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Expression of Green Fluorescent Protein in Both *Spodoptera frugiperda* Cells and *Bombyx mori* Larvae by Ac-Bm Hybrid Virus

Byung Rae Jin[1,2,3], Hyung Joo Yoon[2], Eun Young Yun[2], Seok Woo Kang[2], Eun Sook Cho[3] and Seok Kwon Kang[1]*

[1]The Research Center for New Bio-Materials in Agriculture, and Division of Applied Biology and Chemistry, College of Agriculture & Life Sciences, Seoul National University, Suwon 441-100, Korea [2]National Sericulture & Entomology Research Institute, RDA, Suwon 441-100, Korea [3]College of Natural Resources and Life Science, Dong-A university, Pusan, Korea

=Abstract=

We have expressed GFP in Sf9 and Bm5 cells or *Bombyx mori* larvae by using Ac-Bm hybrid virus capable of replicating in both Bm5 and Sf9 cells. Genomic DNA of Ac-Bm hybrid virus expressing β-galactosidase was cotransfected with baculovirus transfer vector containing GFP gene, pBacPAK-GFP in Sf9 cells. The Ac-Bm hybrid virus harboring GFP was named as Ac-Bm hybrid virus-GFP. The Ac-Bm hybrid virus-GFP-infected insect cells were easily selected by detecting the emission of GFP from each well of cell culture dish on the UV illuminator. GFP produced by Ac-Bm hybrid virus-GFP in Sf9 and Bm5 cells or *B. mori* larvae was confirmed by SDS-PAGE and Western blot analysis using GFP antibody. In addition, *B. mori* larvae infected with Ac-Bm hybrid virus-GFP was apparently appeared fluorescence from the whole body at 5 days postinoculation. The fluorescence of GFP from the hemolymph and fat body of *B. mori* larvae infected with Ac-Bm hybrid virus-GFP was also observed by fluorescence microscope. In conclusion, our results demonstrated that in baculovirus expression vector system, use of Ac-Bm hybrid virus have an additional advantage of expanded host range for producing recombinant proteins.

Key Words: Baculovirus, Expression vector, Silkworm, Insect cells

INTRODUCTION

Baculoviruses have been demonstrated for the abundant expression of a large variety of foreign genes in insect cells [5, 10, 11, 14]. The baculovirus expression vector system mainly utilizes the strong polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) or *Bombyx mori* nuclear polyhedrosis virus (BmNPV), and the recombinant proteins are produced in insect cells or

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*Corresponding author: Seok-Kwon Kang, The Research Center for Nwe Bio-Materials in Agriculture, and Division of Applied Biology and Chemistry, College of Agriculture & Life Sciences, Seoul National University, Suwon 441-100, Korea Tel. 0331-290-2481 FAX. 0331-296-0926 E-mail kskipl@plaza.snu.ac.kr

larvae [2, 10, 12, 15]. The recombinant proteins have been shown, in most cases, to be processed similarly as compared to their authentic counterparts. This feature, together with the high expression levels obtained, has made the baculovirus expression vector system highly attractive. A common insect cell line, *Spodoptera frugiperda*-derived Sf9 or Sf21, is used for AcNPV-mediated expression of foreign genes. *B. mori*-derived Bm5 cell line is used for BmNPV-mediated expression of foreign genes. BmNPV expression vector system has also been frequently utilized for high level expression of foreign genes in silkworm larvae.

Recently, the host range of AcNPV is expanded by homologous recombination with various methods [3, 8, 13, 14]. Extension of AcNPV host range has been shown to be specifically due to nucleotide and amino acid substitutions of the controlling region in the p143 helicase gene. DNA helicase gene product, p143, has been shown to play essential roles in viral DNA replication and host range expansion [3, 7, 8, 13]. In addition, in a previous report [6] we described the construction and expression of Ac-Bm hybrid virus capable of replicating in both Bm5 and Sf21 cells.

In this study we have constructed Ac-Bm hybrid virus-GFP harboring green fluorescent protein (GFP) gene by cotransfection of genomic DNA of Ac-Bm hybrid virus [6] and AcNPV transfer vector containing green fluorescent protein gene [4]. To confirm the efficiency of the Ac-Bm hybrid virus, we have also expressed the GFP in Sf9 and Bm5 cells or *B. mori* larvae.

