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Stably Expressed FIPV Peplomer Protein Induces Cell Fusion and Elicits Neutralizing Antibodies in Mice

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We have established bovine papilloma virus (BPV)-transformed mouse C127 cell lines that synthesize the peplomer protein of the feline infectious peritonitis virus (FIPV) strain 79-1146. For this purpose, a new cassette expression vector pHSL, which carries the Drosophila HSp70 promotor and the polyadenylation signal of the Moloney murine leukemia virus long terminal repeat, was constructed. Cocultivation of the BPV-transformed cell lines with FIPV-permissive feline fcwf-D cells resulted in polykaryocyte formation. Since it depended on the presence of fcwf-D cells, binding of E2 to the cell receptor may be required for membrane fusion. E2 was synthesized as a core-glycosylated protein of 180K which was only slowly transported from the endoplasmic reticulum to the medial Golgi: of the E2-molecules labeled during a 1-hr pulse about half was still completely sensitive to endoglycosidase H after a 2-hr chase, while the remaining E2 had been chased into multiple, partially endoglycosidase H-resistant forms. Immunofluorescence studies also indicated that most E2 was retained intracellularly. Mice immunized with whole lysates of the transformed cells produced FIPV-neutralizing antibodies as shown by plaque reduction.

INTRODUCTION

Coronaviruses, a group of positive-stranded, enveloped RNA viruses, cause considerable economical losses by infecting livestock and other domestic animals. The molecular biology of these viruses is studied not only because of their unusual replication strategy (for review see Siddel *et al.*, 1983; Spaan *et al.*, 1988) but also for the development of effective vaccines by recombinant DNA technology.

Coronaviruses are characterized by large petal-shaped peplomers protruding from the viral membrane. These surface projections, which consist of the peplomer protein E2, play an important role during the infection process, since they mediate receptor binding and membrane fusion (Sturman and Holmes, 1983).

E2 is a large (180K to 200K) glycoprotein (Sturman and Holmes, 1983). Sequence analyses of the E2 genes of several coronaviruses revealed that its protein moiety is 1150 to 1450 amino acid residues in length and contains an N-terminal signal sequence, a C-terminal transmembrane anchor, and 21 to 35 potential N-glycosylation sites (Binns *et al.*, 1985; De Groot *et al.*, 1987b,c; Jacobs *et al.*, 1987; Luytjes *et al.*, 1987; Niesters *et al.*, 1986; Rasschaert and Laude, 1987; Schmidt *et al.*, 1987). Besides glycosylation, synthesis of E2 entails acylation and for some coronaviruses, like mouse hepatitis virus (MHV) and avian infectious bronchitis virus (IBV), cleavage into two subunits (Frana *et al.*, 1985; Stern and Sefton, 1982; Sturman *et al.*, 1985). In the infected cell some E2 is not incorporated into virions but transported to the plasma membrane where it can induce polykaryocyte formation (Sturman and Holmes, 1983, 1985). Cavanagh (1983) provided evidence that each peplomer is a dimer or trimer of E2. The presence of two regions with heptad periodicity in the C-terminal half of the protein suggests that the monomers are held together by a complex interchain coiled coil (De Groot *et al.*, 1987c).

Several observations suggest that E2 is the principal antigen eliciting protective immunity. The N-terminal half of E2 was required for a protective immune response against IBV in chickens (Cavanagh *et al.*, 1986), while mice could be protected against a lethal challenge with MHV by vaccination with purified E2 (Hasony and MacNaughton, 1981) or E2-derived synthetic peptides (Talbot *et al.*, 1988; M. Koolen, personal communication). Passive immunization with E2-specific monoclonal antibodies also provided protection against MHV (Buchmeier *et al.*, 1984; Wege *et al.*, 1984). However, the situation is much more complex in the case of feline infectious peritonitis virus (FIPV), which causes a fatal disease in cats (FIP), involving antibody-mediated early death (Pedersen and Boyle, 1980; Weiss and Scott, 1981).

Mammalian cell lines expressing the FIPV peplomer gene would provide a convenient source of protein to

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dissect the role of E2 in FIP. Moreover, such cell lines could be used to study virus assembly and membrane fusion. Here we report synthesis of FIPV E2 in bovine papilloma virus (BPV)-transformed C127-cells. It is shown that the expression product induces fusion of FIPV-permissive feline cells and is immunogenic in mice. In addition, we describe two new BPV "cassette" expression vectors allowing (i) easy cloning of genes between promotor and termination sequences, (ii) easy replacement of promotor and termination sequences, and (iii) both transient and stable expression.

