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The lectures summarized in this publication were presented by their authors at a workshop held on the 22nd through the 24th of April, 1991, at the Fundación Juan March.

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Fundación Juan March

Workshop on

Transcription and Replication of Negative Strand RNA Viruses

Organized by

D. Kolakofsky and J. Ortín

A. K. Banerjee
M. A. Billeter
P. Collins
M. T. Franze-Fernández
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259 Workshop on Transcription and Replication of Negative Strand RNA Viruses

259

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Serie Universitaria

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I N D E X

POSTERS:

PROGRAMME

1. **Monday 22: First Session.**

A.J. Hay	- Influence of M2 function on the expression of functional HA in influenza A virus infection.
D.F. Summers	- High level expression of influenza A RNA Polymerase subunits (PB1, PB2 and PA) and NP by recombinant baculovirus and the biochemical functions of these proteins.
J. Ortín	- Regulation of influenza virus mRNA splicing.
R.M. Krug	- The influenza virus nonstructural protein 1 regulates the nucleocytoplasmic transport of the viral NS2 mRNA in a sequence-specific manner.
A. Ishihama	- Signals and apparatus for transcription and replication of influenza virus.
P. Palese	- Transcription, replication and packaging signals of influenza virus RNAs.
A. Portela	- Molecular characterization of Thogoto virus (a tick-borne influenza-like virus).
B.L. Seong	- A new method for reconstituting influenza polymerase and RNA in vitro: promoter analysis for cRNA and vRNA synthesis.
M. Krystal	- Study on the mechanism of the anti-influenza virus activity of the murine Mx protein.

2. **Tuesday 23: Second Session.**

M.T. Franze-Fernández	- Transcription termination in an arenavirus.
D. Kolakofsky	- A novel mechanism for the initiation of Tacaribe arenavirus genome replication.
M. Schubert	- Selected topic of VSV replication: polymerase, pathogenesis and pseudotypes.
G.W. Wertz	- A reverse genetic analysis system for VSV.

S.A. Moyer - Transcription of measles and vesicular stomatitis viruses in vitro.

A.K. Banerjee - Phosphorylation states of the phosphoprotein P of vesicular stomatitis virus.

N. Tordo - Transcription mechanisms of Lyssaviruses.

3. **Wednesday 24: Third Session.**

P. Collins - Molecular studies of human respiratory syncytial virus.

J.A. Melero - Genetic mechanisms involved in the generation of antigenic variants of human respiratory syncytial (RS) virus.

R.G. Paterson - RNA editing in paramyxoviruses.

D. Kolakofsky - Sendai virus genome replication via cloned viral genes.

M.A. Billeter - Measles viruses from recombinant cDNAs: a system allowing functional analyses and potentially suitable for vector applications.

A.K. Banerjee - In vitro transcription of human parainfluenza virus type 3: role of cellular actin in mRNA synthesis.

R.M. Elliott - Expression of the Bunyamwera virus L protein using recombinant vaccinia viruses.

K.K. Conzelmann - Complementation of L (Polymerase) deficient rabies virus DI particles by heterologous helper viruses and by rabies virus L expressed in cells.

M. Krystal - Rescue of a foreign gene by Sendai virus.

INTRODUCTION

Negative-strand RNA viruses include "classical" members like influenza , measles or rabies virus, and others that have been studied only more recently, like respiratory syncytial virus, Junin virus, Marburg virus or Thogoto virus. Many of these viruses are the etiological agents responsible of infections important for human and animal health.

Our knowledge about the mechanisms of transcription and replication of negative-strand RNA virus genomes, as well as many other aspects of their basic biology, has traditionally lagged behind that of positive-strand RNA viruses. This situation has been the result, at least in part, of the more complex structure of the responsible enzymes, of the necessary connection of the transcription-replication machinery to the genome packaging mechanism, and especially, of the lack of an experimental setting to introduce *in vitro* generated mutations back into infectious virus. This reverse-genetics approach, so succesfully applied in much more complex biological systems, from bacteria to transgenic animals, was not posible in negative-strand RNA viruses until very recently, when the groups of Martin Billeter and Peter Palese described the recovery of infectious progeny from *in vitro* synthesized measles and influenza virus RNA, respectively.

