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Induction of Antibodies Protecting against Transmissible Gastroenteritis Coronavirus (TGEV) by Recombinant Adenovirus Expressing TGEV Spike Protein

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Ten recombinant adenoviruses expressing either fragments of 1135, 1587, or 3329 nt or the full-length spike gene of transmissible gastroenteritis coronavirus (TGEV) have been constructed. These recombinants produce S polypeptides with apparent molecular masses of 68, 86, 135, and 200 kDa, respectively. Expression of the recombinant antigen driven by Ad5 promoters was inhibited by the insertion of an exogenous SV-40 promoter. Most of the recombinant antigens remain intracytoplasmic in infected cells. All the recombinant-directed expression products contain functional antigenic sites C and B (Gebauer *et al.,* 1991, *Virology* 183, 225–238). The recombinant antigen of 135 kDa and that of 200 kDa, which represents the whole spike protein, also contain antigenic sites D and A, which have previously been shown to be the major inducers of TGEV-neutralizing antibodies. Interestingly, here we show that recombinant S protein fragments expressing only sites C and B also induced TGEV-neutralizing antibodies. The chimeric Ad5–TGEV recombinants elicited lactogenic immunity in hamsters, including the production of TGEV-neutralizing antibodies. The antisera induced in swine by the Ad5 recombinants expressing the amino-terminal 26% of the spike protein (containing sites C and B) or the full-length spike protein, when mixed with a lethal dose of virus prior to administration to susceptible piglets, delayed or completely prevented the induction of symptoms of disease, respectively.

INTRODUCTION

Transmissible gastroenteritis coronavirus (TGEV) infects the enteric and respiratory tissues of newborn piglets resulting in mortality of nearly 100% (Saif and Wesley, 1992). Protection of newborn animals from TGEV infection requires the induction of secretory IgA in milk. Previous studies have shown that precursors of mucosal IgA plasma cells originate in lymphoepithelial structures in the gastrointestinal and respiratory tracts. These precursor cells switch to IgA production in gut- or bronchus-associated lymphoepithelial tissues and migrate to disseminated mucosal effector sites, including gastrointestinal and upper respiratory tracts, as well as to exocrine tissues such as the mammary gland. Recombinant human adenovirus 5 (Ad5) has efficiently been used to induce protection against viral infections (Berkner, 1988; Graham and Prevec, 1992). We have reported that Ad5 infects mucosal tissues of swine (Torres *et al.,* 1995), indicating that recombinant adenoviruses might be used to induce mucosal immunity against TGEV. Helper-independent Ad5-based vectors with the capacity to express foreign genes of up to 4.9 kb have been developed (Bett *et al.,* 1993).

Several viral proteins are important for inducing an immune response to coronaviruses (Spaan *et al.,* 1990; Enjuanes and Van der Zeijst, 1995): the spike protein (S) (Buchmeier *et al.,* 1984; Cavanagh *et al.,* 1986; Daniel *et al.,* 1993; Daniel and Talbot, 1990; Koolen *et al.,* 1990), the membrane protein (Fleming *et al.,* 1989; Laude *et al.,* 1992; Welch and Saif, 1988), and the nucleoprotein (Buchmeier *et al.,* 1984; Laude *et al.,* 1992; Lecomte *et al.,* 1987; Nakanaga *et al.,* 1986; Talbot *et al.,* 1984; Wesseling *et al.,* 1993). The study of the induction of protective immunity to TGEV has focused on S protein because it is the major inducer of TGEV-neutralizing antibodies (Delmas *et al.,* 1986; Jiménez *et al.,* 1986; Laude *et al.,* 1992) and it mediates binding of TGEV to its cellular receptor (Suñé *et al.,* 1990; Godet *et al.,* 1994). A correlation between the antigenic and the physical structure of S protein has been established (Delmas *et al.,* 1986; Jiménez *et al.,* 1986; Suñé *et al.,* 1990). Site A is also involved in the induction of *in vivo* protection (De Diego *et al.,* 1992), but the precise roles of the different antigenic sites in eliciting resistance to TGEV are unknown (Enjuanes and Van der Zeijst, 1995).

In this paper we describe 10 Ad5–TGEV recombinants expressing either full-length TGEV spike protein or three truncated amino-terminal fragments of this protein. These recombinants induced immune responses in hamsters and swine which neutralized TGEV infectivity. In addition, we demonstrate that porcine serum from Ad–TGEV-immune animals protected swine from TGEV infec-

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tion. Finally, we show that virus-neutralizing antibodies are induced in the milk of Ad–TGEV-immune hamsters.

MATERIALS AND METHODS

Eukaryotic cells and viruses

The epithelial swine testicle (ST) cell line (McClurkin and Norman, 1966) and human 293 cells which constitutively express the 5′-end 11% of the Ad5 genome (Graham *et al.,* 1977) were used to grow the recombinant adenoviruses. PUR46-MAD strain of TGEV (Sánchez *et al.,* 1990) was cloned, sequenced, and used as a source of the S gene (Gebauer *et al.,* 1991). Neutralization of TGEV was performed by incubating serial 10-fold dilutions of the virus with a 1/20 dilution of the antibody at 37° for 30 min, and the virus–antibody mixture was plated on ST cells as previously described (Correa *et al.,* 1988). The neutralization index (NI) was defined as the $\log_{10}$ of the ratio of the PFU after incubating the virus in the presence of medium or the indicated antiserum. NI indices are determined rather than titers since in the first procedure virus–antibody mixtures are evaluated in the plaque assay without further dilution of the antibody, providing highly reproducible results and information about the potency of the antibody (the titer reduction expressed in logarithmic units rather than the ability of the serum to neutralize a few PFU).

Ad5 strain dl309 contains a small deletion from 83 to 85 map units and an unknown substitution in the E3 region (Jones and Shenk, 1979). pFG140 is an infectious circularized form of Ad5 dl309 carrying a 2.2-kb DNA insert (pMX2) encoding ampicillin resistance (Apr) and a bacterial origin of replication. Plasmid pFG140 was used as positive control for infectious Ad5 DNA (Graham *et al.,* 1988).

Plasmids and bacteria

The TGEV S gene was cloned into Bluescript (Stratagene) or pYA plasmids (Smerdou *et al.,* 1995) as previously described (Gebauer *et al.,* 1991). *Escherichia coli* DH5 or XL1-blue cells (Stratagene) were transformed with newly constructed plasmids by electroporation (Dower *et al.,* 1988). Plasmid DNA was prepared by the alkaline lysis method (Birnboim and Doly, 1979) and purified by CsCl–ethidium bromide density gradient centrifugation. S gene fragments or the full-length S gene were flanked either by SV-40 promoter (Pr) alone or by both Pr and polyadenylation sequences, as indicated. S gene fragments were first subcloned into pSV2X3 or pSV2X4 plasmids (Prevec *et al.,* 1990). The structures of the three key plasmids (pFG144K3, pAB14, and pFG173) used in the construction of Ad5–TGEV recombinants have been reported previously (Bett *et al.,* 1993; Mittal *et al.,* 1993). Plasmid pFG144K3 was derived from pFG144 (Ghosh-Choudhury *et al.,* 1986) and as essential features contains the 3′-end of Ad5 from the *Xho*I site at 70 map units (m.u.) with a deletion of the *Xba*I D fragment from 78.5 to 84.3 m.u. within the Ad5 E3 coding region. Plasmid pAB14 also contains the 3′-end of Ad5 from map unit 70 to 100 with a 2685-nucleotide deletion in the E3 coding region. Plasmid pFG173 contains a deletion of essential sequences to the left of E3 in the Ad genome that renders it unable to produce infectious Ad5 (Bett *et al.,* 1993; Hanke *et al.,* 1990; Mittal *et al.,* 1993).

Construction of recombinant vectors

The general procedure followed to construct recombinant Ad5 viruses expressing TGEV S gene fragments (Ad–TS) is summarized in Fig. 1. S gene sequences were flanked by SV-40 Pr and polyadenylation sequences when indicated (Fig. 2), by subcloning them into plasmid pSV2X3 or pSV2X4. Cassettes with S gene sequences were inserted into the unique *Xba*I site of the partially deleted E3 gene on plasmid pFG144K3 or pAB14, both of which include the 3′-end of Ad5. Alternatively, S gene fragments were removed from the original plasmid or from pSV2X3-TS vectors without SV-40 Pr signal, or without both Pr and polyadenylation sequences, using the restriction endonucleases indicated in Fig. 1. In this case, fragment ends were blunted with Klenow and T4 DNA polymerase and cloned into the *Xba*I site of pFG144K3 or pAB14 plasmids that were blunted and dephosphorylated according to standard procedures (Maniatis *et al.,* 1989). Each of these plasmids is noninfectious by itself, but can generate infectious virus following cotransfection of 293 cells along with a plasmid, pFG173, which contains the 5′-end of Ad5 (Fig. 1) (Graham and Prevec, 1992; Hitt *et al.,* 1995, 1994). This results in the rescue of genes cloned into the E3 region of viral vectors. Cotransfection was performed essentially as described using the calcium phosphate precipitate technique (Graham and van der Eb, 1973). After 8 to 15 days, plaques were isolated and expanded, and viral DNA was analyzed by *Hin*dIII restriction enzyme digestion. Viruses with the expected DNA pattern were plaque purified three times and the junction of the constructs was sequenced to verify the expected primary structure. Recombinants Ad–TS01 and Ad–TS02 are identical to recombinants Ad–TS5 and Ad–TS6, respectively, except that the first two were constructed using cloning vector pAB14 with the large deletion on E3 gene, while in the construction of the second pair of recombinants plasmid pFG144K3, with the smaller deletion on E3, was used.

