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## Immunogenic characterization and epitope mapping of transmissible gastroenteritis virus RNA dependent RNA polymerase

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### ABSTRACT

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Coronavirus RNA synthesis is a sophisticated process performed by a viral multienzymatic replicase complex, together with cellular factors. A key enzyme of this replication complex is the RNA dependent RNA polymerase (RdRp). To study the replication of coronavirus genome, six monoclonal antibodies (mAbs) specific for transmissible gastroenteritis virus (TGEV) RdRp were generated and characterized. His-tagged RdRp was expressed in baculovirus, purified and used as immunogen to produce mAbs. The TGEV RdRp was recognized by these mAbs in the context of virus infection by immunofluorescence analysis and Western blot. Epitope mapping by Pepscan indicated that RdRp mAbs recognized four non-overlapping linear epitopes located in a 62-amino acid region of the N-terminal domain, suggesting that this region may constitute an immunodominant domain. The availability of TGEV RdRp mAbs will be instrumental to study coronavirus replication and to analyze the function of RdRp in pathogenesis.

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### 1. Introduction

Transmissible gastroenteritis virus (TGEV) is an enveloped, single-strand positive sense RNA virus that belongs to the genus  $\alpha$  of the *Coronaviridae* family within the *Nidovirales* order (de Groot et al., 2010; Enjuanes et al., 2008) (see <http://talk.ictvonline.org/media/g/vertebrate-2008/default.aspx> for official coronavirus taxonomy). Coronavirus (CoV) infections cause a variety of enteric and respiratory diseases relevant in animal and human health, being of special interest the severe acute respiratory syndrome (SARS) in humans (Drosten et al., 2003; Perlman et al., 2000). The CoV genome is about 30 kb in length, the largest RNA virus genome known (Enjuanes et al., 2008; Masters, 2006). The 5' two-thirds of the genome contain the replicase gene that is made up of two overlapping open reading frames (ORFs), ORF 1a and ORF 1b, the latter being translated by a ribosomal frameshift mechanism (Brierley et al., 1987, 1989). Both ORFs are translated into two co-amino-terminal polyproteins, pp1a and pp1ab, which are cleaved autoproteolytically into up to 16 mature

products (nsp1–nsp16) that form the replication–transcription complex, probably together with the participation of cellular factors (Enjuanes et al., 2006; Galan et al., 2009; Masters, 2006; Ziebuhr, 2005; Ziebuhr et al., 2000). CoV replication and transcription are complex processes that take place at cytoplasmic double membrane vesicles (DMVs) and involve coordinated processes of both continuous and discontinuous RNA synthesis (Gosert et al., 2002; Knoops et al., 2008; Masters, 2006; Snijder et al., 2006; Zúñiga et al., 2004).

A key enzyme required for both genome replication and transcription is the RNA dependent RNA polymerase (RdRp) or nsp12. Therefore, the generation of RdRp antibodies may provide an excellent tool to study the precise strategies of CoVs replication and transcription, as well as the role of RdRp in CoV pathogenesis.

In this study, the generation and characterization of a collection of monoclonal antibodies (mAbs) against TGEV RdRp is reported. These mAbs recognized four physically close linear epitopes at the N-terminal domain of the protein, which were conserved in genus  $\alpha$  CoVs.

### 2. Materials and methods

#### 2.1. Ethics statement

Animal experimental protocols were in strict accordance with EU guidelines 2010/63/UE, and Spanish national law RD 1201/2005, on the protection of animals used for experimentation and other

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scientific purposes, and national law 32/2007, on animal welfare in their exploitation, transport, experimentation and sacrifice. The experiments were performed at the animal facility of the Centro Nacional de Biotecnología (CNB-CSIC), Madrid (Permit number 28079-29-A), and were approved by the on site Ethical Review Committee (CEEA-CNB).

## 2.2. Cells and virus

Swine testis (ST) cells (McClurkin and Norman, 1966) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biowhittaker, Berviers, Belgium) at 37 °C. High Five (H5) insect cells (Invitrogen, Barcelona, Spain) were grown in TC100 medium supplemented with 10% FBS at 28 °C. TGEV PUR46-MAD strain (Sanchez et al., 1990) was used to infect ST cells as described previously (Correa et al., 1988).