METHODS

Plasmids and cells

Plasmid pdBPV-MMTneo(342-12) (Law *et al.*, 1983) was obtained from the American Type Culture Collection. Plasmids pA4 LTR and pBN247 were kindly provided by Dr. A. J. M. Berns (Dutch Cancer Institute, Amsterdam). The C127 cells used for transfection experiments were obtained from Dr. P. Howley (NIH, Bethesda). Cells were propagated in DMEM with 10% fetal calf serum. Transfection was carried out using the calcium phosphate precipitation technique (Graham and Van Der Eb, 1973). To enhance the transfection efficiency, the cells were treated with 25% DMSO (Stow and Wilkie, 1976) or 20% glycerol (Frost and Williams, 1978) 4 hr after transfection. Cell lines were established by isolating foci as described by Law *et al.* (1983). *Felis catus* whole fetus (fcwf)-D cells were obtained from Dr. N. C. Pedersen (School of Veterinary Medicine, University of California, Davis).

Nucleic acid analysis

For Northern blot analysis RNA was isolated as described by Spaan *et al.* (1981). Glyoxal-denatured RNA was fractionated on 0.8% agarose gels in 10 m*M* sodium phosphate, pH 8.0 (McMaster and Carmichael, 1977), transferred to nylon membranes (GeneScreen Plus; NEN, Boston), and hybridized according to the manufacturer's recommendations. Standard recombinant DNA techniques were performed as described (Maniatis *et al.*, 1982). Cytoplasmic dot hybridization was performed according to White and Bancroft (1982).

Construction of the expression vectors

The expression vector pHSL was derived from the vector pdBPV-MMtneo(342-12). In order to facilitate the construction of this new vector (see Fig. 1), the large *Bam*HI fragment (7945 bp) representing the entire BPV-1 genome was deleted from pdBPV-MMtneo. The remaining fragment, designated pMTN, was recircularized. As terminator sequences we chose the long terminal repeat (LTR) of Moloney murine leukemia virus (Van Beveren *et al.*, 1980). The 0.6-kb *Pvu*II/*Hin*dIII fragment containing the LTR was isolated from plasmid pA4 LTR. By linker addition the *Pvu*II site was converted into a *Bgl*II site. Similarly, the *Hin*dIII site was changed into a *Bam*HI site. The resulting fragment was ligated to the large fragment of *Bgl*II/*Bam*HI-digested pMTN, yielding plasmid pMTL. The 0.7-kb *Eco*RI/*Bam*HI fragment, carrying the Drosophila heat-shock (HSp70) promotor (Torok and Karch, 1980), was excised from pBN247 and, after converting the *Bam*HI site into a *Bgl*II site by linker addition, ligated to the large *Eco*RI/*Bgl*II fragment from pMTL. The resulting vector, pHSL, was used in our experiments.

A full-length cDNA copy of the E2 gene of FIPV strain 79-1146 (see below) was inserted into the *Bgl*II site of pHSL, while the BPV-1 genome was inserted into the *Bam*HI site. The construct containing the BPV genome in the same transcriptional orientation as the FIPV E2 gene was designated pHSFILB(+); the construct containing the BPV genome in the opposite orientation was named pHSFILB(−). Cell lines made with these plasmids were indicated by RM(+) and RM(−), respectively.

Tailoring of the FIPV peplomer gene

Plasmid pB1 contains a full-length cDNA copy of the E2 gene of FIPV 79-1146 (De Groot *et al.*, 1987b). Before cloning this gene into the expression vectors redundant noncoding sequences located at the 5′ and 3′ ends were removed. For trimming of the 5′ end (extending 350 nucleotides from the E2 initiation codon), 16 ng of the synthetic oligonucleotide 5′ TGTGCCATGATTGTGCT 3′ (corresponding to position −6 to +11 of the E2 gene) was annealed in 10 m*M* Tris–HCl, pH 7.5, 50 m*M* NaCl (total volume 30 μl) to 1 μg of single-stranded DNA from a recombinant M13 mp8 phage containing the (−)sense strand of the 5′ terminal 1.4-kb *Pst*I fragment of pB1. Double-stranded DNA was synthesized by incubation with 0.05 m*M* dNTPs and 3 units Klenow DNA polymerase in a final volume of 40 μl, for 15 min at room temperature. The DNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in 10 μl 10 m*M* Tris–HCl, pH 8, 1 m*M* EDTA. Subsequently, 15 μl of S1 buffer (30 m*M* potassium acetate, pH 4.6, 0.25 *M* NaCl, 1 m*M* $ZnSO_4$, 5% glycerol) containing 7 units S1 nuclease (Pharmacia) was added; digestion was for 20 min at room temperature (Eghterdarzadeh and Henikoff, 1986). After phenol extraction and ethanol precipitation, the DNA was digested with *Pst*I. Thus a 1058-bp fragment was generated corresponding to the 5′ end of the E2 gene. This

