DEVELOPMENT OF PROTECTION AGAINST CORONAVIRUS INDUCED DISEASES

A Review

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INTRODUCTION

Current coronavirus vaccines are classical vaccines, i. e., are based on live attenuated coronavirus. These vaccines provide protection against certain strains of infectious bronchitis virus (IBV) and mouse hepatitis virus (MHV), and with variable results against bovine coronavirus (BCV) and transmissible gastroenteritis virus (TGEV).

Chicken immunized with whole inactivated IBV were protected against virulent challenge¹⁵. In contrast, chicken immunized with the purified spike (S) glycoprotein, nucleoprotein (N), or membrane (M) proteins did not induce protection¹⁷. Immunization with the S1 glycoprotein prevented replication of nephropathogenic IBV in kidney, but not in trachea of immunized chicken³⁶. Protection did not correlate with the presence of virus neutralizing (VN) and haemagglutination inhibiting (HI) antibodies.

In feline infectious peritonitis virus (FIPV), humoral immunity to S protein is not protective, and cell mediated immune responses may be important in immunity⁶⁶. The development of a safe and effective vaccine against FIPV has been very problematic. A variety of approaches have been unsuccessful, including the administration of inactivated FIPV, avirulent FIPV or sublethal doses of virulent FIPV, heterologous live virus vaccines [canine coronavirus (CCV), human coronavirus (HCV)-229E, and TGEV], and a recombinant vaccinia virus expressing FIPV S protein^{98, 66}. Most recently, a temperature-sensitive mutant of FIPV has been developed as an FIPV vaccine. Unfortunately, while testing by the

manufacturer provided 78% protection, independent testing gave from 0 to 50% protection, depending on the strain and dose exposure⁷⁸.

Lactogenic immunity is of primary importance in providing newborn piglets immediate protection against TGEV infection. Effective immunity has only been provided by the administration of virulent virus. Unfortunately, in this case the virus will become enzootic in the farm, preventing its use. Licensed TGEV vaccines have met limited success^{74,26}, CCV and BCV vaccines provide partial protection and have to be improved. In summary, effective protection only to two coronaviruses can be elicited using current vaccines, implying the need of a new vaccine design.

Targets in Protection

The role in protection of the different viral proteins has a variable support. While there are many reports indicating that the S protein elicits protection against coronaviruses of different species^{29, 17, 58, 43} the role of M protein, and particularly of the N protein, is less obvious. There is very little information on the role in protection of the small membrane (sM) protein^{29, 17, 58, 43, 31}, and of the non-structural proteins. Proteins exposed in virus or in virus infected cells have a higher probability of being involved in protection. Therefore, it is of interest to identify them.

S and M proteins have been detected on the surface of infected cells and these proteins have been identified as targets in immune protection in MHV^{29, 35, 17, 48, 64, 82, 27, 58, 105, 43, 51, 70, 32, 89, 31, 20, 44, 102, 34}. S protein also induces protection against IBV and MHV. These proteins are frequently detected in small quantities in the surface of virus infected cells associated to the presence of virions, while they are detected in the cytoplasm of infected cells in large quantities^{29, 35, 17, 48, 64, 82, 27, 58, 105, 43, 51, 70, 32, 89, 31, 20, 44, 102, 34}. These data are in agreement with the concept that coronaviruses assemble at rough endoplasmic reticulum and Golgi membranes. Consequently, most viral proteins are transported through intracellular compartments and only a small proportion, which is not incorporated into virions, is transported to the plasma membrane⁸⁶. Expression of recombinant S, M, sM, haemagglutinin-esterase (HE), and hydrophobic (HP) proteins using poxvirus^{83, 98, 97, 44}, adenovirus^{83, 98, 97, 108, 44}, or baculovirus^{105, 67, 32, 106, 107, 31, 108} has shown that these proteins are found in small amounts on the surface of transformed cells, in the absence of other coronavirus components¹⁰⁵. This result does not necessarily reflect the situation after coronavirus infection, in which, as mentioned above, viral proteins are mainly found associated to virus bound to cell membranes.

M protein induces complement-dependent^{48, 104} and possibly complement-independent neutralizing antibodies^{101, 27} in assays performed *in vitro*. The antibodies elicited *in vivo* failed to protect against MHV challenge in some cases¹⁰² but not in others²⁷. According to the current topological model of coronavirus M protein, only the amino-terminus of the M protein is exposed on the virus surface^{2, 9, 47, 50, 38}. In contrast, presented data support that the carboxy-terminus domain of M protein is exposed on the external virus surface both in TGEV and in MHV^{90, 72} providing a new target for virus or virus-infected cells clearance.

