Instituto Juan March
de Estudios e Investigaciones

12 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Reverse Genetics of Negative Stranded RNA Viruses

Organized by

G. W. Wertz and J. A. Melero

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L. A. Ball
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R. Cattaneo
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REVERSE GENETICS OF NEGATIVE STRANDED RNA VIRUSES

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INTRODUCTION

G. W. Wertz and J. A. Melero

Negative stranded RNA viruses comprise a wide group of viruses of both medical and economic importance. Until recently, the experimental potential of reverse genetics or the ability to engineer changes into negative strand RNA genomes at the cDNA level has not been possible. The main obstacle to the development of such techniques was the prerequisite of primary transcription by the viral transcriptase prior to expression of the gene products that make the replication machinary such that naked genomic RNA transfected into cells, or synthesized intracellularly from transfected cDNA clones, could not be replicated without a source of the viral nucleocapsid and polymerase proteins.

This situation changed dramatically a few years ago with the description by Luytjes et al. (1) of the first system in which a synthetic RNA, containing regulatory sequences of a influenza virus genome segment, could be introduced into cells and amplified with a helper virus. This was followed shortly afterwards by the generation of reassortants containing "in vitro" synthesized RNAs that incorporated site-specific mutations (2). The segmented nature of the influenza genome facilitated the manipulation of a subset of viral genes that, after "in vitro" transcription, could be introduced into cells where reassortement with RNA segments of a helper virus occurred at high frequencies. The advantage of having a segmented genome is shared by some other families of negative stranded RNA viruses, such as bunyaviruses and arenaviruses, where the basic technology developed for influenza virus might

be applied in the future.

The development of reverse genetics systems for the manipulation of the non-segmented genomes of rhabdovirus and paramyxovirus represented, however, another technological challenge. These viruses have large non-segmented negative stranded RNA genomes (12,000-15,000 nucleotides) that had to be cloned and manipulated as a single piece, since recombination frequencies in these virus families are undetectable. It is not surprising then that the first developments with these viruses took advantage of defective VSV genomes, made of smaller RNA segments containing regulatory sequences for replication and encapsidation (3,4). These small RNAs, obtained from viral populations or produced in cells from cDNA copies, could be amplified and encapsidated in cells that expressed the right combination of viral proteins. Alternatively, small synthetic RNAs of Sendai (5) or human RSV (6), that incorporated a reporter gene flanked by regulatory 3' and 5' sequences, could be introduced into cells to be amplified and encapsidaded with the help of wild type virus.

With this scenario, the Workshop on "Reverse Genetics of Negative Stranded RNA Viruses" was an opportunity for experts in the field to gather and discuss recent progress. The meeting was hold in the Madrid premises of the "Instituto Juan March de Estudios e Investigaciones", where we had the invaluable assistance of Andrés Gonzalez and his staff to create the right atmosphere for many fruitfull discussions.

G.W. Wertz and J.A. Melero

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Segmented Genome RNA Virus Replication and Transcription

DIFFERENTIAL REGULATION OF INFLUENZA A VIRUS RNA EXPRESSION BY A NUCLEOTIDE WITHIN THE vRNA PROMOTER

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The genome of influenza A viruses consists of eight negative sense RNA segments. Each RNA segment is packaged in nearly equal molar amounts in viral particles. In contrast, the amount of mRNA expressed by each segment varies dramatically. Sequence analysis reveals that the only difference in the conserved 5'- and 3'-terminal regions is the fourth nucleotide from the 3'-end of genomic RNA (vRNA). Specifically, nucleotide 4 is a U in the NP, HA, M and NS segments, and a C in the PB1, PB2, PA and NA segments. In order to test whether nucleotide 4 is involved in the control of transcription of the influenza A virus genome, we analyzed the level of transcription and replication of different segments of the influenza A virus genome. We then mutated nucleotide 4 of the HA or NA segment by site-directed mutagenesis. This was possible through the use of the ribonucleoprotein transfection system which allows us to introduce cDNA-derived RNA into the genome of influenza viruses. Using viruses in which nucleotide 4 at the 3' end of either the HA or the NA gene has been changed, we found that mRNA expression is indeed modulated by alterations in this region of the promoter. It should be noted, however, that sequences outside of the conserved terminal regions also contribute to regulation of influenza virus gene expression.

A STUDY OF THE INFLUENZA A VIRUS RNA PROMOTER IN VITRO

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The control of transcription and replication in influenza virus is not well understood. Although there is now good evidence that the conserved 12 and 13 nucleotide sequences at each end of the 8 virion RNA segments are required, it is still unclear how exactly these promoter sequences (the 13-long sequence is not actually the promoter but rather the template for synthesis of the cRNA promoter) cooperate with the influenza RNA polymerase complex. This consists of PB1, PB2 & PA and regulates mRNA, cRNA & vRNA, which are synthesized in differing amounts at different times during the influenza life-cycle. In particular, (1) it has been suggested that the nucleoprotein (NP) may regulate the switch from mRNA to cRNA synthesis by acting as an anti-terminator and (2) various authors have proposed that a 'factor', in addition to PB1, PB2 and PA, is required for efficient vRNA synthesis, although as yet this has not been fully characterized.

We are attempting to dissect the promoter sequence, itself, in the hope of finding clues to regulatory sequences. Using influenza cores depleted of endogenous vRNA by micrococcal nuclease digestion, and short usually 14-nucleotide long model RNA templates we observe synthesis of complementary RNA with ApG, with globin mRNA or even without any added primer (albeit at lower efficiency) when vRNA promoter templates are used. In our assay the cRNA promoter is weaker than the vRNA promoter, but it can still be easily assayed with ApG as a primer[1]. The activity of the nuclease-treated cores seems to be superior to polymerase prepared by the classical CsCl centrifugation method, and viral rescue has been achieved by ourselves and others using this newer methodology[1,2].

A detailed study of the effect of mutating each of the 12 nucleotides in the vRNA promoter on promoter strength has shown that only the (5′) CUG (3′) sequence (positions 9-11 inclusive counting from the 3′-end of the template RNA) is crucial for activity. Mutations at other positions decreased promoter strength marginally or not at all[3]. Since the sequence CUG is not present in the cRNA promoter, it was of interest to test if there was a different critical sequence there. The mutational analysis of the 13-long cRNA promoter showed that the related sequence (5′) CUU (3′) at positions 10-12 was crucial for promoter strength. In the cRNA promoter studies there was evidence that position 13 might be important as well. The deletion of a U residue at position 10 of the cRNA promoter, which introduces a (5′) CUG (3′) triplet, strongly increased promoter strength. Our preliminary conclusion is that this different triplet in the vRNA and cRNA promoter explains the decreased promoter strength which we observe in vitro with ApG as a primer.

In our model for transcription and replication of the vRNA promoter, we have proposed that initially one of the polymerase subunits binds to the CUG triplet at nucleotides 9-11 in the promoter. This, we suppose, effectively positions the catalytic site of the polymerase complex and the cap binding protein PB2 at the 3′ end of the template, so as to initiate transcription with capped fragments or to initiate replication. Replication may be the default pathway if a supply of capped fragments is unavailable, possibly due to their binding preferentially to the influenza nucleoprotein, - a non-specific single-stranded RNA binding

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protein synthesised in the infected cell, rather than to PB2. Alternatively, capped fragments might still bind to PB2 but their endonucleolytic cleavage might be blocked by the nucleoprotein.

A specific binding assay, which involves UV cross-linking of the vRNA promoter sequence to micrococcal nuclease treated cores, has been devised and the results of this test, which support the hypothesis of a polymerase binding site within the vRNA promoter, will be presented.

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A REVERSE GENETICS APPROACH TO STUDY THE TRANSCRIPTION AND REPLICATION OF INFLUENZA VIRUS RNA

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The genome of influenza type A virus consists of eight RNA segments of negative polarity. Their transcription and replication takes place in the nucleus of the infected cells, in RNA nucleocapsids that contain at least the three subunits of the polymerase (PB1, PB2 and PA) and the nucleoprotein (NP).

Our group has been interested in the mechanisms of influenza virus RNA transcription and replication, as well as in their consequences on the generation of genetic heterogeneity in virus population. In the past, we had developed *in vitro* systems for viral RNA transcription and replication (del Rio et al., 1985; Lopez-Turiso et al., 1990) and we had determined the mutation rate of the virus to the *mar* genotype (Valcárcel and Ortín, 1989). In order to analyze the structure-function relationships in the transcription/replication complexes and to understand the mechanisms responsible for the regulation of RNA transcription and replication, we have expressed the influenza virus genes from cDNA copies in a variety of expression systems. When the subunits of the viral polymerase and the NP are expressed by means of SV40 recombinant viruses, they are active in the *in vivo* synthesis of a transfected, *in vitro* synthesized virion RNA (de la Luna et al., 1993). These results confirmed that PB1, PB2, PA and NP make up the minimal set of viral genes required for RNA transcription and replication and showed that *in vitro* reconstitution of a viral ribonucleoprotein is not essential for its expression when the gene functions are provided in *trans* by SV40 recombinants.

The availability of monoclonal antibodies specific for the PA polymerase subunit allowed the study of its nuclear transport when expressed by a SV40 recombinant virus, in the absence of influenza virus infection. By means of deletion analysis and site-directed mutagenesis, as well as by the PA-mediated transport of a reporter protein, it could be concluded that the nuclear accumulation of the influenza virus polymerase PA subunit is mediated by an unusual protein structure in which at least two nuclear localization signals, apparently located in different domains of the protein appear to be involved (Nieto et al., submitted). This structural complexity might be related to the PA protein delayed translocation to the nucleus in the influenza virus infectious cycle and to the involvement of other viral genes in the transport process (Nieto et al., 1992).

Finally, the splicing and transport of mRNAs specific for the viral segments 7 and 8 have been studied when cDNA copies of these genes are transcribed *in vivo* by RNA polymerase II. Our results indicate that the M1 mRNA synthesized from a DNA recombinant clone is spliced to both M2 mRNA and mRNA3 and associates efficiently to the nuclear matrix, while the influenza virus transcript does not. The efficiency of splicing to render M2 mRNA is higher in SV40 recombinant virus than in influenza virus infected cells, while the opposite is true for mRNA3. A fraction of the unspliced M1 mRNA is transported to the cytoplasm of recombinant virus-infected cells (Valcárcel et al., submitted).

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SYNTHESIS OF BIOLOGICALLY ACTIVE INFLUENZA VIRUS CORE PROTEINS USING A VACCINIA VIRUS-T7 RNA POLYMERASE EXPRESSION SYSTEM.

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Several procedures that allow reconstituting influenza virus ribonucleoprotein complexes using purified viral core proteins isolated from virions and synthetic RNAs have been described (Luytjes et al, 1989; Yamanaka et al, 1991; Seong and Brownlee, 1992). The reconstituted nucleocapsids have been shown to be: i) functional *in vitro* because the encapsidated RNA can be transcribed by the virus specific replicase and ii) biologically active because the encapsidated RNA can be amplified, expressed and packaged into viral RNA particles when transfected into cells infected with a helper influenza virus. However, in contrast to other negative-stranded RNA virus systems, the synthetic naked RNA was not biologically active when transfected into influenza virus infected cells. In our laboratory, we also described an alternative method to reconstitute functional RNPs, which showed the same properties as those previously reported, using influenza core proteins purified from infected cell extracts (Martín et al, 1992.).

Since then we have been working with the long term aim of developing a system that allows packaging synthetic genes into viral particles without needing purified viral proteins and influenza helper virus. In this regard we have developed a system that allows expression of a synthetic influenza virus-like RNA, containing the antisense coding region of CAT gene flanked by virus genome 5' and 3' termini, using cloned viral genes and a vaccinia recombinant expressing T7 RNA polymerase (vTF7-3). Previously, the four influenza virus genes encoding nucleoprotein, PB1, PB2 and PA polypeptides were cloned under the control of a T7 promoter. Significant levels of CAT activity were detected in cell lysates prepared from cultures infected with vTF7-3 and transfected with the plasmids encoding influenza virus proteins and the synthetic virus-like RNA. Omission of any of the four viral proteins resulted in no detectable CAT activity. However, unlike the situation found when using influenza virus as helper, previous encapsidation of the synthetic RNA with purified viral

proteins was not required for expresion of the transfeced gene. Thus, this system can be used for analyzing the functions of the indivual influenza proteins required for the expression of the transfected RNA.