MATERIALS AND METHODS

Cell lines and viruses

Spodoptera frugiperda (Sf9) and *Bombyx mori* (Bm5) cells were maintained at 27℃ in TC-100 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant baculovirus [6] were propagated in Sf9 or Bm5 cells. In all experiments, the titer of viruses was determined by plaque assays in Bm5 or Sf9 cells as described previously [15, 16].

Virus and DNA purification

Ac-Bm hybrid virus containing β-galactosidase gene under the polyhedrin promoter [6] was obtained from Sf9 cells supernatant by standard methods [16]. The supernatant was clarified at 3,000 rpm for 10 min, and virus particles were then pelleted at 23,000 rpm in an SW28 rotor (Beckman). DNA was extracted with phenol after proteinase K digestion [15].

Construction of Ac-Bm hybrid virus expressing GFP

Cell culture dish (35-mm diameter) was seeded with $1x10^6$ Sf9 cells and incubated at 27℃ for 1 hr to allow the cells to attach. One microgram of Ac-Bm hybrid virus-HE viral DNA [6], 5 μg of AcNPV transfer vector containing GFP gene [4] in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystylene tube. Fifty microliter of 100 μg/ml Lipofectin™ (Gibco) was gently mixed with the DNA solution, and the mixture was incubated at room temperature for 30 min. The cells were washed twice with 2 ml serum-free TC-100 medium. Serum-free TC-100 (1.5 ml) was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubation at 27℃ for 5 hr, 1.5 ml TC-100 containing antibiotics and 10% FBS were added to each dish and the incubation at 27℃ continued. At 5 days postinfection (p.i.), the supernatant was harvested, clarified by centifugation at 2,000 rpm for 5 min, and stored at 4℃. To purify Ac-Bm hybrid virus expressing GFP, $1x10^5$ Bm5 or Sf9 cells were seeded per well on a 24-well plate and the diluted supernatant was added to each well. Ac-Bm hybrid virus expressing GFP was selected from well by UV

irradiation.

SDS-PAGE and Western blot

Bm5 or Sf9 cells were mock infected or infected with wild-type AcNPV or BmNPV, or Ac-Bm hybrid virus expressing GFP in a 35-mm diameter dish ($1x10^6$ cells) at a MOI of 5 PFU per cell. After incubation at 27℃, cells were harvested at 2, and 3 days p.i. For SDS-PAGE of cell lysates, Bm5 or Sf9 cells infected with or without viruses were washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) and mixed with a sample buffer (5% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue, 20% glycerol). Samples were boiled for 5 min and clarified by centrifugation (10,000 x g for 1 min). The total lysates were subjected to 12.5% SDS-PAGE [9], electroblotted and incubated with GFP antibody (Clontech) [17]. Mid-range SDS-PAGE molecular weight standards were from BioRad Laboratories (Richmond, CA).

Expression of GFP in *B. mori* larvae

Fifty μl ($5x10^6$ PFU) of the Ac-Bm hybrid virus expressing GFP was injected into the body cavities of silkworm larvae at the early fifth instar stage, which were then fed on mulberry leaves at 25℃ [2]. Thereafter, the infected larval bodies were pierced with a collecting needle near abdominal appendages and the hemolymph was collected in an ice-cooled Eppendorff tube at 3, 4, and 5 days p.i. The samples were centrifuged at 10,000 rpm for 5 min at 4 ℃. The supernatant was subjected to 12.5% SDS-PAGE, electroblotted and incubated with GFP antibody (Clontech).

Microscopy

B. mori larvae were mock infected or infected with Ac-Bm hybrid virus expressing GFP. The hemolymph and fat body from *B. mori* larvae were collected at 5 days p.i. Microscopy of hemolymph or fat body of *B. mori* larvae was performed with a light and a fluorescent microscope (Axiophot Universal Microscope, Zeiss). The larvae infected with Ac-Bm hybrid virus expressing GFP were also photographed on UV illuminator.

