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Biologically active protease of foot and mouth disease virus is expressed from cloned viral cDNA in *Escherichia coli*

(gene mapping/fusion protein/precursor cleavage/immunoblot)

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ABSTRACT **Foot and mouth disease virus O_1K cDNA had been cloned in *Escherichia coli*. Here we report on *in vitro* recombination of cDNA fragments according to the cDNA restriction map and on expression of viral proteins in *E. coli*. Use was made of the expression vector pPLVP1, which is known to express the virus capsid protein VP1. Recombined cDNAs of various sizes were inserted downstream from the VP1 gene. The constructed plasmids differ from each other in the number of virus genes coding for nonstructural proteins. The effects caused by their expression in *E. coli* are compared. It is shown that the virus protease is expressed in *E. coli* as an active enzyme that recognizes the simultaneously produced virus-specific polyprotein as substrate. The virus protease gene was mapped 5′-adjacent to the virus replicase gene.**

The genome of foot and mouth disease virus (FMDV), a picornavirus, consists of a single-stranded RNA molecule about 8 kilobases (kb) long and of positive polarity. This RNA encodes the four capsid proteins and nonstructural proteins such as the replicase, the protease, VPg, and others of unknown function (1). The coding sequences form a continuous open reading frame of about 7 kb (1), which encodes a single polyprotein of about 250 kilodaltons (kDa). No stable translation product of this size has been observed in virus-infected cells due to posttranslational processing (2, 3) mediated by the viral protease (4) and possibly also host-specific proteases. Initial cleavages have been shown by pactamycin mapping (5) to generate the four precursor proteins P20a, P88, P52, and P100, in 5′–3′ order. The genes encoding the four capsid proteins (6), the replicase (7), and the VPgs (8) have been mapped, whereas the exact position of the viral protease gene is not yet known.

To elucidate the role of the virus protease during maturation of FMDV proteins a system is required that (*i*) separates the virus protease activity from the host-specific protease activities and (*ii*) is suitable for protein processing but not for virus replication. The establishment of such a system is reported here. Use was made of the previously described *Escherichia coli* clones that carry FMDV O_1K-specific cDNA (9). The same report described subcloning of the gene encoding the capsid protein VP1 into a vector that enables the expression of VP1 in *E. coli*.

cDNA fragments were recombined *in vitro* according to the cDNA restriction map so that a series of constructions was obtained (Fig. 1*B*). They differ from each other only in the number of virus genes downstream from the gene encoding protein VP1. After the insertion of these fragments into the VP1 expression vector the effects caused by expression of the genes 3′-adjacent to the VP1 gene were compared *in vivo*. In this system, the protease gene is expressed in *E. coli* and its position is mapped on the viral genome. Furthermore, the enzyme is biologically active. It cleaves the FMDV-specific protein into products of defined size.

