Instituto Juan March
de Estudios e Investigaciones

25 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Genetic Recombination and Defective Interfering Particles in RNA Viruses

Organized by

J. J. Bujarski, S. Schlesinger and J. Romero

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GENETIC RECOMBINATION AND DEFECTIVE INTERFERING PARTICLES IN RNA VIRUSES

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Closing Remarks.

INTRODUCTION

S. Schlesinger

The topics of this meeting- genetic recombination and defective interfering particles in RNA viruses - are subjects that actually have a long history in the study of these viruses. The first report of defective interfering particles is attributed to Von Magnus (1) who, over 40 years ago, reported that undiluted passaging of influenza virus led to the accumulation of noninfectious particles. A more precise characterization of what constitutes defective interfering particles appeared in an article by Huang and Baltimore in which they defined the characteristics of defective viral particles and suggested that "DI particles may be important determinants of the course of acute, self-limiting viral infections and of persistent, slowly progressing viral diseases. In addition many host reactions may alter the production of DI particles and thus influence the outcome of viral infections" (2).

The first report of genetic recombination between animal viruses is more recent and credit is given to George Hirst who in 1962 described recombination with Newcastle disease virus, poliovirus and influenza virus (3). It is important to realize that at that time the nature of the genomes of these viruses was known to be RNA, but the segmented nature of the influenza virus genome and the concept of positive and negative strand RNA genomes had not yet been established. The high level of recombination detected with influenza virus did suggest to Hirst that the RNA of this virus was fragmented. (It is likely that the recombination detected with Newcastle disease virus was due to aggregated virus particles.) It wasn't until the molecular proof for genetic recombination between polioviruses and between foot and mouth disease viruses was demonstrated that the phenomenon of genetic recombination between RNA viruses began to impinge on our thinking about its significance in viral evolution (4).

Mechanisms of recombination between RNA viruses have not been investigated in detail, but template-switching is considered to be the most likely means by which genetic exchange between nonsegmented RNA genomes can occur (5). Distinctions between homologous and nonhomologous recombination between RNA genomes have been made in the literature (6) and at this meeting. (See abstract by Bujarski.) Homologous recombination occurs between related parental RNAs at the same genetic loci but it may or may not be precise. There is as yet no evidence for any enzymatic mechanism to achieve precision. In some crosses a precise crossover may be the only

means of maintaining a functional gene and it would be the only way to obtain viable recombinant progeny. Homologous recombination which is not precise has been termed aberrant or illegitimate homologous recombination. Recombination that occurs between two RNAs which do not show any obvious homology has been referred to as nonhomologous and also as illegitimate. In discussions of recombination between RNA molecules, it is essential to distinguish between mechanisms that require homologous domains and may or may not be precise and those that do not require sequence homology.

The subjects of defective interfering particles and genetic recombination in RNA viruses continue to be of interest and importance. Defective viral RNAs are serving as important tools for defining cis-acting elements in RNA genomes - a topic that receives much attention at this meeting. In addition, a role for defective RNAs in pathogenesis is discussed particularly in the studies with bovine viral diarrhea virus and vesicular stomatitis virus. (See abstracts by Meyers et al. and Huang et al.) Genetic recombination between RNA viruses is much more common than previously appreciated and most likely is an important factor in the formation of new viral species and in RNA genome diversity. Defective interfering particles and RNA recombination can have practical applications as well. The generation of transgenic plants is becoming a reality; the design of virus vectors for gene therapy and drug administration is no longer a remote possibility. The potential for benefit as well as possible risks may depend on our understanding of the propensity of RNA genomes to mutate and generate deletions and the ability of these genomes to undergo recombination.

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I. RNA and Recombination

HIGH FREQUENCY OF DELETIONS AND INSERTIONS DURING REPRODUCTION OF THE PICORNAVIRUS GENOMES

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Reproduction of picornaviruses under optimal (standard) conditions is rarely, if at all, accompanied by obvious rearrangements of the RNA genome (deletions, insertions, duplications). Such stability could in principle have been caused by either the intrinsic accuracy of the viral RNA-dependent RNA polymerases or strong selective pressure against the mutants with the rearranged genomes. When, however, conditions are created under which the acquisition of deletions or insertions is likely to confer a selective advantage, numerous and diverse rearrangements of the viral genome could readily be observed.

For example, engineered poliovirus mutants with an altered spacing between two important cis-acting translational control elements in the 5'-untranslated region (5UTR) of the viral RNA [an oligopyrimidine (box A) and a cryptic AUG-586] generally exhibited a decrease in the growth potential caused by deficient initiation of translation. Such mutants, however, were genetically unstable and produced, upon *in vitro* cultivation, a variety of (pseudo)revertants with at least partially restored reproductive capacities and *in vitro* RNA template activities. A great proportion of these revertants possessed a rearranged genome. The revertant RNAs demonstrated an array of insertions or deletions, all of which tended to restore the wild type box A/AUG distance.

Similarly, engineered 5UTR mutants of Theiler's murine encephalomyelitis virus (TMEV) with an attenuated phenotype might generate, upon reproduction in the mice CNS, virulent variants with diverse deletions.

These data strongly suggest that the picornaviral RNA polymerases are not as processive as it could be thought. A likely explanation for the rearrangements may consist in polymerase "jumping", together with the nascent strand, from one site of a template to another site on the same or another template. The availability of a collection of revertants with differently rearranged genomes provides the basis for a discussion on how the donor and acceptor sites are chosen during such jumps.

FITNESS DECREASE AND INTERFERENCE BY VIRAL QUASISPECIES EVOLVED UNDER STRONG IMMUNE SELECTION.

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The degree of adaptation of a virus to a given environment can be quantitated by its replication ability in competition with some reference, marked virus. The resulting parameter has been termed the relative fitness of a virus in that environment.

The high mutation rates operating during RNA virus replication dictate that the replicating ensemble is not a genetically defined structure but a mutant distribution termed quasispecies (Eigen and Biebricher, 1988). Early work with phage Qβ showed that individual components of the mutant spectrum of a quasispecies manifested decreased fitness relative to the average, parental phage population (Domingo *et al.*, 1978). A gradual decrease of fitness upon repeated plaque-to-plaque isolations (repeated bottleneck events) has been documented for phage Ø6 (Chao, 1990), and for vesicular stomatitis virus (Duarte *et al.*, 1992; Clarke *et al.* 1993). This process of accumulation of deleterious mutations is known as "Muller's ratchet" (Muller, 1964).

We have recently described that antigenic variants of foot-and-mouth disease virus (FMDV), selected with neutralizing polyclonal antibodies directed to a major antigenic site of the virus, may show profound fitness losses (Borrego *et al.*, 1993). However, high fitness clones could be isolated upon dilution and plating of the low fitness populations, suggesting that the latter interfere with normal viruses immersed in the debilitated quasispecies. Fitness loss may be due to the need to cope with the strong selective constraint imposed by antibodies on an icosahedral capsid which shows limited tolerance to amino acid replacements due to structural constraints (Martínez *et al.* 1992; Takeda *et al.* 1994).

We have examined the ability of a debilitated population to interfere either with a replication-competent clone isolated from the same population, or with other FMDVs, or with unrelated RNA viruses. A strong interference was exerted on FMDVs of different serotypes and, to a lesser extent, on

encephalomyocarditis virus and vesicular stomatitis virus. No significant interference was observed on the replication of Semliki forest virus.

The main conclusions of these studies are: (i) The quasispecies population population structure favours frequent fitness changes in RNA viruses. (ii) Strong selection for one genetic trait may lead to virus populations with decreased fitness. (iii) FMDVs selected with polyclonal antibodies directed to a specific antigenic site show poor replication ability and interference with homologous and heterologous RNA viruses.

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FURTHER IDENTIFICATION OF *cis*--ACTING SEQUENCES IN THE GENOME OF SINDBIS VIRUS

Sondra Schlesinger, Barbara Weiss and Ilya Frolov , Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110-1093

My talk will cover our studies on two different aspects of the replication of Sindbis virus: encapsidation signals and how the development of Sindbis virus replicons for expressing heterologous proteins led to the identification of *cis*-acting sequences that affect translation of the capsid protein. We have been investigating the interaction of the capsid protein with Sindbis virus RNA to define the encapsidation signal and had previously identified a 570nt fragment (nt 684-1253) from the 12 kb genome that binds to the viral capsid protein with specificity and is required for packaging of Sindbis virus defective interfering RNAs (1). We have now shown that the capsid binding activity resides in a highly structured 132nt fragment (nt 945-1076) (2). Deletions in the 132-mer all led to a reduction in binding, but the binding of a 5' 67-mer was enhanced by the addition of nonspecific flanking sequences. This latter result suggests that the stability of a particular structure within the 132nt sequence may be important for capsid recognition.

In the second part of the talk I will describe our recent work with Sindbis virus replicons. These RNAs are self-replicating, but lack some or all of the structural protein genes. The RNAs can be packaged into virion particles by co-transfection of cells with defective RNAs that express the structural proteins from the subgenomic mRNA (3). A variety of replicons were tested for their ability to inhibit host cell protein synthesis and cause cytopathic effects (CPE) in infected BHK cells. Our results show that the early steps in viral gene expression are the ones required for the inhibition of host cell protein synthesis in BHK cells (4). In contrast, Sindbis virus and Sindbis virus replicons were clearly distinguished by the time at which CPE became evident. Viruses that synthesized high levels of the two membrane glycoproteins on the surface of the infected cells caused a rapid (12-16 h postinfection) appearance of CPE and those that did not synthesize the glycoprotein spikes showed delayed (30-40 h) CPE. In these studies we have also identified sequences near the 5' terminus of the subgenomic RNA which affect the ability of this RNA to be translated.

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Studies on Bromovirus RNA Replication and Natural and Engineered Bromovirus Recombinants

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The bromoviruses are icosahedral viruses with a three-component, positive-strand RNA genome. Bromoviruses comprise one genus within the alphavirus-like superfamily, a large collection of animal and plant viruses that share conservation in a cluster of RNA replication gene elements, but differ in the number and nature of additional genes, genome organization, virion morphology, host range and other characteristics. Under any divergent model for the evolution of this superfamily, recombination must have played a major role in rearranging conserved gene segments and in recruiting new genes and regulatory elements from host cells or other viruses. These evolutionary possibilities are supported by experiments showing that defective bromovirus RNAs can obtain required genes from co-infecting viral RNAs by natural recombination and that fully functional, hybrid viruses can be created by the engineered assembly of new combinations of genes from such divergent sources as an icosahedral, tripartite bromovirus and a rod-shaped, single component tobamovirus (1).

Bromovirus RNA recombination occurs at a sufficiently high level that, in single cycle infections of transfected cells, the de novo generation and replication of recombinant RNAs can be observed directly by northern blotting, without the use of polymerase chain reaction amplification (2). While other recombination mechanisms may also exist, a range of experimental results suggest that many RNA recombination events may occur as a byproduct of RNA replication, presumably through template switching. Studies of bromovirus RNA synthesis and the machinery that carries it out should thus be instrumental in understanding RNA recombination as well as other central aspects of infection.

Bromovirus RNA replication is carried out by a membrane-associated complex of viral and apparently also cellular factors. Two large bromovirus-encoded proteins are required for RNA replication: the 1a protein (109 kDa) contains a C-terminal helicase-like domain and an N-terminal m^7G-methyltransferase-like domain implicated in viral RNA capping, while the 2a protein (94 kDa) contains a central polymerase-like domain. In vitro, the helicase-like domain of 1a and N-terminal sequences of 2a interact, leading to the formation of a specific 1a-2a complex that may coordinate polymerase, helicase, and capping functions in RNA synthesis (3). In vivo genetic studies show that 1a-2a interaction is not an in vitro artifact, and that compatible interaction between 1a and 2a is essential for synthesis of positive-strand genomic RNA and transcription of subgenomic mRNA (4). The selective RNA synthesis defects associated with heterologous combinations of 1a and 2a genes from different bromoviruses show that at least some aspects of 1a-2a interaction that are required for positive-strand genomic and subgenomic RNA synthesis are distinct from any requirements for negative-strand RNA synthesis. However, the results also suggest that some form of 1a-2a interaction is required for negative-strand RNA synthesis.