Immunoprecipitation of S antigens expressed by recombinant Ad–TS

Subconfluent 293 cells grown in Dulbecco's modified Eagle medium with 5% horse serum (Gibco Europe) were infected with Ad–TS recombinants at a multiplicity of infection (m.o.i.) of 30 PFU per cell. After 1 hr of virus

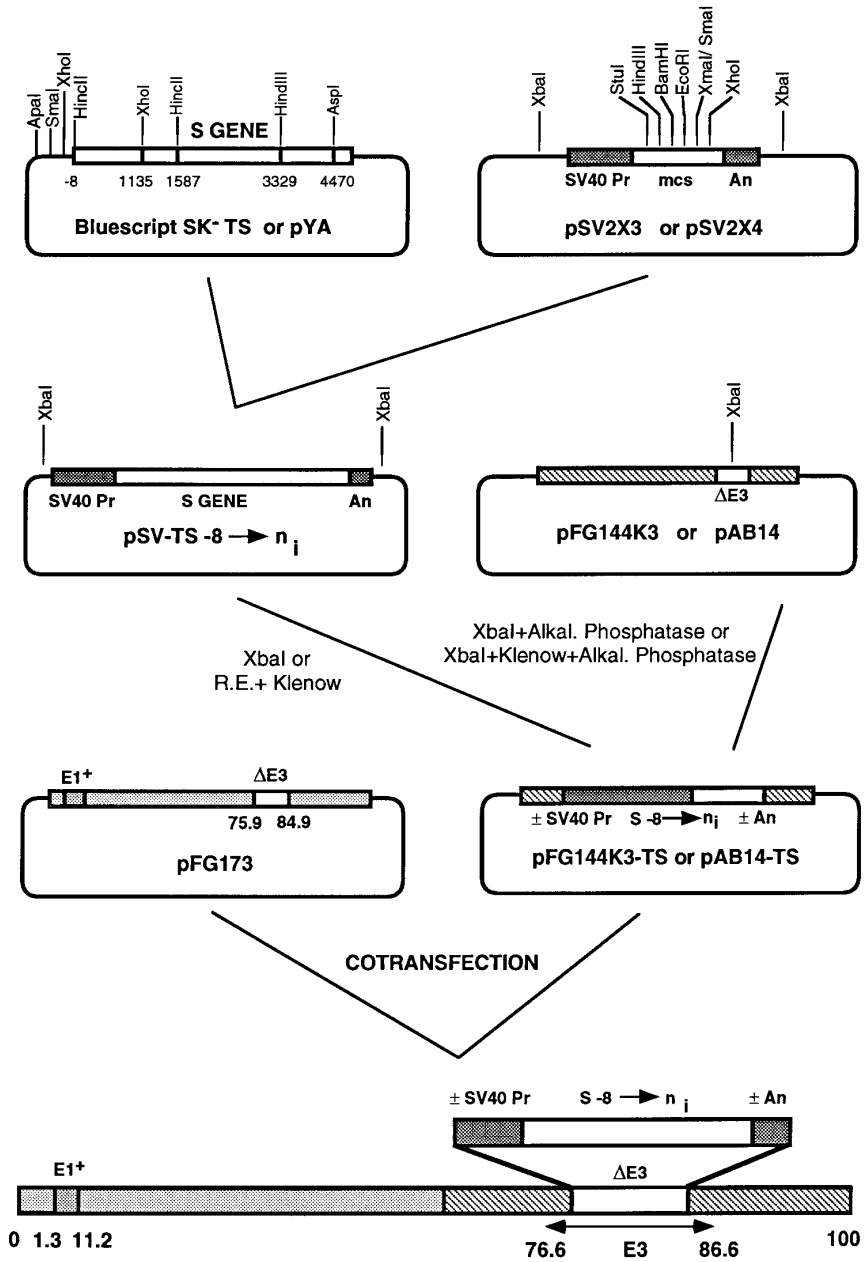

FIG. 1. Construction of plasmid vectors and recombinant adenoviruses carrying S gene. Recombinant plasmids were constructed following standard procedures (Maniatis *et al.,* 1989). S gene sequences previously cloned into Bluescript(SK$^-$) (Promega) or pYA (Smerdou *et al.,* 1995) plasmids were excised using the indicated restriction endonucleases and subcloned into pSV2X3 or pSV2X4, in which the S gene sequences were flanked by SV-40 Pr, polyadenylation sequences, or both. To generate recombinants Ad–TS07, Ad–TS05, Ad–TS9, and Ad–TS06 S gene sequences were cloned directly into plasmid pFG144K3 or pAB14. S gene sequences either alone or flanked by SV-40 sequences were subcloned into the *Xba*I site of pFG144K3 or pAB14, or excised with the indicated restriction endonucleases, blunted using the Klenow polymerase fragment, and cloned into blunted *Xba*I unique site of these vectors. Infectious Ad–TS recombinants expressing S protein fragments were generated by cotransfecting 293 cells with pFG144K3-TS or pAB14-TS (which carry S gene sequences from TGEV and pFG173 plasmids). Diagrams are not to scale. The origins of DNA fragments flanking the S gene are indicated with squares filled with different motifs. Numbers below the bar representing the Ad5 genome (bottom) indicate map units. mcs, multicloning site; Pr, promoter; An, polyadenylation signal; ΔE3, deletion in E3 gene; R.E., restriction endonuclease; TS refers to sequences derived from TGEV spike gene.

adsorption at 37°, fresh medium was added and cells were incubated for 22 hr at 37°. Medium was then replaced by methionine- and cysteine-free medium containing 2% dialyzed serum. Cells were incubated for 1 hr at 37°, washed with methionine- and cysteine-free medium, and refed with fresh medium containing 50 μCi/ml of Pro-Mix: L-[^{35}S] *in vitro* methionine/cysteine labeling mix (1 Ci/mmol, Cod. No. SJQ0079, Amersham Ibérica). Cell monolayers were incubated 1.5 hr, detached with a rubber policeman, washed with cold phosphate-buffered

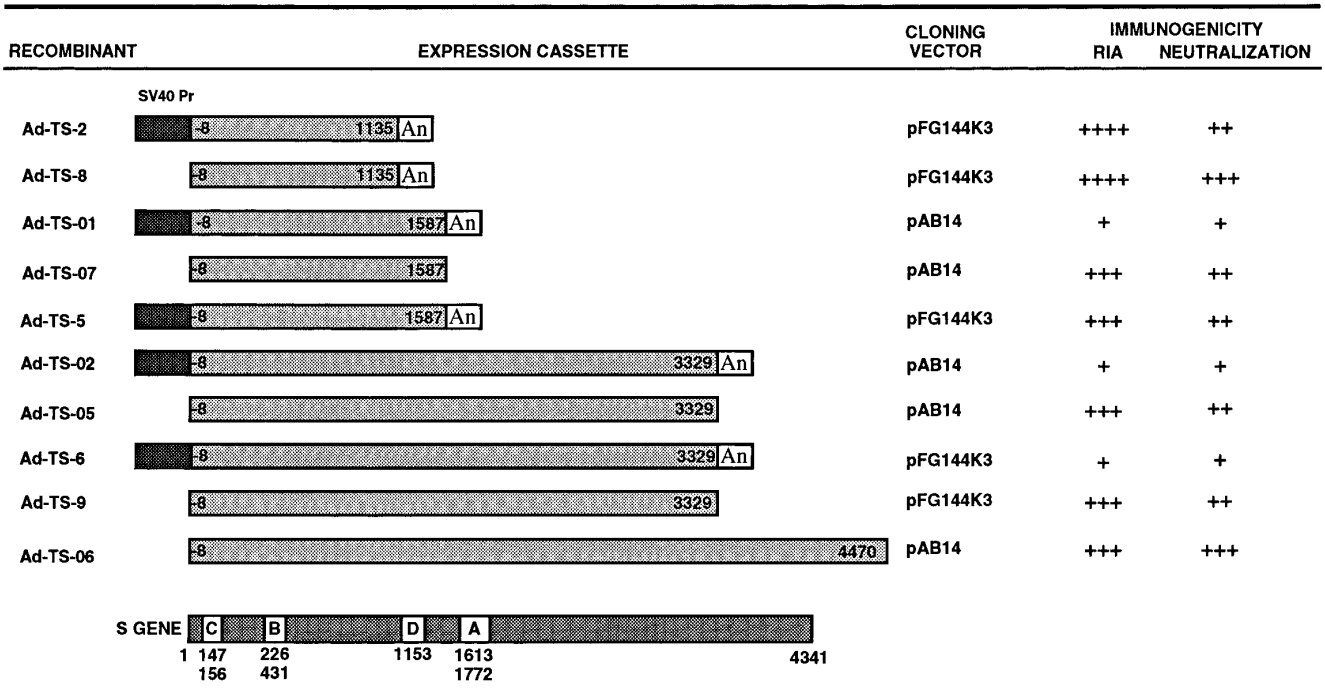

FIG. 2. Structure of the inserts expressed using recombinant Ad5–TGEV. The diagram shows the S gene fragments (light squares) cloned into the E3 gene of Ad5. Numbers inside the squares indicate the nucleotides at both 5′- and 3′-ends of S gene fragment. When indicated, inserts were flanked by SV-40 promoter (Pr) (dark squares) and by polyadenylation sequences (white squares). Several S gene fragments were cloned without either SV-40 Pr or polyadenylation sequences. In the bar shown at the bottom, the positions (nt) of the different antigenic sites of S protein are indicated. The immunogenicity of the recombinant antigens was determined by evaluating the antiserum induced after administering the recombinant Ad5 to hamsters by RIA and neutralization, as described under Materials and Methods. Titers in the RIA were expressed as: +, 0–1000; ++, 1000–3000; +++, 3000–5000; ++++, >5000. Results of virus neutralization were expressed as neutralization index: +, <1; ++, 1–2; +++, 2–3; ++++, >3.

saline, pH 7.2 (PBS), collected by centrifugation at 3000 rpm for 15 min at 4° in a microfuge, and lysed in RIPA buffer (50 m*M* Tris–HCl buffer, pH 7.5, 150 m*M* NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate (SDS), and 0.2 m*M* PMSF). Viscosity was reduced by mixing the tubes in a Vortex mixer and passing the samples through a 0.6-mm needle 10 times. Extracts were centrifuged at 30,000 *g* for 30 min at 4° in a microfuge. Labeled proteins were immunoprecipitated with TGEV-specific porcine serum which had been preadsorbed several times with 293 cells infected with adenovirus Ad5 dl309. Further absorption of the antiserum did not eliminate the nonspecific bands. Antigen–antibody complexes were bound to protein A–Sepharose by overnight incubation at 4°. Sepharose beads were washed three times with RIPA buffer containing 0.2% SDS, and the final pellet was resuspended in electrophoresis sample buffer containing 2.5% SDS and 5% 2-mercaptoethanol (Laemmli, 1970). Samples were boiled for 3 min, the beads were sedimented by low-speed centrifugation, and supernatants were analyzed by polyacrylamide gel electrophoresis and autoradiography. To estimate the amount of protein expressed by each recombinant different dilutions of sucrose gradient-purified ^{35}S-labeled TGEV (used as an standard) and Ad–TS recombinants grown under the same conditions were immunoprecipitated in parallel. The same number of infected cells was analyzed for each recombinant. Similar relative expression levels were obtained in many (>5) experiments. After protein resolution in polyacrylamide gel electrophoresis and autoradiography, the intensity of the immunoprecipitated bands from Ad–TS extracts was compared with that of the reference [^{35}S]TGEV with known protein concentration (determined using BCA Protein Assay Reagent, Pierce) to estimate the amount of S antigen.

