsible for infection of cells in tissue culture is a single enveloped nucleocapsid (budded virus or ECV). The major protein of the AcNPV envelope is the glycoprotein known as gp64 (or alternatively, gp67). The gp64 gene encodes a type I integral membrane glycoprotein with an amino-terminal signal sequence and a carboxy-proximal transmembrane domain (7). The gp64 protein occurs on the viral particle as a disulphide linked oligomer (most likely a trimer, 8) and is responsible for viral cell entry, mediated by acid-triggered membrane fusion (9). Structural studies on the gp64 protein have identified separate domains responsible for oligomer formation and membrane fusion (9). These structural characteristics of gp64 make the protein a good candidate as a presentation platform for the development of a eucaryotic based viral display system.

In this study, we have investigated the suitability of the gp64 protein as a fusion partner to direct the

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0006-291X/97 $25.00

incorporation of heterologous proteins into the baculoviral virion. Our results show that fusion to the amino-terminus of gp64 of either the rubella virus envelope proteins, or the green fluorescent protein (GFP) of *Aequorea victoria*, leads to the incorporation of the product into the baculovirus particle. Such display of GFP on the surface of the baculovirus may lead to an improved approach for monitoring the baculoviral infection process *in vivo*. The ability to individually display the rubella virus envelope proteins, E1 and E2, on the baculovirus surface will facilitate studies with the aim, for example, of identifying the rubella virus receptor. Further, developments to the baculo-display system may allow the use of baculoviruses as a general gene transfer vehicle.

MATERIALS AND METHODS

Cells and virus. *Spodoptera frugiperda* (Sf9 and Sf21AE) cells were grown in monolayer and/or suspension cultures in SF900-II medium (Gibco-BRL, MD, USA) with antibiotics at 27°C. Cells were infected by each recombinant baculovirus at a multiplicity of infection (MOI) of 1-10 and the products analyzed 3 days post infection (p.i.). Viral stocks were produced by infecting cells at MOI 0.1 and harvesting virus after 4 days. Insect cell culture and baculoviral procedures followed standard protocols (5). For storage at 4°C, stock virus samples had FCS added to 2%.

Plasmid construction. Epitope (FLAG)-tagged gp64 was constructed by polymerase chain reaction (PCR) amplification of the gp64 gene from purified AcNPV DNA (5′ primer, GGTGGTGAATTCCGGCCGAACGCGCAAATGAAGACGGG, underlined sequence encodes amino acids 25-31 of AcNPV gp64, 3′ primer, GTTGGGTCTAGATCTTTAATATTGTCTATTACGGTTTC, underlined sequence encodes amino acids 506-512 of AcNPV gp64), and cloning of the EcoRI/BglII-digested PCR product into a EcoRI/BamHI-treated pVL1392-derivative containing the signal sequence of the GluR-D glutamate receptor followed by the FLAG epitope (10). The resulting expression plasmid was designated pFLAGgp64. The predicted amino acid sequence of the FLAGgp64 construct is MRIICRQIVLLFSGFWGLAMG↓DYKDDDDKISRPEFRP**NAQMKTG . . . NRNRQY** (signal peptide cleavage site indicated by an arrow, FLAG peptide underlined and gp64 amino acids 25-512 shown in bold). pGFPgp64 was constructed by PCR of the GFP coding sequence from the plasmid pK410-2 (11) (5′ primer, TCGCAGATCTGTATGAGTAAAGGAGAAGAACTT, 3′ primer, TACCGAATTCTTTGTAGAGCTCATCCATGCC) and cloning of the product into the BglII/EcoRI sites of pFLAGgp64. The resultant plasmid encodes the fusion protein FLAG-GFP-AcNPVgp64. The plasmids pRVE1gp64 and pRVE2gp64 were constructed by PCR amplification of the rubella virus envelope proteins E1 and E2 from the plasmid pGEM2-RV24S (12). The primers used (E1: 5′ primer, CCCCCAGATCTGTGAGGAGGCTTTCACCTA, 3′ primer, TACCGAATTCCTCCGCCCAGGTCTG; E2: 5′ primer, TACCAGATCTTTGGGCTCCAGCCCCGC, 3′ primer, TACCGAATTCATCAAGAGACAGCGC) amplified the DNA encoding the extra-virion regions of both envelope proteins, i.e., the region of the protein displayed on the rubella virus surface. The amplified portions of the rubella virus E1 and E2 proteins were cloned into the BglII/EcoRI sites of pFLAGgp64, the resultant plasmids encoding the fusion proteins FLAG-E1-AcNPVgp64 and FLAG-E2-AcNPVgp64, respectively. In the case of the rubella virus E1 protein, the DNA fragment encoding the FLAG-E1-AcNPVgp64 fusion protein was excised from the plasmid pRVE1gp64 by digestion with EcoRV and HindIII and subcloned into the pFASTBAC 1 vector (Gibco-BRL) digested with StuI and HindIII. The resultant plasmid pFBRVE1gp64 contains two polyhedrin promoters in tandem, followed by the sequence coding for the FLAG-E1-AcNPVgp64 fusion protein.

