

## Expression of the Peplomer Glycoprotein of Murine Coronavirus JHM Using a Baculovirus Vector

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The gene encoding the E2 peplomer glycoprotein of coronavirus mouse hepatitis virus JHM strain (JHMV) has been inserted into the genome of *Autographa californica* nuclear polyhedrosis baculovirus (AcNPV) in lieu of the coding region of the AcNPV polyhedrin gene. This recombinant virus produced E2 protein in insect cells under the control of the baculovirus polyhedrin promoter. The expressed E2 protein was shown in size and antigenic properties to be similar to the E2 protein produced in mouse cells infected by JHMV. The expressed E2 protein was glycosylated and transported to the cell surface; however, no proteolytic cleavage was detected in insect cells. The sera from rats immunized with partially purified E2 protein derived from insect cells reacted in immunoprecipitation and immunofluorescence experiments with the E2 protein produced in JHMV-infected mouse cells. The antiserum failed to neutralize the infectivity of JHMV. These results suggest that the E2 protein expressed by the recombinant baculovirus in insect cells is similar but not identical to the E2 protein produced in JHMV-infected mouse cells. The inability of the E2 protein expressed in insect cells to produce neutralizing antibody is discussed. © 1989 Academic Press, Inc.

### INTRODUCTION

Coronaviruses are pleomorphic, enveloped RNA viruses. The genome of the murine coronavirus mouse hepatitis virus (MHV) is a single plus (+) strand RNA of approximately 27 kb which encodes at least three structural proteins: a nucleocapsid protein (N), a matrix glycoprotein (E1), and a peplomeric glycoprotein (E2), as well as several nonstructural proteins (Siddell *et al.*, 1983; Sturman and Holmes, 1983; Spaan *et al.*, 1988).

The E2 protein forms the projecting spikes or peplomers on the surface of the virus particle (Tyrrell *et al.*, 1967; Sturman, 1977). This protein is involved in the attachment of virus to susceptible cells, the induction of cell-to-cell fusion, and the production of neutralizing antibodies (Holmes *et al.*, 1981a; Collins *et al.*, 1982; Fleming *et al.*, 1983; Wege *et al.*, 1984). Moreover, the E2 protein is important for determining the pathogenic potential of murine coronaviruses (Wege *et al.*, 1982, 1988; Buchmeier *et al.*, 1984; Dalziel *et al.*, 1986; Fleming *et al.*, 1986).

The sequence of the cDNA encoding the JHMV E2 protein predicts a polypeptide of 1235 amino acids with a mol wt of 136,600 (Schmidt *et al.*, 1987). In JHMV-infected mouse cells the E2 protein is synthesized as a 150,000 mol wt cotranslationally glycosylated polypeptide and during transport within the cell,

oligosaccharides are trimmed and terminal sugars are added, resulting in a N-glycosylated polypeptide with a mol wt of 170,000 (Holmes *et al.*, 1981b; Siddell *et al.*, 1981; Niemann *et al.*, 1982). Shortly before or at the time of virus release, a proportion of E2 is cleaved into two polypeptides. The cleavage of the E2 protein is a host cell-dependent event and thought to be a prerequisite for the fusogenic activity of the protein (Niemann *et al.*, 1982; Frana *et al.*, 1985; Sturman *et al.*, 1985).

In order to synthesize enough E2 glycoprotein for structural and functional analyses we have sought to express the E2 gene product in a baculovirus expression vector. The *Autographa californica* nuclear polyhedrosis virus (AcNPV) insect baculovirus vector was chosen because of the high levels of foreign gene expression using the AcNPV polyhedrin promoter (Luckow and Summers, 1988). Also, post-translational modification such as glycosylation, phosphorylation, and cleavage of the signal peptide are known to occur in this system. In this paper, we describe the expression of the JHMV E2 protein by recombinant baculovirus in insect cells and characterization of the polypeptide.

### MATERIALS AND METHODS

#### Viruses and cells

AcNPV and recombinant baculovirus were grown and assayed in confluent monolayers of *Spodoptera*

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*frugiperda* (Sf) cells cultured in TC100 medium containing 10% fetal bovine serum according to the procedures described by Brown and Faulkner (1977). The JHM-SP4 strain (Taguchi and Fleming, 1989) of mouse hepatitis virus (JHMV) was grown in DBT mouse cells.