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Characterization of Bunyamwera Virus L Protein Expressed by Recombinant Vaccinia Virus Systems.

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Bunyamwera virus is the prototype of the family Bunyaviridae and has a tripartite negative-sense RNA genome. The largest genome segment, L, encodes the L protein, the medium-sized segment, M, the two virion glycoproteins G1 and G2, and a nonstructural protein, NSm, and the smallest segment, S, encodes the nucleocapsid protein, N, and a second nonstructural protein, NSs. We have expressed cDNAs representing each RNA segment in recombinant vaccinia virus systems, and have analysed the proteins produced; here the characterization of the expressed L protein will be described. Previously we demonstrated that the recombinant L protein was functional in terms of RNA synthesis activity in a nucleocapsid transfection assay (1): cells infected with recombinant vaccinia viruses expressing L protein were transfected with purified Bunyamwera virus nucleocapsids (prepared by banding in CsCl gradients) and RNA synthesis monitored by Northern blotting. Further work showed that transiently expressed L protein (infection of cells with a recombinant vaccinia virus expressing T7 RNA polymerase followed by transfection with plasmid DNA containing the L gene under T7 promoter control) also had RNA polymerase activity. Site-specific mutations were made in the L cDNA-containing plasmid to change certain amino acids in the putative polymerase domain of the L protein, and the effects of these substitutions on RNA synthesis activity were monitored in the nucleocapsid transfection assay. These experiments showed that residues strictly conserved between the L proteins of different viruses in the family Bunyaviridae were obligatorily required for activity, whereas nonconserved residues could be substituted without abolishing RNA synthesis capability(2).

Analysis of the 5' termini of Bunyamwera virus S segment mRNAs by cloning and sequence analysis revealed the presence of nonviral, heterogeneous sequences 12 -17 bases long. This is similar to reports for other members of the Bunyaviridae and is taken to indicate that mRNA transcription is primed by a "cap-snatching" mechanism. The 3' end of the Bunyamwera virus S mRNA was mapped using an RNase protection assay to 100-110 nucleotides upstream of the 3' end of the template. We analysed the RNAs made by the recombinant L protein in the nucleocapsid transfection system and showed that positive-sense RNAs contain 5' non-viral sequences. Hence the initiation of mRNA transcription by the recombinant L protein resembles that seen during authentic bunyavirus infection and suggests that the L protein has the endonuclease activity which generates the primers. Some of these

positive-sense transcripts terminated at the mRNA termination site but the majority read-through to the end of the template. No primer sequences were found at the 5' termini of negative-sense RNAs synthesized by the recombinant L protein. The recombinant L protein was able to replicate negative-sense RNA supplied by transfected virion-derived nucleocapsids and both positive- and negative-sense RNAs were synthesized. These results indicate that the recombinant L protein has both transcriptase and replicase activities.

The long term aim of our studies is to create a helper-independent system, based on recombinant vaccinia virus expressed proteins, to replicate and package syntheic bunyavirus-like RNAs. Our progress towards this goal will be reported.

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STRUCTURE AND EXPRESSION OF A BUNYAVIRUS (UUKUNIEMI VIRUS) GENOME.

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Uukuniemi virus is a member of the *Phlebovirus* genus within the *Bunyaviridae* family. Like all bunyaviruses, it contains a single-stranded RNA genome split up in three segments, L, M, and S, each coding for specific proteins. The L segment (6423 nucleotides) encodes the RNA-dependent RNA polymerase (2103 amino acids)(1), the M segment (3229 nts) a precursor (p110; 1008 residues) to the G1 and G2 spike proteins (2), and the S segment (1720 nts) the nucleocapsid protein (N; 254 residues), and a nonstructural protein (NSs; 273 residues)(3). While the virion L and M segments are of negative polarity, the S segment is transcribed into two subsegmental mRNAs (encoding N and NSs) in an ambisense manner. All three RNA segments have a stable circular configuration due to inverted complementary sequences at the 3' and 5' ends.

Our recent work has focused on the elucidation of the genome structure and strategy of gene expression, with particular emphasis on the S segment. Analyses of the 5' and 3' ends of the N and NSs mRNAs revealed heterogenous host-derived sequences 8 to 20 nucleotides in length. Primer extension of the M mRNA also indicated extra 5'-end nucleotides. Thus, Uukuniemi virus uses a cap-snatching mechanism for the priming of transcription similar to the one originally described for orthomyxoviruses. The 3' ends of the S segment-derived mRNAs were found to overlap each other by some 100 nucleotides. No clear transcription termination signal could be identified around the termination sites (4).

NSs expressed in insect cells was purified by SDS-PAGE and used to prepare a monospecific affinity-purified antibody. NSs was localized dispersely in the cytoplasm, showing a punctate pattern by immunofluorescence. No NSs was found in the nucleus, nor in purified virions. Sucrose gradient fractionation indicated that NSs expressed either in virus-infected cells, in BHK cells by a T7 RNA polymerase-driven vaccinia virus expression system, in recombinant baculovirus-infected Sf9 cells, or *in vitro* in a reticulocyte lysate associated with the 40 S ribosomal subunit. The association was resistant to a salt concentration below 1.0 M NaCl, suggesting a protein-RNA interaction (5). Despite these new data, no clues as to the function of the NSs have so far been obtained. This is true also for other bunyaviruses. A role in cap-snatching, transcription initiation, replication, or packaging all are plausible possibilities.

It is clear that further advances in understanding the molecular biology of bunyaviruses requires the development of a reverse genetics system. The prime targets for genetic manipulations lie in the 5' and 3' termini of each segment. These sequences are complementary to each other, and they are strictly conserved not only between each segment, but also between viruses within the same genus. The mRNAs of the bunyaviruses so far studied are shorter than the corresponding virion segments and are thus unable to circularize and to become packaged. Therefore, the terminal sequences are likely to be important for the regulation of transcription, replication, and packaging.

We are currently constructing recombinant plasmids encoding the L, p110, N, and NSs proteins under the T7 promoter. Specific antisera are available against the G1,G2, N, and NSs proteins, and antisera against the L protein are at present being produced. Thus, the basic tools for establishing a reverse genetics system are now at hands. Our aim is to introduce the above plasmids in various combinations into cells infected with the T7 RNA polymerase-encoding vaccinia virus, and transfected with a reporter plasmid expressing an artificial RNA segment. This approach has recently been successfully applied to the VSV system (6). The transcribed RNA will have various lengths of the authentic 5' and 3' ends of the S RNA segment, and specify the synthesis of a reporter protein, such as chloramphenicol acetyltransferase (CAT). If successful, this system will hopefully allow us to determine the role of the individual proteins (notably L, N, and NSs) in replication and transcription. Furthermore, mutations in the terminal sequences may unravel the nucleotides crucial for replication/transcription initiation.

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Complex structure of the nuclear translocation signal of influenza virus polymerase PA subunit.

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Transcription and replication of the influenza virus RNA takes place in the nucleus of the infected cells in ribonucleoprotein complexes (RNP). Each of the proteins in the RNP complex, as well as the non-structural proteins NS1 and NS2, accumulate in the nucleus of the infected cells, both in the productive viral infection and when they are independently expressed from cloned versions of the genes.

The nuclear localization signals (NLS) of some of these proteins have been identified, as is the case for PB1, PB2, NP and NS1. We have identified the regions of PA protein involved in its nuclear translocation by a combination of deletion analysis and site-directed mutagenesis of the protein expressed from a SV40 recombinant virus, as well as by the PA-mediated transport of a reporter protein. Three regions appear to play a role in the process: region I (amino acids 124-139) where a nucleoplasmin-like nuclear translocation signal has been identified, region II (amino acids 154-157) which contains a series of casein-kinase II potencial phosphorylation sites and region III (amino acids 186-247). Besides the nucleoplasmin-like NLS present in region I, an additional NLS appears to be present in regions II-III, altough no consensus targeting sequence can be detected. Alterations in any of the regions identified by introducing either point mutations or short deletions, completely prevents nuclear transport, while elimination of the region I or II+III by large amino- or carboxy-terminal deletions, does not abrogate nuclear targeting of the truncated protein. Chimeric plasmids containing amino-terminal sequences of PA fused to ß-galactosidase show that the 280 first aminoacids of PA polymerase subunit are sufficient to direct the translocation of a reporter protein to the nucleus. These results suggest a complex organization of the nuclear translocation signal of the PA subunit in which two independent NLSs are present and must lie in a precise position within the three dimensional structure of the protein to permit nuclear transport.

BIOLOGIC IMPORTANCE OF THE NEURAMINIDASE STALK LENGTH IN INFLUENZA A VIRUS

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To investigate the biologic importance of the neuraminidase (NA) stalk of influenza A virus, we generated viruses with stalks of various lengths (0 - 52 amino acids), using the recently developed reverse genetics system. These mutant viruses, including one that lacked the entire stalk, replicated in tissue culture to the level of the parent virus, whose NA stalk contains 24 amino acid residues. In eggs, however, the length of the stalk was correlated with the efficiency of virus replication: the longer the stalk the better the replication. This finding indicates that the length of the NA stalk affects the host range of influenza A viruses. The NA stalk-less mutant was highly attenuated in mice: none of the animals died even after intranasal inoculation of 10^6 plaque-forming units (PFU) of the virus (the dose of the parent virus required to kill 50% of mice was $10^{2.5}$ PFU). Moreover, the stalk-less mutant replicated only in the respiratory organs, whereas the parent virus caused systemic infection in mice. Thus, attenuation of the virus with the deletion of the entire NA stalk raises the possibility of its use as live vaccines.

ROLE OF CONSERVED CYTOPLASMIC SEQUENCE OF INFLUENZA A VIRUS NEURAMINIDASE

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The neuraminidase (NA) of influenza A virus is a type II glycoprotein anchored in the membrane by its amino terminus. In contrast to the rest of the NA domain, the 6 amino acid cytoplasmic tail is highly conserved in all of the influenza A NA subtypes. This extreme sequence conservation suggests that the sequence is critical to the function of this domain, which is currently unknown. To examine the relevance of the conserved sequence in the cytoplasmic tail, we replaced either a single amino acid residue or the entire cytoplasmic tail of an influenza A virus (A/WSN/33) with influenza B virus tail residues, using the reverse genetics procedure established by Palese and colleagues. All of the "single amino acid" mutants were rescued with the exception of the mutant in which we replaced the conserved proline. This suggests that the proline may play an important role in the structure of the cytoplasmic tail. We also could not rescue the mutant which lacks the cytoplasmic tail. However, we were able to rescue a chimeric WSN NA (FLUBCYT) containing the complete cytoplasmic tail of the influenza B virus NA instead of its own. These results suggest that the conserved cytoplasmic tail sequence of influenza A virus is not critical for virus production. Because the virus with the FLUBCYT NA was more attenuated in mice than the parent virus, we compared the incorporation of the NA molecule into the virion. The amount of the NA in FLUBCYT was significantly reduced as compared with the parent virus. These findings indicate that conservation of the cytoplasmic tail sequence is required for efficient incorporation of the NA molecules into the virion.

Nonsegmented Genome RNA Virus Replication and Transcription

The Conventional Genetics of Negative Stranded RNA Viruses

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A review of the genetic properties of negative stranded RNA viruses may provide information that will assist the development of a universal technology of reverse genetics for these viruses. The characteristics of recombination of RNA genomes *in vivo*, for example, is clearly relevant to the exploitation of any system of reverse genetics. So far reverse genetics has only been achieved with those negative strand RNA viruses which have a nuclear phase in their replication cycle; whether that is relevant or not remains to be seen.

In cultured cells recombination has been observed to occur at moderate to high frequencies among positive stranded RNA viruses belonging to the families *Coronaviridae, Picornaviridae* and *Togaviridae*, and in some instances linear genetic maps concordant with physical maps have been derived by classical methods. *Inter alia* such *in vitro* experiments have revealed; that at any one time only a minority of cells in a culture may be competent to support mixed infection and yield recombinants (*Aphthavirus*); that apparent specific recombination sites in the genome may be the consequence of strong selective forces eliminating the majority of recombinants (*Coronavirus*); and that recombination may involve the negative template strand (*Poliovirus*).