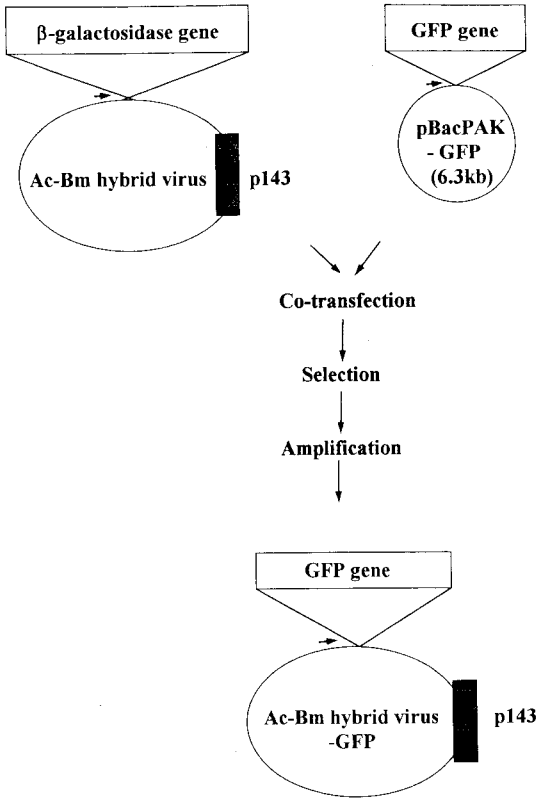

Fig. 1. Flow diagram summarizing procedure used to generate Ac-Bm hybrid virus-GFP. Genomic DNA of Ac-Bm hybrid virus expressing β-galactosidase, Ac-Bm hybrid virus-HE, was cotransfected with AcNPV transfer vector pBacPAK-GFP containing GFP gene in Sf9 cells. The supernatant was collected at 5 days p.i., reinfected into a monolayer of Bm5 cells, and Ac-Bm hybrid virus-GFP expressing GFP was easily selected by the bright glow of GFP on UV illuminator.

RESULTS AND DISCUSSION

Construction of Ac-Bm hybrid virus expressing GFP

The flow diagram summarizing procedure used to generate Ac-Bm hybrid virus expres-

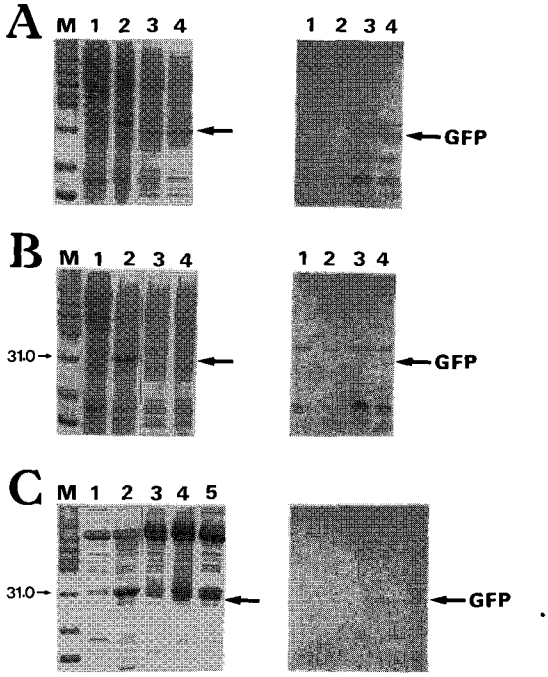

Fig. 2. SDS-PAGE and Western blot analysis of GFP expression of Ac-Bm hybrid virus-GFP in Sf9 and Bm5 cells or *B. mori* larvae. Sf9 (A) and Bm5 (B) cells or *B. mori* larvae (C) were infected with wild-type AcNPV (Lane 2 of panel A) or BmNPV (Lane 2 of panel B and C) or Ac-Bm hybrid virus-GFP (Lanes 3, 4, and 5 of panel A, B, and C). Samples were collected at 2 (Lanes 2 and 3 of panel A and B, or lane 2 of panel C), 3 (Lanes 4 of panel A and B, or lane 3 of panel C), and 5 (Lane 5 of panel C) days p.i. Total cellular lysates or hemolymph were subjected to 12.5% SDS-PAGE (Left panels), electroblotted and incubated with GFP antibody (Right panels). Uninfected Sf9 (Lane 1 of panel A) and Bm5 (Lane 1 of panel B) cells, or *B. mori* larvae (Lane 1 of panel C) and SDS-PAGE molecular weight standards (Lane M of each panel) are also shown. GFP bands are indicated by solid arrows on the right of each panel.

sing GFP is in Figure 1. Genomic DNA of Ac-Bm hybrid virus expressing β-galactosidase was cotransfected with baculovirus transfer vector pBacPAK-GFP containing GFP gene in Sf9 cells. The supernatant was collected at 5 days p.i., reinfected into a monolayer of Bm5 cells, and Ac-Bm hybrid virus expressing GFP was selected. Because the detection of GFP requires only irradiation by UV [1], Ac-Bm hybrid virus expressing GFP was easily selected from each well of the cell culture dish on UV illuminator. The Ac-Bm hybrid virus expressing GFP was named as Ac-Bm hybrid virus-GFP.