#### Insertion of JHMV-E2 DNA into the transfer vector

JHMV-E2 cDNA, which contains no internal *Sma*I or *Hind*III sites, was recovered from the pBluescript<sup>+</sup> (SK+) phagemid (Pfleiderer, 1989) by *Sma*I and *Hind*III digestion. After repair with Klenow enzyme, *Bam*HI linkers were added and the DNA was ligated into the *Bam*HI site of the pAcYM1 transfer vector (Matsuura *et al.*, 1987). After transformation of *Escherichia coli* MC1061 cells, recombinant plasmids were recovered and characterized by restriction enzymes and sequence analysis (Sanger *et al.*, 1977).

#### Cotransfection and selection of recombinant viruses

Sf cells were transfected with mixtures of purified AcNPV DNA and plasmid DNA representing the recombinant transfer vector according to the procedures described previously (Matsuura *et al.*, 1986). After incubation at 28° for 4 days, the supernatant fluids were harvested and used to produce plaques in Sf cell monolayers. Plaque-containing recombinant virus were identified by their lack of polyhedra when examined by transmissible light microscopy. Virus from such plaques was recovered and following three rounds of plaque purification, high-titered ( $10^7$ – $10^8$  PFU/ml) stocks of the recombinant virus were obtained.

#### Immunoprecipitation analyses

Sf cells were infected with recombinant baculovirus at a multiplicity of 5 PFU/cell in 35-mm tissue culture dishes and labeled with 20  $\mu$ Ci of [<sup>3</sup>H]leucine (New England Nuclear, 140.5 Ci/mmol) by the methods described previously (Matsuura *et al.*, 1986). The labeled cells were lysed in 250  $\mu$ l of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris-HCl, 0.01 M EDTA, 0.1% SDS, pH 7.4) and aliquots of 50  $\mu$ l were immunoprecipitated with 3–5  $\mu$ l of anti-JHMV serum using protein A-Sepharose (Pharmacia). In the case of rat sera, protein G-Sepharose (Pharmacia) was used to purify the immunoglobulin component. The immune complexes were disrupted in dissociation buffer (2% SDS, 10% glycerol, 0.1 M dithiothreitol, 60 mM Tris-HCl, 0.01% bromphenol blue, pH 6.8) at 100° for 5 min and analyzed by electrophoresis on 10% SDS-polyacrylamide gels (SDS-PAGE) (Laemmli, 1970). After electrophoresis, the gels were impregnated with 1 M sodium salicylate, dried, and

autoradiographed at –70°. Tunicamycin treatments of Sf cells infected with the recombinant baculovirus and DBT cells infected with JHMV were performed using procedures described by Matsuura *et al.* (1987) and by Taguchi *et al.* (1985), respectively.

#### Immunofluorescence

Sf cells infected with recombinant baculovirus at a multiplicity of 5 PFU/cell were washed three times in PBS and fixed in acetone at 4° for 10 min. The cells were incubated with anti-JHMV serum diluted 1:100 in PBS at 37° for 30 min. The cells were stained with FITC-conjugated goat anti-mouse immunoglobulin (Cappel) for 30 min at 37° and examined under uv illumination. Cells were also incubated with antibodies without prior fixation. In this case, all procedures were performed at 4° to avoid cap formation and pinocytosis of immune complexes.

#### Enzyme-linked immunosorbent assay (ELISA)

Approximately  $10^7$  Sf cells infected with recombinant virus were disrupted by 0.5% Triton X-100 in PBS. After centrifugation, the supernatants were treated with Bio-beads SM-2 (Bio-Rad) to remove the detergent. The cell extracts were diluted with carbonate buffer (20 mM Na<sub>2</sub>CO<sub>3</sub>, 30 mM NaHCO<sub>3</sub>, 250 mM NaCl, pH 9.6) and adsorbed overnight at 4° to flat-bottomed polystyrene ELISA plates (Falcon). After being blocked with 1% BSA in PBS, anti-JHMV serum diluted to 1:100 was added. Alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was added followed by incubation at room temperature for 1 hr and the secondary antibodies were detected with *p*-nitrophenyl phosphate disodium (Sigma) (1 mg/ml in 0.1 M glycine buffer, pH 10.4 containing 1 mM MgCl<sub>2</sub>). Alternatively, horseradish peroxidase-conjugated anti-mouse IgG (Bio-Yeda) together with the substrate, 0.005% H<sub>2</sub>O<sub>2</sub> in 1 mg/ml of 5-amino salicylic acid solution, pH 7.4, were used.