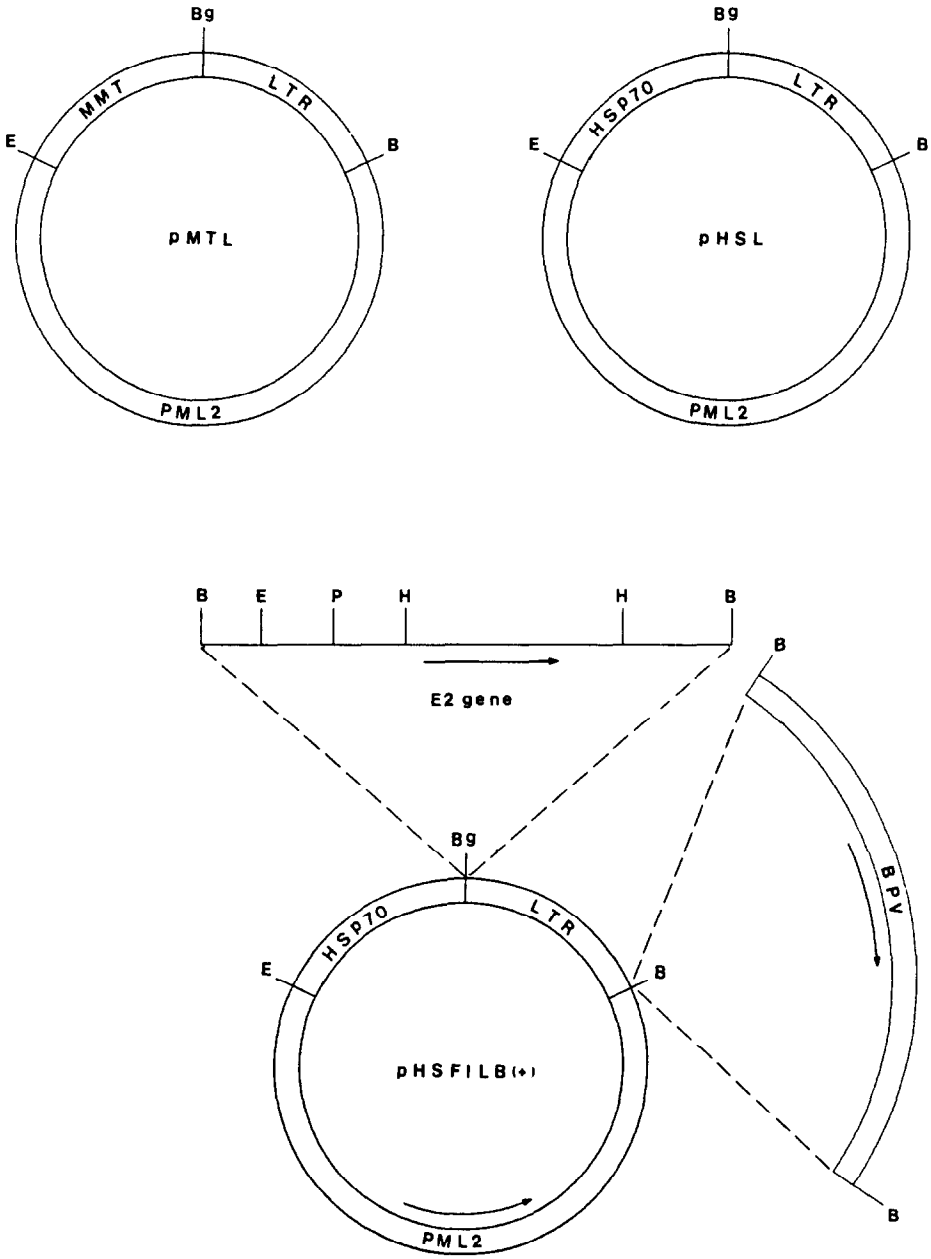

FIG. 1. The structure of the plasmids pMTL, pHSL, and pHSFILB(+); pHSFILB(−) contains the BPV-1 genome in the opposite orientation as compared to pHSFILB(+). The BPV-1 genome, mouse metallothioneine promotor (MMT), Drosophila heat-shock promotor (HSp70), Moloney murine leukemia virus long terminal repeat (LTR), and the bacterial plasmid pML2 are indicated. B, *Bam*HI; Bg, *Bgl*II, E, *Eco*RI; H, *Hind*III, P; *Pst*I. The arrows indicate the directions of transcription of the E2 gene, of the BPV-1 genome, and of the β-lactamase gene in pML2. The plasmids and the details of their construction are described under Materials and Methods. Cell lines generated with pHSFILB(+) and pHSFILB(−) are designated RM(+) and RM(−), respectively.

DNA was cloned into *Hind*III (blunt-ended with Klenow)/*Pst*I-digested pUC8 in the presence of *Bam*HI linkers. The ligation mixture was used for transformation of JM109 according to Hanahan (1983). Recombinant clones were selected by colony hybridization. The FIPV-specific inserts were sequenced. Plasmid p1A contained the 5′ end sequence of the FIPV peplomer gene, starting 5 nucleotides upstream of the AUG codon (1 nucleotide was lost probably by nibbling of the S1 nuclease), preceded by a *Bam*HI linker. Plasmid p1A was digested with *Pst*I and the 3000-bp *Pst*I fragment of B1 was inserted in the correct orientation. The *Acc*I site located 98 nucleotides downstream of the stop codon was used to remove the redundant sequences at the 3′ end. As a result, the complete FIPV E2 gene was contained in a 4.3-kb *Bam*HI fragment.

