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Expression, Processing, and Assembly of Foot-and-Mouth Disease Virus Capsid Structures in Heterologous Systems: Induction of a Neutralizing Antibody Response in Guinea Pigs†

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Plasmids containing the foot-and-mouth disease virus structural protein precursor (P1) and 3C protease genes or the P1 gene alone were expressed in *Escherichia coli*. A recombinant baculovirus containing the P1 gene was also generated and expressed in *Spodoptera frugiperda* cells. Expression of the P1 and 3C genes in *E. coli* resulted in efficient synthesis and processing of the structural protein precursor and assembly into 70S empty capsids. This material reacted with neutralizing monoclonal antibodies which recognize only conformational epitopes and elicited a significant neutralizing antibody response in vaccinated guinea pigs. Expression of the P1 gene in *E. coli* resulted in synthesis of an insoluble product, whereas in insect cells infected with the recombinant baculovirus a soluble product was synthesized. Both soluble and insoluble P1 reacted with a 12S-specific monoclonal antibody, but only soluble P1 elicited a neutralizing antibody response in guinea pigs.

Foot-and-mouth disease (FMD) is a major pathogen of ruminants in many parts of the world. The disease is caused by members of the *Aphthovirus* genus of the *Picornaviridae* family. An inactivated virus vaccine is used in disease control programs, and it has been estimated that worldwide more than a billion doses of the vaccine are used annually (3). Concern about the safety of the vaccine exists, as outbreaks of FMD in Europe have been associated with shortcomings in vaccine manufacture (25). These concerns have led to efforts to develop subunit vaccines as safer alternatives. One of the virus capsid proteins, VP1, either purified from virus particles or produced by recombinant DNA technology, has been successfully used to protect cattle and swine from challenge (4, 26). Likewise, peptides either produced by recombinant DNA technology or chemically synthesized and representing limited regions of VP1 have also been successfully used to protect cattle and swine against challenge with some serotypes of FMD virus (FMDV) (8, 15, 30). However, relatively large amounts of these immunogens were required to produce protection, and often induction of a neutralizing antibody response with these immunogens, as measured by in vitro assays, did not correlate with protection of the host animal (7).

FMDV contains 60 copies each of four proteins (VP1 to VP4) that form an icosahedral particle encapsidating a positive-sense, single-stranded RNA genome of approximately 8,500 nucleotides (3). Examinations of virus structure by monoclonal antibody (MAb) analysis (5, 9, 42, 46), nucleic acid sequencing of neutralization-resistant mutants (6, 14, 44–47), and X-ray crystallographic analysis have revealed that the major immunogenic sites on FMDV (1), as well as on other picornaviruses (22, 38), involve conformational epitopes.

Rweyemamu et al. (41) have shown that FMDV type A empty capsids (subviral particles lacking nucleic acid) are as immunogenic as virions in guinea pigs and that antisera raised against empty capsids have the same serological specificity in neutralization tests as do sera prepared against virions. By MAb analysis, we have demonstrated that empty virus capsids isolated from infected cells antigenically resemble virus particles (21). Furthermore, we and others have shown that, in a cell-free translation system programmed with FMDV RNA or subgenomic RNA transcripts, viral structural proteins self-assemble into various capsid intermediates (12, 18, 21). On the basis of these results, a goal of ours has been to utilize recombinant DNA technology to construct FMDV clones that can express, in an appropriate host, conformationally correct immunogens that lack nucleic acid and could make effective and safe vaccines.

Previously, we described the construction of recombinant cDNA clones representing the complete FMDV coding region (34, 53). By utilizing these constructs in this study, we produced additional clones containing either the structural protein precursor (P1) gene or the P1 gene and the viral 3C gene, which encodes the protease responsible for most of the cleavages within the P1 protein. These clones, under control of a bacteriophage T7 promoter, were expressed in an *Escherichia coli* system. The construct containing only the P1 gene was also used to produce an infectious baculovirus recombinant. In this communication, the synthesis and processing of these gene products are examined. Furthermore, the ability of the gene products to assemble correctly and their immunogenicity in guinea pigs are evaluated.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, the large fragment of DNA polymerase I (Klenow fragment), and calf intestinal alkaline phosphatase were purchased from New England Biolabs, Inc., and Boehringer Mannheim Biochemicals. *E. coli* BL21(DE3) (48) was kindly provided by J. Dunn, Brookhaven National

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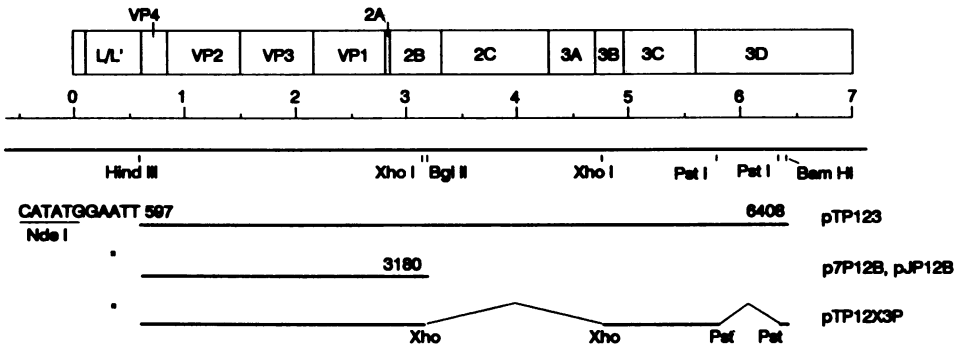

