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Molecular cloning and expression of the *VP1* gene of foot-and-mouth disease virus C_1 in *E. coli*: effect on bacterial cell viability

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Summary. The *VP1* gene of foot-and-mouth disease virus (serotype C_1) has been cloned in *Escherichia coli* Cl^{ts} cells, under the control of the bacteriophage lambda p_L promoter. The expressed VP1 protein was complete and non-fused, and its moleclular weight was indistinguishable from that of the VP1 obtained from virions. Cells harbouring the recombinant vectors exhibited symptoms of plasmid instability and toxicity and died in a few weeks even when never exposed to inducing conditions. A new plasmid clone in which a segment of the *VP1* gene was fused with contiguous genes of the viral genome was very stable. The expressed partial VP1 protein contains the two major immunogenic domains of the virion. This system can be used as a tool to design an immunogenic VP1, and to explore possible synthetic vaccines against foot-and-mouth disease.

Introduction

The foot-and-mouth disease virus (FMDV) is a member of the Picornaviridae family, and the causal agent of one of the economically most important diseases in the world, affecting cloven-hoofed animals (Pereira 1981). Chemically inactivated virions in appropriate adjuvants are currently administered as a vaccine. By means of vaccination programmes and slaughtering of infected and contact animals, the disease has been successfully controlled in Europe. However, outbreaks of A, O and C serotypes occasionally occur, even under strict veterinary control. Some of them have been traced to vaccine production or administration (Beck and Strohmaier 1987), pointing out the risks of using inactivated virions as vaccine components. Poor protection of vaccine strains against antigenically new circulating variants is also a cause of vaccination failures.

Much effort is being made to develop a synthetic vaccine for FMD. Isolated VP1 capsid protein as well as recombinant proteins and synthetic peptides that reproduce the two major antigenic sites of the virus (amino acids 140–160 and 200–213 of VP1) are able to elicit neutralizing antibodies. However, the level of immunoprotection attained has not been sufficient to allow the substitution of the classic vaccines by synthetic antigens. The initial experiences with such new vaccines against FMD and other diseases indicate that protection may be affected by the amino acid sequences around the antigenic sites (van der Werf et al. 1990), by the distribution of the epitopes in the molecule (Cox et al. 1988; Agterberg et al. 1990) and by foreign T-cell epitopes added to the antigen (Francis et al. 1987). Thus, vaccine development requires not only reproducing viral protein sequences, but also testing a large number of modified ones to find the optimal conformation for the immunogenicity of the epitopes. For RNA viruses, the vaccine components should be designed from sequences as similar (or cross-reactive) as possible to those of the circulating, relevant variants.

In this work, we have obtained stable *Escherichia coli* clones that express the carboxy-terminal half of VP1 of FMDV serotype C_1 (isolate C-S8, 1970) (Sobrino et al. 1986; Martínez et al. 1988; Villaverde et al. 1988). Due to the information accumulated in recent years on the antigenic properties of C-S8 (Mateu et al. 1990, and references therein), and to the fact that the last FMDV C_1 viruses circulating in Spain and other locations in Europe were closely related to C-S8, we took the sequence of VP1 from C-S8 as the starting point to develop clones of *E. coli* expressing antigens of serotype C_1. In anticipation of the necessity for medium- or large-scale production of this plain or later modified antigens, we decided to use a temperature-inducible system that could be easily adapted to a fermentation process. These recombinant plasmids are stable, produce protein reactive with specific anti-FMDV monoclonal antibodies, can be genetically manipulated, and can serve for producing large amounts of antigen to be evaluated as a possible vaccine component.

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Materials and methods

Viruses, plasmids and bacterial strains. FMDV was prepared as described (Mateu et al. 1987). Plasmids pAZe3 and pAZe3ss (Zaballos et al. 1987) are expression vectors carrying the lambda p_L promoter, a ribosome binding site and an unique *Nco*I site. In pAZe3 the *lacZ* gene downstream of the p_L promoter permits screening of recombinant clones in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates. Digestion of both plasmids with *Nco*I and filling in the resulting cohesive ends with DNA pol I Klenow fragment, yields a terminal ATG codon able to initiate translation of the protein encoded by the contiguous sequence. pBR-VFA-C_1B75 is a cDNA clone representing 4.2 kb of the C-S8 genome, and contains some structural and non-structural genes (Villanueva et al. 1983). *E. coli* strain M72 is a *lacZam Δbio-uvrB Δtrp-EA2* lysogen of lambda *Nam7Nam53c*I857 (ts) *ΔH1*. BF1000 is a Tn*10*::*recA142* derivative of M72, obtained by transduction with bacteriophage P1 as previously described (Miller 1972). Luria-Bertani (LB) and M9 (Maniatis et al. 1982) culture media were used to grow the cells.