Cocultivation fusion assay

To monitor E2-induced cell fusion, 10^6 fcwf-D cells and 10^5 BPV-transformed C127 cells were seeded into a 35-mm-diameter petri dish and allowed to adhere and grow for 16 hr at 37°. For induction of the heat-shock promotor the cells were incubated for 2 hr at 42° and then reincubated at 37°. Cell fusion was assessed by light microscopy.

Protein analysis

FIPV 79-1146-infected cells were labeled with [^{35}S]methionine (Amersham) from 6 to 6.5 hr after infection as described previously (De Groot *et al.*, 1987a). After 2 hr incubation at 42°, RM(+)19 cells were labeled at 37° by growing in cysteine-free DMEM, containing 100 μCi/ml [^{35}S]cysteine (Amersham). Cells were lysed in PBS, 0.5% Triton X-100. E2 was immunoprecipitated using ascitic fluid from an FIPV-infected cat (De Groot *et al.*, 1987a). Digestion with endoglycosidase H was performed as described by Rose and Bergmann (1983). Proteins were denatured in Laemmli sample buffer, containing 5% 2-mercaptoethanol, and subsequently analyzed on 5% SDS–polyacrylamide gels.

Immunofluorescence

Indirect surface immunofluorescence was carried out with RM(+)19 cells, fixed in 3% paraformaldehyde as described by Rose and Bergmann (1983). For intracellular immunofluorescence, the cells were fixed for 20 min at −20° in 95% methanol, 5% acetic acid. Sera (1:100) obtained from a cat before and after experimental infection with FIPV 79-1146 were used as the primary antibodies, followed by FITC-conjugated rabbit anti-cat immunoglobulin G (1:80, Nordic).

Immunization

RM(+)19 and AM2 cells were grown to confluence, subjected to heat shock, followed by a 5-hr incubation at 37°. The cells were washed three times with PBS and harvested with a cell scraper. They were then suspended in PBS (10^8 cells/ml) and disrupted by freeze–thawing followed by 10-min sonification (bath sonicator, Type RK102H, Bandelin, West Germany). The cell suspensions were stored at −70°.

The concentration of the cell lysates was adjusted to an equivalent of 10^7 cells/mouse/dose. The first dose, consisting of lysate mixed with an equal volume of complete Freund's adjuvant, was applied subcutaneously. The mice were boosted intraperitoneally on Days 10 and 21 with lysates suspended in PBS (Marchioli *et al.*, 1987).

Plaque reduction assay

Twofold serial dilutions of heat-inactivated sera (150 μl) were mixed with about 120 PFU of FIPV 79-1146 in an equal volume of DMEM, 10% fetal calf serum. Incubation was for 1 hr at 37°. These samples were used to infect monolayers of fcwf-D cells. After 1 hr absorption at 37°, the cells were washed twice with PBS and finally maintained for 20 hr under an agar overlay. Plaques were counted after staining with 0.02% neutral red in PBS. Antibody titers, expressed as the reciprocal serum dilution causing 50% plaque reduction, were estimated according to the method of Reed and Münch (1938).

RESULTS

Construction of cassette expression vectors

At the time we started this research, no convenient BPV expression vectors were available. Therefore, we constructed the expression vector pMTL. This vector carries the mouse metallothioneine (MMT) I promotor (Hamer and Walling, 1982), while the Moloney murine leukemia LTR (Van Beveren *et al.*, 1980) provides a polyadenylation signal. pMTL has been designed with the aim to allow for easy exchange of promotor and termination sequences. To construct vector pHSL the MMT promotor was replaced by the Drosophila HSp70 heat-shock promotor (Torok and Karch, 1980) (Fig. 1). Any protein coding sequence can be cloned into the unique *Bgl*II site downstream of the promotor. Vectors for stable expression were constructed by inserting the BPV-1 genome (Law *et al.*, 1981; Sarver *et al.*, 1981) into the unique *Bam*HI site downstream of the LTR. The versatility of these vectors was demonstrated by stable expression of the vesicular stomatitis virus (VSV) membrane glycoprotein and the nucleocapsid protein of MHV (not shown). A BPV-transformed cell line AM2, which expresses the MHV nucleocapsid protein under the control of the MMT promotor (Bredenbeek *et al.*, unpublished), served as a negative control in the experiments described below.

Selection of E2-expressing cell lines

A full-length cDNA copy of the FIPV E2 gene was obtained from plasmid pB1 (De Groot *et al.*, 1987b). To avoid possible interference with transcription and translation, noncoding sequences at the 5′ and 3′ end of the gene were removed.