It is also clear that recombination plays a role in the evolution of these viruses in nature. For example, intratypic recombinants of poliovirus have been recovered from the faeces of children soon after adminstration of live triple vaccine. The alphavirus, Western equine encephalitis, appears to be a recombinant of Eastern equine encephalitis virus and Sindbis virus. And cytopathic strains of the pestivirus, bovine viral diarrhoea virus, carry specific insertions of a host genomic sequence.

Reassortment of genome sub-units is universal in negative stranded RNA viruses of the families *Arenaviridae*, *Bunyaviridae* and *Orthomyxoviridae*. In the *Bunyaviridae*, which is the most diverse family of the segmented genome negative stranded RNA viruses, reassortment of the tripartite genome is restricted to serologically related viruses. Even in crosses of genetically compatible viruses the pattern of reassortment of genome subunits may diverge dramatically from the random expectation (1). Elucidation and circumvention of these restrictions to allow controlled reassortment of subunits would provide a limited form of reverse genetics in these viruses. Likewise in mixed infections of influenza viruses gene reassortment is restricted to viruses of the same serotype. Although convergent evolution cannot be excluded, in evolutionary terms more complex genetic interactions appear to have occurred. For example, the 6/7 segmented acarine orthomyxoviruses, Thogoto and Dhori, have four

genes that exhibit homology with the P, NP and M1 genes of influenza viruses, whereas their single glycoprotein shows similarity to the gp64 of baculoviruses. Rare instances of *in vivo* intermolecular recombination have also been recorded in the orthomyxoviruses; the clearest example being the influenza A virus possessing an HA gene with an insertion of nucleotides apparently derived from 28S ribosomal RNA.

By contrast, recombinant viruses have never been identified in the progeny of mixed infections of the non-segmented genome negative stranded RNA viruses. The three families of negative stranded RNA viruses with non-segmented RNA genomes, the *Filoviridae*, *Paramyxoviridae* and *Rhabdoviridae*, exhibit common structural and organisational features which have allowed them to be grouped as the Order *Mononegavirales*. The absence of recombinants is usually attributed to the rapid association of nucleoprotein and template RNA during replication in the cytoplasm which may reduce opportunity for intermolecular interactions. Nonetheless the prevalence of defective viruses with internal deletions or permuted quasi-recombinant genomes in the progeny of negative stranded RNA viruses propagated at high multiplicity suggests that recombination is possible and that other mechanisms may operate to prevent recovery of viable recombinants. The mechanisms may include adverse selection, superinfection exclusion, and host cell competence.

Comparative studies of viral genome sequences also suggest that recombination generating non-defective progeny may occur as a rare event. One possible example of such an event was the abrupt appearance and rapid dissemination of a novel avian paramyxovirus, turkey rhinotracheitis virus, in Europe in 1985. This virus exhibited a gene order different from the highly conserved gene order of all other members of the family *Paramyxoviridae* so far characterised (2). Turkey rhinotracheitis virus proved to be a pneumovirus with a translocation of the SH and G gene pair in the gene order. The only other recognised pneumoviruses, the respiratory syncytial viruses infecting man and ungulates and pneumonia virus of mice, are endemic in their natural hosts and retain the characteristic gene order of paramyxoviruses. The appearance of this novel pneumovirus may be a consequence of some unique genetic event resulting in an abrupt expansion of the host range of pneumoviruses.

Analysis of the components of the genomes of non-segmented negative stranded RNA viruses has depended on the classification of conditional lethal mutants into groups by complementation, followed by assignment of phenotypes (3.4). Subsequently, complementation tests making use of mutants with assigned phenotypes have been employed as a convenient procedure for establishing the functional integrity of products expressed from recombinant vectors. (An example is the rescue of the tsN1 M-defective mutant of respiratory syncytial virus by superinfection with vaccinia virus/M gene recombinants in the presence of phosphonoacetic acid). Conversely, the phenotypes of unassigned conditional lethal mutants could be established by

complementation with infectious viral vectors carrying individual non-mutant genes; but this approach remains unexploited so far.

More commonly the functions of individual gene products have been investigated by expression from normal and specifically mutated sequences *in vitro*. In favourable circumstances fortuitous sequence changes generated during the course of the cloning and amplification of genes by reverse transcription and PCR can be utilised to explore gene functions by *in vitro* transcription and translation (5). However, the error proneness of specific template sequences limits the universal applicability of this approach. For example, the carboxy-terminal third of the attachment (G) protein gene of respiratory syncytial virus has been found to be unusually prone to transcription errors *in vivo* and *in vitro*, resulting predominantly in frameshfts due to insertion or deletion of adenosine residues in some but not all runs of such residues (6).

Mastery of reverse genetics will facilitate the analysis of individual gene functions and assist the rational development of new vaccines. A prerequisite for the latter, however, will be knowledge of the genetic determinants of virulence. Although achievable for animal pathogens by direct experimentation, this type of information is rarely available for human viruses and the potential for manipulation of the genome of human viruses by reverse genetics in vaccine development may be limited. In anticipation of the development of reverse genetics, an attempt is being made to identify determinants of virulence by nucleotide sequencing the entire genome of a modified strain of respiratory syncytial virus. This modified strain, which exhibits reduced virulence and near-normal immunogenicity in adults, was developed by stepwise induction of three temperature-senstive mutations at diminishing restrictive temperatures. Provisional data suggests that this approach is feasible.

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REPLICATION OF POSITIVE AND NEGATIVE STRANDED VIRAL RNAs SYNTHESIZED INTRACELLULARLY FROM SPECIALIZED TRANSCRIPTION PLASMIDS

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The application of reverse genetics to RNA viruses requires a method for the intracellular delivery of RNA transcripts that are competent to replicate. Our approach to this problem was to design and construct specialized DNA plasmids which could be transcribed in cells to yield RNAs that resembled as closely as possible the authentic genomic RNAs of positive and negative stranded RNA viruses. Two types of such plasmids were developed: in the first, a promoter site for bacteriophage T7 RNA polymerase was followed immediately by a full-length viral cDNA, and this was followed in turn by sequences encoding a *cis*-acting autolytic ribozyme and a transcriptional terminator. When transfected into cells that expressed T7 RNA polymerase from a vaccinia virus recombinant, circular plasmids of this structure directed transcription of RNAs whose 5′ and 3′ ends were determined by the position of the T7 promoter and the site of autolytic cleavage, respectively.

Using the ribozyme from the antigenomic strand of hepatitis delta virus (HDV), we made plasmids that yielded RNAs of the positive strand virus flock house virus (FHV) and of a DI particle of the negative strand virus vesicular stomatitis virus (VSV) which had perfect 3′ termini. These RNAs replicated efficiently when provided with the appropriate *trans*-acting factors that were necessary to catalyze RNA replication in the two systems, opening the way to analyses of the *cis*-acting sequences required for RNA genome replication in these two viruses.

In the second type of transcription plasmid, viral cDNAs were flanked by inverted copies of the HDV ribozyme and placed downstream of a T7 promoter site. In this arrangement, autolytic cleavage generated perfect 3′ ends both for the primary transcript and, after RNA replication, for its complement. Efficient replication was observed for FHV RNAs expressed from this type of plasmid. The development and application of this approach to studies of the mechanism of RNA replication in FHV and VSV will be presented.

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INFECTIOUS DEFECTIVE INTERFERING PARTICLES OF VSV GENERATED FROM TRANSCRIPTS OF A cDNA CLONE: ANALYSIS OF CIS AND TRANS-ACTING FACTORS IN VSV RNA REPLICATION AND VIRUS ASSEMBLY

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Negative stranded RNA virus genomes not only require an RNA dependent RNA polymerase for infectivity but also the RNA genome itself must be encapsidated with the viral nucleocapsid protein in order to be an efficient template for the polymerase. These considerations have severely hindered the application of reverse genetic methods to the analysis of cis-acting signals in RNA replication and in the regulation of gene expression of these viruses.

We carried out transcription of a complete negative stranded genomic RNA from a cDNA clone of a VSV DI particle directly in cells that were simultaneously expressing the five VSV proteins from separately transfected cDNA clones. By this means, the negative stranded transcript was encapsidated with N protein to form a functional template and was replicated by the VSV polymerase. No helper VSV was required. Replication occurred at high levels and could be assayed by direct biochemical means. Efficient encapsidation of transcripts required the expression of both the N and P protein. Replication of the encapsidated transcript to produce progeny plus strand RNA and subsequent amplification to produce negative strand RNA required expression of the VSV N, P and L proteins. The molar ratios of the proteins relative to one another were critical for efficient replication. Furthermore, the replicated RNAs were assembled and budded to yield infectious DI virions. Budding of infectious virions required the expression of all five VSV proteins. If M or G were omitted, infectious particles were not budded.

An exact 3' terminus of the initial transcript was generated by autolytic cleavage using a ribozyme from hepatitis delta virus and was found to be critical for replication. In contrast, up to four additional, heterologous nucleotides could be present at the 5' end of the initial transcript without compromising replication. Importantly, however, these extra nucleotides were not present at the 5' ends of the RNA products replicated from the initial transcript, indicating that the VSV RNA

polymerase repaired the extension either by termination of synthesis of the initial positive strand RNA prior to the extra nucleotides or by initiation of synthesis of the new negative strand internally, directly at the first VSV specific nucleotide. The effects of alterations of the terminal sequences of the RNA on encapsidation and replication were analyzed. The results of these experiments will be discussed.

The minimum requirements for assembly and budding of infectious RNA-containing particles were analyzed by deletion of increasing regions of the genome RNA. The deleted versions of the genome were analyzed for their ability to: 1) replicate and 2) assemble and bud infectious virions. The implications of these results for virus assembly will be discussed.

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The Mechanism of Paramyxovirus P Gene mRNA Editing.
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Almost all paramyxoviruses modify their P gene mRNAs by G insertions during synthesis, to create frameshifts which fuse downstream overlapping ORFs to an N-terminal portion of the gene. This process is referred to as mRNA editing. Different viruses have different editing patterns which appear to be matched to their ORF expression requirements. For example, Sendai and measles virus predominantly insert a single G to access the V ORF, bPIV3 inserts 1 to 6 Gs at equal frequency to accesss both the V and D ORFs, and SV5 and mumps virus predominantly insert 2Gs to access the C-terminal portion of the P ORF.

Based on editing site sequence comparisons, and mRNA synthesis experiments <u>in vitro</u> in which the NTPs were varied, a stuttering model has been proposed for this form of editing. For Sendai virus, where the G is inserted opposite a run of 3 Cs on the template, we propose that the transcriptase would pause after copying the middle C, then slip back or upstream by 1 base still carrying the 3'end of the nascent chain. When synthesis resumes, one G will be inserted as the middle template C will now be copied twice. For bPIV3, the slippage-insertion will be repeated 1 to 6 times. Since SEN and bPIV3 use basically the same editing sequence, this suggests that the difference is due to a counting mechanism which determines the number of slippage-insertion events. For SV5 and mumps virus, a 1 base slippage is disfavored because an A:C pair would be formed in the mis-alignment intermediate, whereas a 2 base slippage creates only U:G pairs; hence a 2 base slippage is then the predominant event.

To test this model, we have made a DNA plasmid which expresses a SEN mini-genome which itself is designed to express a mini-mRNA containing the editing site. The 5' end of the mini-genome is defined by a T7 promoter and the 3'end by a ribozyme. Viral encapsidation and expression of the mini-genome is provided via cloned and T7-promoted NP, P, and L genes, and T7 polymerase via a recombinant vaccinia virus. One construct, containing 103 nt of the P gene, was found to produce mini-mRNAs which were edited identically to those in a natural infection (50% of the mRNA containing a 1G insertion, 2G insertions representing <5% of the mRNAs). Results of experiments in which the sequence at the insertion site were altered by point mutations, and by substituting the editing regions of mumps virus and bPIV3 for that of SEN, will be discussed.