FIG. 1. Schematic representation of FMDV expression plasmids. A map of the coding region of the FMDV genome, using the picornavirus nomenclature system (40), is given at the top, and selected restriction sites used to construct the plasmids are shown. The labeled pTP and p7P plasmids contain a bacteriophage T7 promoter and a Shine-Dalgarno sequence upstream of the FMDV coding region, while plasmid pJP contains the FMDV sequence in baculovirus transfer vector pJV(NheI). pTP123 contains the P1, P2, and P3 coding regions up to a *Bam*HI site at base 6408 in the 3D gene. p7P12B and pJP12B contain the P1 and P2 regions up to a *Bgl*II site at base 3180 in the 2B gene. pTP12X3P contains the P1 and P2 regions up to an *Xho*I site at base 3142 in the 2B gene and the P3 region from an *Xho*I site at base 4753 in the 3B gene to a *Pst*I site at base 5790 in the 3D gene. Thus, pTP12X3P contains the complete 3C protease gene. The bridged areas on pTP12X3P represent deletions of the FMDV coding region, as described in the text.

Laboratories, and expression vector pT7-7 was a gift of M. Esteban, State University of New York Health Science Center at Brooklyn. Cells were grown in 2× YT (2) or M9 medium (2), each containing 100 μg of ampicillin per ml. *Autographa californica* nuclear polyhedrosis virus was provided by M. Summers, Texas A&M University, College Station; pJV(NheI) was the gift of C. Richardson, McGill University; and *Spodoptera frugiperda* (Sf9) cells were obtained from the American Type Culture Collection.

Construction of plasmids. Recombinant plasmids were constructed by standard cloning procedures (2) and used to transform HB101 or XL1-Blue cells. Relevant constructs are diagrammed in Fig. 1. Plasmids were constructed from pTP123 (53), which contains FMDV sequences from bases 592 to 6408 (VP0 to the middle of 3D) in T7 expression vector pET-3c (48). pTP1A was made by digestion of pTP123 with *Bam*HI and *Bgl*II and ligation. p7P12B was constructed by digestion of pTP123 with *Nde*I and *Eco*RV, gel isolation of the FMDV fragment (bases 592 to 3180), and ligation of this DNA into expression vector pT7-7 (50), which had been digested with *Nde*I and *Sma*I and treated with calf intestinal alkaline phosphatase. pTP12X3P was made by digestion of pTP123 with *Xho*I, ligation, digestion with *Pst*I, and ligation. Both constructs p7P12B and pTP12X3P contain three codons upstream of the amino terminus of VP4 and a methionine codon for initiation of protein synthesis. p7P12B contains 21 codons of pET-3c downstream of the coding region of FMDV before an in-frame stop codon, and pTP12X3P contains three codons downstream of the annealed *Pst*I site. Plasmid DNA was recovered from transformed HB101 cells and checked by restriction enzyme analysis and nucleic acid sequencing of the 5′ junction by the dideoxy-chain termination method (43).

Construction of recombinant baculovirus. To construct transfer plasmid pJP12B, FMDV sequences were removed from pTP1A by digestion with *Nde*I and *Eco*RV, blunt ended with the Klenow fragment, gel isolated, and ligated into β-galactosidase gene-containing vector pJV(NheI) (54), which had been digested with *Nhe*I, blunt ended with the Klenow fragment, and treated with calf intestinal alkaline phosphatase. Recombinant virus JP12B was made by cotransfecting pJP12B and *A. californica* nuclear polyhedrosis virus DNAs into Sf9 cells and plaque purifying the recombinant virus as previously described (49).

Induction in *E. coli*. BL21(DE3) cells containing plasmid pTP12X3P or p7P12B were grown in 2× YT to an A_{600} of approximately 0.6. At this time, expression was induced by addition of 125 μg of isopropyl-β-D-thiogalactopyranoside (IPTG) per ml. Cells were harvested prior to and at various times after induction. Cells were pelleted, washed twice with phosphate-buffered saline (PBS), suspended in sample preparation buffer (0.63 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.68 M 2-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue), boiled for 3 min, and pelleted. The supernatant (total) was examined by SDS-polyacrylamide gel electrophoresis (PAGE) (28), and the gels were analyzed by silver staining (55). To radiolabel proteins, cells were grown in M9 medium and at an A_{600} of 0.6 they were induced with IPTG as described above. [^{35}S]methionine (50 to 100 μCi/ml) was added to 0.5 ml of cells prior to and at various times after induction, and labeling was continued for 30 min. To inhibit *E. coli* protein synthesis, rifampin, at 200 μg/ml, was added 30 min after induction and the radioisotope was added 30 min later. Cells were pelleted and lysed in sample preparation buffer as described above. Alternatively, cells were pelleted, washed twice in PBS, suspended in 100 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (TE) plus 4% glycerol, incubated with 100 μg of lysozyme per ml at 37°C for 15 min, and frozen at −70°C. The cell lysate was thawed, adjusted to 10 mM $MgSO_4$, treated with 25 μg of DNase per ml for 15 min at 37°C, and pelleted by centrifugation in an Eppendorf microcentrifuge for 5 min (37). The pellet was suspended in 100 μl of TE–4% glycerol, and both the pellet and supernatant fractions were analyzed by PAGE or sucrose gradient centrifugation.

Protein synthesis in Sf9 cells. Monolayers of Sf9 cells were infected at 0.1 PFU per cell with recombinant virus JP12B. Cells were harvested after 5 days (unless otherwise noted), pelleted, suspended in 0.15 M NaCl–0.002 M EDTA–0.01 M Tris-HCl (pH 7.5) (NET) containing 0.05% Nonidet P-40, and frozen at −20°C.