#### Partial purification of the expressed E2 protein

Approximately 3 to 5  $\times 10^7$  Sf cells infected with the recombinant virus at a multiplicity of 5 PFU/cell were collected, washed with PBS three times, and disrupted with 1% Triton X-100 in TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8.0). After centrifugation, the supernatant was loaded on a 10 to 50% (w/w) continuous sucrose gradient containing 0.1% Triton X-100 above a cushion of 65% sucrose. The gradients were centrifuged at 38,000 rpm for 20 hr at 4° in a Hitachi RPS40T rotor. Fractions (0.5 ml) were collected and treated with Bio-beads SM-2 to remove the detergent. After dilution with carbonate buffer, each fraction

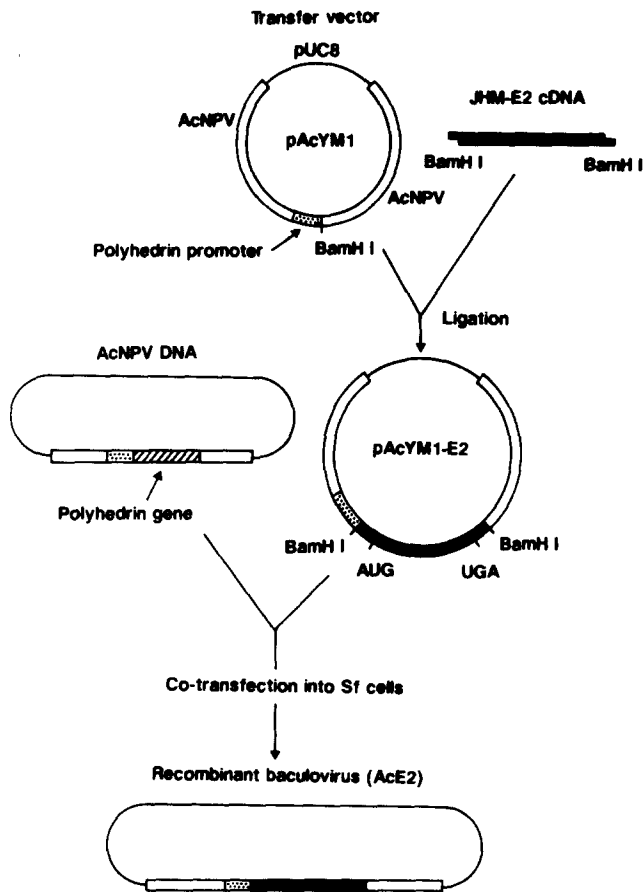


FIG. 1. Strategy to produce the recombinant baculovirus transfer vector containing the E2 gene of JHMV (pAcYM1-E2) and the recombinant baculovirus (AcE2). Details of the preparations of the recombinant transfer vector and the recombinant virus are given under Materials and Methods.

was adsorbed to microtiter wells overnight at 4° and ELISA using anti-JHMV serum was performed to identify positive fractions. The pooled samples were dialyzed to remove sucrose and concentrated by freeze-drying. Protein concentrations were assayed according to Lowry's method (Lowry *et al.*, 1951).

#### Production of antibodies to the expressed E2 protein

Wistar rats (4 weeks old) and BALB/c and C57BL mice (4 to 5 weeks old), serologically proven to be free from MHV infection, were purchased from a commercial breeder and used for immunization of the E2 protein expressed in insect cells. Animals received one intramuscular injection of partially purified E2 protein in Freund's complete adjuvant (Day 0) followed by one intraperitoneal injection (Day 70). Intraperitoneal injections of the intact insect cells carrying expressed E2

protein or the cell extracts were also performed and repeated two or three times. The animals were bled one week after the last injection, and the serum sample was tested for the presence of antibodies by immunofluorescence and immunoprecipitation using DBT cells infected with JHMV. Radiolabeled JHMV polypeptides were obtained as previously described (Taguchi *et al.*, 1985). Neutralization tests (50% plaque reduction) were performed as previously reported (Taguchi *et al.*, 1980).

## RESULTS

### Isolation of a recombinant baculovirus containing the JHMV E2 gene

The strategy for the construction of the baculovirus transfer vector containing the E2 gene of JHMV is shown in Fig. 1. The cDNA was inserted into the *Bam*HI site of the transfer vector pAcYM1 (pAcYM1-E2). The orientation and sequence of the E2 gene relative to the AcNPV polyhedrin leader was determined by restriction enzymes and DNA sequence analyses. The AUG codon of the E2 gene was determined to be 75 bases downstream of the linker region.