## 2.3. Construction of a recombinant baculovirus expressing TGEV RdRp

A recombinant baculovirus expressing TGEV RdRp, fused to a 6-His tag at the N-terminal region (rBV-His-RdRp), was generated using the Bac-to-Bac expression system (Invitrogen, Barcelona, Spain), according to the manufacturer's instructions. A DNA fragment containing the TGEV full-length RdRp coding sequence (nts 12,309–15,094; gi:13399293), flanked by SfoI and Sall restriction sites, was generated by PCR from a TGEV-derived replicon (Almazan et al., 2004) using the forward oligonucleotide 5'-GCACTCAAGGCGCCAGTTTACTGTTGATCAAAGTTA(C)TT(G)AA(T)CGAGTGGC(C)GG(A)TC(A)AGTGCAGCTCGACTAGAACC-3' (SfoI site is underlined) and the reverse oligonucleotide 5'-GGCTGGGTGCGACTATTATTGCAAGACAGTGGACTTTTCATAGAGGC-3' (Sall site is underlined and the termination codons are in bold). The 5' end of the RdRp coding sequence contains the slippery sequence and the pseudoknot structure responsible for the -1 ribosomal frameshift that allow the expression of ORF 1b. Therefore, the forward oligonucleotide was designed to eliminate the frameshift by the introduction of 6 silent mutations in the slippery sequence and the pseudoknot (showed in parenthesis) and to restore the reading frame by insertion of a C (showed in bold) just downstream of the slippery sequence. The PCR product was digested with SfoI and Sall and cloned into the same restriction sites of the intermediate plasmid pFastBacHT (Invitrogen, Barcelona, Spain) to generate the plasmid pFB-His-RdRp, where the RdRp is fused to a His tag at the N-terminal region. This plasmid was transformed into DH10Bac cells (Invitrogen, Barcelona, Spain) to generate a recombinant bacmid by transposition of pFB-His-RdRp expression cassette. Finally, H5 insect cells were transfected with the purified recombinant bacmid using Cellfectin (Invitrogen, Barcelona, Spain) to generate the recombinant baculovirus rBV-His-RdRp. The recombinant virus was amplified in H5 cells and then high-titer virus stocks were obtained. All constructs were confirmed by sequencing.

## 2.4. Expression and purification of TGEV His-tagged RdRp

H5 cells were grown in 15-cm diameter dishes to 90% confluence and infected at a multiplicity of infection (MOI) of 2 with rBV-His-RdRp. At 60 h post-infection (hpi), infected cells were collected, washed with cold PBS and centrifuged at 1000 × g for 5 min at 4 °C. The cell pellet was resuspended in 1 ml of binding buffer (50 mM sodium-phosphate buffer, pH 8, 300 mM NaCl, 4.5 mM imidazole) per 1.5 × 10<sup>7</sup> cells, and lysed by three freeze-thaw cycles. After that, DNA was sheared by passing it 6 times through an 18-gauge needle, and the insoluble cellular material was discarded by centrifugation at 5000 × g for 15 min at 4 °C. The soluble recombinant protein was

**Table 1**  
Properties of TGEV RdRp mAbs.

mAb	Titer <sup>a</sup>	Isotype	H5 <sup>b</sup>		ST <sup>c</sup>	
			WB	IFA	WB	IFA
4D10	1:10,000	IgG1	+++	+++	+++	+
2C11	1:50,000	IgG2a	+++	+++	++	+++
3C12	1:15,000	IgG2a	+++	+++	++	++
2B5	1:3,000	IgG2a	+	++	ND	+
5H1	1:2,500	IgG2a	+	++	ND	+
3F9	1:25,000	IgG2b	+++	+++	++	+

WB, Western blot assay; IFA, immunofluorescence assay; ND, not detected.

<sup>a</sup> Antibody titer determined by ELISA.

<sup>b</sup> Insect H5 cells infected with rBV-His-RdRp.

<sup>c</sup> Porcine ST cells infected with TGEV PUR46-MAD.

purified by metal chelate affinity chromatography using Ni-NTA agarose (Sigma-Aldrich, Madrid, Spain), according to the manufacturer's instructions with some modifications. Briefly, the recombinant protein was bound to the resin by incubation at 4 °C for 2 h. Then the resin was washed 3 times with binding buffer, twice with wash buffer 1 (50 mM sodium-phosphate buffer, pH 8, 300 mM NaCl, 9 mM imidazole), and once more with wash buffer 2 (50 mM sodium-phosphate buffer, pH 8, 300 mM NaCl, 15 mM imidazole). Finally, the recombinant protein was eluted with elution buffer (50 mM sodium-phosphate buffer, pH 8, 300 mM NaCl, 150 mM imidazole), and desalted on PD-10 columns (GE Healthcare, Madrid, Spain) with PE buffer (50 mM sodium-phosphate buffer, pH 8, 100 mM NaCl). The purified protein was analyzed by SDS-PAGE and then quantified by a BCA protein assay (Pierce Biotechnology, Rockford, USA) according to the manufacturer's instructions.