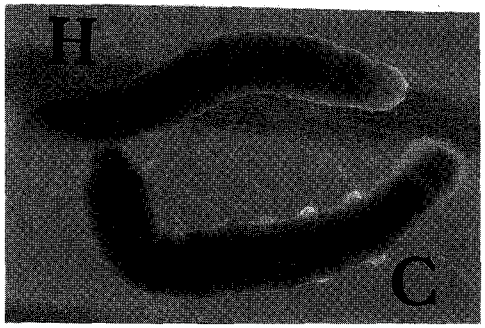

Fig. 3. *B. mori* larva infected with Ac-Bm hybrid virus-GFP. *B. mori* larvae were mock infected (C) or infected with Ac-Bm hybrid virus-GFP (H). Photography of *B. mori* larvae was performed on UV illuminator.

Expression of GFP by Ac-Bm hybrid virus-GFP in Sf9 and Bm5 cells or *B. mori* larvae

To examine the expression of GFP of Ac-Bm hybrid virus-GFP in Sf9 and Bm5 cells or *B. mori* larvae, the cells infected with viruses were initially analyzed by SDS-PAGE and Western blot (Fig. 2). Wild-type baculovirus-infected cells showed a pattern of protein synthesis typical of baculovirus-infected cells. GFP band was not detected in cells infected with wild-type baculoviruses or from mock-infected cells. As expected, the 27 KDa GFP band expressed by Ac-Bm hybrid virus-GFP in Sf9 cells was observed at 2 and 3 days p.i. (Fig. 2A). In addition, although GFP expression level of Ac-Bm hybrid virus-GFP in Bm5 cells was significantly lower than that expressed in Sf9 cells, GFP band was apparently observed in Bm5 cells infected with Ac-Bm hybrid virus-GFP (Fig. 2B). GFP produced by Ac-Bm hybrid virus-GFP in Sf9 and Bm5 cells was confirmed by Western blot analysis using GFP antibody.