Production and purification of recombinant baculoviruses. The recombinant baculoviruses AcFLAGgp64, AcGFPgp64 and AcRVE2gp64 were produced by homologous recombination after co-transfection of the recombinant transfer plasmids pFLAGgp64, pGFPgp64 and pRVE2gp64, respectively, with linear wild-type AcNPV DNA (Invitrogen, CA, USA). The recombinant viruses encoding the AcNPVgp64 fusion proteins were plaque purified according to standard procedures (5). The recombinant baculovirus AcRVE1gp64 was produced using the Bac-to-Bac system (Gibco-BRL). In brief, the FLAG-E1-AcNPVgp64 expression cassette was transferred from the plasmid pFBRVE1gp64 into a baculovirus shuttle vector (bacmid) by a site-specific transposition event (13). The resultant recombinant bacmid was introduced into *Spodoptera frugiperda* cells by Insectin (Invitrogen) mediated transfection, followed by collection of the recombinant AcRVE1gp64 virus in the transfected cell medium.

Concentration and sucrose gradient purification of budded virus. Baculovirus samples for analysis by sucrose gradient sedimentation were prepared from infected cell supernatants by centrifugation of culture medium (100,000 × g, 30 min at 4°C) which had previously been clarified by low speed centrifugation (6,000 × g, 15 min at 4°C). The viral pellet obtained was resuspended on ice in PBS, and loaded onto a 25-60% sucrose gradient prepared in PBS. Viral samples were banded in the gradient by centrifugation at 100,000 × g for 18 h at 4°C and fractionated from the top. Sucrose gradient fractions were analyzed for the presence of gp64 fusion proteins by western blotting.

SDS-PAGE and western blots. Protein extracts from baculovirus infected insect cells were prepared by the solubilization of infected Sf9 or Sf21 cells in SDS-PAGE sample buffer with 2-ME and boiled for 5 min before electrophoresis in a 7.5% SDS-PAGE gel. Baculovirus samples for immunoblot analysis were prepared from infected cell supernatants by centrifugation of medium (100,000 × g, 30 min at 4°C) which had previously been clarified by low speed centrifugation (6,000 × g, 15 min at 4°C). The viral pellet obtained was directly solubilized in SDS-PAGE sample buffer with 2-ME and boiled before electrophoresis. After electrophoresis, cell and baculoviral extracts were transferred to nitrocellulose and probed with either the anti-AcNPV gp64 mAb B12D5 (Dr. Loy Volkman) or the anti-FLAG mAb M1 (14, IBI Kodak, CT, USA) as indicated, and developed with an alkaline phosphatase-conjugated goat anti-mouse antibody according to the manufacturer's instructions (Bio-Rad, CA, USA). Alternatively, nitrocellulose membranes were probed with a biotinylated anti-FLAG mAb BioM2 (IBI Kodak) and developed with a streptavidin-alkaline phosphatase conjugate (Amersham, UK).

