Molecular cloning, sequencing and expression in *Escherichia coli* of the capsid protein gene from rabbit haemorrhagic disease virus (Spanish isolate AST/89)

J. A. Boga,¹ R. Casais,¹ M. S. Marin,² J. M. Martin-Alonso,¹ R. S. Carmenes,¹ M. Prieto³ and F. Parra¹*

¹ Departamento de Biología Funcional, Area de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, 33071 Oviedo, Spain, ² NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, U.K. and ³ Laboratorio de Sanidad Animal, 33299 Gijón, Spain

We describe the cloning, nucleotide sequencing and expression in *Escherichia coli* of the major capsid component (VP60) from the Spanish field isolate AST/89 of rabbit haemorrhagic disease virus (RHDV). The sequence of the 3'-terminal 2483 nucleotides of the genome was found to be 95.4% identical to the German RHDV strain, showing ten changes in the deduced VP60 amino acid sequence. The gene coding for this structural polypeptide has been expressed in bacteria as a β-galactosidase fusion protein or using a T7 RNA polymerase-based system. The VP60 fusion protein showed only partial antigenic similarity with native VP60 and did not confer protective immunity. The recombinant VP60 produced in the T7 RNA polymerase-based system was antigenically similar to the viral polypeptide as determined using polyclonal and monoclonal antibodies. When used to immunize rabbits the recombinant VP60 was able to protect the animals against a lethal challenge using purified RHDV.

Rabbit haemorrhagic disease (RHD) was first described in China (Liu et al., 1984) as an acute lethal sickness characterized by a haemorrhagic syndrome. The particles of the causative virus (RHDV) are icosahedral, 27 to 35 nm in diameter and lack an envelope. They contain a single capsid protein of M₉ 60K (VP60) and a positivestranded ssRNA genome approximately 7.5 kb in length. These characteristics suggest that RHDV is a member of the Caliciviridae (Meyers et al., 1991a; Ohlinger et al., 1990; Parra & Prieto, 1990). Despite the structural similarity to well characterized members of this family, RHDV shows a relevant difference concerning its VP60 gene expression strategy. In RHDV the VP60 protein is encoded at the 3' end of a large open reading frame (ORF1) extending over the majority of the genome (Meyers et al., 1991a; Parra et al., 1993), whereas feline calicivirus (FCV) and hepatitis E virus (HEV) produce their major structural polypeptides from independent ORFs (Carter et al., 1992; Tam et al., 1991). In spite of this and in common with FCV (Carter, 1990) and HEV (Tam et al., 1991), RHDV produces a subgenomic mRNA 3' coterminal with the genomic RNA (Meyers et al., 1991b) that codes for VP60 protein when translated in *vitro* (Boga et al., 1992). Considering these findings the VP60 protein found in the virions could be produced by processing of a polyprotein precursor or by translation of the subgenomic mRNA. Direct N-terminal amino acid sequencing of VP60 protein revealed that this viral polypeptide is most probably derived from the subgenomic mRNA (Parra et al., 1993).

The first studies on the role of VP60 protein in the immune response against RHD revealed that the presence of antibodies to VP60 protein in convalescent serum correlated with acquired immunity against RHDV, indicating that VP60 protein could be a candidate for a subunit vaccine against RHD (Parra & Prieto, 1990). In this paper we describe the cloning, nucleotide sequencing and expression in *Escherichia coli* of the major antigen of RHDV (isolate AST/89), as well as the preliminary antigenic and immunogenic characterization of the recombinant products.

We have determined the nucleotide sequence (Sanger et al., 1977) of 2483 nucleotides from the poly(A) tail at the 3' end of the Spanish RHDV isolate AST/89. The nucleotide sequence of the RHDV AST/89 isolate was 95.4% identical to that described for the German RHDV strain and the deduced VP60 amino acid sequence (Mount & Conrad, 1986) differed in 10 residues from that of the German RHDV (Meyers et al., 1991a). Some
of the substitutions produced relevant changes in the predicted hydrophilicity profile of the VP60 protein (not shown).

Analysis of the nucleotide sequence of the AST/89 isolate also revealed the presence of an ORF2 (predicted polypeptide product 117 amino acid residues) placed downstream of the VP60 coding sequence that was highly similar to that predicted for the German RHDV strain (Meyers et al., 1991a) and other European isolates of the virus (Milton et al., 1992).

In view of the potential of VP60 protein as a vaccine for RHD, we have cloned and overexpressed this gene as a fusion protein with bacterial β-galactosidase or in the T7 RNA polymerase system using as the initiator codon the ATG at position 351 (Fig. 1) which has been shown to encode the amino-terminal methionine residue of the virion-purified VP60 protein (Parra et al., 1993).