Enzymes. Restriction enzymes, DNA polI Klenow fragment, RNase inhibitor, alkaline phosphatase, reverse transcriptase, T4 DNA ligase, polynucleotide kinase, nuclease S1, RNase A, X-gal, 2-nitrophenyl-β-D-galactopyranoside (ONPG) and antibiotics were from Boehringer Mannheim (FRG) and were used as directed by the manufacturer. Radiolabelled nucleotides were from Amersham International (UK). The β-galactosidase assay was done as described peviously (Miller 1972).

Construction of plasmids for the expression of the complete and non-fused VP1 gene. For this construction two synthetic oligonucleotides were used as primers to obtain a VP1 protein with the same sequence as in the virus, the only difference being an additional methionine at the N-terminal end, encoded by the ATG codon contained in the *Nco*I site of the plasmid. AVcons1 is a 24-mer oligonucleotide (5′-TCATCACAGCAGTTGTTTTGCAGG-3′) carrying at the 5′ end a sequence complementary to two stop codons, not present in the viral genome. The other 18 residues are complementary to the 3′ end of the VP1 gene. AVcons2 is a 21-mer oligonucleotide (5′-ACTACGACCACTGGTGAATCT-3′) that reproduces the 5′ end sequence of the VP1 gene from its first codon (compare with the viral sequence in Martínez et al. 1988). Synthesis of the first strand cDNA was performed using 2 μg of RNA template and 8 μg of primer AVcons1 hybridized by boiling for 2 min followed by slow cooling in 100 mM TRIS, pH 8.3, 140 mM KCl, 16 mM $MgCl_2$, in the presence of 20 units of ribonuclease inhibitor. The hybrid was recovered by ethanol precipitation. Polymerization was done for 135 min at 42° C, in 0.1 M TRIS, pH 8.3, 10 mM $MgCl_2$, 20 mM KCl, 0.1 M EDTA, 1 mM dithiothreitol (DTT), 0.8 mM each of dATP, dTTP, and dGTP, 0.4 mM dCTP, 25 units of reverse transcriptase, 40 units of ribonuclease inhibitor and 10 μCi of [α-^{32}P]dCTP (approx. 3000 Ci/mmol), in a final volume of 70 μl. The synthesis was stopped by heating the tube at 90° C for 5 min. Phenol-chloroform extraction, ethanol precipitation, RNAse treatment and second-strand synthesis with Klenow fragment using AVcons2 as a primer were performed according to standard procedures (Maniatis et al. 1982). Any remaining overhanging 3′ ends resulting from the first strand synthesis were removed by digestion of 8 ng cDNA with 3 units of S1 exonuclease at 37° C in 200 μM NaCl, 50 mM sodium acetate, 1 mM $ZnSO_4$, and 0.5% glycerol for 30 min in 30 μl final volume or with mung bean nuclease treatment as described previously (Jobling et al. 1988). Before use, DNA was incubated at 16° C for 30 min with 10 units of Klenow enzyme in the presence of 4 mM of the four deoxyribonucleotides, to ensure the absence of cohesive ends that could have been generated by an extended action of the single strand exonucleases. cDNA was ligated with pAZe3, prepared as described above, at a molar ratio of 1:1. M72 Cl^{ts} competent cells were prepared according the protocol number 3 of Hanahan (1985) and transformed to ampicillin resistance. Heat shock was done at 34° C for 2 min. White colonies in LB X-gal plates were replica-plated in duplicate in LB plates and transferred to nitrocellulose filters, where they were probed separately with both 5′-^{32}P-radiolabelled AVcons1 and AVcons2 oligonucleotides as described in Maniatis et al. (1982). The hybridization temperatures were 65° C and 57° C, respectively.