The bromovirus RNA replication complex also is associated with host membranes and host proteins. Immunological and functional analysis of RNA-dependent RNA polymerase extracts from bromovirus-infected cells (5), the repeated occurrence of conserved cellular RNA sequence motifs within the cis-acting replication signals of bromovirus RNAs, and other results implicate host factors as active participants in bromovirus RNA replication. Further studies of host factors in bromovirus replication are being assisted by the recent observation that bromovirus RNAs can replicate in yeast (6).

A variety of genetic and biochemical evidence suggests that many if not most of the protein factors in the bromovirus RNA replication complex interact with the viral RNA. The probable existence of multiple RNA binding sites in this multi-protein RNA replication complex may contribute to template switching in RNA recombination by allowing both donor and acceptor templates to bind simultaneously to adjacent components of the same RNA replication complex, facilitating template exchange.

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RECOMBINATION AND POLYMERASE ERROR CONTRIBUTE TO SEQUENCE RESTORATION IN BMV RNA.

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Both homologous and non-homologous recombination have been reported (Bujarski and Kaesberg, 1986; Rao et al., 1990) for the genomic RNAs of brome mosaic virus (BMV). Sequence changes in the viral genome also occur as a result of the relatively high error rate of RNA-dependent RNA polymerase resulting from the lack of a proof-reading function.

Although the 3' 200 nt of each of the genomic RNAs of BMV are similar, they contain a limited number of site-specific substitutions that are characteristic for each RNA. Although the 3' end of RNA-3 exhibits the strongest (-)-strand promoter activity in barley transfection experiments, progeny levels from inocula containing RNAs-1, -2 and -3 all bearing the RNA-3 3' end were only 35% of wild type (Duggal et al., 1992). When all three genomic RNAs bore the RNA-1 sequence, progeny accumulation was reduced to less than 15% of wild type. Experiments are underway to determine if selection pressure for the specific nucleotide substitutions in the 3' sequences is sufficient to regain the characteristic wild type sequences and, if so, how rapidly the discrete sequences are restored.

To explore the relative contributions of polymerase error and recombination mechanisms to sequence alteration, we (Rao and Hall, 1993) have studied four mutants of BMV RNAs -2 and -3 that greatly debilitate (-) strand replication as a result of single (GUA) or double (CAA, GAA, UAA) nucleotide substitutions in a non-structured (loop) element of the 3' region that was thought to represent a tyrosine tRNA (AUA) anticodon (Dreher and Hall, 1988). The presence of additional, putatively neutral, base changes both upstream and downstream of the mutated sequence provided markers to detect whether sequence changes in the progeny resulted from recombination or polymerase error.

When BMV RNA-3 bearing the "anticodon" mutations was present in the inoculum, reversion to the wild type AUA sequence occurred via both polymerase error and recombination. A pseudorevertant sequence (AAA) was obtained relatively frequently as a result of polymerase error, suggesting that this sequence provided enhanced function over the supplied mutant sequences. For inoculations containing the RNA-2 mutants, the AUA sequence was shown to be restored by recombination processes in several lesions (in *Chenopodium hybridum*). Only one correction by polymerase error was observed, and this yielded the AAA pseudorevertant sequence. No revertants were obtained in barley, the systemic host, using RNA-2 inocula bearing the anticodon mutations. However, a recombination event restored the wild type AUA sequence when RNA-2 bearing the AAA mutation was inoculated on barley in combination with wild type RNAs -1 and -3.

It appears that, when the "anticodon" mutations were present on RNA-3, sufficient replication occurred to permit sequence amelioration (to AAA) or reversion (to AUA) in both the local lesion and systemic hosts. In this circumstance, polymerase error was seen to be the more frequent sequence restoration system, although recombination also provided a functional correction mechanism. RNA-2 encodes a polypeptide vital for viral replication, and mutations introduced into this RNA greatly debilitated replication, reducing opportunities for sequence correction by polymerase error. Indeed, the only inoculum combination sufficiently infectious to support any sequence restoration in the systemic host was that containing the AAA sequence. In this situation, recombination was the only repair process observed.

Findings from the above experiments support the concept that RNA viruses frequently exist as quasispecies (Domingo et al., 1978; Eigen and Biebricher, 1988). They confirmed that both polymerase error and recombination events occur routinely and that both provide powerful strategies for restoring the consensus genome.

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II. Defective Interfering I

CORONAVIRUS DI RNAs: A TOOL TO STUDY REPLICATION, TRANSCRIPTION AND RECOMBINATION, Robbert G. van der Most, Guido van Marle, Willem Luytjes and Willy J.M. Spaan, department of Virology, Institute of Medical Microbiology, Faculty of medicine, Leiden University, PO Box 320, 2300 AH Leiden, The Netherlands.

Coronavirus are a group of enveloped viruses that cause infections in man, other mammals and birds. The viral genome is a single-stranded (+)-sense RNA molecule of exceptional length, e.g. the mouse hepatitis virus (MHV) has a genome of 32 kb. Coronaviruses have attracted attention because of their unusual replication strategy which involves leader primed transcription of subgenomic mRNAs, subgenomic-size replicative forms and homologous RNA recombination.

Homologous RNA recombination in coronavirus infected cells presumably occurs via template switching ('copy-choice'). As proposed for picornavirus RNA recombination, polymerase complexes containing nascent RNA dissociate from their original template, and re-initiate transcription on a different template. Such polymerase jumping is probably not only responsible for the high frequency homologous RNA recombination, but also for the formation of defective genomes as a result of non-homologous recombination events, and for the synthesis of leader containing subgenomic RNAs. In contrast to both homologous recombination and defective genome formation which are not sequence specific, subgenomic mRNA synthesis can be explained as the result of a sequence-specific polymerase jump. Current data indicate that a leader RNA first binds to a sequence motif of at least 9 nucleotides (nt), the promoter, located in the intergenic regions on the negative stranded template and then re-initiates transcription. Basepairing of the leader RNA to the promoter is possible because a sequence complementary to the promoter is present near the 3' end of the leader RNA. Possibly, the promoter functions not only as an acceptor site for leader transcripts, but also as a polymerase recognition signal.

The high frequency homologous RNA recombination of MHV has been exploited as a means to generate defined genetic changes in the viral genome. Although a full-length infectious cDNA clone would provide the most powerful tool to study the recombination and transcription of coronaviruses, the extreme length of the genome poses an obvious technical problem. The alternative strategy involves recombination between a synthetic DI RNA and the standard virus genome. For this purpose, we have developed an RNA vector based upon MHV defective interfering (DI) particles (Van der Most *et al.*, J. Virol., 1991, *65*, 3219-3226). The MHV A59 RNA vector MIDI consists of three genome fragments, derived from the replicase open reading frames (ORF) 1a and 1b, and the nucleocapsid (N) ORF. During generation of the DI genome these fragments have been fused in frame, forming a single "full-length" ORF. RNA transcribed from a full-length DI cDNA clone is efficiently replicated in MHV infected cells. It was shown that marker mutations introduced into the DI RNA are replaced by wild type sequences during replication in MHV infected cells. More importantly however, these mutations were incorporated into the genome of MHV-A59. The potential of DI-directed mutagenesis was illustrated by the rescue of the temperature-sensitive mutant Albany-4 (Van der Most *et al.*, Nucl. Acids Res. 1992, 20, 3375-3381). Currently, we explore the possibilities to introduce mutant spike and heterologous genes in the coronavirus genome by means of homologous recombination. Progress will be discussed.

The recombination studies have also revealed that the MHV DI RNAs require a functional open reading frame (ORF) for efficient propagation, i.e. DI RNAs carrying nonsense mutations have a strong selective disadvantage and escape mutants that have restored the ORF emerge during propagation of such DI RNAs (De Groot *et al.*, J. Virol. 1992, *66*, 5898-5905). To determine whether the DI polypeptide or translation perse is involved we designed additional DIs containing deletions, insertions and 3' non-coding regions of different lengths. Analysis of these DIs in a transfection/propagation experiment, yielded the following results. (i) Truncation of the ORF at the 1b/N junction alone did not significantly reduce the fitness of DI RNA. Thus, translation of N sequences was not required for efficient propagation in these DIs. (ii) Truncation of the ORF at the 1a/1b junction, preventing the translation of 1b and N sequences, was not lethal but consistently led to a lower accumulation of DI RNA. In addition, escape mutants containing the full length ORF were detected, suggesting that translation of ORF1b sequences was still required despite the deletion. (iii)

Introduction of a large in frame insertion upstream of the stopcodon at the 1a/1b junction resulted in a DI mutant that was efficiently propagated.

From these data we conclude that the relative portion of the DI RNA that is translated determines its fitness.

To unravel the mechanism of leader primed transcription, we have inserted a double-stranded oligonucleotide comprising the mRNA7 promoter into the DI cDNA clone. Transcripts from this construct contain the RNA promoter downstream of the ORF. Following RNA transfection into MHV-infected cells, this DI RNA was replicated efficiently and a 0.4 kb subgenomic RNA was synthesized. An extensive mutational analysis of the promoter resulted in the following conclusions: (i) The extent of basepairing between the leader and the promoter is not the sole determinant for transcription efficiency, (ii) The 10 nt RNA 3 promoter is much more vulnarable to mutations then the RNA 7 promoter. (iii) Promoter recognition by the transcriptase is not mediated by base pairing alone. (iv) Nucleotide substitutions at the 3' end of the promoter were copied more often into the subgenomic DI transcripts then mutations at the 5' end.
A new model for the leader primed transcription will be presented.

Requirement for translation of an internal region of the poliovirus genome in cis; possible effects on DI particle maintenance in positive-strand RNA viruses.

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Poliovirus, like many other positive-strand RNA viruses, replicates in the cytoplasm of its host cells. The possibilities for interactions between translation and genome replication are therefore comparable to those in prokaryotic cells, where there are several examples of coupling between translation and DNA synthesis. For poliovirus and several other positive-strand RNA viruses, the hypothesis that translation of a particular RNA genome is required in cis in order for that RNA genome to replicate has been supported by experiments in several laboratories. Such a requirement, if true, could provide a powerful defense for these RNA viruses against the accumulation of mutant genomes.

To test this hypothesis, nonsense and frameshift mutations have been introduced into the coding regions of RNA genomes. However, these mutations also introduce changes into the RNA sequence that can themselves result in cis-dominant mutant phenotypes, making it difficult to conclude that it was the effect of the mutations on translation, and not on RNA sequence or structure, that resulted in the inability of the mutated RNAs to replicate.

To demonstrate unambigously the existence of a requirement for translation of the poliovirus genome in cis, we tested the replication of poliovirus genomes in the presence and absence of translation without altering their RNA sequence. By comparing the replication of poliovirus RNA genomes containing amber mutations in amber-suppressing (Sedivy et al., Cell 50, 379-389, 1987) and nonsupressing cell lines, we could study the identical genomes in the presence and absence of translation in cis. We identified amber mutants that can replicate in amber-suppressing cells, showing they had no cis-dominant RNA defects. However, in nonsuppressing cells, even in the presence of all viral proteins provided in trans, mutant viruses that could not translate the terminal 3789 nucleotides of the coding region could not replicate, whereas mutant viruses that could translate the entire poliovirus genome except the terminal 1300 nucleotides could replicate efficiently. A region of the poliovirus genome termed the CTR (cis-translation required) region was thus identified. RNase protection experiments demonstrated that viral RNA synthesis is the process that requires translation in cis.

This requirement for translation in cis results either from a requirement for cis-acting proteins encoded by the CTR or for a requirement for ribosomal passage through the CTR. Regardless of the mechanism, a requirement for translation of RNA viruses in cis can serve as a late proofreading mechanism, allowing selection against RNA genomes with mutations that alter translational reading frame. We suggest that this can provide a mechanism by which many positive-strand RNA viruses are less plagued by defective genomes than their negative-strand counterparts.