To anticipate possible adverse effects related to the transcriptional orientation of the BPV sequences in the vector, we used both pHSFILB(+) and pHSFILB(−) (Fig. 1) for transfection of C127 cells. Three weeks after transfection, 48 transformed foci [32 RM(+) and 16

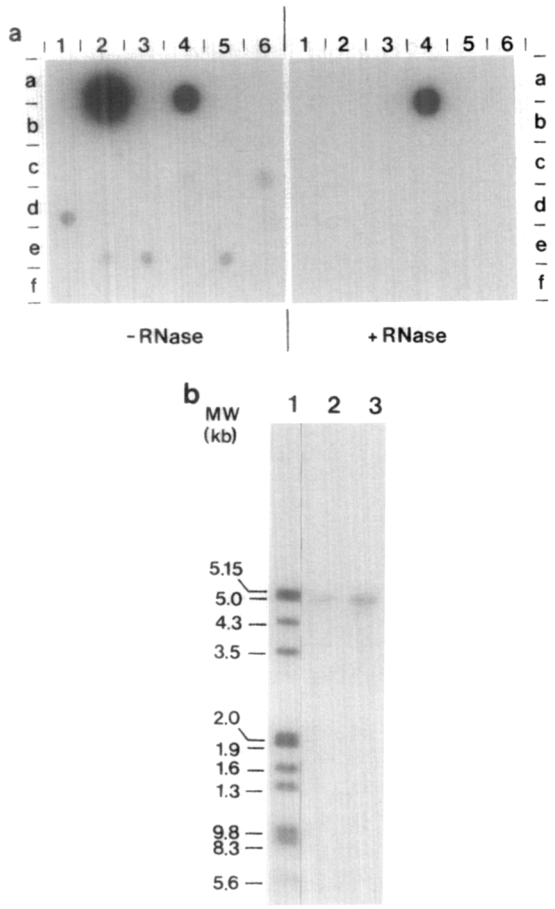

FIG. 2. (a) Screening for E2 transcripts. Cytoplasmic RNA was extracted from 2×10^5 BPV-transformed cells and spotted on nitrocellulose as described by White and Bancroft (1982) (rows b–f). About 1 μg of RNA isolated from FIPV-infected cells and 50 ng of an 1800-bp *Hin*dIII fragment derived from the FIPV E2 gene were spotted as positive controls (a2 and a4, respectively). A duplicate filter was incubated for 30 min at 37° in 2× SSC, containing 10 μg/ml RNase A. Filters were hybridized to a nick-translated FIPV E2-specific probe. (b) Glyoxal-denatured RNA extracted from noninduced (lane 2) and heat-shock-induced (lane 3) RM(−)17 cells was separated on 0.8% agarose gels, transferred to a nylon membrane, and hybridized to a nick-translated 4.5-kb *Bam*HI fragment containing the complete FIPV E2 gene. To estimate molecular weights a glyoxal-denatured *Eco*RI/*Hin*dIII digest of phage λ DNA was induced (lane 1).

RM(−)] were isolated, established as continuous cell lines, and tested for accumulation of E2-specific RNA by cytoplasmic dot hybridization (White and Bancroft, 1982) after a heat shock (Fig. 2a). About 20% of the cell lines gave a positive reaction. There was no difference in the percentage of positive cell lines generated by pHSFILB(+) or (−).

To characterize the E2 transcripts, cytoplasmic RNA was extracted from some of these cell lines and analyzed by Northern blotting. The nick-translated 4.3-kb *Bam*HI restriction fragment containing the complete E2 gene (Fig. 1) was used as a probe. A prominent RNA species of 4.9 kb in length was detected. This size is consistent with a full-length transcript initiated at the heat-shock promotor and terminated at the polyadenylation site in the LTR. The 4.9-kb RNA species was also observed in noninduced cells, indicating that the heat-shock promotor is leaky (Fig. 2b). Transcription was enhanced about two- to fivefold after induction. In one cell line, RM(+)15, the 4.9-kb RNA was not present; instead an E2-specific RNA of about 2 kb was found (not shown).

Expression of E2

To test for E2 synthesis and its transport to the cell surface, we used an assay based on E2-induced fusion activity. The eight "best hybridizing" cell lines were cocultivated with fcwf-D cells (FIPV permissive feline cells) and found to induce cell fusion (Fig. 3). Syncytia were only formed after addition of fcwf-D cells. Fusion did not occur in fcwf-D cells cocultivated with AM2 cells (which express the MHV nucleocapsid protein) or RM(+)15 cells (which produce an aberrant E2 transcript; not shown).

For most cell lines heat shock was required to induce cell fusion; after 16 hr of cocultivation at 37° only few syncytia were observed. Syncytia appeared and increased in number 4 to 5 hr after heat shock. In contrast, one cell line RM(+)19 caused widespread cell fusion even without induction. Differences in fusion potential were taken to reflect differences in the level of E2 expression. Using this criterium the RM(+)19 cell line was considered the best producer and therefore selected for further study. The low-producing cell line RM(−)17 was included for comparison.

To analyze E2 synthesized in these cell lines, [^{35}S]cysteine-labeled cell lysates were subjected to immunoprecipitation with an FIPV-specific antiserum. A protein of 180K was detected in RM(+)19 cells. This product was also present in RM(−)17 cells, although in very low amounts, but it was not detected in lysates of AM2 cells (Fig. 4a). The 180K protein comigrated with pulse-labeled E2 produced in FIPV-infected fcwf-D cells (Fig. 4b). These results indicated that the 180K product was indeed the E2 protein. The bands >200K were observed in both the lysates of E2-producing cell lines and the AM2 cell line indicating that these products are nonspecifically precipitated by the FIPV polyclonal antiserum.