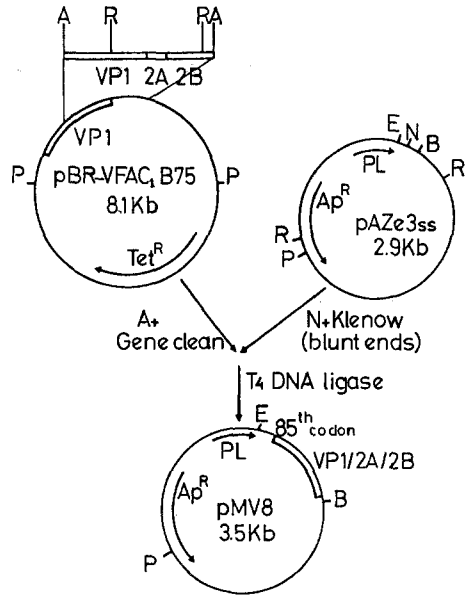

Fig. 1. Construction of plasmid pMV8. Details of the manipulations are given in Materials and methods: *A*, *Alu*I; *B*, *Bam*HI; *E*, *Eco*RI; *N*, *Nco*I; *P*, *Pst*I; *R*, *Rsa*I

Construction of plasmids for the expression of a partial VP1 protein. pBR-VFA-C1B75 was digested to completion with *Alu*I. Two restriction fragments of about 0.6 kb co-migrated in 2% agarose gels, and were recovered using Geneclean. One of them spanned the *VP1* gene from nucleotide 255 (numbering of Martínez et al. 1988), the 2A gene (encoding a peptide of 16 amino acids) and a segment of 234 nucleotides from the 2B gene. Position 255 of the *VP1* gene is the first nucleotide of the 85th codon of the gene (Fig. 1). The eluted DNA was ligated with pAZe3ss, opened with *Nco*I, filled in with Klenow fragment, and transformed into BF1000 cells. The presence of the *VP1 Alu*I-*Alu*I fragment in the correct orientation was verified by restriction analysis using *Rsa*I, which has two asymmetric target sequences in the insert and two additional ones in the vector.

Western blot of recombinant proteins. Whole cell extracts of induced and non-induced cultures were prepared by boiling the pellet of 1.5 ml of culture in denaturing sample buffer for 5 min. Western blot procedures and 7FC12 and SB10 monoclonal antibodies recognizing the 140–160 VP1 epitope have been described elsewhere (Mateu et al. 1987, 1988). Heat induction was done by shifting to 42° C during 90 min when cultures reached an optical density at 550 nm of 0.20.

Results and discussion

Cloning and expression of the C-S8 VP1 gene

We first attempted to obtain a cDNA clone representing the entire *VP1* gene, to express a complete and non-fused VP1 protein. The only restriction was given by the fact that the viral RNA is translated into a polyprotein of 260 kDa, which is self-cleaved by several internal viral proteases. Thus, an additional ATG codon

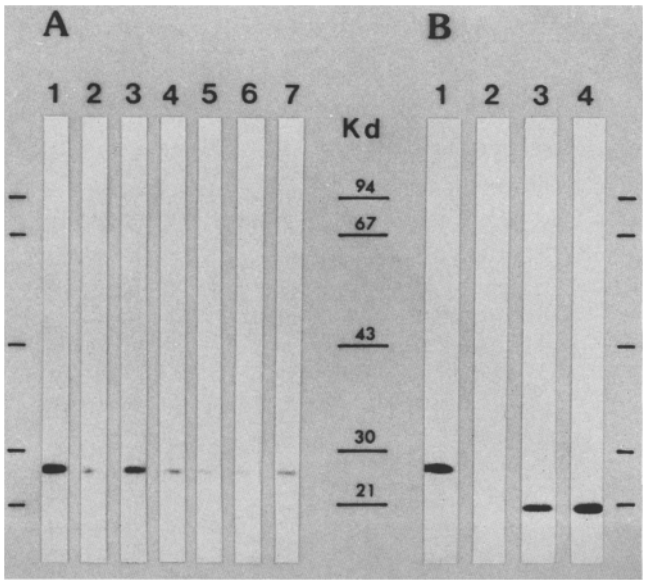

Fig. 2 A, B. Reactivity of cell extracts with monoclonal antibodies against foot-and-mouth disease virus 140–160 epitope in Western blot assays. **A** C-S8 virions (*lane 1*); different clones expressing the complete and non-fused VP1 protein (*lanes 2–7*). **B** C-S8 virions (*lane 1*); BF1000/pMV8 growing at 30° C (*lane 2*), after 1 h at 42° C (*lane 3*) and after 2 h at 42° C (*lane 4*)

needed to be supplied at the 5′ side of the first nucleotide of the VP1 gene. This resulted in an extra methionine residue at the N-terminal end of the predicted molecule. A total of 276 white transformant colonies were probed for the presence of both the *VP1* cDNA ends. In six positive clones, Western blot analysis revealed the presence of a protein of about 29 kDa, indistinguishable from the VP1 of virions, that reacted with the monoclonal antibody 7FC12 (Fig. 2A). A unique band suggests the absence of proteolytic activity against the expressed VP1.