EVOLUTION OF TRANSMISSIBLE GASTROENTERITIS CORONAVIRUSES: RECOMBINATION AND GENERATION OF DEFECTIVE GENOMES

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Transmissible gastroenteritis coronavirus (TGEV) causes an enteric disease associated with high mortality in neonates. Non-pathogenic respiratory isolates (PRCV) derived from TGEV have appeared independently in Europe and North America. The genetic relationship among TGEVs and PRCVs has been based on their RNA sequences. An evolutionary tree relating 13 enteric and respiratory isolates has been proposed. According to this tree a main virus lineage evolved from a recent progenitor which was circulating around 1941. From this, by accumulation of point mutations and the introduction of deletions, respiratory isolates and viruses with different degrees of pathogenicity were originated. A constancy in the mutation fixation rate from origin was observed. This rate was $7x10^{-4}$ nucleotide substitutions per site and per year, and falls in the range reported for other RNA viruses. Evidence for recombination in coronaviruses of the TGEV group has being identified. All PRCVs have a deletion of 621 to 678 nt at the 5' end of S gene, which codes virus spikes. Inter or intramolecular recombination at non-homologous sequences probably introduced the deletions. *In vitro* evolution of TGEV by serial passage of the virus at high moi showed the generation of stable defective interfering subgenomic RNAs of 10, 12, and 24 kb. These RNAs contained sequences of the 5' and 3'-ends of the *wt* genome, and internal sequences mapping at the 3'-end of ORF 1b, and were probably originated by recombination. Coinfection of ST cells in culture by enteric PUR46*wt* and respiratory mutants (PTV-*ts-dmar*) yielded recombinant progeny. Since the recombination frequency was low, selective pressure (high temperature and simultaneous selection with two monoclonal antibodies) had to be used. Forty percent of the characterized recombinants had the crossing over in the 5' end of the S gene, the same area where recombination was taking place to generate the PRCVs.

The subgenomic 10 kb RNA was encapsidated and was separated from the *wt* virus by isopycnic centrifugation. A cDNA copy of this RNA has been cloned. Spike genes from TGEV with enteric and respiratory tropism are being engineered into the defective genomes to study the relationship between tropism, virulence, and immunoprotection in coronaviruses related to TGEV. Studies on the genetic structure and tropism of the recombinants selected between enteric and respiratory coronaviruses is being used to determine the molecular bases of TGEV tropism.

The mechanism of generation of cymbidium ringspot tombusvirus defective interfering RNA and requirements for replication

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Defective interfering (DI) RNAs are deletion mutants of viral genomes which have lost all essential genes required for viral functions such as movement, replication and encapsidation. They are heterogeneous in size, ranging from c. 0.4 to 0.7 kb and they need the presence of the helper virus for replication. DI RNAs replicate at the expense of the helper genome from which they derived as a result of a series of progressive deletions.

There are many aspects of the biology of CyRSV DI RNAs which are still poorly understood. For instance, neither the mechanism of DI RNA generation, the sequence and structural requirements for viral replicase recognition have been identified, nor it is understood how DI RNA is translocated in infected plants. To study the mechanism of DI RNA generation, *Nicotiana clevelandii* plant were inoculated with wild type and mutant *in vitro* transcripts of DI RNAs (0.7 kb). It was shown that new smaller DI RNAs were formed from the larger DI RNA (0.7 kb) *in planta* and evidence will be provided, that the locally occured highly base paired structure in the longer DI RNA is responsible for the generation of the smaller DI RNA. The obtained results suggest, that the viral replicase is not always able to unwind this base paired region and copies it, but circumvents it without releasing and produces colinear deletion mutants. This deletion event is not sequence but rather structure specific and can be reproduced if the appropriate DI RNA sequence is substituted with foreign sequence carrying strong secondary structure. The mechanism responsibile for DI RNA generation strongly resembles the heteroduplex mediated RNA recombination described by Nagy and Bujarski (1993. *Proc Natl. Acad. Sci. USA* 90, 6390-6394)

DI RNAs are excelent naturally formed molecules to learn sequence and structural requirements for theirs recognition and replication by the viral replicase. Some dosen of deletion mutants of the known smallest DI RNA were prepared to establish the essential sequence and structural elements for the replication. Single-base mutants were also created to understand the role of computer-predicted stem-loop structures located at the termini of DI RNA.

Defective Interfering RNAs of Plant Tombusviruses: Analysis of essential sequence elements and a model explaining DI RNA generation and evolution.
By T. Jack Morris & K.A. White

Previous research from our lab led to the first molecular characterization of a defective interfering RNA (DI RNAs or DIs) in association with tomato bushy stunt virus (TBSV), a small RNA plant virus (Hillman *et al.*, 1987). We showed as well that different Tombusvirus strains varied in virulence as a result of the presence of these linear deletion mutants which competed with parental virus replication and reduced the severity of disease symptoms. The DIs of several Tombusviruses have now been cloned and sequenced offering the opportunity to identify cis-acting sequences important in both virus multiplication and packaging. Comparative sequence analysis demonstrates that although tombusvirus DI RNA components may vary in size, they all contain four distinct conserved regions of sequence derived from 5', internal and 3' regions of the viral genome similar to the sequenced TBSV-DI RNAs (Knorr *et al.*, 1991; White and Morris, 1993). This suggests that these conserved regions may contain all important *cis*-elements required for replication of their tombusvirus helpers and may well explain why DI RNAs derived from TBSV will replicate upon co-infection with other distantly related tombusviruses. Infectious cDNA clones of TBSV, TBSV-DI RNA, CNV (Rochon and Johnston, 1991), and CNV-DI RNAs have been used to do a comparative functional analysis of the conserved regions in representative DI species derived from both viruses. Results of this analyses will be described showing essential sequence elements present in the four conserved sequence motifs of all of the functional DI RNAs of these two distinct tombusviruses and a conserved structural motif essential for replication in both types of DI RNAs.

We have also been interested in understanding the mechanisms involved in the generation and evolution of the DI RNAs. Infections initiated in protoplasts using synthetic transcripts of naturally occurring and artificially constructed TBSV defective RNA have been analyzed for competitiveness by co-inoculation with helper-genomic RNA transcripts. The ability of various defective RNA to evolve to alternative forms was tested by serially passaging products of these protoplast infections. These studies indicate that: (i) replication competence is a major factor dictating DI RNA competitiveness and is likely a primary determinant in DI RNA evolution; (ii) DI RNAs are capable of evolving to both smaller and larger forms; (iii) DI RNA recombination and/or rearrangement is responsible for the formation of the evolved RNA molecules; and (iv) sequence complementarities between positive and negative-sense strands in the regions of the junctions suggest that base pairing between an incomplete replicase-associated nascent strand and acceptor template may mediate selection of recombination sites. A step-wise deletion model to describe the temporal order of events leading to the formation of tombusvirus DI RNA will be described.

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III. Recombination I

REASSORTMENT AND RECOMBINATION IN DOUBLE-STRANDED RNA VIRUSES

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Viruses with double-stranded (ds) RNA genomes are a large group infecting both prokaryotes and eukaryotes. The dsRNA genome can be unichromosomal or segmented. The life cycles of these viruses are unique in that both the parental and progeny dsRNA genomes are particle-associated throughout the life cycle and the only genetic information free and accessible for genetic interactions are messenger-sense transcripts produced by the particle-associated RNA-dependent RNA polymerase (transcriptase). Generation of new combinations of genetic information by reassortment of genome segments is a high frequency event in the segmented genome dsRNA viruses, and has been documented in members of virus families (number segments, hosts): *Birnaviridae* (2, vertebrates, invertebrates), *Partiviridae* (2, fungi), *Cystoviridae* (3, bacteria), and *Reoviridae* (10-12, vertebrates, invertebrates, plants). However, the mechanism(s) of reassortment are not understood. Recombination by traditional breakage-reunion or copy-choice mechanisms has not been detected among the segmented-genome dsRNA viruses, although the generation of rearranged genome segments (deletions, duplications) suggests that a copy-choice-like mechanism may be possible. Recently, one report appeared documenting inter-segmental recombination in *Cystoviridae*, most likely by a copy-choice type mechanism. Among the unichromosomal dsRNA viruses of family *Totiviridae* (1, fungi, protozoa), the possibility of recombination has not been systematically investigated.

Reassortment in the segmented-genome dsRNA viruses will be quickly reviewed, with emphasis on the genetics of reassortment and passing mention of the usefulness of reassortment as a tool for analysis of viral gene function. Newly developed in vitro RNA replication systems for the *Totiviridae*, *Cystoviridae*, and *Reoviridae* will be described, and preliminary results from these systems bearing on possible mechanisms of reassortment will be discussed.

Rearrangement of sequences within genome segments of various members of the *Reoviridae* will be described, and their possible generation by a copy-choice-like mechanism will be discussed. The limited information available on recombination between heterologous genome segments of the *Cystoviridae* will presented.

Natural Recombinants and Laboratory Chimeras of Alphaviruses

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Some years ago, we determined from limited sequence analysis that Western equine encephalitis virus (WEE) was a natural recombinant between Eastern equine encephalitis virus (EEE) and a Sindbis-like progenitor. WEE had always been somewhat puzzling, since it was strongly serologically cross-reactive with Sindbis virus, but with the disease potential of the characteristic New World encephalitic viruses. From the sequence, it was clear that two recombination events had occurred, such that the Sindbis-like glycoprotein genes (with short flanking sequences) had been substituted for the glycoproteins of EEE. The remainder of the genome, including the capsid protein and the upstream nonstructural proteins, was closely related to EEE, as was the extreme 3' end of the RNA. However, within the cytoplasmic domain of glycoprotein E2 and the carboxyterminal domain of the capsid, amino acid changes had occurred which were postulated to "better adapt" these two proteins to one another (Hahn et al., 1988).

In an attempt to delineate just what interactions occur between the capsid protein and E2 during budding and assembly, we have now constructed several chimeric viruses, using full length cDNA clones of Ross River (RR) and Sindbis (SIN) viruses from which infectious RNA can be transcribed in vitro. In one of these chimeras, the entire genome with the exception of the capsid protein is from RR, but the capsid is from SIN (RR/SINc). This chimera was almost nonviable; although nucleocapsids were produced in normal amounts, very little budding virus was produced. Within the cytoplasmic tail of E2 there are 11 amino acid differences between RR and SIN, and these were systematically altered by site-specific mutagenesis. Change of two of these to the SIN equivalent improved the yield of chimeric virus >1000-fold, and change of 9 out of 11 improved it still more, but not to the wild type levels (Lopez et al., 1994). These results, as well as those from sequence analysis of WEE, clearly show that during budding the alphavirus nucleocapsid interacts with the carboxy-terminal cytoplasmic domain of glycoprotein E2 in a sequence-dependent manner.

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STUDIES OF CORONAVIRUS RNA TRANSCRIPTION USING A DEFECTIVE-INTERFERING RNA: IMPLICATION FOR THE MECHANISM OF HOMOLOGOUS AND ABERRANT HOMOLOGOUS RECOMBINATION

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Coronavirus mRNA transcription involves the binding of a leader RNA to distant intergenic sequences and initiation of transcription using the leader RNA as a primer, resulting in mRNAs with a fused leader sequence. This is analogous to homologous recombination, which involves the switching of nascent RNA products to a homologous site on a new RNA template. The mechanism of precise sequence recognition is not clear. We have utilized a defective-interfering (DI) RNA of mouse hepatitis virus (MHV) as a vector and inserted a reporter gene (chloramphenicol acetyltransferase) to study the mechanism of coronavirus transcription and recombination. We show that MHV RNA transcription is regulated by 3 components: an intergenic sequence (IS), which serves as a promoter; a cis-acting 5'-end sequence, which serves as an enhancer-like element; and a trans-acting leader sequence, which serves as a primer. This study established unequivocally that the leader RNA present in coronavirus mRNAs is derived in trans, thus requiring polymerase jumping during RNA transcription. Therefore, coronavirus transcription serves as a legitimate model for RNA recombination. We have also detected cellular proteins binding to both the leader sequence and IS. We propose that these proteins could be transcription factors, whose functions are analogous to those of DNA-dependent RNA transcription factors. We have also shown that, in a particular MHV strain, the leader-mRNA fusion sites are not precise and frequently occur at sites far away from the homologous sequence present between the leader and IS. This is analogous to aberrant homologous recombination seen in other RNA viruses. The occurrence of this phenomenon in this particular MHV strain suggests that the ability to undergo aberrant homologous recombination is genetically determined. Since the leader sequence and intergenic sequence of this MHV are identical to the corresponding sequences of other MHV strains, except for a 9-nucleotide deletion downstream of the leader sequence, this result suggests that either the viral polymerases or RNA sequences other than the homologous region influence the leader-fusion site selection and also aberrant homologous recombination. Therefore, direct RNA-RNA binding between the homologous sequence may not be the primary determinant of cross-over site selection. We propose that RNA transcription and recombination is mediated primarily by protein-protein or protein-RNA interaction.