In order to obtain recombinant baculovirus containing the E2 gene, pAcYM1-E2 DNA (10–50 µg) and infectious AcNPV DNA (1 µg) were cotransfected into Sf cells (Fig. 1). Approximately 100 plaques produced by the progeny viruses from the transfection were screened and four clones of polyhedrin-negative recombinant progenies were obtained. Three of these clones were revealed to produce E2 protein by immunofluorescence and SDS-PAGE.

### Expression of the E2 protein by recombinant baculovirus in insect cells and characterization of the polypeptide

In order to analyze the time course of synthesis of E2 protein by the recombinant baculovirus, ELISA was undertaken. Extracts of Sf cells infected with the recombinant baculovirus 1 to 4 days before were adsorbed to microplates, and then the amount of expressed E2 protein was determined by anti-JHMV serum. As a control, wild-type AcNPV-infected cell extracts were employed. The results showed that immunoreactive E2 protein could be detected as early as 2 days postinfection and that the amount of E2 protein was maximal at 3 days postinfection (Fig. 2). Radiolabeled E2 protein expressed in insect cells was initially detected at 24 hr postinfection, and its synthesis was still detectable at 72 hr postinfection (data not shown).

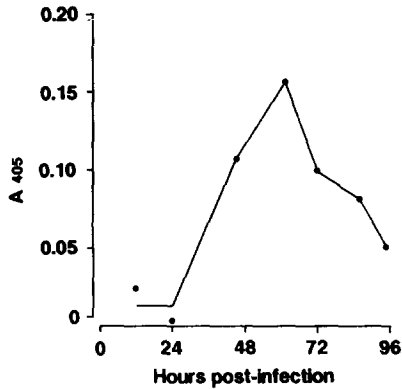


Fig. 2. Time course of production of the E2 protein in insect cells by the recombinant baculovirus. Sf cells infected with recombinant baculovirus were disrupted by 0.5% Triton X-100 in PBS at 12 to 96 hr postinfection and the lysates were adsorbed to microtiter plates. ELISA using anti-JHMV serum was done by the procedure described under Materials and Methods. Background OD values from Sf cells infected with wild-type baculovirus were subtracted.

The size of the protein expressed by recombinant baculovirus was determined in a pulse-label experiment using [ $^3$ H]leucine (Fig. 3a). This experiment showed that the recombinant virus synthesized a protein that migrated with an estimated mol wt of 150,000, similar to that of the cotranslationally glycosylated JHMV E2 protein synthesized in infected DBT cells. A protein of this molecular weight was not observed in cells infected with wild-type AcNPV or mock-infected Sf cells.

The 150,000 mol wt protein was precipitated from cells infected with the recombinant virus by anti-JHMV antibodies (Fig. 3b, AcE2). As expected, the anti-JHMV serum also contains a component which reacts strongly with the 60,000 mol wt nucleocapsid protein synthesized in JHMV-infected cells (Fig. 3b, JHMV). In wild-type AcNPV-infected Sf cells a strong band with a mol wt of 33,000 was detected, which has been shown to be nonspecifically precipitated polyhedrin protein resulting from incomplete solubilization of the occlusions in the immunoprecipitation buffer (Miyamoto *et al.*, 1985) (Fig. 3b, AcNPV).

In order to determine whether carbohydrate chains were attached to E2 protein produced in insect cells, Sf cells infected with recombinant virus were treated with 10  $\mu$ g/ml of tunicamycin and immunoprecipitated with anti-JHMV serum. As shown in Fig. 4, the molecular weight of the E2 protein expressed in the presence of tunicamycin was reduced to approximately 130,000 similar to that of the unglycosylated E2 protein in JHMV-infected DBT cells. These results indicated that glycosylated E2 protein was expressed during the recombinant virus infection.