Immunofluorescence

ST cells at a density of approximately 1.5×10^5 cells/cm^2 in microslide culture chambers (Miles Scientific) were infected with adenovirus Ad140 which contains no S gene insert, or with Ad–TS recombinants, at a m.o.i. of 3 PFU/cell. At 24 hr postinfection, cell monolayers were washed and fixed either with methanol:acetone (1:1) at −20° for 15 min or with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were washed three times with PBS and once with 0.3% bovine serum albumin (BSA) in PBS for 10 min at room temperature. The cells

were incubated with hybridoma supernatants containing a mixture of MAbs 1D.B12, 5B.H1, and 1D.G3 (specific for S protein sites B, C, and D, respectively) or with MAb H2-19 specific for a 70K Ad5 antigen. After three additional washings with PBS, cells were covered with a 1:200 dilution of fluoresceinated goat anti-mouse immunoglobulins (Cappel Laboratories) in 0.3% BSA in PBS, incubated for 40 min at room temperature, washed five times for 10 min each with PBS, and mounted on glycerol–PBS (9:1).

Binding of ^{125}I-labeled MAbs to 293 cells infected with recombinant Ad–TS

Confluent ST cell monolayers plated on 24-well microplates were infected (m.o.i. 40 PFU/cell) with recombinant Ad–TS viruses. At 24 hr postinfection, cells were washed with PBS and fixed in methanol:acetone (1:1) for 15 min at −20° or in 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were washed three times with PBS and for 2 hr with 0.5% BSA in PBS. Aliquots of 0.25 ml of ^{125}I-labeled purified MAbs (1×10^6 cpm/well; 1.5×10^7 cpm/μg) (Greenwood *et al.,* 1963) in PBS with 0.2% BSA were added to each well and incubated for 1 hr at room temperature, and the cell monolayers were washed six times with PBS. MAb binding was determined by collecting the cells in 0.25 ml of 0.5 *N* NaOH and counting the radioactivity in a gamma counter.

Immunization of hamsters and swine

Eight-week-old golden Syrian hamsters were immunized with infectious Ad–TS recombinants by three routes: oral (4×10^8 PFU in 0.2 ml of PBS), nasal (2×10^8 PFU/0.1 ml), and intraperitoneal (1×10^9 PFU/0.5 ml). The virus was administered at Days 0, 32, 60, and 90, and orbital plexus puncture bleedings were performed at Days 0, 32, 47, 87, 105, and 115. Females with highest titers of TGEV-specific antibodies were crossed with nonimmune males, and 8 days later another dose of the homologous Ad–TS recombinant was administered. Twenty-four hours after delivery, hamsters were subcutaneously administered 10 IU of oxytocin. The milk was collected 1 hr later by applying vacuum with a syringe. Milk was diluted fourfold in PBS and stored at −20°.

One-month-old swine, from crossing Large White and Belgium Landrace, were immunized three times at 0, 28, and 56 days, each time by three routes: oral (1×10^9 PFU), nasal (1×10^9 PFU), and intraperitoneal (1×10^9 PFU per dose). Serum was collected 14 days after the last immunization.

Radioimmunoassay (RIA) and competitive RIA (cRIA) with ^{125}I-labeled MAbs

RIA was performed using purified TGEV as antigen (0.1 μg/well) as previously described (Jiménez *et al.,* 1986). Titers in RIA were defined as the inverse of the highest dilution giving a binding threefold higher than background.

Detection of the different antigenic sites in the S protein fragments, encoded by recombinant Ad–TS, was carried out by cRIA using the antiserum elicited in hamsters by the different recombinants. The binding of ^{125}I-labeled MAbs to purified TGEV bound to microplates was performed as previously reported (Correa *et al.,* 1988) with some modifications. Briefly, purified TGEV (0.1 μg/well) was plated, remaining binding sites were saturated with 5% BSA in PBS, and ^{125}I-labeled MAbs (sp act 1.5×10^7 cpm/μg; 4×10^5 cpm/well) were added and incubated for 2 hr at 37° in the presence of fivefold dilutions of the competitor antiserum prepared in PBS with 0.1% BSA. Microplates were washed six times with 0.1% BSA and 0.1% Tween-20 in PBS. Well bottoms were cut and bound radioactivity was determined in a gamma counter. The percentage of radioactivity bound was determined in relation to the radioactivity bound in the absence of competitor MAb. Purified homologous MAbs were used as positive controls in the cRIA.

Protection of swine by immune serum

The virulent TGEV strain PUR46-SW11-ST2 (1×10^7 PFU/swine) was mixed with 3 ml of the porcine antiserum induced by recombinants Ad–TS8 or Ad–TS06, incubated at 37° for 60 min, and administered using a gastric tube to 2-day-old miniswine born from TGEV-seronegative sows. Inoculated animals were fed three times per day with milk formula for newborns (Nidina 1, Nestlé) containing 3 ml of the antiserum. Control animals were treated following the same procedure but using serum induced by wt Ad5. Virus titers after 1, 2, and 3 days in animals challenged with virus treated with control serum and 1, 2, or 5 days postinoculation in animals challenged with TGEV immune serum-treated virus were determined in tissue extracts from jejunum and ileum, lungs, mesenteric, and mediastinal lymph nodes. Tissue homogenization was performed at 4° using an OMNI 2000 homogenizer (Omni International).

RESULTS

Ad5–TGEV recombinants

Ten Ad5–TGEV recombinants expressing TGEV S gene fragments were constructed using vectors with different deletions on E3 gene or combinations of SV-40 promoter and polyadenylation signals. Using these recombinants S protein fragments of four different sizes were expressed. The recombinants were obtained by replacing the E3 gene of the Ad5 genome with S gene sequences starting from nt −8 and the first 5′-end 1135, 1587, 3329, or 4341 nt of the S gene. These recombinants code for fragments of 378, 529, 1109, and 1447 amino acids (aa) extended from the amino-terminus (Fig. 2). The

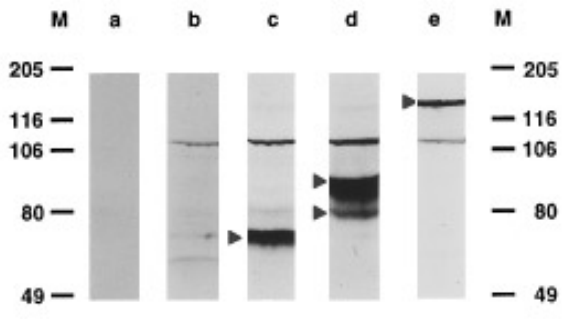

FIG. 3. Analysis of the S protein fragments expressed by Ad–TS recombinants. Recombinant antigens expressed by the indicated Ad5–TS viruses were metabolically labeled with [^{35}S]methionine, immunoprecipitated with TGEV-specific antiserum, and analyzed by SDS–PAGE (Materials and Methods). Specific bands are indicated by arrows. One or two bands with molecular masses around 60 and 110 kDa were nonspecifically precipitated in Ad5 virus (without insert)-infected but not in uninfected cells, and probably correspond to Ad5 proteins. Lanes correspond to representative immunoprecipitates from extracts of: a, uninfected 293 cells; b, cells infected with Ad5 virus with no insert; c, Ad–TS2 or Ad–TS8; d, Ad–TS07 or Ad–TS5; e, Ad–TS9. Recombinants Ad–TS02, Ad–TS05, and Ad–TS6 give bands in the same position as that of Ad–TS9 but of lower intensity (not shown). A very faint specific band was observed after immunoprecipitation of extracts from cells infected with recombinant Ad–TS01 and Ad–TS06 (not shown). M, molecular weight markers (numbers indicate kDa $\times$ 10^{-3}).

last product represents the full-length spike protein. The constructs were obtained using either plasmid pFG144K3 or plasmid pAB14 (Fig. 1), with deletions of 1.88 or 2.69 kb, respectively, in E3 (Bett *et al.,* 1993). Recombinant plasmids were constructed as summarized (Fig. 1). When indicated, the S gene fragments were flanked by Pr and polyadenylation signals (Fig. 2) by cloning them into vector pSV2X3 or pSV2X4. Inserts were subcloned into plasmid pFG144K3 or pAB14 containing the 3′-end half of Ad5. Human 293 cells were cotransfected with one of these plasmids and pFG173, which contains almost the entire Ad5 genome with a lethal deletion across the E3 region. Fully infectious Ad–TS viruses were recovered following recombination in cotransfected 293 cells. Recombinant viruses were plaque purified. The DNA from all the recombinants gave the pattern and sequence expected for each insert by *Hin*dIII restriction endonuclease analysis and sequencing of DNA junctions (results not shown).

