Instituto Juan March
de Estudios e Investigaciones

143 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Engineering RNA Virus Genomes as Biosafe Vectors

Organized by

C. M. Rice, W. J. M. Spaan and L. Enjuanes

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Introduction

C. M. Rice, W. J. M. Spaan and L. Enjuanes

Engineering RNA virus genomes as biosafe vectors for vaccine development and gene therapy has been the title of a meeting that has recently taken place at the "Instituto Juan March de Estudios e Investigaciones". Progress on human genome knowledge is leading to the identification of gene defects responsible for human diseases. These defective genes can be replaced by using somatic gene therapy, but it is essential to develop vectors that express the healthy gene with a predefined timing and tissue distribution.

Viral and bacterial pathogens are responsible for the major diseases and death worldwide. A recent report of the World Health Organization has shown that AIDS, malaria, tuberculosis, measles and acute respiratory and enteric infections are responsible for 90% death in humans less than 44 year old. To prevent these infectious diseases it is important to improve the current vaccination and gene therapy strategies and design new ones. These strategies have a common element, the administration of drugs that interfere with the replication of the microorganism. Frequently this intervention is mediated by genes encoding proteins that elicite responses inhibiting pathogen replication. The efficient introduction of genetic material into tissues requires to overcome the defenses that the organism has set up to counteract the entrance of foreign materials, to drive these genes to the target organ, and to control their expression. Viruses have evolved through many generations to overcome these barriers and frequently show high tissue specificity, making viruses ideal tools to target expression of heterologous genes. The use of viruses as vectors for the tissue specific expression is essential for both vaccine design and gene therapy.

During the meeting, emphasis was focused on RNA viruses because generally their genomes are not integrated within the host chromosome, except the retroviruses, making RNA virus vectors safer. In addition, vectors based on RNA viruses displaying a variety of tropisms have been emerging during recent years. As reported through the meeting, these viruses are being very helpful both in vaccination and gene therapy since tissue specific expression is essential for the success of the treatment. The engineering of infectious cDNA clones for most viruses, including those with RNA genomes of positive and negative polarity has facilitated studies by reverse genetics with these viruses in order to precisely control their virulence. The construction of replication-competent propagation-deficient viruses, and of non-cytopathic replicons based on viral genomes derived from several virus families, has facilitated the study of virus replication and the use of viruses as vehicles for vaccine development and gene therapy.

The continuous emergence of new viral pathogens was reported. These pathogens frequently cross the species barrier due to their high evolution rates that facilitate the adaptation of viral genomes to new ecological nitches. The existence of viruses as quasispecies possesses some uncertainty on the safety of any live virus vector. Therefore it was concluded that strong safety guards must be engineered in these vectors, in order to increase the ratio benefit to risk to acceptable levels. Due to the risk involved in the generation of new virus entities the use of appropriated containment facilities and professional behavior of the scientists involved in the generation of new autoreplicative genomes was considered essential.

It must be expected that in the new Millennium the administration of a viral vector to plants, animals or human beings, will require that this type of interventions are made in a safe way. Although any intervention in human beings has associated a risk, it is also possible to

design strategies that reduce this uncertainty to levels further lower that those associated with common life. Accordingly, biosafety on virus vector design must be a priority.

The meeting was intense, permitted close interaction between scientists working on different aspects of virus replication and, hopefully, will stimulate the opening of new research avenues.

Charly M. Rice, Willy J. M. Spaan and Luis Enjuanes

Session 1: Vectors based on positive strand RNA virus genomes I
Chair: Paul Ahlquist

RNA vectors based on Sindbis virus

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Alphaviruses like Sindbis virus encode extremely efficient RNA amplification machines. In tandem with unraveling the molecular details of alphavirus replication, these viruses have been harnessed for expression of foreign antigens. Advantages include ease of manipulation, high level or regulated expression, a broad host range, and selection of mutations that allow non-cytopathic replication in some cell types. Recombinant RNA replicons can be engineered that are infectious and capable of spread or as defective "one-way" vectors that require helper functions for packaging. Replication competent RNAs can be launched by cell-free transcription and transfection or in cells by using host transcription machinery. These vectors are being employed for basic research, production of protein-based therapeutics, and for vaccination against a variety of human and animal pathogens.

Poliovirus as a mucosal vaccine vector and its potential use to develop an AIDS vaccine

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Poliovirus is a member of the picornavirus family of positive strand RNA viruses. Ongoing efforts have been focused to develop picornaviruses as gene therapy (3) or suicide vectors (7), however, the vast majority of vector development with picornaviruses has focused on the engineering and use of poliovirus based vaccine vectors.

Viruses are strong inducers of cellular and humoral immune responses. Thus, activation of the immune responses by vaccination with recombinant viruses expressing relevant antigens derived from other pathogens or tumors is a promising approach for the prevention and treatment of infectious diseases and cellular malignancies. Viral vaccine vectors that have been successfully used in experimental models include poxviruses, adenoviruses, alphaviruses, picornaviruses, and influenza viruses. Vectors based on currently used human vaccine viruses [i.e. poliovirus (the Sabin oral poliovirus vaccine) and vaccinia (the smallpox vaccine)] are particularly appealing due to their well characterized safety profiles in millions, if not billions, of vaccinees, as well as the extensively characterized ability of these vaccines to induce strong long-term immune responses in humans.

We have demonstrated that recombinant polioviruses express the inserted antigenic protein in readily detectable amounts (2, 4, 10, 11). Inserts encoding up to 200 additional amino acids can be successfully accommodated in recombinant polioviruses. As the introduced antigens are expressed within infected cells, stimulation of both cell-mediated and humoral immunity is achieved (2, 4, 5, 8-11). Although the recombinants express the foreign sequences during the replicative cycle, these proteins are not included in the mature virus particle. As such, the virion structure, antigenicity, and host range of the recombinant polioviruses is not altered (1, 2, 6). Importantly, we have recently constructed Sabin poliovirus vectors expressing a defined library of overlapping SIV protein fragments representing the entire sequences of SIV Gag, Pol, Env, and Nef. These Sabin recombinants stably express the SIV antigenic sequences and they were able to induce protective immunity against a challenge with pathogenic SIV in macaques (5).

RNA viruses such as poliovirus have multiple beneficial attributes as well as problems that should be overcome. We will discuss the advantages and disadvantages of the polio system. We will focus particularly on the utilization of poliovirus based vaccine vectors as mucosal vaccines, particularly with respect to the development of an effective AIDS vaccine, as the development of a vaccine to prevent AIDS is the most active area of vaccine research in the world today.

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Coronavirus reverse genetics: viral based vectors for gene expression

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The construction of virus vectors derived from RNA viruses is a comprehensive process that for optimum performance requires: (i) the availability of an infectious cDNA clone; (ii) knowledge on the transcription mechanism to optimize expression levels; (iii) determination of the essential and non-essential genes to create room for heterologous genes; (iv) understanding of the molecular basis of virus tropism, in order to control the species and tissue-specificity of the vector; (v) control of the virus virulence, in order to generate attenuated virus vectors; and (vi) design a strategy for vector safety. Progress on these aspects using transmissible gastroenteritis virus (TGEV) genome will be summarized.

We have developed a safe virus vector using a cDNA clone encoding a full-length coronavirus RNA (1). The cDNA was constructed as a bacterial artificial chromosome (BAC) that facilitated coronavirus reverse genetics. This BAC encodes a TGEV that infects both the enteric and respiratory tract of swine. The stable propagation of the TGEV full-length cDNA in bacteria for, at least, 200 generations has been considerably improved by the insertion of an intron to disrupt a toxic region identified in the viral genome (2). The viral RNA was expressed in the cell nucleus under the control of the cytomegalovirus (CMV) promoter and the intron was efficiently removed during translocation of this RNA to the cytoplasm.

Partially overlapping TGEV genes have been separated by duplicating sequences at the 5' flank of each gene and by introducing unique restriction endonuclease sites between each gene pair. ORF separation allowed gene deletions showing that genes 3a, 3b, and 7 are non-essential for virus replication.

In order to increase expression levels, coronavirus transcription regulatory sequences (TRSs) have been characterized. The minimal TRS was a core sequence (CS) consisting of six nucleotides (5'-CUAAAC-3'). Transcription efficiency was increased by extending the sequences flanking the CS 5' upstream and also my modification of the 3' downstream sequences (3). CS flanking sequences modulate mRNA levels and can cause complete silencing of expression from a canonical CS. Modulation of mRNA levels has also been achieved by CS point mutagenesis. Furthermore, the engineering of an insertion site for the heterologous gene, between genes N and 7, led to a new genetic organization of the 3' end of recombinant viruses. As a consequence, a major species of subgenomic mRNA (sgmRNA) was generated from a TRS with the non-canonical CS 5'-CUAAAA-3'. Studies using these non-canonical CSs, or those generated by point mutagenesis, showed that the sequence of the sgmRNA leader-body junctions included the mutated CS sequences, suggesting that the discontinuous step of coronavirus transcription occurs during minus strand synthesis. Extension of the complementarity between the CS flanking sequences in the negative RNA and the viral leader RNA was associated to the transcriptional activation of non-canonical CSs. This observation indicates that additional base-pairing between the leader RNA and sequences flanking the CS motif compensates for the absence of a canonical CS.

TGEV derived expression vectors expressed high amounts of the heterologous gene (50 μg/10^6 cells) and were very stable (>20 passages) (4). Interestingly, a specific lactogenic immune response against the heterologous protein has been elicited in sows and their progeny. Replication-competent propagation-deficient virus vectors, based on TGEV genome deficient in the essential gene E, have been developed by complementation with E^+ packaging cell lines. Cell lines transiently expressing TGEV E protein were established using the non-cytopathic Sindbis virus replicon pSINrep21. In addition, cell lines stably expressing the E gene under the CMV promoter have been developed. Recombinant TGEV deficient in E gene were successfully rescued ($1x10^7$ pfu/ml) in cells stably expressing porcine aminopeptidase N (BHK-pAPN), the cellular receptor for TGEV, and transiently expressing TGEV E protein. Infectious TGEV was also recovered ($5x10^5$ pfu/ml) from porcine cells stably expressing E protein. Virus titers were proportional to E protein expression levels. The virions produced in the packaging cell lines showed the same morphology and stability to pH and temperature as virus derived from the full-length TGEV genome. These viruses were stably grown for more than 10 passages in the E^+ packaging cell lines.

TGEV species-specificity has been extended to infect canine and human cells by replacing the S gene of TGEV by S genes from canine and human coronaviruses. In this case, a replication-competent propagation-defective TGEV has been used for safety. TGEV derived vector virulence has been modulated by three procedures: (i) reducing virus growth in the enteric tract by introducing modifications at the 5' end of the S gene; (ii) modification of the TRSs at the 5' end of each gene; (iii) deletion of non-essential genes such as gene 7. These alterations of TGEV genome led to the generation of a collection of vectors with different attenuation degrees.

Application of virus vectors to human requires risk reduction to levels below those of conventional medical interventions. Coronavirus vectors based on TGEV genome have been engineered to infect human cells by replacing the S gene by that of human coronaviruses. To increase the safety of the human adapted vector, a replication-competent propagation-deficient virus is being modified by introducing mutations that abrogate the activity of the RNA encapsidation signal (ε), and relocating an active ε next to deleted genes. A recombination event leading to the recovery of the essential genes will most likely lead to the loss of the packaging signal, generating a non-viable virus and, therefore, to a safe vector. These results indicate that coronavirus could be used as flexible vectors with high potential for vaccine development and, possibly, for gene therapy.