The role of N protein in protection is being understood¹⁰². Antibodies to N protein have no virus neutralizing activity *in vitro*. An N protein fragment has been detected in the surface of infected cells (S. Stohlman, personal communication), and it has been shown that a monoclonal antibody (MAb) to the N protein prevented the cytopathic effect of MHV in L cell⁵². This antibody, as well as another non-neutralizing MAb, protected mice from acute disease⁵². In contrast, other anti-N protein antibodies did not provide *in vivo* protection^{33, 13, 88, 52}. The mechanism by which the N protein protects is unclear. It may involve a cell-mediated defense mechanism, e.g. cytotoxic T lymphocyte (CTL) activity⁸⁵, T helper cells¹⁰³, or T cell-derived antiviral cytokines¹⁰². N-reactive helper T cells supporting virus-

specific B cell responses have been demonstrated in TGEV¹. This type of response might account for the enhancement of protection observed in MHV after combined immunization with the N and S proteins¹⁰².

HE protein is also exposed on the surface of both BCV particles and infected cells¹⁰⁸. HE expressed using recombinant Ad5 induced haemagglutination. Small membrane protein, an integral membrane protein of TGEV having Cexo-Nendo orientation is exposed on the surface of virions. The protein is also exposed on the surface of virus infected cells or insect cells transformed with baculovirus coding for sM³¹. The 9 kDa HP protein encoded by TGEV ORF7, or the equivalent protein of FIPV²¹ is another accessible membrane-associated protein. The cellular location of HP suggests that it may play a role in the membrane association of replication complexes or in virus assembly⁹².

In summary, there is clear evidence that S, M, HE, sM, and HP proteins are exposed on the surface of virus infected cells, mostly virion associated, while there is limited evidence on the exposure of N protein. Nevertheless, all these proteins constitute targets in protection against coronaviruses induced diseases.

B and T Cell Epitopes Involved in Protection

Antigenic sites in the major structural coronavirus proteins, S, M, N, and HE have been previously described⁸². Extensive studies on B cell epitopes have been done on the S protein of MHV, TGEV, IBV, FIPV, and BCV^{13, 86, 23, 25, 59, 19, 18, 30, 53, 92, 20}. The location of antigenic sites inducing virus neutralizing antibodies has been summarized for TGEV, MHV, BCV, and IBV (Figure 1). Most of the information known on FIPV is coincident with that of TGEV⁷⁵. Several S protein antigenic sites have been involved in neutralization: 3 in TGEV, 8 in MHV, 2 in BCV, and 3 in IBV. Most antigenic sites are located on the amino-terminal

Figure 1. Antigenic sites on aligned coronavirus peplomer protein sequences. The location of dominant antigenic sites (dark squares), of antigenic sites recognized by neutralizing antibodies (arrows) and fusion inhibiting antibodies (arrows with plus sign), and of peptides eliciting protection (lines with star) is shown approximately to scale (small numbers below bars indicate amino acid number in hundreds). TGEV, capital letters below bar indicate the names of the antigenic sites^{24,30}. MHV, roman numbers indicate names of defined antigenic sites^{59, 58, 73, 99, 20, 91}. BCV, capital letters below bar indicate defined antigenic sites¹⁰⁷. IBV, HR₁ and HR₂ indicate the location of hypervariable regions^{16,54}.

half domain of S protein. TGEV, MHV, and IBV have sites inducing neutralizing antibodies in the first 150 amino acids of S protein. Other sites inducing neutralizing antibodies on the amino-terminal half of S protein are approximately located between amino acids (aa) 200 and 400 (TGEV, MHV, BCV) and between aa 500 and 700 (TGEV, MHV, and BCV) (see Figure 1 for a more precise location). In the S2 half of IBV a site involved in neutralization has been defined next to the right of the trypsin cleavage site. In the S2 half of MHV two domains have been involved in virus neutralization. One contains several epitopes located between aa 800 to 900, and the other one is located between amino acids 1000 to 1220. Generally, monoclonal antibodies specific for S1 are more neutralizing than those S2 specific⁸³. No antigenic site critical in TGEV neutralization has been identified in the stem portion of TGEV S protein.