After infection of 293 cells with Ad–TS recombinants, S protein antigens remained cell associated. Tris buffer containing 1% SDS was used to solubilize them. The estimated size of recombinant S antigen expressed by Ad–TS vectors was evaluated by immunoprecipitation and representative results are shown (Fig. 3). S polypeptides were detected with a polyclonal TGEV-specific porcine serum. Good specific immunoprecipitation bands were systematically obtained with all recombinants except Ad–TS01, Ad–TS02, and Ad–TS06, which gave a faint band (results not shown). Recombinant products with apparent molecular masses of 68 and 135 kDa (Fig. 3, lanes c and e, respectively) were obtained for recombinant S protein fragments of 378 and 1109 aa, respectively. Recombinants Ad–TS07 and Ad–TS5, both coding for polypeptides of 529 aa, gave a main band of 86 kDa and a minor band of 80 kDa (lane d), which probably corresponds to an underglycosylated form of the antigen or to a degradation product. The difference between the expected and the apparent molecular mass of the recombinant products suggests that these are heavily glycosylated, as occurs during S protein synthesis after TGEV infection (Delmas and Laude, 1990). A band with M_r 110K was also precipitated in cells infected with Ad5 with or without S gene insert (Fig. 3, lanes b to e), but not in uninfected cells (lane a) suggesting that it was a nonspecifically bound Ad5 antigen. This band was not eliminated after extensive serum adsorption with extracts from Ad5-infected 293 cells.

Antigen expression levels

Recombinant antigen expression levels were estimated by immunoprecipitation with TGEV-specific antiserum using extracts from 293 cells infected with the chimeric Ad–TS viruses (Fig. 3). The amount of S protein was based on the comparison of band intensity after immunoprecipitation and autoradiography of ^{35}S-labeled recombinant antigens and reference sucrose gradient-purified ^{35}S-labeled TGEV with known protein concentration. Both reference virus and recombinant antigens were labeled and analyzed in parallel using the same experimental conditions. Since the distribution of the methionine and cysteine in the different fragments was similar, no significant correction of band intensity was necessary in the analysis. The expression levels ranged from 0.1 to 10 μg of S protein per 10^6 infected cells. Maximum expression levels (5 to 10 μg/10^6 cells) were obtained for recombinants Ad–TS5, Ad–TS8, and Ad–TS07, intermediate levels (1 to 3 μg/10^6 cells) for Ad–TS9, Ad–TS2, Ad–TS05, and Ad–TS6, and minimum (around 0.1 μg/10^6 cells) for recombinants Ad–TS01, Ad–TS02, and Ad–TS06. Relative expression levels were highly reproducible in different experiments. All the recombinants, including those expressing minimum amounts of antigen, were also consistently positive in the immunofluorescence and ^{125}I binding assays and in the induction of TGEV-specific antibodies (see below).

When indicated, the S gene fragment cloned into Ad5 was flanked by Pr and polyadenylation signals (Fig. 2). Comparison of the expression levels in constructs with S gene fragments of the same size indicated that Ad5 recombinants made using pFG144K3 plasmids expressed higher levels of antigen than those based on plasmid pAB14, although in some cases (i.e., recombinant Ad–TS07 compared with Ad–TS5) the level of ex-

pression was similar (results not shown). In recombinants with the same E3 deletion it was also observed that removal of SV-40 Pr yielded Ad–TS recombinants with higher expression levels (results not shown).

Cellular location of the S antigens coded by the Ad–TS recombinants

To study the cellular location of recombinant S antigen, we used immunofluorescence analysis of ST cells infected with four selected recombinants each coding for S fragments of different size: 387, 529, 1109, and 1447 (full-length S protein) amino acids. A bright fluorescent signal was observed in the cytoplasm of methanol–acetone-fixed cells infected with recombinants Ad–TS8, Ad–TS5, and Ad–TS9 (results not shown). Highest fluorescence intensity was seen with TGEV-infected cells and lowest intensity with cells infected with Ad–TS06 recombinant expressing the full-length S protein. In infected cells fixed with paraformaldehyde processed in parallel, the intensity of the staining was considerably weaker. In this case, cells infected with TGEV showed a clear staining of the plasma membrane, while very weak fluorescence was observed in cells infected with the four Ad–TS recombinants. When immunofluorescence was performed with a human Ad5-specific MAb (which binds 72K protein) bright fluorescence was observed on discrete areas of the nucleus, but not in the cytoplasm (results not shown), in contrast to the cytoplasmic fluorescence observed with TGEV-specific MAbs.

An estimation of the relative amount of S antigen located in the cytoplasm or accessible on the surface of Ad5-infected ST cells was determined by studying the binding of ^{125}I-labeled MAb 1D.B12 (site B-specific) to methanol- or paraformaldehyde-fixed cells (results not shown). This MAb was selected because it recognizes an epitope present in all Ad–TS recombinants. Cells infected with recombinants Ad–TS8, Ad–TS5, and Ad–TS9 permeabilized with methanol–acetone expressed the highest amount of S antigen, which ranged between 60 and 66% of the amount expressed on ST cells infected with TGEV. In cells infected with these recombinants the binding of site B-specific MAb to exposed antigen was around 10% of the binding to cytoplasmic S antigen of TGEV-infected cells. That is, the amount of S antigen detected on the surface of the infected cells was at least sixfold lower than that seen in the cytoplasm. The recombinant products were not detected in the supernatants of infected cells, although the media were not concentrated to detect small antigen amounts.

Proper folding of the S protein fragments expressed by the four selected recombinants was evaluated by determining the amount of ^{125}I-labeled MAb specific for antigenic sites A, B, C, and D bound to infected ST cells (Fig. 4). All recombinants expressed sites C and B. Recombinant Ad–TS9, in addition, expressed sites D and A. Although amino acids 380 to 387 of S protein site D are coded by recombinant Ad–TS5, this site was poorly recognized by MAb 1D.G3 specific for D site on Ad–TS5-infected cells (Fig. 4). The four antigenic sites were weakly detected in cells infected by recombinant Ad–TS06, probably due to the low replication level of this recombinant.

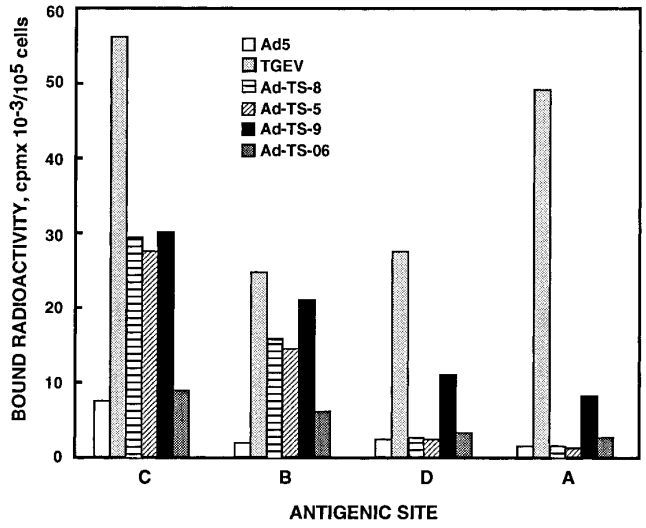

FIG. 4. Binding of MAbs specific for S protein antigenic sites to ST cells infected with Ad–TS recombinants. ^{125}I-labeled MAbs specific for antigenic site C (5B.H1), B (1D.B12), D (1D.G3), and A (6A.C3) (Gebauer *et al.,* 1991) were incubated on methanol–acetone-fixed ST cells infected with the indicated recombinant or with TGEV. Cell monolayers were washed and the bound radioactivity was determined as indicated under Materials and Methods. Mean values have been represented. Standard deviations were lower then 25% of the mean in all cases and are not shown.

Immunogenicity of the recombinants

Immune responses elicited by the different recombinants were studied by inoculating hamsters both oronasally and intraperitoneally (Fig. 5). Seven of the ten recombinants summarized in Fig. 2 elicited titers in RIA higher than 2500 and NI between 1 and 3. The best inducers of TGEV-neutralizing antibodies were recombinants Ad–TS8, Ad–TS2, and Ad–TS06, expressing either the smallest protein fragment or the full-length protein (Fig. 2).

Four recombinants (Ad–TS8, Ad–TS5, Ad–TS9, and Ad–TS06), each expressing S gene fragments of different lengths (Fig. 2) were selected to study the induction of an immune response to sites A, B, and D by cRIA (Fig. 6). Site C was not included in the study because the amino acid sequence PNSD recognized by MAbs specific for this site (Gebauer *et al.,* 1991) is present in proteins of the immunoglobulin superfamily and other serum proteins (Correa *et al.,* 1988; I. Correa and L. Enjuanes, unpublished results). Recombinant Ad–TS9 induced an immune response to antigenic sites B, D, and A (Fig. 6).