## 2.5. Generation of monoclonal antibodies against TGEV RdRp

Murine mAbs specific for RdRp were obtained by standard procedures (Harlow and Lane, 1988). Eight-week-old BALB/c mice were immunized subcutaneously with 30 µg of purified His-RdRp protein in complete Freund's adjuvant (Difco, Madrid, Spain), followed by similar injections in incomplete Freund's adjuvant at 4-week intervals. Ten days after the second immunization in incomplete Freund's adjuvant, serum was collected from each animal and the anti-RdRp response analyzed by enzyme-linked immunosorbent assay (ELISA). The best responder mouse was boosted intraperitoneally with 70 µg of His-RdRp in PBS and 3 days later the spleen cells were fused with the murine P3X63-Ag8.653 myeloma cell line (CRL 1580, American Type Culture Collection) using polyethylene glycol 4000 (Merck, Madrid, Spain), as described previously (Galfe and Milstein, 1981; Kremer and Márquez, 2004). The resulted hybridomas were selected for RdRp-specific antibody response by ELISA, Western blot and indirect immunofluorescence. Selected antigen-specific antibody-secreting hybrids were stabilized by limiting dilution in two cloning steps, and the immunoglobulin subclass determined by ELISA (Table 1) using specific peroxidase-conjugated antibodies against the heavy chain of mouse immunoglobulins (IgG1, IgG2a, IgG2b, IgG3 and IgM).

## 2.6. Western blot analysis

Cell lysates or purified His-RdRp protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C Extra Nitrocellulose, GE Healthcare, Madrid, Spain) with a Bio-Rad Mini protean II electroblotting apparatus at 100 V for 50 min. Membranes were blocked for 1 h with 5% dried skimmed milk in TTBS (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% Tween 20) and then probed with the mouse antibodies anti-polyHistidine-Peroxidase tag (Sigma-Aldrich, Madrid, Spain)

**Table 2**  
Oligonucleotide used for the construction of recombinant plasmids.

Primer name <sup>a</sup>	Primer sequence 5'–3' <sup>b</sup>
Frag1VS	GCACGCTCGAGATGAGTTTTACTGTGTGATCAAAAGTTACTTG
Frag1RS	GGCTGGGGATCCTAATTAATAATCAGAACCATAGATGTCAC
Frag2VS	GCACGCTCGAGATGGATCTTAATGGCAATTTCTACGATTTCCGG
Frag2RS	GGCTGGGGATCCTAATTAAGATCCCTCTTCAAAGAATCCACGC
Frag3VS	GCACGCTCGAGATGCCAGGTCACCTTAACAAGGATTTCTACG
Frag3RS	GGCTGGGGATCCTAATTAATTATGTGTACAACAACCAACATGC
Frag4VS	GCACGCTCGAGATGGATAATGGTTGTTGATGGGATGGG
Frag4RS	GGCTGGGGATCCTAATTAATTGCAAGACAGTGGACTTTTCATAGAGGC

<sup>a</sup> Vs, virus sense; RS, reverse sense.

<sup>b</sup> Restriction endonuclease sites used for cloning are underlined (BamHI, GGATCC; XhoI, CTCGAG). Translation initiation (ATG) and termination (CTA and TTA) codons are shown in bold.

(diluted 1:20,000), the anti-RdRp mAbs generated (diluted 1:200), or the mAb 3DC10 specific for TGEV nucleocapsid (N) protein (diluted 1:2000) (Martín-Alonso et al., 1992) in TTBS containing 3% dried skimmed milk. Bound primary antibodies were detected with horseradish peroxidase conjugated rabbit anti-mouse IgG (Sigma–Aldrich, Madrid, Spain) diluted 1:30,000 in TTBS with 3% dried skimmed milk, followed by incubation with the Immobilon Western chemiluminescent substrate (Millipore Ibérica, Madrid, Spain), according to the manufacturer's recommendations.

### 2.7. Indirect immunofluorescence microscopy

Subconfluent H5 and ST cells grown on glass coverslips were mock-infected or infected at an MOI of 2 with rBV-His-RdRp and TGEV PUR46-MAD, respectively. At different hpi, cells were washed with PBS, fixed by incubation with 4% paraformaldehyde (PFA) in PBS for 30 min at RT, and permeabilized with 0.1% Triton X-100 for 15 min at RT. Alternatively, cells were fixed and permeabilized with methanol for 10 min at  $-20^{\circ}\text{C}$ . After washing with PBS, cells were incubated in blocking solution (10% FBS in PBS) for 45 min at RT, incubated with the RdRp mAbs diluted 1:20 in blocking solution for 90 min at RT, washed extensively with PBS, and then incubated with alexa-488 fluorochrome-conjugated goat anti-mouse IgG (Invitrogen, Barcelona, Spain) at a 1:500 dilution in blocking solution for 1 h at RT. The coverslips were washed four times in PBS, incubated with 1 mg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 20 min at RT to stain the cell nucleus, and mounted on glass slides with Prolong Gold anti-fade reagent (Invitrogen, Barcelona, Spain). Samples were examined with a ZEISS Axiophot fluorescence microscope. Confocal microscopy was performed using a Leica SP5 laser scanning microscope, and images were collected and processed with LAS AF (Leica, Wetzlar, Germany) and Adobe Photoshop (CS3) software.