When I had preliminary discussions with Andrés González about the possibility of organizing a workshop within the International Meetings in Biology Program of the Fundación Juan March, I immediately felt that time was particularly appropriate for those research groups interested in negative-strand viruses to get together and discuss the state of the art and future prospects in the field. Since the workshops within this Program have to include a necessarily very limited number of participants, I decided to restrict the topics to those related to control of gene expression and RNA replication. This decision was not completely arbitrary. It was my feeling, and I hope that this is a general one,

that a better understanding of the transcription and RNA replication mechanisms would lead to generalized procedures for *in vitro* reconstitution systems and marker rescue methods. These, in turn, should further our possibilities to ask relevant questions about not only RNA replication, but also virus-cell interactions, pathology of the infections, etc.

After three days of very intensive exchange of ideas, I think that the results of the workshop exceeded my already optimistic expectations. This was so because of the high scientific value of the work presented, both by invited speakers and by younger participants. It was very easy for Dan Kolakofsky and myself to arrange the program after the exceedingly good response we had among the scientists in the field.

Of course, it would have been imposible for us to organize this workshop without the expert, kind and elegant dedication of Andrés González and his co-workers in the Fundación Juan March. They managed to overcome Murphy's law down to the smallest detail and allowed us to completely dedicate ourselves to our job in this island of quietness that the Fundación Juan March represents in the midst of the busy city of Madrid. I should like to express, on behalf of everyone at the meeting, our gratitude to them.

FIRST SESSION

A. J. HAY
D. F. SUMMERS
J. ORTÍN
R. M. KRUG
A. ISHIHAMA
P. PALESE
A. PORTELA
B. L. SEONG
M. KRYSTAL

Influence of M2 function on the expression of functional HA in influenza A virus infection.

A.J. Hay, R.J. Sugrue, S. Grambas and M.S. Bennett.

The M2 protein is a minor component of the envelope of influenza A viruses which plays a role both in virus uncoating and the initiation of infection and in the maturation and release of progeny virus particles. In the latter respect, the M2-mediated conformational change in H7 HA to the low pH form induced by the action of amantadine hydrochloride has provided evidence that the M2 protein has a role in regulating the pH within vesicles of the trans Golgi network, thereby protecting the structural integrity of the glycoprotein during its transport to the cell surface. Structural features of the tetrameric molecule suggest that the M2 protein forms a transmembrane channel which may be involved directly in proton translocation.

The important relationship between HA and M2 is evident from the influence of mutations in either component on the expression of native functional HA. Mutations in HA which increase the pH of the conformational transition cause increased expresssion of low pH HA on the cell surface and impair virus growth as do certain mutations in M2 which impair its function. The incompatibility in this regard between the HA and M genes of different viruses, as indicated by the phenotypes of genetic reassortants, is another factor which limits the degree of genetic diversity among influenza A viruses.

HIGH LEVEL EXPRESSION OF INFLUENZA A RNA POLYMERASE SUBUNITS (PB1, PB2, and PA) AND NP BY RECOMBINANT BACULOVIRUS AND THE BIOCHEMICAL FUNCTIONS OF THESE PROTEINS. J.M. Galarza+, A. Sowa+, D. Segal+, V.M. Hill+, B. Szewczyk*, R. Skorko§, and D.F. Summers+. Department of Cellular, Viral and Molecular Biology, University of Utah School of Medicine, Salt Lake City, Utah 84132+, Department of Biochemistry, University of Gdansk, Gdansk, Poland*, Department of Pharmacologic Microbiology, Medical Academy of Gdansk, Gdansk, Poland§.

Our laboratory has been engaged in studies to define the factor(s) involved in modulating RNA synthesis by negative-strand animal virus polymerases between the transcriptive and replicative modes. To this end we have successfully established a completely reconstituted *in vitro* influenza A virus RNA synthesis system from the three purified polymerase subunits (PB1, PB2, PA), NP and virion RNAs. The constituents were derived from large amounts of virion RNPs provided by Dr. Graeme Laver, Canberra, Australia. Purification and renaturation of the four influenza proteins was accomplished by using SDS-PAGE separation, electroblotting to Immobilon membranes, elution of the separated proteins and renaturation using *E. coli* thioredoxin. Although this procedure was successful using components purified from virus, it provided only a few micrograms of polymerase.

