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VIROLOGY 188, 168–174 (1992)

Antigenic Structure of Transmissible Gastroenteritis Virus Nucleoprotein

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Received September 19, 1991; accepted January 29, 1992

A group of 11 monoclonal antibodies (MAbs) raised against transmissible gastroenteritis virus (TGEV) was used to study the antigenic structure of the virus nucleoprotein (N). To identify the regions recognized by MAbs, DNA fragments derived from the N-coding region of the TGEV strain FS772/70 were cloned into pUR expression plasmids and the antigenicity of the resulting fusion proteins was analyzed by immunobloting. A major antigenic domain was identified, covering the first 241 amino acid residues of N, within which an epitope (residues 57–117) was also found. A second antigenic domain extended from residues 175 to 360 of the nucleoprotein, within which a subsite was characterized within the region covering residues 241–349. MAb DA3 recognized a linear epitope which mapped within residues 360 and 382 at the carboxy terminus of the nucleoprotein. The binding of the majority of the MAbs (8 out of 11) to large fusions, but not to smaller fragments included in them, suggests a conformational dependence of the MAb binding sites. Our data show that the use of fusions in Western blot experiments is a useful approach to map not only linear epitopes but more complex antigenic structures found in the nucleoprotein of TGEV.

INTRODUCTION

Transmissible gastoenteritis virus (TGEV) is a coronavirus that causes a highly contagious enteric disease in pigs, resulting in a high mortality among neonates and severe economical losses to the affected farms (Garwes, 1982).

The viral particle is composed of a single-stranded RNA genome (Brian *et al.*, 1980) and three major structural polypeptides: a surface glycoprotein (S, spike, or peplomer) with a monomeric M_r 200,000; a glycosylated integral membrane protein (M) of M_r 28,000–31,000; and a basic phosphorylated protein (N or nucleoprotein) of M_r 47,000 associated with the viral RNA (Garwes and Pocock, 1975).

TGEV-infected cells have, in addition to the genomic RNA, seven or eight species of subgenomic mRNAs (Jacobs *et al.*, 1986; Britton *et al.*, 1986; Sethna *et al.*, 1989; Wesley *et al.*, 1990). The region of each mRNA responsible for the expression of its gene product appears to correspond to the 5′-terminal sequences that are absent from the next smaller species (Sturman and Holmes, 1983; Stern and Sefton, 1984). *In vitro* translation studies have shown that mRNA 7 (1.7 kb) directs the synthesis of the viral nucleoprotein (Britton *et al.*, 1986; Jacobs *et al.*, 1986). The N gene has been sequenced from several TGEV strains (Britton *et al.*, 1988; Kapke and Brian, 1986; Rasschaert *et al.*, 1987), allowing the identification of an open reading frame that encodes a polypeptide of 382 amino acids with a calculated M_r of 43,483. The amino acid sequence of the nucleoprotein from the British field strain FS772/70 and American Purdue strains of TGEV differed by 2.1%, most of the changes being conservative.

It has been demonstrated in another coronavirus, mouse hepatitis virus (MHV), that the nucleoprotein has a variety of functions in addition to being a structural component of the helical nucleocapsid (Sturman and Holmes, 1983). It plays important roles in viral pathogenesis, replication (Nakanaga *et al.*, 1986; Compton *et al.*, 1987), and transcription (Baric *et al.*, 1988; Stohlman *et al.*, 1988). In contrast, very little is known about the antigenic structure of the N protein. The production of monoclonal antibodies (MAb) specific for N (Jiménez *et al.*, 1986) and antigenic homology among N proteins of coronaviruses have been reported (Sánchez *et al.*, 1990).

In this paper we report the use of β-galactosidase-N fusion proteins to establish the location of MAb epitopes on the amino acid sequence of the TGEV nucleoprotein. This approach has been used successfully by several laboratories to locate MAb-binding sites on the S glycoprotein (Luytjes *et al.*, 1989) and the M glycoprotein of MHV (Tooze and Stanley, 1986).