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Analysis of Recombination Hot Spots in Genomic and Subviral RNAs of Turnip Crinkle Virus

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TCV is an icosahedral virus with a monopartite RNA genome that infects a broad range of dicot plants. Based on sequence comparison studies, TCV has been classified as a member of the carmoviruses in virus supergroup II. The genomic RNA, which is capped at the 5'-end and contains a free hydroxyl group at the 3'-end, consists of 4054 bases specifying five open-reading frames. TCV supports the replication of numerous subviral RNAs including: (i) satellite (sat-) RNAs such as sat-RNA D, which share no sequence similarity with the genomic RNA beyond seven 3'-terminal bases; (ii) DI RNAs, whose sequence is completely or nearly completely derived from the genomic RNA; (iii) chimeric species, containing full-length or nearly full-length sat-RNA D at the 5'-end and one or more segments from the 3'-end region of the genomic RNA at the 3'-end.

Examination of sequences at the junctions of DI RNAs, chimeric RNAs, and recombinants derived *de novo* from portions of sat-RNA D and the chimeric sat-RNA C or TCV genomic RNA, revealed that one of three motifs is nearly always located at the right side of the crossover sites. Motif I (the sequence in sat-RNA C that is found at the junction of sat-RNA D/sat-RNA C recombinants) resembles a sequence 10 bases from the 5'-end of TCV genomic RNA; motif II resembles the sequence at the 5'-ends of sat-RNAs D and one DI RNA; motif III is similar to sequence at the 5'-end of the one mapped TCV subgenomic RNA (1.45 kb). Based on the junction sequences, we proposed a non-processive mechanism whereby the RdRp jumps to these particular motifs (or structures that include these sequences) following complete or nearly complete transcription of sat-RNA D. This model suggests that the RdRp recognizes these motifs as internal initiation sites, and that template switching occurs during (+)-strand synthesis.

Based on mutagenesis studies and nuclease and chemical mapping of the secondary structure of the motif I region in sat-RNA C, we proposed that recombination at motif I requires a stem-loop and an upstream single-stranded region. The crossover junctions are located upstream of the hairpin at the base of the stem. We have now extended our analysis to include two consecutive motif III sequences (IIIA and IIIB) located in a non-coding region of TCV genomic RNA, about 250 bases from the 3'-end. These two motifs are the main targets for recombination between TCV genomic RNA and sat-RNA D. Computer modeling suggests that a stable hairpin with an upstream single stranded region can be formed in the motif IIIA&B region that would place the major recombination site upstream of the hairpin at the base of the stem, with a secondary site in the hairpin loop. We have nearly completed an analysis of junction positions using genomic RNA templates containing an extensive series of point mutations and deletions in the motif IIIA&B region. Our results, which involve the examination of over 1000 independent recombinants, suggest that a portion of the stem is important in targeting reinitiation of synthesis at the base of the stem. Furthermore, this preferred junction site can be shifted to the secondary site in the loop by

single point mutations that potentially increase the size of the loop.

The sequence in sat-RNA D that comprises the left side of the junctions was also examined. Over 50% of the sat-RNA D segments were either full-length or terminated 13 nucleotides upstream of the 3'-end. Non-template nucleotides (mainly uracil residues) were found at about 30% of all junctions; however, the presence of non-template nucleotides was influenced by the sequence at the end of the sat-RNA D segment.

We have also found that motif IIIA or B appears to be required for normal accumulation of the genomic RNA in turnip plants. Plants inoculated with genomic RNA transcripts containing a deletion of one complete motif and a portion of the second accumulate < 2% of wild-type levels. The lack of accumulation *in vivo* apparently involves replication since genomic RNA transcripts containing deletions of greater than one motif do not replicate as well as wild-type transcripts in single plant cells (protoplasts). A link between recombination and replication may explain why these untranslated sequences have persisted in the genome of the virus.

As part of this study, we have come across an unusual recombinant between sat-RNA D and the TCV genomic RNA. This recombinant molecule has three different junctions and is composed of both (+)-strand and (-)-strand genomic RNA sequence. The sequence at the right sides of the junctions are the typical motif sequences described above. The appearance of this recombinant species suggests that recombination can occur between (+)-strands and (-)-strands of the TCV genomic RNA.

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Genetic recombination in brome mosaic virus - homologous and nonhomologous RNA-RNA crossing overs re-defined.

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Brome mosaic virus (BMV), a plant tripartite RNA virus, has available efficient genetic recombination mechanisms among its RNA segments (Bujarski and Kaesberg, 1986; Bujarski *et al.*, 1994). Our data reveal the existence of two types of recombination events in BMV, which utilize different structure of recombination intermediates. In one type, crossing overs require homologous sequences (Nagy, P. D. and Bujarski, J. J., unpublished results) while in another one the crosses occur at double-stranded regions formed between recombining molecules (Bujarski and Dzianott, 1991; Nagy and Bujarski, 1993). Although the molecular mechanism of both recombination types seems to be different (Nagy and Bujarski, 1992; Bujarski and Nagy, 1994), a common feature is that their functional sequences operate as recombinationally-active 'cassettes', while the flanking sequences play a limited role in crossover events.

These results prompt us to re-define homologous and nonhomologous RNA recombination. A 'classical' (genetic) definition considers homologous RNA recombination as occurring "between related parental RNAs at the same genetic loci, so preserving any reading frame and regenerating a basically similar recombinant molecule. In nonhomologous recombination neither of these restrictions applies (King, 1988)". We propose to define homologous RNA recombination as the one which occurs between homologous domains. Depending on flanking sequences, such homologous crossovers may or may not re-generate similar recombinant molecules. To distinguish between these two possibilities, we propose to use the terms of, respectively, 'legitimate' homologous and 'illegitimate' homologous recombination. We further propose to name other recombination events as nonhomologous recombination, simply because they do not require sequence homology. According to this definition, the heteroduplex-driven events between BMV RNAs are nonhomologous. Since these crosses did not regenerate parental BMV RNA molecules, they should be further named as illegitimate nonhomologous recombinants. One possibility for legitimate heteroduplex-driven (nonhomologous) recombinants is if the crossing overs occur at a central site within the double-stranded palindromic region between two related RNA sequences. Finally, our results reveal that both homologous and nonhomologous recombination events in BMV may, or may not cause sequence insertions or deletions at the sites of crossovers. We propose the name of 'precise' and 'imprecise' recombination, respectively, to describe these two categories.

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THE ROLE OF RECOMBINATION IN GENERATING THE DIVERSITY OF TOBRAVIRUSES

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The tobraviruses, tobacco rattle virus (TRV) and pea early-browning virus (PEBV), have bipartite single-stranded RNA genomes. RNA-1 codes for replication and movement proteins, and can replicate independently in plants. The nucleotide sequence of RNA-1 is similar for all strains of each virus, but is largely different between the two viruses (1). In contrast, the coding sequences of RNA-2, which include the particle protein gene and up to two other RNA-2 specific ORFs, are highly diverse among strains of each virus. However, at the 3' and 5' ends, RNA-1 and RNA-2 of naturally occuring strains are homologous, although the length of the homologous region is variable and may include at the 3' end the whole or part of one or more ORFs (2).

In pseudo-recombination experiments, isolates consisting of RNA-1 and RNA-2 from different strains of the same virus are readily obtained, but RNA-1 of TRV cannot support replication of PEBV RNA-2, nor RNA-1 of PEBV that of TRV RNA-1. However, isolates that have a combination of properties of the two viruses are found in nature (2). These isolates have host ranges and RNA-1 sequences typical of TRV, but their particle proteins and at least one other RNA-2 coded protein are typical of PEBV. Pseudo-recombinant isolates in which one genome part is from one of these atypical isolates and the other from TRV can be produced, and the 3' and 5' ends of their RNA-2 have some TRV-like sequences. The RNA-2 species of these atypical isolates are therefore recombinant molecules that contain sequences derived from both TRV and PEBV.

The mechanism by which the identity of the 3' ends of RNA-1 and RNA-2 of natural isolates is maintained probably involves copy-choice at a sequence motif that resembles the 5' ends of genomic and sub-genomic RNA species. However, in a pseudo-recombinant isolate comprising RNA-1 and RNA-2 from different TRV isolates, the 3' ends may be non-identical and remain so over several passages in plants (3). Furthermore, the 3' end of RNA-2 of the atypical TRV isolate I6 is almost identical to that of PEBV RNA-2, and the TRV-like sequences are limited to the 25 residues that are common to both viruses. Thus, 3'-terminal identity does not seem to determine the compatibility of the genome parts, or to be essential to the viability of the virus.

At the 5' end, although the sequence of RNA-2 shows considerable homology with that of RNA-1, it contains an insertion of about 26 bases (4). The 5' end of RNA-2 of isolate I6 also has this insertion, and this molecule seems therefore to have resulted from recombination between TRV RNA-2 and PEBV RNA-2. However, between the identifiably TRV-like and PEBV-like sequences are about 100 residues of uncertain origin, which prevent identification of the precise

recombination junction. None of the regions in RNA-2 whose sequence is conserved among several tobravirus strains seem likely candidates.

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RNA Recombination Between Viroids

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Many studies have shown that various combinations of up to a dozen different viroids can be found to replicate together in individual plants such as especially in citrus, orange, grapefruit, lemon and also in grapevine. The sequence of most of these viroids is characterized by the presence of sequence motifs which are evidently derived from two or three other viroids present or absent in the corresponding plant. Because these different motifs are interspaced by motifs specific for the chimeric viroid all of these are characterized by a mosaic-type sequence. This finding has led to the hypothesis that in the past a series of RNA recombination and mutation events must have taken place which gave rise to these viroid chimeric.

Direct evidence for the essential role of RNA recombination in the evolution of viroids has come from our recent study of the Coleus viroid system. In certain cultivars of the ornamental plant Coleus blumei a unique system of three Coleus viroids (CbVd) of different chain length was detected. These mechanically transmissible new viroids are named CbVd 1 (249 and 250 nts), CbVd 2 (302 nts), and CbVd 3 (362 nts). Our analysis revealed that CbVd 1 and CbVd 3 are the parental viroids of the system whereas CbVd 2 represents a truely chimeric viroid offspring that arose by RNA recombination between them. Thus, the Coleus viroid system is actually a viroid family in the colloquial sense. The chimeric CbVd is composed of two virtually unchanged parental sequences which are connected at a sharply defined boundary. In its rod-shaped secondary structure model the left hand part is identical to the corresponding left hand part of CbVd 3, whereas its central part and its right hand part is identical to the corresponding regions of CbVd 1.

The analysis of the distribution of the three viroids in a vegetatively propagated and hence biologically cloned Coleus plant population revealed that in 1991 about 20% of the plants contained the chimeric viroid, whereas in 1992 it was found in about 60% of them. Thus the recombination between the parent viroids is not only of recent origin but still in progress today. All these features render the Coleus viroid system, in particular the unique CbVd 2, a kind of missing link in support of the repeatedly presumed involvement of RNA recombination between different viroids during viroid evolution. The perspectives and the possible mechanisms of viroid RNA recombination will be discussed.

IV. Defective Interfering II

Host effect in removal, *de novo* generation and accumulation of defective interfering RNAs in broad bean mottle virus.

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Broad bean mottle virus (BBMV) is a positive strand sense RNA virus that has three genomic RNAs. Two strains of BBMV encapsidate defective-interfering RNAs (DI-RNAs) which exacerbate the severity of symptoms when inoculated on pea seedlings. These additional RNAs are generated by internal deletions in the RNA-2 component, preserving the open reading frame and coding for a truncated 2a protein (Romero et al 1993). In order to understand the molecular function of DI-RNAs in BBMV infection, we have studied the accumulation and encapsidation of DI-RNAs in several BBMV-strains and hosts. Our results demostrate that DI-RNAs are found in BBMV-infected broad bean plants and seems it is an inherent feature of BBMV , because *de novo* generation of DI-RNAs has been observed after subsequent serial passages of BBMV through different plant hosts.