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Engineering arterivirus genomes as vectors

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The development of infectious cDNA clones has opened the possibility to genetically engineer Arteriviruses (Snijder & Meulenberg, 2001) into expression and/or vaccine vectors. An especially interesting feature of Arteriviruses (and other Nidoviruses) is their polycistronic genome organization and the synthesis of an extensive nested set of subgenomic transcripts from the 3' end of the viral genome (Pasternak et al., 2000). Thus, in theory, multiple foreign genes could be inserted in an Arterivirus genome and each of those could be expressed from a different viral subgenomic mRNA. On the other hand, the compact organization of the 3' end of the Arterivirus genome poses some serious problems: all reading frames overlap with neighboring genes and transcription-regulating sequences are located within coding regions. Furthermore, the Arterivirus particle is poorly characterized in terms of structural proteins, virus assembly, and tropism.

Using the Equine Arteritis Virus (EAV) infectious cDNA clone (van Dinten et al., 1997), we have explored various possibilities to engineer the Arterivirus genome for expression of foreign sequences. As a first step, the requirements for virion production were investigated, to explore the possibility of replacing nonessential genes with foreign sequences. A novel structural protein was discovered (Snijder et al., 1999) and (by making knockout viruses) it was shown that the products of each of the 7 genes in the 3' end of the EAV genome are required for the production of infectious progeny (Molenkamp et al., 2000). The same study revealed that the EAV replicase suffices for genome replication and subgenomic mRNA synthesis. In addition, indications were obtained that, in contrast to the constructs lacking the major structural proteins (N, M, and GP5), the knockouts for the minor structural proteins (E, GP2b, GP3, and GP4) produce noninfectious subviral particles.

The two main Arterivirus envelope proteins, GP_5 and M, play an essential role in virus assembly and infectivity. The formation of a disulfide-linked heterodimer is an important feature of GP_5-M function. Using the EAV infectious cDNA clone, we have analyzed GP_5-M heterodimerization in more detail. We concluded that (surprisingly) GP_5 glycosylation is not essential for EAV infectivity in cell culture and that GP_5-M dimerization is based on an interaction between GP_5 Cys-34 and M Cys-8. Subsequently, the possibility to exchange envelope protein ectodomains between Arteriviruses was explored. We have engineered chimeric EAV vectors in which the ectodomains of GP_5 and/or the M protein were replaced by the corresponding sequences of either the porcine Arterivirus (PRRSV) or the mouse Arterivirus (LDV). To this end, the overlaps between the ORFs 4, 5, and 6 in the 3'-terminal part of the EAV genome were first removed to allow the free exchange of sequences without affecting other genes. Interestingly, EAV vectors carrying either the PRRSV or the LDV GP_5 ectodomain produced reasonable levels of chimeric virus particles. Surprisingly, both chimeric viruses were still able to infect BHK-21 cells. These are routinely used to grow EAV, but cannot be infected with either PRRSV or LDV. This implies that the ectodomain of the Arterivirus GP_5 protein, which was considered as the most probable receptor-binding membrane protein, is not the main determinant of Arterivirus tropism.

The possibility to generate defective EAV vectors and specific complementing cell lines was explored using the Sindbis virus-based, noncytopathogenic SinRep19 RNA expression vector. Pilot studies had indicated that Sindbis virus (replicating at endosomal membranes and budding from the plasma membrane) and EAV (replicating at ER membranes and budding from intracellular membranes) could replicate and assemble in the same infected cell. For the minor glycoproteins GP_{2b}, GP_3, and GP_4, complementing BHK-21 cell lines could be readily established which were found to be able to complement knockout viruses lacking the corresponding gene. The SinRep19 cell lines stably expressed the complementing gene for at least 3 months and produced knockout virus titers that approached those of the wild-type virus. These results opened the possibility to design defective single-cycle (DISC) EAV vectors, whose biosafety is guaranteed by the fact that the defective genome can only be packaged into an infectious virion upon replication in the specific complementing cell line.

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Multigene expression using coronavirus-based vector RNA

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A characteristic feature of coronaviruses is their unique transcriptional strategy which results in the synthesis of 6-8 subgenomic mRNAs. To exploit this transcriptional strategy, we have developed a new type of eukaryotic, multi-gene RNA vector. We have constructed a 24.7 kilobase RNA (vec-CLG RNA) containing the 5` and 3` ends of the human coronavirus (HCoV) genome, the HCoV replicase gene and three reporter genes (CAT, LUC and GFP); each located downstream of a HCoV transcription associated sequence. After co-electroporation of vec-CLG RNA and N protein mRNA into BHK-21 cells, we demonstrated the expression of CAT, LUC and GFP. Sequence analysis of the unique mRNA leader-body junctions confirmed the synthesis of coronavirus specific transcripts. In further experiments we have asked whether vec-CLG RNA can be incorporated into coronavirus-like particles (CVLPs) in order to transduce eukaryotic cells expressing the HcoV receptor, aminopeptidase N (CD13). Vec-CLG RNA was co-electroporated into BHK-21 cells with HCoV genomic RNA and N protein mRNA. After 3 days the tissue culture supernatant was transferred to MRC-5 cells. The supernatant from the MRC-5 cultures was then used to transduce both immature and mature human dendritic cells (DCs) which could be identified by GFP expression. In summary, we have shown that it is possible to use the unique characteristics of coronavirus transcription to produce a vector RNA that can coexpress several heterologous proteins. In addition we have shown that human coronavirus-based vector RNA can be packaged into CVLPs that can be used to transduce human DCs. These data encourage us to develop the coronavirus vector system for immune therapy applications.

Session 2: Vectors based on positive strand RNA virus genomes II
Chair: Charles M. Rice

Cytopathogenic and noncytopathogenic pestivirus replicons

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The genus Pestivirus within the family Flaviviridae comprises the species Bovine viral diarrhea virus 1 (BVDV-1), BVDV-2, Classical swine fever virus and Border disease virus. The pestivirus genome consists of a positive-stranded non-polyadenylated RNA molecule with a size of approximately 12.3 kb which contains one large open reading frame (ORF) flanked by 5′ and 3′ nontranslated regions (NTR). This ORF encodes a polyprotein of approximately 3900 amino acids which is co- and posttranslationally processed by viral and cellular proteases leading to the mature viral proteins. The first third of the ORF encodes a non-structural autoprotease and four structural proteins, while the 3′ part of the RNA genome codes for the other nonstructural (NS) proteins. Two biotypes of pestiviruses, cytopathogenic (cp) and noncytopathogenic (noncp) viruses, are distinguished by their ability to cause a cytopathic effect in tissue culture. One important difference between cp and noncp BVDV is the expression of NS3, which is colinear to the C-terminal part of NS2-3. While NS2-3 is expressed in both cp- and noncp BVDV infected cells, NS3 is found exclusively after infection with cp BVDV. Accordingly, NS3 is regarded as the marker protein for cp BVDV strains (1).

In pregnant animals, diaplacental infection with noncp BVDV can result in the birth of persistently infected animals with an acquired immunotolerance to the infecting BVDV strain. Such persistently infected animals may come down with mucosal disease (MD), a particularly severe clinical manifestation of BVDV infection. In addition to the persisting noncp virus, cp BVDV can always be isolated from animals with MD.

Molecular analyses of several BVDV pairs isolated from field cases of MD indicated that the cp viruses can evolve from the respective noncp viruses by RNA recombination. The mutations identified in the genomes of the cp viruses include insertions of cellular sequences and genomic rearrangements with duplications of viral sequences. The cellular sequences present in the genomes of cp pestiviruses analysed so far encode ubiquitin, the ubiquitin-like proteins SMT3B and NEDD8, ribosomal protein S27a together with an N-terminally truncated ubiquitin, light chain 3 (LC3) of microtubule associated proteins 1A and 1B, GATE-16, GABA(A)-RAP and the J-domain protein Jiv (previously termed cINS) (1, 2, 3, 4, 5). Interestingly, Jiv triggers NS2-3 cleavage in cis and in trans.

Moreover, defective interfering particles (DIs) of bovine viral diarrhea virus (BVDV) have been identified and shown to be cytopathogenic (cp) in the presence of noncytopathogenic (noncp) helper virus (6). Interestingly, a subgenomic (sg) RNA corresponding in its genome structure to one of those BVDV DIs was replication competent in the absence of helper virus (7). On the basis of two infectious BVDV cDNA clones, namely, BVDV CP7 (cp) and CP7ins- (noncp), bicistronic replicons expressing proteins NS2-3 to NS5B were established (8). These replicons express, in addition to the viral proteins, the reporter gene encoding ß-glucuronidase; the release of this enzyme from transfected culture cells was used to monitor cell lysis. Applying these tools, we were able to show that the

replicon derived from CP7ins- does not induce cell lysis. Accordingly, neither N^{pro} nor any of the structural proteins are necessary to maintain the noncp phenotype. Furthermore, these sg RNAs represent the first pair of cp and noncp replicons which mimic complete BVDV CP7 and CP7ins- with respect to cytopathogenicity. Pestiviral replicons and complete pestiviral genomes will be useful for studies concerning cytopathogenicity, RNA recombination and vector development.

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Novel chimeric flavivirus vaccine viruses (ChimeriVax™) based on yellow fever 17D virus as a vector

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Yellow Fever (YF) 17D is an ideal vector for genes encoding protective antigens of flaviviruses. Chimeric vaccines were constructed by replacing the viral envelope prM and E genes of YF 17D with the corresponding genes of other flaviviruses [e.g. Japanese encephalitis (JE)(1-3), dengue (DEN) (4-6), West Nile (WN) (7), and St. Louis encephalitis (SLE) viruses. The E protein of the donor virus contains protective antigens, while replicative enzymes are encoded by YF nonstructural genes. The JE prME donor was an attenuated vaccine strain (SA14-14-2), whereas wild type DEN sequences were used. Chimeric virus vaccines were evaluated in preclinical and clinical studies. All chimeras grew to high titers in Vero cells (intended for c-GMP manufacturing), were less neurovirulent than YF 17D vaccine in mouse and monkey models, were markedly restricted in their ability to replicate in mosquitoes (8, 9), and were immunogenic and protective after a single subcutaneous inoculation. Prior YF immunity did not preclude successful vaccination. Simultaneous immunization of monkeys with a multivalent vaccine containing multiple ChimeriVax™ viruses against the four DEN serotypes was performed.

Three Phase I/II clinical trials were conducted in healthy naïve or YF- immune adults to assess safety, dose response and immunogenicity of ChimeriVax™-JE. The vaccine was well tolerated and the profile of adverse events was similar to that of YF 17D vaccine. The seroconversion rate was 100% in both naïve and YF-immune subjects after a single immunization (10). The vaccine appears well suited for rapid immunization of travelers against JE and for residents of JE-endemic regions of Asia and Australia. A Clinical trial of ChimeriVax™-DEN2 monovalent vaccine was completed in Q1 2002 and data is expected to be unblinded in December 2002. A tetravalent vaccine for dengue is prepared under cGMP manufacturing and is scheduled to be tested in early 2003 in human volunteers.

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Recombinant yellow fever virus expressing HCV E1 and E2

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The attenuated yellow fever virus strain 17D (YFV-17D) has been widely used as a safe and effective vaccine for over 60 years. The recent construction of a stable, full-length infectious YFV-17D cDNA facilitates the development of YFV-17D as a human carrier vaccine for foreign antigens. The advantages of recombinant YFV-17D based vaccines include the ability to induce long-lasting immunity, safety, affordability, efficacy, and approved use in humans.