RESCUE OF SYNTHETIC ANALOGS OF RESPIRATORY SYNCYTIAL VIRUS GENOMIC RNA. P. Collins*, L. Kuo, K. Dimock, M. Mink, D. Stec, and H. Grosfeld, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, 20892 USA

Respiratory syncytial virus (RSV) differs substantially from more-extensively studied nonsegmented negative strand RNA viruses such as vesicular stomatitis and Sendai viruses in numerous aspects of its proteins and genomic RNA (vRNA). A vaccine for this important pediatric pathogen remains to be developed. We will present a progress report on work with the previously-described[1] vRNA analog RSV-CAT.

RSV-CAT vRNA is a cDNA-encoded, truncated version of vRNA in which nt 87-15043 of the 15,222-nt vRNA have been deleted, removing all of the viral orfs, and have been replaced with the orf for bacterial chloramphenicol acetyl transferase (CAT). The CAT orf is flanked on the upstream end by the leader region and the gene-start and nontranslated sequences of the NS1 gene and on the downstream end by the nontranslated and gene-end sequences of the L gene and the trailer region. RSV-CAT vRNA was synthesized *in vitro* by run-off transcription from linearized cDNA (or from a cDNA encoding a ribozyme motif adjacent to the 3' end to mediate cleavage of the product RNA). When transfected into tissue culture cells and complemented by superinfection with standard RSV, RSV-CAT was "rescued" as evidenced by the expression of CAT and by the production of infectious particles which could be passed multiple times onto fresh cells.

Reverse transcription in conjunction with the polymerase chain reaction provided evidence that the predicted polyadenylated subgenomic mRNA is produced, supporting the idea that the so-called gene-start and gene-end sequences indeed are transcriptive signals. A positive-sense version of RSV-CAT RNA, corresponding to replicative intermediate RNA, was equally active in rescue.

The *cis*-acting sequences in the leader region and flanking NS1 nontranslated gene sequence have been mapped by (i) "scanning" mutagenesis involving the insertion of a foreign hexanucleotide at intervals of 3-5 nt, (ii) "saturation" mutagenesis involving changing each sequence position in turn to each nucleotide alternative (the 3'-terminal 27 nt have been analyzed), and (iii) the deletion of segments from, or insertion of variously-sized heterologous segments into, the leader region.. Results to date have identified two circumscribed signals (called "boxes") within the leader region. The gene-start signal that immediately follows the leader region was found to be a third insertion-sensitive sequence, whereas the remaining adjacent NS1 nontranslated sequence was tolerant of insertions. Also, the leader region sequence immediately preceding the gene start signal could be deleted with minimal effect. Thus, the A residue which has been noted to precede almost all gene start signals is not

obligatory.

We anticipated that the 3'-terminal 26 nt of vRNA would be highly sensitive to mutagenesis because its sequence is well-conserved (83% identical) with that of the 3' end of replicative intermediate RNA. Indeed, the above-mentioned two boxes are within these 26 nt. However, there were four surprising findings. First, the 3'-terminal four nt of RSV-CAT vRNA could be removed with little effect on rescue and, at the other (5') end (which encodes the 3' end of replicative intermediate RNA), a single nt could be removed with little effect. It may be (i) that these terminal nt indeed are not needed, or (ii) that they are repaired by some highly efficient mechanism (we have not yet examined the rescued vRNAs for evidence of repair), or (iii) that the effects are of a subtle nature that remain to be revealed. The effect of deletion from both ends was equal to the sum of the individual deletions, suggesting that the two ends were affected independently. This spoke against a model in which a deletion at the 3' end might be repaired using the 5' end as template. The second finding was that the spacing of the two above-mentioned boxes relative to each other and to the 3' end could tolerate some variability and thus did not appear to be critical. Third, within this 26-nt conserved 3'-terminal sequence, nt 10-20 were tolerant of insertions, deletions and substitutions. Fourth, two substitutions at leader position 4 were "up-mutations" (10-20 fold increases in CAT expression and passage). The corresponding changes in the trailer region were without similar effect, indicating that, despite their functional interchangeability[1], the *cis*-acting signals in the 3' ends of vRNA and replicative intermediate RNA are not identical. It seems odd that these up-mutations do not occur naturally (or, at least, were not in the sequence that we determined for the parental vRNA). Perhaps there are deleterious effects that have not yet been detected. Or, there may be something about the mechanism of synthesis of the first four nt that constrains their mutability.

Dicistronic versions of RSV-CAT (containing CAT and the luciferase orf) have been constructed to analyze the functions of transcription signals and intergenic regions in stop-start transcription and readthrough replication.

In an accompanying paper, we describe similar studies with CAT-containing vRNA analogs based on human parainfluenza virus type 3 (PIV3), a representative of the second subfamily of paramyxoviruses. RSV and PIV3 share some common themes with regard to their *cis*-acting RNA signals. For example, the fact that biologically-active CAT-containing vRNA analogs can be constructed for both viruses indicates that in each case the *cis*-acting signals are located in the leader, trailer and flanking sequences at the vRNA termini and can be attached to foreign sequences without loss of function. However, mutational analyses in progress indicate that the distribution and sequences of the signals within these regions are different for the two viruses.

One difficulty of these systems is that the expression and replication of the cDNA-encoded vRNA analog occurs in the context of, and competes with, the much-more-extensive expression and replication of the helper virus. This has complicated the direct analysis of the analog-encoded RNAs. We attempted to improve the efficiency of the system by synthesizing RSV-CAT vRNA intracellularly from transfected cDNA using a vacccina virus recombinant as the source of T7 RNA polymerase. However, we found that vaccinia virus itself directed extensive expression of the transfected CAT gene. Expression appeared to be directed by vaccinia virus gene products because it was (i) independent of RSV helper virus coinfection, (ii) independent of expression of T7 RNA polymerase, (iii) independent of a T7 promoter, and (iv) independent of RSV-specific sequences. The ability of vaccinia virus to express a transfected CAT gene by seemingly illegitimate means has also been noticed by others[2]. We are now working on the use of (i) other expression systems to supply the T7 polymerase and (ii) other reporter genes.

We also are working to replace the RSV helper with expression vectors to supply the viral proteins required for complementation. This would allow for direct identification of the minimum constellation of expressed proteins required for nucleoencapsidation, transcription, replication and virion assembly.

Also, cDNA encoding the complete vRNA is being constructed to test for the ability to direct expression of replication-competent RSV. Successful rescue and passage was achieved for an RSV vRNA analog that is 49.3% of full parental length. This argues that a nascent status for vRNA is not an absolute requirement for its encapsidation. And, this supports the idea that it ultimately will be possible to rescue complete, nondefective RSV from cDNA-encoded RNA, perhaps even by our current, inefficient method of RNA transfection.

The fact that functional chimeric RSV (and PIV3) gene cassettes and chimeric vRNAs can be constructed indicates that these viruses are surprisingly amenable to engineering and also bodes well for the idea of producing virus from engineered cDNA. The information from the mutagenesis studies and from sequence comparison of different RSV strains gives the picture of a genome organization in which the individual genes are self-contained, mRNA-coding cassettes that are separated by intergenic regions that appear to be without function. This sort of modular organization might make it possible to engineer individual genes without affecting the other genes, and might even allow the insertion new mRNA-encoding modules containing foreign orfs. If so, the engineering of nonsegmented negative strand viruses would be easier than is the case with, for example, adenovirus, which has overlapping transcriptional units, or poliovirus, which encodes a large polyprotein whose processing is sensitive to pertubation. Finally, these attributes suggest the idea that these viruses eventually might find use as expression vectors for certain purposes such as immunization or

gene therapy involving the respiratory tract.

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Abstract : Efficient replication of Sendai virus defective RNA molecules from plasmid DNA.

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A copy back defective interfering (DI) RNA was cloned and inserted into a pSP65 plasmid (pSV-DIH4) in such a way that the positive strand DI RNA was exactly synthesized by the T7 RNA polymerase after linearization of the plasmid at a BsmI site. The linearized pSV-DIH4 was subsequently transfected into CV1 cells in conjunction with pGem plasmids containing the Sendai virus NP, P/C and L genes under the control of the T7 RNA polymerase. These cells were equally infected with a vaccinia virus recombinant expressing the T7 RNA polymerase. Under these conditions the DI RNA expressed from the pSV-DIH4 was properly encapsidated and replicated by the Sendai virus proteins (see Calain et al., 1992. Virology 191, 62-71).
Subsequently, the delta virus ribozyme was introduced in the pSV-DIH4 in place of the Bsm1 site (pSV-DIH4/Rbz) to generate the exact 3' end of the DI RNA transcript. This procedure was shown to significantly increase the reproducibility and the efficiency of the DIH4 RNA expressed from the plasmid in such a system.

Attempts to modify the sequence of the DIH4 RNA by making internal deletions showed unequivocally that efficient DI RNA replication obeyed to precise rules as for the total length of the RNA transcript. Attempts to apply these rules to the replication of an internal deletion DI RNA will also be presented.

CIS-ACTING SIGNALS IN THE HUMAN PARAINFLUENZA VIRUS 3 GENOME.

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Analysis of *cis*-acting sequences in the genomic RNA (vRNA) of negative-strand RNA viruses has been hampered by the lack of methods for direct genetic manipulation. We constructed a cDNA encoding an internally-deleted version of the vRNA of human parainfluenza virus type 3 (PIV3) in which the viral genes were replaced by the bacterial chloramphenicol acetyl transferase (CAT) orf. The CAT orf was flanked upstream (3' relative to the encoded vRNA) by 111 PIV3 nucleotides including the 3' leader region, the NP gene-start signal, and the 5' nontranslated region of the NP gene, and was flanked downstream by 115 PIV3 nucleotides including the 3' nontranslated region of the L gene, the L gene-end signal, and the 5' trailer region. The T7 promoter was placed adjacent to the trailer region and an *Hga*1 site adjacent to the leader region such that run-off transcription from linearized plasmid would yield a negative-sense RNA, PIV3-CAT vRNA. In a second version of the cDNA, the positions of the T7 promoter and *Hga*1 site were reversed so that transcription would yield a positive-sense RNA, PIV3-CAT(+) RNA, representing replicative intermediate RNA. Each PIV3-CAT RNA contained correct termini, was 5.8% the length of PIV3 vRNA, and contained 226 nt of PIV3-specific sequence.

Transfection of PIV3-infected 293 cells with either RNA resulted in CAT expression and the incorporation of PIV3-CAT RNA into infectious particles. For PIV3-CAT(+) RNA, some of the CAT expression was independent of PIV3 and presumbly reflected direct translation of the input RNA, but the production of infectious PIV3-CAT particles was strictly dependent on helper PIV3. Passage of PIV3-CAT to fresh cells was blocked by incubation with PIV3-neutralizing antibodies. CAT expression by PIV3-CAT vRNA increased more than 3000-fold during nine serial passages, establishing that the synthetic RNA contained all of the *cis*-acting sequences required for efficient replication and transmission.

To further define the PIV3 sequences necessary for rescue of PIV3-CAT vRNA, mutational analysis was performed. Rescue was ablated by deletion of nontranslated NP sequences (excluding the gene-start signal) but was unaffected by deletion of nontranslated L gene sequences. This was not due to differences in translatability or stability of the predicted subgenomic mRNAs: thus, the NP nontranslated region appears to contain a sequence critical for replication and/or transcription. "Scanning" mutagenesis was performed by insertion of a heterologous dinucleotide at different sites within the leader and NP nontranslated regions. The leader region and leader-proximal part of the NP nontranslated region were highly sensitive to insertional inactivation, suggesting that the PIV3 *cis*-acting signals are more dispersed than are those of respiratory syncytial or influenza virus. "Saturation" mutagenesis is in progress in which, beginning at the 3' end of leader, each sequence position is changed systematically to the other nucleotide possibilities.