Observations with the optical microscope revealed the presence of long filaments in some of the bacterial cultures. After a few days, the colonies of the recombinant strains carrying the *VP1* gene had difficulties in growing at 30° C both in solid and liquid medium in the presence of ampicillin. Titres of viable cells were from 1% to 10% lower in the presence than in the absence of ampicillin. Plasmid instability appeared to be associated with the presence of the VP1 DNA or protein. We were not able to obtain tertiary cultures producing VP1. Ampicillin-resistant cells were not recovered from glycerolates of primary cultures of these strains, stored at −20° C or −70° C, although normal growth took place in the absence of the antibiotic. It was surprising that those cells were never grown at temperatures higher than 30° C, and so the VP1 expression was expected to be repressed.

Such plasmid instability disqualifies this construction for further studies. In consequence, new constructions were made deleting the apparently less immunogenically relevant VP1 sequences. A segment including the 3′ half of the *VP1* gene that encodes the two major antigenic sites of VP1 protein and a small portion of the contiguous genes was removed from pBR-VFA-C_1B75 by *Alu*I digestion, and cloned in the *Nco*I site of pAZe3ss (Fig. 1). Strain BF1000, a RecA142 derivative of M72 was used to prevent the possible RecA-mediated cleavage of the Cl^{ts} repressor under non-inducing conditions. The resulting pMV8 plasmid is very stable in both BF1000 and M72 strains. The fused protein was expressed by temperature induction in BF1000, and a unique protein band with a molecular weight of 20 kDa was detected by Western blot analysis (Fig. 2B). The expected molecular weight according to the nucleotide sequence was about 30 kDa. This difference could be the result of an internal 2A-mediated cleavage at the 2A/2B junction (Clarke and Sangar 1988), which would remove the 2B fragment from the C-terminal end of the fused protein. Truncated VP1 is estimated to be about 1% of the total protein 2 h after induction. No symptoms of toxicity, or filamentation or loss of viability in the presence of ampicillin have been observed in any such cultures grown at appropriate temperatures.

Effect of VP1 gene expression on cell viability

The dramatic behaviour displayed without exception by all of the clones expressing the entire VP1, could be explained if we assume that:

1. Some domains of the C-S8 VP1 protein are toxic for *E. coli* cells (as reported for other eucaryotic and viral proteins).
2. A low basal level of *VP1* transcription in the presence of active CI857 repressor is enough to kill the cells even under non-inducing conditions.

In fact, the β-galactosidase assay of the M72/pAZe3 strain revealed a very low (but detectable) level of transcription from the p_L promoter at 30° C (results not shown), although we were not able to detect VP1 antigen in cell extracts at this temperature. The results of recent studies indicate that the full repression of the cI857-p_L system is only achieved at temperatures up to 29° C, and that at 32° C it is possible to find small amounts of the gene product (Lowman and Bina 1990). Since the *VP1* gene sequence is present in pBR-VFA-C_1B75 (a stable, non-toxic plasmid), it is unlikely that the DNA sequence itself was toxic. Instability in the absence of inducer has been also reported in cells harbouring an adenovirus gene controlled by the *lac* promoter (Houde and Weber 1990).

In previous studies on the expression of FMDV VP1 (Kleid et al. 1981; Küpper et al. 1981; Winther et al. 1986) or of large regions of the viral genome (Klump et al. 1984; Strebel et al. 1986; Strebel and Beck 1986; Vakharia et al. 1987; Clarke and Sangar 1988) no toxic effects for bacterial cells were reported. Among others, a possible explanation could be the considerable divergence between the FMDV of O and A serotypes (used by most other workers) and C_1 (Villanueva et al. 1983). The toxic domain of C-S8 VP1 protein must be located in the first N-terminal 84 amino acids, because the absence of its encoding sequence in pMV8 renders the host strains stable. In fact, one of the hypervariable re-