Two hypervariable regions identified in IBV S protein at amino acid positions 38-51 and 99-115 from the N-terminus (excluding the signal sequence) are involved in the induction of neutralizing antibodies^{61, 43}. In MHV a 13-mer synthetic peptide derived from S protein (aa 846 to 858) coupled to an influenza virus T-cell epitope protects against lethal MHV infection^{43, 12}. A second peptide of 10-mer (aa 993 to 1002) of S protein, coupled to KLH elicited neutralizing antibodies and a protective immune response in BALB/c mice⁸⁷. One MHV neutralizing MAb which selected escaping mutants with changes at amino acids 255 and 1116, also inhibited fusion activity^{65, 41}. The information accumulated on the antigenic and functional structure of S protein in all coronaviruses, indicates that it has a complex three-dimensional structure in native state with interactions between distant domains in the primary structure^{48, 30, 20, 41}. S protein fragments extended from the amino terminus, provided recombinant antigens inducing TGEV neutralizing antibodies⁷⁶.

Universal coronavirus vaccines based on a conserved domain of 124 aa found in the C terminal portions of TGEV, CCV, and FCV S protein have been patented. The report claims that this fragment is capable of eliciting a protective immune response against FIPV, FECV, TGEV, BCV, HCV, and IBV⁸¹. Further work is required to confirm these findings.

Less information is available on T-cell epitopes (Figure 2). In MHV virus-specific MHC class II-restricted cytotoxic CD4⁺ cells recognize an immunodominant epitope com-

Figure 2. T cell epitopes precisely located in coronavirus proteins. The approximate location of T cell epitopes on the S, N, and M proteins is indicated by white boxes. IF, interferon inducer epitope; CTL, Cytotoxic T cell determinant; Th, T helper cell epitope^{45, 7, 8, 49, 1, 34, 100}.

prising the amino acids 329-343 of the viral S-glycoprotein. The epitope is recognized by polyclonal and virus specific cytotoxic T cell clones³⁴. No evidence for the activation of the classical CD8⁺ MHC class I-restricted CTL was found by these authors. MHV-JHM immune CD4⁺ Th1 T cells transferred to MHV-JHM lethally infected mice results in protection from death, but provides little or no protection from demyelination⁴⁵. CD8⁺ cytotoxic T cells specific for MHV-JHM are involved in clearance of virus from CNS and thereby provide specific immunological protection. These CTL are specific for both S and N protein, but not for M or HE proteins. A subset of CD4⁺ cells is also involved in virus clearance (S. Stohlman, personal communication). Most (15 out of 20) cell lines established from the brains of infected animals were specific for the N protein^{45, 84}. MHV-JHM infection of rats induced immunodominant CD4⁺ T-cells which recognized with high prevalence amino acids 388 to 563 of the carboxy-terminal domain of N protein. The same results were obtained from rats immunized with bacterially expressed N-protein or from animals vaccinated with a stable N-protein expressing vaccinia recombinant. Transfer of N specific CD4⁺ T-cells to MHV-JHM infected rats mediated protection against acute disease¹⁰⁰.

T cell responses to the three major TGEV structural proteins: S, M, and N have been identified in haplotype defined TGEV immune miniswine¹. Four immunodominant T cell epitopes were identified using 15-mer synthetic peptides, three located in the nucleoprotein (N46, aa 46 to 60; N272, aa 272 to 286; and N321, aa 321 to 335) and one on the membrane protein (M196, aa 196 to 210). T lymphocytes from N321 peptide-immune miniswine reconstituted the *in vitro* synthesis of TGEV-specific antibodies by CD4⁺ TGEV-immune B cells. The antibodies synthesized were directed at least against all three major structural proteins and neutralized TGEV infectivity, indicating that N321 peptide defines a functional T helper epitope which elicits T cells capable of collaborating with B cells specific for different proteins of TGEV. An interesting epitope has been defined on the M protein of TGEV which induces interferon in naive lymphocytes, and may be relevant in protection⁴⁹.

In IBV, protection is induced by generating cytotoxic and helper T cell responses that augment the activity of B cells, in producing virus neutralizing antibody. MHC class II-restricted CD4⁺ T-cell hybridomas responded to IBV N protein amino acid sequence 71 to 78^{6,8}. In summary, N protein seems to be a dominant antigen for T cell responses, although S and M proteins are also targets for cellular immunity.