MATERIALS AND METHODS

Strains and media

Escherichia coli XL1-Blue {endA1, hsdR17 (r_k^-, m_k^+), supE44, thi$^-$, recA1, gyrA96, relA1, lac-, [F′, proAB,

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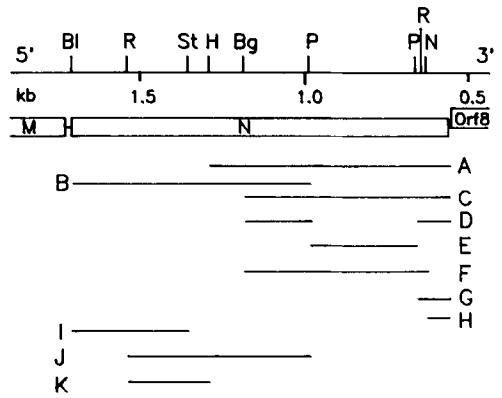

FIG. 1. Schematic representation of the fragments (A to K) of the TGEV nucleoprotein gene cloned into pUR vectors. The upper line indicates the restriction sites used for cloning (Bl, *Bal*I; Bg, *Bgl*II; H, *Hin*dIII; N, *Nsi*I; P, *Pst*I; R, *Rsa*I; St, *Sty*I). The boxes indicate the position of relevant coding regions in the TGEV genome.

$lacIq^{ZM15}$, Tn*10*(tet^R)]} from Stratagene was used in routine plasmid construction and expression experiments. *E. coli* cells were transformed using the RbCl method (Hanahan, 1985). Transformants were selected on LB plates containing ampicillin (100 $\mu g \cdot ml^{-1}$).

cDNA and plasmid vectors

The nucleoprotein cDNA from mRNA 7 was obtained as described elsewhere (Britton *et al.*, 1988). A TGEV N gene *Bam*HI cassette, pBNP5, reconstructed from that cDNA and cloned into the *Bam*HI site of pBR322 (Britton *et al.*, 1988) was used as the source of the nucleoprotein gene.

To produce β-galactosidase fusion proteins in *E. coli*, the pUR expression vectors (290, 291, and 292) (Rüther and Müller-Hill, 1983) were used.

Construction of recombinant plasmids

To map the antigenic domains of TGEV nucleoprotein, 11 restriction fragments (Fig. 1, A to K) of the N gene were cloned, in frame, into the appropriate pUR expression vector. In the pUR expression system foreign gene fragments are inserted at the 3'-end of the lacZ gene, resulting in a hybrid gene product which has the complete β-galactosidase amino acid sequence followed by the residues encoded by the in frame cloned DNA fragment. A detailed list of restriction endonucleases, generated fragments, and pUR plasmids that were used is shown in Table 1. When necessary for correct in frame ligation, blunt ends were generated by treatment with the Klenow fragment of DNA polymerase (i.e., pUR vector in fusions J and K). The *Bam*HI restriction site at the 5'-end of fragments B and I was made by adding the appropriate linkers to the blunt ends generated after digestion of the N gene with endonuclease *Bal*I (Fig. 1). Fusion I was constructed from fusion B by generating a stop codon in fusion B, cutting this fragment with restriction endonuclease Sty I (see Fig. 1 and Table 1), end-repair with Klenow and blunt-end ligation. Fusion D was the result of deleting a *Pst*I fragment from fusion C (Fig. 1).

Four of these restriction fragments (B, I, J, K) contained sequences from the 5'-half (residues 2 to 241) of the N gene. The remaining seven fragments (A, C, D, E, F, G, H) covered different regions of the 3'-half (residues 174 to 382).

Competent *E. coli* XL-1 cells were transformed with each construct and the recombinant plasmids were selected on LB-ampicillin plates at 37°. Plasmid DNA was purified and the insertions were checked for the correct orientation and for subcloning artifacts (data not shown) by restriction analysis. When convenient, the junction area of the construct was also sequenced.

Digestion and analysis of plasmid DNA

Use of restriction enzymes, DNA-modifying enzymes, plasmid preparation, and digestion procedures were as specified by the manufacturers or as described in standard manuals (Maniatis *et al.*, 1982). The enzymes were provided by Boehringer-Mannheim (Germany) or New England Biolabs (UK). DNA fragments were purified from low melting point agarose gels by the CTAB method (Langridge, 1980) or using the GeneClean kit from BIO 101 (USA).

Antibodies

The murine monoclonal antibodies 3B.B5, 3B.B6, 3B.D8, 3B.D10, 3B.H3, 3C.D8, 3C.E4, 3D.C10, and 3D.H10 (Jiménez *et al.*, 1986) were used in Western blotting experiments as hybridoma culture supernatants at 1/50 dilutions. MAbs DA3 (Garwes *et al.*, 1987) and 25-H7 (L. Saif, Ohio University) were used as ascites at 1/10,000 dilution.