BBMV DI-RNAs are supported by some hosts, e.g. broad bean, but not by others e.g. bean. Hence, one can remove them from the virus by passing through bean plants. Full lengh cDNA clones of BBMV and DIs were synthesized (Pogany et al. 1994; Romero et al., 1993)) and the transcripts were obtained from these clones. They were used to examine accumulation and infeccions of DI-RNAs in homologous and heterologous BBMV strains. The elimination of the translation initiation codon in BBMV DI transcripts by *in vitro* mutagenesis resulted in the reduction of the accumulation of mutant DI RNA under the detectable level *in planta* , suggesting a specific role of coding capacity in DI RNA accumulation.

In conclusion, our results reveal that host plants influence the accumulation and encasidation of BBMV DI RNAs. The latter affect symptom formation in some BBMV-infected hosts.

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Soon a method to assess genetic recombination for paramyxoviruses with the use of programmed defective RNAs ?

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Generation of defective interfering (DI) viral genomes results from a recombination event between two non contiguous genome sequences. For paramyxoviruses, as well as for negative stranded viruses in general, the favoured mechanism proposed involves the leap of an acting RNA polymerase carrying the nascent RNA genome from its original nucleocapsid template to another position on this template, to another position on another template or even back to the same nascent genome. These leaps result in the generation of defective RNA genomes that lack various internal portions of the viral genome with, however, conservation of both genomic ends (internal deletion DI), or of defective genomes that in place of the 3' genomic end contain a copy of the 5' genomic end (copy back DI). The frequency of such a recombination event is unknown but is probably similar to that estimated for the generation of a successfully amplified vesicular stomatitis virus DI (10^{-8}, Holland et al., J. Virol. 17 : 805-815, 1976), if one compares the rate at which the DI RNAs of both virus families appear in viral infections initiated with a DI free virus stock.

The analysis of primary sequences of 4 Sendai virus (SV) and 3 measles virus (MV) DI RNAs led to little information regarding the RNA sequence properties involved in either the fall off of the RNA polymerase or the reattachment site. At this latter, the reinitiation requires a limited (1 or 2), or even non existent base pairing between the nascent RNA and the template. Assuming that the RNA sequences present on the successfully amplified DI RNAs reflect those present at the time of DI RNA generation, these results support, in the present understanding of the RNA replication mechanism, the randomness of this process. Once randomly generated, the DI RNAs would, however, be selectively amplified according to rules directing RNA replication and interference.

In the past two years, attempts to reconstruct RNA DI genomes from RNA originating form DNA have been successful for influenza, vesicular stomatitis (VSV), respiratory syncitial, human parainfluenza, rabies and Sendai viruses. In our laboratory, a natural copy back SV DI RNA (DI-H4) was cloned (Calain et al., Virology, 191 : 62-71, 1992). Transcription of this RNA in cells expressing the SV NP, P, and L lead to proper encapsidation of this DI RNA. The nucleocapsids generated in this way were efficiently replicated by the viral functions provided in trans and were eventually propagated in SV viral stocks . By limited nucleotide deletions and/or insertions, it was shown that the ability to efficiently replicate this DI RNA was tied to the total nucleotide number being a multiple of six (Calain et al, 1992, Calain and Roux, J. Virol. 67 : 4822-4830, 1993) This "rule of six" was interpreted as the

indication that the NP protein makes contact with exactly six nucleotides, and that, at the DI RNAs 3' ends, only an exact encapsidation leaving no dangling nucleotides or no NP protein contacting less than six nucleotides would represent a proper replication promoter.

An internal SV deletion DI (E307), containing a partial NP gene sequence fused to the end of the L gene, was then cloned (Engelhorn et al., J. Gen. Virol. 74 : 137-141, 1993). This DI RNA sequence contains the genomic 3' and 5' ends and therefore is expected to transcribe as well as to replicate. Indeed the original E307 DI RNA, present in the natural viral stock, produces a fused NP/L mRNA encoding a truncated NP protein. When the cloned DI RNA was transcribed in a system identical to that described above for the copy back DI, *bona fide* viral nucleocapsids were produced and replicated. When confronted to the rule of six, the internal DI RNA also exhibited the requirement for containing a total number of nucleotides as a multiple of six. The propagation of this DI RNA generated from plasmid in a viral stock showed production of the truncated NP protein confirming the transcribing ability of the DI RNA.

When compared to the DI-H4 RNA, the E307 RNA always showed a lower replication efficiency by a factor of 20-30. This opened the question as to whether the replication was hampered by the ability to transcribe. To test this hypothesis a variety of constructs were produced where the 3' end of the deletion DI was gradually replaced by that of the copy back DI and conversely. When assayed, the replication ability of both sets of constructs correlated with their putative ability to transcribe. Confirmed assessment of this transcription ability , when expected, has still to come. Other explanations like encapsidation ability of the T7 transcript, or more trivial the stability of the various encapsidated and/or replicated constructs are also to be investigated.

In conclusion, defective SV RNAs can be generated from plasmid DNA, and introduced into regular viral stocks. Depending on their 3' end primary sequences these RNAs exhibit different replication ability, possibly tied to their transcribing ability. This system therefore opens the possibility to have a close look to the cis sequences involved in transcription and replication.

As far as RNA recombination is concerned, this system may provide tools to assess some basic features involved. For instance, two DI RNAs with poor replication ability can be built in such a way that only a recombinant between the two will yield a construct with high replication ability.

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Immunopathologic and Encephalitic Changes in Mice Infected with Vesicular Stomatitis Virus (VSV) and It's Defective Interfering (DI) Particles A. S. HUANG, I. V. PLAKHOV, C. S. REISS, and C. AOKI, New York University, New York, N.Y.

Previous studies have suggested that the use of DI particles were problematical for controlling or ameliorating viral infections. To further examine the role of defective interfering particles in neuropathogenesis, BALB/c mice were infected intranasally with purified standard VSV and DI preparations. Using immunohistochemical staining techniques neurons in the olfactory bulb and other cells involved with the olfactory system were found to express viral antigens shortly after infection. Specific locales of the brain and neural tracts were consistently positively-stained with antiviral antibody, whereas adjoining regions were completely negative. Coinfection with DI particles reduced this spread and limited viral antigen expression to those parts of the olfactory system closest to the site of inoculation. Mice that recovered showed no residual viral antigen in any parts of the brain and were resistant to challenge by a lethal dose of VSV. Careful quantitation and control inoculations with UV-irradiated DI preparations suggest that previously reported exacerbations of the infection by DI particles were due to contamination of the preparations by standard VSV. These studies also suggest that VSV spreads in the brain following specific pathways without random viral release and diffusion. Therefore, DI particles are potentially useful for limiting viral infections and VSVprovides an important tool for determining neuronal pathways.

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Pathogenesis of Mucosal Disease - Characterization of Cytopathogenic Pestiviruses

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Bovine viral diarrhea virus (BVDV) represents one member of the genus pestivirus. Two biotypes of BVDV, noncytopathogenic (ncp) and cytopathogenic (cp), are known. Antigenically closely related cp and ncp BVDV can be isolated from cattle suffering from fatal mucosal disease (MD) and are called a BVDV pair.

The positive stranded RNA genomes of several cpBVDV strains contain insertions highly homologous to cellular sequences. For five of these the cellular homologue was shown to be a ubiquitin-coding sequence. The host cell derived inserts are either integrated between formerly neighbouring nucleotides or are flanked by large duplications of viral sequences. The insertions are always located within the genomic region coding for the nonstructural protein p125.

Analysis of additional BVDV pairs revealed that the respective cp strains do not contain insertions of cellular sequences. However, two of such genomes show dramatic rearrangements of viral sequences including duplication of the p80 coding sequence, the C terminal region of p125. Interestingly, a totally different change of a BVDV genome was found for the cp strain from another pair. We detected only a cp specific insertion of 27 viral nucleotides in the p125 coding region. In spite of the genomic variations, all these viruses are replicating autonomously. However, we have recently characterized a completely novel kind of BVD cytopathogenic agent, which is an internal deletion type defective interfering particle (DI). Expression of DI encoded proteins conferred cytopathogenicity while the helper virus alone was non cytopathogenic.

Insertions as well as duplications are missing in the genomes of the ncp counterparts even though the genomic sequences from the members of each pair are almost 100% identical. Our data show that cpBVDV develops from ncp BVDV in a RNA recombination process which leads to either integration of additional sequences or to loss of internal sequences.

The generation of cpBVDV in an animal persistently infected with an ncp virus is regarded as causative for development of MD. Cytopathogenicity of BVDV strains is always correlated with the appearance of BVDV p80 which is generated by (1) cleavage of p125, (2) expression from a duplicated region in the viral genome or (3) translation from the defective genome. In contrast, only p125 but not p80 can be observed after infection with ncp strains. Our data show that very different kinds of rearrangements of the viral genome and/or insertions of cellular sequences can lead to production of p80. Occurrence of p80 is apparently the crucial step towards cytopathogenicity. Our results strongly suggest a linkage between RNA recombination, generation of p80, cytopathogenicity and pathogenesis of MD.

Characterization of CNV DI RNA dimers generated during co-infection of synthetic CNV DI RNA transcripts and helper transcripts. Renée Finnen[1] and D'Ann Rochon[2]. [1]Department of Microbiology and Immunology, University of British Columbia, Vancouver, B.C., CANADA, V6T 1Z3 and [2]Agriculture Canada, 6660 Northwest Marine Dr., Vancouver, B.C., CANADA, V6T 1X2.

Cucumber necrosis tombusvirus (CNV) is an icosahedral virus with a monopartite, single-stranded, positive sense RNA genome of about 4.7 kb in length. Symptom-attenuating defective interfering RNAs (DI RNAs) of CNV can be generated *de novo* in *Nicotiana clevelandii* and DI RNAs are also associated with our laboratory culture of CNV (CNV-Lc). CNV DI RNAs generated *de novo* and those associated with CNV-Lc have been cloned and completely sequenced. Upon testing the biological activity of transcripts generated from the DI RNA clones, prominent, higher molecular weight RNAs were noted in *N. clevelandii* which had been co-infected with transcripts generated from a full-length clone of CNV and transcripts generated from the DI RNA clones. Based on their mobility in denaturing agarose gels, these RNA species appeared to be single-stranded RNA approximately twice the size of the CNV DI RNA used for co-infection ("dimer" sized). We speculated that these "dimer" sized RNAs could be head-to-tail dimers of CNV DI RNA sequence such as those observed with certain satellite RNAs and turnip crinkle virus DI RNAs. RNA sequencing was used to confirm that the "dimer" sized RNAs were in fact head-to-tail dimers. Junction sequences were determined using reverse transcriptase polymerase chain reactions and direct sequencing of the amplified products. The junctions were the same for all CNV DI RNA dimers tested and consisted of a precise fusion of 3' and 5' sequences, probably with no non-templated additions. In order to further investigate modes by which these dimers are generated and their biological significance, cDNA clones corresponding to dimers of CNV DI RNA sequence with precise junctions were constructed. Initial studies on the biological activity and possible autocatalytic capability of dimer transcripts will be discussed and models for the generation and replication of CNV DI RNA dimers will be proposed.

SEQUENCES ESSENTIAL FOR THE ACCUMULATION OF DEFECTIVE INTERFERING RNAs OF CUCUMBER NECROSIS AND TOMATO BUSHY STUNT TOMBUSVIRUSES

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Tombusvirus Defective Interfering (DI) RNAs attenuate the plant symptoms caused by the virus inhibiting the viral genomic RNA replication. The DI RNAs are co-linear deletion mutants derived from the parental genome. Four conserved regions (I through IV) are always present.