Also in progress are mutational analyses of the gene-end, intergenic and gene-start sequences that constitute the PIV3 gene junctions. These studies involve bicistronic vRNA analogs that encode a second marker enzyme, luciferase (luc). The CAT and luc coding sequences were placed separately under the control of PIV3 gene-start and gene-end signals, were separated by an intergenic region, and were flanked by the leader and trailer regions. Two versions were made that differed in gene order: 3'-CAT-luc-5' and 3'-luc-CAT-5'. To date, we have compared the different naturally-occurring junction sequences and also have tested the effects of deleting the intergenic triplet. Removal of the intergenic triplet ablated expression of the downstream gene, suggesting that it is critical for reinitiation during stop-start transcription. Remarkably, the same deletion elevated (5 to 10-fold) expression of the upstream gene: this might be explained by models in which (i) the polymerase recycles in the absence of the intergenic triplet, or (ii) the intergenic triplet is responsible for polymerase pausing such that its ablation results in increased mRNA completion. Disruption of the gene-end signal by the insertion of 8 nucleotides, mimicking the natural situation for the M gene, did not affect expression of the downstream gene but enhanced that of the upstream gene: the gene-end signal also might be involved in recycling or pausing such that its disruption increases transcription. Thus, the sequences at the junctions of PIV3 genes regulate the expression of the upstream gene as well as the downstream gene.

ANALYSIS OF THE PROMOTER OF ENCAPSIDATION OF THE RABIES GENOME

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We have developed different *in vitro* techniques to appreciate the interactions between RNA and proteins which are the essence of any biological activity in RNA viruses. This tool would help to undertake a functional analysis of the *Mononegavirales* genome. Based on the rabies *Lyssavirus* model, we have studied the promoter of encapsidation recognized by the first molecules of nucleoprotein (N) at the 5' end of the genome and antigenome.

By gel retardation assay, a leader RNA probe, produced by *in vitro* transcription, was shown to interact specifically with viral ribonucleocapsids (RNP) purified from infected cell extracts. By covalent photo-cross-linking with L.A.S.E.R. and subsequent SDS-PAGE, we characterized the polypeptide responsible for the interaction: it has the molecular weight of the viral N protein and is specifically immuno-precipited by an anti-N monoclonal antibody. The same specific interaction is obtained with a total cytoplasmic extract from infected cells.

Trying to localize more exactly the promoter, we found that the N protein binds with high affinity to the first 23 nucleotides of the leader RNA, and with less affinity to the 22 following nucleotides. In addition, we demonstrated a specific interaction between the rabies leader RNA and heterologous RNP from a very divergent *Lyssavirus*, Mokola virus. This allowed us to confine the promoter of encapsidation within the 15 extreme nucleotides, since the two viral genomes are only identical up to this position. However, the existence of an accessory site of lower affinity mapping in the region 20-40 cannot be totally ruled out. RNAse protection experiments and site directed mutagenesis are currently in progress to clarify these questions. These methods for studying interactions between RNA and proteins would be extended to identify viral or cellular (tissue-specific) factors regulating the expression of rabies virus genes.

RABIES VIRUS REPLICATION AND TRANSCRIPTION STUDIED BY EXPRESSION OF RECOMBINANT VIRAL PROTEINS.

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A rabies virus in vivo replication and transcription system was developed which takes advantage of naturally ocurring defective interfering particles (DIs). Helpervirus-free DIs are produced in cell lines complementing the DI deficiency and are then used to study the contribution of particular proteins to replication and transcription.
SAD DI1 is a genetically stable internal deletion type DI deficient for the L gene. The DI RNA is replicated and transcribed slowly in cells constitutively expressing low levels of rabies virus L protein. In order to determine the amount of protein necessary for optimal DI replication and transcription, L protein and in addition all other rabies virus proteins were expressed from recombinant Vaccinia virus and from plasmids using the Vaccinia/T7-Polymerase system in DI infected cells. After coexpression of recombinant N and NS proteins in addition to L, a dramatical increase of RNA synthesis rate and production of infectious DIs was observed indicating that the DI encoded N and NS proteins are suboptimal in function. Expression of high amounts of recombinant M protein led to inhibitory effects on DI replication. Using an "optimal replication mix" of recombinant proteins, we are now investigating the functions of engineered L proteins. In addition, attempts are being made to rescue synthetic DI RNAs from cells expressing recombinant rabies virus proteins.

FAITHFUL AND EFFICIENT IN VITRO RECONSTITUTION OF VESICULAR STOMATITIS VIRUS TRANSCRIPTION USING PLASMID-ENCODED L AND P PROTEINS

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In contrast to host cell gene transcripts which are synthesized by complex multisubunit polymerases and capped and methylated by additional polypeptides, non-segmented negative strand RNA viruses rely on only two viral polypeptides to achieve the same result: a large multifunctional polymerase protein (L) and a smaller accessory phosphoprotein (P). We have developed here an in vitro system which specifically and faithfully reconstitutes the vesicular stomatitis virus (VSV) transcription process using plasmid-encoded L and P proteins. The assay is based on adding purified nucleocapsid templates (N-RNA complex) to extracts of cells transfected with plasmids expressing viral proteins via the vaccinia-T7 RNA polymerase recombinant virus system. Under optimal conditions, the reconstituted system and the native virion core reaction behaved identically for all aspects of synthesis examined: ratio of transcripts produced, polyadenylation, and net synthesis per template. Moreover, the amounts of L and P proteins relative to template in the optimized reconstituted system were similar to those in virion cores and the complexes displayed similar specific infectivities suggesting that the stoichiometry of the transcription complex was also faithfully reconstructed. Notably, reconstitution was not dependent on cotranslation of P and L proteins in the same cell. About half as much activity was obtained by mixing equivalent amounts of extracts expressing each protein individually and this difference was probably due to stimulation of L protein accumulation by cotransfection with P plasmid (up to 5-fold in some experiments). Surprisingly, deleting a small region in the C-terminal half of the L polymerase protein (amino acids 1638 to 1673) abolished transcription as well as stimulation by P coexpression. Since the catalytic domain of the L polymerase almost certainly lies in the N-terminal half of the protein, these results suggest that the putative nucleotide-binding motif in the deleted segment may be involved in an accessory function essential for the transcription process.

Protein Function and Assembly

SELECTION AND CHARACTERIZATION OF A NEURAMINIDASE-MINUS MUTANT OF INFLUENZA VIRUS AND ITS RESCUE BY CLONED NEURAMINIDASE GENES

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A neuraminidase (NA) deficient mutant, designated NWS-Mvi, of the reassortant influenza virus A/NWS/33_{HA}-A/tern/Australia/G70C/75_{NA} (H1N9), was selected by passaging virus in MDCK cells in a medium containing neuraminidase from the bacterium *Micromonospora viridifaciens* and polyclonal antiserum against the influenza NA. Growth of the resulting mutant virus is dependent on addition of neuraminidase to the medium. Western blot analysis showed that the neuraminidase protein was absent from the mutant virus particles, and Northern hybridization showed that RNA segment 6, which contains the coding information for the NA, had undergone massive deletion. Viral protein synthesis in cells infected with the mutant virus was not dependent on addition of neuraminidase. In the absence of a functional NA, the NWS-Mvi mutant virus can infect MDCK cells with normal cytopathic effects. This neuraminidase-minus influenza virus serves as an excellent source of parent virus for reverse genetics experiments involving genes that encode a functional neuraminidase.

Assembly and replication of vesicular stomatitis virus -John Rose, Rebecca Burdine, Lisa Chong, Randall Owens, and Michael Whitt. Departments of Pathology and Cell Biology, Yale University School of Medicine, New Haven, Ct. 06510.

We investigated the interaction of the vesicular stomatitis virus (VSV) matrix (M) protein with cellular membranes *in vivo*. Approximately 90% of M protein, expressed without other VSV proteins, was soluble, while the remaining 10% was tightly associated with cellular membranes. The same distribution of M protein was observed in VSV infected cells. Preliminary membrane fractionation experiments indicated that M was associated mainly with the plasma membrane. Conditions known to release peripherally associated membrane proteins (2M KCl, pH 11.0, EDTA) did not detach the membrane-bound M protein from isolated membranes. Membrane associated M protein was soluble in the detergent Triton X-114 while soluble M protein was not, suggesting a chemical or conformational difference between the two forms. Co-expression with the VSV glycoprotein (G) and nucleocapsid protein (N) did not alter the fraction of M protein binding to membranes *in vivo*. Membranes containing associated M protein were able to bind RNP cores while membranes lacking M protein were not, suggesting that the bound M protein molecules are required for attachment of RNP cores to membranes during normal virus budding.

To study the domains of M protein required for membrane and nucleocapsid binding, we expressed truncated versions of M lacking the amino terminal basic domain, or the carboxy-terminal domain. M proteins lacking the amino terminal domain retained the ability to bind membranes *in vivo*, although the interaction was disrupted more easily than that of wild-type M. In addition, membranes containing the truncated M protein were unable to bind nucleocapsids, suggesting a role for the N-terminus in this interaction. Carboxy-terminal deletions had little or no effect on binding of membranes or nucleocapsids.

To examine the involvement of the cytoplasmic domain of the VSV G protein in viral assembly we studied incorporation of human immunodeficiency virus (HIV) envelope proteins into VSV virions. This study employed the system described by Whitt et al. (1989) which allows expressed envelope proteins to rescue phenotypically a temperature-sensitive

mutant of VSV (tsO45). A hybrid HIV-1/VSV protein containing the extracellular and transmembrane domains of the HIV-1 envelope protein fused to the cytoplasmic domain of VSV G protein was able to rescue the tsO45 mutant lacking the G protein, while the wild type HIV-1 envelope protein or an HIV-1 envelope protein with a truncated cytoplasmic domain the same size as the VSV cytoplasmic domain was not. The VSV(HIV) pseudotypes obtained infected only $CD4^+$ cells and were neutralized specifically by anti-HIV-1 sera. These results combined with our earlier results indicate that the cytoplasmic tail of the VSV glycoprotein contains a signal that is both necessary to direct the VSV G protein into virions, and sufficient to direct a foreign protein into virions.

To obtain a system in which VSV assembly and other aspects VSV replication can be studied in greater detail, we have been attempting to recover infectious VSV by expression of a DNA encoding the full length RNA genome in the presence of VSV proteins. These studies as well as attempts to obtain replication of subgenomic fragments of VSV will be discussed briefly.

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A REVERSE GENETIC APPROACH TO STUDY THE ORGANIZATION OF INCLUSION BODIES AND THE MATURATION OF SURFACE GLYCOPROTEINS OF HUMAN RESPIRATORY SYNCYTIAL (RS) VIRUS

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The expression of RS virus genes by a T7 polymerase-driven vaccinia virus system has been used to study the properties of some viral products independently of the replicative cycle. Three examples will be presented:

1.- Formation of inclusion bodies.- It has been known for long time that inclusion bodies are formed in the cytoplasm of cells infected with human RS virus (1). Their composition and significance were unknown. Immunofluorescence staining and immunoelectron microscopy of RS virus infected cells with specific antibodies indicated that the nucleoprotein (NP), the phosphoprotein (P) and the 22kd protein were components of the cytoplasmic inclusions. The expression of these viral genes, either individually or in different combinations, demonstrated that co-expression of the NP and P proteins was necessary and sufficient to induce the formation of cytoplasmic aggregates that resembled the inclusions of RS virus infected cells. Co-expression of the 22kd protein with either NP or P did not induce the formation of cytoplasmic aggregates. However, the 22kd protein was incorporated to the inclusions when co-expressed with both NP and P.

Immunochemical data revealed the presence of NP-P and NP-22kd protein complexes in extracts of RS virus infected cells. The same complexes were also detected in extracts of transfected cells that expressed the appropriate gene product combinations. This system is now being used to locate the protein domains of the different components involved in the formation of the cytoplasmic inclusions.

2.- Mutations that alter the maturation pathway of the F glycoprotein.- The expression of the RS virus F protein using vaccinia recombinants has been used to test its effectiveness in the induction of a protective immune response (2,3). To study the contribution of individual epitopes to protective immunity, we have expressed the F protein of mutants that escape neutralization by monoclonal antibodies. During the course of these studies, a mutant F protein was accidentally cloned that contained six amino acid changes. These mutations inhibited the proteolytic processing of the Fo precursor and its transport to the cell surface. Different vaccinia recombinants were then prepared to study the effect of the different mutations upon F protein maturation. The replacement of Phe237 by Ser, either alone or in combination with other mutations, was sufficient to inhibit the F protein transport to the cell surface. This mutant phenotype resembles that of a double mutant, recently described by Anderson et al. (4), with

the replacements Val301 to Ala and Val447 to Met.