The hyperimmune serum against TGEV strain FS772/70, raised in gnotobiotic piglets, was kindly provided by Dr. P. Britton (AFRC Institute for Animal Health, Compton, UK).

Rabbit anti-mouse immunoglobulins, RAM/Ig (GMADE), were provided by Nordic Immunological Laboratories (The Netherlands).

SDS–PAGE and immunoblotting

The cell extracts of recombinant *E. coli* cultures, induced with 200 μ*M* isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hr at 37°, were fractionated in a 8% SDS–polyacrylamide gel electrophoresis system (Laemmli, 1970) and transferred onto nitrocellulose

membranes (Millipore Code HAHY00010, pore size 0.45 μm), using a Mini Protean II, Bio-Rad electroblotting apparatus at 100 V for 1 hr in 25 m*M* Tris–192 m*M* glycine buffer, pH 8.3, containing 20% methanol (Burnette, 1981). After the transfer, the nitrocellulose membranes were blocked in 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, 2.7 m*M* KCl, 140 m*M* NaCl (PBS), containing 1% gelatin and 0.1% Tween 20 (PTG) for 1 hr at room temperature. The membranes were then incubated for at least 1 hr at room temperature in the appropriate dilution of the specific monoclonal antibody, followed by 1-hr incubations with rabbit anti-mouse antibodies (1/2000 in PTG) and 0.1 μCi/ml [^{125}I]-Protein A (Amersham International plc, 32 mCi mg^{-1}).

Swine polyclonal antibodies against TGEV antigens were directly detected with [^{125}I]-Protein A in PTG buffer.

RESULTS

Antigenicity of the fusion proteins

Prior to the antigenic studies, the correct translational reading frame of the β-galactosidase–nucleoprotein fusions was investigated. This was done by studying the size of the resulting hybrid proteins in SDS–PAGE relative to native β-galactosidase. For this purpose, overnight recombinant cultures were induced using IPTG and analyzed in a 8% SDS–PAGE as described in methods. After Coomassie blue staining most tracks (Fig. 2a) showed major protein bands larger than the β-galactosidase (116K) marker, corresponding to size increases over the native β-galactosidase from 9 to 29K (Table 1). Fusion proteins G and H, which included fragments of N protein coding for 34 and 23 amino acid residues, respectively, gave a major expression product of size similar to that of β-galactosidase in SDS–PAGE, since the expected increases of 3.6 and 2.5 kDa, respectively, could not be detected (Table 1).

To investigate the antigenic similarity between native TGEV nucleoprotein and the fragments of N protein expressed in the β-galactosidase-chimeras, the cell extracts of induced recombinants were analyzed by SDS–PAGE and electrotransferred onto nitrocellulose membranes. The antigenicity of the fusion proteins was determined by studying their reactivity with swine polyclonal antibodies against TGEV strain F772/70. The results in Fig. 2b (lanes A–K) showed that all fragments were recognized by the pig serum, indicating that all regions of the nucleoprotein fused to β-galactosidase were antigenic. The efficient detection of

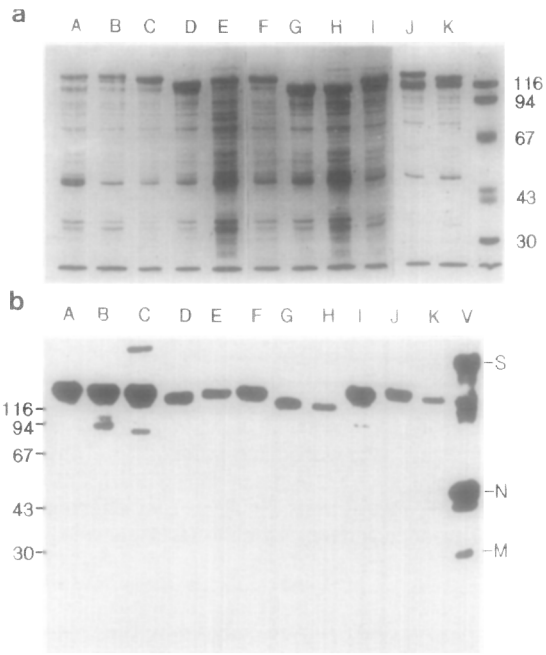

FIG. 2. Polyacrylamide gel electrophoresis of *E. coli* cell extracts expressing TGEV nucleoprotein fusions (A to K) stained with Coomassie blue (a) and Western blot immunoscreening of the expression products with a polyclonal anti-TGEV pig serum (b). The sizes of markers are indicated in kilodalton scale. Lane V of the immunoblot contains purified TGEV virions. S, N, and M indicate the positions in SDS–PAGE of the structural proteins of TGEV.

the small fusion proteins G and H (34 and 23 residues, respectively) indicated that the C-terminus of N protein must be very immunogenic. Extracts from induced cells transformed with pUR vectors without insert or not induced recombinants did not bind (data not shown) to the pig serum.