RESULTS

To enable fusion of heterologous proteins to the baculovirus AcNPV major envelope glycoprotein, gp64, we constructed the vector pFLAGgp64 (Fig. 1). This vector has the amino-terminus of gp64 fused to the FLAG epitope tag (14) followed by restriction sites for BglII and EcoR1. Between these sites we have inserted the cDNAs for the green fluorescent protein (GFP) of *Aequorea victoria* (15), or either of the envelope proteins, E1 or E2, of rubella virus (16) (Fig. 1). These proteins were chosen to test the suitability of gp64 to direct the incorporation of the resultant fusion protein into the baculovirus particle. The placement of the FLAG tag at the amino-terminus of the gp64 fusion proteins was designed to allow detection of the full length product

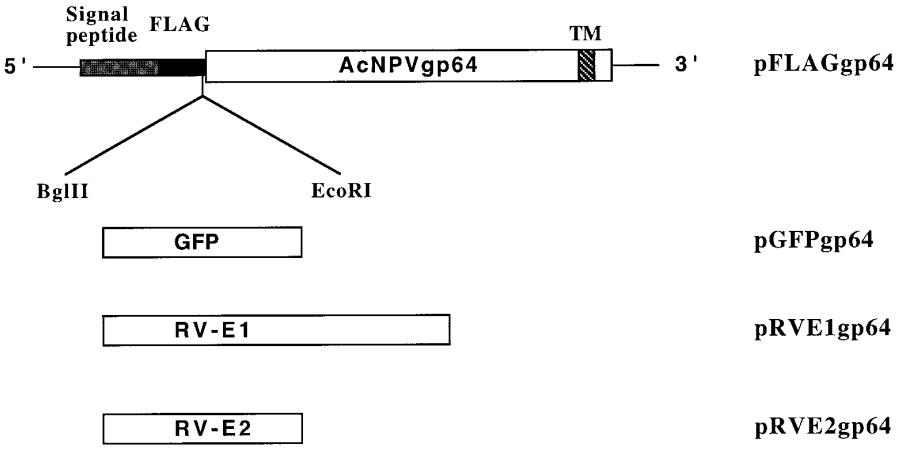

FIG. 1. Expression cassette structure of the different AcNPVgp64 fusion vectors used in this study. The pFLAGgp64 vector was used for the construction of the other gp64 fusion vectors (pGFPgp64, pRVE1gp64, and pRVE2gp64) by insertion of the different PCR amplified DNAs between the BglII and EcoRI sites. The signal peptide used in the pFLAGgp64 vector is derived from the glutamate receptor GluR-D (10). The FLAG peptide sequence (DYKDDDDK) (14) was included at the amino terminus of all the fusion proteins. TM, transmembrane segment of the AcNPVgp64 gene; GFP, green fluorescent protein; RV-E1, rubella virus E1 envelope protein; RV-E2, rubella virus E2 envelope protein.

and potentially allows for selection of recombinant viruses by binding to a FLAG specific antibody.

The gp64 fusion plasmids represented in Fig. 1 were used to generate the recombinant baculoviruses, AcFLAGgp64, AcGFPgp64, AcRVE1gp64 and AcRVE2gp64. These viruses have two copies of the gp64 coding sequence, one being the wild type gp64, essential for viral infectivity, and the second being the gp64 fusion protein, under the control of the polyhedrin gene promoter. These viruses were used to infect Sf21 insect cells, and the resulting cell pellets were analyzed by SDS-PAGE followed by probing with specific monoclonal antibodies (Fig. 2). When the infected cell extracts were probed with the M1 antibody, specific for the FLAG epitope tag, a band was obtained for each sample consistent with the full length fusion protein, i.e. FLAGgp64: 66kDa, GFPgp64: 98kDa, RVE1gp64: 120kDa, and RVE2-gp64: 105kDa. These same products reacted with the anti-gp64 antibody, B12D5, consistent with them representing the gp64 fusion proteins. In the anti-gp64 immunoblots a 64kDa band is also detected, due to the presence of the wild type gp64. As control viruses we have used wild type AcNPV and a recombinant baculovirus, AcRVE1, encoding the rubella virus E1 envelope protein with an amino terminal FLAG tag (17). The GFP protein retained activity when fused to the amino terminus of gp64, demonstrated by the green fluorescence of infected insect cells when observed by fluorescence microscopy (data not shown).