To assess proteolytic cleavage of the E2 protein expressed in insect cells, Sf cells infected with the recombinant virus as well as DBT cells infected with JHMV were pulse-chase labeled and cell lysates were immunoprecipitated with anti-JHMV serum. Although the

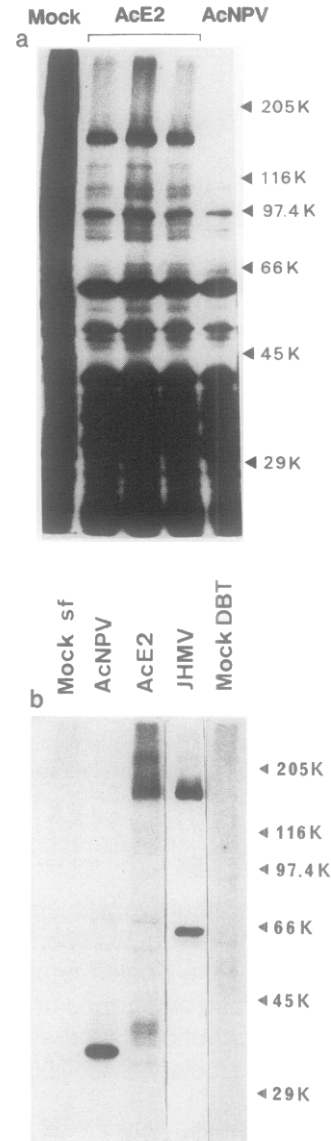


Fig. 3. Expression of the E2 protein in insect cells by the recombinant baculovirus. (a) The proteins synthesized in Sf cells infected with three different viral clones of recombinant baculovirus (AcE2), Sf cells infected with wild-type baculovirus (AcNPV), or mock-infected Sf cells (Mock) were labeled 1 hr with [ $^3$ H]leucine at 24 hr postinfection. Cell lysates were analyzed by 10% SDS-PAGE. (b) Immunoprecipitation with anti-JHMV serum of [ $^3$ H]leucine-labeled proteins from Sf cells infected with recombinant baculovirus (AcE2), Sf cells infected with wild-type baculovirus (AcNPV), mock-infected Sf cells (Mock Sf), JHMV-infected DBT cells (JHMV), and mock-infected DBT cells (Mock DBT). Immune precipitates were analyzed by 10% SDS-PAGE as described under Materials and Methods.

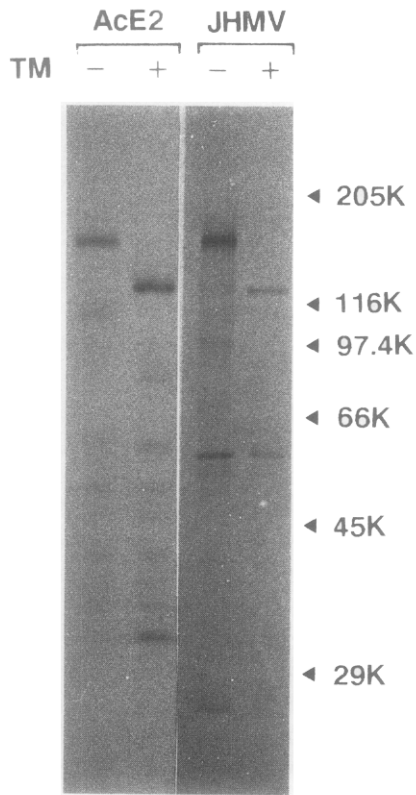


Fig. 4. Effects of tunicamycin on the expression of the E2 protein in insect cells by the recombinant baculovirus. Sf cells infected with recombinant baculovirus (AcE2) were treated with 10  $\mu$ g/ml of tunicamycin (TM) as described under Materials and Methods. The cells were disrupted and precipitated with anti-JHMV serum. As control, JHMV-infected DBT cells (JHMV) were treated with 4  $\mu$ g/ml of tunicamycin and similarly immunoprecipitated. Immune precipitates were analyzed by 10% SDS-PAGE.

90,000 mol wt cleavage product of the E2 protein was detected in DBT cells infected by JHMV within 30 min (Fig. 5b), no cleavage products were detected in the insect cells during 4-hr chase (Fig. 5a). Sf cells carrying expressed E2 protein were treated with various concentrations of trypsin; however, no cleavage products were obtained (data not shown).

To study the localization of the E2 protein expressed in insect cells, immunofluorescence was performed. Sf cells were infected with the recombinant virus and 3 days later, cells were fixed with acetone. The fixed cells were incubated with anti-JHMV antibodies and stained with FITC-conjugated anti-mouse IgG. As shown in Fig. 6a, the E2 protein expressed in insect cells could be localized on the cell surface. No JHMV-related antigen was detected within the AcNPV-infected cells (Fig. 6b) or mock-infected cells (Fig. 6c). The surface location of the E2 protein found in cells infected with the recombinant virus was confirmed by immunofluorescence us-

ing unfixed cells (data not shown). Further, immunofluorescence analyses showed that five monoclonal antibodies which recognize different epitopes on the E2 protein of cl-2 variant of JHMV (Taguchi *et al.*, 1985) all reacted with the expressed E2 protein (data not shown).