MAb epitope mapping

To define antigenic domains in the N protein of TGEV, extracts of IPTG-induced recombinant cells expressing chimeras B, C, E, G, H, I, J, and K, representing in total the full-length of N, were analyzed in 8% polyacrylamide gels and transferred to nitrocellulose filters. Similar membranes containing the same set of fusion proteins were incubated with each of the 11 N-specific monoclonal antibodies in the form of hybridoma supernatants or ascites (see Materials and Methods). The analysis of the differential reactivities of a panel of 11 MAbs with the set of 11 N-fusion proteins allowed the elaboration of an antigenic map of this protein.

Monoclonal antibodies 3B.B5, 3B.B6, 3B.H3, 3C.E4, 3D.C10, and 3C.D8 showed the same Western blot patterns. These MAbs identified fusion B (residues

TABLE 1

TGEV NUCLEOPROTEIN RESTRICTION FRAGMENTS CLONED INTO pUR VECTORS

Fragment	pUR	Restriction sites		Residues[b]		Mr[c] (kd)
		5′	3′	NH_2	COOH	
A	290	*Hin*dIII	*Hin*dIII[a]	138	382	26.8
B	292	*Bal*I	*Pst*I	2	241	26.4
C	291	*Bgl*II	*Hin*dIII[a]	174	382	22.9
D	291	*Bgl*II	*Pst*I	174	241	
		*Pst*I	*Hin*dIII[a]	349	382	9.9
E	292	*Pst*I	*Pst*I	241	349	12
F	291	*Bgl*II	*Nsi*I	174	360	20.6
G	292	*Pst*I	*Hin*dIII[a]	349	382	3.6
H	292	*Nsi*I	*Hin*dIII[a]	360	382	2.5
I	292	*Bal*I	*Sty*I	2	117	12.6
J	291	*Rsa*I	*Pst*I	57	241	20.2
K	291	*Rsa*I	*Hin*dIII	57	138	8.9

[a] The *Hin*dIII site is in the pBR322 cloning vector, 3′-downstream of the nucleoprotein gene.
[b] Residue number 1 is the amino terminal methionine of the TGEV nucleoprotein.
[c] The molecular weights of the N fragments inserted.

2–241) and failed to react with the rest of the fusions, including fusions I (2-117), J (57-241), and K (57-138), whose amino acid sequences are included in fusion B. Figure 3 shows the experimental results obtained with MAb 3C.E4, an example of this group of antibodies. Interestingly, MAb 25-H7 which also bound to fusion B reacted to the smaller fusions I, J and K included in B (Fig. 3, 25-H7) and defined in this way an epitope within the major antigenic domain. We conclude from this result that the epitope of MAb 25-H7 must lie between residue 57, which is the first N-residue represented in fusions J and K, and residue 117, which is the last N-residue expressed in fusion I.

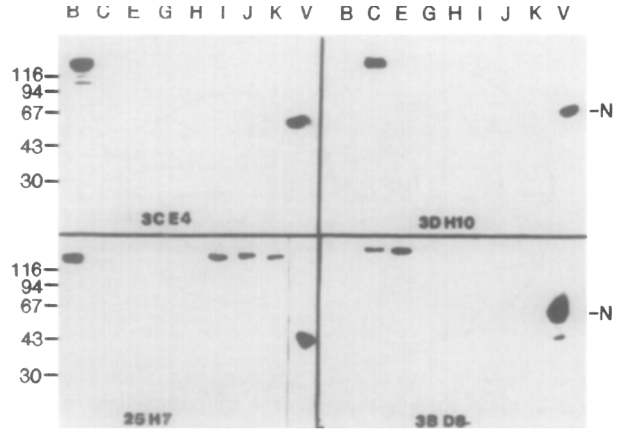

FIG. 3. Western blot analysis of *E. coli* cell extracts expressing TGEV N-fusions (B, C, E, G, H, I, J, K) using MAbs 3C.E4, 25-H7, 3D.H10, and 3B.D8. The sizes of markers are indicated in kilodaltons. Lane V contained purified TGEV virions.