Analysis of capsid structures. Supernatant fractions from lysed pTP12X3P-containing *E. coli* were diluted to 1% Nonidet P-40 and 1% sodium deoxycholate and centrifuged

on a 10 to 50% (wt/vol) sucrose gradient in NET buffer in an SW41 rotor at 17,000 rpm for 17 h at 4°C.

Preparation of FMDV-infected cell lysates. Bovine kidney (BK) cells or baby hamster kidney (BHK-21) cells were infected with FMDV type A12 strain 119ab at a multiplicity of approximately 20 PFU per cell and labeled with [^{35}S]methionine after the appearance of cytopathic effects. After labeling, the cells were washed and lysed as previously described (13).

In vitro translation. Preparation of rabbit reticulocyte lysates and the conditions for in vitro protein synthesis were as previously described (19).

Western blotting (immunoblotting). FMDV proteins were detected immunologically following Western blotting as described by Towbin et al. (51), by using a semidry blotter and nitrocellulose membranes. The blots were blocked by using 3% nonfat dry milk in PBS and incubated overnight at 4°C with rabbit or bovine serum against inactivated FMDV or bovine convalescent serum (1:100 or 1:200). Antibody binding was detected by incubation for 2 h with approximately 500,000 cpm of ^{125}I-labeled acid-treated FMDV per ml or with iodinated recombinant protein A/G (17, 33).

MAbs. The MAbs were produced at our Center by following protocols previously detailed (31, 45, 46). In brief, the immune cells were elicited in BALB/c mice by either inoculation of killed virus or virion fragments or by FMDV infection and fused with the 6X63-Ag8.653 myeloma cells of Kearney et al. (24). The MAbs were classified by epitope location (5, 9, 42, 46), which was based upon determination of the least structurally complex particle with which the MAb reacted (i.e., the 140S virion, the 12S protein subunit [12Sps], or individual viral proteins).

Sandwich enzyme-linked immunosorbent assay (ELISA). Anti-FMDV antibodies were purified (i.e., by ammonium sulfate precipitation and protein A-Sepharose affinity chromatography) from the sera of rabbits that had been given four 100-μg subcutaneous inoculations of purified FMDV A12 at 4-week intervals (26). Cross-reactions between the rabbit anti-FMDV and the mouse MAbs were eliminated by immunoadsorption. A 1:200 dilution of rabbit anti-FMDV antibodies (catcher) in 0.05 M Na_2CO_3–$NaHCO_3$ (pH 9.6) was adsorbed onto plastic overnight at 4°C (Nunc Immunoplates II; GIBCO-Europe Ltd). The remaining protein-binding capacity of the plastic was saturated with blocking buffer composed of 3% bovine serum albumin and 0.05% Tween 20 in PBS for 1 h at 37°C. Antigens were bound to the coated plastic by incubation overnight at 4°C and then reacted with undiluted tissue culture supernatants of various MAbs for 30 min at 37°C and 15 min at 4°C. The MAbs bound to the plastic were detected by an ELISA procedure performed as previously described (45).

Vaccination. Mature Dunkin-Hartley guinea pigs were vaccinated under the loose skin just off the dorsal midline over the rib cage with 2 ml of an antigen preparation per animal. A booster vaccination was administered 96 days after the primary vaccination. The antigen preparations contained equal volumes of lysed cells at a protein concentration of 3 to 4 mg/ml, emulsified in Freund's complete adjuvant. On the basis of the results obtained (see Fig. 3), approximately 1% or less of the total cell protein represents FMDV products.

Serologic evaluation. Blood samples were obtained by cardiac puncture, and serum was prepared by standard procedures and stored at −20°C until tested. A 50% mouse protective dose assay was performed with dilutions of serum as previously described (46).

RESULTS

Construction of plasmids containing FMDV genes. To study the in vivo expression, processing, and assembly of FMDV proteins, plasmids containing cDNA from the type A12 genome were constructed in several vectors. The regions of the FMDV polyprotein encoded by these plasmids are represented in Fig. 1. Two of the plasmids were constructed by placing the FMDV sequences in an expression vector under the control of a bacteriophage T7 promoter. Plasmid pTP12X3P contains the gene for the FMDV structural protein precursor (P1), the 2A gene, most of 2B and 3B genes, the complete 3C gene, and a small portion of the 3D gene, while plasmid p7P12B contains the P1 and 2A genes and most of the 2B gene but lacks the 3C gene. These plasmids were used to transform BL21(DE3) cells which contain the T7 RNA polymerase gene inserted in the host chromosome, where its expression is inducible by IPTG (48). Plasmid pJP12B contains the same FMDV genes as p7P12B cloned into baculovirus transfer vector pJV(NheI) under the control of the polyhedrin promoter. Plasmid pJP12B, along with wild-type *A. californica* nuclear polyhedrosis virus DNA, was used to cotransfect Sf9 cells, and a recombinant virus, JP12B, was selected (23, 49).