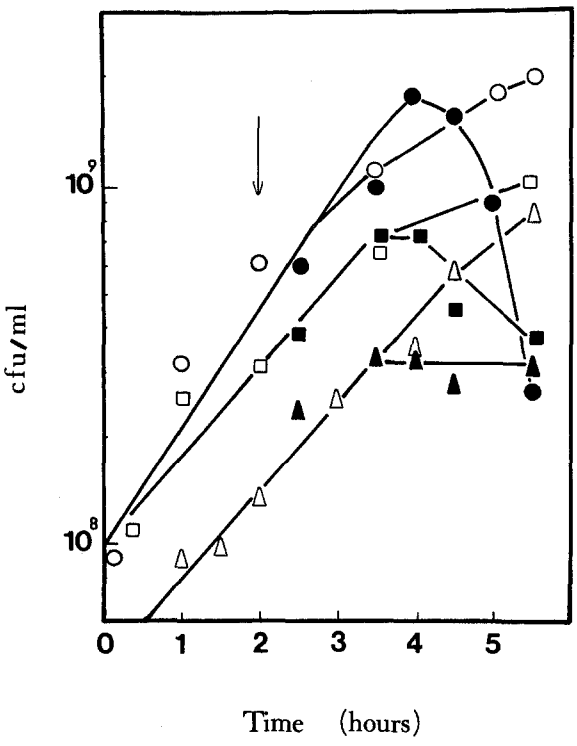

Fig. 3. Cell viability (colony-forming units, cfu) in M72 (●, ○) and BF1000 (■, □) cultures carrying the plasmid pMV8, and in BF1000/pAZe3ss (▲, △). Cells were grown at 30° C (○, □, △) in Luria-Bertani (LB)-ampicillin medium (at 100 μg/ml), and were shifted to 42° C at the time indicated by the *arrow* (●, ■, ▲). Plating was done at 30° C in LB-ampicillin plates

gions of the *VP1* gene is found between nucleotides 120 an 170 (see Fig. 1 in Dopazo et al. 1988).

A prediction of the secondary structure of some C, A and O VP1 proteins was done using the sequence analysis software package (version 6.1) from the University of Wisconsin Genetics Computer Group (Devereux et al. 1984). A predicted α-helix region extending along amino acids 48 to 59 shows a high index of antigenicity and a very great surface probability in C-S8 protein. This short stretch is predicted to be folded into a β-sheet in C_3 Indaial, O_1 Kaufbeuren and A_{24} Cruzeiro VP1 proteins, in which these two parameters have very low values (for the sequences, see Makoff et al. 1982; Kurz et al. 1981). Another C_1 isolated, non-epidemiologically related to C-S8, C_1O (Beck et al. 1983), shows the same pattern of secondary structure prediction as that of C-S8. This observation suggests that a highly hydrophilic domain in the N-terminal half of the FMDV VP1 protein could be exclusive to serotype C_1. No information on the folding of the VP1 peptide expressed in *E. coli* can be inferred from the three-dimensional structure of FMDV virions (Acharya et al. 1989).

The accumulation of the protein expressed from plasmid pMV8 also showed minor toxic effects on cell viability, both in $RecA^+$ and in RecA142 strains (Fig. 3), 2 h after heat induction. However, this fact does not affect normal growth and expression upon induction, even in large volume cultures up to 1 l. These data should be taken into account to subsequently establish fermentation conditions.

Toxicity to *E. coli* cells during expression of recombinant eucaryotic or viral genes has been frequently reported. In some cases, by changing the medium composition (Chatellard and Chroboczek 1989) or cloning the gene into another vector (Maley et al. 1990) cell stability and product yield are improved. When the products are enzymes, the cell death process is associated with the interference in their activity by cell metabolism or damage to cell molecules (Pham and Coleman 1985; Bedouelle et al. 1990). Competence of foreign proteins with ribosomes for binding to mRNA (Miller et al. 1989), and sequence homology with *E. coli* cell enzymes (Yudkin and Harrison 1987) are also proposed mechanisms. Some toxic domains of proteins lacking defined catalytic properties (such as structural proteins) have been mapped by obtaining mutant or truncated gene products that have lost the lethal character (Tsai et al. 1989; Couto and Friedberg 1989; Isaacs et al. 1989). No structural features seem to be shared by all those removed or inactivated domains, including the N-terminal 84 amino acids of C_1 VP1. This fact suggests that in each case, cell death is produced by different mechanisms.

Large-scale production of bioengineered chimeric proteins by means of industrial fermentation is less costly than the large-scale synthesis of peptides. Thus, the analysis reported here on the toxicity of the expressed FMDV proteins to the bacterial system are a prerequisite for the design of possible synthetic antigens that could be stably produced by fermentation procedures.

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