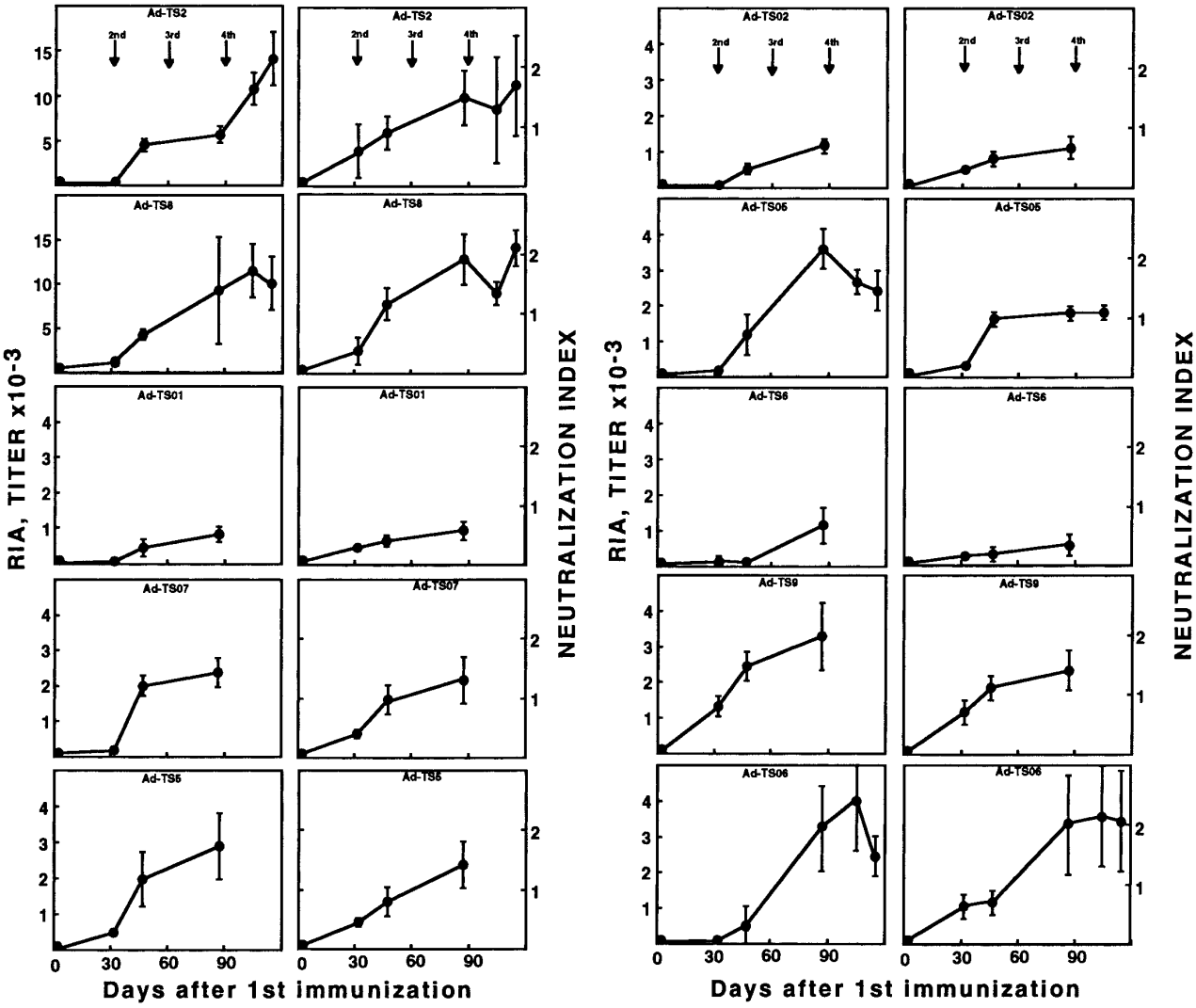

FIG. 5. Immune response induced by Ad–TS recombinants in hamsters. Groups of four golden Syrian hamsters were immunized at Time 0 and at times indicated by arrows (see Materials and Methods) with the indicated recombinants. Sera collected at 0, 32, 47, 87, and, in some cases, at 105 and 115 days postinfection were evaluated by RIA and neutralization against TGEV. Mean serum titers and standard deviation errors are represented for each time point. The titer by RIA was defined as the inverse of the highest antibody dilution giving a binding three times higher than the background in the RIA assay. The NI was defined as the $\log_{10}$ of the ratio of the PFU after incubating the virus in the presence of medium or the indicated antiserum.

All recombinants induced a strong response to site B (Fig. 6A) which is conformation and glycosylation dependent (Gebauer *et al.,* 1991). As expected, site A was only reconstituted by recombinants Ad–TS06 and Ad–TS9, expressing the full-length S protein or the 135-kDa S antigen, but not by recombinants which do not include the residues implicated in this site (Fig. 6C).

Induction of lactogenic immunity by Ad–TS recombinants

Female hamsters immunized twice with recombinants Ad–TS8, Ad–TS9, and Ad–TS06 were crossed with non-immune males and administered a third dose of the homologous Ad–TS recombinant 10 days before delivery. The presence of TGEV-specific antibodies in the sera and milk was determined between Days 1 and 2 during lactation (Fig. 7). The three recombinants induced antibodies in serum with titers in RIA ranging from 5×10^3 to 1.5×10^4 and in milk from 2×10^3 to 3×10^3 (Fig. 7A). Serum and milk antibodies neutralized TGEV with NIs ranging from 2 to 4 and around 1, respectively (Fig. 7B). As expected, recombinants with no insert did not elicit TGEV-specific antibodies. While antibody titers in sera decreased with insert size, the NI increased, suggesting that antibodies to site A contributed significantly to the neutralization of TGEV.

Induction of immune response in swine by recombinants Ad–TS8 and Ad–TS06

The Ad–TS8 and Ad–TS06 recombinants expressing the smallest insert and the full-length spike protein, re-

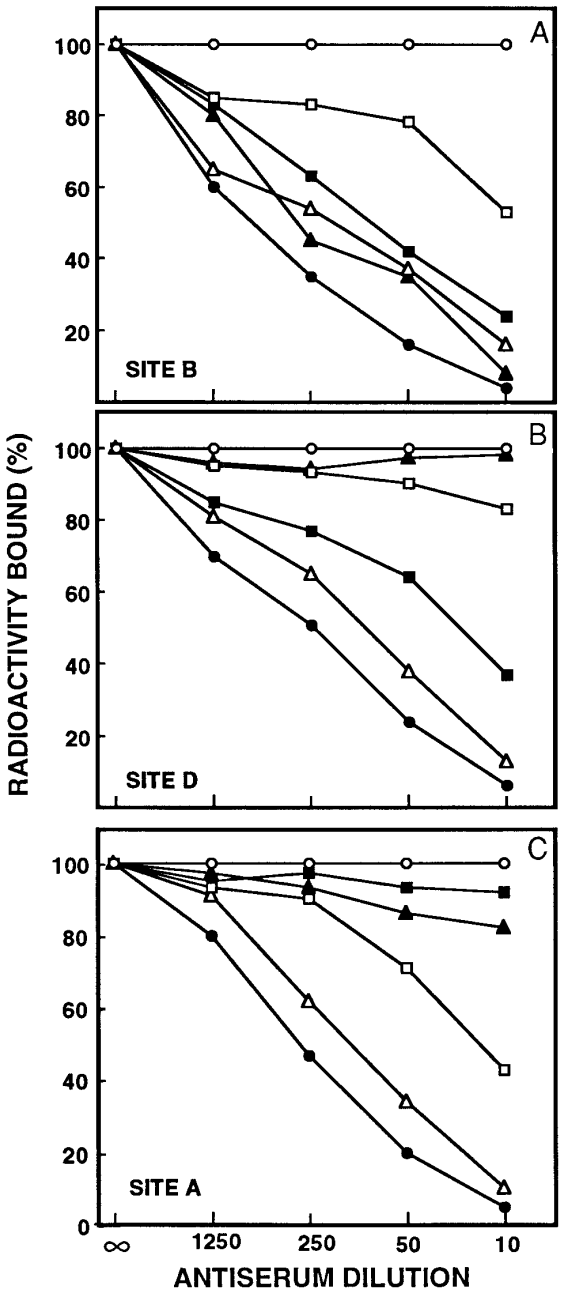

FIG. 6. Antigenic site specificity of the antisera induced in hamsters by Ad–TS recombinants. The binding to purified TGEV of ^{125}I-labeled MAbs specific for antigenic sites B, D, and A of TGEV S protein (A, B, and C, respectively) was inhibited in a cRIA (see Materials and Methods) by (●) the homologous unlabeled MAb, by (○) a control antisera with no reactivity to TGEV, or by sera from hamsters immunized with Ad-TS recombinants: (▲) Ad–TS8; (■) Ad–TS5; (△) Ad–TS9; (□) Ad–TS06.

spectively, were selected to study the induction of TGEV-neutralizing antibodies in swine. Although the level of recombinant antigen produced in ST cells was high for recombinant Ad–TS8 and low for Ad–TS06 (Figs. 3 and 4), both recombinants induced high titers of TGEV-specific antibodies in swine as determined by RIA (1×10^4 and 5×10^4, respectively) and by neutralization (NI of 2 and 5, respectively). To study the potential of these antisera for protection against TGEV, sera induced by these recombinants were examined for the ability to prevent TGEV infection. Virulent TGEV (PUR46-SW11-ST2 strain, 1×10^7 PFU/dose) was mixed with the antibody induced by each recombinant, incubated at 37° for 60 min, and administered to highly susceptible 2-day-old miniswine. Virus titers were determined in jejunum and ileum, lungs, mesenteric, and mediastinal lymph nodes at 1, 2, 3, and 5 days postinoculation. The results (Fig. 8) indicated that virus titers found in the enteric tissues were between 10^2 and 10^3-fold lower when virus was premixed with antiserum induced by recombinant Ad–TS8 (Fig. 8D), and very low titers ($<5 \times 10^2$ PFU/g of tissue) of infectious virus were detected in the small intestine of newborn pigs that were administered the antibody elicited by recombinant Ad–TS06 (Fig. 8F). In contrast, titers ranging between 7×10^3 and 1×10^7 PFU/g of tissue were detected in the tissues of control animals to which serum induced by wt Ad5, used as a control, was administered (Fig. 8B). In addition, neither mortality nor clinical symptoms were observed in animals treated with serum induced by recombinant Ad–TS06 (Fig. 8E), while control animals presented diarrhea 24–30 hr postinfection and died around Day 3 postinfection (Fig. 8A).

DISCUSSION

Ten Ad5–TGEV recombinants have been constructed and screened for their ability to express spike protein fragments of TGEV. Four recombinants expressing the full-length spike protein or truncated fragments spanning different lengths of S protein from the amino-terminus have been selected, and their ability to induce virus-neutralizing antibodies was determined. These Ad–TS viruses induced lactogenic immunity in hamsters, and the recombinant expressing the full-length S protein elicited antisera that, when mixed with a lethal dose of virus prior to administration to susceptible piglets, prevented the induction of disease symptoms.