Systematic deletion mutagenesis of both cucumber necrosis virus (CNV) and tomato bushy stunt virus (TBSV) DI cDNA clones was performed in order to determine the function of the conserved regions.These analyses revealed that region III of both DI types was totally dispensable for the DI accumulation in *N benthamiana* protoplasts and plants. Although the interference effects against viral RNA were less pronounced in the mutants that lacked region III than in the wild type DI RNAs. Large deletions in regions I and IV generally yielded inactive CNV and TBSV DIs, except for a 75 nucleotide (nt) deletion within region I of the CNV DI which was tolerated. In addition comparable segments in the midle region II of the TBSV-DI RNA (38 nt) and the CNV-DI RNA (34 nt) could be deleted without abolishing their infectivity. A conserved stem-loop structure could be predicted in this area of region II for both DI's. Infectivity experiments indicated that the dispensable sequences comprise the external portion of the loop, whereas the indispensable sequences make up the base of the stem.

Our comparative functional analysis of CNV and TBSV DIs supports the idea that the conserved regions of sequence in segments I, II and IV represent essential cis-elements generally important for the replication of all tombusvirus DI RNAs.

V. Recombination II

Aura Alphavirus Packages the Subgenomic mRNA

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Aura alphavirus is a South American virus that is serologically related to Sindbis virus [1-3]. Alphaviruses package their 12 kb genome into a nucleocapsid that possesses T=4 icosahedral symmetry, which requires 240 molecules of nucleocapsid protein [4]. The assembled nucleocapsid then buds through the plasma membrane to acquire an envelope containing two virus-encoded glycoproteins, which form a T=4 icosahedral lattice on the surface of the virus. Aura virus, unlike other alphaviruses, also packages the viral 4 kb subgenomic mRNA, and we suggest that a packaging signal is found within the region of the genome transcribed into subgenomic RNA [5]. The subgenomic RNA is packaged not only into particles that appear to be identical to that of the virion (T=4), but also into particles that are smaller and have T=3 symmetry, which requires 180 molecules of capsid protein. Thus the Aura capsid protein is able to form different sizes of nucleocapsids depending upon the size of RNA that is encapsidated. Examination of the published literature [6-8] suggests that other alphaviruses are also capable of forming T=3 structures when DI RNA is encapsidated, and thus that this is a general feature of alphavirus packaging. Aura virus appears to be unique among the alphaviruses in possessing an encapsidation signal within the region transcribed into subgenomic RNA, however, as all other alphaviruses examined appear to have a packaging signal in the 5' region not transcribed into subgenomic RNA [9] and do not package the subgenomic RNA [5].

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Title:
Generation of replication-competent retroviruses by recombination: Implications for construction of safe retroviral vector systems

Retroviruses are in wide and increasing use as vectors for the delivery of therapeutic genes in man. "Packaging" cell lines have been constructed for the production of replication-defective virion particles that transduce viral vector RNAs. A significant problem is the tendency of these virus preparations to lead to the formation of replication-competent virus during transduction. These viruses seem to arise by recombination events between copackaged viral RNAs during reverse transcription. The donor RNAs for these recombination events are probably the RNAs encoding the virion particle proteins and the vector RNAs themselves. Several examples of such reversion events will be presented, and various means to prevent the formation of wild-type virus for gene therapy will be considered.

In many settings, two replication-defective viral genomes co-expressed in the same cell can complement to form a functional virion, and during infection these genomes will recombine. Similar recombination events occur to mediate reversion of deletion mutations in retroviral genomes. Murine cell lines expressing a deletion mutant of a given retrovirus can ultimately yield replication-competent viruses by permitting "patch repair" of the deletion; the patch seems to be derived from endogenous retroviral sequences present in the host genome. These reversion mechanisms serve to limit the selective pressure that can be applied to the study of defective retroviruses, and highlight the resourcefulness of the viruses in self-transmission.

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HIV sequence variation represents a genetic big bang, with mutations being dependent on the intracellular dNTP pools, and frequent recombination occurring through strand slippage and aberrant splicing

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The tempo of HIV sequence variation is of truly astronomical proportions. Typically intrapatieint amino acid sequence divergence of 5-20% for certain regions can be observed within several years. Longitudinal studies in monkeys infected by molecularly cloned simian immunodeficiency virus (SIV) has well documented the remorseless accumulation of variants with time. Occasionally very rapid fixation occurred, up to 8% amino acid variation in as little as 20 weeks. Given the point mutation rate/base/cycle, hundreds to thousands of sequential cycles are necessary to produce such variation. If the burst size was merely 2 per round the whole universe would be transmuted into HIV ($2500\sim10^{150}$). Only if the burst size is severely restricted several hundred fold, presumably be the immune system, can proviral loads similar to those observed ($\sim10^{7}$-10^{10}) be achieved. Note that massive bottlenecking must ensue.

Among viruses, retroviruses are uniquely sensitive to fluctuations in intracellular dNTP concentrations. Indeed HIV replication may be enhanced or restricted by modulation of intracellular dNTP pools, notably dCTP. A catastrophic loss of dCTP is probably the cause of G->A hypermutation, a most remarkable feat whereby up to 60% of all G residues may be monotonously substituted by A. Many deletions and insertions occur via slippage of the nascent primer strand with respect to the template. Deletions may be trivial, several kilobases and may occur at frequencies of 5-10% per replication cycle.

Retroviruses may transduce cellular sequences with remarkable ease (mutant frequencies of $\sim10^{-3}$). This can occur by aberrant splicing between HIV donor and cellular acceptor sites, so giving rise to chimeric transcripts. It is inevitable that novel, replication competent HIVs with transduced cellular sequences will be identified in the next few years.

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POSTERS

RNA recombinations in the Qβ phage system

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Our previous study revealed recombinant RNAs in the products of so-called "spontaneous" RNA synthesis that occurs in the cell-free Qβ replicase reactions not provided with template RNAs (1-3). We have also found that these RNAs are formed in the Qβ phage-infected *E. coli* cells, are encapsidated by the phage, and can propagate *in vivo* in a number of phage generations displaying all the traits of virus satellite RNAs (4). These RNAs may (1) or may not contain (2) fragments of the Qβ phage genome indicating that viral sequences are not indispensable for either formation or replication of the recombinant molecules. In laboratory, single molecules of these RNAs can be disseminated through air and can invade cell-free Qβ replicase reactions resulting in the apparently template-independent RNA synthesis. This fact has been demonstrated by utilizing a novel technique that allows RNA molecules to be cloned *in vitro* by growing them as colonies in a layer of agarose containing the purified Qβ replicase and nucleotide substrates (4,5). We believe that the RNA colony technique can be a powerful tool for studying RNA recombination *in vitro*, as it allows even solitary recombinant molecules to be detected and analyzed. New data will be presented suggesting that (i) all known Qβ phage satellite RNAs have been formed by RNA recombination; (ii) there are "hot spots" in RNA molecules where recombination is highly probable; (iii) cross-over sites display a bias towards higher content of A or U; (iv) some naturally occurring recombinant RNAs undergo a site-specific self-cleavage and/or are attacked by RNase P presumably at the cross-over sites, indicating that these activities can play a role in RNA recombination; and (v) Qβ phage satellite RNAs containing fragments of the Qβ phage genome can suppress replication of the genomic RNA via antisense mechanism.

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RAPID GENERATION OF DIMERIC DEFECTIVE INTERFERING RNA OF CYMBIDIUM RINGSPOT TOMBUSVIRUS

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Cymbidium ringspot tombusvirus (CyRSV) supports the replication of defective interfering (DI) RNAs. CyRSV DI RNA was shown to consist of mosaic molecule made up entirely of noncontignous regions of genomic RNA. Coinoculation of *Nicotiana clevelandii* plants with *in vitro* transcripts of both genomic and DI RNAs results a rapid accumlation of a new DI like RNA species larger than the input synthetic DI RNA. This RNA was suggested to be a dimer of input DI RNA inoculum. The dimeric nature of this new RNA species was clearly demonstrated by cloning and sequencing of the putative junction regions. A study was therefore undertaken to ascertain whether the formation of DI RNA dimers has any biological role, in particular whether dimers could be the forms in which DI RNA molecules move in inoculated leaves.

However, the generation of dimeric forms is not characteristic of all CyRSV DI RNAs. The smallest (DI-2, 4o4 nt) and a medium size (DI-3, 484 nt) DI RNA produce dimers, contrary to the largest DI-13 RNA (679 nt) which is almost unable to form dimers during replication. The shortening of DI-13 RNA molecules resulted the appearance of dimers and the insertion of foreign sequences (ranging from 34 to 24o nt) into DI-2 RNA abolishes the dimer formation. These results indicates that the generation and/or replication of dimers depends only on the size of the monomer molcule.

Coinoculation of synthetic genomic RNA with both DI-2 and DI-3 RNAs resulted only homologous dimer DI RNAs except one out of 30. In addition sequence analysis of progeny molcules showed that a recombinat molecule was occured between the two different DI RNAs.

Inoculation of plants with either homologous or heterologous dimeric DI RNA transcripts led to an efficient replication of dimer DI RNA but produced also a significant amount of monomer. This result shows that the lack of heterologous dimers is not a consequence of the unability of these molecules to replicate, but likely they are not generated at all. The mechanism which is responsible for the generation of dimers will be discussed.

CHARACTERIZATION OF MULTIPLE CIRCULAR RNAs DERIVED FROM A CARNATION VIROIDLIKE RNA BY SEQUENCE DELETIONS AND DUPLICATIONS.

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We have characterized recently a small circular RNA from carnation whose viroid or satellite nature still remains to be established. The analysis of the sequence of this viroidlike RNA (275 nucleotides) has revealed that it can adopt hammerhead structures in the strands of both polarities (1). Moreover, the corresponding plus and minus RNA transcripts self-cleavage *in vitro* as predicted by those structures, although in the case of the plus strand a double hammerhead structure appears to be more efficient than a single one (1).

Northern blot hybridization experiments using a double PAGE system to resolve small circular RNAs together with a radioactive RNA probe transcribed from a cDNA clone of the 275 nt viroidlike RNA, revealed that carnation extracts contained lower amounts of other nine homologous circular RNAs with apparently smaller and larger sizes. These RNAs were purified and then characterized molecularly by reverse transcription and PCR amplification using two pairs of adjacent primers of opposite polarities. Following this approach, only sequences corresponding to the full-length circular RNAs were amplified. Sequencing of the corresponding cDNA clones confirmed that the RNA species moving faster in the denaturing gel had deletions affecting different extensions of three of the four arms of the cruciform structure of lowest free energy proposed for the 275 nt viroidlike RNA (1). These results can be explained by a copy choice mechanism similar to that proposed in viral RNA recombination and particularly in the generation of DI RNAs, which involves a template switching during RNA synthesis. This switching could be caused by the low degree of processivity of the RNA polymerase and by regions of strong secondary (and perhaps tertiary) structure where RNA polymerases usually pause (2). On the other hand, the RNA species moving slower in the denaturing gel had sequence duplications, one of them almost coincidental with one of the deleted regions observed previously, and another one encompassing the arm of the cruciform structure of the 275 nt viroidlike RNA which is conserved in all the smaller RNA species. This arm, therefore, appears to be involved in some critical function since it is not dispensable. A copy choice mechanism most probably also operates in the generation of the repeated sequences.

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BIOLOGICAL PROPERTIES OF CHIMERIC PLUM POX POTYVIRUS OBTAINED BY *IN VITRO* RECOMBINATION

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Plum pox potyvirus is the agent responsible for one of the most important diseases of trees of the *Prunus* genus. Its genome is formed by a 10 kb single stranded, positive-sense RNA molecule, which is expressed through its translation into a unique polyprotein that undergoes extensive proteolytic processing. Genetic manipulation of the PPV genome has been facilitated by the availability of a full-length cDNA clone from the Rankovic isolate, from which infectious RNA can be synthesized *in vitro* (Riechmann *et al.*, 1990).

cDNA from the PPV PS has been cloned and the complete genomic sequence of this isolate has been determined. Analysis of the reported sequences of PPV isolates indicated the existence of three PPV strains. Two of them, one including the Rankovic isolate and the other the PS one, seemed to be responsible for the two kinds of disease observed in the field. The sequence analysis showed that one of the PPV isolates could be originated by a natural homologous recombination event from viruses of these two strains. A full-length PS cDNA clone yielding infectious RNA transcripts has been obtained. This clone, together with that of the Rankovic isolate previously prepared, has allowed the construction of hybrid molecules containing sequences of two PPV strains, with the aim of localizing the determinants of their biological properties. The chimeric viruses were infectious, indicating that the proteins and the regulatory elements of the two viruses are compatible and that hybrid proteins encoded by the chimeric viruses are functional. PPV-PS and PPV-Rankovic caused similar kind of symptoms in several herbaceous hosts but largely differed in the symptom severity. The analysis of the infections caused by the parental and the different hybrid viruses indicated that the degree of severity seems not to be directly related with the level of virus accumulation, and that the determinants of pathogenicity involved in the different pattern of infection observed reside in the central region of the PPV genome. The capsid protein, the most divergent one between the PS and Rankovic isolates, and that has been shown to contain important pathogenicity determinants in other cases, and the 3' noncoding region seemed to be not relevant for the type of PPV infection in the herbaceous plants employed. Experiments are in course to map more precisely the PPV factors responsible for the differences observed in the infections of the herbaceous plants and to analyze the determinants of pathogenicity of the disease in the natural woody hosts.