Having demonstrated that one could recover the activity of the purified subunits, we then turned out efforts to obtaining large amounts of the polymerase subunits and NP using an heterologous expression system. Using recombinant baculovirus we have successfully expressed, at high levels, the three polymerase subunits, NP and M1 in Sf9 cells.

PA and PB2 have been purified and renatured using the Immobilon/thioredoxin technique. We have shown that PB2 will endonucleolytically cleave radiolabelled capped AIMV4RNA, but will not cleave uncapped AIMV4RNA. The cleavage reaction was inhibited by the addition of 0.5 mM ApG and polyU. PA was shown to be a protein kinase which phosphorylated casein and CIP-dephosphorylated NP protein.

NP protein expressed in Sf9 cells was purified by immunoaffinity chromatography. Purified NP was shown to autophosphorylate and to phosphorylate casein in a cAMP-independent reaction. Furthermore, purified NP was able to bind single-stranded RNA as demonstrated by a mobility shift of ssRNA in non-denaturing gels. The binding of NP to ssRNA caused a diminution of its kinase activity in proportion to binding. Dephosphorylation of NP with CIP resulted in a loss of >95% of its kinase activity.

REGULATION OF INFLUENZA VIRUS mRNA SPLICING

J.Ortín, J. Valcárcel, A. Portela and P. Fortes.
Centro Nacional de Biotecnología (CSIC). Universidad Autónoma. Cantoblanco. 28049 Madrid.

Influenza virus RNA segment 7 generates three poly-A^+ RNAs, M1 mRNA, M2 mRNA and mRNA3, the latter of which contains almost no coding capacity. M2 mRNA and mRNA3 derive from M1 mRNA by removal of a single intron. The kinetics of M1 and M2 mRNA accumulation in the cytoplasm of productively infected cells was studied by means of a quantitative RNA protection assay. The relative proportion of M2 to M1 mRNA increased during the course of the infection by a factor of 2.7. To analyze the basis for this change, the kinetics of M1 and M2 mRNAs synthesis and nuclear accumulation, their stabilities and nucleocytoplasmic transport were studied. Under the experimental conditions used, the synthesis of segment 7-specific RNA showed a peak at 4 hpi and continued later at a slower rate. The half-lives of M1 and M2 mRNAs were indistinguishable (2.73h for M1 mRNA and 2.70h for M2 mRNA) and the kinetics of nucleocytoplasmic transport *in vivo* or *in vitro* showed no preferential transport of either mRNA at early or late times in the infection. Consequently, a regulation at the level of mRNA splicing is proposed. Using the mRNA synthesis and stability data, a simulation was performed to predict the change in splicing efficiency required to account for the mRNA accumulation results. The best fit was obtained when the splicing efficiency changed about 20 times at a time when viral gene expression was maximal.

To analize the possible mechanisms responsible for this regulation, the splicing of M1 mRNA derived from a cDNA cloned into a SV40 recombinant was studied. The splicing was more efficient than in influenza virus infected cells, but nevertheless incomplete. Inactivation of the M2 cistron did not affect the efficiency of splicing, but inactivation of M1 cistron reduced it by a factor of ~3. Studies on the effect that *cis*-mutations in the M1 mRNA or *trans*-activity of other viral genes could have on the efficiency of M1 mRNA splicing are in progress.

The Influenza Virus Nonstructural Protein 1 Regulates the Nucleocytoplasmic Transport of the Viral NS2 mRNA in a Sequence-Specific Manner, Robert M. Krug, Firelli V. Alonso-Caplen, and Martin E. Nemeroff, Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ 08855-1179.

The influenza virus nonstructural protein 1 (NS1 protein), which was first identified in 1971, accumulates in the nucleus, but its function in the nucleus has not yet been determined. We tested the possibility that the NS1 protein has a function similar to that of the nuclear Rev protein of HIV-1, namely the facilitation of the transport of unspliced viral pre-mRNA from the nucleus to the cytoplasm. Splicing is regulated in influenza virus-infected cells. A portion of NS1 mRNA, the mRNA that encodes the NS1 protein, is spliced to form NS2 mRNA that encodes another nonstructural protein. We inserted the NS1 gene into a transient transfection vector and determined whether the NS1 protein mediated the nuclear export of unspliced NS1 mRNA. Surprisingly, rather than facilitating the transport of NS1 mRNA, the NS1 protein inhibited the transport of spliced NS2 mRNA. Our results indicate that the NS1 protein, like the HIV Rev protein, regulates the nuclear export of RNA in a sequence-specific manner. This regulation, however, does not facilitate the expression of an unspliced pre-mRNA, but rather inhibits the expression of a spliced mRNA. These results identify a novel function of the influenza virus NS1 protein.