### 2.8. Plasmid construction and *in vitro* transcription/translation

Four overlapping DNA fragments (F1–F4) covering the full-length RdRp coding sequence (Fig. 4), flanked by XhoI and BamHI restriction sites, were generated by PCR using the plasmid pFB-His-RdRp as template and specific oligonucleotides (Table 2). Fragment F1 encodes the first 263 aa of the RdRp, F2 the region from aa 200 to 424, F3 from aa 402 to 640 and F4 from aa 606 to 929. The PCR products were digested with XhoI and BamHI and cloned into the same restriction sites of the plasmid pcDNA-3.1(–) (Invitrogen, Barcelona, Spain) under the control of the cytomegalovirus promoter to generate the plasmids pcDNA-F1, F2, F3 and F4. All constructs were confirmed by sequencing. The *in vitro* transcription/translation reactions were performed from 1  $\mu\text{g}$  of each plasmid using the TNT<sup>®</sup> coupled reticulocyte lysate system (Promega Biotech Ibérica, Madrid, Spain), according to the manufacturer's instructions. Biotinylated Lys-tRNA (Promega Biotech Ibérica, Madrid, Spain) was used in translation reactions

for detection of the expressed proteins with peroxidase-conjugated streptavidin.

### 2.9. Pepsican analysis

A collection of 146 peptides of 10 aa each, overlapping in 8 residues, and covering RdRp fragment F1, was synthesized with the SPOT-synthesis method (Frank, 2002) on a ASP222 (Intavis AG, Koeln, Germany), linked to an Amino-PEG<sub>500</sub>-UC540 cellulose membrane (Intavis AG, Koeln, Germany) and probed with the RdRp mAbs by immunoblotting.

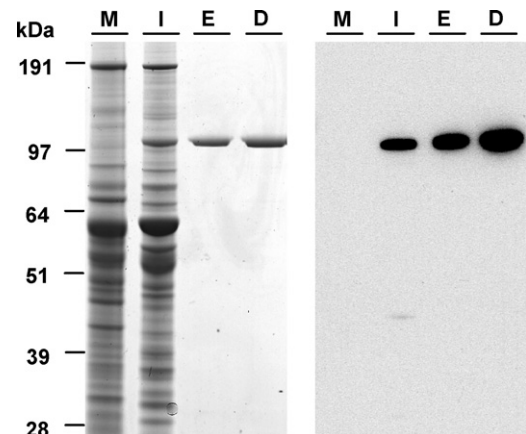
## 3. Results

### 3.1. Expression and purification of TGEV His-tagged RdRp

To generate TGEV RdRp mAbs, a recombinant baculovirus expressing the RdRp fused to a His tag on its amino terminus (rBV-His-RdRp) was generated using the Bac-to-Bac system (Invitrogen, Barcelona, Spain). H5 insect cells were infected with the recombinant virus rBV-His-RdRp. At 60 hpi, the expression of the His-tagged RdRp was analyzed by SDS-PAGE (Fig. 1, left panel) and Western blot using a polyHis specific antibody (Fig. 1, right panel). An abundant polypeptide of about 109 kDa, corresponding to the expected size of the His-RdRp fusion protein, was clearly detected in extracts from infected cells but not in mock-infected cells. The His-tagged protein was further purified under native conditions by nickel affinity chromatography, desalted using a PD10 Sephadex column, and analyzed by SDS-PAGE and Western blot. A highly pure (>95% of purity) His-RdRp protein was obtained (Fig. 1) with a concentration of 340  $\mu\text{g}/\text{ml}$ .

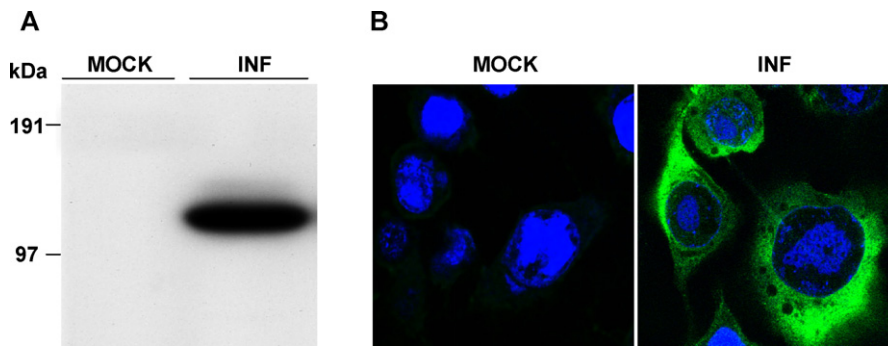
### 3.2. Production of monoclonal antibodies against TGEV RdRp