Helper-independent Ad5 viruses with a deletion in the E3 gene have been constructed, and the S gene was inserted into the E3 gene. Two types of Ad5 recombinants with deletions of 1.88 and 2.69 kb in the E3 gene have been used to express S protein, based on plasmids pFG144K3 and pAB14, respectively (Bett *et al.,* 1993). Since the large deletion affected the splicing acceptor site after gene L4 and most of the E3 gene, it was of interest to determine the comparative levels of expression in these two plasmids. Expression levels were always higher using Ad5 viruses with the smaller deletion in E3, independent of the insert size, suggesting that removal of the splicing acceptor site after the L4 gene might have reduced E3 gene expression. Sequences inserted without an exogenous polyadenylation signal were successfully expressed, indicating that the polyadenylation signal of the E3 gene has probably been used.

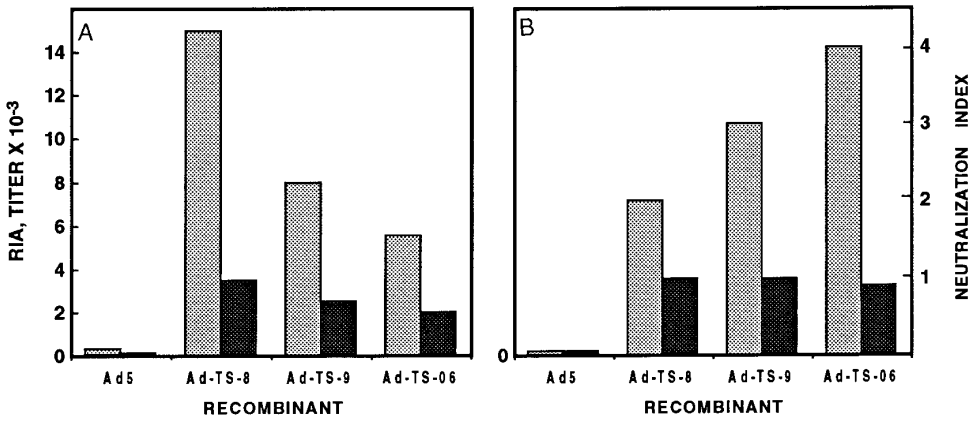

FIG. 7. TGEV-specific antibodies induced by Ad–TS recombinants in the serum and milk of lactating hamsters. Titers in (A) RIA and (B) neutralization in the serum (light columns) and milk (dark columns) of hamsters immunized with Ad5 virus or the indicated Ad–TS recombinants. Titers in RIA and neutralization were defined as described in Fig. 5. Mean values of four hamsters have been represented. Standard deviations were lower than 30% in all cases and are not shown.

In general, recombinants with relatively small inserts (1135, 1587, and 3329 nt) expressed larger amounts of S polypeptide than those with larger (4470-nt) inserts. The recombinants with smaller inserts gave Ad5 titers in cell culture between 3×10^8 and 1×10^9 PFU/ml, while Ad5–TS virus with an insert of 4470 nt consistently gave titers lower than 10^7 PFU/ml. Thus, the level of expression in these recombinants correlates well with their level of replication. The three recombinants (Ad–TS8, Ad–TS5, and Ad–TS9) with genome sizes lower than 104% of wt Ad5 were stable after 10 passages, while the recombinant with a genome size close to 105% of wt Ad5 (Ad–TS06) was unstable (results not shown). These results are in line with previous work suggesting that the Ad5 virion has the ability to package approximately 105% of the wt genome length. This value is generally considered to be the maximum working capacity of the system (Ghosh-Choudhury *et al.,* 1986; Berkner, 1988; Bett *et al.,* 1993).

Viruses in which the inserted gene was flanked by an SV-40 Pr always showed lower expression levels than those not flanked by this Pr (Fig. 3). This suggests that the SV-40 Pr, in the context that has been used in this work, is inhibiting and transcription is probably driven from the nearby Ad5 E3 Pr. The transcription could also be driven from the major late protein Pr that is located far to the left at m.u. 16. Similar observations have been made with other Ad5-based vectors containing analogous E3 substitutions (Schneider *et al.,* 1989; Graham and Prevec, 1992; Both *et al.,* 1993).

Antigenic sites C, B, D, and A (starting from the amino-terminal end) have been defined on S protein (Correa *et al.,* 1988; Gebauer *et al.,* 1991). Sequences coding for sites C and B were included in all recombinants and, in fact, S polypeptides with these two sites were detected after infection with all Ad–TS viruses. The recombinant coding for the full-length S protein (Ad–TS06) expressed low levels of S antigen and, accordingly, of all antigenic sites (A, B, C, and D), probably due to low replication levels. Nevertheless, antigenic sites A and B were properly folded after infection with Ad–TS06 virus since high antibody levels against these sites were elicited in hamsters, as detected by cRIA (Fig. 6). S protein trimer formation easily explains the dichotomy between low expression levels and high efficiency in eliciting a high immune response. S protein trimers (the native form of the glycoprotein in the virus) probably are more stable and better represent the peplomer in the native virion. Although recombinant Ad–TS5 contains the sequences coding for site D core (located in S protein from aa 377 to 390) (Gebauer *et al.,* 1991; Lenstra *et al.,* 1991; Posthumus *et al.,* 1990), it was very weakly detected by site D-specific MAbs, while sites C and B, also encoded in this recombinant, were well represented. Site D may have been hidden by incorrect folding of the S protein in this area. Site A, the major inducer of TGEV-neutralizing antibodies, was detected in larger amounts after infection by recombinant Ad–TS9 (expressing S protein without the membrane anchor domain) than by recombinant Ad–TS06 (which expresses the full-length S protein). This may be a consequence of the higher expression levels provided by Ad–TS9, since it has been previously shown (Godet *et al.,* 1991) that the full-length spike forms trimers and reconstitutes site A better than truncated S proteins missing the membrane anchor domain. In fact, one of the two major inducers of TGEV-neutralizing antibodies was Ad–TS06 virus, in spite of the low amount of S protein produced by this recombinant.

Seven of ten Ad–TS recombinants expressing S fragments induced TGEV-neutralizing antibodies in hamsters. Recombinant Ad–TS8, expressing a truncated form of S protein spanning 378 aa from the amino-terminus (which includes sites C and B but not site A), induced virus-neutralizing antibodies. Since site C does not in-

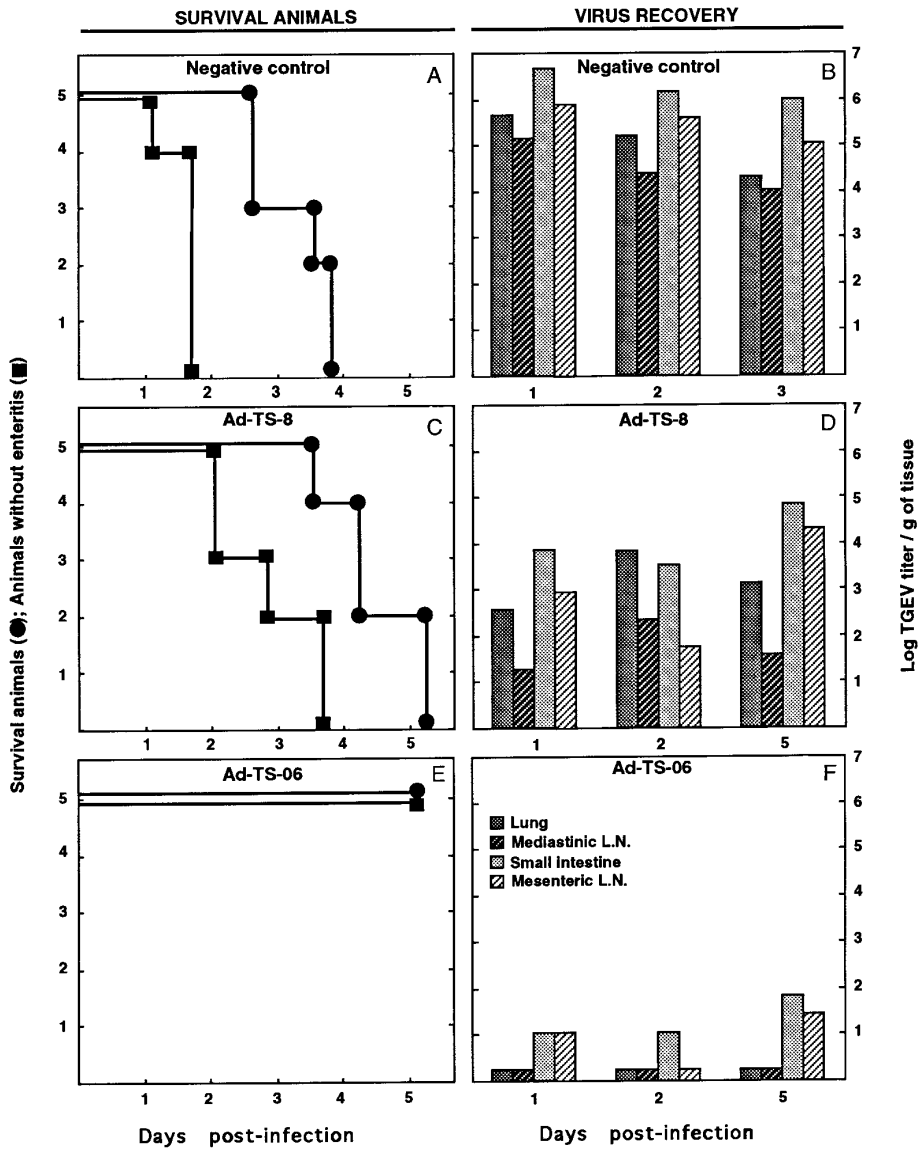

FIG. 8. Protection of swine with porcine sera elicited by Ad–TS recombinants. TGEV-specific swine antiserum was elicited by administration of wt Ad5 virus, Ad–TS8, or Ad–TS06 recombinants (see Materials and Methods). The number of swine surviving after the oral administration of 1×10^7 PFU of the virulent strain PUR46-SW11-ST2 of TGEV mixed with antisera induced by (A) wt Ad5 or by the recombinants (C) Ad–TS8 or (E) Ad–TS06 expressing the 1135 amino-terminal nt or the full-length spike protein, respectively, is shown. The recovery of infectious virus was determined 1, 2, and 3 or 5 days postinfection (when the animals either died or were sacrificed) in the indicated tissue homogenates, in animals administered the virulent virus with serum from (B) Ad5, (D) Ad–TS8, or (F) Ad–TS06 immune swine. Three groups of five swine were used to follow the survival rate. The infectious virus was followed in three groups of three animals each. Mean values have been represented. Standard deviations were lower than 25% in all cases and are not shown.