To initially determine if the gp64 fusion proteins are incorporated into the AcNPV virion, we collected budded virus from infected Sf21 cells and prepared viral pellets by centrifugation. The purified viral pellets were analyzed by SDS-PAGE and probed with the M1 or anti-gp64 antibodies (Fig. 3). The M1 antibody detected products of the expected size for the full length fusion proteins, i.e. FLAGgp64: 66kDa, GFPgp64: 98kDa, RVE1gp64: 120kDa, and RVE2gp64: 105kDa. These same bands were also detected with the anti-gp64 antibody. No M1 antibody reactive material was detected in the budded virions from the AcRVE1 virus, demonstrating that incorporation of a heterologous protein into the baculovirus particle is dependent on fusion with the gp64 envelope protein.

Attempts to demonstrate the display of the FLAG epitope tag on the surface of the gp64 fusion baculoviruses by electron microscopy of immunogold-labeled budded virus were not successful due to the occurrence of damaged virus particles (the fragile nature of the AcNPV budded virus has been previously noted, 18). However, we did achieve specific immunogold-labeling with the M1 antibody of baculovirus particles budding from the AcFLAGgp64 infected cell surface (data not shown). Although such labeling was sparse, the bound gold particles were always associated with virus particles, and no labeling was observed in the case of wild type AcNPV infected cells. To further confirm the viral incorporation of the gp64 fusion proteins, the gp64 fusion viruses were analyzed by sedimentation through a 25%-60% sucrose gradient, and the fractions subjected to western analysis (Fig. 4). The level of the GFPgp64 fusion protein in the sucrose gradient peaked at 42-47% sucrose, the same fractions as the peak of infectious virus, as determined by plaque assay. Furthermore, in similar

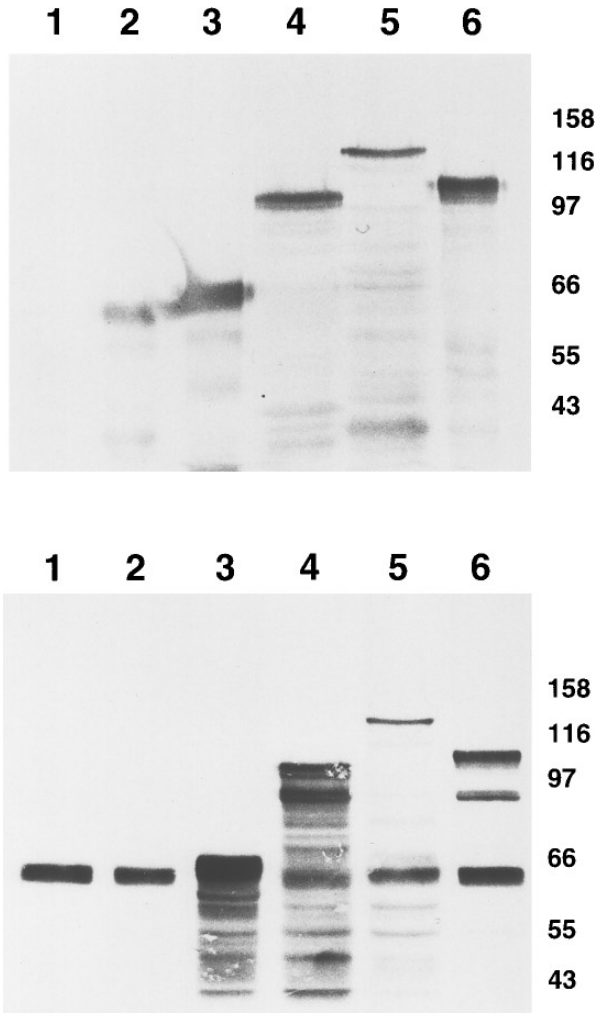

FIG. 2. Western blots of gp64 fusion baculovirus infected Sf21 cells. Nitrocellulose membranes were probed with either the FLAG epitope specific mAb, M1 (upper panel), or the anti-gp64 mAb, B12D5 (lower panel). The viruses used were (lane 1) AcNPV wild type, (lane 2) AcRVE1, a virus encoding the rubella virus envelope protein E1 with an amino-terminal FLAG tag, (lane 3) AcFLAGgp64, (lane 4) AcGFPgp64, (lane 5) AcRVE1gp64, and (lane 6) AcRVE2gp64. Position of the protein molecular mass standards is indicated on the right in kilodaltons.

sucrose gradients the level of the FLAGgp64, RVE1gp64 and RVE2gp64 fusion proteins also peaked at 42-47% sucrose.

