CLONING AND EXPRESSION OF THE cDNA FOR THE MAJOR ANTIGEN OF FOOT AND MOUTH DISEASE VIRUS TYPE ASIA I 63/72

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ABSTRACT

Double stranded cDNA for the foot and mouth disease virus (FMDV) type Asia I 63/72 was prepared and cloned in the expression vector pUR222. The recombinant DNA was transferred into *E. coli* RRI-ΔT5 and the transformants were identified as white colonies in the presence of the dye x-gal on agar plates. Many of them gave strong positive signals on hybridization with ^{32}P-labelled viral RNA. The middle *Bam*HI fragment of the cDNA is known to carry the sequence for a few nonstructural proteins and for the major antigen, VP1. The clones containing the *Bam*HI fragments of the cDNA and the nearly full length cDNA produced proteins which gave positive signals in sandwich ELISA, showing that they cross-reacted with the antibodies raised against the virus. This showed that the major antigen is expressed by the cloned cDNA for the FMDV type Asia I.

INTRODUCTION

FOOT and mouth disease virus (FMDV) of the family of Picorna virus has a positive sense single-stranded RNA genome of about 8 kb[1]. In infected cells this RNA codes for a single primary translation product which is cleaved to generate precursors for structural proteins, P88, protease and VPg, P100, polymerase and other noncharacterised proteins. The virus-induced protease is responsible for hydrolyzing the primary translation product into P88, P52 and P100 proteins. The P88 protein is further processed into four structural capsid proteins which arearranged into the precursor in the order H_2N-VP_4-VP_2-VP_3-VP_1-COOH[2, 3]. The VP_1 protein is the main immunizing antigen and the variations in the amino acid sequence are probably responsible for the antigenicvariations of the virus which result in 7 sero types and over 60 sub-types[4]. To facilitate the detailed study of the viral genome and its products and to produce VP_1 in large quantities, a double-stranded cDNA of the virus was prepared and cloned in an expression vector pUR222 at the *Bam*HI and *Pst*I sites by following three different methods. The expression of the major antigen was demonstrated by the sandwich enzyme linked immunosorbant assay (ELISA).

METHODS

Isolation of Viral RNA

From a single plaque, FMDV type Asia I 63/72 Mukteswar isolate[5] (stock vaccine strain, Indian Veterinary Research Institute, Bangalore), was grown in baby hamster kidney (clone 21) monolayers in 5l Provitsky bottles at 37°C for 10–12 hr. The virus in the culture fluid was precipitated with polyethylene glycol-6000 and purified according to the method of Grubman *et al*[6]. The yield of the virus was 180 μg/l. The RNA was extracted from the virus using sodium dodecyl sulphate (SDS) proteinase-K method and purified[6]. The RNA was precipitated with 2.5 volumes of ethanol at −70°C and further purified by oligo (dT) cellulose chromatography. The yield of the poly A$^+$ RNA was 40 μg from 1 mg of virus.

Preparation of cDNA

The cDNA was prepared according to the procedure of Maniatis *et al*[7] with some modifications. The purified viral RNA (10 μg in 10 μl of water) was heated at 70°C for 3 min and the cDNA was synthesised by the reverse transcriptase reaction using oligo (dT) as the primer. The RNA was hydrolysed by treatment with NaOH and the single-stranded cDNA was converted into double strand by the DNA polymerase reaction using Klenow fragment of *E. coli* DNA polymerase I. The gaps in the cDNA were filled by the reverse transcriptase reaction in the absence of primer and RNasein. The hair-pin loop and the single-strand regions were removed by treatment with S1 nuclease followed by the DNA polymerase I reaction using Klenow fragment to fill up the gaps in the blunt ended cDNA.

Isolation of plasmid DNA

Plasmid pUR222 DNA was isolated from the overnight culture of *E. coli* RRI-ΔT5 harbouring the plasmid' grown in Luria Broth (LB) in the presence of ampicillin (200 μg/ml) using alkali lysis method'. The plasmid DNA was further purified by passing through the Sepharose 4B column and precipitated with ethanol, washed and dried in vacuum and dissolved in 10 mM tris-HCl, pH 8.0 containing 1 mM EDTA. The yield of plasmid was 600 μg/l of the culture.

Kinasing of DNA and RNA

The DNA and RNA were kinased by using T4 phage polynucleotide kinase and ATP. The RNA was kinased by the forward reaction[10] while the DNA was kinased by the exchange reaction[11]. The deoxyoligonucleotide was kinased by the forward reaction as described by Maniatis *et al*[12].