TGEV RdRp mAbs were generated in mice using the purified His-RdRp protein produced in baculovirus as immunogen. After cell fusion, the hybridomas secreting RdRp specific mAbs were identified by ELISA using the purified His-RdRp protein as the antigen. Thirty five positive hybridomas were selected and their supernatants retested by Western blot and immunofluorescence. Six out of the 35 hybridomas with the highest reactivity



**Fig. 1.** SDS-PAGE and Western blot analysis of His-RdRp expression and purification. TGEV RdRp was expressed using baculovirus as a His-tagged protein and purified by nickel affinity chromatography. Samples from the expression and purification steps were resolved by 10% SDS-PAGE and analyzed by coomassie blue staining (left panel) and Western blot (right panel) using a mouse anti-polyHis antibody (Sigma–Aldrich, Madrid, Spain). M, mock-infected H5 whole-cell extract; I, rBV-His-RdRp infected H5 whole-cell extract; E, fraction eluted with 150 mM imidazole; D, desalted protein. Molecular size markers are indicated in kDa.





**Fig. 2.** Characterization of RdRp mAb 4D10. Insect H5 cells were mock-infected (MOCK) or infected with rBV-His-RdRp (INF) at an MOI of 2 and analyzed at 60 hpi. (A) Western blot analysis. Cell lysates were resolved by 10% SDS-PAGE and analyzed by immunoblotting with mAb 4D10. Molecular size markers are indicated in kDa. (B) Immunofluorescence analysis. Cells were fixed with PFA, permeabilized with Triton X-100, incubated with mAb 4D10 followed by Alexa-488 fluorochrome-conjugated goat anti-mouse IgG (Invitrogen, Barcelona, Spain), and analyzed by confocal microscopy. Cell nuclei were stained with DAPI.

by both techniques were cloned twice by limiting dilution and the isotype of the secreted immunoglobulin determined (Table 1). Four of them (2C11, 5H1, 3C12, and 2B5) were IgG2a, one (3F9) IgG2b, and one (4D10) IgG1. Western blot and immunofluorescence analysis demonstrated that all mAbs specifically recognized both the SDS-denatured and native recombinant protein in H5 cells infected with rBV-His-RdRp (Table 1), suggesting that the epitopes recognized by the RdRp mAbs were linear. Among these mAbs, 5H1 and 2B5 presented the lowest binding activity in both techniques, in agreement with their antibody titers that were lower than those of the other four mAbs (Table 1). As an example, the ability of mAb 4D10 to recognize the recombinant His-RdRp protein by Western blot and immunofluorescence is illustrated (Fig. 2).

### 3.3. Characterization of RdRp mAbs in TGEV infected cells

After demonstrating the specific binding of the selected mAbs to the His-RdRp protein produced in the baculovirus system, the ability of these mAbs to recognize the viral RdRp in TGEV infected ST cells was analyzed by Western blot and immunofluorescence analysis (Table 1).

Western blot of extracts from infected ST cells (12 hpi), using the mAb 4D10, demonstrated that the antibody recognized a specific band of about 105 kDa, corresponding to the viral RdRp, which was absent in mock-infected cells (Fig. 3A, left panel). In addition to the monomeric form, the antibody specifically recognized higher molecular weight polypeptides that might represent either RdRp oligomers or pp1ab polyprotein processing precursors (Fig. 3A, left panel). Similar results, with a slightly higher background, were obtained with mAbs 2C11, 3C12 and 3F9 (Fig. 3A, middle panel). In the case of mAbs 5H1 and 2B5 no binding was detected (Table 1), most likely due to the low titer of both antibodies and the low expression level of RdRp during infection, compared with the expression level of other viral proteins, such as the N protein (Fig. 3A, right panel).

Immunofluorescence analysis of infected ST cells (12 hpi) was first performed using PFA and Triton X-100 as fixation and permeabilization reagents, respectively, but only a faint perinuclear signal was detected. As an example, the staining obtained with the mAbs 4D10 and 2C11 is illustrated (Fig. 3B, upper panels). To increase the accessibility of the antigen, cells were fixed and permeabilized with methanol. Under these conditions, a specific and stronger staining was detected in infected cells with all RdRp mAbs. Among these, mAbs 2C11 and 3C12 provided the highest signal (Fig. 3B, lower panels and Table 1). The RdRp presented a perinuclear vesicular pattern that may correspond to the DMVs where viral RNA synthesis takes place (Fig. 3B).