**Production and characterization of antisera to expressed E2 protein in rats**

To assess the ability of the E2 protein produced by the recombinant baculovirus to induce antibodies, rats

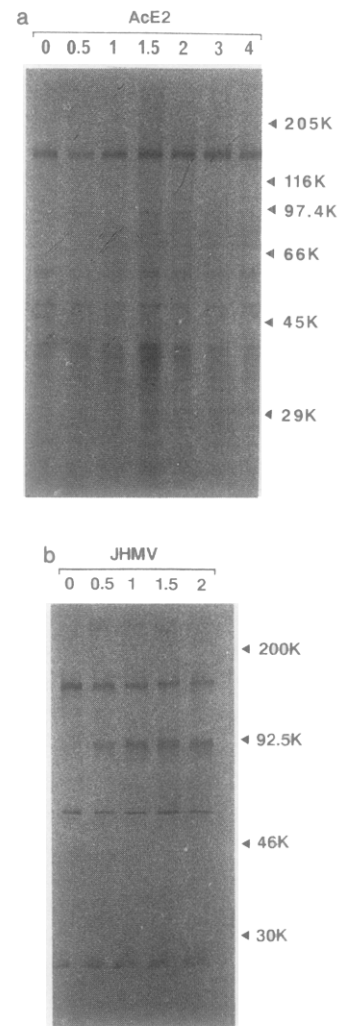


Fig. 5. Pulse-chase label of the E2 protein expressed in insect cells by the recombinant baculovirus. (a) Sf cells infected with recombinant baculovirus (AcE2) were labeled with [<sup>3</sup>H]leucine as described under Materials and Methods, and chases (0, 0.5, 1, 1.5, 2, 3, and 4 hr) were done after removing [<sup>3</sup>H]leucine from the medium. (b) As control, JHMV-infected DBT cells were labeled with [<sup>35</sup>S]methionine for 30 min and chases (0, 0.5, 1, 1.5, and 2 hr) were done (JHMV). Lysates of the cells were immunoprecipitated with anti-JHMV serum and analyzed by 10% SDS-PAGE.

were immunized with sucrose gradient purified material. Lysate of Sf cells infected with recombinant baculovirus was loaded onto a 10 to 50% (w/w) sucrose gradient and centrifuged as described under Materials and Methods. A single broad peak of ELISA-reactive E2 protein in the gradient was pooled, which finally showed the ELISA titer 1:625 and 3 mg/ml of protein concentration. Sera collected from the animals immunized with this material were serologically tested. As shown in Fig. 7, the rats immunized with partially purified E2 protein produced antibodies which reacted by immunoprecipitation with the E2 protein synthesized in DBT cells infected with JHMV. In immunofluorescence as well, DBT cells infected with JHMV were stained by the antisera (data not shown). However, in the plaque reduction assay the antisera failed to neutralize the infectivity of JHMV (neutralization titer < 1:5).

Mice immunized with E2 protein expressed by the recombinant baculovirus in insect cells did not produce detectable level of antibodies against E2 protein in immunofluorescence, immunoprecipitation, and viral neutralization experiments.

## DISCUSSION

The E2 protein of MHV plays a central role in the attachment of the virus to susceptible cells, the induction of cell-to-cell fusion, the production of neutralizing antibodies, and the determination of the pathogenic potential of the virus (Collins *et al.*, 1982; Wege *et al.*, 1984, 1988). In order to further study these biological functions, it is necessary to purify the E2 protein free of other MHV proteins. For this purpose, we have chosen the pAcYM1 baculovirus expression system, which allows high levels of foreign gene expression.

The E2 protein expressed by recombinant baculovirus in insect cells was shown in size and antigenicity to correspond to the cotranslationally glycosylated E2 protein produced in mouse cells infected with JHMV. Immunofluorescence analysis indicates that the expressed E2 protein is located on the cell surface similar to in virus-infected cells. No protein bands corresponding to unglycosylated E2 protein (mol wt 136,000) or intermediate species representative of partial glycosylation were detected.