Expression of FMDV proteins. Bacterial cells containing pTP12X3P and p7P12B were induced with IPTG, and at 1 h after induction they were radiolabeled with [^{35}S]methionine. Lysates were prepared and analyzed by SDS-PAGE. Induction of cells containing pTP12X3P resulted in synthesis of proteins that comigrated with FMDV proteins P1-2A, P1, VP3-VP1, VP0, VP3, VP1, and 3C, while induction of cells harboring p7P12B resulted in synthesis of P1-P2′ (presumably the uncleaved precursor) and P1-2A (Fig. 2). These results suggest that FMDV proteins are expressed in *E. coli* and that the presence of the 3C protease resulted in correct and efficient processing of the P1 precursor. *E. coli*-expressed VP0 migrates slightly slower than marker VP0, presumably because of the presence of four additional codons in plasmid pTP12X3P, including an initiating methionine codon, upstream of the amino terminus of VP4 (see Materials and Methods). Processing between P1-2A and P2 is inefficient in *E. coli* cells containing p7P12B, while efficient processing to P1-2A was observed in Sf9 cells infected with recombinant baculovirus JP12B (Fig. 3, 4, and 5).

Host transcription can be selectively inhibited with rifampin, a drug that inhibits *E. coli* RNA polymerase while not affecting T7 RNA polymerase (48). Since FMDV proteins are translated from genes under the control of a T7 promoter, their synthesis should continue in the presence of this drug. As shown in Fig. 2, proteins that comigrate with FMDV proteins continue to be synthesized while synthesis of most other proteins is inhibited (lanes 5 and 9).

The expression of viral proteins in bacterial cells and in insect cells infected with recombinant baculovirus JP12B was also examined by SDS-PAGE of cell lysates followed by silver staining. As shown in Fig. 3, a low level of P1-P2′ is present upon induction of cells containing p7P12B and proteins P1, VP3-VP1, VP0, VP3, VP1, and 3C are synthesized in pTP12X3P-containing cells. A protein comigrating with FMDV protein P1-2A is observed after infection of Sf9 cells (Fig. 3B).

Immunological identification of FMDV proteins. To demonstrate that the proteins synthesized in induced cells containing plasmids pTP12X3P and p7P12B are virus specific, lysates were examined by Western blotting using antiserum prepared against either inactivated virus or convalescent

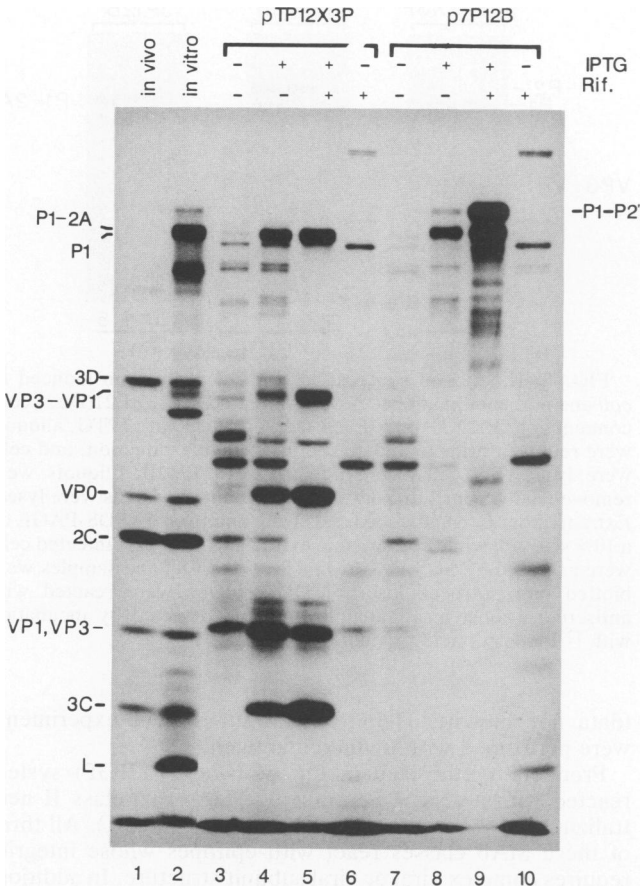

FIG. 2. Expression of FMDV proteins in *E. coli* cell lysates containing plasmids pTP12X3P and p7P12B prior to and after induction with IPTG. Aliquots of BL21(DE3) cells containing plasmid pTP12X3P or p712B were radiolabeled with [^{35}S]methionine for 30 min prior to or at 1 h after induction with IPTG. To inhibit *E. coli* RNA polymerase-directed RNA synthesis, 200 μg of rifampin (Rif.) per ml was added 30 min after IPTG induction and the radioisotope was added 30 min later. Cells were lysed in sample preparation buffer and examined by SDS-PAGE on a 15% slab gel. Lanes: 1, [^{35}S]methionine-labeled FMDV A12-infected BK cell cytoplasmic extract; 2, [^{35}S]methionine-labeled FMD A12 virion RNA-directed in vitro protein synthesis lysate; 3 to 6, lysates containing pTP12X3P radiolabeled without (−) or with (+) IPTG or rifampin; 7 to 10, lysates containing p7P12B radiolabeled without or with IPTG or rifampin. Truncated proteins are indicated by a prime.