DISCUSSION

Our results indicate that fusion of a protein to the amino-terminus of gp64 is sufficient to achieve incorporation of the product into the baculovirus virion. Surface expression of a protein in *Spodoptera frugiperda* insect cells is not enough to achieve such incorporation, as we have demonstrated in this study with the E1 envelope protein of rubella virus. This contrasts with the situation for the vesicular stomatitis virus (VSV), which has recently been shown to non-specifically incorporate heterologous membrane proteins into the virus particle (19). The fusion of a protein to gp64 does not appear to alter the growth of the resultant virus, since we have been able to produce high titre stocks of all our gp64 fusion viruses. However, it still remains a possibility that the fusion of some proteins to gp64 may adversely effect viral production and/or infectivity. In the work reported here we have utilized sucrose gradient sedimentation analysis to distinguish between viral incorporation and secretion of the gp64 fusion proteins. It is particularly important to make this distinction as gp64 has been found in the culture medium of baculovirus infected insect cells prior to the budding of virus (20).

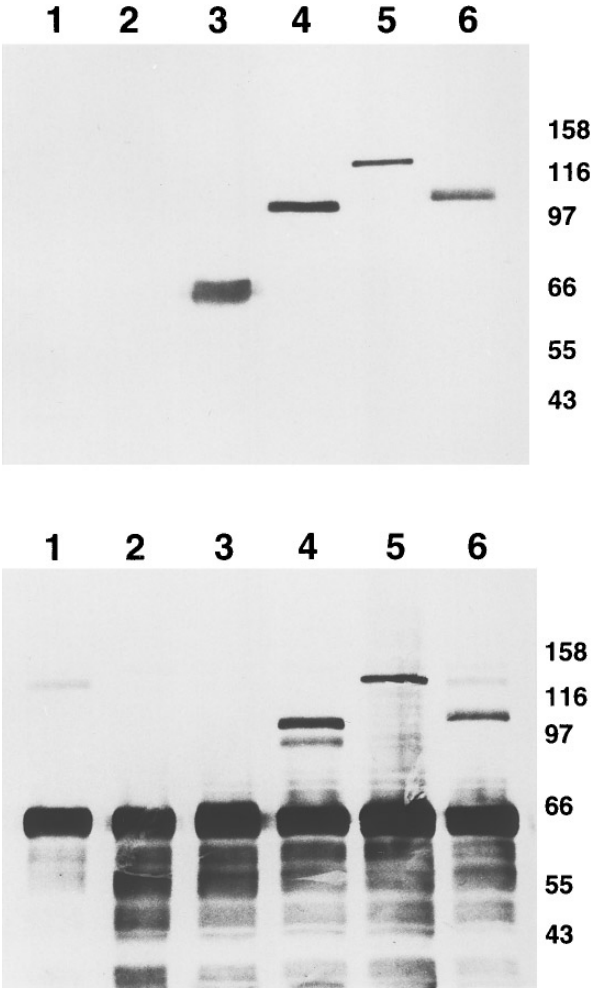

FIG. 3. Western blots of gp64 fusion baculoviruses. Nitrocellulose membranes were probed with either the FLAG epitope specific mAb, M1 (upper panel), or the anti-gp64 mAb, B12D5 (lower panel). The viruses used were (lane 1) AcNPV wild type, (lane 2) AcRVE1, (lane 3) AcFLAGgp64, (lane 4) AcGFPgp64, (lane 5) AcRVE1gp64, and (lane 6) AcRVE2gp64. Position of the protein molecular mass standards is indicated on the right in kilodaltons.