When the vaccinia recombinant that expressed the F mutant Phe237-Ser was used to immunize BALB/c mice, these were fully susceptible to a RS virus challenge. In contrast, animals immunized with recombinants expressing either the wild type or other mutant F proteins were protected. The failure to induce protection correlated with the absence of serum antibodies that recognized the native protein and neutralized the virus. Thus, limited amino acid changes may have very profound effects upon the biological and immunological properties of the RS virus F glycoprotein. This should be of special concern when considering the design or evaluation of safe and effective RS virus vaccines.

3.- Hypermutatios, reiterative A-G substitutions, in the G protein gene of escape mutants.- Two escape mutants ($R_{16}C/1$ and $R_{16}C/10$) selected with a monoclonal antibody that recognize a conserved epitope of the G glycoprotein contain multiple nucleotide sustitutions (A-G) in the middle part of the G protein gene. These reiterative A-G changes resemble those found in other RNA viruses, in particular the multiple U-C transitions (in the positive sense) found in the matrix gene mRNA of measles virus from SSPE patients (5). Evidence will be presented to suggest that the multiple A-G changes in the G protein gene were generated by an alternative mechanism to that of misincorporations by an error prone polymerase.

The two escape mutants had lost one of the four cysteine residues which are located in the middle of the G protein ectodomain and that are conserved among all RS virus isolates. We are now trying to clone and express the mutated G proteins using the vaccinia-T7 transient expression system. This will allow us to explore the role of individual cysteines in both the formation of disulfide bridges and the maturation of the G glycoprotein.

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TYPE-SPECIFIC FUNCTIONAL INTERACTIONS BETWEEN PARAMYXOVIRUS HN AND F GLYCOPROTEINS

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We have been investigating the role of the fusion glycoprotein (F) and hemagglutinin-neuraminidase (HN) genes of parainfluenza viruses in the induction of cell fusion. Using the vaccinia-T7 transient expression system, a high level expression of the F and HN proteins was obtained in HeLa-T4 cells, which are relatively resistant to vaccinia virus-induced cytopathology.

The glycoproteins of parainfluenza viruses type 1 (PI1), type 2 (PI2), type 3 (PI3), Sendai virus (SN) and simian virus (SV5) have been expressed either singly or in combination in HeLa-T4 cells. Consistent with our previous results (Hu et al., 1992), cell fusion was observed in cells transfected with homologous F/HN combinations. With one exception, coexpression of heterologous combinations of F and HN proteins of any of these viruses did not result in cell fusion. The single exception was observed with the closely related PI1 and Sendai virus HN glycoproteins, either of which could interact with the Sendai F protein to induce cell fusion upon coexpression.

Cell fusion was abolished when cells cotransfected with F and HN were treated with exogenous neuraminidase, indicating that the attachment function of HN to sialic acid receptors is critical to cell fusion. Examination of the effect of the neuraminidase activity of HN on the F protein revealed that sialic acid was removed from the F protein by coexpression of either homologous or heterologous HN proteins, excluding the possibility that the effect of the HN neuraminidase activity on F could be involved in the F/HN type-specific interaction. To determine whether potential soluble molecules play a role in fusion, we transferred the supernatant from F-expressing cells to HN-expressing cells or vice versa. However, no effect of soluble factors was detected.

By cross-linking and immune precipitation of proteins expressed on the cell surface, we observed that anti-PI2 HN antiserum coprecipitated the PI2 F protein but did not precipitate coexpressed F proteins of other paramyxoviruses, suggesting that the homologous F and HN are closely associated with each other. A capping assay also indicated that the homologous F and HN may be physically associated, since PI3 HN was found to cocap with PI3 F but not with PI2 F. Taken together, these results suggest that the homologous F and HN glycoproteins may form a complex on the cell surface which is involved in cell fusion.

We have also begun to investigate the molecular domains of F and HN which are involved in their functional interaction. It was observed that truncation of the cytoplasmic tail of the PI3 F protein significantly decreased its cell fusion activity. Thus the intracytoplasmic domain of F appears to play a role in the biological functions exhibited by the protein's external domain.

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CELL FUSION BY THE ENVELOPE GLYCOPROTEINS OF PERSISTENT MEASLES VIRUSES WHICH CAUSED LETHAL BRAIN DISEASE

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Measles virus (MV) is one of the primary causes of infant death in third world countries, and a recent MV epidemic has caused more than one hundred deaths in North American inner cities. On rare occasions, MV persistence induces lethal syndromes of the central nervous system known as subacute sclerosing panencephalitis and measles inclusion body encephalitis (SSPE and MIBE) which are characterized by reduced expression of the viral envelope proteins, and by lack of viral budding. The MV envelope contains two integral membrane proteins, fusion (F) and hemagglutinin (H), and a membrane-associated matrix (M) protein. Reduced production of the viral envelope proteins in human brain infections can be ascribed partly to a steep gradient of transcription resulting in low level expression of the distally located genes M, F and H (2). On the other hand, in MVs replicating in brains of SSPE and MIBE patients, numerous mutational alterations of the envelope genes have been identified (1): Characteristically, certain mutations alter drastically the entire M protein and the F protein intracellular domain (5), whereas other more subtle mutations distinguish the extracellular domains of the F and H proteins (3).

Here we present evidence that truncation of the F protein intracellular domain does not impair fusion function and we suggest that this alteration interferes with viral budding. Unexpectedly, certain combinations of functional F and H proteins were unable to induce syncytia formation, an observation suggesting that specific F-H protein interactions are required for cell fusion. We also found that three of four H proteins of persistent MVs are defective in intracellular transport, oligosaccharide modification, dimerization, and fusion-helper function. Thus, MVs replicating in brains at the terminal stage of infection are

typically defective in M protein and in the two integral membrane proteins.

A plausible scenario for the role of mutational alterations of the MV envelope proteins in the development of SSPE or MIBE is the following. During acute disease a fully functional MV may cross the blood-brain barrier. Then, a mutation impairing viral assembly occurs. This mutation may be a prerequisite for lethal MV persistence since all characterized MVs from SSPE and MIBE show mutations throughout the entire M protein or the cytoplasmic domain of the F protein, or both (1). It appears likely that additional, more subtle mutations, like those in the extracellular domains of the F and H proteins, are necessary to allow a defective virus not only to persist in one cell or in a localized brain area, but to spread throughout the entire brain, overgrowing the wild type virus (4; 6).

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ACTIVATION OF INFLUENZA AND PARAMYXOVIRUS GLYCOPROTEINS BY SUBTILISIN-LIKE EUKARYOTIC ENDOPROTEASES

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Posttranslational proteolytic cleavage by arginine-specific proteases is essential for the infectivity of influenza virus and an important determinant for spread of infection and virus pathogenicity. Cleavage depends on the presence of an appropriate host protease and on the cleavability of the hemagglutinin. High cleavability requires the motif Arg-X-Lys/Arg-Arg at the cleavage site (1). The ubiquitous enzyme responsible for the activation of the highly cleavable hemagglutinin of fowl plague virus, the prototype of the pathogenic avian influenza virus strains, has been identified as furin, a member of a family of subtilisin-like endoproteases of eukaryotic cells (2). Furin is calcium-dependent and is localized in the Golgi apparatus. It is specifically inhibited by peptidyl-chloromethylketones containing the correct cleavage site motif. Other viral glycoproteins containing this motif, such as HIV1 gp (3) the F protein of NDV (4) and human parainfluenza virus 3, are also activated by furin. In contrast, the highly cleavable F-protein of a pantropic variant of Sendai virus (5) appears to be activated by a protease different from furin.

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FREQUENT REVERSION OF FRAME-SHIFT MUTATIONS SELECTED IN ESCAPE MUTANTS OF HUMAN RESPIRATORY SYNCYTIAL (RS) VIRUS: GENETIC INSTABILITY OF OLYGO-A SEQUENCES OF THE G PROTEIN GENE

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We have previously reported the isolation of RS viruses resistant to MAb 63G that contained reading frame shifts in the G protein gene, generated by deletions or insertions of single adenosines in short A runs (1). Mutants R63/1/2/3 and R63/2/4/8 had lost an A after nucleotides 588 and 623, respectively. Mutant R63/2/4/1 had also lost an A after nucleotide 623 but had an extra A seven triplets later.

The frame shifts of mutants with single deletions introduced drastic sequence changes in the G protein C-terminal third. Since these changes could impair the G protein function, we evaluated the stability of these viruses after serial in vitro passage. Only the double mutant R63/2/4/1 was stable after passage in the absence of MAb. Moreover, this mutant competed out the wild type virus when the progeny of mixed infections was passed serially in vitro.

The single mutants R63/1/2/3 and R63/2/4/8 reverted to the wild type phenotype after few passages in the absence of MAb 63G. This phenotypic reversion correlated with the insertion of an extra A at the site of the original deletion. No other sequence changes were detected in the revertants. However, mutant R63/2/4/8 was also unstable in the presence of MAb 63G, as indicated by the emergence of a positive reaction with antibodies (except 63G) that were lost in the original mutant. Sequence analysis indicated that the insertion of an extra A after nucleotide 648 was responsible of the antigenic changes. Thus, this "pseudorevertant" has two reading frame shifts in the G protein gene at exactly the same positions as in mutant R63/2/4/1.

In summary, the results obtained indicate that insertions and deletions of single adenosines in short A runs of the G protein gene is a frequent mutational event. Pathak and Temin (2) have reported that frame shifts in As or Gs runs of the spleen necrosis virus genome occur at frequencies comparable to that of misincorporations. Thus, slippage of the viral polymerases when copying runs of identical nucleotides may be another mechanism that RNA viruses could utilize to generate genetic diversity. The report by Sullender et al. (3) of two human RS virus isolates in which most sequence differences in the G protein gene could be explained by two reading frame shifts (analogous to those of mutant R63/2/4/1) underlines the significance of these genetic changes for the natural history of RS virus.

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JUNIN ARENAVIRUS NUCLEOCAPSID PROTEIN BEHAVES AS A TRANSCRIPTIONAL ANTITERMINATOR *IN VIVO*

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Junín virus is the etiological agent of Argentine hemorrhagic fever (AHF). The genome of this arenavirus is composed of two single stranded RNA species designated L and S. The 3.4 kilobases long S RNA encodes the major structural polypeptides, i.e. the nucleocapsid protein N and the glycoprotein precursor GPC. The non-overlapping open reading frames for these products have opposite polarities and are separated by a 91 nucleotides-long non-coding sequence. The potential secondary structure of this intergenic region is composed of two very stable hairpin-loops (ΔG^{o}: -39 and -57 kcal/mole, respectively) [1].

Northern blot analyses indicated that infected cells contain genomic and antigenomic copies of the S RNA. In addition, GPC and N mRNAs are subgenomic species corresponding to the 5' half and complementary to the 3' half of the viral S RNA, respectively.

The 5' termini of the subgenomic mRNAs were examined by primer extension, PCR amplification and sequence analysis. These experiments have shown several extra nucleotides extending beyond the copy of the 3' end of the S RNA template, suggesting a cap-snatching mechanism.

In order to define the transcription termination, the 3' termini of the mRNAs were determined in RNase protection experiments. The results indicated that the terminal sequences correspond to the copy of the first hairpin structure encountered by the RNA polymerase during the transcription of the intergenic region.

When cells were infected in the presence of protein synthesis inhibitors, the N mRNA was the only detectable transcription product [2]. These results suggested that the synthesis of a full-length antigenomic copy of the S RNA requires an antiterminator function. This function could be supplied by N, some other viral protein or a cellular polypeptide with a high turnover rate. To test the first hypothesis, BHK cell clones were generated expressing N under the control of an SV40 promoter [3]. These cells were preincubated with translation inhibitors and infected with Junín virus. The experiments indicated that the cells containing N prior to infection supported transcription of full-length S RNA in the absence of protein synthesis.

The transcription termination mechanism might be the result of the formation of a hairpin-loop structure at the 3' end of the N mRNA at the time when the intergenic region is being transcribed, leading to the release of the RNA polymerase. In the frame of this hypothesis, the binding of N might destabilize the secondary structure allowing the polymerase to proceed in order to complete a full length transcript.