Based on the recently established full-length infectious YFV-17D cDNA we have constructed a recombinant YFV expressing the HCV E1, E2 and p7 proteins. The genes encoding these HCV structural proteins were cloned in frame between the YFV E and NS1 genes. Despite the fact that this insertion enlarged the length of the viral genome with approx. 1.8 kb, transfection of in vitro transcibed RNA resulted in viral RNA synthesis, protein expression, and production of recombinant virus. In addition to the YFV proteins, the HCV E1 and E2 proteins were efficiently expressed.

The non-covalently linked E1E2 heterodimer was detected, but appeared to be retained in the ER.

More detailed analysis of the replicative properties of this recombinant YFV/HCV virus in tissue culture showed that it replicated less efficient as compared to the YFV-17D control virus. Analysis of the kinetics of virus production in cells transfected with the recombinant YFV/HCV RNA demonstrated that the onset and peak of virus production was similar as compared to the parental YFV-17D. However, the titers were generally 10-fold lower and the plaque size was significantly smaller.

106 pfu of purified recombinant virus was used to immunise HLA A2.1 transgenic mice. The qualitative and quantitative aspects of the cellular and humoral immune response was analysed. The results of these studies will be included and discussed.

Kunjin replicon-based gene expression and delivery system

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We have been developing a heterologous gene (HG) expression and delivery system based on a noncytopathic replicon RNA of the flavivirus Kunjin (KUN). The system directs high level expression of a number of HGs *in vitro* and *in vivo* after delivery of the corresponding recombinant KUN replicons in the form of either naked RNA (RNA-based vectors, SP6 promoter-driven), or plasmid DNA (DNA-based vectors, CMV promoter driven), or virus-like particles (VLPs). VLPs with titres of up to 10E7 per ml were generated by co-transfection of KUN replicon RNA with Semliki Forest virus replicon RNA expressing KUN structural genes required for packaging.

Selectable RNA-based and DNA-based replicon vectors were constructed by insertion of antibiotic resistance genes and were used to establish a wide range of cell lines from different mammalian hosts stably expressing a number of HGs. Immunization of mice with KUN replicon vectors in the form of plasmid DNA, naked RNA, and VLPs encoding murine polyepitope and HIV Gag resulted in induction of antibody and protective $CD8^+$ T cell responses. Current studies are focused on improvement and simplification of a packaging system for VLP production and on development of KUN replicon-based HIV and cancer vaccines. In summary, KUN replicons have high potential to be used for large-scale protein production in mammalian cells, as vaccine vectors, and possibly as vectors for gene therapy.

Nodavirus RNA replication and new parallels in positive-strand RNA, retro- and dsRNA virus replication

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Nodaviruses are positive-strand RNA animal viruses with small, ~4.5 kb genomes. Previously we showed that the best-studied nodavirus, flock house virus (FHV), replicates its RNA and forms infectious virions in the model yeast, Saccharomyces cerevisiae (Price et al., 1996). FHV RNA2 encodes the viral capsid proteins while RNA1 encodes RNA replication protein A and an inhibitor of antiviral RNA silencing, protein B. Protein B is translated from a subgenomic mRNA, RNA3.

We have mapped RNA1 regulatory sequences that control RNA1 replication and RNA3 production in cis and RNA2 replication in trans (Lindenbach et al., 2002). Subgenomic RNA3 production required ~500 nt overlapping and just upstream of the RNA3 start site and a distal ~13 nt sequence 1.5 kb farther upstream. Base pairing between these distal elements was essential for RNA3 production, as shown by targeted mutation and directed evolution. These and other experiments (Price et al. 2000) suggest that, unlike many other viruses, subgenomic RNA3 may be synthesized from a subgenomic rather than a genomic (-)RNA template. When protein A was provided in trans, cis-acting RNA1 mutations that blocked or restored synthesis of subgenomic RNA3 also blocked or restored in parallel the replication of FHV genomic RNA2. Thus, RNA2 replication depends on RNA1 not only to provide protein A but also for functions encoded at the RNA level.

As well as studying viral cis / trans functions in FHV replication, we are using yeast genetics to identify host genes affecting virus replication. In one approach, we are using an ordered set of yeast deletion strains, each with a single known gene knocked out, to systematically test the effects of ~5000 of the 6200 yeast genes on FHV RNA replication and gene expression.

Like all other positive-strand RNA viruses, nodavirus RNA replication is associated with intracellular membranes. By confocal microscopy, cell fractionation and other approaches, we localized FHV RNA replication protein A and FHV RNA synthesis in Drosophila and yeast cells to outer mitochondrial membranes (Miller et al., 2001; Miller and Ahlquist, 2002). We have also mapped protein A sequences sufficient for mitrochondrial targeting and used protease digestion, selective permeabilization, and epitope tagging to characterize the nature and topology of protein A association with mitochondrial membranes.

By electron microscopy, we found that FHV RNA replication is associated with 40-60 nm spherular invaginations of the outer mitochondrial membrane. Recently we found that another positive-strand RNA virus, brome mosaic virus (BMV), forms very similar RNA replication complexes on ER membranes (Schwartz et al., 2002). This work shows that BMV RNA replication factors 1a, 2a, and a specific cis-acting replication signal recapitulate the functions of Gag, Pol and RNA packaging signals in retrovirus cores. Prior to RNA replication, 1a forms spherules budding into the ER membrane, sequestering viral positive-

strand RNA templates. When expressed, 2a polymerase co-localizes in these spherules, which become the sites of viral RNA synthesis and retain (-)RNA templates for (+)RNA synthesis. These results explain many features of replication by many positive-strand RNA viruses and reveal that these viruses, reverse transcribing viruses, and dsRNA viruses share fundamental similarities in replication and likely have common evolutionary origins.

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Engineering nodavirus genomes as vectors

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Several features make the nodaviruses of insects attractive systems for harnessing as expression vectors (1, 2). Foremost is the fact that expression of the viral RNA-dependent RNA polymerase (RdRp), which results from translation of the larger segment of the bipartite viral genome (3), leads to abundant cytoplasmic RNA replication in a wide variety of cell types: from insects, several vertebrates, plants, and even the yeast S. cerevisiae. The viral RdRp is highly template-specific, and during natural virus infection yields only the two segments of the viral genome (RNAs 1 and 2) and a small subgenomic RNA (RNA3) that is templated by RNA1 and suppressed by RNA2. The three viral RNAs encode: RNA1, the RdRp itself; RNA2, a proteolytic precursor to the two viral capsid proteins; and RNA3, one or two small non-structural proteins, one of which (B2) has been implicated as a suppressor of RNA interference.

In order to be able to harness the full potential of the nodavirus system for the expression of heterologous genes, we have examined three different nodaviruses: Flock house virus (FHV), Nodamura virus (NoV), and Pariacoto virus (PaV). Each virus is of particular interest for a different reason: FHV because it is the best-studied system of nodaviral RNA replication (4); NoV because its RdRp is the most active in vertebrate cells and uniquely retains its RNA replicase activity at 37°C (5); and PaV because the high-resolution 3D structure of its virions reveals the internal organization of about 35% of the viral RNA (6, 7).

Using vertebrate cells that expressed T7 RNA polymerase, we reconstructed nodavirus RNA replication from infectious cDNA clones that transcribed the genomic RNAs. Each RNA1 segment expressed a functional RdRp that replicated its cognate genome segments but not those of the other two viruses. However, in addition to this straightforward template specificity, a more complex mechanism of regulation was discovered when wild-type FHV RdRp was translated from a version of RNA1 that could not replicate because it lacked both RNA termini. Surprisingly, this RdRp also failed to replicate FHV RNA2. The inhibition was traced to the lack of subgenomic RNA3, because RNA2 replication could be restored by providing RNA3 exogenously in trans (8). The RNA3-dependence was mapped to the 3' end of the positive strand of RNA2, which was shown to contain a cis-acting replication signal that required the presence of RNA3 for activity. In contrast, the RNA1 3' end was RNA3-independent. RNA3 itself appeared to be responsible for transactivation since the small non-structural proteins that it encodes were not required. These results establish that RNAs 2 and 3 counter-regulate one another: RNA3 is required for RNA2 replication, which in turn suppresses RNA3 synthesis. We propose that this mechanism serves to coordinate the synthesis of the two genome segments which are needed in equimolar amounts for optimum co-encapsidation.

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Alphavirus replicon vaccines

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Replicon vectors based on Venezuelan equine encephalitis virus (VEE) contain a self-replicating RNA encoding the VEE replicase proteins and expressing a gene of interest substituted for the VEE structural protein genes. The replicon RNA is encapsidated into VEE replicon particles (VRP) by supplying VEE capsid and envelope glycoproteins in trans. VRP expressing immunizing genes from a wide variety of pathogens have proven to be safe and effective vaccines in animal models, including primates.

Macaques immunized with these vectors expressing genes of simian immunodeficiency virus (SIV) were examined in an intrarectal challenge experiment with highly pathogenic SIV-E660. A portion of the gag gene, the full length gp160 gene and a gene for gp160 truncated immediately prior to the membrane spanning domain (gp140) of the smH4 clone of SIV were cloned and packaged into separate VRP. These were administered to macaques subcutaneously at a dose of 107 infectious units at 0, 1 and 4 months. At 5 months the animals were challenged with SIV-E660 intrarectally. The immunization induced neutralizing antibodies in 6/6 vaccinees and CTL in 4/6. Upon challenge, the vaccinated groupexperienced reduced peak virus loads compared to controls which received VRP expressing influenza HA. Virus load was reduced at set point and at 41 weeks post-challenge, and CD4 counts were preserved and even increased in the vaccinated animals. Therefore, immunization with a VRP based vaccine formulation provided significant protection from intrarectal challenge with a highly pathogenic SIV strain. VRP expressing HIV clade C gag are being prepared for use in a human phase I trial in the U.S. and Africa. An additional primate challenge experiment immunizing with antigens of SHIV89.6P has been initiated, and preliminary immunological and protection data will be presented.

Session 3: Vectors based on negative strand RNA virus genomes
Chair: Robert A. Lamb

Plasmid-based reverse genetics for influenza virus

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The development of reverse genetics techniques allowing the genetic manipulation of influenza viruses has led to the generation of recombinant viruses with specific insertions, deletions and substitutions in their genomes. Influenza viruses expressing foreign antigens have shown preclinical efficacy as vaccines against different infectious diseases and as anti-tumor agents in mice. In addition, it is also possible to attenuate influenza viruses using these reverse genetics techniques. Interestingly, recombinant influenza viruses containing specific deletions in the viral NS1 gene show different degrees of attenuation. These attenuated NS1 mutant viruses are impaired in their ability to evade the type I interferon system of the host. Recombinant NS1 mutant influenza viruses with appropriate levels of attenuation and immunogenicity may represent a new generation of effective live influenza virus vaccines.

Recombinant live-attenuated vaccines and vaccine vectors against human respiratory syncytial virus (HRSV) and the human parainfluenza viruses (HPIVs)

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The ability to produce complete infectious negative strand RNA viruses from cDNA provides a method for engineering viral genomes to produce optimized, highly-characterized live viral vaccines. We are using this approach to develop vaccines against the paramyxoviruses HRSV and HPIV serotypes 1, 2 and 3, which in aggregate account for nearly half of hospitalizations for respiratory tract disease early in life. Worldwide, acute respiratory tract infections are responsible for a large burden of disease and remain the greatest cause of death due to infectious disease. We will outline aspects of the design of recombinant vaccines and vaccine vectors using HRSV and HPIV3 as examples.