SIGNALS AND APPARATUS FOR TRANSCRIPTION AND REPLICATION OF INFLUENZA VIRUS

Akira Ishihama

Department of Molecular Genetics, National Institute of Genetics

Mishima, Shizuoka 411, Japan

Influenza virus contains eight negative-strand RNA segments as the genome and an RNA polymerase as a virion component. The RNA polymerase is believed to play a major role in not only transcription but also replication of viral RNA. I will summarize our studies on the structure-function relationship of the RNA polymerase.

The RNA polymerase is associated with the putative promoter/origin located at the double-stranded stem of each panhandle-structured vRNA segment and, together with N protein, forms ribonucleoprotein (RNP) cores. In viral particles, M protein is associated with the RNP cores via N protein, and represses the expression of RNA polymerase activity. The RNA polymerase can be isolated from RNP cores by stepwise centrifugation in CsCl and CsTFA. The purified RNA polymerase is composed of each one of the three P proteins (PB1, PB2 and PA), and is able to catalyze both capped RNA endonuclease and primer-dependent RNA synthesis only in the presence of vRNA. Small-sized single-stranded synthetic RNA templates can be fully transcribed by the purified RNA polymerase provided that such model templates contain the promoter/origin sequence. On naked vRNA, however, transcription is abolished soon after initiation. Ordered association of NP is needed for efficient elongation of RNA chains.

On transfection assay, RNP (P proteins-NP-vRNA complexes) cores

produce infectious viruses but P-RNA complexes (P proteins-vRNA complexes) lack this activity. Foreign RNA of minus-polarity can be transcribed (and perhaps replicated) *in vivo*, when the promoter/origin sequence is linked to RNA termini and, after formation of RNP structure, transfected into cells carrying the viral RNA polymerase.

Replication of vRNA takes place via two-step reactions, *i.e.*, vRNA-dependent synthesis of cRNA (complementary RNA) and cRNA-directed synthesis of vRNA. The P protein complex is involved in these processes but a switching factor(s) exists in infected cells, which converts the RNA polymerase into a putative replicase complex that is able to support the preferential synthesis of cRNA over mRNA.

I will also describe some results of our detailed analysis on the function of each P protein and the functional domain(s) on each P protein, using P proteins overproduced from respective cDNA clones.

TRANSCRIPTION, REPLICATION AND PACKAGING SIGNALS OF INFLUENZA VIRUS RNAs

P. Palese, M. Enami, G. Luo, W. Luytjes, T. Muster and X. Li

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One Gustave L. Levy Place, New York, NY 10029

Biologically active influenza virus ribonucleoprotein (RNP) complexes can be reconstituted *in vitro* using synthetic RNAs and purified viral proteins. Transfection of RNPs in the presence of influenza viruses then leads to amplification, expression and packaging of the RNAs by the helper virus (Luytjes et al., Cell 59, 1107, 1989; Enami et al., Proc. Natl. Acad. Sci. USA 87, 3430, 1990). In addition, this system allowed us to ascertain that the eleven 3' terminal nucleotides of influenza viral RNAs are required for transcription from minus sense RNA to plus sense RNA. The conserved twelve 5' terminal nucleotides are required for replication of minus sense RNA from plus sense template RNA. The 3' and 5' terminal residues of viral RNAs are partially complementary and the resulting double-stranded panhandle structure provides the features required for polyadenylation of mRNA as well as for packaging of the negative strand virion RNA. We also succeeded in engineering an infectious influenza virus into which nine different RNA segments were packaged (rather than the usual eight).

MOLECULAR CHARACTERIZATION OF THOGOTO VIRUS (A TICK-BORNE INFLUENZA-LIKE VIRUS).

A.Portela[1], L.D. Jones[2], P. Nuttall[2].