E2 could not be detected on the plasma membrane of RM(+)19 cells by immunofluorescence microscopy using the serum of an FIPV-infected cat (not shown), but in permeabilized RM(+)19 cells perinuclear and granular cytoplasmic staining was observed (Fig. 5); staining was not observed in AM2 cells (not shown).

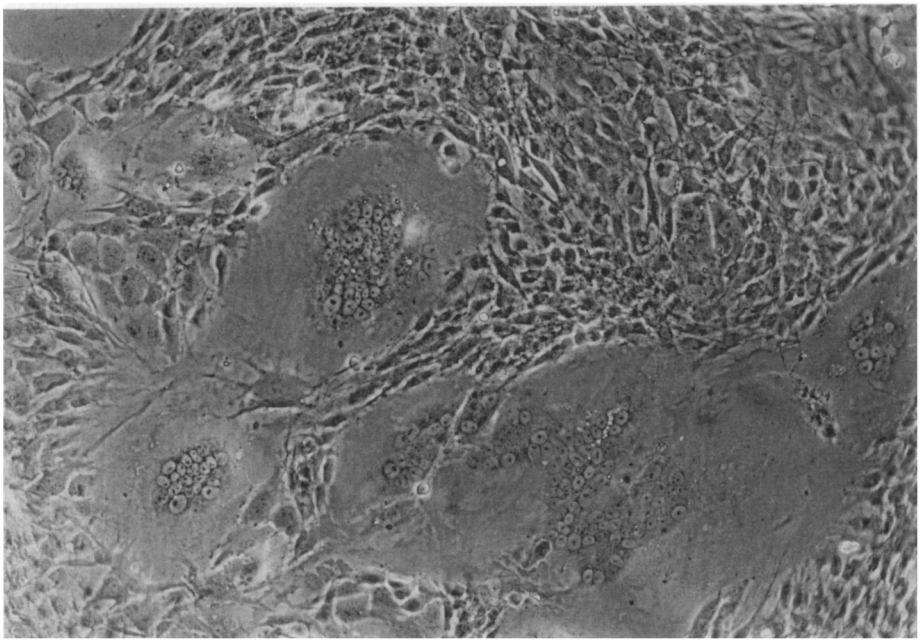

FIG. 3. Fusion of fcwf-D cells induced by RM(+)19 cells. Details are explained under Materials and Methods.

These results suggested that most E2 was retained intracellularly.

To study the transport of E2 in the transformed cells, we analyzed the sensitivity of its oligosaccharide chains to endoglycosidase H (endo H). Endo H-sensitive high-mannose oligosaccharides are added to the protein backbone during translocation across the membrane of the endoplasmic reticulum (ER). These precursor sugar chains are processed to endo H-resistant "complex" oligosaccharides in the medial Golgi (Dunphy and Rothman, 1985; Hubbard and Ivatt, 1981). Figure 4b shows that E2 produced during a 1-hr pulse-labeling of RM(+)19 cells did not contain complex oligosaccharide side chains. Digestion with endo H yielded a 160K product comigrating with pulse-labeled, endo H-treated E2 from FIPV-infected cells. About 50% of E2 in RM(+)19 cells was still fully endo H-sensitive after a 2-hr chase. The remaining E2 molecules appeared to be chased into products ranging in length from 160K to 180K after endo H-treatment. Prolonged chasing did not result in the appearance of a discrete endo H-resistant product. Rather, the material accumulated as multiple diffuse bands ranging in length from 180K to 200K (not shown).

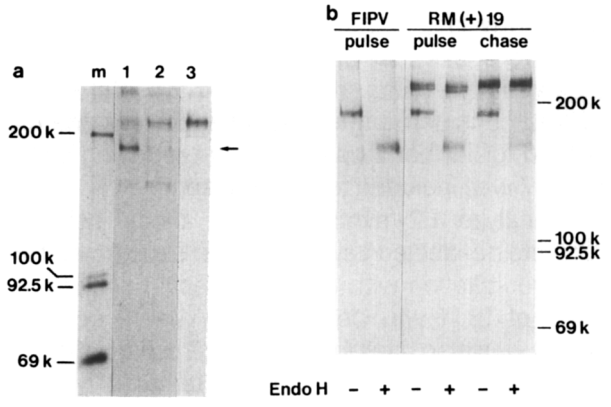

FIG. 4. (a) Analysis of immunoprecipitates of RM(+)19 cells (1), RM(−)17 cells (2), and AM2 cells (3). Cells were labeled with [^{35}S]cysteine from 0 to 5 hr after heat shock. Lysates were subjected to immunoprecipitation using an E2-specific serum. Samples were analyzed on 5% SDS–polyacrylamide gels. The 180K product is indicated by an arrow; m: molecular weight marker. (b) Pulse-chase labeling of E2 in RM(+)19 cells. Cells were labeled from 1 to 2 hr after heat shock with [^{35}S]cysteine. Subsequently, cells were either lysed immediately ("pulse") or after a 2-hr chase with DMEM containing 4 m*M* unlabeled cysteine ("chase"). E2 was immunoprecipitated. Half of each sample was digested with endo H (+); the other half was not digested (−). Samples were analyzed on 5% polyacrylamide gels. E2 immunoprecipitated from pulse-labeled FIPV-infected cells was used as marker. Relative amounts of E2 were estimated by densitometry of the autoradiogram using a scanning densitometer (Type DD2, Kipp and Sons, The Netherlands).