Recombinant HRSV and HPIV3 vaccine viruses and vectors can be attenuated (i) by introduction of attenuating point mutations such as ones identified in existing biologically-derived attenuated strains, (ii) by deletion of nonessential accessory genes, and (iii) by the "transfer" of attenuating mutations identified in one virus, such as HRSV, into a heterologous virus, such as HPIV3, using sequence alignment as a guide. In addition, both HRSV and HPIV3 have bovine (B) counterparts that are attenuated in primates due to a natural host range restriction. Thus, the antigenic determinants of HRSV or HPIV3 can be combined with one or more "internal" protein genes of the respective bovine counterpart to produce attenuated chimeric viruses.

Vaccine development can be expedited by swapping the major protective antigens of an attenuated HRSV or HPIV3 strain with ones representing, respectively, a heterologous HRSV antigenic subgroup or HPIV serotype so as to create new vaccine viruses. Alternatively, protective antigens from one or more heterologous pathogens can be expressed as additional, supernumerary genes. HPIV3 is preferred as a vector over HRSV for this application due to its more efficient growth and gene expression as well as its greater physical stability. One particularly attractive application of this strategy is to express the protective G and F antigens of HRSV from HPIV3, thus obviating the difficulty of working with HRSV and combining two needed vaccines. A vectored HRSV vaccine might be particularly useful for boosting an initial immunization by an attenuated version of complete HRSV.

The immunogenicity of a protective antigen can be increased by expression from a promoter-proximal location, either by inserting one or more supernumerary genes into the promoter proximal position or by rearranging the gene order. Another strategy is to express immunomodulatory molecules from genes inserted into the recombinant virus. For example, expression of granulocyte macrophage colony-stimulating factor from an attenuated HPIV3 vaccine candidate resulted in a 3- to 6-fold increase in virus-specific serum antibodies induced in rhesus monkeys.

The development of live, cDNA-derived vaccine viruses for widespread use offers the opportunity to apply information from basic studies to improve human health.

Attenuation of Sendai viruses by swapping replication promoters

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There are many ways to attenuate viral virulence, and in many cases the mechanisms that operate are poorly understood. Leader (***le***) and trailer (***tr***) RNAs are short, promoter-proximal transcripts generated during abortive antigenome and genome synthesis, respectively. Recombinant Sendai viruses (rSeV) that express ***tr***-like RNAs from the leader region are non-cytopathic, and moreover, prevent wild-type SeV from inducing apoptosis in mixed infections. These rSeV thus appear to have gained a function. We have found that ***tr*** RNA binds to a cellular protein with many links to apoptosis (TIAR) via the AU-rich sequence 5' UUUUAAAUUUU. Duplication of this AU-rich sequence alone within the ***le*** RNA confers TIAR binding on this ***le**** RNA and a non-cytopathic phenotype to these rSeV in cell culture. Transgenic over-expression of TIAR during SeV infection promotes apoptosis and reverses the anti-apoptotic effects of ***le**** RNA expression. Moreover, TIAR over-expression and SeV infection act synergistically to induce apoptosis. These short, promoter-proximal RNAs apparently act by sequestering TIAR, a multivalent RRM-family RNA-binding protein involved in SeV-induced apoptosis. In this view, ***tr*** RNA is not simply a by-product of abortive genome synthesis from the antigenomic promoter, but is also an antigenome transcript that functions to modulate the cellular innate antiviral response. Why these promoter-swap mutants have lost virulence in mice, however, is unclear.

A Newcastle disease virus-based assay for the identification of interferon antagonists

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Through the use of reverse genetics, an influenza virus lacking the NS1 protein was generated. It was shown that this protein is an auxiliary (virulence) factor which plays a crucial role in inhibiting the host's interferon (IFN) response (Garcia-Sastre et al. 1998). Subsequently, other negative strand RNA viruses were also shown to code for specific interferon antagonists. For example, the VP35 protein of Ebola virus (belonging to the Filoviridae) was shown to complement the growth (in MDCK cells) of the influenza virus lacking the NS1 protein (Basler et al. 2000). This finding suggests that Ebola virus VP35 has an anti-interferon activity similar to that of the NS1 protein.

We have now developed a Newcastle disease virus (NDV) assay based on the expression of a transgene, the green fluorescence protein (GFP). The recombinant NDV is sensitive to IFN, and the transfection of chick embryo fibroblast (CEF) cells with a plasmid expressing different proteins allows the identification of interferon antagonists. Using this novel assay with a GFP read-out, it was shown that the V protein of NDV and the homologous V protein of the Nipah virus both possess interferon-antagonist activity (Park et al.). Surprisingly, the anti-interferon activity of the NDV protein is associated with the carboxy perminal region of the protein, while that of the Nipah V protein is located in its amino terminal region. This finding suggests that the mechanism by which these two V proteins exert their anti-interferon activity must be different. Finally, we postulate that all negative strand RNA viruses have evolved mechanisms to counteract the host's interferon response to viral infection.

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Regulation of rhabdovirus transcription and replication: Another function of the rabies virus matrix protein

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We are studying factors regulating rhabdovirus gene expression (1-3) and virus assembly (4,5) by using engineered Rabies virus (7). Gene expression of RV involves the synthesis of subgenomic mRNAs ("transcription") and of full length RNA-containing ribonucleoprotein complexes (RNPs; "replication"). During virus assembly, RNPs are enwrapped into an envelope containing the internal amphipathic Matrix protein (M) and the transmembrane spike glycoprotein G. The M protein mediates coiling of RNPs into skeleton-like structures and their recruitment to the membrane. RNP condensation by M is associated with a general shut down of viral RNA synthesis ("freezing" of RNPs). Recombinant RVs lacking the M gene are not able to condense RNPs and to bud off rhabdovirions (6).

In search of factors regulating gene expression from RV RNPs, we now again identified the M protein as a crucial virus protein. In artificial transcribing or non-transcribing RV minigenome systems, a selective inhibition of transcription, but not of replication was caused by M provided in trans. Supplementing non-deficient wt RV with extra M, in cells expressing M in an inducible fashion (Teton-M), also led to a decrease in transcription rates and virus mRNA accumulation. To analyze RNA synthesis in the absence of M and of virus assembly and efflux of RNPs, a recombinant full length RV clone in which both M and G genes were replaced with placeholder reporter genes, NPgrL, was generated. Surprisingly, accumulation of replication products from NPgrL was strongly diminished, indicating the loss of a replication stimulatory factor. This took place in spite of a highly increased transcription rate and in abundance of virus proteins. The NPgrL phenotype was reversible in a dose dependent manner by providing M in trans. The ability of M in altering the balance of RV transcription and replication was exploited for generation of an autonomously replicating RV vector with highly increased transcription rates. This was achieved by attenuating M transcription through moving the M gene to the 5' terminal genome position.

A role of M in regulation of RNA synthesis independent of its role in virus assembly can be explained by direct or indirect activity of M on either (i) the polymerase, by causing it to switch from transcriptase to replicase, or (ii) on the RNP, rendering it a template for transcription or replication. Recombinant RVs carrying mutant or heterologous M genes are now being analyzed to dissect M domains relevant for assembly and RNA synthesis regulation and to dissect the mechanism involved.

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Attenuation of negative strand RNA viruses by gene rearrangements or insertions

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Viruses of the order *Mononegavirales* encompassing the four families *Rhabdoviridae, Paramyxoviridae, Filoviridae and Bornaviridae* are responsible for a wide range of infections in animals, plants and fish. Gene expression in these viruses is controlled primarily at the level of transcription by gene location relative to the single 3' proximal polymerase entry site. We have shown that it is possible to manipulate the phenotype of *Vesicular stomatitis virus* (VSV), the prototypic Rhabdovirus, by rearranging the order of genes and thereby taking advantage of the obligatorily sequential nature of transcription wherein position of a gene relative to the single promoter determines level of expression. For example, we have shown that translocation of the N gene to successive positions away from the promoter results in a progressive decrease in N expression, in replication in cultured cells and in lethality for mice. In addition, moving the G gene towards the promoter yielded increased G expression and a more rapid and higher antiviral immune response. By combining these two alterations, we generated viruses with rearranged genomes that were attenuated in mice and swine, but could nevertheless stimulate a protective immune response.

We investigated the basis for this attenuation in the mouse by examining the ability of viruses to travel to and replicate in the olfactory bulb (OB) and brain after intranasal inoculation. The results showed that viruses with rearranged genomes differed dramatically from the WT virus in the distribution and intensity of neuropathogenic lesions and in the type and levels of cytokines induced. All viruses reached the OB and brain but the outcome of these infections was markedly different: extensive meningitis and lethal encephalitis in the case of the WT as compared to clearance and survival with various rearranged viruses. The rearranged viruses were not defective in the ability to cause disease because if inoculated directly into the brain they caused lethal encephalitis as did the WT virus. These findings indicate that gene rearrangement can alter the viral spread and cytokine response to intranasal infection, even without any changes to the gene sequences. Analysis of the competitive fitness of viruses with rearranged genomes showed that even though they replicated as well or better than wild type virus in cell culture, they were less fit when assayed under competitive conditions. In spite of this, the rearranged gene orders remained stable with repeated passage both in cell culture and in animals.

As predicted from the mechanism of transcription, the expression of additional heterologous genes inserted into the VSV genome was largely determined by the position of insertion. However, although added genes were stably maintained in the genome during repeated passages, their stability of expression depended on where they were inserted.

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Stability of transgene expression by measles virus vectors

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Many measles virus (MV) recombinants have been engineered expressing transgenes from transcription units added either upstream of the first (N), the third (M) or the sixth (L) MV gene, mediating relative mRNA expression levels of about 10 : 3 : 1. Not only single, but two and even three transgenes encompassing more than 5 kilobases can be expressed from a single vector. Transgenes include marker genes (for sensitive MV detection) and genes encoding proteins of other pathogens (1, 2) or cytokines (3) (for vaccination purposes). In addition, MV genes can be exchanged by genes with similar functions even from distantly related viruses (4).

In most cases vector propagation in cultured cells or in animals is only slightly delayed, titers similar to standard vaccine strain MV are obtained, and transgene expression is surprisingly stable over many viral generations. However, when the product of a transgene strongly interferes with MV propagation, which can occur with large membrane anchored proteins, and in particular with envelope glycoproteins of related viruses (2), highly delayed growth and genetic instability may be observed: point mutations, mainly leading to stop codons in the added reading frames, abolish expression of the transgenes, thus conferring a propagation advantage to the MV recombinant, which rapidly overgrows the unaltered vector. This problem may be largely overcome by an alternative expression strategy by which the added reading frame is expressed upstream of a resident MV gene to which it is linked by a picornavirus-derived self-processing device.

In contrast to the positive strand vectors, transgene inactivation has never been observed so far to occur by deletion of RNA segments. The reasons underlying the highly stable transgene expression by MV (shared by other representatives of the *Paramyxovirinae*, but likely to a somewhat minor degree by *Pneumovirinae* and *Rhabdoviridae*) will be discussed. On one hand the adherence to the "rule of six" appears important, as reflected by the notion that also internal cis-acting elements appear to be optimally recognized in the context of hexameric RNP, as revealed by constructed phase-shift mutants (A. Zuniga, unpublished results). The extremely tight RNP structure covering the entire genome without any irregularity likely counteracts with particular efficiency homologous and heterologous RNA copy-choice recombination. On the other hand, the propagation of MV in cell culture (and also within infected animals) from cell to cell or by polyploid virions tends to suppress the outgrowth of unaltered recombinants by mutated, "fast" viruses. Highly relevant incidental observations by the group of Roberto Cattaneo (5) have corroborated these notions.