It is not known whether the balance of the transcription/replication processes depends on the accumulation of N and its use in nucleocapsid assembly. Phosphorylation of N and/or a more complex relay involving interactions with other viral products (e.g. Z protein) might also participate in the regulation of RNA synthesis.

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THE HYPERVARIABLE C-TERMINAL TAIL OF THE SENDAI VIRUS NUCLEOCAPSID PROTEIN IS REQUIRED FOR TEMPLATE FUNCTION, BUT NOT FOR RNA ASSEMBLEY.

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The paramyxovirus nucleocapsid proteins (NP) are relatively well conserved, except for the C-terminal 20% (or ca. 100 aa), referred to as the tail. We have examined whether this hypervariable tail is required for genome synthesis, both in vitro where synthesis is predominantly from the input templates, and in vivo where multiple rounds of amplification occur. In these viruses, genome synthesis and assembly of the nascent chain are coupled. We find that the tail is required in-vivo, but not in-vitro, and interpret these results as indicating that the tail is not required for RNA assembly, but is required for the template to function in RNA synthesis. Deletions within the conserved N-terminal 80% of the protein, on the other hand, rendered the protein nonfunctional in either system. Another interesting observation was that an NP C-terminal deletion mutant was capable, in-vivo, of encapsidating cellular RNA's at high efficiency and had apparently lost all template selectivity (both in the absence and the presence of co-expressed P protein). This may indicate that the C-terminal tail also plays a role in the template specificity of assembley.

POSTERS

Rift Valley Fever virus L Polymerase: Comparison with the polymerases of other segmented negative strand RNA viruses.

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To determine the complete genome organization of RVF virus we cloned and sequenced the L segment (Muller et al., in Nucl. Acid Res. 1991). However, the published sequence was erroneous in the region from nucleotide 6015 to the 3' end of the viral complementary sense molecule. After recloning, we found that the correct sequence of the RVF L-segment is 6404 nucleotide long, coding for a polypeptide of 237.7 kDa in the viral complementary sense. No open reading frame could be detected in the 5' region of the virion sense RNA, indicating that, contrary to the previous prediction, the RVFV L segment has no ambisense strategy.

In the last two years, enough L segment sequences of segmented negative strand viruses have become available to carry out valid sequence comparisons between the polymerases of the Bunyaviridae and those from other viruses. In addition to the four functional motifs which are postulated to define the polymerase activity, we found four new regions which are conserved within the L proteins. Two of them are located in close proximity to the functional domains and are strictly conserved within the group of segmented negative strand RNA viruses (Bunyaviridae, Arenaviridae and Orthomyxoviridae). The two other regions correspond to the N terminal part of the L proteins and are conserved only within the Arena- and Bunyaviridae. The possible role(s) of these domains will be discussed.

Use of MV/CDV chimeric proteins to study structure/function relationships in the Paramyxovirus Fusion protein.

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We have previously used vaccinia virus recombinants to show that for efficient measles virus (MV) fusion to occur, expression of both the hemagglutinin (HA) and Fusion (F) glycoproteins is required (Wild et al. 1991). In this experimental system, infection of HeLa cells by a vaccinia virus recombinant expressing the MV HA gene is followed by transfection of a pgptΔ6 recombinant plasmid expressing the MV F protein. Fusion however does not occur with heterologous paramyxovirus glycoproteins : e.g. MV HA plus canine distemper virus (CDV) F does not give fusion. Thus in the present study, by the construction of chimeras between the MV and CDV F proteins expressed from pgptΔ6, we aim to identify the amino acids on the MV F responsible for the functional interaction with the MV HA necessary for fusion to occur.
Preliminary results suggest that both the F1 and F2 components of the MV F contact the MV HA : one interaction site is within the cysteine-rich region of the F1 and the other is situated in the carboxyterminal region of the F2 between AAs 60 and 82.
The Leucine Zipper structure found in all Paramyxovirus Fusion proteins that we have shown to be essential for fusion (Buckland et al. 1992) does not play a role in this association : exchange of the MV zipper for the CDV zipper does not abrogate fusion.
We are also using these chimeras to map anti-MVF monoclonal antibodies in order to further investigate functional domains in the MVF.

"REVERSE GENETICS OF NEGATIVE STRANDED RNA VIRUSES".

Defining Mutations Involved in Resistance to 2'-Deoxy-2'-Fluoroguanosine, a Specific Inhibitor of Influenza Transcriptase.

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2'-Deoxy-2'-Fluoroguanosine (2'fluoro-dGuo) inhibits the replication of all influenza A and B strains tested in tissue culture and in the lungs of mice.* Previously we have shown that the compound is phosphorylated by cellular deoxycytidine kinase and that the triphosphate selectively inhibits the influenza transcriptase in vitro.+ We have extended these studies by determining the activity of 2'-fluoro-dGuo in cell culture on primary and secondary virus transcription and on virus and cell protein synthesis. In addition, we have examined the potential for development of resistance with A/X31 virus by serial passage in CEF cells in increasing concentrations of the drug. Virus with a 5 fold shift in sensitivity to 2'fluor-dGuo was isolated after 10 passages. Comparative kinetic studies with the influenza transcriptase from purified parent and resistance viruses revealed significant changes in both the Ki and the GTP substrate Km of the mutant virus. Sequencing of the viral PB1 polymerase gene revealed 3 sequence changes. One of which was found in two different drug-resistant viruses generated in different cell lines (CEF or MDCK) using different drugs (2'fluoro-dGuo or 2'-fluor-dCyt). This change at aa 492 from a phenylalanine to leucine arose from a codon change from TTC to CTC or TTA in the two resistant viruses. We are now using reverse genetics to produce recombinant viruses with mutated PB1 sequences to determine the significance of various sequence changes in drug resistance.

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mRNA ANALYSIS OF INFLUENZA VIRUS SEGMENTS 7 AND 8.

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Influenza virus RNA segment 7 encodes two proteins, M1 and M2, depending on the removal of an intron from its primary transcript. As these proteins are essential for viral viability this process must be somehow regulated. To investigate the mechanism of splicing regulation, an influenza virus segment 7 cDNA was cloned under the control of a funtional SV40 early promoter and appropriate polyadenylation signals in a SV40 recombinant virus (SVM) and expressed in COS-1 cells. RNA analysis of SVM recombinant infected cells showed that (i) the appropriate splicing events to generate M2 mRNA occur and (ii) significant amounts of unspliced M1 mRNA, but less in comparison with influenza virus infected cells, are transported to the citoplasm. Besides that, expression of both M1 and M2 proteins was detected in these cells. Analysis of the relative proportion of M2 mRNA to mRNA3, an alternative spliced mRNA which uses a distal donor site of M1 mRNA, indicated that proximal 5' splicing site is preferently used in SVM recombinant virus infected cells while distal donor is the mayor choice in influenza virus infected ones. In addition, a different mRNA intranuclear distribution was found in SVM or influenza virus infected cells: the SVM segment 7 transcript associate preferently to the nuclear matrix, where splicing seems to take place, while the influenza virus one do not. We speculate that these differences in splicing efficiency and splice site choice might be related to different subnuclear localization of segment 7 transcripts synthesized by the different transcriptional machineries.

EVOLUTION OF THE G PROTEIN GENE OF HUMAN RESPIRATORY SYNCYTIAL (RS) VIRUS DURING SIX CONSECUTIVE EPIDEMICS FROM MONTEVIDEO (URUGUAY)

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To study the antigenic and genetic variation of the RS virus G protein among viruses from the same geographical area, 41 subgroup A isolates, obtained in Montevideo between 1987 and 1992, were subjected to the following analysis:

1.- **Reactivity of viruses with anti-G monoclonal antibodies (MAbs) and RNase A fingerprinting.-** Dot-tests with a panel of anti-G MAbs indicated that the Montevideo isolates could be classified into two main antigenic categories. This classification correlated with two RNase A fingerprint patterns of the G protein gene (1). However, the RNase A fingerprints could be subdivided into multiple sub-patterns, after comparative analysis of some diagnostic bands. Thus, a high degree of genetic variation was found among the isolates of the same antigenic subgroup, as previously reported for more limited sets of RS virus strains (2,3,4).

2.- **Sequence of the G protein gene from individual isolates.-** A number of isolates were selected, according to their antigenic and genetic profiles, for a further in-depth characterization. The RS virus G protein gene showed a high level of sequence divergence (up to 12-14%) among the selected isolates. The sequence differences were accounted by nucleotide substitutions that were either silent, translated into amino acid changes or introduced alternative termination codons. Viruses of the same or different antigenic and genetic categories could be isolated during the same epidemic. Sequence comparison of viruses isolated in Montevideo with viruses isolated either in Birmingham (U.K.) or Madrid (Spain), indicated that the RS virus G protein from subgroup A isolates could be classified into two main evolutionay lineages. These two lineages were widely spread in distant geographical areas and co-circulated in different epidemics. The high level of nucleotide changes (>50%) that resulted in amino acid changes and the accumulation of those changes in certain areas of the G protein, antigenically relevant, support the notion that immune selection may be a factor determining the RS virus natural history.

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CONFORMATIONAL CONSTRAINTS OF CONSERVED NEUTRALIZING EPITOPES FROM A MAJOR ANTIGENIC AREA OF THE HUMAN RESPIRATORY SYNCYTIAL VIRUS FUSION (F) GLYCOPROTEIN.

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Recently, we have located a major conserved antigenic area on the F glycoprotein between amino acids 260-275 [1] by sequencing of escape mutants selected with neutralizing monoclonal antibodies (MAbs). The amino acid changes in the escape mutants were clustered around residues 262, 268 and 272. The vicinity of these amino acids and the reactivity of these MAbs with the F_1 subunit in western blots suggested that the antibodies recognized sequential epitopes. However, most epitopes of this area were not reproduced with short synthetic peptides (~ 20 residues) covering essential amino acids. In addition, amino acid changes at distant positions of certain epitopes affected the binding of their MAbs. These results suggested that those epitopes needed a certain conformation.

In order to determine the conformational constraints of the epitopes we have synthesized overlapping peptides of increasing length containing essential amino acids for their integrity. The peptides have been used as antigens in ELISAs to test the binding of MAbs as well as polyclonal antisera (human convalescent antisera and rabbit hyperimmune serum). The binding of most antibodies was dependent on the peptide length; thus, most epitopes of both monoclonal and polyclonal antibodies were only reproduced in 41 and 61mer peptides that included residues 255 to 275 of the F_1 subunit (peptides F235-275 and F215-275 respectively). Some of these epitopes were only present in the longer peptide F215-275. Considering the length of this peptide, structural features other than primary structure should play a role in antibody recognition.

The binding of MAbs to the peptides correlated with the structure adopted by the peptides in solution, as determined by circular dichroism (CD) spectroscopy and susceptibility of the peptides to trypsin digestion. The effect of denaturing agents on the reactivity of antibodies with peptide F215-275 also indicated that higher order structures are needed to reproduce certain epitopes.

We have evaluated the immunogenicity of these peptides either free or coupled to keyhole limpet hemocyanin (KLH). All peptides induced high titre antisera that reacted with the peptides; however, these antisera failed to react with the mature F molecule. Thus, larger peptides reproduced most MAbs epitopes but failed to induce antibodies cross-reactive with the native F glycoprotein. These results illustrate the difficulty of reproducing a meaningful immune response against a native antigen using synthetic peptides as immunogens.

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Phenotype analysis of influenza virus PA protein mutants

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We have developed an expression system to assay the functionality of the influenza virus polymerase subunits and other viral gene products, in influenza virus RNA synthesis. The influenza virus genes (including PB1, PB2, PA, NP and non-structural proteins) were expressed in COS-1 cells by infection with SV40 recombinant viruses. The biological activity of these proteins was tested *in vivo* by transfection of an influenza virus-like RNA that contains either the chloramphenicol acetyl transferase (CAT) or influenza virus hemagglutinin (HA) genes in negative polarity. Expression of transfected RNA is thus dependent on amplification and transcription by an influenza virus RNA polymerase activity.

The system was very efficient in inducing CAT activity or HA expression by transfection of both artificial influenza virus-like RNPs or naked RNAs. The set of PB1, PB2, PA and NP genes was shown to be the minimal and sufficient one for the expression of transfected viral RNA.