duce virus-neutralizing antibodies, site B, or neighboring antigenic domains involved in virus neutralization, have been reconstituted in a functional form. It has been proposed that factors mapping in the S segment which has been deleted in the porcine respiratory coronavirus (PRCV) (from aa 21 to 241) (Callebaut *et al.,* 1988; Sánchez *et al.,* 1990), and more precisely alterations in amino acid 219 (or residues close to it) might be involved in the loss of enteric tropism (Sánchez *et al.,* 1992). These factors might be the presence of a second receptor binding site recognized by a putative second receptor (Enjuanes and Van der Zeijst, 1995) or, alternatively, other factors similarly to those described in mouse hepatitis virus system (Fazakerley *et al.,* 1992; Yokomori *et al.,* 1993). Recombinant adenoviruses expressing only the 378 amino-terminal residues of the S polypeptide (which are mostly deleted in PRCV strains) provide partial protection against TGEV. These data indicate that the amino-terminal S protein fragment might be relevant to confer enteric tropism by complementing the binding of *N*-aminopeptidase (identified as a major TGEV receptor) to an S protein domain mapping close to antigenic site A (Delmas *et al.,*

1992; Godet *et al.,* 1994). Another observation supports this hypothesis. Ad5 vectors have been used to express the amino-terminal 564 aa of PRCV S protein, resulting in production of TGEV-neutralizing antibodies (Callebaut *et al.,* 1994) which did not protect against challenge with virulent TGEV (Callebaut and Pensaert, 1995). By contrast, the Ad–TS recombinant eliciting antiserum providing passive protection against challenge with virulent TGEV carries S sequences derived from TGEV instead of PRCV. The presence of 224 aa (from residue 21 to 244) in recombinant Ad–TS06, which are deleted in the PRCV, might have been critical to achieve the observed protection. This interpretation is in agreement with the partial protection seeing with the antiserum elicited in swine by recombinant Ad–TS8, which includes the sequences deleted in PRCV, but at the same time indicates that larger spike protein fragments (as those including site A) are needed to elicite full protection.

Protection by recombinant adenoviruses expressing S protein fragments lacking site A extends the results recently reported (Tulboly *et al.,* 1994) on S protein expression using baculoviruses. These authors showed induction of TGEV-neutralizing antibodies only with recombinants expressing S protein fragments spanning 745 aa or more from the amino-terminus, that is, with S protein fragments including site A, but not with S protein fragments lacking this site. Several factors may account for this difference, such as the use of a live vector in this study, versus immunization with nonreplicating antigens expressed in insect cells.

Recombinant Ad–TS06 expressing the full-length S protein was used to study the induction of antibodies providing protection in swine, the natural host of TGEV. Porcine serum elicited by this recombinant, when mixed with a lethal dose of virus prior to administration to susceptible piglets, prevented the replication of virulent TGEV administered orally as virus–antibody mixtures and fully protected swine from clinical signs and death. This is the first time that *in vivo* protection against virulent TGEV has been shown with serum induced by recombinant S protein. The S antigen used in previous experiments (Garwes *et al.,* 1978) was prepared by disrupting TGEV with 1% NP-40 and purifying S protein on sucrose gradients. The use of vectors expressing S protein, described in this paper, clearly establishes that S protein alone elicites protection against TGEV. Experiments with Ad–TS recombinant-immune pregnant sows will be made to test the protection of neonates through lactogenic immunity. The data presented indicate that passive protection can be achieved by the administration of antiserum and that cellular immune response was not essential to abrogate virus replication and symptoms of disease. This result facilitates the end goal of this work targeted to vaccine development for the induction of neutralizing antibodies in sows.

The Ad–TS recombinants induced antibody responses with higher titers in swine than in hamsters, although both species were permissive to virus infection. S protein has been previously expressed using *E. coli* (Hu *et al.,* 1984, 1987) or poxviruses (Pulford and Britton, 1991), but TGEV-neutralizing antibodies were only elicited with recombinant poxviruses. Expression of S antigenic site D, as a fusion protein on the surface of *E. coli* led to induction of TGEV-neutralizing antibodies when purified recombinant antigen was used as immunogen, but not when live vector was administered (Bousquet *et al.,* 1994). Using *Salmonella typhimurium,* site D has been expressed and TGEV-neutralizing antibodies have been elicited in serum and in mucosal areas using live recombinant bacteria (Smerdou *et al.,* 1995) but protection experiments using these systems have not been reported. Ad5 vectors have a high probability of inducing effective mucosal immunity against TGEV, since this virus showed tropism for mucosal tissues in pigs, and the animals infected with this virus experienced neither respiratory nor intestinal disorders (Callebaut and Pensaert, 1995; Callebaut *et al.,* 1994; Torres *et al.,* 1995).

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REFERENCES

Berkner, K. L. (1988). Development of adenovirus vectors for the expression of heterologous genes. *BioTechniques* 6, 616–629.

Bett, A. J., Prevec, L., and Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* 67, 5911–5921.

Birnboim, H. C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7, 1513–1517.

Both, G. W., Lockett, L. J., Janardhana, V., Edwards, S. J., Bellamy, A. R., Graham, F. L., Prevec, L., and Andrew, M. E. (1993). Protective immunity to rotavirus-induced diarrhea is passively transferred to newborn mice from naive dams vaccinated with a single dose of a recombinant adenovirus expressing rotavirus VP7sc. *Virology* 193, 940–950.

Bousquet, F., Martin, C., Girardeau, J. P., Mechin, M. C., Vartanian, M. D., Laude, H., and Contrepois, M. (1994). CS31A capsule-like antigen as an exposure vector for heterologous antigenic determinants. *Infect. Immun.* 62, 2553–2561.

Buchmeier, M. J., Lewicki, H. A., Talbot, P. J., and Knobler, R. L. (1984). Murine hepatitis virus-4 (strain JHM)-induced neurologic disease is modulated *in vivo* by monoclonal antibody. *Virology* 132, 261–270.

Callebaut, P., and Pensaert, M. (1995). Expression of immunogenicity of the spike glycoprotein of porcine respiratory coronavirus encoded in the E3 region of adenovirus. *Adv. Exp. Med. Biol.* 380, 265–270.

Callebaut, P., Correa, I., Pensaert, M., Jiménez, G., and Enjuanes, L.

(1988). Antigenic differentiation between transmissible gastroenteritis virus of swine and a related porcine respiratory coronavirus. *J. Gen. Virol.* 69, 1725–1730.

Callebaut, P., Pensaert, M., and Enjuanes, L. (1994). Construction of a recombinant adenovirus for the expression of the glycoprotein S antigen of porcine respiratory coronavirus. *Adv. Exp. Med. Biol.* 342, 469–470.

Cavanagh, D., Davis, P. J., Derbyshire, J. H., and Peters, R. W. (1986). Coronavirus IBV: Virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. *J. Gen. Virol.* 67, 1435–1442.

Correa, I., Jiménez, G., Suñé, C., Bullido, M. J., and Enjuanes, L. (1988). Antigenic structure of the E2 glycoprotein from transmissible gastroenteritis coronavirus. *Virus Res.* 10, 77–94.

Daniel, C., and Talbot, P. J. (1990). Protection from lethal coronavirus infection by affinity-purified spike glycoprotein of murine hepatitis virus, strain A59. *Virology* 174, 87–94.

Daniel, C., Anderson, R., Buchmeier, M. J., Fleming, J. O., Spaan, W. J. M., Wege, H., and Talbot, P. J. (1993). Identification of an immunodominant linear neutralization domain on the S2 portion of the murine coronavirus spike glycoprotein and evidence that it forms part of a complex tridimensional structure. *J. Virol.* 67, 1185–1194.

De Diego, M., Laviada, M. D., Enjuanes, L., and Escribano, J. M. (1992). Epitope specificity of protective lactogenic immunity against swine transmissible gastroenteritis virus. *J. Virol.* 66, 6502–6508.

Delmas, B., and Laude, H. (1990). Assembly of coronavirus spike protein into trimers and its role in epitope expression. *J. Virol.* 64, 5367–5375.

Delmas, B., Gelfi, J., and Laude, H. (1986). Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. *J. Gen. Virol.* 67, 1405–1418.

Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Norén, O., and Laude, H. (1992). Aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV. *Nature* 357, 417–420.

Delmas, B., Rasschaert, D., Godet, M., Gelfi, J., and Laude, H. (1990). Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike protein. *J. Gen. Virol.* 71, 1313–1323.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16, 6127–6145.

Enjuanes, L., and Van der Zeijst, B. A. M. (1995). Molecular basis of transmissible gastroenteritis coronavirus (TGEV) epidemiology. *In* "Coronaviruses" (S. G. Siddell, Ed.), pp. 337–376. Plenum, New York.

Fazakerley, J. K., Parker, S. E., Bloom, F., and Buchmeier, M. J. (1992). The V5A13.1 envelope glycoprotein deletion mutant of mouse hepatitis virus type-4 is neuroattenuated by its reduced rate of spread in the central nervous system. *Virology* 187, 178–188.

Fleming, J. O., Shubin, R. A., Sussman, M. A., Casteel, N., and Stohlman, S. A. (1989). Monoclonal antibodies to the matrix (E1) glycoprotein of mouse hepatitis virus protect mice from encephalitis. *Virology* 168, 162–167.

Garwes, D. J., Lucas, M. H., Higgins, D. A., Pike, B. V., and Cartwright, S. F. (1978). Antigenicity of structural components from porcine transmissible gastroenteritis virus. *Vet. Microbiol.* 3, 179–190.