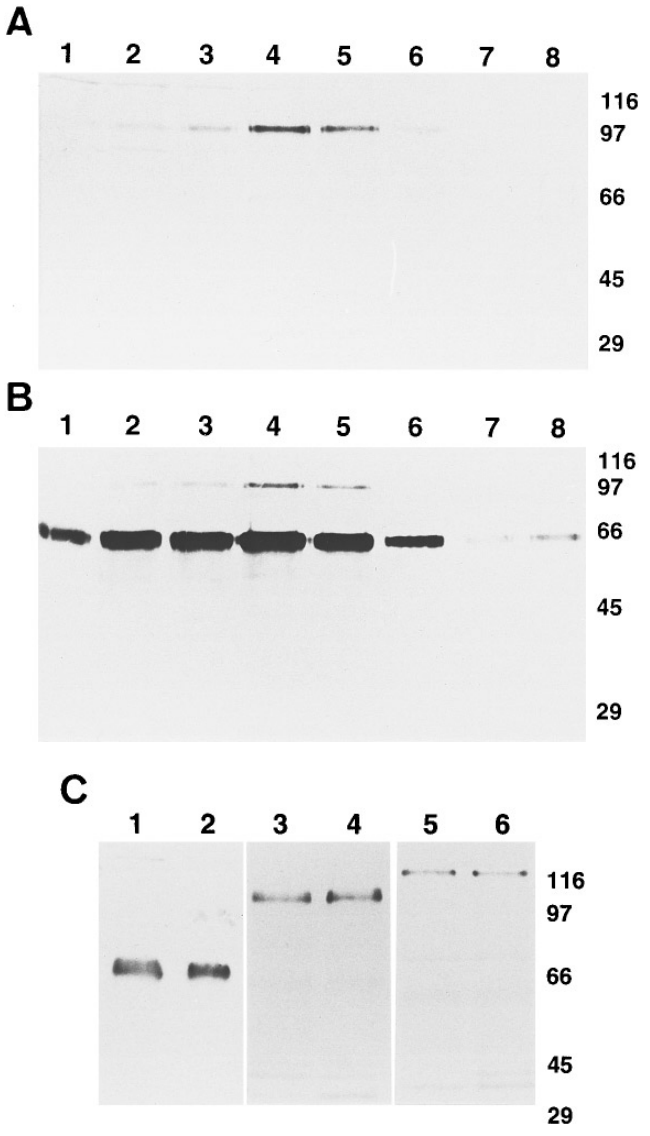

FIG. 4. Sucrose gradient analysis of gp64 fusion baculoviruses. A and B show western blots of the AcGFPgp64 virus sucrose gradient fractionation, probed with a biotinylated anti-FLAG mAb, BioM2 (A), or the anti-gp64 mAb, B12D5 (B). Fractions (1-8) were collected from the top of the sucrose gradient (25-60%). (C) Western blots of the peak fractions (fractions 4 and 5 for all gradients) from the AcFLAGgp64 (lanes 1 and 2), AcRVE2gp64 (lanes 3 and 4), and AcRVE1gp64 (lanes 5 and 6) virus sucrose gradients. AcFLAGgp64 virus samples (lanes 1 and 2) were probed with the biotinylated anti-FLAG mAb, BioM2. AcRVE2gp64 and AcRVE1gp64 virus samples were probed with the FLAG epitope specific mAb, M1. Position of the protein molecular mass standards is indicated on the right in kilodaltons.

Similar results to those presented here have recently been reported by Boublik et al. (21). These workers fused either glutathione-S-transferase (GST) or the HIV gp120 protein to the amino terminus of gp64 and achieved incorporation of the product into the virus particle. It is difficult to compare the efficiency of virion incorporation of these gp64 fusion proteins with those which we have produced. Although we have not quantitated the level of incorporation of our different gp64 fusion proteins relative to native gp64, the intensity of the signal obtained in western blots of the AcFLAGgp64 virus, would suggest a higher level of incorporation of the FLAGgp64 protein compared to the other gp64 fusion proteins. This is possibly to be expected, as the larger the protein fused to gp64 the more likely it would interfere with gp64 trimer formation, and hence incorporation into the virus particle. GFP and the rubella virus envelope proteins E1 and E2, were all incorporated into the virus particle to a similar level.