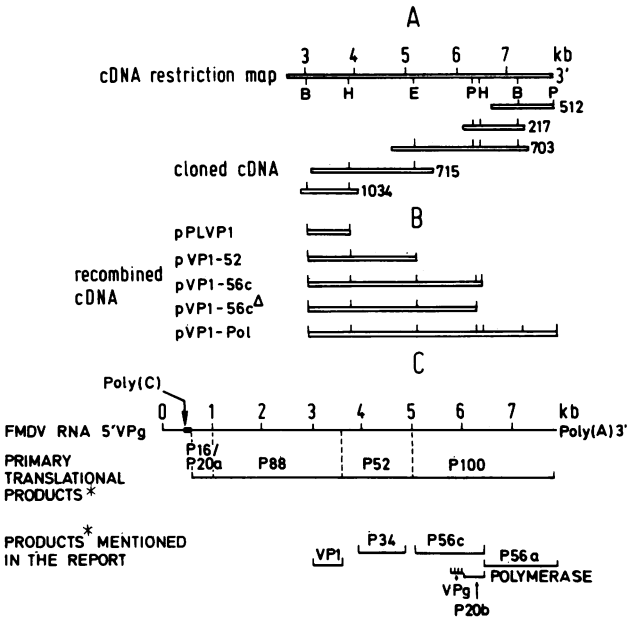

FIG. 1. Correlation of FMDV cDNA fragments to the physical map of FMDV. (*A*) Localization of the cDNA fragments present in *E. coli* clones according to the restriction map of FMDV cDNA essentially as described (9). (*B*) Recombination of genome fragments outlined in Fig. 2. (*C*) FMDV cDNA restriction map is correlated to a recent version (27) of the physical map of FMDV (1). Lengths of RNA and cDNA are given in kb, using the numbering system of Küpper *et al.* (9). The relevant cleavage sites for restriction enzymes (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I) are indicated.

*Applying the picornavirus nomenclature proposed at the third Meeting of the European Study Group on Molecular Biology of Picornaviruses (1983, Urbino, Italy). The new notation is given in parentheses following the current name of the protein: P20a (L); P16 (L′); P88 (P1); P52 (P2); P100 (P3); VP1 (1d); P34 (2c); P56c (3abc); VPg (3b); P20b (3c); and P56a (3d).

MATERIALS AND METHODS

Expression System. Plasmid pPLVP1 has been constructed from pPLc24 (10) by insertion of the cloned cDNA fragment that encodes the major part of capsid protein VP1 and has been found to produce VP1-specific antigenic determinants (9). The FMDV reading frame is in phase with that of the 5′-adjacent gene fragment of phage MS2 replicase, which has all signals necessary for initiation of translation, and is continued into the 3′-adjacent vector DNA for 13 additional codons. Transcription depends on the leftward promoter (p_L) of bacteriophage λ but is blocked when the λ repressor cI is

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Abbreviations: FMDV, foot and mouth disease virus; kb, kilobase(s); kDa, kilodalton(s); bp, base pair(s).

present. Repression of gene expression was desired when recombinant DNA had to be cloned and isolated. These experiments were done in *E. coli* K-12 ΔH1 Δ*trp* (11) that contain the gene for the repressor cI integrated in the genome. Expression assays were done with *E. coli* K-12 C600 transformants that harbor plasmid pcI857 (12). These transformants produce the λ repressor cI857 (13) at 28°C but not at 42°C.

Plasmid Constructions. See Fig. 2. We applied standard procedures for transformation (14) (except that it was done at 28°C), isolation of plasmids (15), restriction enzyme analysis (as recommended by the suppliers), and *in vitro* DNA recombination (16). DNA fragments were separated in standard agarose gels (1%). Fragments electroeluted from agarose into dialysis bags were concentrated by precipitation with ethanol, dissolved (30 mM Tris·HCl/5 mM EDTA/50 mM NaCl, pH 7.5) and stored at 4°C or below until use. Colonies were screened by *in situ* hybridization (17) using ^{32}P-labeled (18) FMDV-specific DNA fragments as probes. Correct orientation of inserts was determined by restriction endonuclease analysis.

The cloning and growth of recombinant bacteria were carried out under L3B1 safety conditions as advised by the German Zentrale Kommission für biologische Sicherheit.

Radiolabeling of Proteins. *E. coli* cells (30 ml) were cultured overnight at 28°C, washed twice with chilled M9 buffer (18.7 mM NH_4Cl/22 mM KH_2PO_4/33 mM Na_2HPO_4/0.2 mM $CaCl_2$) and suspended in 5 ml of M9 buffer warmed to 42°C. ^{14}C-Labeled histidine, leucine, threonine, phenylalanine, and proline (Amersham; 1 μCi each; 1 Ci = 37 GBq) were added at 40 nmol each and the bacterial suspension was incubated at 42°C for 30 min. The cells were collected, resuspended in 1% $NaDodSO_4$/phosphate-buffered saline (phosphate-buffered saline is 137 mM NaCl/3 mM KCl/6 mM Na_2HPO_4/1 mM KH_2PO_4, pH 7.2) and boiled for 10 min. Aliquots were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (PAGE) (19) as described (20). The gels were dehydrated in dimethyl sulfoxide, soaked in a solution of