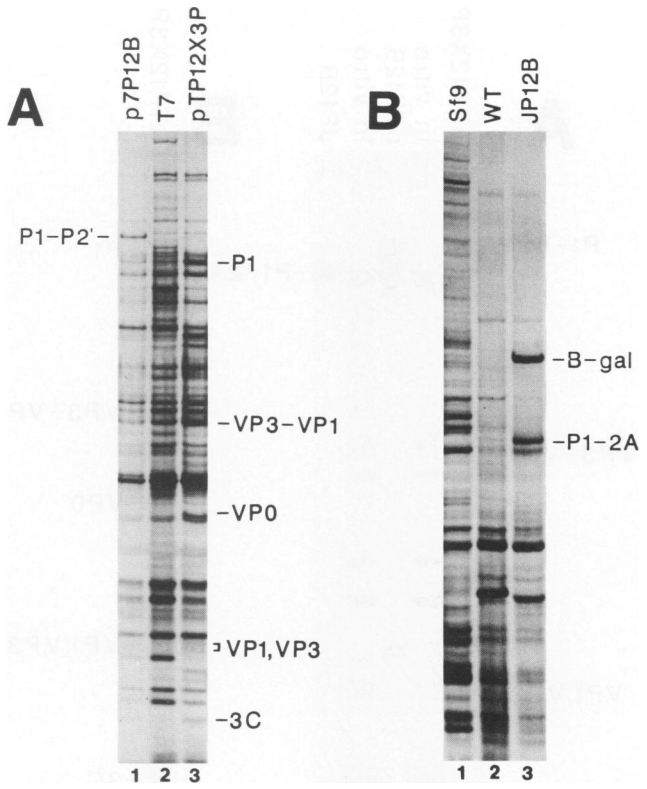

FIG. 3. Expression of FMDV proteins in induced *E. coli* cells and in recombinant baculovirus-infected cells. (A) Aliquots of BL21(DE3) cells containing p7P12B (lane 1) or pTP12X3P (lane 3) were induced with IPTG for 4 or 2 h, respectively. Cells were lysed, and proteins were examined by SDS-PAGE on a 12.5% slab gel after silver staining. Control cells containing plasmid pT7-7 lacking FMDV sequences (lane 2) were induced and examined as described in the text. (B) Sf9 cells were mock infected (lane 1), infected with wild-type baculovirus (lane 2), or infected with recombinant baculovirus JP12B (lane 3). Three days after infection, cells were lysed and cytoplasmic extracts were examined by SDS-PAGE on a 7.5% slab gel after silver staining. WT, wild type; B-gal, β-galactosidase.

serum. Upon induction, cells containing pTP12X3P produced immunologically reactive proteins comigrating with P1, VP3-VP1, VP0, VP3, VP1, and 3C, while cells containing p7P12B produced reactive P1-P2′ and lesser amounts of P1-2A (Fig. 4A and B). In Sf9 cells infected with JP12B, only a protein comigrating with P1-2A was reactive by Western blot analysis (Fig. 4A), although this recombinant virus contains the same FMDV coding region as p7P12B.

The kinetics of induction of FMDV proteins in *E. coli* and in infected Sf9 cells were analyzed by Western blot analysis (Fig. 5). FMDV proteins induced in *E. coli* containing pTP12X3P reproducibly accumulated for only 2 h, and subsequently the cells eliminated this plasmid (data not shown). FMDV proteins produced by cells containing p7P12B generally accumulated for at least 4 h (Fig. 5). In Sf9-infected cells, P1-2A was first observed 3 days after infection and accumulated for at least 2 additional days (Fig. 5).

The solubility of the FMDV proteins expressed in *E. coli* and in infected Sf9 cells was examined (data not shown). In cells containing pTP12X3P, P1, VP0, and 3C were mainly insoluble and VP3 and VP1 were mainly soluble, while in cells containing p7P12B, all of P1-P2′ and P1-2A were insoluble. In contrast, in Sf9-infected cells, P1-2A was approximately 50% soluble.

Assembly of FMDV structural protein complexes. To determine whether the processed structural proteins expressed in *E. coli* cells containing pTP12X3P are able to assemble into empty capsids, cells were radiolabeled 1 h after induction and the soluble fraction of cell lysates was examined by sucrose gradient centrifugation (Fig. 6). A peak of radioactivity migrated in the region of the empty capsid marker (70S) (Fig. 6A). SDS-PAGE analysis of this peak showed that it contained only structural proteins VP0, VP3, and VP1 (Fig. 6B). The identities of these proteins were confirmed by Western blotting (data not shown).

Immunological demonstration of capsid assembly. A series of MAbs against FMDV serotypes A12, A5, Asia-1, and 01