The AcNPV virus is not the only virus which has been investigated as the basis of a eucaryotic display system. Examples include, poliovirus (22), rhinovirus (23), and sindbis virus (24) from animals, and cowpea mosaic virus (25) and tobacco mosaic virus (26) from plants. However, these viruses have only been engineered to display peptide epitopes as vehicles for vaccine development (reviewed in 27). Recently, it has been reported that the green fluorescent protein (GFP) can be displayed on the surface of the potato virus X by fusion to the amino-terminus of the coat protein (28, 29). In this case the presence of the native coat protein was also required to enable the assembly of infectious virus particles. However, the suggested uses for the potato virus X display vehicle have centered around applications such as heterologous protein production, vaccine development and the non-invasive study of virus multiplication and spread (29). In contrast, gp64 fusion baculoviruses may be of use in the development of eucaryotic display libraries and in the targeting of baculoviruses to novel host cell types.

Recent developments in the baculovirus expression system (reviewed in 30) are opening up the possibility of constructing display libraries in recombinant baculoviruses. Traditionally, recombinant baculoviruses are produced by co-transfection of a transfer vector containing an expression cassette with baculoviral DNA into *Spodoptera frugiperda* cells. *In vivo* recombination between the transfer vector and the baculoviral genome results in the production of recombinant viruses. The use of linearized baculoviral DNA for transfection (31) and the development of a lethal deletion virus (32) has greatly increased the efficiency of recombinant virus isolation. Further developments with particular relevance for library generation include the production of recombinant viruses *in vitro* by the Cre-lox system of bacteriophage P1 (33), the ability to directly clone into the AcNPV genome (34) and improvements to the transposon-mediated bacmid system (35).

Targeting of baculoviral vectors to novel cell types via the display on the viral vector surface of a cell type specific ligand, may have applications in the developing field of gene therapy. Recently it has been demonstrated that suitably modified baculoviral vectors effi-

ciently transfer and express genes in mammalian cells of liver origin (36-38). Expression of the reporter gene in such modified baculoviruses was dependent on the presence of a promoter active in mammalian cells (37). The lack of reporter gene expression in cell types of non-liver origin does not appear to be due to a block in baculovirus internalization (36). Possibly the pathway of baculoviral internalization is different in liver cells compared with cells of non-hepatic origin. By modifying the surface of viral vectors it is possible to target specific cell types at the level of cell binding and entry (39, 40). Hence, by modifying the baculovirus surface via engineering of the gp64 protein, it may be possible to specifically alter the pathway of internalization such that baculovirus-mediated gene transfer is possible to various different cell types of therapeutic interest. We are currently investigating these proposals.

ACKNOWLEDGMENTS

We are grateful to Dr. Loy Volkman for providing the anti-gp64 antibody (B12D5), Dr. Martin Chalfie for providing the gene coding for the green fluorescent protein (GFP), and Ms. Anja Pallas and Tuula Kuurila for excellent technical assistance. We also acknowledge Dr. Verne Luckow and Dr. Deborah Murrey for providing access to the Bac to Bac system. This study was funded by the Academy of Finland and the European Union (EU Contract BIO-2CT94-3069).