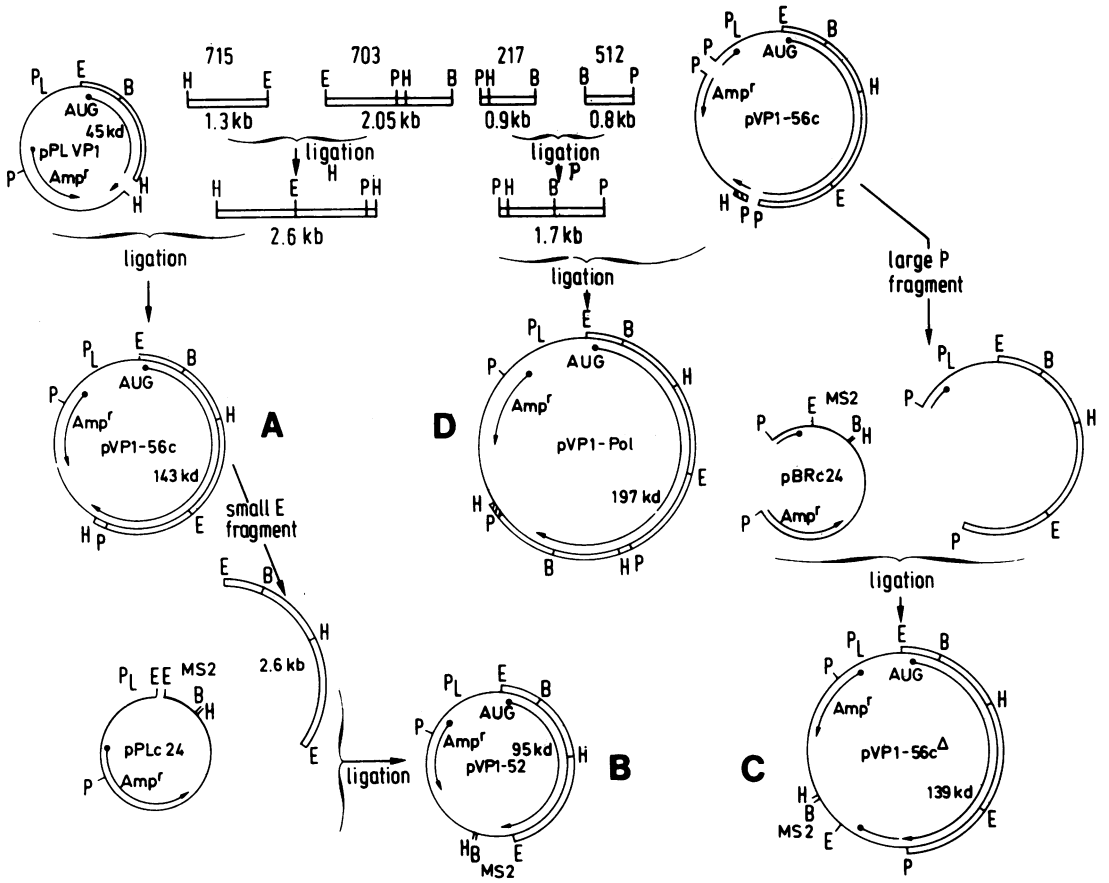

FIG. 2. Construction of recombinant plasmids used in this work. FMDV O_1K-specific DNAs are indicated by double lines, vector DNAs are single lines. The lengths of relevant DNAs are indicated in kb. Letters used to designate restriction sites are the same as in Fig. 1, as well as correlation of FMDV O_1K-specific DNA fragments to the cDNA restriction map. Arrows indicate the orientation of open reading frames. Those designated Amp^r encode β-lactamase. The initiation codon AUG indicates that transcription depends on p_L. E-B segments designated MS2 are identical to those containing initiation codons but are disconnected from p_L. (*A*) Construction of pVP1-56c; the *Hin*dIII/*Eco*RI fragment isolated from plasmid pFMDV715 (Fig. 1*A*) was ligated to the *Eco*RI/*Bam*HI fragment from plasmid pFMDV703. The resulting high molecular weight concatemers were digested with *Hin*dIII. Among others, molecules should be present that consist of *Hin*dIII/*Eco*RI fragments from both plasmids pFMDV715 and pFMDV703, joined via the *Eco*RI site. Without purification the DNA fragments were ligated into the *Hin*dIII site of vector pPLVP1. (*B*) Construction of pVP1-52; the small *Eco*RI fragment of pVP1-56c containing MS2 DNA and FMDV DNA encoding VP1 and P52 was isolated and inserted into the *Eco*RI site of pPLc24. (*C*) Construction of pVP1-56cΔ; plasmid pBRc24 derives from pPLc24 (*B*) by replacing the small *Pst* I/*Eco*RI fragment with the 750-base-pair (bp) *Pst* I/*Eco*RI fragment of pBR322 (25). Thus p_L is eliminated and the β-lactamase gene is restored. The large *Pst* I fragment of pVP1-56c containing p_L, MS2 DNA, and all FMDV DNA except the 3′-terminal 148-bp *Pst* I/*Hin*dIII fragment was isolated and inserted into the *Pst* I site of pBRc24. (*D*) Construction of pVP1-Pol; the FMDV-specific *Pst* I/*Bam*HI fragments were isolated from pFMDV217 and pFMDV512. They represent the 3′-end of the cDNA (Fig. 1). They were ligated at their common *Bam*HI site and the product was subcloned as a *Pst* I insert, using pBR322 as vector, and subsequently inserted into the *Pst* I site of the FMDV-specific sequence in pVP1-56c. This plasmid contains the FMDV genes from the VP1 gene continuous with the 3′-end, including the RNA-dependent RNA polymerase gene, the stop codon of the viral reading frame, and the poly(A) tract. The FMDV O_1K-specific P–H segment that is out of frame is shown hatched. kd = kDa.