1.Instituto de Salud Carlos III, Centro Nac. de Microbiología, Majadahonda 28220, Madrid, Spain. 2.Institute of Virology, Mansfield Road, Oxford OX1 3SR, United Kingdom.

Thogoto (THO) virus, an unclassified tick-borne virus, has been repeatedly isolated from ticks and various vertebrate species. The virus shares morphological and genetic features with members of the Orthomixoviridae family. It is an enveloped virus that contains a genome consisting of six segments of single stranded negative sense RNA (Clerx et al,1983, Virology 127, 205-219). The 3'and 5' ends of the genomic RNA are complementary and show similarities to those of influenza viruses. In addition, RNA sequencing of segment 3 viral RNA has revealed sequence similarities between regions of the predicted protein encoded by this segment and regions of the PA protein of influenza A and B viruses (Staunton et al,1989, J. Gen. Virol. 70, 2811-2817).

To analyze whether the virus exhibits other properties similar to those of influenza viruses we have initiated the molecular characterization of the structural components of the virion. It was found that highly purified virus preparations contain, at least, 6 viral encoded proteins with molecular weights ranging from 29 to 92 Kd. One of them is the glycoprotein (75 Kd). This protein was characterized, as an envelope-associated protein, by detergent and salt dissociation studies, and by proteolitic degradation of the exposed proteins of the virion. In the same way, the nucleoprotein and the matrix protein, were identified as the 52 and 29 kd molecular weight proteins. Inmunofluorescence studies, using monoclonal antibodies, located the glycoprotein on the infected cell membrane, and the nucleoprotein in the nucleus, showing, therefore, that the replication of the virus involves a nuclear phase. In addition, we found that the virus has pH-depedent haemaglutinating and hemolitic activities, with an optimum at pH 5.9. These activities are associated with the viral glycoprotein since they could be inhibited by monoclonal antibodies anti-glycoprotein. All these results will be discussed regarding the taxonomic classification of this virus.

A new method for reconstituting influenza polymerase and RNA *in vitro*: Promoter analysis for cRNA and vRNA synthesis.

Baik L. Seong and G.G. Brownlee
Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.

A new method of reconstituting the influenza RNP complex *in vitro* is presented, where two steps of influenza replication (vRNA→cRNA and cRNA→vRNA) as well as the cap dependent transcription (vRNA→mRNA) are compared using a model system. Influenza polymerase proteins were prepared from the viral core by depletion of viral RNA with micrococcal nuclease, which was subsequently inactivated by EGTA. This polymerase preparation efficiently transcribed model vRNA and cRNA templates. Using RNA templates with progressive deletion from the 5' end, we found that the 13 long and 12 long 3' conserved sequences of cRNA and vRNA of influenza A virus are the minimal promoters and by themselves are sufficient to promote vRNA and cRNA synthesis *in vitro*. Panhandle structures are not required for either vRNA or cRNA synthesis. Studies are in progress to assess the effect of subtle changes in sequence in the promoters with their activity in order to understand how the polymerase complex carries out and controls its transcription and replication.

STUDY ON THE MECHANISM OF THE ANTI-INFLUENZA VIRUS ACTIVITY OF THE MURINE Mx PROTEIN, Taosheng Huang*, Jovan Pavlovic+, Otto Haller@, Peter Staehli+ and Mark Krystal*

* Dept. of Microbiology, Mt. Sinai Medical Center, N.Y.,N.Y.; + Institute fur Immunologie and Virologie, Universitat Zurich, Zurich Switz.; @ Abteilung Virology, Institut fur Medizinische Mikrobiologie und Hygeine, Freiberg, Germany

The murine Mx1 gene is expressed after interferon induction and confers to animals selective resistance to influenza virus. The antiviral effect is also observed in tissue culture but the molecular basis for the virus inhibition is poorly understood. We have recently developed a completely artificial replication system for influenza virus using recombinant vaccinia vectors expressing influenza polypeptides along with reconstituted RNP molecules. This system has been used to analyze the antiviral actions of Mx1. Vaccinia viruses expressing the influenza virus polymerase can overcome the block in viral gene expression caused by Mx1 in a dose dependent manner. In addition, experiments utilizing various RNP constructs or subsets of the recombinant vaccinia vectors needed for replication has contributed to the elucidation of the antiviral mechanism of the murine Mx1 protein.