Preparation of antibodies

Antibodies against purified Asia I vaccine strain virus in hamsters and hyperimmune serum in guinea pigs against the vaccine strain virus were produced[13, 14]. The immunoglobulins from the serum were precipitated with 40% $(NH_4)_2SO_4$. The antibodies against the contaminating *E. coli* proteins in the serum were removed by adsorbing to the *E. coli* cell extract proteins immobilized on cyanogenbromide activated Sepharose. The gel was centrifuged off and the immunoglobulins in the supernatant fraction was repeatedly purified by adsorption.

Antibodies against guinea pig IgG were produced in rabbits and were separated from the whole serum by using guinea pig IgG immobilized on Sepharose affinity column. The antibodies so purified were conjugated to the horseradish peroxidase enzyme using periodate method as described by Wilson and Nakane[15] and modified by Suryanarayana, Banumati and Rao (unpublished results).

Cloning

The double-stranded cDNA preparation was divided into three equal portions. The cDNA in one portion was restricted with *Bam*HI, deproteinised and ligated to the *Bam*HI restricted, dephosphorylated pUR222 DNA as reported by Thomas *et al*[16]. Only the internal fragments of the cDNA will be ligated to the plasmid DNA to form circular recombinant molecules by this method. To clone full length cDNA and to regenerate the *Bam*HI fragments of the inserted DNA, *Bam*HI adapters were attached to both ends of the cDNA in the second portion and cloned at the *Bam*HI in the plasmid[17]. The cDNA in the third portion was tailed with dC residues using terminal transferase[18]. Similarly the *Pst*I digested plasmid DNA was tailed with dG residues and annealed to the dC tailed cDNA, to produce circular recombinant molecules.

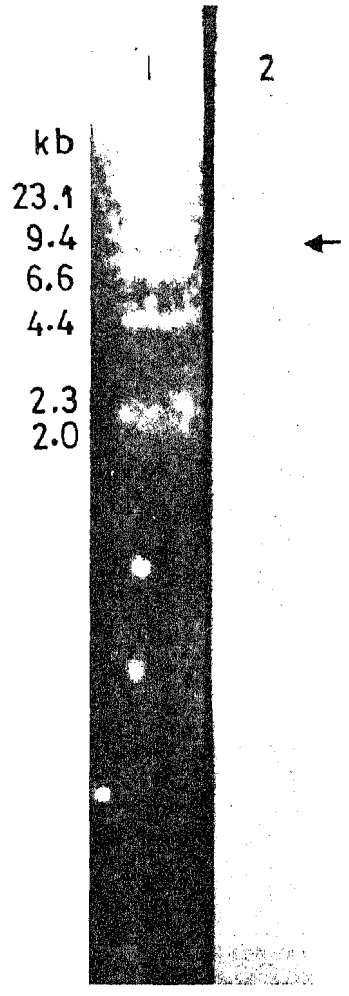

Figure 1. Size of the double-stranded cDNA. The double-stranded cDNA for the FMDV RNA was prepared, purified by gel filtration through Sephadex G-150. The 5' ends of the DNA were labelled with ^{32}P and the cDNA (400 cpm) was analysed by electrophoresis on 0.6% agarose gel (100 x 10 x 2 mm) at v/cm for 5 hr using 50 mM tris-acetate 1 mM EDTA buffer, pH 8.3, *Hind*III digested ADNA fragments (2 μg) being used molecular weight markers. The DNA was stained with ethidium bromide (0.5 μg/ml) and photographed in UV light. Then, the gel was exposed to x-rayfilm in Dupont Casette with intensifying screens and autoradiographed at −70°C for 48 hr. The arrow indicates the position of the radioactive band. Lane 1–Hind111 digested ADNA and 2–^{32}P-cDNA.

Preparation of cell extract

The bacteria harbouring the plasmid with or without the cDNA was grown overnight in 250 ml of L. broth containing 1mM of isopropyl-β-thio-galactoside as inducer. The cells were treated with 2% $CHCl_3$ for 5 min at 37°C, centrifuged at 4,000 g for 10 min. The pellet was washed once with phosphate buffered saline (PBS) and frozen at −20°C. The pellet was thawed, suspended in 3ml PBS kept on ice and sonicated twice with 30 sec pulse. The lysate was centrifuged at 30,000 g for 20 min and the supernatant was collected and stored at −70°C.

RESULTS AND DISCUSSION

Size of cDNA

The ^{32}P-labelled cDNA was analysed by electrophoresis in 0.6% agarose using *Hind*III digested λ-DNA fragments as molecular weight markers. A band corresponding to about 8 kb size was observed (figure 1). The smaller cDNA fragments might have been removed by the Sephadex G-150 gel filtration during the purification of cDNA. The RNA of FMDV strain O_1K has a length of 8kb including the poly C and the poly A tracts[19]. The length of 8kb indicates the near full length cDNA for the FMDV RNA obtained in the reverse transcriptase reaction.