In order to confirm the fact that Ac-Bm hy-

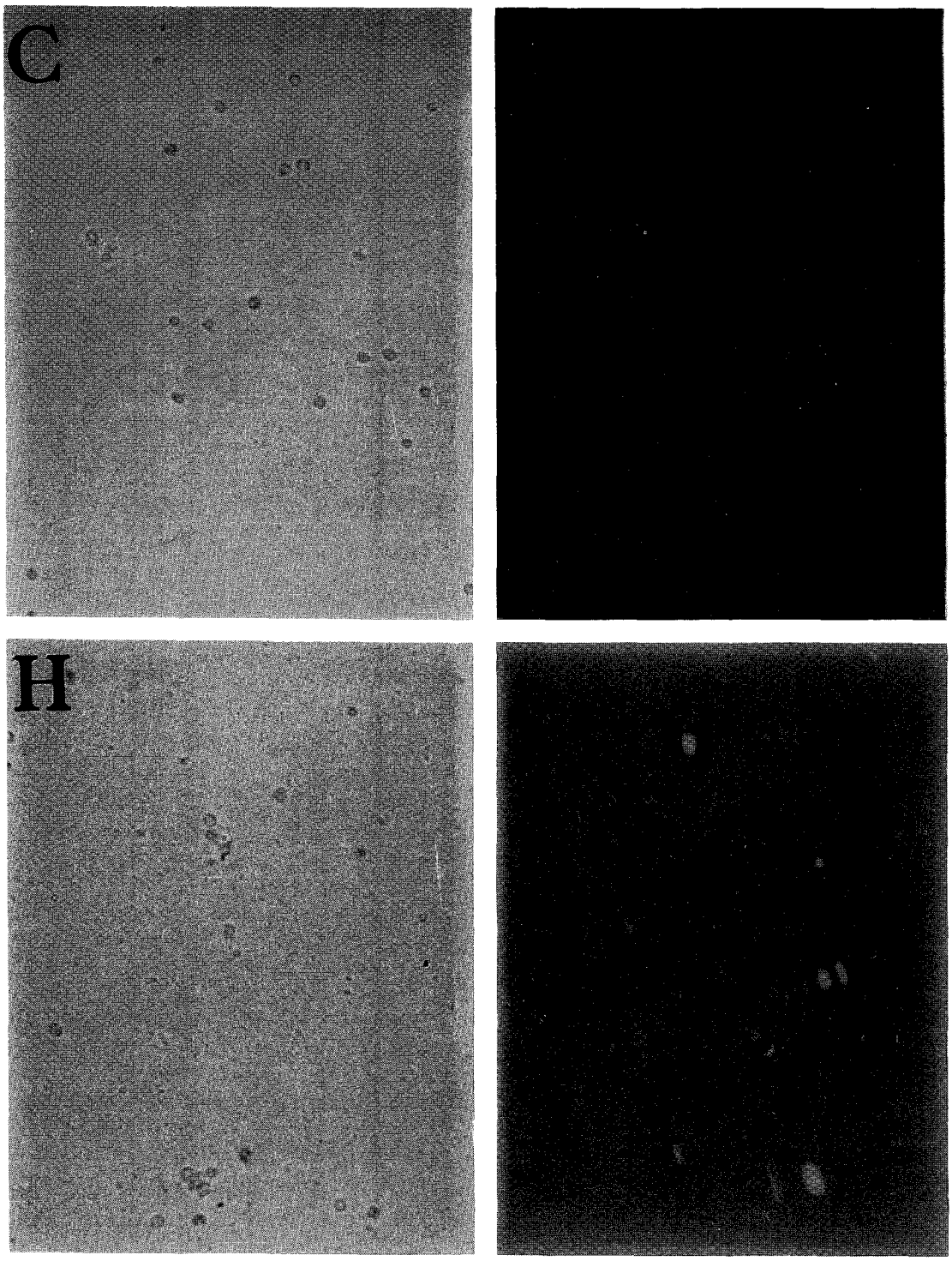

Fig. 4. Hemolymph of *B. mori* larva infected with Ac-Bm hybrid virus-GFP. *B. mori* larvae were mock infected (C) or infected with Ac-Bm hybrid virus-GFP (H). The hemolymph was collected at 5 days p.i. Microscopy of hemolymph of *B. mori* larvae was performed using light (Left panels) and fluorescent (Right panels) microscope (x200).