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Session 4: Vectors based on retroviruses
Chair: Gail W. Wertz

Lentiviral vectors and liver-based gene therapy

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Lentiviral vectors can mediate the efficient delivery, stable integration and long term expression of transgenes in a wide range of cells irrespective of their cell cycle status, thus opening exciting perspectives for the genetic treatment of human diseases. Our laboratory has been exploring the potential of lentiviral vectors for the genetic treatment of liver and lympho-hematopoietic disorders. In the first area, we have developed protocols that allow for the efficient transduction and reimplantation of human hepatocytes. We are also taking advantage of "lentimmortalized" human hepatocytes, cells that can grow continuously while exhibiting the properties of primary hepatocytes, in particular the ability to home to the liver and provide hepatic functions in immunodeficient mice. In the second area, we use chronic granulomatous disease (CGD), which results from a hereditary defect of neutrophils and macrophages, as a model system, and hematopoietic stem cells as targets for transduction. Selected aspects of this research will be presented and discussed.

Spumaretroviruses as vectors

Axel Rethwilm

Spumaretroviruses (foamy viruses [FV]) are a particular subgroup of retroviruses which make use of a replication strategy different from all other retroviruses (orthoretroviruses). In recent years many aspects of this replication pathway have been uncovered although we are far from having a complete picture.

Aberrant features of the FV replication strategy include the reverse transcription of an RNA pregenome essentially late in the infection cycle before virus is released, the way how capsid and glycoproteins interact, and the method how Pol protein is incorporated into the capsid.

Because of more or less theoretical grounds arguments have been made in favor of FV vectors compared with conventional retroviral vectors which are much further developed. Among these arguments are first of all a lack of pathogenicity upon natural animal infections or accidental human infections by primate FVs. A probably high capacity to accomodate foreign genes and the lack of inactivation by human serum can be added further to the list of favorable reasons to develop FV vectors. However, until recently no practicle advantage of FV vectors compared to orthoretroviral vectors could be demonstrated.

This situation has now changed. A careful comparative analysis of the ability to transduce unstimulated human hematopoietic stems cells (HSCs) and HSCs being able to repopulate NOD/Scid mice revealed that FV-derived vectors are superior to vectors derived from murine leukemia virus and as good as lentivirus vectors pseudotyped with the VSV-G glycoprotein.

These results prompt further research into the FV molecular biology, into the development of applicable vectors, and the establishment of packaging cell lines allowing the production of high titer vector viruses.

Turning retrovirus replication on and off; the design of a safer live-attenuated HIV-1 vaccine

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No safe and effective vaccine has been developed for the prophylaxis of AIDS. Live-attenuated viruses are the most effective vaccines tested thus far, but such variants are considered unsafe because continued replication can lead to the selection of faster-replicating variants that are pathogenic (1). We therefore designed a novel vaccine strategy in which an HIV-1 virus is used that replicates exclusively in the presence of the non-toxic effector doxycycline (dox). This was achieved by replacement of the viral TAR-Tat system for transcriptional activation by the E. coli-derived Tet-system for inducible gene expression (dox-regulated rtTA transcriptional trans-activator and the tetO DNA binding site). These designer 'HIV-rtTA' viruses replicate in a strictly dox-dependent manner both in a T cell line and in primary blood cells (2). Furthermore, the rate of replication can be fine-tuned by simple variation of the dox-concentration. These HIV-rtTA viruses may represent improved vaccine strains because their replication can be turned on and off at will. Moreover, we will present evidence that the Tet-system is fine-tuned by spontaneous virus evolution to optimize its new function in virus replication in human cells (3, 4). We identified rtTA protein variants with improved potency and increased dox-sensitivity. Thus, virus evolution can be exploited to select for novel Tet-systems with modified specificity and increased sensitivity. These reagents have proven very useful for optimization of our HIV-rtTA vaccine candidate. In addition, the modified reagents may be of particular use in other applications of the Tet-system, e.g. the regulation of transgene expression in transgenic animals and gene therapy vectors. For instance, a vector with a more sensitive Tet-system may allow gene therapy in the brain, where much lower dox-levels can be reached.

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Session 5: Biosafety of the virus vectors
Chair: Didier Trono

Biosafety of emerging and genetically modified viruses

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In the past ten years, several viruses have emerged which are highly pathogenic for humans, and because no licensed control measures (eg vaccines or drugs) are so far available to protect laboratory personnel and others who might come into contact with them, they need to be handled under conditions of maximum laboratory containment (biosafety level 4). Unfortunately, even experienced virologists may not take all possible safety precautions when handling a newly emerged virus. Such an incident occurred in 1994 when a virologist studying Sabiá, (a South American arenavirus known to have caused two severe infections, one of which was fatal), had an accident in his laboratory at the Yale Arbovirus Research Unit, infecting himself through an aerosol consequent on a leaking centrifuge tube. Though he survived the infection, he placed numerous members of the general public at risk before he realized the seriousness of his illness and sought medical treatment (Barry, M et al New Engl. J. Med. 333, 294, 1995). Although he was dealing with an emerging virus, it was known to be a rodent-borne pathogenic arenavirus, and these viruses (such as Lassa, Machupo, Junin, Guanarito and field strains of LCM) are notorious for causing laboratory-acquired infections. Thus one must ascribe a degree of blame for this incident to the research worker, whose fundamental error lay in not reporting the laboratory accident when it first occurred. It is an axiom that biosafety involves openness and trust among workers in the laboratory.

Recently, the threat of bioterrorism in the USA has prompted considerable tightening of controls over the possession as well as the use of potentially harmful viruses.

The CDC has been charged with establishing and maintaining a list of biological agents and toxins that have potential to pose a severe threat to public health and safety. This list of "Select Agents" being considered for adoption includes 12 bacteria, 2 fungi, 11 toxins, and more than 20 viruses. A US federal law signed on June 12, 2002, "Enhancing Controls on Dangerous Biological Agents and Toxins", requires that all facilities possessing, using or transferring Select Agents must register with the Secretary of Health and Human Services, requires the facilities to provide information on the Select Agent that can be used to characterize and facilitate its identification, and requires that registered facilities only provide access to Select Agents to named individuals who can be checked by the federal government using criminal, immigration, national security and other electronic databases to enquire whether the named individual scientist is a restricted person, who would then be denied access to the Select Agent.

These Select Agent restrictions also apply to the use of full length nucleic acids (synthetic or naturally derived) of any of the listed viruses, as well as nucleic acids that encode any virus protein that is known to be a determinant of virulence or can cause disease if expressed in vivo or in vitro, in an expression vector or host chromosome, or in a carrier plasmid. The period during which the public or concerned scientists could comment on this new law ended on September 17th, 2002, and CDC anticipates publishing an interim final rule on Select Agents in co-ordination with the US Department of Agriculture on or before December 9th, 2002.

The implications of these new biosafety regulations for the practice of virology, and the exploration and engineering of RNA viruses in the USA will be discussed.

Genetic rescue of RNA viruses: safety issues and possible solutions

Richard E. Randall

Any manipulation which alters the tissue tropism or host range of a virus is potentially generating a novel pathogen. Whilst this possibility is not unique to RNA viruses, and has thus been considered over the years by health and safety committees, there are a number of concerns which have to be specifically addressed when manipulating the genomes of RNA viruses. For example, the plasticity of the virus envelope of many negative strand RNA viruses has allowed the substitution of glycoprotein genes between viruses. In situations were the recombinant virus is forced to use the inserted gene for its own replication, whilst the initial recombinant virus may grow poorly, given the high mutation rates of RNA viruses, the recombinant viruses may rapidly evolve to replicate more efficiently and thus may become potentially more pathogenic. There are however a number of potential approaches to guard against the generation of novel pathogens. One approach would be to ensure that any virus generated is sensitive to interferon (IFN) as such viruses would be attenuated and unlikely to cause to disease.

IFNs are produced in response to virus infections and are powerful biological mediators, inducing an anti-viral state in cells and influencing the subsequent immune response. To survive in nature, it appears that all viruses must have some strategy for circumventing the IFN response. Viruses usually achieve this by producing proteins which either interfere with the ability of IFNs to induce an anti-viral state within cells, or by blocking the activity of anti-viral enzymes which have the potential to inhibit virus replication. Many paramyxoviruses at least partially circumvent the IFN response by blocking IFN signalling. Simian virus 5 (SV5), for example, blocks IFN signalling by targeting STAT1 (a host cell protein essential for IFN signalling) for proteasome-mediated degradation, and this is solely a property of the virus V protein. Furthermore, the ability of viruses to circumvent the IFN response may limit their host range, and this is a theme that will further be explored in the talk.

Since the virus proteins involved in circumventing the IFN response are usually luxury proteins, it is often possible to delete their genes without having deleterious effects on virus replication in tissue culture. For example, a recombinant bunyawera virus that is deleted in its NSs gene is sensitive to IFN, grows equally well on Vero cells (which do not produce IFN) to wild type virus, but is attenuated in vivo. Not only are such IFN sensitive viruses potential attenuated virus vaccines, they may also prove safe vectors for studies on gene function. For example, if gene manipulations and substitutions were performed on bunyaviruses to study the function of the virus glycoprotein it would be possible to use the NSs deleted virus as the vector secure in the knowledge that any virus generated would be attenuated. The theme of generating IFN-sensitive viruses for producing attenuated virus vaccines and safe vector will be discussed.

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Paramyxovirus assembly studied using reverse genetics and biochemistry

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Efficient assembly of enveloped viruses at the plasma membrane of virus-infected cells requires coordination between cytosolic viral components and viral integral membrane glycoproteins. This coalescence is believed to require interactions between the cytoplasmic tails of surface glycoproteins and the matrix (M) protein. For the paramyxovirus SV5 we have investigated the importance of the cytoplasmic domains of the fusion (F) protein and the hemagglutinin-neuraminidase (HN) protein in virus assembly and budding. By using reverse genetics, we have generated a series of recombinant viruses which contain truncations to the F and HN cytoplasmic tails. Analysis of the F cytoplasmic tail truncated viruses indicated that the cytoplasmic tail of the F protein was dispensable for normal virus replication and budding in tissue culture. In contrast the HN cytoplasmic tail-deleted viruses were shown to be replication-impaired, as judged by small plaque size, reduced replication rate, and lower maximum titers when compared to wt SV5. Release of progeny virus particles from cells infected with HN cytoplasmic tail-truncated viruses was inefficient as compared to wt virus, but syncytia formation was enhanced. Furthermore, accumulation of viral proteins at presumptive budding sites on the plasma membranes of infected cells was prevented by HN cytoplasmic tail truncations. We interpret these data to indicate that formation of budding complexes, from which efficient release of SV5 particles can occur, depends on the presence of an HN cytoplasmic tail. To investigate further the requirements for assembly and budding of SV5, we generated two double mutant recombinant viruses that lacks 8 amino acids of the predicted 17 amino acid HN protein cytoplasmic tail in combination with truncation of either 10 or 18 amino acids from the predicted 20 amino acid F protein cytoplasmic tail. Both of the double mutant recombinant viruses displayed a replication defect in tissue culture and a budding defect, the extent of which was dependant on the length of the remaining F cytoplasmic tail. Our data suggest a redundant role for the cytoplasmic tails of the HN and F proteins in virus assembly and budding.