Reconstruction and cloning of the VP60 gene in pUR290 (Rüther & Muller-Hill, 1983) and pT7.7 (Studier et al., 1990) expression vectors is summarized in Fig. 1. For cloning purposes a 884 bp fragment corresponding to the 5' region of VP60-coding sequence from cDNA clone pT35 was amplified by PCR. The upstream primer (O-5) contained added BglII and NdeI sites. These restriction sites facilitate selection of the correct translational reading frame for the constructs
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Fig. 2. SDS-PAGE of E. coli cell extracts expressing pUR290 and pURVP60 recombinant products. These were stained with Coomassie blue (a) and detected by immunoblotting with an anti-RHDV serum (b). Lanes 1, Bacteria harbouring the plasmid pUR290; lanes 2, bacteria harbouring the plasmid pURVP60.

The cell extracts of induced transformants separated by SDS-PAGE were electrotransferred onto nitrocellulose membranes (Burnette, 1981). The blots were then incubated with anti-RHDV rabbit serum (Parra & Prieto, 1990) or specific anti-VP60 mouse monoclonal antibodies (MAbs) 1H8 and 6G2. The results shown in Fig. 2(b) demonstrate that the fusion was recognized by the rabbit serum, indicating antigenic similarity to VP60 protein. Nevertheless, immunoblotting using MAbs 1H8 and 6G2 (not shown) gave no detectable reaction in conditions where both antibodies reacted to VP60 protein purified from whole virus. These results indicated antigenic differences with respect to the native protein. Moreover, rabbits immunized using purified β-galactosidase–VP60 protein developed VP60-reactive antibodies but were not protected (not shown) against a lethal challenge using purified RHDV (100 LD₅₀ per rabbit).

Using the same type of experimental approach as described above we have investigated the production of full-length VP60 protein in a T7 RNA polymerase system as well as the antigenic and immunogenic properties of the recombinant product. For this purpose the cell extracts of induced or uninduced transformants, as well as purified RHDV virions, were analysed in parallel lanes by SDS–PAGE (Fig. 3a) and electrotransferred onto nitrocellulose membranes. The blots were then incubated with anti-RHDV rabbit serum (Fig. 3b) or with the specific anti-VP60 MAbs 1H8 (Fig. 3c) and 6G2 (Fig. 3d). The results showed that the recombinant VP60 protein was antigenically similar to RHDV VP60 protein considering that both were identified by the polyclonal and monoclonal specific antibodies.

To investigate the immunogenicity of recombinant VP60 this antigen was purified from the insoluble fractions of IPTG-induced cultures and used to immunize two rabbits. For this purpose the bacterial cells were suspended in PBS pH 7.5 and disrupted in a French...
produced in 48 h after the challenge showing the clinical signs of immunization. The two rabbits immunized with the RHDV were evaluated by challenging the rabbits using a lethal dose of purified virus (100 LD_{50} per animal), which indicated a gradual seroconversion to VP60 in the immune rabbit serum used in the immunoblotting experiments. Fig. 4 shows the resulting anti-VP60 antibody levels in preimmune or immunized rabbits using purified recombinant VP60 protein which was over 95% pure by SDS-PAGE analysis.

Two rabbits were immunized twice, with a 1 week interval, using 100 µg of purified VP60 protein mixed 1:1 (v/v) with complete Freund’s adjuvant. Two control animals were also inoculated with complete Freund’s adjuvant only. Blood samples were taken from all four animals before and after the immunizations and the level of specific anti-VP60 antibodies was analysed in an indirect ELISA test using purified RHDV as the antigen. For comparative purposes the dotted bar in Fig. 4 indicates the anti-VP60 antibody level of the anti-RHDV rabbit serum used in the immunoblotting experiments (Fig. 2 and 3).

The protection of the two immunized animals against RHDV was evaluated by challenging the rabbits using a lethal dose of purified virus (100 LD_{50} per animal), which was administered intranasally 7 days after the last immunization. The two rabbits immunized with the recombinant VP60 protein survived whereas the controls died 48 h after the challenge showing the clinical signs of RHD.

The data in this paper demonstrate that VP60 protein produced in E. coli could be an effective immunogen able to protect against RHD and that the correct immunogenicity of the recombinant antigen could be significantly altered by adding large carrier proteins at its amino terminus. Shorter amino-terminal extensions (not shown) did not exhibit the same effect as β-galactosidase on the antigenic properties of the resulting fusions considering that they were equally identified in immunoblotting experiments using polyclonal and monoclonal specific antibodies. Although the immunogenicity of those constructs has not been evaluated the VP60 fusions carrying short amino-terminal extensions could also be effective immunogens against RHD. This could be hypothesized taking into account the previously reported neutralization of other caliciviruses such as FCV by rabbit antiserum to the recombinant capsid protein produced using a T7-based system (Guiver et al., 1992) which gave rise to a fusion protein carrying 12 added amino acids.

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