Immunization of mice

To determine whether the BPV-transformed cell lines synthesize enough E2 for use as immunogen, mice were immunized on Day 1 and boosted on Days 10 and 21 with lysates of RM(+)19 and AM2 cells. Sera collected at Days 1 (preserum), 21, 28, and 40 were tested for the presence of E2-specific antibody in a plaque reduction assay. Table 1 shows that mice immunized with RM(+)19 cell lysates developed rising titers of FIPV neutralizing antibodies. No plaque reduc-

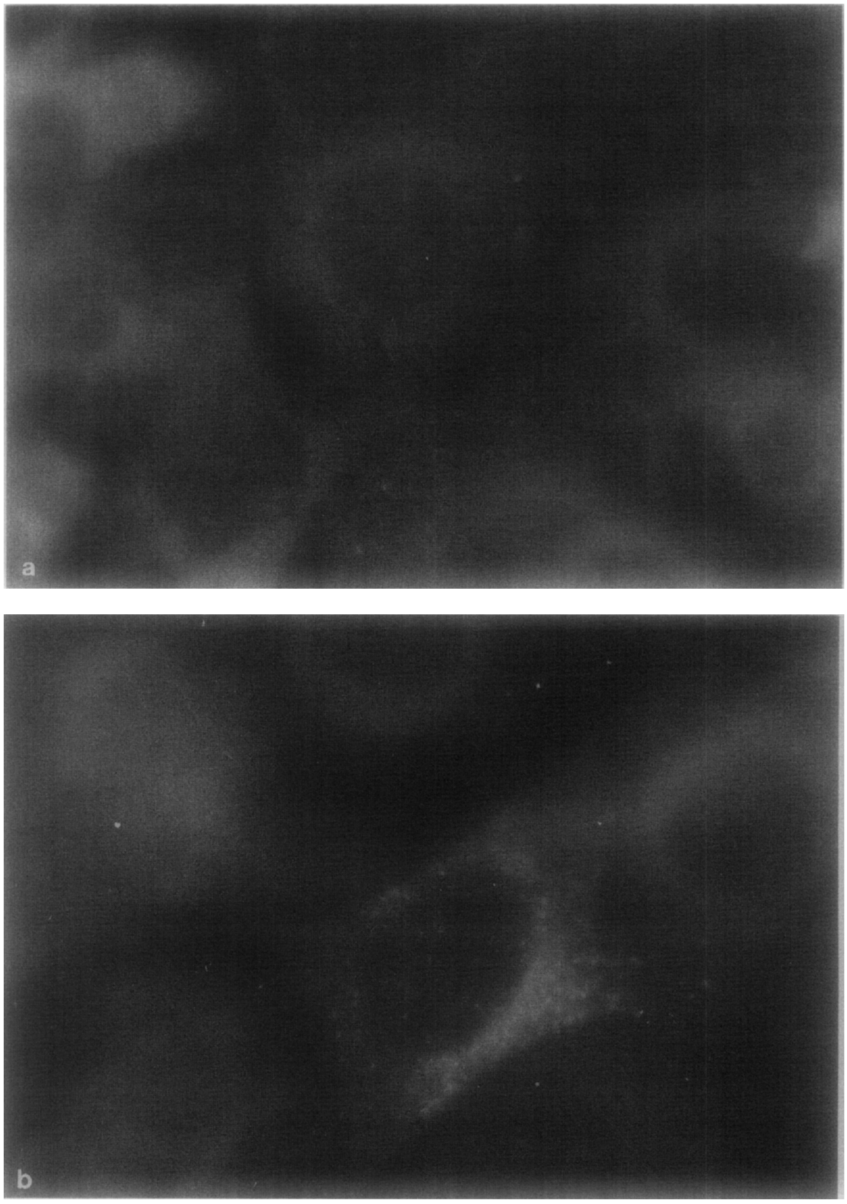

FIG. 5. Detection of the E2 protein by indirect immunofluorescence microscopy. RM(+)19 cells grown on coverslips were fixed with 95% methanol, 5% acetic acid and incubated with serum obtained from a cat before (a) and after (b) experimental infection with FIPV 79-1146.

tion was observed with sera from mice immunized with AM2 cell lysates.