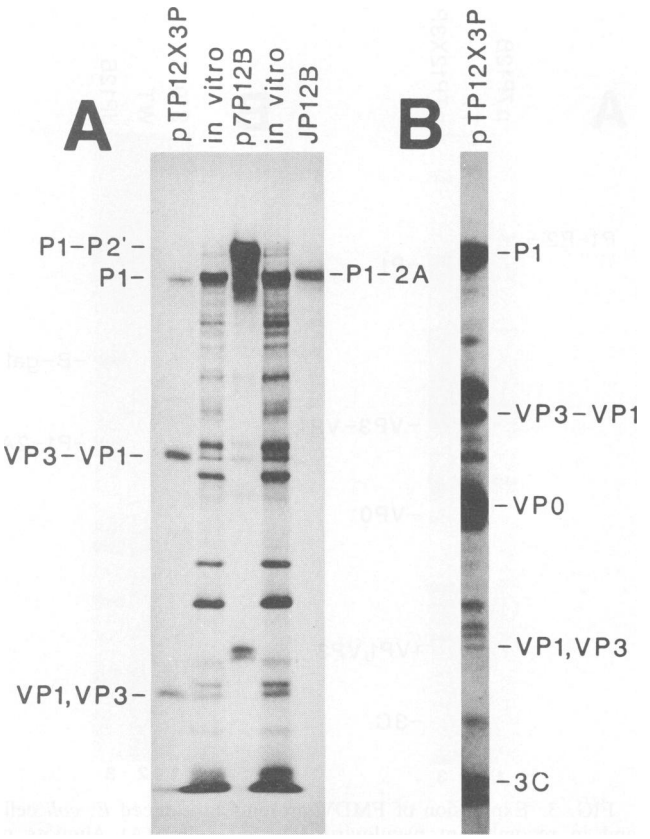

FIG. 4. Immunoblot analysis of FMDV proteins expressed in induced *E. coli* cells and in recombinant baculovirus-infected cells. (A) Aliquots of BL21(DE3) cells containing pTP12X3P or p7P12B were induced with IPTG for 4 or 2 h, respectively, and cells were lysed, while a cytoplasmic extract was prepared 4 days after infection of Sf9 cells with JP12B. The samples were separated by SDS-PAGE on a 12.5% slab gel, blotted onto nitrocellulose, reacted with antiserum against inactivated FMDV, and detected with ^{125}I-labeled protein A/G. A [^{35}S]methionine-labeled, FMD A12 virion RNA-directed in vitro protein synthesis lysate was used as a marker. (B) An aliquot of BL21(DE3) cells containing pTP12X3P was induced for 2 h, lysed, analyzed by SDS-PAGE on a 12.5% slab gel, blotted onto nitrocellulose, reacted with bovine convalescent antiserum against FMDV, and detected with ^{125}I-labeled protein A/G.

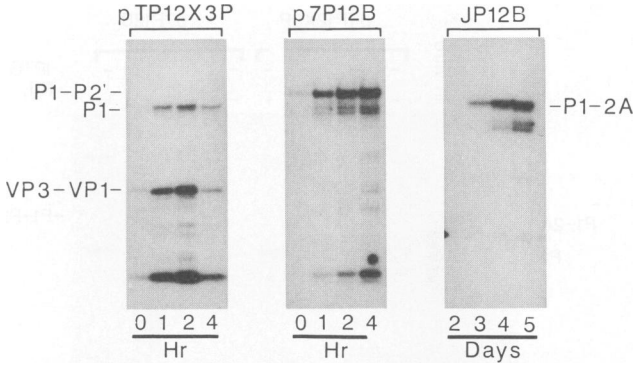

FIG. 5. Kinetics of synthesis of FMDV proteins in induced *E. coli* and in recombinant baculovirus-infected cells. BL21(DE3) cells containing pTP12X3P or p7P12B were induced with IPTG, aliquots were removed prior to and at 1, 2, or 4 h after induction, and cells were lysed. Sf9 cells were infected with JP12B, aliquots were removed daily from 2 to 5 days after infection, and cells were lysed. Extracts from *E. coli* induced cells were analyzed by SDS-PAGE on a 10% slab gel, while cytoplasmic extracts from JP12B-infected cells were analyzed by SDS-PAGE on a 7.5% slab gel and samples were blotted onto nitrocellulose. FMDV proteins were reacted with antiserum against inactivated FMDV and detected by incubation with ^{125}I-labeled, acid-treated FMDV.

Brugge have been characterized and classified (5, 9, 21, 31, 35, 45, 46). Five classes of MAbs were used for analysis of pTP12X3P-, JP12B-, and p7P12B-expressed products (9). Class I MAbs are specific for 140S virus, class II MAbs react with both 140S virus and 12Sps, class III MAb epitopes are on VP1 and are exposed on the virion and 12Sps, class IV MAbs react only with 12Sps, and class V MAbs react with 12Sps and VP1. MAbs of classes I, II, and III are neutralizing antibodies. Epitopes recognized by class IV and V MAbs are not available on the virion, and thereby these MAbs are not neutralizing. The MAbs were used in a sandwich ELISA with total lysed cells or the soluble or pellet fractions from induced *E. coli* containing pTP12X3P or p7P12B and a cytoplasmic extract from JP12B-infected Sf9 cells. Essentially identical results were obtained with all of the cell fractions of a particular antigen (data not shown). In initial assays, cell extracts were titrated at various dilutions and the highest titers were obtained with undiluted antigen (data not shown). Therefore, all subsequent experiments were performed with undiluted antigen.

Products synthesized in the *E. coli*(pTP12X3P) system reacted with a class I neutralizing MAb, two class II neutralizing MAbs, and two class IV MAbs (Table 1). All three of these MAb classes react with epitopes whose integrity requires complex viral or viral subunit structure. In addition, several class III neutralizing antibodies (sequential epitopes) and a class V nonneutralizing antibody reacted with the products (Table 1). FMDV protein P1-2A produced in JP12B-infected Sf9 cells reacted with one class IV MAb (and weakly with a second) and also with a class V MAb (Table 1). FMDV P1-P2′ synthesized in induced *E. coli* containing p7P12B reacted weakly with the same class IV and V MAbs as P1-2A (Table 1). A class II MAb, 23FB4, and two class IV MAbs, 12AF4 and 31IH7, were elicited with FMDV serotypes A5 Spain, 01 Brugge, and Asia-1, respectively. The structures recognized by these MAbs are common to at least several other serotypes (9, 44).

Vaccination of guinea pigs. The five guinea pigs vaccinated with lysed cells expressing pTP12X3P demonstrated a neutralizing antibody response after one vaccination, and the titers of the surviving animals increased significantly after the booster vaccination (Table 2). Only two of five animals demonstrated a serologic response when vaccinated with lysed JP12B-infected cells, and the titers also increased after the booster vaccination. Animals vaccinated with lysed cells expressing p7P12B failed to seroconvert, even after the second vaccination.