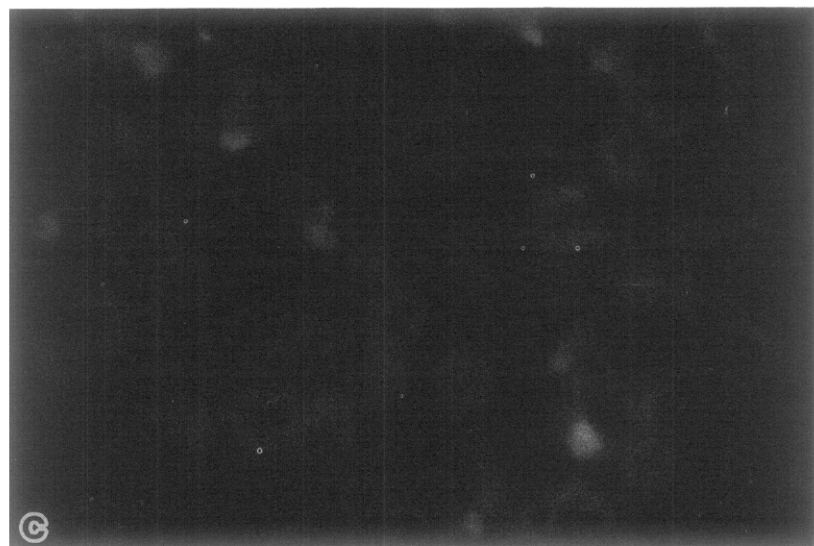
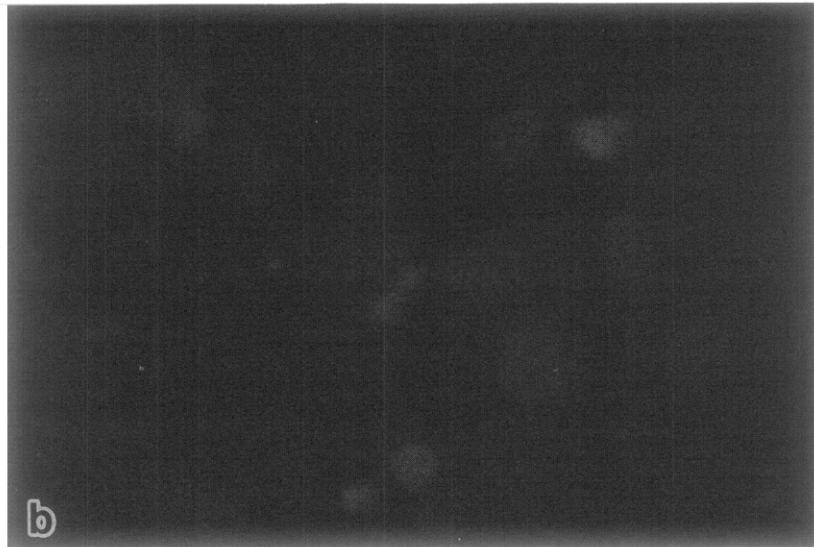
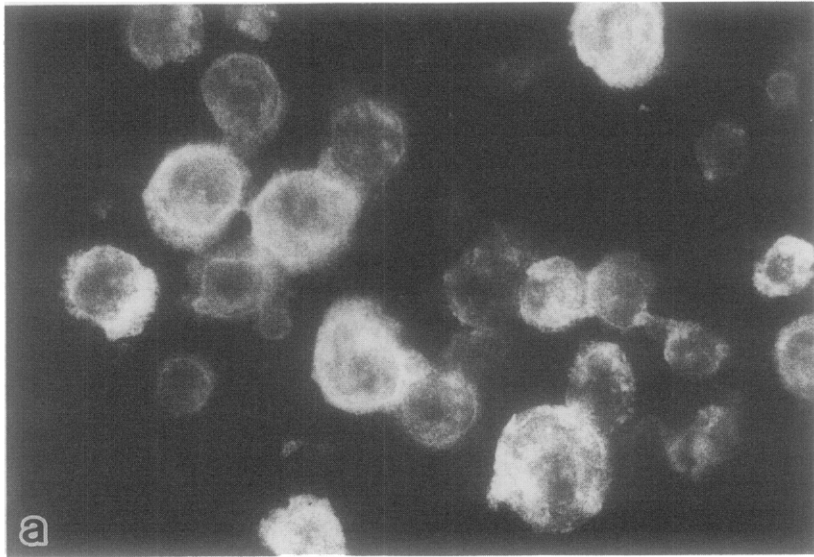
In contrast to these cotranslational modifications, we have evidence that the post-translational processing of the E2 protein expressed in insect cells differs

from that in JHMV-infected mouse cells. For example, proteolytic cleavage, which is believed to be a prerequisite for the fusogenic activity of the JHMV E2 protein (Sturman *et al.*, 1985), does not occur. Also, further studies will be necessary to determine if the complex post-translational processing of the JHMV E2 carbohydrate components is identical in insect and mouse cells.