SECOND SESSION

M. T. FRANZE-FERNÁNDEZ
D. KOLAKOFSKY
M. SCHUBERT
G. W. WERTZ
S. A. MOYER
A. K. BANERJEE
N. TORDO

TRANSCRIPTION TERMINATION IN AN ARENAVIRUS

Tacaribe virus (TV), a member of the Arenaviridae family, is an enveloped virus with genetic information encoded in two segments of single-stranded RNA called S and L. We have just completed the TV genome structure. It was found that the TV S RNA -like the S RNA of a number of arenaviruses- encodes the major structural proteins: i.e. the glycoprotein precursor (GPC) and the nucleoprotein (N) in an ambisense coding arrangemen. Analysis of the structure of TV L RNA revealed a genetic organization similar to that of the S RNA. The open reading frame, which codes for the L protein is located at the 3' region and is complementary to the L genome. The other open reading frame at the 5' region has the same sense as the genome and codes for a polypeptide with a "zinc finger-like" sequence that we have called P_{11}. The nucleotide sequence of the intergenic region in both the S RNA and the L RNA leads to the prediction of a strong secondary structure. This peculiar genomic organization together with the identification of subgenomic RNAs suggested that the intergenic region would function as a transcription terminator. To have an insight into the signals involved in the termination of transcription, the 3' ends of the TV four putative mRNAs have been characterized. It was found that the transcripts terminate within the intergenic region in each RNA segment. No special sequences that might function as termination signals were evident. The 3' end sequences of the four putative mRNAs can be predicted to adopt GC-rich stable hairpin configurations ($\Delta G \geq -25$ kcal). These observations suggest that the transcript structure rather than particular sequences might be the signal involved in the termination of arenavirus transcription.

María T. Franze-Fernández - Centro de Virología Animal
Serrano 661, 1414 Buenos Aires, Argentina.

A Novel Mechanism for the Initiation of Tacaribe Arenavirus Genome replication. Dominique Garcin and Daniel Kolakofsky. Univ. of Geneva, Switzerland.

The vast majority of (-) RNA virus genomes contain a 3' terminal U_{OH} residue (position +1), and genome replication begins with ATP at this position. In contrast, both genome segments of arenaviruses contain a 3'G residue, and although they could start antigenome synthesis with CTP, there is no precedent for this nucleotide being used for initiation by viral RNA polymerases.

When the 5'ends of Tacaribe virus antigenomes are mapped by primer extension, they are found at position -1, and this nucleotide appears to be not pppC, but (p)ppG. The position of the 5'end does not appear to be an artefact of the mapping method. The 5'ends of bunyavirus antigenomes are correctly mapped to position +1 in parallel experiments. More importantly, when ds-RNA is made from genomes and antigenomes, the 5'end of antigenomes (and genomes) is found to be a single-stranded G residue, whereas the C at +1 is in a ds-structure (again by primer extension). A G is also found at position +1 when antigenome clones are examined.

How arenaviruses initiate genome synthesis is far from clear, but an attractive interpretation of the apparently non-templated G at position -1 is that pppGpC was used to start the chains, as there is also evidence that (capped) primers are used to initiate arenavirus mRNAs. To test this possibility, we examined RNA synthesis in vitro with infected cell extracts. The dinucleotide GpC was found to stimulate RNA synthesis (both mRNAs and genomes) ca. 20-fold, and ApApC ca. 10-fold, whereas 5 other di- and trinucleotides were either inactive or mostly inactive. The 5'ends of the GpC and ApApC stimulated N mRNA made in vitro were mapped by a novel procedure, and found to be at positions -1 and -2 respectively, indicating that the oligonucleotides were acting as primers. Insufficient RNA was made in the absence of primers for their 5'ends to be examined.

Selected Topics of VSV Replication: Polymerase, Pathogenesis and Pseudotypes.

Manfred Schubert, Ph.D.
Chief Viral Replication Section
Laboratory of Viral and Molecular Pathogenesis
NINDS, NIH
Bethesda, MD, U.S.A.

The presentation will focus on four aspects of VSV replication studied in our lab which are important for the future generation and the use of recombinant VSV particles.

1. The roles of the viral polymerase proteins will be discussed in light of observations which underline the importance of the carboxyterminal domain of the NS protein for transcription and also point to a possible key role of the NS protein for the mechanism of homotypic and heterotypic autointerference by defective interfering particles.