An attempt to immunoprecipitate the RdRp in TGEV infected ST cells using the RdRp mAbs was unsuccessful, probably due to the low expression level of RdRp during the infection (data not shown).

### 3.4. Identification of the epitopes recognized by RdRp mAbs

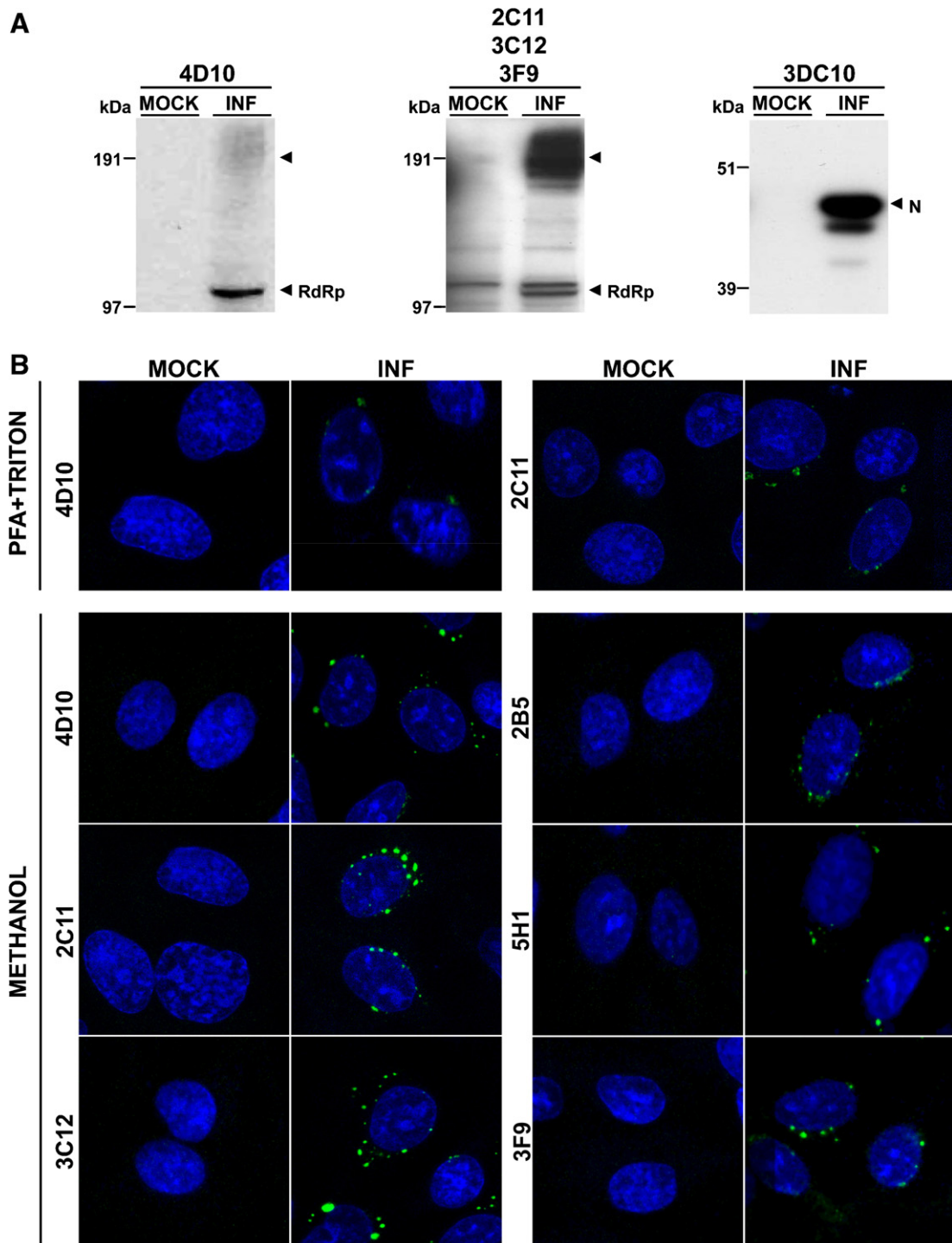
Due to the large size of the RdRp, the epitope identification was performed in two steps. In the first step, the RdRp was subdivided into 4 overlapping fragments (F1–F4) of around 250 aa according to the predicted RdRp domains (Fig. 4A). The coding sequence of the four fragments was obtained by PCR using specific primers (Table 2). These fragments were cloned into the mammalian expression vector pcDNA-3.1(–), expressed using an *in vitro* transcription/translation system, and the binding of the mAbs to the RdRp fragments was analyzed by Western blot. All six RdRp specific mAbs recognized the fragment F1 that contains the first 263 aa of the RdRp (Fig. 4A). Interestingly, computer analysis of the RdRp sequence, using the DNASTAR software (DNASTAR Inc., Madison, USA), showed that fragment F1 had the highest surface probability and antigenic index values of the entire RdRp (data not shown). In the second step, the specific epitopes were identified by Pepscan analysis of the RdRp fragment F1. To this end, a synthetic peptide array containing 146 peptides of 10 aa each, overlapping in 8 residues, was generated using the SPOT-synthesis method (Frank, 2002) and probed with the RdRp mAbs by immunoblotting. Four linear epitopes located in a 62 aa region from residues 97–158 were identified (Fig. 4B). The epitope sequences were 97-DFFTYK-102 for 2C11 and 3C12, 117-TKYTMM-122 for 4D10, 125-CYAIRN-130 for 5H1, and 153-FFENKD-158 for 3F9 and 2B5. These data suggest that the RdRp N-terminal region covering residues 97 to 158 may constitute an immunodominant domain.