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INCREASE OF FITNESS ASSOCIATED TO A RECOMBINATION EVENT THAT RESTORES THE HOMOLOGOUS 3'END IN PSEUDORECOMBINANTS BETWEEN CUCUMBER MOSAIC AND TOMATO ASPERMY CUCUMOVIRUSES.

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Cucumber mosaic virus (CMV) and tomato aspermy virus (TAV) are two closely related viruses with a tripartite, single-stranded, plus sense RNA genome. Viable pseudorecombinants can be obtained by exchanging genomic segments 1+2 (encoding replication functions) and 3 (encoding movement and structural functions) of the two viruses. We obtained in this way pseudorecombinants between hungarian aisolates of CMV and TAV. The analysis of the pseudorecombinant having RNAs 1+2 of CMV and 3 of TAV (C1C2T3) shows that the 3'end of TAV RNA3 has been substituted for the 3'end of CMV, becoming homologous with those of RNAs 1 and 2. The exchanged portion includes the tRNA-like structure, that differs between TAV and CMV, and is the recognition site for the viral replicase. Competition experiments showed that the reombinant T(c) RNA3 had an increased fitness, when replicated by CMV replicase (that is, in the context of CMV RNAs 1+2) over non recombinant TAV RNA3.

RNA recombination is probably a common phenomenon in nepoviruses.

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Two features of the genomic organization of tomato black ring nepovirus (TBRV) and its close relative grapevine chrome mosaic virus (GCMV) suggest the existence of RNA recombination as a common phenomenon in nepoviruses.

The genomic RNA-2 of a english isolate (ED) of TBRV has been sequenced in its cloned cDNA form, and comparison of the predicted amino-acid sequence of its encoded polyprotein with that of GCMV shows a strong sequence homology in an internal domain whereas homology to TBRV strain S is not significantly higher in this region than anywhere else. A close look at the nucleotide sequence in this region indicates that this could be the result of a recombination event between these two virus species. Such a possible rearrangement in the region coding for a polyprotein has also been described between two strains of another picorna-like virus, Plum Pox Potyvirus (PPV, Cervera et al., *J. Gen. Virol.* **74**, 329-334, 1993).

A striking feature of the genomic organization of nepoviruses is the virtual identity in the extremities of their two genomic RNAs. This is the case both in the 5' and 3' regions, including parts of the coding region, in viruses such as tomato ringspot virus (Rott et al., *Virology* **185**, 468-472, 1991), in the 3' non coding region only of viruses such as TBRV and GCMV (Dodd & Robinson , *J. Gen. Virol.* **68**, 973-978, 1987) and is not observed in viruses such as grapevine fanleaf or arabis mosaic viruses. Previous results recently obtained in our laboratory show that the 3' region of pseudorecombinants obtained by exchange of genomic segments between GCMV and TBRV is rearranged after few generations.

Analysis of defective viral genomes in circulating populations of the hepatitis C virus (HCV).

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Hepatitis C virus (**HCV**) is a positive single-stranded RNA virus (10.000 bases) that replicates through an error-prone RNA polymerase laking proofreading activity and consequently has a high mutation rate. The rate of fixation of mutations has been estimated to be in the order of $1.8x10^{-3}$ substitution/nt/year, similar to that occurring in other RNA virus like HIV, Influenza, etc...). Analysis of the sequence populations from single isolates has shown the simultaneous presence of closely related, but non-identical sequences revealing the **quasispecies** nature of HCV genome.

In a recent study performed in our laboratory, we have observed the presence of viral sequences carryng in frame **"STOP"** codons, indicating the existence of cocirculating defective genomes; the frecuency of this phenomena has been found to be as high as 10% of analysed sequences.

The existence of these defective genomes might play an important role in the modulation of desease activity, and in the establishment of viral persistence (which occurs in more than 80% of infected individuals). One of the major questions regarding the presence of these STOP codons is to know whether they are mantained in the population by a complementation mechanism, or their presence merely reflects extremely high mutation rates and they represent essentially lethal mutations doomed to extinction.

In this work we have optimized a method for screening the existence of STOP codons in different genomic regions of cocirculating **HCV** populations.

Briefly, PCR amplified products corresponding to the **Core** (369 nts) and **NS3** (659 nts) regions were cloned in the expression vector pET5c in which the inserted fragment is under control of the T7 promoter which is inducible by IPTG in the bacterial strain *E.Coli BL21* (F^-, ompT, lon, $r^-_B m^-_B$). The size of the recombinant protein produced by each one of the bacterial clones, after induction by IPTG, are then analyzed by SDS-PAGE and western-blot.

The finding of truncated proteins represented by bands of a molecular weight lower than expected in comparison to the full-lengh protein indicate the presence of a STOP codon in their coding sequence. The clones corresponding to those truncated proteins are subsequently sequenced by the Sanger method.

Thusfur **sixty-two** clones corresponding to the **CORE** region have been analysed using this strategy; two of them (3%) expressed truncated peptides clearly lower than the MW of the cloned fragment obtained in all other clones (MW 18000) . From the **NS3** region, **sixty-five** clones have also been studied and four of them (6%) has been found to encode truncated proteins than the one obtained in the rest of the clones (MW 27000). Sequencing of these defective clones which currently under way will allow to determine whether these sequences coding for truncated proteins accumulate mutations at a higher frecuency than other sequences of the viral population with respect to the average sequence, this would support the hypotesis that a complementation mechanism helps to mantain the defective sequences in the viral populations.

DEFECTIVE INTERFERING MUTANTS FROM CUCUMBER MOSAIC VIRUS RNA3

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We are interested in the protection of plants against virus diseases. One possible strategy is to prepare transgenic plants that express defective interfering (DI) RNA that could interfer and reduce the virus replication. In that way Cucumber Mosaic virus (CMV) is a choice virus because it is a positive sense RNA plant virus with a broad host range. CMV contains 3 genomic RNA 1, 2 and 3. RNA 3 has two open reading frames encoding 3a protein and coat protein separated by a inter-cistronic region that contains a subgenomic promoter for production of RNA 4.
The first step in this aim consisted in the cloning of several kind of DI to define the sequence liable to reduce virus replication so that outcome RNA accumulation Using CMV RNA 3 cloned dowstream from T_7 DNA pol. promoter some DI were obtained. I*n vitro* DI transcripts were co-electroporated with CMV virus in Tobacco protoplast. But the Boccard and Baulcombe (1993) publication describing the characterization of cis-acting sequences necessary for RNA 3 accumulation lead us to prepare directly DI in a binary vector pKYLX71. The cloning in pKYLX71 is current dowstream from the 35S CaMV promoter.
The RNA 3 DI in pKYLX71 will be transfered in tobacco plant via Agrobacterium. The transformed plant will be tested for their resistance to virus infection.

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IN VITRO EVOLUTION OF TGE CORONAVIRUS

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Porcine transmissible gastroenteritis virus (TGEV) is an enteropathogenic coronavirus that infects pigs causing a transient diarrhoea in adults and a mortality close to 100% among newborn animals. The objective of this communication is to study the *in vitro* evolution of TGE virus. TGEV was isolated for the first time in 1946. Non-enteropathogenic PRCVs have been derived from TGEV. The genetic relationship among PRCVs and TGEV has been determined based on their RNA sequences. An evolutionary tree relating 13 enteric and respiratory isolates has been proposed. According to this tree a main virus lineage evolved from a recent progenitor which was circulating around 1941. From this, secondary lineages originated enteric and respiratory isolates. Least squares stimation of the origin of TGEV-related coronaviruses showed a significant constancy in the mutation fixation rate. This rate was $7\pm2 \times 10^{-4}$ nucleotide substitutions per site and per year and falls into the range reported for other RNA viruses. In vivo, TGEV causes persistent infections in lung cells.

In vitro evolution of the virus was studied in order to determine genetic variability and molecular bases of persistence. Enteric isolates (PUR 46), as well as respiratory isolates (NEB 72, HOL87) can induce persistence in swine testis cell cultures. In these persistently infected cells aproximately 50% of the cells produced viral structural antigens, as has been seen by immunofluorescence. Infectious virus production was 10^7-10^8 PFU/ml. Northern blot analysis of viral mRNAs produced in persistently infected cells showed no difference with viral mRNAs of the original cytophatic virus. Cloning of persistently infected cells led only to negative clones for the production of virus, and the expresion of viral antigens. Persistently infected cells were resistant to superinfection by respiratory and enteric isolates. This fact could the base for the induction of intracelular immunity.

In vitro TGEV evolution was also studied by serial undiluted passages of enteric (PUR 46, PUR 46 mar 1CC12) and respiratory isolates (HOL87) in ST cells. After 30 passages at high multiplicity of infection, three new subgenomic RNA species of 10.5, 12 and 24 kb were detected in one of the three isolates (PUR 46). These RNAs were stable after 15 undiluted passages in ST cells and significantly interfered replication of wt virus. These RNAs contained sequences of the 5' and 3' -ends of the wt genome, indicating that they could be defective genomes. These RNAs were also encapsidated and it was possible to separate wt and defective virions in a sacarose gradient centrifugation. cDNAs from these RNA defective genomes are being synthesized, cloned and sequenced. All defective genomes lacked structural protein genes, and contained at least 400 nucleotides of the 3' -end wt genome. The potencial of these defective virus as coronavirus-based vectors to induce mucosal immunity without producing disease will be discussed.

Mechanism of RNA-RNA Recombination in Brome Mosaic Virus: Role of Sequence Homology and Complementarity

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Mutations introduced into the 3' noncoding region of RNA3 of brome mosaic virus (BMV), a plant tripartite RNA virus, are efficiently repaired by intersegment RNA-RNA recombination (reviewed by Bujarski et al., Adv. Virus. Res., 43, 1994). Both homologous and nonhomologous crossover events were observed. Comparison of sequences at the site of crossovers for a large number of independently generated BMV recombinants suggested different structure of recombination intermediates for the two types of recombinants.

To investigate structural requirements of nonhomologous recombination in BMV, a unique RNA3-based recombination vector was constructed. Short sequences derived from RNA1 were inserted in 'antisense' orientation into this vector. These inserts were found to facilitate the formation of RNA1 - RNA3 nonhomologous recombinants in C. quinoa. The crossover sites clustered at one side of the heteroduplex formed between the complementary sequences of the vector RNA3 and the wt RNA1 substrates. Modifications within this region affected the location of crossovers. This could be explained by template switching of the replicase at strong double-stranded regions (Nagy and Bujarski, Proc. Natl. Acad. Sci. USA, 90: 6390-6394, 1993).

A 60 nt 3' conserved sequence derived from BMV RNA2 was inserted into the recombination vector in 'sense' orientation. This facilitated the generation of homologous RNA2-RNA3 recombinants in C. quinoa. Subsequent deletions in this sequence revealed that sequence identity between RNA2 and RNA3 (as short as 5 nt) facilitated homologous recombination between the RNAs, but the incidence of recombination was greatly reduced. The effect of mismatches on homologous recombination was studied by introducing point mutations into the above 60 nt RNA2-derived sequence. We observed that one or two mismatches did not detectably affect the efficiency of recombination or the selection of sites of crossovers. However, three or more mismatches decreased the efficiency of homologous recombination. In addition to precise homologous recombinants, in which recombination did not result in addition or deletion of nucleotides at the crossover sites, aberrant homologous recombinants were observed. The latter had one or two nucleotides deleted or inserted at the crossover sites when compared with the homologous parental sequences. In contrast to nonhomologous recombinants, the aberrant homologous recombinants had homologous parental sequences flanking the sites of crossovers. In conclusion, three different types of recombinants were identified in BMV. Models for generation of these recombinants will be presented.