According to the phenotypes of *ts* mutants, the PA polymerase subunit has been implicated in viral RNA synthesis. Since very little information could be obtained by comparisons of the PA protein sequence with the data banks, we have started a dissection of the functional domains of PA protein by the analysis of the phenotypes of mutant proteins generated *in vitro*. The results obtained with a series of mutants including amino and carboxy-terminal deletion mutants, mutants with internal deletions and point mutants will be presented.

BIASED HYPERMUTATIONS, REITERATIVE A-G SUBSTITUTIONS, IN TWO HUMAN RESPIRATORY SYNCYTIAL (RS) VIRUS ESCAPE MUTANTS SELECTED WITH A MONOCLONAL ANTIBODY DIRECTED AGAINST THE G GLYCOPROTEIN

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Two escape mutants ($R_{10}c/1$ and $R_{10}c/10$) of the human RS virus Long strain were selected after ten serial "in vitro" passages in the presence of monoclonal antibody (MAb) c793. This antibody recognizes an epitope of the G protein which is conserved in all strains of the two antigenic subgroups in which human RS virus isolates have been subdivided (1). Both mutants contained multiple A-G transitions (U-C in the positive sense) in the middle region of the G protein gene. Six of the nucleotide changes in each mutant were translated into amino acid substitutions that included the replacements Cys182 to Arg in mutant $R_{10}c/1$ and Cys186 to Arg in $R_{10}c/10$. These two amino acid residues are part of the four cysteines cluster (located in the middle of the G ectodomain) which is conserved in all human and bovine RS virus isolates sequenced to date. In fact, the escape mutants reported here represent the first examples of RS viruses containing only three cysteines in the G protein ectodomain.

The reiterative U-C changes in the G mRNA of the escape mutants have precendents in other RNA viruses. The closest example is the multiple U-C transitions in the matrix gene mRNA of measles virus present in biopsies of SSPE cases (2). The generation of this hypermutated measles viruses was best explained by a mechanism involving unwinding/modification of double-stranded RNA (3). If the same mechanism operated in the generation of mutants $R_{10}c/1$ and $R_{10}c/10$, then multiple U-C transitions should be observed at earlier passages of the isolation process.

When viruses from passage 6 were analyzed for binding of MAb c793 they showed an intermediate reactivity between Long and the mutants. After plaque purification, individual viral clones displayed either the wild type or the mutant phenotype (binding of MAb c793). Those plaque purified viruses that lack epitope c793 contained all the sequence changes detected in passage 10. In addition, the mutants from passage 10 were stable after 5 more passages in the absence of antibody and all the sequence changes were maintained.

The simultaneous appearence of the U-C transitions in mutants resistant to antiboy c793 and their stability after, at least, 9 passages (6 to 15) support the notion that these changes were generated by an alternative mechanism to the sequential addition of misincorporations by an error prone polymerase.

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EXPRESSION AND PURIFICATION OF INFLUENZA VIRUS REPLICASE SUBUNITS IN A PROCARIOTIC VECTOR.

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During the influenza virus infection three types of specific RNAs are synthesized in the nucleus of the infected cell: mRNA, cRNA and vRNA. This process is mediated by the action of at least four viral proteins: PA, PB1, PB2 (the three subunits of the transcription-replication complex), and NP.

In order to study the individual roles of these proteins and the proteic interactions that lead to the formation of the true active complex of replication, we decided to overproduce the three subunits of the replicase in E. coli, to obtain sufficient amounts of recombinant products for in vitro assays.

The vector used (pMal-c) allows the expression of the cloned gene as a fusion protein with the bacterial maltose binding protein, with the advantages of high inducible expression, solubility of the synthesized product, easy single-step purification by affinity cromatography and possibility to obtain the heterologous polypeptide alone by digestion with a target-directed protease.

The genes coding for PA, PB1 and PB2 proteins were cloned in pMal-c vector, and expression of the recombinant fusion protein was induced by adding 0.3 mM IPTG to the cultures. Accumulation of a protein of the expected MW was observed in each case when bacterial total extracts at 2 hours post-induction were analized by SDS-PAGE with Coomasie brilliant blue staining or by Western-blot with specific antibodies .

Purification of the inducible protein from the soluble fraction (about 30% of total expressed protein) was carried out by affinity cromatography in an amylose resin, rendering purified fractions of fusion proteins.These fractions will be used in the production of monoclonal antibodies against the replicase subunits. At present we are carrying out preliminary assays to test the presence of enzymatic activities associated with PA protein, which function in the viral cycle is unknown.

SUMMARY AND PERSPECTIVES

G. W. Wertz and J. A. Melero

The papers presented at the workshop on Reverse Genetics of Negative-Stranded RNA Viruses and summarized in the preceding abstracts describe numerous advances that have been made toward understanding the molecular biology of the negative-stranded RNA viruses. This meeting reported work using traditional methods as well as the rapidly evolving new techniques for manipulation of negative-stranded RNA genomes at the cDNA level followed by recovery of these engineered RNAs by transcription. A great deal of progress has been made in the past two years yet a great deal of work remains before crucial areas of genome replication, transcription and assembly are understood. In evaluating the progress reported at the workshop, the segmented and the non-segmented RNA viruses will be considered separately as the achievements in the two areas differ at present.

In the case of the segmented, negative-stranded RNA viruses, the original work pioneered by Palese and coworkers who used *in vitro* transcription of individual influenza gene segments followed by transfection, amplification and expression of the RNP in cells coinfected with helper virus, has been followed by numerous important advances in understanding the molecular biology of influenza. This work, coupled with the *in vitro* approach employed by Brownlee and coworkers and the alternate approach of plasmid-supported replication developed by Ortin and his colleagues, have provided insights into the protein requirements for replication, the sequences essential for signalling replication and transcription, and have identified signals involved in controlling mRNA abundances and polyadenylation. Additionally, with influenza virus, it has been possible to recover reassortant viruses, termed "transfectant" viruses, that contain altered individual gene segments, which can be examined for the effects of the alterations on virus replication in cells and in animals. In particular, alterations of the neuraminidase gene have been exploited to examine the effects of altering the structure of this protein on infectivity and pathogenity of virions (Palese and colleagues; Kowaka and colleagues; Air and colleagues). At present, not all gene segments of influenza have been altered and recovered in transfectant virions. The use of the influenza reverse genetic system for generation of transfectant virions still requires live helper virus, as a system has not yet been developed for the segmented viruses whereby all gene functions can be supplied by cDNA-derived RNAs. Nevertheless, because of the ability to generate transfectant viruses by reassortment of segments, workers with the segmented RNA viruses are now in a position to address almost any area of the molecular biology of these viruses, be it the role of *cis*-acting or *trans*-acting factors in replication, transcription, assembly or pathogenesis.

For the non-segmented, negative-stranded RNA viruses, the recovery of a complete, genome length infectious RNA from a cDNA clone has yet to be achieved, but, despite this, significant progress has been made using two distinct approaches. On the one hand, for the paramyxoviruses, the approach used has been similar to that described above for influenza virus: transcription *in*

vitro from a cDNA containing the terminal portions of Sendai virus, RS virus or parainfluenza virus (PIV3) surrounding a reporter gene, followed by transfection of the RNA transcripts into cells infected with wild-type virus as helper. Assays of reporter gene activity show that these transcripts have been replicated, transcribed, and incorporated into infectious particles (Krystal and colleagues; Collins and colleagues; Dimock and colleagues; Kolakofsky and colleagues). With parainfluenza virus (PIV3) and RS virus, it has been possible to investigate *cis*-acting sequences important in expression of reporter genes, and the interesting results emerging indicate that tolerance of changes in the terminal sequences of RS virus is much greater than the tolerance of changes made in of the PIV3 genome (Collins and Dimock). The first efforts to investigate the effect of alterations on intergenic and gene end and gene start sequences were reported by Dimock and Collings. These studies involved a bicistronic vRNA analog containing the CAT an luciferase coding sequences both placed under control of PIV3 gene start and gene end signals, separated by an intergenic region, and flanked by the PIV3 leader and trailer regions. Their results showed that deletion or disruption of the intergenic or gene start and gene end sequences has the potential to affect both upstream and downstream gene expression. Gene expression using this approach, however, is at a level that is detectable only by the use of reporter genes; direct biochemical analysis of RNA has not been possible. Thus, effects on RNA replication cannot easily be distinguished from effects on transcription and the ability to detect RNA will be necessary before it will be possible to demonstrate at what level of RNA synthesis alteration of *cis*-acting signals acts.

A second approach for the non-segmented viruses involves transcription from a specialized transcription vector designed to yield discrete RNA transcripts *inside* the cells (Ball and colleagues). The genomes of defective interfering particles of the rhabdovirus, VSV, and the paramyxovirus, Sendai virus were expressed from cDNAs and shown to replicate efficiently when expressed in the presence of the N,P and L proteins also expressed in the cell from cDNAs (Wertz and colleagues, Roux and colleagues). In this approach, the transcript was synthesized by bacteriophage T7 polymerase expressed from a vaccinia virus recombinant in cells which were simultaneously expressing the N,P and L genes from other transfected plasmids. In the case of VSV, replication of the RNA was measurable by direct biochemical means, demonstrating that the replication of the intracellularly transcribed genomes occurred at high levels.

Complete infectious VSV defective interfering virus particles were assembled and budded from cells supported entirely by cDNAs using the vaccinia T7 system to generate DI genomic RNA transcripts in cells simultaneously expressing all five viral proteins from cDNA. Thus, it is possible to assay the role of each *trans*-acting gene product in encapsidation, replication, assembly and budding to produce infectious particles, thereby making each step in the virus life cycle accessible to investigation (Wertz and colleagues).

With the defective interfering particle replication systems, it is also possible to assay directly the *cis*-acting signals important in genome encapsidation and replication, and to determine the minimal genome requirements for replicable RNAs. With VSV, Wertz and colleagues were able to delete 90% of the VSV DI genome and obtain encapsidation, replication, assembly and budding of a genome of 192 nucleotides. Additionally, Roux and colleagues made the intriguing observation while generating deletions in the Sendai genome, that only RNAs whose length was an even multiple of six could replicate. To account for this result, they proposed an encapsidation "spacer" rationale, the "rule of six".

In summary, excellent progress has been made in development of reverse genetic systems for both the segmented and the non-segmented, negative-strand RNA viruses, opening new avenues for investigation of the role of *cis*- and *trans*-acting factors in transcription, replication and assembly of these viruses. A major goal remaining for the segmented RNA viruses is recovery of transfectant virions containing altered segments of each gene, both individually and in combinations. A major goal remaining for the non-segmented viruses is the development of methods to recover complete, genome length infectious transcripts from cDNA clones.

G.W. Wertz and J.A. Melero

List of Invited Speakers

Workshop on

REVERSE GENETICS OF NEGATIVE STRANDED RNA VIRUSES

List of Invited Speakers

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Workshop on

REVERSE GENETICS OF NEGATIVE STRANDED RNA VIRUSES

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246 **Workshop on Tolerance: Mechanisms and implications.**
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247 **Workshop on Pathogenesis-related Proteins in Plants.**
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251 **Lecture Course on Approaches to Plant Development.**
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254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
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256 **Workshop on Chromatin Structure and Gene Expression.**
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8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

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10 **Workshop on Engineering Plants Against Pests and Pathogens.**
Organized by G. Bruening, F. García-Olmedo and F. Ponz. Lectures by R. N. Beachy, J. F. Bol, T. Boller, G. Bruening, P. Carbonero, J. Dangl, R. de Feyter, F. García-Olmedo, L. Herrera-Estrella, V. A. Hilder, J. M. Jaynes, F. Meins, F. Ponz, J. Ryals, J. Schell, J. van Rie, P. Zabel and M. Zaitlin.

11 **Lecture Course on Conservation and Use of Genetic Resources.**
Organized by N. Jouve and M. Pérez de la Vega. Lectures by R. P. Adams, R. W. Allard, T. Benítez, J. I. Cubero, J. T. Esquinas-Alcázar, G. Fedak, B. V. Ford-Lloyd, C. Gómez-Campo, V. H. Heywood, T. Hodgkin, L. Navarro, F. Orozco, M. Pérez de la Vega, C. O. Qualset, J. W. Snape and D. Zohary.

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