Recombinant Vaccines to Protect against Coronavirus

Vaccinia virus (VV) recombinants expressing coronavirus proteins have been successfully used in preventing MHV induced disease, but not in diseases induced by other coronaviruses as FIPV or TGEV. A strong protection against acute encephalomyelitis was mediated in Lewis rats which were immunized with recombinant VV expressing a fusogenic S-protein²⁸. By contrast, a VV recombinant encoding a non fusogenic S-protein variant or the N-protein was not capable to confer protection. In addition, MHV-JHM S-specific IgG antibodies elicited before MHV-JHM challenge modulated the disease process, changing it from an acute disease to subacute demyelinating encephalomyelitis. Immunization with recombinant VV expressing spike (S) protein of FIPV or CCV, induced early death syndrome (EDS)⁹⁸. The epitope responsible for EDS in CCV is found on the first 700 amino-acids of the S protein.

Empty parvovirus capsids expressing linear epitopes of MHV have also been used to protect against MHV infections. The MHV protective epitope designated site A of strain A59 S protein⁵⁴ was inserted in the major structural protein (VP2) of human parvovirus and expressed using baculoviruses¹². The peptide (H2N-846-SPLLGCIGSTCAE-858-COOH) has 13 amino acids. The 58 kDa protein assembled into capsids resembling native B19 virion, when expressed in insect cells. Immunoelectron microscopy indicated that the epitopes

inserted in the loop were exposed on the surface of the chimeric particles. Antibodies specific for the inserted sequences were induced, which recognized the native virus. Mice immunized with the chimeric capsids were partially protected against a lethal challenge infection with MHV. It will be interesting to determine whether expression of linear epitopes from other coronavirus will also provide protection against the homologous virus.

Vectors to Induce Mucosal Immunity

Coronavirus often infect respiratory or enteric mucosal areas. Then, a strategy that could elicit protection against coronavirus infections is the induction of mucosal immunity. Extensive studies that span more than 20 years from many laboratories have shown that the precursor for mucosal IgA plasma cells originate in the lymphoepithelial structures in the gastrointestinal and respiratory tracts. This precursor, which switch or commits to IgA in gut, or bronchus-associated lymphoepithelial tissues (BALT) migrate from gut associated lymphoid tissues (GALT) to mesenteric lymph nodes, upper respiratory tracts or to exocrine tissues such as the mammary gland, where terminal differentiation into IgA secreting plasma cells takes place. Prokaryotic and eukaryotic vectors with tropism for GALT have been used to elicit immune response to coronavirus.

Human and bovine enteropathogenic strains of *Escherichia coli* have a multimeric surface protein CS31A that has been used as a carrier for foreign antigenic determinants¹⁰. Site D (named site C by H. Laude's group) of TGEV S protein includes a continuous epitope which can be represented by a synthetic peptide able to induce virus neutralizing antibodies^{69, 30}. Sequences coding for antigenic site D^{69, 30, 68} have been cloned in the V3 region of CS31A protein. The recombinant hybrid protein was purified and used to immunize mice with 20 µg of antigen per dose. Significant antibody titers and neutralization of the virus was demonstrated. In contrast, antibody titers to TGEV induced by the recombinant bacteria were low¹⁰. Site D from TGEV S protein fused to *E. coli* heat-labile toxin B (LT-B) subunit was expressed in attenuated *Salmonella* providing a collection of 6 recombinant antigens with a variable number of site D⁸⁰. LT-B-site D fusion products were purified and subcutaneously inoculated into rabbits, in order to determine their immunogenicity. One of the hybrid proteins induced TGEV neutralizing antibodies. The corresponding plasmid was used to transform the vaccine strain *S. typhimurium* Δ*cya* Δ*crp* Δ*asd* χ3987. Constitutive expression of LT-B-site D protein led to the selection of bacteria expressing a recombinant product that formed pentamers and showed high stability *in vitro*. The recombinant bacteria also elicited TGEV specific antibodies *in vivo*⁸⁰. Expression of TGEV S protein in avirulent *S. typhimurium* yield recombinant antigens with variable toxicity in bacteria. Transformed bacteria expressing a 53 kDa amino-terminal fragment of the S protein and the whole protein (144 kDa), respectively, showed an acceptable stability. Recombinant bacteria expressing the amino-terminal fragment of S protein was selected as a potential bivalent vector to induce both immunity to *Salmonella* and to TGEV. The bacteria colonized swine and induced an antibody response to *Salmonella*, but the response against TGEV was weak. Prokaryotic vectors developed so far, do not seem the ideal candidates to induce protective immunity against coronaviruses, possible because S protein is an essential antigen in protection against coronaviruses, and the conformation of this antigen is highly dependent on glycosylation^{23, 19}.