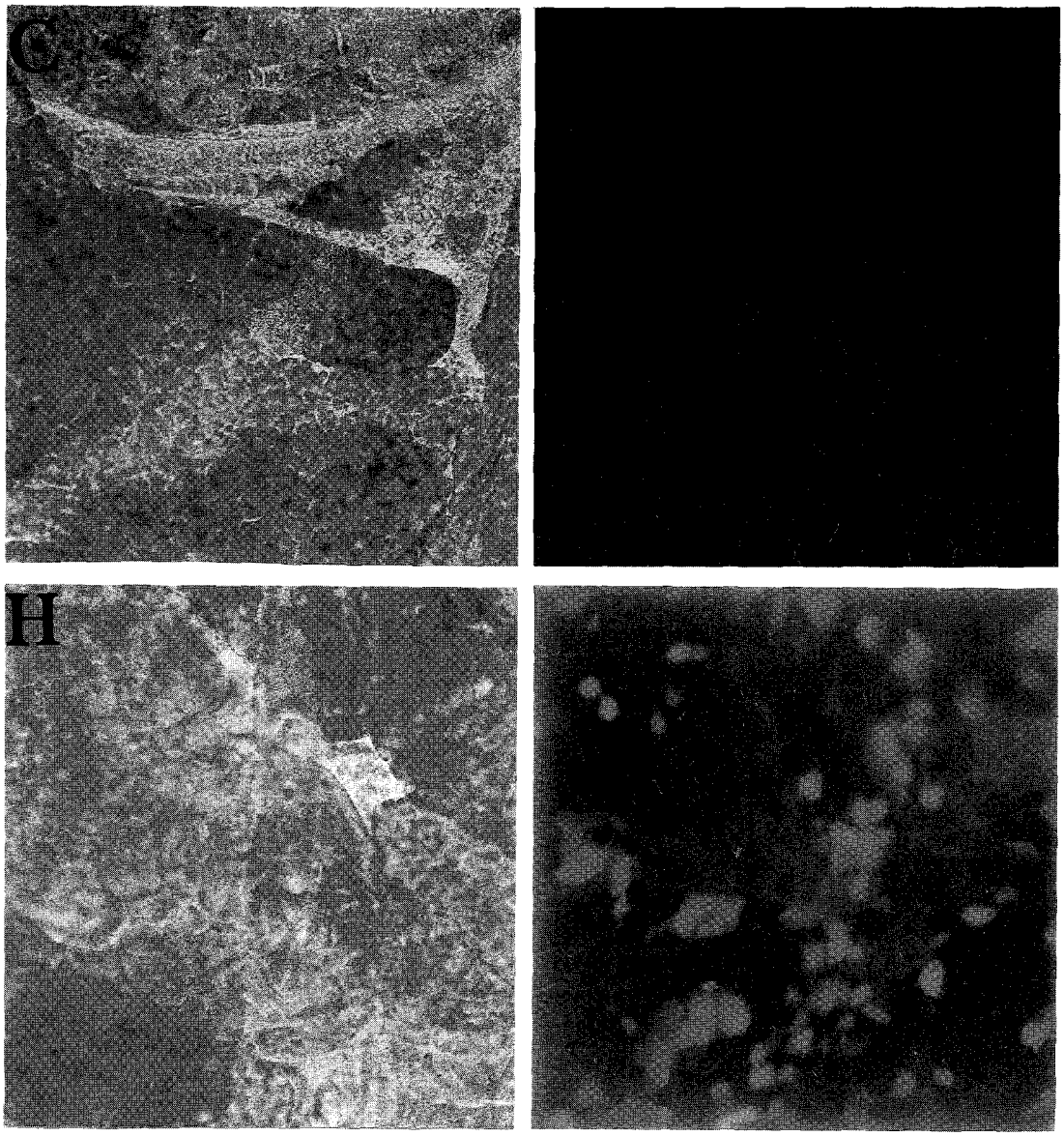

Fig. 5. Fat body of *B. mori* larva infected with Ac-Bm hybrid virus-GFP. *B. mori* larvae were mock infected (C) or infected with Ac-Bm hybrid virus-GFP (H). The fat body was collected at 5 days p.i. Microscopy of fat body of *B. mori* larvae was performed using light (Left panels) and fluorescent (Right panels) microscope (x200).

brid virus can effectively express GFP in silkworm larvae, Ac-Bm hybrid virus-GFP was injected in the fifth instar larval stage of the silkworm. Hemolymph of *B. mori* larvae infected with Ac-Bm hybrid virus-GFP was collected at 3, 4, and 5 days p.i. The 0.5 μl hemolymph collected from the infected larvae was subjected to SDS-PAGE and Western blot analysis (Fig. 2C). The GFP band was clearly detected from hemolymph of *B. mori* larvae at 4 days p.i. The result indicated that Ac-Bm hybrid virus-GFP effectively expressed GFP in silkworm larvae.

In the present study, Ac-Bm hybrid virus-

GFP effectively expressed GFP in Sf9 and Bm5 cells or silkworm larvae by virus replication. The expression of Ac-Bm hybrid virus-GFP was more effective in the silkworm larvae rather than in the Bm5 cells. These results clearly demonstrate that Ac-Bm hybrid virus-GFP has an additional advantage of expanded host range for producing recombinant protein in comparison with the recombinant AcNPV.

Microscopy of hemolymph and fat body of the silkworm larvae infected with Ac-Bm hybrid virus-GFP

When *B. mori* larva early in the fifth instar stage was injected with $5x10^6$ PFU of Ac-Bm hybrid virus-GFP, fluorescence clearly appeared from the whole body at 5 days p.i. on UV illuminator (Fig. 3). To examine the expression of GFP in the tissue of the silkworm larvae infected with Ac-Bm hybrid virus-GFP, *B. mori* larvae were mock infected or infected with Ac-Bm hybrid virus expressing GFP. The hemolymph of *B. mori* larvae collected at 5 days p.i. was observed with a light and a fluorescent microscope (Fig. 4). The result showed that fluorescence of the GFP was clearly detected in the hemocytes of the hemolymph from the infected silkworm larvae. In addition, in the fat body of *B. mori* larvae collected at 5 days p.i., the bright glow of GFP was also clearly observed (Fig. 5). The result revealed that the replication of Ac-Bm hybrid virus-GFP in the tissues of the infected silkworm larvae occurred normally, and GFP was also expressed.

SUMMARY

In conclusion, we furthermore provide evidence that Ac-Bm hybrid virus was effectively expressed foreign genes in Sf9 cells or silkworm larvae and that it may play a major role in the improvement of baculovirus expression vector system.

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