RNA recombination between replication impaired mutants of the associated RNA of the ST9 strain of beet western yellows virus

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Encapsidated RNA of the ST9 strain of the luteovirus beet western yellows virus (ST9-BWYV) is composed of not only a 5.6 kb genomic RNA (ST9gRNA) but also an additional 2.8 kb associated RNA (ST9aRNA). Full length cDNA clones of both RNAs were constructed and transcripts (tST9gRNA and tST9aRNA) were shown to be infectious in tobacco protoplasts. tST9aRNA also infects leaves on which it is rub inoculated, but does not spread in the plant. The presence of ST9aRNA has been shown to increase the accumulation of ST9gRNA in both protoplasts and plants and is directly correlated with an increase in symptom severity when compared to infections initiated by ST9gRNA alone. The ST9aRNA has three large open reading frames (ORFs). Frame-shift mutations were made individually in each of these three ORFs the result of which is to introduce stop codons within a few amino acid residues from the site of the mutation. Transcript RNAs from these three mutations still replicate when electroporated into tobacco protoplasts, but at very reduced levels when compared to wild type ST9aRNA. We are currently infecting tobacco protoplasts with combinations of these mutant ST9aRNAs and analyzing them for recombination. This analysis is by a PCR method we developed which can discriminate between the wild type and mutant sequences.

Studies on the RNA-binding properties of the influenza virus Nucleoprotein. A. Portela, and C. Albó. Instituto de Salud Carlos III. Centro Nacional de Microbiología. 28220 Majadahonda. Madrid. Spain.

In the viral particle, the influenza virus genome is covered over its entire length by a viral encoded protein -the Nucleoprotein (NP)- to form ribonucleoprotein (RNP) complexes. To gain insight into the RNP structure we decided to study the RNA-binding properties of the NP. To this end, the NP was purified from RNP complexes by centrifugation in CsCl-glycerol gradients, and the purified protein, essentially devoid of RNA, was then tested in two different assays. In the first assay, the RNA-binding specificities of the NP to ribonucleotide homopolymers poly(G), poly(A), poly(U), and poly(C) at various salt concentrations were investigated. The purified protein bound to all RNA homopolymers at 0.1 M NaCl. However the protein displayed a clear binding preference for poly(U) since most of the NP remained bound to this homopolymer at 1 M NaCl.

We have also examined the capability of the protein to protect the RNA from the action of different RNases. A synthetic labeled RNA was incubated with either NP or BSA, treated with several ribonucleases and the protected RNA fragments were analyzed by electrophoresis in acrylamide gels. The results obtained showed that the NP was able to protect the RNA, at least partially, against digestion by RNase A, RNase ONE, and micrococcal nuclease.

Currently, we are preparing a series of mutant cDNAs encoding truncated versions of the influenza virus NP. We intend to test the RNA-binding activity of the mutated polypeptides and map the RNA binding domain of the protein.

COMPLEMENTATION STUDIES ON TRANSGENIC PLANTS OF A LETHAL MUTANT OF TOBACCO VEIN MOTTLING POTYVIRUS.

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We have recently generated a collection of four-codon insertion mutants of a potyvirus (tobacco vein mottling virus, TVMV) by manipulation of a cDNA clone from which infectious RNA can be transcribed in vitro. The inserted sequences do not interrupt the potyviral polyprotein open reading frame. The inoculation of tobacco protoplasts and plants with RNA transcribed from this collection of mutant plasmids has allowed us to identify a number of apparently lethal mutations: these include each of the ten mutants obtained in the P3/CI/6K/NIa/NIb region of the TVMV genome and, in adition, one mutant in the $P1^{Pro}$ gene and one mutant in the HC^{Pro} gene (1).

We are interested in assesing whether the expression of TVMV genes in transgenic tobacco plants can complement the defects in replication of the mutant viral RNAs. This type of experiment will provide information on the ability of some of the proteins to perform their function(s) in trans and also on the possibility of recombination between a defective potyviral genome and a transgenically produced transcript.

In this study we have used a transgenic tobacco line (the gift of David Thornbury, University of Kentucky) that expresses the $P1^{Pro}/HC^{Pro}$/P3 coding regions of TVMV, in conjunction with our lethal $P1^{Pro}$(616) TVMV mutant. The results show that mutant $P1^{Pro}$(616) can generate infections in about 20% of inoculated transgenic tobacco plants where it may be able to be replicated to a sufficient level to generate a number of sequence variants some of which are able to restore wild-type-like infectivity.

RECOMBINATION BETWEEN DEFECTIVE TOMBUSVIRUS RNAs GENERATES FUNCTIONAL HYBRID GENOMES.

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RNA recombination is recognized as a process capable of mediating both the repair and evolution of RNA virus genomes. For plant viruses, only the bromovirus and carmovirus groups have been definitively shown to undergo *trans*-RNA recombination. The tombusviruses, a group of small RNA plant viruses, generate replication-competent deletion mutants of their genomes during infections [*i.e.* defective interfering (DI) RNAs]. To test whether tombusvirus RNAs were capable of recombining in *trans*, protoplasts were co-inoculated with *in vitro*-generated transcripts of a nonreplicating 3'-truncated genomic RNA (gRNA) of cucumber necrosis tombusvirus (CNV) and DI RNAs of tomato bushy stunt tombusvirus (TBSV). Two dominant recombinant viral RNA populations (ca. 3.0 and 4.8 kb) composed of various large contiguous 5' segments of the CNV gRNA fused to 3'-terminal regions of the TBSV DI RNA were generated. The 3.0 kb recombinants were capable of autonomously replicating in protoplasts, but could not infect whole plants. Some of the 4.8 kb recombinants were able to systemically infect plants and induced wild type symptoms. A functional recombinant (ca. 4.8 kb) was also generated *in planta* after direct coinoculation of whole plants with the two defective RNA components. These results definitively demonstrate that (i) recombination can occur between tombusvirus RNAs (ii) nonreplicating RNAs can take part in recombination and (iii) DI RNAs can participate in recombination-mediated repair of virus genomes. Possible mechanisms for the formation of the recombinants are proposed and evolutionary implications are discussed.

Expression in transgenic plants of the 3' untranslated region of the turnip yellow mosaic virus (TYMV) genome or of defective interfering RNA derived from the TYMV genome

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Turnip yellow mosaic virus (TYMV) is the type member of the tymovirus group; its genome is composed of a single (+) sense RNA molecule. It has been shown that synthesis of TYMV (-) genomic RNA *in vitro* is inhibited by RNAs bearing the 3'-terminal 100 nucleotides of the TYMV genome that include the initiation site for (-) strand synthesis. Our aim was to evaluate the potential of RNAs that include the initiation site for synthesis of (-)-strand viral RNA to protect transgenic plants against virus infection by interference with viral RNA replication.

We have introduced into *Brassica napus* genes coding for transcripts containing the 3' untranslated region of the viral genome. This sequence expressed in transgenic plants confers protection against mechanically-inoculated virus or viral RNA. One interesting feature of the protection is that a proportion of the inoculated transgenic plants does not become infected. Moreover, this sequence is recognized by the viral replicase in infected plants and serves to initiate synthesis of an RNA complementary to the initial transcript.

We have also constructed and introduced genes coding for artificial defective interfering RNA (DI RNA) derived from the TYMV genome. These DI RNA are constructed with replication origin of the genome initiating (-) strand and subgenomic RNA synthesis. Transgenic plants expressing artificial DI RNA have been regenerated and are currently tested for their reaction to viral infection.

List of Invited Speakers

Workshop on

GENETIC RECOMBINATION AND DEFECTIVE INTERFERING PARTICLES IN RNA VIRUSES

List of Invited Speakers

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Workshop on

GENETIC RECOMBINATION AND DEFECTIVE INTERFERING PARTICLES IN RNA VIRUSES

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11 **Lecture Course on Conservation and Use of Genetic Resources.**
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Organized by C. S. Goodman and F. Jiménez. Lectures by J. Bolz, P. Bovolenta, H. Fujisawa, C. S. Goodman, M. E. Hatten, E. Hedgecock, F. Jiménez, H. Keshishian, J. Y. Kuwada, L. Landmesser, D. D. M. O'Leary, D. Pulido, J. Raper, L. F. Reichardt, M. Schachner, M. E. Schwab, C. J. Shatz, M. Tessier-Lavigne, F. S. Walsh and J. Walter.

18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
Organized by C. Nathan and A. Celada. Lectures by D. O. Adams, M. Aguet, C. Bogdan, A. Celada, J. R. David, A. Ding, R. M. Fauve, D. Gemsa, S. Gordon, J. A. M. Langermans, B. Mach, R. Maki, J. Mauël, C. Nathan, M. Röllinghoff, F. Sánchez-Madrid, C. Schindler, A. Sher and Q.-w. Xie.

19 Workshop on Viral Evasion of Host Defense Mechanisms.
Organized by M. B. Mathews and M. Esteban. Lectures by E. Domingo, L. Enjuanes, M. Esteban, B. N. Fields, B. Fleckenstein, L. Gooding, M. S. Horwitz, A. G. Hovanes-sian, M. G. Katze, I. M. Kerr, E. Kieff, M. B. Mathews, G. McFadden, B. Moss, J. Pavlovic. P. M. Pitha, J. F. Rodríguez, R. P. Ricciardi, R. H. Silverman, J. J. Skehel, G. L. Smith, P. Staeheli, A. J. van der Eb, S. Wain-Hobson, B. R. G. Williams and W. Wold.

20 Workshop on Genomic Fingerprinting.
Organized by McClelland and X. Estivill. Lectures by G. Baranton, D. E. Berg, X. Estivill, J. L. Guénet, K. Hayashi, H. S. Kwan, P. Liang, M. McClelland, J. H. Nadeau, D. L. Nelson, M. Perucho, W. Powell, J. A. Rafalski, O. A. Ryder, R. Sederoff, A. J. G. Simpson, J. Welsh and H. Zischler.

21 Workshop on DNA-Drug Interactions.
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

22 Workshop on Molecular Bases of Ion Channel Function.
Organized by R. W. Aldrich and J. López-Barneo. Lectures by R. W. Aldrich, C. M. Armstrong, P. Ascher, K. G. Beam, F. Bezanilla, S. C. Cannon, A. Castellano, D. Clapham, A. Ferrús, T. Hoshi, A. Konnerth, R. Latorre, J. Lerma, J. López-Barneo, R. MacKinnon, G. Mandel, A. Marty, C. Miller, B. Sakmann, B. Soria, W. Stühmer and W. N. Zagotta.

23 Workshop on Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids.
Organized by C. M. Thomas, E. M. H. Wellington, M. Espinosa and R. Díaz Orejas. Lectures by J. C. Alonso, F. Baquero, P. A. Battaglia, P. Courvalin, F. de la Cruz, D. K. Chattoraj, E. Díaz, R. Díaz Orejas, M. Espinosa, J. C. Fry, K. Gerdes, D. R. Helinski, B. Hohn, E. Lanka, V. de Lorenzo, S. Molin, K. Nordström, R. W. Pickup, C. M. Thomas, J. D. van Elsas, E. M. H. Wellington and B. Wilkins.

24 Workshop on Deterioration, Stability and Regeneration of the Brain During Normal Aging.
Organized by P. D. Coleman, F. Mora and M. Nieto-Sampedro. Lectures by J. Avila, Y.-A. Barde, P. D. Coleman, C. W. Cotman, Y. Lamour, M. P. Mattson, A. Matus, E. G. McGeer, B. S. Meldrum, J. Miquel, F. Mora, M. Nieto-Sampedro, V. H. Perry, M. P. Rathbone, G. S. Roth, R. R. Sturrock, H. B. M. Uylings and M. J. West.

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