Transformation

The recombinant DNA molecules obtained by insertion of *Bam*HI fragments of the cDNA, the adaptor ligated cDNA annealed to *Bam*HI sticky ends at the *Bam*HI site and the dC/dG tailed cDNA at *Pst*I site were transferred into the *E. coli* RRI-ΔT5 according to the method of Mandel and Higa[20] as Followed by Thomas *et al*[16]. Since pUR222 plasmid is an expression vector with β-galactosidase gene and its promoter, any recombinant will generate white colonies on x-gal plates due to lack of production of the portion of β-galactosidase enzyme[21]. Recombinant DNA having inserts at *Bam*HI site and at *Pst*I site gave rise to 72 and 27 white colonies respectively. There were a few blue colonies showing the absence or rejection of the foreign DNA in the plasmid vector.

Identification of colonies

The clones containing the recombinant DNA were grown on nitrocellulose filter and hybridized to 5′ end labelled ^{32}P-FMDV RNA[22] and autoradiographed (figure 2). There were 13 colonies showing strong signals on hybridization, 2, 5 and 6 colonies containing

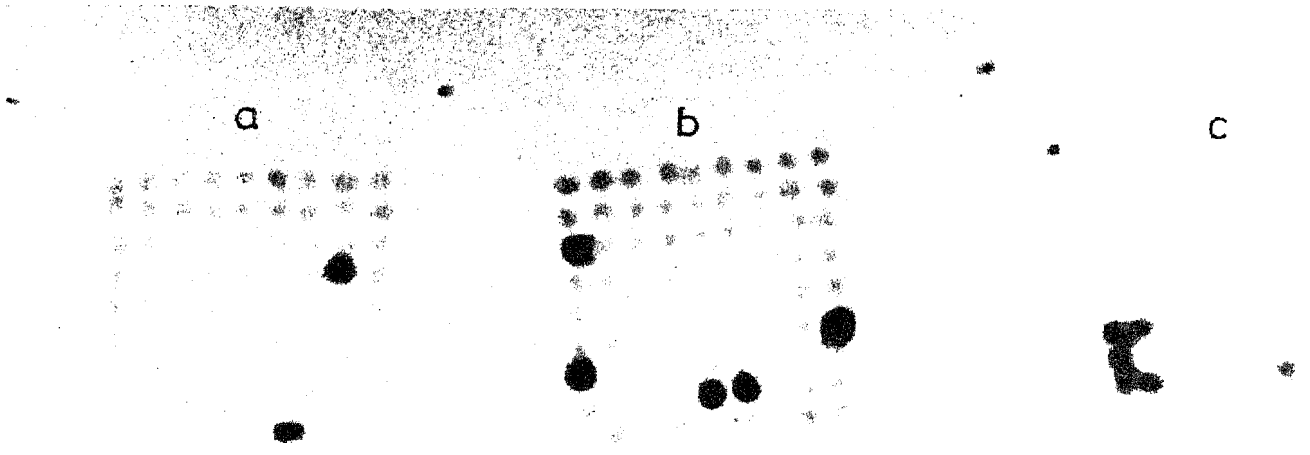

Figure 2. Colony hybridization of the cloned DNA. The white colonies were grown on nitrocellulose filters placed on LB agar containing ampicillin (200 μg/ml) for 24 hr, the cells were lysed, the DNA was denatured and the filters were dried, prehybridized for 3 hr and then hybridized with ^{32}P-5′ end labelled FMDV RNA (15×10^6 cpm) in hybridization buffer at 40°C for 2 days as described by Thomas *et al*[16]. The filters were washed, dried and subjected to and autoradiography at −70°C for 12 hr.

*Bam*HI restricted cDNA fragments, *Bam*HI adapter ligated cDNA and dC tailed cDNA, respectively.

Size of the inserted DNA

Colonies were grown overnight in L. broth containing ampicillin (200 μg/ml) and the plasmid DNA was isolated. Plasmid containing *Bam*HI fragments of cDNA for FMDV was restricted with *Bam*HI while the clones containing dC tailed cDNA was restricted with *Pst*I, and analysed by electrophoresis on 1% agarose. The size of the *Bam*HI insert was about 4 kb. The internal *Bam*HI fragment of cDNA of O_1K strain was reported to be about 4 kb in length[3]. The existence of about 4 kb fragment of the cloned *Bam*HI fragment of Asia I strain shows that there may not be any change in the *Bam*HI sites on the cDNA of O_1K and Asia I type strains. The PstI fragment was slightly more than 4 kb which corresponds to the internal PstI fragment as reoported in case of O_1K strain. The cDNA of the O_1K strain contains another 2 kb *Pst*I internal fragment which carries the gene for polymerase[3]. This fragment was not identified in the *Pst*I digest of the plasmid carrying the dC tailed cDNA of Asia I strain, under the experimental conditions employed. The fragment may be used to study the expression and the regulation of the polymerase gene. The clones containing the *Bam*HI adapter DNA carry the near full length cDNA for the viral RNA and hence carries most of the viral genes.