## 4. Discussion

After the SARS outbreak in 2002 (Drosten et al., 2003), the study of CoV replication has acquired significant relevance in order to develop effective strategies to prevent and control CoV infections. The RdRp is a key protein involved in both genome replication and transcription. Therefore, the availability of RdRp mAbs will significantly facilitate the study of CoV replication and transcription mechanisms.

In this study, a set of six mAbs against the TGEV RdRp was generated and characterized. Most of mAbs recognized both the recombinant His-RdRp protein expressed in insect cells and the viral protein in TGEV infected ST cells, by both Western blot and immunofluorescence analysis (Table 1).

Analysis of infected ST cells by Western blot showed a specific band of 105 kDa. Additionally, higher molecular mass polypeptides

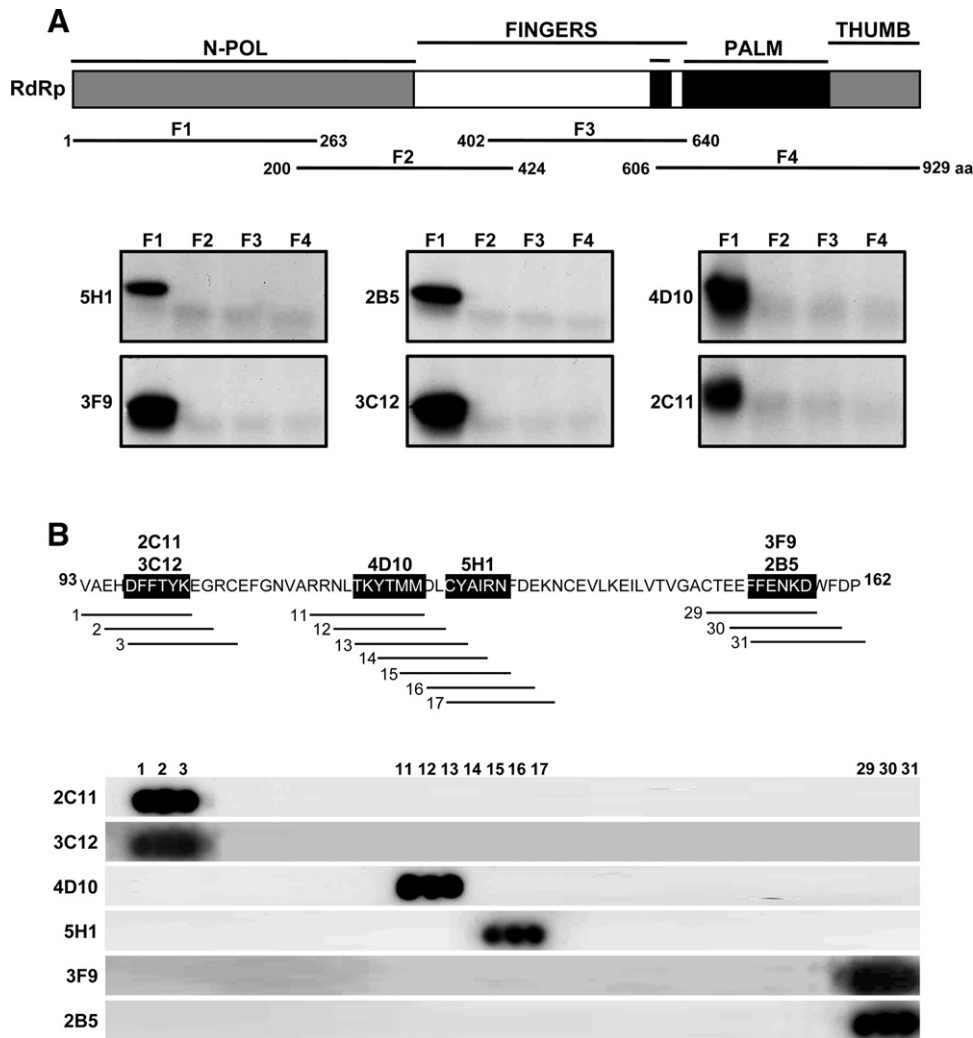


**Fig. 3.** Identification of RdRp in TGEV infected ST cells. Porcine ST cells were mock-infected (MOCK) or infected (INF) with TGEV at an MOI of 2 and RdRp expression analyzed at 12 hpi. (A) Western blot analysis. Total cell extracts were resolved by 4–12% SDS-PAGE and analyzed by immunoblotting with the indicated RdRp mAbs (left and middle panels) and the N protein mAb 3DC10 as a control of TGEV infection (right panel). Specific bands are indicated by arrowheads. Molecular size markers are shown in kDa. (B) Immunofluorescence analysis. Cells were fixed and permeabilized with PFA and Triton X-100 or with methanol, incubated with the indicated RdRp mAbs followed by Alexa-488 fluorochrome-conjugated goat anti-mouse IgG (Invitrogen, Barcelona, Spain), and analyzed by confocal microscopy. Cell nuclei were stained with DAPI.

of around 150kDa were also detected, which could represent RdRp homo- or hetero-oligomeric structures or pp1ab polyprotein processing precursors. Similar results have been reported in MHV, where RdRp protein precursors of about 150 and 300kDa were detected (Brockway et al., 2003). RdRp was also recognized by immunofluorescence, showing a perinuclear vesicular pattern that is typical for CoV DMVs, where viral RNA synthesis takes place

(Gosert et al., 2002; Knoops et al., 2008; Snijder et al., 2006). From these results, it could be concluded that RdRp mAbs recognize both the unfolded and the native protein and most likely they recognize linear epitopes.

The epitopes recognized by the RdRp mAbs were identified by Pepscan analysis. Four different linear epitopes were found in a 62 aa region at the RdRp N-terminal domain, indicating that