DISCUSSION

Expression in *E. coli* of a construct containing the FMDV structural protein and 3C protease genes resulted in efficient synthesis and processing of the FMDV structural protein precursor P1. Biochemical and immunological analyses demonstrated that variable portions of the processed structural proteins were assembled into capsid intermediate complexes, including 70S particles, and were able to react with

TABLE 1. Anti-FMDV MAb reactions in a sandwich ELISA with the expressed FMDV proteins[a]

MAb	Eliciting FMDV antigen	MAb class[b]	Specificity	S/C[c]		
				pTP12X3P	JP12B	p7P12B
2PD11.12.8.1	A12	I	140S	4.1	1.1	0.9
2FF11.1.4	A12	II	140S, 12Sps	3.1	1.2	1.2
23FB4.1.1[d]	A5 Spain	II	140S, 12Sps	4.2	1.4	1.1
6EE2.1.2	A12	III	140S, 12Sps, VP1	4.6	1.0	1.5
6HC4.1.3	A12	III	140S, 12Sps, VP1	7.0	1.0	1.4
12AF4.1.1[e]	01 Brugge	IV	12Sps	8.7	4.8	2.7
31IH7.1.1[f]	Asia-1	IV	12Sps	7.7	1.6	1.3
6AE9.1.1	A12	V	12Sps, VP1	9.6	2.9	2.1

[a] Samples include undiluted supernatant fractions from lysed *E. coli* containing pTP12X3P or p7P12B which were induced for 2 h or cytoplasmic extracts from lysed Sf9 cells which were infected for 5 days with recombinant baculovirus JP12B. Controls include undiluted supernatant fractions from lysed *E. coli* containing pT7-7 induced for 2 h or cytoplasmic extracts from lysed Sf9 cells infected with wild-type baculovirus.
[b] See reference 9.
[c] Sample-to-control ratio.
[d] Reacts with serotypes A, O, and C.
[e] Reacts with serotypes A and O.
[f] Reacts with serotypes Asia-1, A, and C.

conformation-dependent MAbs. Furthermore, these proteins-complexes induced a significant neutralizing antibody response in guinea pigs.

A second construct expressed in *E. coli* or as a recombinant baculovirus in Sf9 cells contained only the P1 and 2A genes and part of the 2B gene. Expression in *E. coli* resulted in synthesis of an insoluble polyprotein, P1-P2′, which was not efficiently processed at the 2A-2B cleavage site. In contrast, this construct was expressed as a soluble protein in insect cells and was very efficiently processed to P1-2A. Both products reacted with a class IV, 12S-specific MAb (Table 1), but only the soluble product was able to induce a neutralizing antibody response in guinea pigs (Table 2). This is the first demonstration that a picornavirus structural protein precursor is able to elicit a neutralizing antibody response in animals.

Assembly of picornavirus structural proteins into virus particles is a complex process involving many steps. The initial step is synthesis of a protomer which contains one copy of each of the four structural proteins. Five protomers then assemble into a pentamer. It is postulated that process-

TABLE 2. Serologic response elicited by vaccination of guinea pigs with FMDV-expressed proteins

Antigen[a]	MPD_{50}[b] on PVD[c]:	
	39	126[d]
pTP12X3P	2.0	2.4
pTP12X3P	1.4	>4.0
pTP12X3P	1.7	3.0
pTP12X3P	1.6	
pTP12X3P	0.4	
JP12B	0.9	1.0
JP12B	0.7	0.5
JP12B	<0.3	2.9
JP12B	<0.3	0.7
JP12B	<0.3	
p7P12B	<0.3	<0.3
p7P12B	<0.3	<0.3
p7P12B	<0.3	
p7P12B	<0.3	
p7P12B	<0.3	

[a] Groups of five guinea pigs were each vaccinated with a different antigen preparation that consisted of 1 ml of lysed cells, containing 3 to 4 mg of total cell protein, emulsified in an equal volume of Freund's complete adjuvant. Approximately 1% or less of the total cell protein represents FMDV products.
[b] Antibody titers are expressed as the 50% mouse protective dose (MPD_{50}), derived as the $\log_{10}$ of the reciprocal dilution protecting half of the mice inoculated with 100 mouse lethal doses.
[c] Groups of five guinea pigs were vaccinated on day 1 and boosted on day 96. The animals were bled on postvaccination days (PVD) 39 and 126, and the sera were subsequently tested in a mouse protection assay. By postvaccination day 126, a number of animals had died.
[d] The titers of the surviving animals bled at postvaccination day 126 do not necessarily correspond to those of particular animals within an antigen group at postvaccination day 39.