20% 2,5-diphenyloxazole in dimethyl sulfoxide, and washed with water (21). Dried gels were exposed to Kodak X-Omat AR film at −70°C.

Antibody against FMDV O_1K. Goat antisera to FMDV O_1K were a generous gift of K. Strohmaier (Tübingen). IgG was purified according to the protocol of Harboe and Ingild (22). From 4 mg of goat IgG thus obtained, *E. coli*-specific antibodies were removed by incubation (20 hr/20°C) with 8 mg of total cell extracts prepared from *E. coli* cotransformed with pcI857 and pPLc24 that had been coupled to cyanogen bromide-activated Sepharose 4B (23) in a 1-ml reaction volume. Nonbound antibodies were present in the supernatant after centrifugation. It was used directly for immunoassays.

Immunoblotting. Total cell extracts separated by NaDodSO_4/PAGE were analyzed by immunoblotting essentially as described (ref. 24; all steps were performed at room temperature). The gels were electrophoretically blotted for 12–16 hr onto nitrocellulose filters (Schleicher & Schüll, 0.45 μm pore diameter). The filters were soaked in 1% gelatin/50 mM Tris·HCl/150 mM NaCl/5% bovine serum albumin fraction V (Sigma)/0.02% Nonidet P-40 (Bethesda Research Laboratories), pH 7.5, for 20 min with one change of buffer to saturate additional protein-binding sites. They were incubated for 1 hr with purified goat anti-O_1K IgG diluted with 3% bovine serum albumin/0.25% gelatin/50 mM Tris·HCl/150 mM NaCl/0.025% Nonidet P-40, pH 7.5. The filters were washed in 0.1% bovine serum albumin/0.25% gelatin/50 mM Tris·HCl/150 mM NaCl/0.025% Nonidet P-40, pH 7.5, for 45 min (the buffer was changed three times) and incubated for 1 hr with anti-goat IgG coupled with peroxidase (Miles, diluted as above). The filters were washed as above and once with phosphate-buffered saline and then incubated with 0.05% 3,3′-diaminobenzidine/0.03% H_2O_2/0.1 M sodium phosphate, pH 7.2. The reaction was stopped by washing the filter twice with 0.1 M sodium phosphate, pH 7.2.