DISCUSSION

Expression vectors containing either the complete genome or subgenomic fragments of BPV-1 have been used to establish continuous cell lines producing large quantities of exogenous proteins (Hsiung *et al.*, 1984; Pavlakis and Hamer, 1983; Reddy *et al.*, 1987; Sambrook *et al.*, 1985; Sarver *et al.*, 1985, 1987; Zinn *et al.*, 1982). In these vectors the BPV sequences carry the functions for plasmid replication and stable maintenance in the transformed cells (Law *et al.*, 1981; Sarver *et al.*, 1981).

We have set out to establish a number of BPV-transformed mammalian cell lines which express coronavirus structural proteins. Such cell lines may provide a continuous source of protein for use as vaccine, but may also aid in the study of protein function and virion assembly. To facilitate plasmid construction we have developed the cassette vectors pMTL and pHSL containing the mouse MMT-1 and Drosophila HSp70 promotor, respectively. Both vectors possess the Moloney murine leukemia virus LTR to provide a polyadenylation signal. The unique *Bgl*II site downstream of the promo-

TABLE 1

Immunizing agent[a]	Neutralizing titer[b] at Day 1	21	28	40
AM2	<8	<8	<8	<8
RM(+)19	<8	10	87	153

[a] Mice were immunized with disrupted RM(+)19 or AM2 cells (five mice per group). Doses of either preparation were given on Days 0 (subcutaneously), 10, and 21 (intraperitoneally). Heat-inactivated sera were pooled and tested in a plaque reduction assay.

[b] The neutralizing titer is indicated as the reciprocal of the serum dilution causing 50% plaque reduction when incubated with 120 PFU of FIPV 79-1146.

tor elements can be used to insert any gene of interest. For stable expression, the BPV genome can be introduced into the unique *Bam*HI site. However, the vectors are equally suited for transient expression; e.g., by inserting the SV40 origin into the *Bam*HI site they can be used for expression in COS cells (Subramani and Southern, 1983).

Here we have used pHSL-derived plasmids to establish cell lines synthesizing the peplomer protein of FIPV strain 79-1146. Southern blot analysis of total cellular DNA extracted from our best producing cell line RM(+)19 showed the presence of 100–150 integrated copies of pHSFILB(+) DNA per equivalent of diploid mouse genome (not shown).

The cell lines induced syncytium formation when cocultivated with FIPV-permissive feline cells. This effect was concluded to be E2 specific, since it was not induced by the BPV-transformed cell line AM2 synthesizing the MHV nucleocapsid protein nor by the RM(+)15 cell line producing truncated E2 transcripts. In addition, syncytia formation was inhibited in the presence of FIPV antiserum; preimmune serum from the same cat did not inhibited cell fusion (data not shown). Fusion did not occur between murine cells but depended upon the presence of feline cells, suggesting that for membrane fusion to occur binding of E2 to the FIPV cell receptor is required. A similar observation was made for cell lines expressing the envelope glycoprotein of human immune deficiency virus: polykaryocyte formation was restricted to cells bearing the CD4 receptor (Sodroski *et al.*, 1986).

In RM(+)19 cells E2 was synthesized as a core-glycosylated protein of 180K. Removal of the oligosaccharide side chains by endo H reduced the size of the expression product to 160K (about the size of the FIPV E2 apoprotein) (De Groot *et al.*, 1987b). As shown by the fusion assay some of the E2 was transported to the plasma membrane. However, immunofluorescence studies suggested that most E2 was retained intracellularly. This was confirmed in pulse-chase experiments: of the E2 molecules labeled during a 1-hr pulse about half was still completely sensitive to endo H treatment after a 2-hr chase. The remaining material was chased into immature endo H-resistant products 160K to 180K in length, probably representing partially processed medial Golgi forms (Dunphy and Rothman, 1985). These results indicate that in RM(+)19 cells E2 is only slowly transported from ER to Golgi. For comparison, the VSV glycoprotein expressed in BPV-transformed C127 cells is processed to the mature endo H-resistant form in less than 30 min (Florkiewicz *et al.*, 1983). The slow maturation of E2 in RM(+)19 cells could be host cell determined: the influenza virus hemagglutinin (HA) acquired terminal glycosylation with a half-time of more than 2 hr in BPV-transformed C127 cells (Sambrook *et al.*, 1985), while in simian CV-1 cells terminal glycosylation was completed within 40 min (Copeland *et al.*, 1986). However, the slow rate of transport was also observed in various cell lines infected with a vaccinia virus recombinant expressing E2 of FIPV (Vennema *et al.*, submitted). Since coronaviruses bud through pre-Golgi membranes (Tooze *et al.*, 1988) a delayed intracellular transport of free E2 is likely to be a prerequisite for efficient virus assembly.

To explore the potential of our BPV-transformed cell lines as immunogen, we immunized mice with lysates of RM(+)19 cells. We did not determine the amount of E2 in these lysates, since quantitative assays are not yet available. However, there was enough E2 to elicit FIPV neutralizing antibodies. Although the titers were modest, we trust that higher titers can readily be obtained by using larger amounts of cell lysate and optimized immunization protocols. We would like to use RM(+)19 cells for immunization experiments in cats to further study the role of E2 in immunopathology and protection.

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