Gebauer, F., Posthumus, W. A. P., Correa, I., Suñé, C., Sánchez, C. M., Smerdou, C., Lenstra, J. A., Meloen, R., and Enjuanes, L. (1991). Residues involved in the formation of the antigenic sites of the S protein of transmissible gastroenteritis coronavirus. *Virology* 183, 225–238.

Ghosh-Choudhury, G., Haj-Ahmad, P., Brinkley, J., Rudy, J., and Graham, F. L. (1986). Human adenovirus cloning vectors based on infectious bacterial plasmids. *Gene* 50, 161–171.

Godet, M., Rasschaert, D., and Laude, H. (1991). Processing and antigenicity of entire and anchor-free spike glycoprotein-S of coronavirus TGEV expressed by recombinant baculovirus. *Virology* 185, 732–740.

Godet, M., L'Haridon, R., Vautherot, J. F., and Laude, H. (1992). TGEV coronavirus ORF4 encodes a membrane protein that is incorporated into virions. *Virology* 188, 666–675.

Godet, M., Grosclaude, J., Delmas, B., and Laude, H. (1994). Major receptor-binding and neutralization determinants are located within the same domain of the transmissible gastroenteritis virus (Coronavirus) spike protein. *J. Virol.* 68, 8008–8016.

Graham, F. L., and Prevec, L. (1992). Adenovirus-based expression vectors and recombinant vaccines. *In* "Vaccines: New Approaches to Immunological Problems" (R. W. Ellis, Ed.), pp. 363–385. Butterworth-Heinemann, Stoneham, MA.

Graham, F. L., and van der Eb, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456–467.

Graham, F. L., Prevec, L., Schneider, M., Ghosh-Choudhury, G., McDermott, M., and Johnson, D. C. (1988). Cloning and expression of glycoprotein genes in human adenovirus vectors. *In* "Technological Advances in Vaccine Development" (L. Laskey, Ed.), pp. 243–253. A. R. Liss, New York.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, 59–72.

Greenwood, F. C., Hunter, W. M., and Glover, J. W. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* 89, 114–123.

Hanke, T., Graham, F. L., Lulitanond, V., and Johnson, D. C. (1990). Herpes simplex virus IgG Fc receptor induced using recombinant adenovirus vectors expressing glycoproteins E and I. *Virology* 177, 437–444.

Hitt, M., Bett, A. J., Addison, C., Prevec, L., and Graham, F. L. (1995). Techniques for human adenovirus vector construction and characterization. *In* "Methods in Molecular Genetics" (K. W. Adolph, Ed.), Vol. 7. Academic Press, Orlando.

Hitt, M., Bett, A. J., Prevec, L., and Graham, F. L. (1994). Construction and propagation of human adenovirus vectors. *In* "Cell Biology: A Laboratory Handbook" (J. E. Celis, Ed.), pp. 109–128. Academic Press, Orlando.

Hu, S., Bruszewski, J., Boone, T., and Souza, L. (1984). Cloning and expression of the surface glycoprotein gp195 of porcine transmissible gastroenteritis virus. *In* "Modern Approaches to Vaccines. Molecular and Chemical Basis of Virus Virulence and Immunogenicity" (R. M. Chanock and R. A. Lerner, Eds.), pp. 219–223. Cold Spring Harbor Laboratory, New York.

Hu, S., Bruszewski, J., Smallig, R., and Browne, J. K. (1987). Studies of TGEV S protein gp195 expressed in *E. coli* and by a TGE-vaccinia virus recombinant. *In* "Immunobiology of Proteins and Peptides. III. Viral and Bacterial Antigens" (M. Zouhair Attasi and H. L. Bachrach, Eds.), pp. 63–82. Plenum, New York.

Jiménez, G., Correa, I., Melgosa, M. P., Bullido, M. J., and Enjuanes, L. (1986). Critical epitopes in transmissible gastroenteritis virus neutralization. *J. Virol.* 60, 131–139.

Jones, N., and Shenk, T. (1979). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 17, 683–689.

Koolen, M. J. M., Borst, M. A. J., Horzinek, M. C., and Spaan, W. J. M. (1990). Immunogenic peptide comprising a mouse hepatitis virus A59 B-cell epitope and an influenza virus T-cell epitope protects against lethal infection. *J. Virol.* 64, 6270–6273.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

Laude, H., Gelfi, J., Lavenant, L., and Charley, B. (1992). Single amino acid changes in the viral glycoprotein M affect induction of alpha interferon by the coronavirus transmissible gastroenteritis virus. *J. Virol.* 66, 743–749.

Lecomte, J., Cainelli-Cebera, V., Mercier, G., Mansour, S., Talbot, P.,

Lussier, G., and Oth, D. (1987). Protection from mouse hepatitis virus type 3-induced acute disease by an anti-nucleoprotein monoclonal antibody. *Arch. Virol.* 97, 123–130.

Lenstra, J. A., Erkens, J. H. F., Zwaagstra, K. A., Posthumus, W. P. A., Meloen, R. H., Gebauer, F., Enjuanes, L., and Stanley, K. K. (1991). Selection of mimotopes from a random sequence expression library by monoclonal antibodies against transmissible gastroentritis coronavirus. *J. Immunol. Methods* 152, 149–157.

Maniatis, T., Fritsh, E. F., and Sambrook, J. (1989). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McClurkin, A. W., and Norman, J. O. (1966). Studies on transmissible gastroenteritis of swine. II. Selected characteristics of a cytopathogenic virus common to five isolates from transmissible gastroenteritis. *Can. J. Comp. Vet. Sci.* 30, 190–198.

Mittal, S. K., McDermott, M. R., Johnson, D. C., Prevec, L., and Graham, F. L. (1993). Monitoring foreign gene expression by a human adenovirus based vector using the firefly luciferase as a reporter gene. *Virus Res.* 28, 67–90.

Nakanaga, K., Yamanouchi, K., and Fujiwara, K. (1986). Protective effect of monoclonal antibodies on lethal mouse hepatitis virus infection in mice. *J. Virol.* 59, 168–171.

Posthumus, W. P. A., Meloen, R. H., Enjuanes, L., Correa, I., van Nieuwestadt, A., and Koch, G. (1990). Linear neutralizing epitopes on the peplomer protein of coronaviruses. *Adv. Exp. Med. Biol.* 276, 181–188.

Prevec, L., Campbell, J. B., Christie, B. S., Belbeck, L., and Graham, F. L. (1990). A recombinant human adenovirus vaccine against rabies. *J. Infect. Dis.* 161, 227–230.

Pulford, D. J., and Britton, P. (1991). Intracellular processing of the porcine coronavirus transmissible gastroenteritis virus spike protein expressed by recombinant vaccinia virus. *Virology* 182, 765–773.

Saif, L. J., and Wesley, R. D. (1992). Transmissible gastroenteritis. *In* "Diseases of Swine" (A. D. Leman, B. Straw, W. L. Mengeling, S. D'Allaire, and D. J. Taylor, Eds.), pp. 362–386. Iowa State Univ. Press, Ames.

Sánchez, C. M., Gebauer, F., Suñe, C., Méndez, A., Dopazo, J., and Enjuanes, L. (1992). Genetic evolution and tropism of transmissible gastroenteritis coronaviruses. *Virology* 190, 92–105.

Sánchez, C. M., Jiménez, G., Laviada, M. D., Correa, I., Suñé, C., Bullido, M. J., Gebauer, F., Smerdou, C., Callebaut, P., Escribano, J. M., and Enjuanes, L. (1990). Antigenic homology among coronaviruses related to transmissible gastroenteritis virus. *Virology* 174, 410–417.

Schneider, M., Graham, F. L., and Preveck, L. (1989). Expression of the glycoprotein of VSV by infectious adenovirus vectors. *J. Gen. Virol.* 70, 417–427.

Smerdou, C., Antón, I. M., Plana, J., Curtiss, R., and Enjuanes, L. (1995). Expression of a continuous epitope from transmissible gastroenteritis coronavirus S protein fused to *E. coli* heat-labile toxin B subunit in attenuated *Salmonella* for oral immunization. Submitted for publication.

Spaan, W., Cavanagh, D., and Horzinek, M. C. (1990). Coronaviruses. *In* "Immunochemistry of Viruses. II. The Basis for Serodiagnosis and Vaccines" (M. H. V. Regenmortel and A. R. Neurath, Eds.), pp. 359–375. Elsevier, Amsterdam.

Suñé, C., Jiménez, G., Correa, I., Bullido, M. J., Gebauer, F., Smerdou, C., and Enjuanes, L. (1990). Mechanisms of transmissible gastroenteritis coronavirus neutralization. *Virology* 177, 559–569.

Talbot, P. J., Salmi, A. A., Knobler, R. L., and Buchmeier, M. J. (1984). Topographical mapping of epitopes on the glycoprotein of murine hepatitis virus-4 (strain JHM): Correlation with biological activities. *Virology* 132, 250–260.

Torres, J. M., Alonso, C., Ortega, A., Graham, F. L., and Enjuanes, L. (1995). Tropism of human adenovirus Ad5 based vectors in swine and their use in protection against transmissible gastroenteritis virus. Submitted for publication.

Tulboly, T., Nagy, E., Dennis, J. R., and Derbyshire, J. B. (1994). Immunogenicity of the S protein of transmissible gastroenteritis virus expressed in baculovirus. *Arch. Virol.* 137, 55–67.

Welch, S. K. W., and Saif, L. J. (1988). Monoclonal antibodies to a virulent strain of transmissible gastroenteritis virus: Comparison of reactivity with virulent and attenuated virus. *Arch. Virol.* 101, 221–235.

Wesseling, J. G., Godeke, G. J., Schijns, V. E. C. J., Prevec, L., Frank, F. L., Horzinek, M. C., and Rotier, P. J. M. (1993). Mouse hepatitis virus spike and nucleocapsid proteins expressed by adenovirus vector protect mice against a lethal infection. *J. Gen. Virol.* 74, 2061–2069.

Yokomori, K., Asanaka, M., Stohlman, S. A., and Lai, M. M. C. (1993). A spike protein-dependent cellular factor other than the viral receptor is required for mouse hepatitis virus entry. *Virology* 196, 45–56.