RESULTS

Construction of pVP1-56c and pVP1-52. The *Hin*dIII/*Eco*RI cDNA fragments of pFMDV715 and pFMDV703 were inserted adjacent to the VP1 gene into pPLVP1 (Fig. 2*A*) so that the FMDV O_1K-specific DNA fragments were aligned according to the FMDV cDNA restriction map. The recombinant plasmid contains the FMDV genes encoding capsid protein VP1 and the precursor proteins P52 and P56c (Fig. 1). It is designated pVP1-56c.

Plasmid pVP1-52 was constructed by inserting the 2.6-kb *Eco*RI fragment of pVP1-56c into the *Eco*RI site of plasmid pPLc24 (Fig. 2*B*). The plasmid pVP1-52 contains the FMDV genes encoding VP1 and the precursor P52 (Fig. 1).

Molecular Weight Calculation for the Virus-Encoded Proteins. Compared to pPLVP1 the reading frame in pVP1-52 is elongated by 1.3 kb and that of pVP1-56c by 2.6 kb (Fig. 2 *B* and *A*). From the known nucleic acid sequences (25, 26) it is concluded that translation is continued into the vector for at most 50 codons. Thus the coding capacity of pVP1-52 corresponds approximately to a 95-kDa protein and that of pVP1-56c to a 143-kDa protein (Fig. 3). The presence of the gene encoding the virus protease in one or both constructions is suggested because its locus is reported to be somewhere in the middle of the FMDV genome (4). Gene expression therefore may result in a protease that, if active in *E. coli*, should be able to process its polyprotein substrate as it does in eukaryotic FMDV expression systems (2, 3). In this case cleavage of virus-specific protein to products lower in molecular weight is suggested.

Gene Expression. Protein synthesis depending on pPLVP1, pVP1-52, pVP1-56c, or pPLc24 in transformed *E. coli* was induced by shifting the temperature from 28°C to 42°C. Aliquots of each culture were subjected to NaDodSO_4/PAGE and proteins in the gel were visualized by staining with Coomassie brilliant blue (Fig. 4*A*). Compared to cells carrying pPLc24 (background control) pPLVP1-containing cells exhibit increased amounts of 45-kDa protein. An extra 95-kDa protein was observed in pVP1-52-containing cells and a 34-kDa protein in pVP1-56c-containing cells. Whether these proteins exhibit virus specificity was analyzed by immunoblotting of a parallel gel with goat anti-O_1K as a probe (Fig. 4*B*). In fact, the 45-kDa (pPLVP1), the 95-kDa (pVP1-52), and the 34-kDa protein (pVP1-56c) contain VP1-specific antigenic determinants in contrast to the control (pPLc24).

Evidence for an Additional Virus-Specific Cleavage Product. The production of a 95-kDa VP1-specific protein in pVP1-52 cells confirms the calculation from the open reading frame (Fig. 3). This is not the case with pVP1-56c, since a 34-kDa VP1-specific protein was obtained by expression of a sequence with the capacity to code for a 143-kDa protein. A defect in the reading frame of pVP1-56c is excluded by the fact that pVP1-52 has been constructed from pVP1-56c (Fig. 2*B*) and produces a 95-kDa protein. This suggests that (*i*) pVP1-56c contains the protease gene and (*ii*) its expression results in an active enzyme. This gene is 3′-proximal to the genes already present in pVP1-P52. The experiment shows further that expression of adjacent genes 3′ to the protease gene is not required for the expression of active protease.