**Fig. 4.** Epitope mapping of RdRp mAbs. (A) Identification of RdRp domains recognized by the mAbs. Four overlapping fragments (F1–F4) covering the full-length RdRp were obtained by PCR, expressed in a transcription/translation system, and probed with the RdRp mAbs by Western blot. The RdRp domains (N-pol, Fingers, Palm and Thumb) and the location of the 4 fragments within the RdRp sequence are shown in the upper part of the figure. (B) Pepscan analysis of RdRp fragment F1. A synthetic peptide array, covering fragment F1, was generated as described in Section 2 and probed with the RdRp mAbs by immunoblotting. The position in the array and the sequence of the peptides recognized by the mAbs are indicated. The deduced epitope sequence for each mAb is shown in black boxes.

this region constitutes an immunodominant domain. These epitopes are conserved among TGEV related members of the CoV genus  $\alpha$ , such as the canine and feline CoVs (CCoV and FCoV) (Fig. 5). Interestingly, the epitope recognized by the mAb 4D10 is highly conserved in the majority of CoVs from genus  $\alpha$ , in

infectious bronchitis virus (IBV) from genus  $\gamma$ , and in SARS-CoV from genus  $\beta$  (Fig. 5). In fact, the mAb 4D10 recognized the SARS-CoV RdRp by Western blot (data not shown), indicating that this mAb, at least, may be used to identify the RdRp of other CoVs.



**Fig. 5.** Conservation of TGEV RdRp epitopes in other CoVs. The RdRp region covering residues 93–162 of CoVs from the three genera ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) were aligned using the Clustal-W program. The sequence of the TGEV RdRp epitopes and the residues conserved in other CoVs are boxed. TGEV, transmissible gastroenteritis PUR46-MAD (gi:13399293); HCoV229E, human CoV 229E (gi:12175747); NL63, human CoV NL63 (gi:93004453); CCoV, canine CoV (gi:214027098); FCoV, feline CoV (gi:302877212); PEDV, porcine epidemic diarrhea virus (gi:121722566); BtCoV-512, bat CoV 512 (gi:152994037); MHV, mouse hepatitis virus (gi:6625761); SARS-CoV, severe acute respiratory syndrome virus (gi:30027617); IBV, infectious bronchitis virus (gi:33569220).

Although polyclonal antibodies recognizing the RdRp of genus  $\beta$ -belonging SARS-CoV and mouse hepatitis virus (MHV) have been described previously (Prentice et al., 2004; Shi et al., 1999), this is the first time that mAbs recognizing the RdRp from CoVs of genus  $\alpha$  have been generated and their epitopes identified. The availability of these mAbs will be very useful to study the CoV replication and transcription mechanisms.

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