Four monoclonal antibodies had their binding sites between residue 174 and the C-terminal end of the nucleoprotein. The monoclonal antibodies 3B.D10 and 3D.H10 reacted with fusion C (residues 174–382) (Fig. 3, 3D.H10) and fusion F (residues 174–360) (not shown), but not with smaller fusions E (241–349), G (349–382), and H (360–382), all of which are included in fusion protein C. The reaction of these MAbs defined an antigenic domain between residues 174 and 360 of the TGEV nucleoprotein. MAb 3B.D8, which recognized fusion C in Western blots also reacted to fusion E (241–349) (Fig. 3, 3B.D8), thus identifying an epitope within this amino acid sequence.

Using the same type of experimental approach the epitope of MAb DA3 could be mapped between residues 360 and 382 of the nucleoprotein. The immunoblot used in this case included extracts of induced and uninduced strains transformed with a pUR vector without insert and fusions A, B, C, D, E, F, G, and H. This monoclonal antibody reacted (Fig. 4) with all chimaeras (A, C, D, G, and H), which included the 23 C-terminal residues (360–382) of the nucleoprotein, but did not bind to fusion protein B (or the smaller derivatives I, J, and K), nor to fusion proteins E and F.

The results in Fig. 4 also showed that no recombinant products were detected in tracks corresponding to uninduced cultures (–), in contrast to the high detection level of the protein in the induced culture (+). The localization of the DA3 epitope within the 23 C-terminal residues of N was further confirmed by the results of Western blots of fusion proteins C (174–382) and F (174–360) (Fig. 4, lanes C and F), which differed

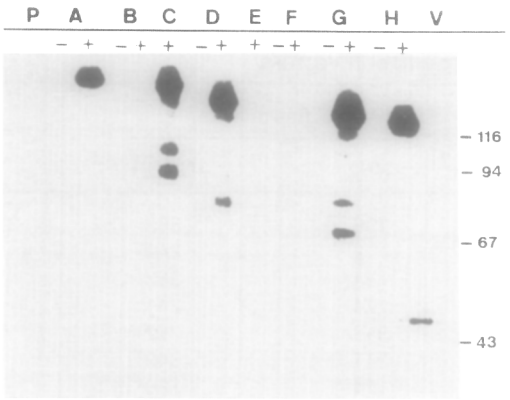

FIG. 4. Western blot analysis of *E. coli* cell extracts expressing TGEV N-fusions (A, B, C, D, E, F, G, H) using MAb DA3. Lane P contained a cell extract from an induced *E. coli* culture transformed with a pUR vector. Symbols (+, –) indicate IPTG-induced (+) or uninduced (–) cultures. Lane V contained purified TGEV virions. The sizes of markers are indicated in kilodaltons.

only in the C-terminal amino acid residues, and by the strong reactivity of H, which only expressed the differential residues (360–382) between fusions C and F.

DISCUSSION

According to the reactivity of the 11 MAbs in Western blot experiments, two major antigenic domains, A and B, can be defined in the N protein of TGEV (Fig. 5). Domain A was located in the amino half of the protein (residues 1–241) and domain B, within the C-terminal half, including residues 174 to 360 of N. Domain A appeared to be immunodominant and induced the synthesis of seven of the 11 MAbs tested (3B.B5, 3B.B6, 3B.H3, 3C.D8, 3C.E4, 3D.C10, and 25-H7). All of these MAbs recognized fusion protein B, but only 25-H7 reacted to smaller fusions (I, J, and K), whose sequences are part of fusion B. It can thus be concluded that the epitopes of the former six MAbs are most probably dependent on local protein conformation which is present in fusion protein B, but absent in the smaller derivatives of B (Fig. 5). Similar results indicating the implication of local conformation have been described for other viral proteins (Lenstra *et al.*, 1989; Jacobs *et al.*, 1990). However, MAb 25-H7 appears to be more independent of structure and binds to the shorter chimaeras (I, J, and K) included in B. The smallest fragment (K) recognized by this antibody is 82 residues long. Using these data, it can be postulated that the binding site of Mab 25-H7 is probably located between residues 57 and 117 of the nucleoprotein, which is the only region common to fusions I, J, and K. This sequence thus defined an epitope (residues 57–117) within the major domain A (Fig. 5).