Among the eukaryotic vectors with tropism for GALT or BALT, adenoviruses are nowadays widely used. Two types of adenovirus vectors have been developed: non-defective (vectors with substitutions in E3) which can replicate in many permissive cells, and induce high level expression of the foreign insert, and defective vectors (with E1 gene deleted) which only replicate in 293 cells that provide E1 gene functions *in trans*. Non-defective and

defective adenovirus vectors allow the expression of heterologous genes with up to 4,5 and 8 kb, respectively. These vectors are highly stable and induce strong responses^{5, 76}.

MHV S, N, and M proteins have been expressed using human adenovirus serotype 5 (Ad5). Mice intraperitoneally inoculated with these recombinants elicited serum antibodies which specifically recognized the respective proteins. Only antibodies to S protein neutralized MHV *in vitro*, but titers were low. N protein also induced detectable antibodies as determined by ELISA. By contrast, upon intracerebral challenge with a lethal dose of MHV, a significant fraction of animals vaccinated with adenovirus vectors expressing either the S or the N protein were protected. This protective effect was significantly stronger when the animals were immunized with recombinants expressing both S and N protein, as compared to survival after single immunizations¹⁰².

TGEV spike protein has been expressed using Ad5. The whole protein, or fragments of different lengths: 378, 529, and 1109 amino acids extended from the amino-terminus have been expressed. The recombinant antigens included different sets of the antigenic sites C, B, D, and A, previously defined in the TGEV S protein⁷⁶. These Ad5-TGEV-S recombinants infect hamsters and swine⁹¹, and induce TGEV neutralizing antibodies. Furthermore, Ad5-TGEV-S immune hamsters secrete TGEV neutralizing antibodies in the milk during lactation⁷⁶, indicating that these recombinants might have a potential in protection against enteric diseases caused by coronaviruses. Vectors based on porcine adenoviruses are being developed^{62, 71}.

HE of BCV has been expressed using Ad5. The recombinant HE polypeptide existed in monomeric (65K) and dimeric form (130k). Mice inoculated intraperitoneally with live recombinant Ad5-HE elicited a significant level of BCV-neutralizing antibodies¹⁰⁸. Protection experiments are being performed with live recombinant virus.

Development of Coronavirus Based Vectors

To immunize a determined animal species against a coronavirus it might be advisable to use a vector based on coronaviruses isolated from the corresponding specie. This would provide several advantages: i) The coronavirus is a multiantigenic system which could elicit a polyvalent immune response that may prevent the appearance of escaping mutants; ii) virus vectors can be selected with a tropism identical to the one exhibited by virulent wild type (*wt*) virus, inducing a local immune response where required; and, iii) vector approval for use in a given species by regulatory agencies may be easier when vaccines are derived from viruses affecting the same species. Then, it may be convenient to develop vectors based in coronaviruses. The ultimate goal is to develop a replication competent coronavirus RNA vector (Figure 3, C). From this, safer vectors could be derived by introducing a deletion removing an essential virus protein, which could be provided *in trans* by a packaging cell line (Figure 3, C). Also suicide vectors could be derived by deleting the encapsidation signal from the helper virus. Unfortunately, the coronavirus genome (around 30 kb) is too large to be cloned and expressed as a single infectious RNA for technical reasons. The construction of these vectors is based on genetic manipulation of single-strand positive-sense RNA genomes, which today can be done by recombination between *wt* genomes and genetically engineered defective interfering (DI) RNAs of small size.