REFERENCES

1. Clackson, T., and Wells, J. A. (1994) *Trends Biotechnol.* **12,** 173–184.
2. Makowski, L. (1994) *Curr. Opin. Struct. Biol.* **4,** 225–230.
3. Lowman, H. B., and Wells, J. A. (1993) *J. Mol. Biol.* **234,** 564–578.
4. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994) *Annu. Rev. Immunol.* **12,** 433–455.
5. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) Baculovirus Expression Vectors, a Laboratory Manual, Freeman, New York.
6. Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M., and Possee, R. D. (1994) *Virology* **202,** 586–605.
7. Whitford, M., Stewart, S., Kuzio, J., and Faulkner, P. (1989) *J. Virol.* **63,** 1393–1399.
8. Oomens, A. G. P., Monsma, S. A., and Blissard, G. W. (1995) *Virology* **209,** 592–603.
9. Monsma, S. A., and Blissard, G. W. (1995) *J. Virol.* **69,** 2583–2595.
10. Kuusinen, A., Arvola, M., Oker-Blom, C., and Keinänen, K. (1995) *Eur. J. Biochem.* **233,** 720–726.
11. Oker-Blom, C., Orellana, A., and Keinänen, K. (1996) *FEBS Lett.* **389,** 238–243.
12. Suomalainen, M., Garoff, H., and Baron, M. D. (1990) *J. Virol.* **64,** 5500–5508.
13. Luckow, V. A., Lee, S. C., Barry, G. F., and Olins, P. O. (1993) *J. Virol.* **67,** 4566–4579.
14. Hopp, T. P., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L., and Conlon, P. J. (1988) *Bio/Technology* **6,** 1204–1210.
15. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) *Science* **263,** 802–805.
16. Frey, T. K. (1994) *Adv. Virus Res.* **44,** 69–160.
17. Orellana, A., Mottershead, D. G., van der Linden, I., Keinänen, K., and Oker-Blom, C. Manuscript in preparation.
18. Summers, M. D., and Volkman, L. E. (1976) *J. Virol.* **17,** 962–972.
19. Schnell, M. J., Buonocore, L., Kretzschmar, E., Johnson, E., and Rose, J. K. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11359–11365.
20. Jarvis, D. L., and Garcia, A., Jr. (1994) *Virology* **205,** 300–313.
21. Boublik, Y., Bonito, P. D., and Jones, I. M. (1995) *Bio/Technology* **13,** 1079–1084.
22. Rose, C., Andrews, W., Ferguson, M., McKeating, J., Almond, J., and Evans, D. (1994) *J. Gen. Virol.* **75,** 969–977.
23. Resnick, D. A., Smith, A. D., Geisler, S. C., Zhang, A., Arnold, E., and Arnold, G. F. (1995) *J. Virol.* **69,** 2406–2411.
24. London, S. D., Schmaljohn, A. L., Dalrymple, J. M., and Rice, C. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 207–211.
25. Porta, C., Spall, V. E., Loveland, J., Johnson, J. E., Barker, P. J., and Lomonossoff, G. P. (1994) *Virology* **202,** 949–955.
26. Turpen, T. H., Reinl, S. J., Charonenvit, Y., Hoffman, S. L., Fallarme, V., and Grill, L. K. (1995) *Bio/Technology* **13,** 53–57.
27. Lomonossoff, G. P., and Johnson, J. E. (1996) *Curr. Opin. Struct. Biol.* **6,** 176–182.
28. Oparka, K. J., Roberts, A. G., Prior, D. A. M., Chapman, S., Baulcombe, D., and Santa Cruz, S. (1995) *Protoplasma* **189,** 133–141.
29. Santa Cruz, S., Chapman, S., Roberts, A. G., Roberts, I. M., Prior, D. A. M., and Oparka, K. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6286–6290.
30. Davies, A. H. (1994) *Bio/Technology* **12,** 47–50.
31. Kitts, P. A., Ayres, M. D., and Possee, R. D. (1990) *Nucleic Acids Res.* **18,** 5667–5672.
32. Kitts, P. A., and Possee, R. D. (1993) *Biotechniques* **14,** 810–817.
33. Peakman, T. C., Harris, R. A., and Gewert, D. R. (1992) *Nucleic Acids Res.* **20,** 495–500.
34. Ernst, W. J., Grabherr, R. M., and Katinger, H. W. D. (1994) *Nucleic Acids Res.* **22,** 2855–2856.
35. Leusch, M. S., Lee, S. C., and Olins, P. O. (1995) *Gene* **160,** 191–194.
36. Boyce, F. M., and Bucher, N. L. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 2348–2352.
37. Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P., and Strauss, M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10099–10103.
38. Sandig, V., Hofmann, C., Steinert, S., Jennings, G., Schlag, P., and Strauss, M. (1996) *Hum. Gene Ther.* **7,** 1937–1945.
39. Cosset, F.-L., and Russell, S. J. (1996) *Gene Therapy* **3,** 946–956.
40. Miller, N., and Vile, R. (1995) *FASEB J.* **9,** 190–199.