The antigenic domain B involved residues 174 to 360 of the nucleoprotein and was defined by the specific reaction of MAbs 3B.D10 and 3D.H10. These antibodies only identified chimeric proteins C and F but not their smaller derivatives (D, E, G, and H). These results also point out the involvement of local conformation on antigen recognition. The binding site of MAb 3B.D8 was further mapped between residues 241 and 349 (Fig. 5), which is represented in fusion E, since this is the smallest fragment which reacted to MAb 3B.D8. This antibody thus defined an epitope (residues 241–349) within domain B (Fig. 5).

It will be necessary to perform a more refined epitope mapping for MAbs 3B.D8 and 25-H7, either using smaller fusions or by the Pepscan method, to elucidate their linear or conformational nature (Barlow *et al.*, 1986).

The epitope of MAb DA3 was mapped between residue 360 and the carboxyl end (residue 382) of the nucleoprotein. This antibody reacted in Western blots with all fusion proteins expressing the carboxy terminus of the protein (Fig. 4). Preliminary data were able to map this epitope to between residues 360 and 373.

The results obtained in Western blot experiments with the panel of 11 MAbs revealed, in most of the cases, that a minimum length of N in the fusion proteins was required for Mab recognition. This clearly indicated the involvement of distant nucleoprotein residues which after SDS–PAGE and subsequent electroblotting folded into the correct structures to be recognized by the specific MAbs. The conformation of the coronavirus nucleocapsid has not yet been clearly elucidated; nevertheless, electron microscopic data suggest helical or tubular structures (Oshiro, 1973; Caul *et al.*, 1979; Kennedy and Johnson-Lussenburg, 1976). The results of the immunoblots indicate that the frag-

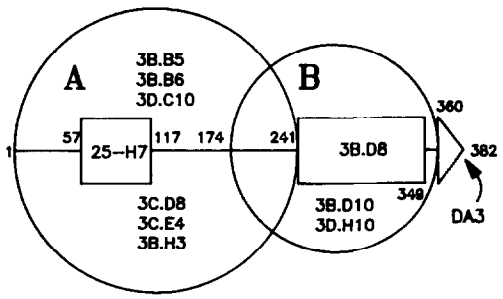

FIG. 5. Antigenic map of TGEV nucleoprotein. Circles represent the two antigenic domains A and B of N. The site-specific MAbs are indicated in the corresponding circle. The squares represent subsites based on the specific reaction of the MAbs shown inside. The triangle represents the epitope of MAb DA3. The numbers indicate the first and last nucleoprotein residues involved in the antigenic sites, subsites, and DA3 epitope.

ments of nucleoprotein expressed as β-galactosidase fusions (i.e., B and C) fold into a similar conformation to the one that triggered the synthesis of the murine MAbs.

Irrespective of the reasons why 8 out of 11 MAbs reacted only to large fusions (expressing more than 200 residues of N) and not to smaller derivatives, our results show the advantage of using gene fusions for epitope mapping, over other methods using oligopeptides, which may fail to give clear results when complex antigen structures are involved.

Our mapping results are consistent with the published antigenic relationship among coronaviruses (Sánchez *et al.,* 1990) and showed that the antigenic heterogeneity reported for the N protein of porcine respiratory coronavirus (PRCV) relative to all TGEV isolates is restricted to domain B, defined by MAbs 3B.D10 and 3D.H10. These antibodies did not bind to 5 of 8 PRCV isolates studied, one of which also did not react to MAb 3B.D8, a marker of an epitope defined within this antigenic determinant. These three MAbs failed also to react to other coronaviruses except to canine coronavirus (Sánchez *et al.,* 1990). In contrast, most of the MAbs reacting to the major domain A of TGEV nucleoprotein also reacted to all PRCV isolates, showing a high degree of conservation of this domain between these two porcine coronaviruses.

ACKNOWLEDGMENTS

We are grateful to P. Britton for providing hyperimmune TGEV-antiserum, and to L. Saif for MAb 25-H7. J.M.M.A. and M.B. are recipients of fellowships from Ministerio de Educación y Ciencia and Fondo de Investigaciones Sanitarias, respectively. This research was supported by the Biotechnology Action Programme of the Commission of the European Communities Contracts BAP-0219-E (Oviedo, Spain), BAP-0464-E (Madrid, Spain), and BAP-0235-UK (United Kingdom). This work in the Oviedo laboratory was also supported by Fundación Ramón Areces.

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