At least two types of vectors based on coronaviruses can be anticipated as intermediate steps towards the construction of a non-defective coronavirus vector: i) a helper-dependent RNA-replication defective virion, which requires the cooperation of a helper virus for its replication (Figure 3, A); ii) a helper independent (replicase +) defective virus, capable of self replicating the RNA, but dependent for assembly on structural proteins provided *in trans* by a second RNA molecule derived from a DI genome (Figure 3, B). The replicase, as defined today, is coded by a 20 kb fragment, which makes difficult the construction of an

Figure 3. Models of potential coronavirus-derived expression constructs. A. Summarized structure of helper virus (upper part) and a DI genome engineered as a vector to express foreign genes (lower bar). B. Structure of a potential coronavirus based replicon (upper bar) and an engineered DI carrying information to code for structural proteins and foreign antigens (lower construct). C. Single RNA genome with the information for the replicase, essential proteins, and heterologous genes (upper bar). A defective genome can be derived by the introduction of a deletion (Δ) in an essential gene (lower bar). PS, promoter sequences; ES, encapsidation signal; IPS, internal promoter sequences; An, poly A. Triangles above bars indicate promoter position.

RNA replicon, to be expressed from a DNA vector, as shown for nodavirus, alphaviruses, and other small RNA viruses^{56,11,4}. These vectors are safer, but their immunogenicity usually is lower than that of vectors which replicate and encapsidate. These vectors could be passaged several times before their ability to replicate is lost. The replication is maintained as far as both the replicon containing RNA and the RNA coding for structural proteins are passed together.

Key contributions to progress in this area have been the studies on the minimum *cis*-signal requirements for DI RNA replication^{61,60,93,39}. Most advanced work is coming from laboratories dealing with MHV. An MHV DI RNA-based expression system (Figure 4A)⁵⁷ represents one of the first MHV-based expression vectors derived from a coronavirus. This vector has low packaging efficiency, although it includes the packaging signal described so far.

Expression in these vectors relies on an internal ribosome entry site (IRES) before the heterologous gene (Figure 4A), or on the transcription of a subgenomic RNA by introducing a coronavirus derived internal promoter sequence (IPS) at the 5' end of the heterologous gene (Figure 4B). Chloramphenicol acetyltransferase (CAT), luciferase, and MHV hemagglutinin-esterase HE proteins have been expressed using this type of vectors (Figure 4B)⁵⁵. Both the wild-type and recombinant HE proteins are incorporated into viral particles, thus generating a pseudo-recombinant virus. The recombinant DI RNA can be passaged at least four times. This DI RNA is thus an effective expression vector for delivering foreign genes into virus-infected cells. Another RNA vector based on MHV DI particles has been obtained (Figure 4C)⁹⁴. Studies on the replication of this vector have revealed that the MHV DI RNAs require a functional open reading frame (ORF) for efficient propagation²². The DI-vector could be used to modify the helper virus genome by homologous recombination to express heterologous genes. Site directed recombination has been shown between full length RNA genomes containing a *ts* mutation on the N gene, and a DI RNA genome,

Figure 4. Coronavirus derived expression constructs.

A. Structure of a MHV DI genome used as a vector to express a foreign gene (CAT) using an IRES⁵⁷. B. DI genome engineered to express a heterologous gene (HE) under the control of an internal promoter sequence (IPS)⁵⁵. C. Recombination between a helper virus and a MHV DI genome (MIDI-C) to modify N protein of helper virus. This technique can be used to introduce foreign genes that could be expressed by coronavirus based vectors⁹⁶. pT7, phage T7 promoter, ES, encapsidation signal; IRES, internal ribosome entry site; CAT, chloramphenicol acetyl transferase reporter gene; IPS, internal promoter sequence; HE, haemagglutinin-esterase; N, N protein; *ts*, temperature sensitive mutation.

containing a *wt* N gene⁴⁰. The frequency of site directed homologous recombination was significantly increased with the use of encapsidated co-replicating RNAs⁹⁶.

The aims are directed to the development of a system which obviates the need of a helper virus. A vector containing the gene encoding the RNA dependent RNA polymerase, and the replication signals will be required. Mapping the replication signals and polymerase gene reconstruction are priority goals. Such a vector can only be rescued into virus particles in the presence of the structural proteins. Assembly of DI particles in the absence of helper virus using transient expression of the structural proteins S, M, N, and sM, and the synthetic DI RNA has been achieved (W. Spaan, personal communication).