Translation of the protease gene is possible only after translation of the 5′-preceding genes (VP1 and P52; see Fig. 1). Therefore, the VP1-specific 34-kDa protein cannot be the only translation product. Since antibodies directed against virus-specific nonstructural proteins were not available, it was investigated whether these proteins can be specifically labeled *in vivo*. ^{14}C-labeled amino acids were added to cultures of cells carrying pVP1-56c after induction of expression. Cultures of cells carrying pPLc24, pPLVP1, and pVP1-52 served as controls. Aliquots of each culture were subjected to NaDodSO_4/PAGE. Fluorography of the gel (Fig. 4*C*) revealed incorporation of ^{14}C-labeled amino acids into the pPLVP1-specific 45-kDa protein, the pVP1-52-specific 95-kDa protein, and the pVP1-56c-specific 34-kDa protein. Here, protein in the 26-kDa position is more intensively radiolabeled than protein in the background control (pPLc24), indicating that more than one protein bands at the same position. The preferential synthesis after induction of gene expression strongly suggests the virus specificity of one of the 26-kDa proteins. Due to the background of radiolabeled proteins and the unknown size of protease products in the bacterial system, it is hard to judge which other bands expressed from pVP1-56c might be virus specific.

Mapping of the Protease Gene. By recombination of DNA the 148-bp *Pst* I/*Hin*dIII fragment was deleted from the 3′ end of the FMDV-specific insert in pVP1-56c (pVP1-56cΔ; Fig. 2*C*). It was of interest to see whether the deletion affects the protease gene. In this case the molecular weight of the pVP1-56cΔ-specific translation product should be very similar to that deduced from the open reading frame in pVP1-56c (Fig. 3). Induction of pVP1-56cΔ-dependent gene expression resulted in the production of a 139-kDa VP1-specific protein as shown by immunoblotting of a NaDodSO_4/polyacrylamide gel with goat anti-O_1K as probe (Fig. 4*D*). The experiment with cells containing pVP1-56c served as a control. Hence, deletion of the 148-bp *Pst* I/*Hin*dIII fragment accounts for the loss of virus protease activity. Correlation with the physical map (Fig. 1) leads to the conclusion that the COOH-terminal part of protein P56c is the protease.

The above result was controlled with another construction. The 148-bp *Pst* I/*Hin*dIII fragment was separated from the 5′-preceding FMDV cDNA in pVP1-56c by insertion of a 1.7-kb *Pst* I fragment into the *Pst* I site. The 1.7-kb *Pst* I

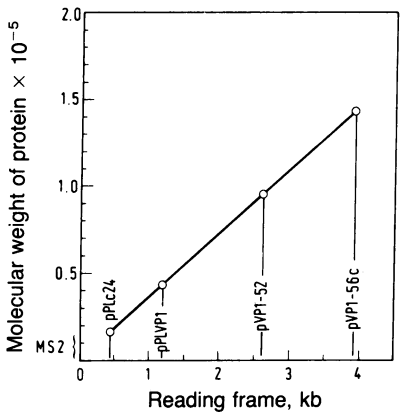

FIG. 3. Correlation of open reading frames to the size of translated proteins. The molecular weight of the protein encoded by vector pPLc24 (10) is deduced from the nucleic acid sequence (26). The first 99 amino acid residues are expressed from the gene that encodes the replicase protein of bacteriophage MS2. Plasmid pPLVP1 has been shown to exhibit an open reading frame of 396 codons and to encode a 45-kDa protein (9). Insertion of additional 1.3-kb FMDV-specific DNA in the case of pVP1-52 (Fig. 2*B*) and 2.6-kb FMDV-specific DNA in the case of pVP1-56c (Fig. 2*A*) creates open reading frames of 829 and 1262 codons. The open reading frame is continued into 3'-adjacent DNA (25, 26) but does not exceed 50 codons. The molecular weights of the gene products thus can be predicted by assuming a mean molecular weight of 110 for each amino acid. pVP1-52 should encode a virus-specific protein of about 95 kDa and pVP1-56c one of about 143 kDa.