We have also developed a system to analyze the components necessary for virus budding by analyzing the requirements for the efficient formation of virus-like particles. When the SV5 matrix (M) protein was expressed in mammalian cells it was found that SV5 M alone could not induce vesicle budding and was not secreted from cells. Coexpression of M with the viral glycoproteins HN or F also failed to result in significant VLP release. It was found that M protein in the form of VLPs was only secreted from cells, with an efficiency comparable to authentic virus budding, when the M protein was coexpressed with one of the two glycoproteins, HN or F, together with the nucleocapsid (NP) protein. The VLPs appeared similar morphologically to authentic virions by electron microscopy. CsCl density gradient centrifugation indicated that almost all of the NP protein in the cells had assembled into nucleocapsid-like structures. Deletion of the F and HN cytoplasmic tails indicated an important role of these cytoplasmic tails in VLP budding. Furthermore, truncation of the HN cytoplasmic tail was found to be inhibitory towards budding, as it prevented coexpressed wt F protein from directing VLP budding. Conversely, truncation of the F cytoplasmic tail was not

inhibitory and did not affect the ability of coexpressed wt HN to direct budding of particles. Taken together, these data correlate exactly with the data obtained from the analysis of recombinant viruses. Furthermore, the VLP studies suggest that multiple viral components, including assembled nucleocapsids, have important roles in the paramyxovirus budding process.

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Biosafety of morbillivirus vectors

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The morbilliviruses form a closely, serologically related group of viruses which are distinguished primarily by their host range. Overall sequence homologies are in the order of 70 % with some genes such as those for the attachment proteins at much lower homologies (~40-50%) than the internal genes that encode the proteins involved in the basic evolving unit of the virus which consists of the genomic negative stranded RNA, the nucleocapsid protein and the replicase (L protein) and its associated phospho-(P) protein. These viruses have been given separate names depending on their host but in essence in any other virus group they would be called by a single name with an indication of the host e.g. human morbillivirus (for measles virus-MV); canine morbillivirus (canine distemper virus-CDV) or bovine morbillivirus (rinderpest virus- RPV). Until the mid 80's four species were known (MV, CDV, RPV and peste-des-petits ruminants virus- PPRV) but in the late 80's and early 90's two new members (phocine distemper virus- PDV from seals) and the cetacaean morbillivirus (of dolphins and porpoises) have been discovered as a result of epizootics involving high levels of mortality in the hosts species. The closeness of the relationships of these viruses necessitates that biosafety considerations on the use of these viruses as vectors cannot be divorced from environmental safety for other hosts. In order to study barriers that may or may not exists to cross-species infection with the various morbilliviruses we are making chimaeric viruses in which the glycoproteins of one morbillivirus are replaced by those of another. So far we have done this using genomes of vaccine viruses as backbones. Our tests system consists of RPV-CDV and CDV-RPV chimaeras because experimental animals are available as natural hosts for these viruses. The results obtained so far indicate that glycoproteins are not the only determinants for replication in specific cells. We now wish to study this in fully virulent viruses, which have been generated for MV [(1)] and for RPV [(2)], but not yet for canine distemper virus. Only in virulent viruses will we be able to assess the true levels of replication of chimaeras and infer the barriers to cross-species infection that may exist.

Recently, Yanagi's group [(3)] reported that SLAM (CD150) was a receptor for all three morbilliviruses that were tested (MV, CDV and RPV), that each only showed a small preference for its own host SLAM and that SLAM homologues of other species could be used as receptors. If this were the case, barriers to cross-species infection would appear to be low.

RPV vectors have been shown to be able to induce an immune response to foreign proteins but these have not yet been used in the field to assess their safety and efficacy [(4)].

MV vectors have been considered for different uses e.g. as delivery vehicles for immunogens; antigens, (anti)-genes, proteins or cytotoxic agents or to act as cytocidal agents themselves. The biosafety considerations associated with each of these uses are potentially different as the infection of various cell types and tissues with the vector may be desirable in one case and not in another. Proper evaluation is hampered by a lack of knowledge of MV pathogenesis, determinants of attenuation and host cell factors that control replication. It appears that vaccine virus strains are able to use CD46 as a receptor to down-regulate its cell surface expression and render the cell sensitive to complement lysis but that wild-type viruses

are not able to do so. However, the use of CD46 [5] and the ability to down regulate the receptor [6] and the interaction with the TLR2 receptor [7] in the innate immune system all appear to be linked to a small number (or single) of amino acid changes in the haemagglutinin. Potential reversion to wild type is thus a distinct possibility particularly as the error rate of the polymerase appears to be similar to that of other RNA viruses [8] though the stability of measles virus genotypes in the field is remarkable [9]. Other receptors may become apparent in the near future since the tissue distribution of CD46 (for vaccines) and SLAM does not adequately explain the pathogenesis of the virus [10].

MV even in attenuated form does give rise to immuno-suppression, all be it very much reduced in comparison to wild type viruses. Viruses that contain inserts that effect immuno-modulation (for example viruses that express cytokines or other known proteins that might affect the Th1/Th2 balance) should be subjected to very stringent safety assessments. Similarly viruses with a different receptor repertoire should be assessed guided by the precautionary principle.

We are in the paradoxical situation that nowadays many more questions about viral pathogenesis and immunology can be posed than can be answered. Hence, the use of viruses as vectors in humans will pose problem for the regulatory authorities. These are conservative as none are willing to take risks with the public's confidence in vaccinology for the sake of vectoroly unless the benefits are very high and clear cut.

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Evolution of cell recognition by viruses and vaccine biosafety

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Because of high mutation rates, RNA viruses replicate as complex distributions of related genomes termed viral quasispecies (Eigen and Biebricher, 1988; Drake and Holland, 1999; Domingo et al 2001). Modifications of cell recognition by viruses is increasingly recognised as one of the biological consequences of viral genetic heterogeneity that can impact viral pathogenesis and disease emergence (Baranowski et al. 2001). We have studied evolution of clonal population of foot-and-mouth disease virus (FMDV) with regard to modifications of host cell tropism. FMDV contains a protruding and mobile loop (G-H loop of VP1) on the capsid surface which includes an Arg-Gly-Asp (RGD) motif for recognition of integrins for cell entry, and also several epitopes for antibody binding (Verdaguer et al.1995; Mateu, 1995). Passage of FMDV clone C-S8C1 resulted in dispensability of the RGD (Martínez et al. 1997) because the virus acquired the capacity to enter cells by at least two different alternative receptors: heparan sulfate and a third, still unidentified, pathway (Baranowski et al. 2000). This expansion of receptor usage resulted in a modification of host cell tropism in that the multiply passaged virus could infect a number of human and monkey cell lines (Baranowski et al. 2000). Chimeric FMDV cDNA constructs documented that the expansion of host cell tropism was associated with several amino acid substitutions at the capsid surface (Baranowski et al. 2000, 2001). Dispensability of the RGD resultd in an expansion of the repertoire of antibody-resistant mutants which now could include replacements within the RGD, thus constituting an example of coevolution of cell tropism and antigenicity (Ruiz-Jarabo et al.,1999). Dispensability of the RGD has permitted also the construction of chimeric FMDVS in which several residues of the G-H loop of VP1 (including the RGD) were replaced by an unrelated FLAG marker. This construct was also infectious in cell culture (Baranowski et al. 2001a). These altered FMDVS are candidate, marked vaccines, and the immune responses they generate in animals are under study.

Many studies with other viruses are in line with the results with FMDV in showing that:(i)One virus may modify its receptor specificity as a result of one or a few amino acid replacements on its surface; (ii) Because frequently there is an overlap between receptor-binding sites and antibody-binding sites on virus surfaces, a coevolution of cell tropism and antigenicity can take place. Flexibility in cell recognition associated with the quasispecies dynamics of RNA viruses may have implications for viral pathogenesis, virus-mediated gene delivery and the emergence of viral disease (Baranowski et al. 2002).

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POSTERS

Contribution of mutagenicity and ribonucleotide pool imbalances in the antiviral effect of ribavirin and mycophenolic acid

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Foot-and-mouth disease virus (FMDV) is an aphthovirus that normally causes a cytolytic infection, and occasionally a persistent infection *in vivo* and *in vitro*. We have studied a persistently infected cell line (R cells) that can be cured of FMDV by treatment with the antiviral agent ribavirin. R cells were subjected to various treatments with ribavirin, mycophenolic acid (MPA) and guanosine. After each treatment, 10 molecular clones of the genes coding for the viral proteins VP1 and 3D were sequenced, amounting to a total of 152860 nucleotides. We found that ribavirin causes a 6- to 8-fold increase in mutation frequency. Treatment with MPA, that has the same decreasing effect on guanine nucleotide pools as ribavirin but no other known effects, resulted in a 4-fold increase in mutation frequency and had an antiviral effect comparable to but weaker than that of ribavirin treatment. Guanosine addition increased intracellular GTP concentrations and decreased the antiviral effects in all treatments, but had only a slight reducing effect in mutation frequencies due to ribavirin. Our results show that the antiviral effect of ribavirin is due both to nucleotide pool imbalances and mutagenesis, the first one also facilitating the latter. These two mechanisms are equally antiviral as themselves, and the combination of the two, possibly together with additional mechanisms, forms the full antiviral effect of ribavirin.

Engineering and stabilization of a full-length infectious cDNA clone of transmissible gastroenteritis coronavirus by insertion of an intron

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Recently, we have engineered a full-length cDNA clone of transmissible gastroenteritis coronavirus (TGEV) in *E. coli* cells as a bacterial artificial chromosome (BAC) (F. Almazán et al., 2000. Proc. Natl. Acad. Sci. USA. 97, 5516-5521). This cDNA facilitates reverse genetics of coronavirus genomes for the study of gene function and their use as expression vectors. Although this strategy led to the rescue of infectious TGEV from the cDNA, a residual toxicity of the cDNA involving sequences at the 3' end of ORF 1a was observed. This instability was detected by replicating the plasmid for more than 80 generations and led us to design a cloning strategy based on the manipulation of a cDNA without the toxic sequence and the insertion of this sequence just before transfection into cells. The stability of the infectious BAC clone in *E. coli* has been considerably improved by the insertion of a 133-nucleotide synthetic intron to disrupt the sequence responsible of the toxicity. The viral RNA was expressed in the cell nucleus under the control of the cytomegalovirus promoter and the intron was efficiently removed by splicing during RNA translocation from the nucleus to the cytoplasm. The intron insertion in two different positions allowed stable plasmid amplification for at least 200 generations and efficiently recovering of infectious TGEV from cells transfected with the modified cDNAs.

In addition, optimization of infectious BAC clone transfection has improved the recovery efficiency of infectious TGEV from cDNA to levels between 107 to 108 pfu/ml.

Amplification of the full-length Feline Calivirus genome by long RT PCR and transcription of infectious RNA directly from the amplicon

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Feline calicivirus (FCV), a major agent of respiratory illness in cats, is classified in the genus Vesivirus of the family Caliciviridae. The virions (30-40 nm in diameter), have a 7.7 kb single-stranded positive sense RNA genome, are non-enveloped and icosahedral. The genomic RNA comprises a 3'poly(A) tail and is linked to a small protein (VPg) covalently attached at the 5´end.