Optimization of the expression in coronavirus requires information on transcription regulation, which nowadays is under debate. The extent of base pairing between the promoter and the leader complementary promoter sequence has been proposed as the control of subgenomic RNA abundance^{82, 46}. In fact, certain nucleotide substitutions in the 3' consensus sequence impair promoter activity. However, other data from MHV, IBV, and FIPV indicates that there is no correlation between potential base pairing and mRNA abundance^{42, 95}. Mutations disrupting the RNA duplex do not reduce promoter activity more than conservative substitutions in the same position⁹⁵. One interpretation of all the data is that successful transcription initiation requires a promoter-leader duplex of minimal stability. Once this condition is met, extending the base pairing does not increase the rate of transcription initiation⁹⁵.

Coronavirus mRNAs are synthesized in MHV infected cells at a rate inversely related to their length⁷⁹. In contrast, in FIPV and TGEV infected cells the shortest mRNAs (RNAs 7 and 6, respectively) are produced in much smaller quantities than the next larger mRNAs encoding the nucleocapsid protein. These data suggest that vectors based on different coronaviruses will have to use various promoters for optimum expression. Fusion of leader and body is imprecise. To explain the generation of this heterogeneity it has been proposed that leader RNAs are trimmed by a nuclease to remove the single-stranded RNA sequence from the 3' end after the leader complementary promoter sequence is annealed to the promoter³. Alternatively, it has been proposed³⁷ that a sequence-specific nuclease cleaves the leader RNA at position 1 or 2 of the consensus sequence. More recently it has been proposed⁹⁵ that transcription is initiated at multiple, possibly random, sites within the IPS. A 3' to 5' nuclease activity present in many RNA polymerases could remove 3' residues in the IPS causing the heterogeneity.

Resistance to Virus Infections: Intracellular Interference and Transgenic Animals

Two strategies in the construction of transgenic animals resistant to viral infection could be distinguished: i) "intracellular immunity", which could have been more appropriately named intracellular interference, and ii) development of transgenic animals providing immunoprotection. Intracellular interference includes: i) anti-sense RNA and ribozyme approaches; ii) trans-dominant negative mutant viral proteins; iii) molecular decoys; and, iv) intracellular expression of antibody to viral proteins. Antisense oligonucleotides containing sequences complementary to leader RNA induced a significant effect on the multiplication of MHV-JHM. A vector which expressed the antisense mRNA⁷ of MHV decreased virus multiplication in cell cultures. In transfected cell lines expressing a ribozyme against the 5' end of MHV genome, MHV-multiplication was inhibited by more than 98% at 12 h post infection⁷⁷. Transgenic mice expressing antisense RNA against the nucleocapsid protein gene of MHV, under the control of RSV LTR, were more resistant to the lethal challenge of MHV than non-transgenic mice⁶³.

Interference in plant and mammalian viruses is provided by expression of the coat protein or the replicase of a virus in the cells susceptible of infection. Little has been done in coronaviruses in this subject. cDNAs engineered using DI RNAs might be used to study the interference in coronavirus replication, mediated by defective products of different ORFs.

Single-chain antibody molecules specific for surface S glycoprotein of MHV have been constructed to interfere with coronavirus induced diseases⁴¹. The single chain antibody was derived from a murine MAb that has a high binding affinity and neutralizes virus infectivity. Single chain antibodies have been expressed in CRFK and DBT cells using eukaryotic expression vectors with human cytomegalovirus or metallothionein promoters⁴¹. The resistance of these cells to virus infection and S protein-mediated membrane fusion is being evaluated.

Transgenic pigs secreting TGEV neutralizing antibodies in the milk, to passively protect the progeny, are being constructed. The project is based on the protection of newborn animals by oral administration of IgG or IgA antibodies purified from immune sows, previously reported. The genes coding for the light and heavy chain of TGEV neutralizing MAb 6A.C3, which recognizes a conserved epitope of S protein⁷⁵ have been expressed under the control of the whey acid protein (*wap*) promoter. This promoter is activated by peptidic and steroid hormones present in stimulating concentration in the mammary gland during lactation. Activation of *wap* promoter results in the synthesis of WAP protein very abundant in the milk. The recombinant antibody has been expressed under the control of both human cytomegalovirus and *wap* promoters in cell culture¹⁴. Recombinant antibody had titers of 10⁴ as determined by RIA, and neutralization index of 4 log units, using TGEV antigen or infectious virus, respectively. Transgenic mice carrying the genes coding for both the heavy and light chains of 6A.C3 MAb have been developed. The presence of TGEV specific antibody in the milk of transgenic mice during lactation is being determined. Constructs providing optimum levels of TGEV neutralizing antibody will be used to produce transgenic pigs.

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