fragment consists of FMDV 3'-specific cDNA. The 148-bp *Pst* I/*Hin*dIII fragment is now contributed by pFMDV217 (Figs. 1*A* and 2*D*) and not by pFMDV703 as in pVP1-56c. Plasmid pVP1-Pol contains a 5-kb insert of FMDV cDNA that encodes all nonstructural proteins additional to VP1. The VP1-specific proteins expressed by pVP1-Pol and pVP1-56c were compared by immunoblotting using anti-O_1K as probe. As can be seen, both constructions produce 34-kDa VP1-specific proteins (Fig. 4*E*). The control (pPLVP1) produces the 45-kDa protein. Detailed analysis of the products in *E. coli* carrying pVP1-Pol will be described elsewhere.

All VP1-specific proteins are detected in the membrane fraction of bacterial extracts (data not shown). This may explain why these proteins are protected from rapid degradation by *E. coli* proteases.

DISCUSSION

It is shown in this report that the FMDV O_1K-specific protease is expressed as an active enzyme from cloned FMDV O_1K-specific cDNA in *E. coli*. Activity of the protease is demonstrated by conversion of its natural substrate, which is produced simultaneously. One of the products is a 34-kDa protein. It was detected by the use of antibodies directed against VP1-specific antigenic determinants present in this protein. Another protein of 34 kDa has been described as being generated from the precursor P52 (27). Due to comigration with the VP1-specific 34-kDa protein in the gels (Fig. 4) its possible presence cannot be demonstrated by this technique. However, presence of a further virus-specific protein that is 26 kDa is highly suggested from the *in vivo* ^{14}C-labeling experiment. On the basis of the construction of pVP1-56c (Fig. 2*A*), a 26-kDa protein can be explained as a fusion protein consisting of protein P20b (Fig. 1) and a vector-encoded COOH terminus.