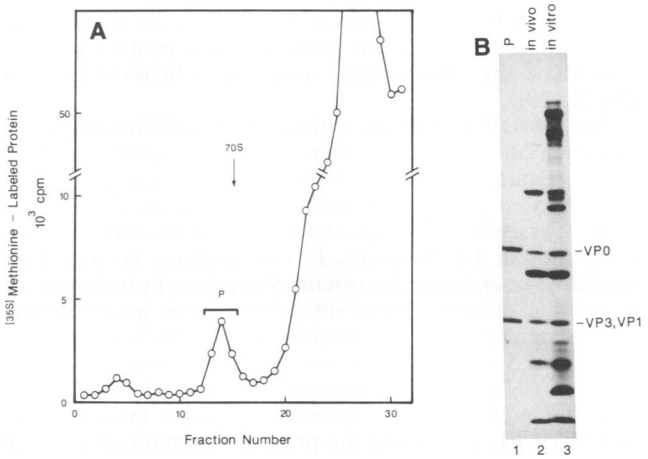

FIG. 6. Formation of FMDV empty capsids in *E. coli* cells containing plasmid pTP12X3P. An aliquot of BL21(DE3) cells containing plasmid pTP12X3P was induced with IPTG and radiolabeled 1 h later for 30 min with [^{35}S]methionine. Cells were lysed, and a supernatant fraction was prepared, treated with Nonidet P-40 and sodium deoxycholate, and centrifuged on a 10 to 50% (wt/vol) sucrose gradient as described in Materials and Methods. A [^{35}S]methionine-labeled FMDV-infected BK cell cytoplasmic extract was centrifuged on a parallel gradient and used as a marker. Samples from each fraction were assayed for acid-insoluble radioactivity. Fractions from the 70S region were pooled (P), acetone precipitated, and analyzed by SDS-PAGE on a 15% slab gel. Lanes: 1, pooled samples (P) from the gradient; 2, [^{35}S]methionine-labeled FMDV-infected BK cell cytoplasmic extract; 3, [^{35}S]methionine-labeled FMD virion RNA-directed in vitro protein synthesis lysate.

ing of the protomer to VP0, VP3, and VP1 occurs immediately upon synthesis and, thus, prior to its assembly into a pentamer (39). Empty capsids are formed by assembly of pentamers and entrance of an RNA molecule and processing of VP0 to VP4 and VP2 results in formation of mature virions. The role, if any, that an unprocessed protomer, or perhaps an unprocessed pentamer, might have in inducing an immune response in animals has not been evaluated. Our results obtained with recombinant baculovirus JP12B suggest that folding of P1 occurs, since it was recognized in a solid-phase radioimmunoassay by a MAb which reacts only with acid-derived 12S structures and not with any of the individual structural proteins (45, 47). Furthermore, P1, either as a protomer or as a more complex structure, was able to elicit a neutralizing antibody response in guinea pigs.

Our results obtained with the bacterial expression system containing plasmid pTP12X3P demonstrate the assembly of FMDV empty capsids and, perhaps, 14S pentamers. However, the process is clearly inefficient. This is in contrast to the efficient morphogenesis of FMDV in infected tissue culture cells. Thus, we have been unable to produce sufficient amounts of empty capsids or other complexes to allow us to purify these antigens and directly examine their immunogenic potential in experimental animals.

There are a number of possible reasons for inefficient assembly of FMDV capsid structures in the *E. coli* expression system. For example, it has recently been shown for all picornaviruses examined, including FMDV, that the amino-terminal glycine of VP4 is myristoylated (11, 32). This modification occurs initially on the P1 precursor protein (11, 32). The importance of this modification has been examined with poliovirus myristoylation-negative mutants. In this system, the late steps of virus assembly are inhibited and the reduced level of mature virions produced is not infectious (27, 29). Thus, it has been speculated that myristoylation of P1 and VP0 functions as an anchorage site for maintenance of a high concentration of protomers and pentamers at the membrane site where capsid assembly is believed to occur (10, 27, 29).

Our FMDV constructs contain four additional codons, including an initiating methionine codon, upstream of the amino-terminal glycine of VP4. Thus, in these constructs, the proposed consensus myristoylation signal, glycine-X-X-X-Ser/Thr, where X represents any amino acid (11), has been altered. Furthermore, *E. coli* contains no detectable myristoyl-coenzyme A:protein *N*-myristoyltransferase activity, the enzyme responsible for protein myristoylation (16). Recently, however, Duronio et al. (16) have reconstituted the *Saccharomyces cerevisiae* myristoyl-coenzyme A:protein *N*-myristoyltransferase system in *E. coli*. Therefore, it is now feasible to determine whether myristoylation of FMDV P1, containing the proposed amino-terminal consensus myristoylation signal, results in more efficient assembly of FMDV capsid intermediate structures in an *E. coli* expression system containing the *S. cerevisiae* myristoyl-coenzyme A:protein *N*-myristoyltransferase gene.

The apparent toxic effect of the 3C protease in our bacterial expression system also clearly limits the level of synthesis and assembly possible. Alternative strategies to circumvent this problem include placing the P1 and 3C genes under the control of different promoters and expressing the 3C gene product only after substantial P1 has been synthesized. In addition, isolation of a recombinant baculovirus containing both the P1 and 3C genes may also result in higher levels of production of FMDV empty capsids than possible in the *E. coli* system. For example, poliovirus empty capsids have been purified from Sf9 cells infected with a recombinant baculovirus containing the entire coding region of poliovirus type 3 (52). However, in contrast to the level of production of P1-2A in our system, Roosien et al. (36) have been able to synthesize only very low levels of FMDV capsid proteins in their recombinant baculovirus expression system.

The concept of utilizing biosynthetically produced complex FMDV capsid structures as vaccines appears to be worth pursuing. However, a number of fundamental questions concerning virus assembly and the level of capsid complexity necessary to induce maximum production of a protective antibody response in animals remain unanswered. Nevertheless, our initial studies suggest that complex FMDV antigens, including the unprocessed P1 structural protein precursor, are able to induce a neutralizing antibody response. Furthermore, results from ongoing challenge experiments with swine indicate that animals vaccinated with pTP12X3P-expressed products are protected against virulent virus challenge, while JP12B-expressed P1 is able to prevent FMDV-induced disease but not virus infection. However, p7P12B-expressed P1-P2′ affords no protection (20).

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