The genetic study of RNA viruses is greatly facilitated by the availability of infectious cDNA clones. However, their construction has often been difficult, involving the cloning and ligation of several cDNA fragments. In this study, we have applied the "long RT-PCR" technique to the synthesis of a full-length DNA amplicon from the RNA of Feline Calicivirus (FCV). Primers were synthesized to match the extremities of the FCV genome. The antisense primer, homologous to the 3' end, was used in both the reverse transcription (RT) and the PCR steps. With these primers, a full-length amplicon (7.7 kb) is obtained. Further, since we engineered a T7 promoter in the sense primer, RNA could be transcribed directly from the amplicon with T7 RNA polymerase. Following transfection of cultured feline kidney cells with the RNA transcripts, infectious virus was recovered displaying the same cytopathic effect of the original virus.

In this communication we report the application of this method for generating cDNAs of FCV genomes to the analysis of the role of the VPg and the poly(A) tail in infectivity.

Role of phosphoprotein P in chandipura virus transcription-replication transition

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Recent development of mononegalovirus based vector as a tool for gene therapy and vaccination emphasized detail understanding of steps involved in viral life cycle. Although macromolecules associated with the two major steps in the viral life cycle, namely transcription and replication, is well documented, the mechanism by which virus switches from transcriptive to replicative phase is still an enigma. We took Chandipura virus, a member of rhabdoviridae family, as our model system to address this problem.

During transcription viral RNA dependant RNA polymerase transcribes the genome to produce leader RNA and five discrete viral mRNA. However, in replication the same polymerase antiterminates at the gene end sequences and eventually synthesize an exact complement of negative sense genome.

This replication intermediate serves as a template for further round of replication to synthesize many more copies of progeny genome molecules.

Our previous studies demonstrated that Phosphoprotein P in its CKII phosphorylated form acts as a transcriptional activator.

We have employed biochemical and cell biological means to further verify role of Phosphoprotein in transcription-replication transition. Our recent observations can be summarized as:

1. Unphosphorylated P binds to leader RNA sequence forming two different complexes whereas phosphorylation totally abrogates its RNA binding ability.
2. Phosphorylation defective mutant of P protein inhibits viral transcription and promotes readthrough at the gene boundaries both *in vitro* and *in vivo*.
3. Cellular factors in conjunction with viral gene products maintain a subset of P protein in unphosphorylated state in the infected cell to support optimum replication.

These observations led us to propose a new model explaining transcription-replication switch in negative stranded RNA viruses.

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Oncoretroviral and lentiviral bicistronic vectors based in ΔhGHR, a biosafe surface labelling molecule for the positive selection of gene-modified transplantable human cells

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We describe a novel selectable marker for retroviral transduction and selection of human cells. The molecule expressed in the cell surface is a truncated version of the human growth hormone receptor (ΔhGHR), capable of ligand (hGH) binding but devoid of the domains involved in signal triggering. We demonstrated that the engineered molecule is stably expressed in the target cells as an inert molecule unable to trigger proliferation, or rescue from apostosis upon ligand binding. This new marker will have a tentative wide application spectrum because hGHR in the adult is only expressed in high levels in liver cells. The ΔhGHR label has a potential improved biosefaty over previously described surface labelling molecules. It belongs to a well characterized hormonal system non-essential in adults and there is extensive clinical experience with hGH administration in humans. This record allow us to anticipate the lack of relevant clinical consequences of massive expression of the transgene as a result of successful replacement of a large tissue with genetically transduced cells. Bicistronic oncoretroviral and lentiviral vectors that use this marker have been engineered for the manipulation of lymphohematopoietic and epidermal stem cells and compared with other conventional cell-surface or cytometric markers. Examples of expression/selection profiles as well as a antibody free method to enrich transduced cells will be discussed. In addition, using bicistronic retroviral vectors encoding distinct cell markers we have established experimental conditions for the efficient single-step multiple transduction of human primary T lymphocytes. Transduction efficiencies were evaluated by flow cytometry, and double- and triple-transduced cells were isolated by fluorescence cell sorting. This multiple transduction procedure could be of interest for the development of new gene therapy approaches that involve the simultaneous expression of more than one gene in a single cell.

Shedding light on coronavirus heterologous gene expression

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Coronaviruses are enveloped, positive-stranded RNA viruses with the largest known RNA genomes. They are considered promising vectors for vaccine development and gene therapy. To study their expression of heterologous genes we inserted, at different positions in the murine coronaviral genome using targeted recombination, luciferase expression cassettes containing coronaviral transcription regulatory sequences (TRSs). Position-dependent expression levels and the viruses' genetic stabilities were investigated. Both the renilla and firefly luciferase genes were expressed to high levels in culture cells, while coronaviral replication was hardly affected. The stability of expression was clearly dependent on the gene inserted. While expression of the renilla gene from different genomic positions was stable, expression of the firefly gene gradually declined as the result of large deletions. This instability was not due to packaging capacity being limited: insertion of the firefly luciferase gene into a genome from which the non-essential genes had been deleted did not improve stability. Luciferase expression levels increased when the renilla gene was inserted closer to the 3'end of the genome.

Insertions generally reduced the expression levels of upstream genes. Rearrangements of the coronaviral gene order demonstrated that it is not the distance per se between the expression cassette and the genomic 3' end that affects the transcription efficiency. The combined results are consistent with coronavirus transcription models in which the transcription levels from upstream TRSs are negatively affected by downstream TRSs. Altogether, our results shed new light on the regulation of coronavirus transcription and on the potential of coronaviruses as multivalent live vaccines.

Generation of yeast narnavirus 23S RNA from a vector

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Yeast 23S RNA virus belongs to the family *Narnaviridae*. Like other members of the family, 23S RNA is a non-infectious, non-encapsidated plus single-stranded RNA virus (1). The complete nucleotide sequence (2891 nt) of 23S RNA genome has been determined (2,3). It only encodes its RNA polymerase p104. Although the virus has no protein capsid, the genomic RNA forms a ribonucleoprotein complex with p104 in a 1:1 stoichiometry and resides in the host cytoplasm (4). We have begun to analyze the RNA polymerase-viral RNA interactions in detail in order to understand the replication mechanism and the virus-host interactions. It is thus essential to develop an *in vivo* launching system in which these interactions are criticallly assesed by reverse genetics. Furthermore, such a system has a great potential for an industrial use since the viral RNA accumulates to amounts equivalent to those of rRNAs under defined conditions, without harming the host. Recently, we have succeded to generate 23S RNA virus in yeast from a cDNA vector containing the complete sequence of 23S RNA genome. The launching required the active RNA polymerase p104 and an *in vivo* processing to produce the precise viral RNA 3' end.

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The use of reverse genetics to study the replicative cycle of human respiratory syncytial virus

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Human respiratory syncytial virus (HRSV) is the main cause of severe lower respiratory infections in infants and very young children. Its genome is a negative single-stranded RNA molecule that codes for, at least, 11 proteins. To study the function of each gene product in the replicative cycle of HRSV we have used the following reverse genetics modalities: i) Individual gene products have been expressed from cloning vectors, purified and their biochemical properties studied. As an example, the fusion (F) protein of HRSV has been expressed from vaccinia recombinants and two different forms of F have been identified that correspond to the pre- and post-activated states of this molecule (1). Triggering of activation requires cleavage of the F polypeptide at two distinct furin sites (2). ii) A minigenome system has been used to analyze the activity of proteins required for transcription and replication of HRSV. For instance, the interaction of the phosphoprotein (P) with the 22k protein was originally analyzed by changes in the electrophoretic mobility after SDS-PAGE and in a two-hybrid assay. The biological significance of this interaction was tested in the minigenome system. A strict correlation was found with mutants of the 22k protein that interacted with P and that were active in the minigenome assay (3). iii) Finally, viruses have been rescued from full-length cDNA copies of the viral genome. Viruses with site-specific mutations have been generated and their phenotypes are being characterized. For example, the infectivity of viruses with deletions in the G protein gene is being evaluated in different host cells. An overview of these studies will be presented, with emphasis in the development of HRSV as a biosafe vector.

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Anti-methylation protection of Rous sarcoma virus-based vectors

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CpG islands are important in protection of adjacent housekeeping genes from de novo DNA methylation and for keeping them in transcriptionally active state. However, little is known about their capacity to protect heterologous genes and assure position-independent transcription of adjacent transgenes or retroviral vectors. To tackle this question, we have used the mouse aprt CpG island to flank a Rous sarcoma virus (RSV)-derived reporter vector and followed the transcriptional activity of integrated vectors. RSV is an avian retrovirus which does not replicate in mammalian cells due to several blocks at all levels of the replication cycle. We show that our RSV-derived reporter proviruses linked to the mouse aprt gene CpG island remain undermethylated and keep its transcriptional activity after stable transfection into both avian and non-permissive mammalian cells. This effect is most likely caused by the protection from de novo methylation provided by the CpG island and not by enhancement of the promoter strength. Our results are consistent with previous finding of CpG islands in proximity to active but not to inactive proviruses and support further investigation of protection the gene transfer vectors from DNA methylation. We started experiments with a CpG island core sequence of some 120 bp in length that is better manageable for modification of replicating retrovirus, not merely a proviral DNA.

Biochemical probing of the FMDV IRES structure

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Initiation of translation driven by internal ribosome entry site (IRES) elements depends upon the structural organization of this cis-acting element. Foot-and-mouth disease (FMDV) IRES is organized in 5 domains numbered 1 to 5, or G to L. Domains 1-2 and 4-5 interact with several cellular proteins, including PTB, eIF4B, eIF3 and eIF4G. Domain 3 (or I) has been shown to interact in a sequence specific, ionic strength, temperature and RNA concentration manner, with distant IRES regions.

Additionally, domain 3 contains two conserved motifs (GNRA and RAAA) located at its apical region which are essential for IRES activity. These motifs are candidates to participate in the organization of IRES structure.

The tertiary structure of IRES elements is still unknown. To elucidate the involvement of the apical region of domain 3 in the organization of the IRES structure, we have studied the structural organization of the FMDV IRES using several complementary approaches. First, a mutational analysis carried out in the FMDV IRES showed that the GNRA motif is not functionally substituted by a UNCG motif, previously reported to stabilize the secondary structure in other RNA molecules. Second, primer extension analysis of chemically modified RNAs was performed in the presence and absence of magnesium ions, known to stabilize the formation of tertiary interactions. Third, the interpretation of results from DMS, kethoxal and DEPC modification assays has been aided of primer extension analysis of AMT-psoralen induced crosslink, as well as enzymatic probing.

Comparison of results obtained from structural probing of the wild type RNA with mutants in the GNRA structural motif allowed us to identify residues contributing to tertiary interactions within the central domain of the FMDV IRES. Thus, sequences in the apical region of domain 3 play a crucial role in the organization of IRES structure, with important effects on activity. Consistently, domains 4-5 are not sufficient to support internal translation, albeit they are responsible for eIF4G interaction.

In band-shift assays, the apical region 3ABC interacts with domain 3 and its proximal region, STEM3, four times more efficiently than with domain 1-2. We have found that the RAAA motif is essential for the maintenance of RNA structure, as its disruption leads to rearrangement of the FMDV IRES structural organization affecting intradomain interactions. Moreover, it also mediates RNA-protein interactions, as the pattern of RNA-binding proteins was modified by the CGCCC substitution in the RAAA motif. We have identified three proteins, p39, p34 and p29, that interact specifically with domain 3. Two of them, p39 and p29, recognize discontinuous structural motifs in domain 3. While the apical part by itself does not interact with any of these proteins, the proximal part, STEM3, is efficiently recognized by p34. Remarkably, binding of p29 requires the AAAA sequence at the RAAA motif, together with the integrity of domain 3, strongly supporting the existence of RNA-RNA interactions mediated by the RAAA motif.