Rats immunized with the E2 protein expressed in insect cells produced antibodies against the E2 protein. In previous reports, antibodies raised against viral surface glycoproteins expressed in the baculovirus system have been shown to neutralize infectivity of the virus (Kuroda *et al.*, 1986; van Wyke Coelingh *et al.*, 1987; Ray *et al.*, 1989). Unexpectedly, the antibodies which we obtained failed to neutralize the infectivity of JHMV, although these antibodies bound to the E2 protein produced in mouse cells. There are several possible explanations. First, as suggest above, there might be differences in the carbohydrate structure between the E2 protein expressed in insect cells and that produced in mouse cells. Such differences may influence epitopes involved in eliciting neutralizing antibodies. Second, the uncleaved form of the E2 glycoprotein might not be able to induce neutralizing antibodies in animals. Third, it is possible that the E2 cDNA clone used (or the RNA molecule from which it was produced) has acquired mutations critical to the epitopes responsible for eliciting neutralizing antibodies. We consider this possibility unlikely, because of five different monoclonal antibodies directed against the E2 protein, three of which are neutralizing antibodies, all reacted well with the E2 protein expressed in insect cells. In our view, many of these questions can be further investigated by expressing the E2 glycoprotein in mouse cells using the vaccinia virus system.

In summary, we have demonstrated the high-level expression of the JHMV E2 protein in insect cells infected with a recombinant baculovirus, although the expressed E2 protein was proven to be antigenically somehow different from authentic E2 protein produced in mouse cells as a result of JHMV infection. To explain such differences, we need to investigate the difference of glycosylation of E2 protein in insect cells and mouse cells. Experiments are now in progress to study in detail glycosylation modification and the antigenic structure of E2 protein synthesized in insect cells.

Fig. 6. Immunofluorescence by anti-JHMV serum of insect cells infected with recombinant or wild-type baculovirus. Infected Sf cells were treated with anti-JHMV serum and stained with FITC-conjugated anti-mouse immunoglobulin for fluorescence microscopy as described under Materials and Methods. (a) Cells infected with the recombinant AcE2. (b) Cells infected with wild-type AcNPV. (c) Uninfected cells.



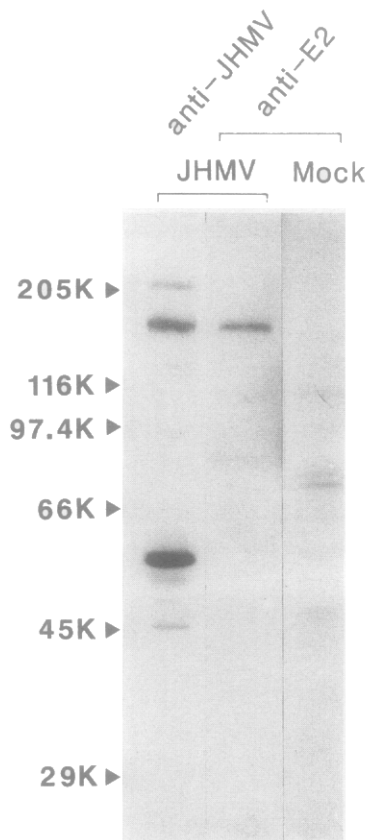


FIG. 7. Detection of anti-E2 antibodies in sera of rats immunized with the E2 protein expressed in insect by the recombinant baculovirus. JHMV-infected (JHMV) and mock-infected DBT cells (Mock) were labeled with [ $^3$ H]leucine and the extracts were precipitated with the serum of immunized rat (anti-E2) as described under Materials and Methods. As control, the extract of DBT cells infected with JHMV was precipitated with anti-JHMV serum (anti-JHMV). Immune precipitates were analyzed by 10% SDS-PAGE.

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