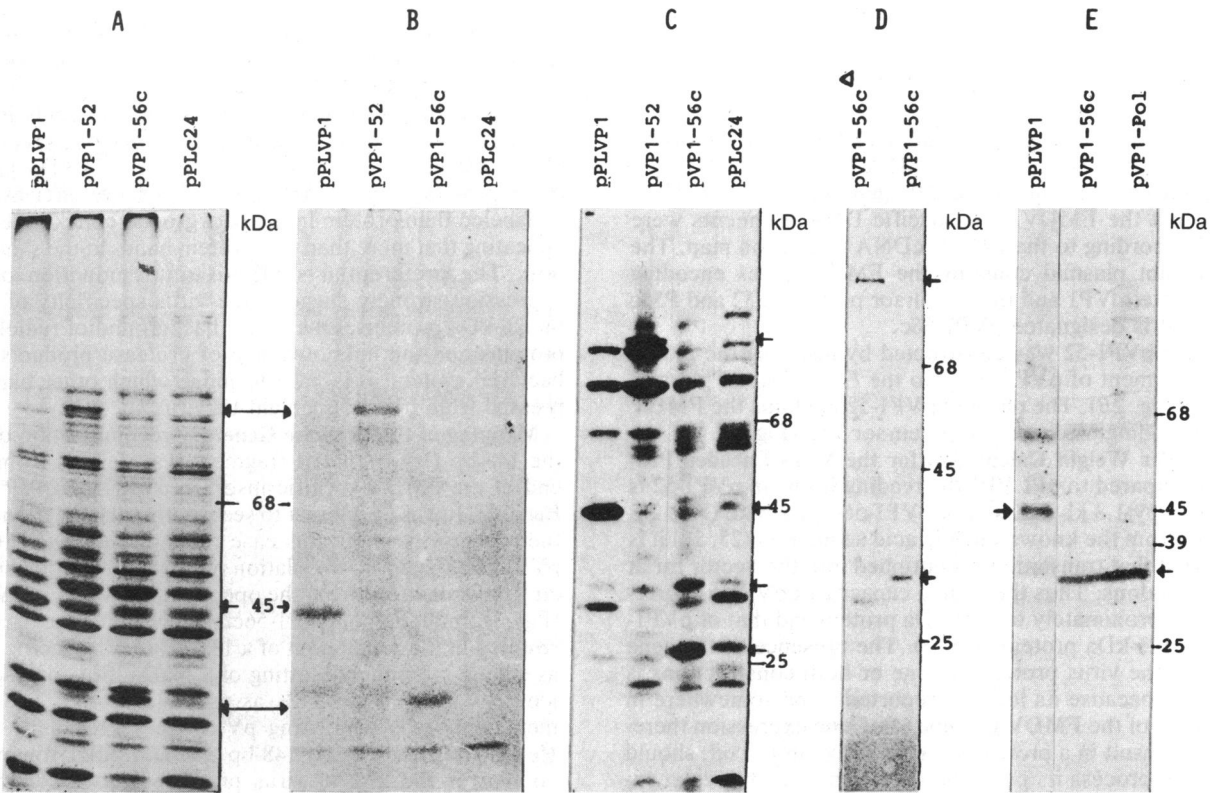

FIG. 4. Size determination of translation products. Cells were cultured overnight at 28°C. Expression was induced by 1:5 dilution with medium (1% Bacto-tryptone/0.7% yeast extract/0.5% NaCl) prewarmed to 42°C and was stopped 30 min later by chilling the cultures in ice. Total *E. coli* proteins were subjected to $NaDodSO_4$/PAGE (11% gel). (*A*) Coomassie brilliant blue pattern; (*B*, *D*, and *E*) Immunoblot patterns obtained by using goat anti-O_1K as probe. (*C*) Fluorogram of ^{14}C-label incorporated *in vivo*. The positions of marker proteins are indicated and their molecular masses are given in kDa. Arrows indicate relevant proteins.

Expression of the protease activity which is inhibited by Zn ions (data not shown) depends on pVP1-56c and pVP1-Pol. It is not expressed by pVP1-56cΔ, which differs from pVP1-56c by a 148-bp deletion. It accounts for the loss of viral protease activity. An essential part of the protease gene must be here. The location of the 148-bp DNA fragment in the FMDV cDNA is known from the restriction map (Fig. 1). Its correlation to the physical map (27) suggests that the protein expressed from this gene is P20b. The products expressed from the 3'-proximal virus genes therefore are $(VPg)_3$–protease–polymerase (7, 8). The protease gene has been mapped 5'-adjacent to the polymerase gene also in other picornavirus genomes: encephalomyocarditis virus (28) and poliovirus (29). Data obtained earlier (30), however, have suggested the poliovirus protease to be positioned elsewhere.

pVP1-56c encodes, at least partially, the precursor proteins P88, P52, and P100 (Fig. 1). The translation product therefore exhibits the primary cleavage sites between these precursors (27) additional to sites that are cleaved during processing of the precursors into the mature proteins. The VP1-specific 34-kDa protein expressed from pVP1-56c has to be related to the COOH-terminal part of precursor P88 since this is also VP1 specific. As deduced from the nucleic acid sequence (26, 31) and the physical map (27), a fused protein consisting of the MS2 replicase NH_2-terminal part and the VP1 COOH-terminal part is 34 kDa and excised from the 143-kDa total protein if sites known to be cleaved in the viral polyprotein are used. All other explanations for generation of a VP1-specific protein from the 143-kDa protein have to assume either different molecular mass or cleavage at sites that are not used during processing of the polyprotein. Attention therefore should be drawn to the possibility that the precursors P88 and P52 may be disconnected by action of the viral protease.

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