Use of Plum Pox Potyvirus (PPV) based vectors for the production of immunopeptides in plants

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Plum pox potivirus (PPV) is a positive-strand RNA virus that infects trees of the Prunus genus and different herbaceous hosts. Its genome consists of a single 10kb molecule that codes for a polyprotein that is processed by three virus-encoded proteinases. The full-length cDNA of the PPV genomic RNA has been engineered to obtain several vectors that have been used for the expression of antigenic peptides and full-length proteins.

Two different vectors based on PPV have been proved to produce large amounts of foreign proteins in plants. The insertion sites of these vectors are placed near the N-terminal end (between P1-HC) or near the C-terminal end (between NIb-CP) of the polyprotein and are flanked by restriction sites that facilitate cloning. Specific viral proteases recognition sites that will release the foreign peptide from the virus polyprotein flank the insert sequence. A PPV-derived vector is now available that include both the P1-HC and NIb-CP insertion sites in a single cDNA molecule. To improve the use of PPV for the expression of extracellular-targeted proteins, as the antibody chains, a new vector, in which the insertion site will be at the end of the polyprotein, is under construction.

We are using these PPV-based vectors for the production in plants of immuno-therapeutic antibodies against an important enteric disease of farm animals, caused by coronaviruses (TGEV). Antibody sequences encoding either the separate L and H chains of the recombinant full-length IgA molecules or the single chains of specific SIPs (scFv), able to neutralize TGEV, have been inserted into the expression vectors, and the efficiency of these expression systems to produce the different immunopeptides in plants is currently under analysis.

To facilitate the use of the different vectors, the full-length cDNA has been introduced into a binary vector derived from *Agrobacterium tumefaciens*. *Agrobacterium* transfected with the transforming vector, in which PPV-containing the foreign sequence has been included, is then used for the expression of the immunopeptide in plants. In this way, both the advantages of the virus for the high level expression of proteins and the high efficiency of *Agrobacterium* to introduce foreign DNA in plants are combined. This agro-inoculation of PPV-constructs has proved to work for the expression of the antibodies or SIPs in plants.

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Functional studies of PA subunit of influenza virus polymerase using rescued viruses

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Influenza virus polymerase is a complex of three protein subunits; PB1, PB2 and PA. This complex carries out both transcription and replication of the viral RNA. PA subunit is a phosphorylated protein that induces its own proteolysis and that of coexpressed proteins. Potential casein kinase II phosphorylation sites were mutated in PA protein. Mutations affecting position 157 produced an underphosphorylated protein and almost completely eliminated proteolysis induction, whereas a mutation at position 162 produced a modest decrease on proteolysis induction. Viral-like RNPs were reconstituted in vivo with these PA mutants. They resulted defective in viral RNA replication to the same extent they were deficient on proteolysis induction. Influenza viruses were rescued with PAwt and with PA157 and PA162 mutations using a plasmid cDNA transfection system. The phenotype of these viruses was analysed in culture cells and mice. PA157 mutant virus showed a delay on viral protein synthesis and PA nuclear transport. The rate of protein synthesis of this mutant was moderately decreased as compared with rescued wild type virus and gave rise to almost normal protein accumulation levels. The analysis of cRNA and vRNA synthesis using real time RT-PCR indicated that PA162 virus was similar than rescued PAwt whereas rescued PA157 virus showed a decreased synthesis of both types of RNAs. The study of the rescued viruses on animal models showed that PA157 virus had a reduced pathogenicity in mice.

Development of alphavirus vectors for gene therapy: antitumoral effect of recombinant SFV viral particles expressing cytokines

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Alphaviruses are a group of enveloped viruses which contain a single positive strand RNA genome. Alphaviral vectors consist of a defective viral genome in which the genes coding for the structural proteins have been substituted by the foreign gene to be expressed. Recombinant RNA genomes can be synthesized *in vitro* and packaged into viral particles by cotransfection of cells with a helper RNA providing the structural genes of the virus in trans. In the case of Semliki Forest virus (SFV) a two-helper RNA system has been developed in which the capsid and the spikes proteins are expressed from two independent RNAs, reducing the generation of wild type virus through recombination to undetectable levels. In this system high expression levels of the spikes are achieved by fusing these proteins to the first 34 aminoacids of the capsid which functions as a translation enhancer. The enhancer is removed from the spikes by the 2A autoprotease from FMDV, used as a linker between the enhancer and the spikes.

In this study we have produced SFV viral particles encoding murine interleukin-12 gene (SFV IL-12) with the two-helper RNA system, and tested their ability to eliminate tumoral nodules upon intratumoral injection in a mouse model of colon carcinoma. Correct expression of the transgene was tested in supernatants of infected BHK cells by metabolic labeling with S^{35}-methionine and western-blot. Recombinant IL-12 produced by SFV IL-12 showed to be biologically active *in vitro*, inducing the production of IFN-γ in murine splenocytes at levels of 4.5 ng/mL. Single tumor nodules were implanted in C57BL/6 mice by subcutaneous injection of MC38 colon carcinoma cells. When tumor size was 5 mm in diameter, nodules were treated with a single intratumoral injection of SFV IL-12, SFV LacZ or saline as control. Treatment with SFV IL-12 resulted in a significant inhibition of tumor growth in a dose-dependent manner. With a dose of 10^8 viral particles of SFV-IL-12, 80% of treated mice experienced a complete tumor regression with long-term tumor-free survival. All mice that rejected the tumors showed a specific protection against tumor rechallenge, indicating the induction of immunological memory. These data show that alphavirus vectors can be useful to enhance antitumor immunity by local delivery of cytokines, such as IL-12.

Engineering transmissible gastroenteritis virus genome as an expression vector provides novel information about the mechanism of transcription

Isabel Sola, Javier Ortego, Sara Alonso, Sonia Zúñiga, Cristina Riquelme and Luis Enjuanes

Basic knowledge on the molecular basis of coronavirus: (i) transcription; (ii) essential genes; (iii) virulence; and (iv) packaging cell lines has been applied to the engineering of a coronavirus vector. The genome of the transmissible gastroenteritis coronavirus (TGEV) has been engineered as an expression vector using an infectious cDNA (4). The vector led to the efficient (>40 µg/106 cells) and stable (>20 passages) expression of a heterologous gene, driven by the transcription regulatory sequences (TRS) (1, 2) of the ORF 3a inserted in the site previously occupied by the non-essential ORFs 3a and 3b. Interestingly, a specific lactogenic immune response against the heterologous protein has been elicited in sows and their progeny. The engineering of an additional insertion site for the heterologous gene between the N and 7 viral genes led to instability and to a new genetic organization of the 3' end of recombinant viruses. As a consequence, major species of subgenomic mRNAs (sgmRNA) were generated from TRSs with non-canonical core sequences (CS). Similar results have been obtained from the systematic mutagenesis study of ORF 3a CS, indicating that extension of the complementarity between the CS flanking sequences and the viral leader RNA was associated to the transcriptional activation of non-canonical CSs. Replication-competent propagation-deficient TGEV derived virus vectors, that are deficient in the essential gene E have been developed by complementation within E+ packaging cell lines. Cell lines transiently expressing TGEV E protein from the non-cytopathic Sindbis virus replicon pSINrep21 or stably expressing the E gene under the CMV promoter have been established (3).

Recombinant TGEV deficient in the non-essential 3a and 3b genes, and the essential E gene (rTGEV-*3ab*E) was successfully rescued in these cell lines. rTGEV-*3ab*E reached high titers (1x107 pfu/ml) in cells transiently expressing the TGEV E protein and in packaging cell lines stably expressing protein E (5x105 pfu/ml). The availability of packaging cell lines will significantly facilitate the production of safe TGEV derived vectors for vaccination and, possibly, gene therapy.

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Recombinant Newcastle Disease Virus (NDV) as a Vaccine vector

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Newcastle disease virus (NDV) is a member of the *Rubulavirus genus* in the *Paramyxoviridae* family and is categorized into three pathotypes depending on the severity of the disease that it causes in birds: lentogenic, mesogenic, or velogenic. NDV has a negative sense single stranded RNA genome of 15,186 nucleotides in lenght. The genome codes for six proteins, the nucleoprotein (NP), phosphoprotein (P), matrix (M) proten, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein and the RNA dependent RNA polymmerase (L). The surface glycoproteins of the virus are the HN, which is viral receptor binding protein, and F having virus-cell membrane fusion activity. The F protein is activated upon proteolytic cleavage of a precursor (F0) to produce disulfide-linked F1 and F2 polypeptides.

The ability to genetically engineer negative-strand RNA viruses has led to extraordinary advances in understanding their biology. An important application of reverse-genetic techniques is the generation of recombinant viruses for use as vaccine vectors. One advantage of RNA virus vectors-based vaccines, with the exception of retroviruses, is the impossibility of chromosomal integration of the viral genome into the host cell, since these viruses, do not have a DNA step in their replication cycle.

In our work, we apply a reverse genetics approach to rescue a recombinant NDV expressing a foreing protein. A complete cDNA clone of the NDV avirulent strain Hitchner B1 (ATCC VR108) was constructed and infectious recombinant virus (rNDV/B1) was generated. Also, an influenza virus hemagglutinin (HA) gene was inserted into the recombinant virus between P and M genes. The rescued virus (rNDV/B1-HA) efficiently expesses the HA protein on the cell surface of infected cells and the HA was also incorporated into NDV particles. rNDV/B1-HA virus replicated slightly slower and to lower titers than rNDV/B1 in embryonated chicken eggs and showed increased attenuation properties when compared to the parent virus. rNDV/B1-HA virus was non-pathogenic in mice but induced a strong humoral antibody response against influenza virus as well as against NDV. Finally, this recombinant virus provided complete protection against an otherwise lethal challenge with influenza virus, demonstrating the potential of recombinant NDV as a vaccine vector.

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Lentiviruses as vectors for gene repair

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Genetic diseases are caused by mutations in genes whose function is required for the normal development or stasis of the human body. With few exceptions, current treatments for these diseases are palliative, not curative. Gene therapy by augmentation has recently scored successes in haematopoietic diseases, and it is likely that these will be followed by trials in other disorders where animal models are showing promise. However, gene augmentation cannot correct dominant diseases. Thus, alternative strategies are required to address such disorders, and one of them is gene repair by homologous recombination (gene targeting). This would be an ideal form of gene therapy because it allows permanent correction of the damaged gene. Homologous recombination can repair point mutations, small inversions and deletions of up to a few kilobases and this could lead to correction of multiple diseases, both dominant and recessive. Unfortunately, in practice gene repair is hindered by the low frequency of homologous recombination. Several studies have pointed to the fact that the method of cell transfection impacts greatly on the frequency of gene targeting. We have reasoned that the use of virus vectors allowing optimum transduction of the target cell of interest might affect positively the frequency of homologous recombination. We are currently developing an *ex vivo* system based on the use of integration-deficient HIV vectors for homologous recombination in haematopoietic cells, using *HPRT* as a model target gene. Results will be presented illustrating the efficiency and fidelity of the system.

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