Expression of the antigen

The antigenic protein in the cell extract was detected by following sandwich indirect ELISA where the antigen is caught on specific antibodies that are adsorbed on polystyrene[23]. The presence of antigen was detected by using antibodies against the antigen and double antibodies conjugated with horseradish peroxidase enzyme (figure 3). The type A virus did not give any positive ELISA reaction (well 1). The $A_{400\,nm}$ of the reaction mixture was 0.12. These indicated that the absence of crossreactivity of the viral proteins with antibodies generated against type Asia I inactivated virus as reported[13]. Also the extracts from the *E. coli* cells harbouring the plasmid pUR222 did not show significant crossreaction ($A_{400\,nm}$ = 0.2) (well 2, 3). The two different clones obtained from dC tailed cDNA of FMDV Asia I, gave positive ELISA ($A_{400\,nm}$ = 0.77 and 0.69) (well 4 & 5) indicating the expression of the major antigen. The clones having the *Bam*HI internal fragment of the cDNA gave positive signal ($A_{400\,nm}$ = 0.57) (well 6). The *Bam*HI internal fragment of the cDNA of O_1K strain contains the nucleotide sequence for the major antigen, VPI, P34 and VPg proteins[24]. In all FMDV strains studied so far, the major antigen is identified as VPI protein. Hence the positive ELISA reaction as well as the high $A_{400\,nm}$ of the reaction mixture show the transcription and translation of the major antigen by the cloned cDNA. It is likely that the protein is a fused product with a part of β-glactosidase protein. The precursor protein may or may not be processed by viral specific protease to produce the capsid proteins. The cloning of the *Bam*HI restricted cDNA fragment in an expression vector seems to be a

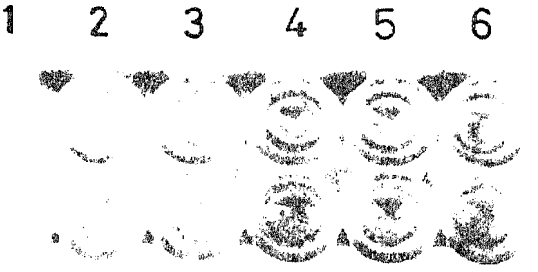

Figure 3. ELISA for the detection of the antigen. The polystyrene wells of 0.3 ml capacity were coated with 2 μg of anti Asia I hamster immunoglobulins in 0.2 ml of 0.1 M carbonate buffer, pH 9.5, overnight at 4°C. The wells were washed thrice, for 3 min each time, with PBS containing 0.05% Tween-20. The leftover sites in the wells were saturated with 3% BSA in PBS and kept overnight. The bacterial extract (50 μl, 800 μg protein) was diluted to 100 μl with PBS-Tween-20 containing 1.5% BSA, added to the wells and incubated at 37°C for 3 hr. The wells were washed thrice, 100 μl of anti Asia I guinea pig IgG, pretritrated in ELISA test and diluted to 1:600 with PBS-Tween containing 3% BSA, was added and incubated at 37°C for 1 hr. The wells were washed as before, 100 μl of anti-guinea pig rabbit IgG conjugated with horseradish peroxidase diluted to 1:600 in PBS-Tween containing 3% BSA was added and incubated at 37°C for 1 hr. The solution in the wells was decanted, the wells were washed with PBS-Tween and incubated with 0.2 ml of 0.02% 3, 3′-orthodianisidine dihydrochloride in 50 mM sodium acetate buffer, pH 5.0 containing 0.02% H_2O_2 for 1 hr at room temperature. The reaction was stopped by adding HCl to the final concentration of 0.5 M. The colour developed was followed by $A_{400\,nm}$ and photographed. Well 1, culture fluid (100 μl) containing type A virus. Cell extracts (50 μl, 800 μg protein) from *E. coli* containing: 2 & 3, pUR222 plasmid; 4 & 5, pUR222 plasmid carrying dC/dG tailed cDNA from different clones and 6, pUR222 plasmid carrying *Bam*HI fragment of the cDNA.

straight forward and efficient method for production of the major antigenic protein of FMDV in large quantities.

22 May 1985

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