2. Besides its role in the regulation of transcription and viral assembly, a unique role of the VSV matrix protein M in cytopathogenesis was identified, which involves a disruption of the cytoskeleton, resulting in the typical rounding of the cells after infection. Expression of cloned temperature sensitive matrix protein, as well as infections with the ts mutant demonstrated a reversibility of this cytopathic effect. Reversibilty was independent of protein synthesis, and the absence of the cytopathic effect correlated not only with a redistribution of the M protein in the cell from a diffuse to a dotted staining pattern but also with an inhibition of viral assembly.

3. Conditions were established for the efficient generation of VSV pseudotypes using mixed infections with recombinant vaccinia viruses expressing viral and cellular glycoproteins, like the HIV receptor CD4. Our future goal is to target virus particles to specific cell types. We focus on an HIV infected cell as a model target cell. The data demonstrate a lack of an exclusion of the cellular glycoprotein CD4 during viral assembly. This is compared to the insertion of similar levels of a chimeric CD4/VSV G protein, consisting of the ectodomain of CD4 precisely fused to the transmembrane and cytoplasmic domains of the VSV glycoprotein. The data suggest that when the VSV G protein is expressed at normal levels, the

transmembrane and cytoplasmic domains of the VSV glycoprotein are not required for the insertion of the cellular glycoprotein CD4 into the viral envelope.

4. The strategy and the results of our efforts towards the generation of "new age" recombinant VSV particles will be summarized.

A REVERSE GENETIC ANALYSIS SYSTEM FOR VSV

Asit K. Pattnaik and Gail W. Wertz

We have developed an alternative approach to structure-function analysis of vesicular stomatitis virus (VSV) gene products and their interactions with one another during each phase of the viral life cycle.

Transfection of plasmid DNAs containing VSV genes under the control of a T7 RNA polymerase promoter into cells infected with a recombinant vaccinia virus that expresses the gene for bacteriophage T7 RNA polymerase results in high-level expression of the VSV proteins. Coexpression of the N, NS, and L proteins of VSV in cells allows replication and amplification of defective interfering (DI) particle RNA of VSV. We demonstrate now that by transfecting plasmids containing cDNA clones for all five of the VSV genes into cells, it is possible to express all five VSV proteins such that they can support replication, assembly, and budding of infectious DI particles from these cells. This system will allow us to study detailed structure-function aspects of each viral protein and its role in each step of the viral replicative cycle.

Transcription of Measles and Vesicular Stomatitis Viruses In Vitro. Sue A. Moyer, Department of Immunology and Medical Microbiology, University of Florida, Gainesville, FL 32610.

A transcription system with detergent-disrupted purified measles virus has been developed. Synthesis of authentic, full-length measles virus N, P, M, and F mRNAs by purified virus occurred as identified by dot blot hybridization analysis to individual measles virus clones and gel electrophoresis. The addition of the soluble protein fraction from uninfected A549 cells stimulated overall viral RNA synthesis, but did not alter the relative abundance of each of the mRNAs. Measles virus synthesized in vitro, a leader RNA of ~55 nucleotides in length, suggesting that like other negative-strand viruses, transcription initiated only at the 3' end of the genome RNA. Purified measles virus also catalyzed RNA editing during the synthesis of the P mRNA as shown by modified primer extension analysis of the mRNA products and by translation of the modified RNA into the V protein in rabbit reticulocyte lysates. These data suggested that the RNA editing activity was virus encoded.

Negative-strand RNA viruses require a nucleocapsid (N) protein encapsidated template for transcription by the viral RNA polymerase. We have developed methodology for the encapsidation of small, synthetic VSV RNAs with purified N protein. These synthetic nucleocapsids can be specifically transcribed with purified VSV RNA polymerase. Templates as short as 22 nucleotides can be transcribed. Synthetic nucleocapsids containing potential cis signals found at the gene junctions thought to mediate capping, methylation and polyadenylation are ignored during in vitro transcription. Since these signals are recognized in vitro with wild-type nucleocapsids, additional sequences and/or structures distant from the gene junctions might be important for RNA processing. Encapsidated RNAs with the 3' 50 terminal nucleotides of both the (+) and (-) strand sense (leader genes) were transcribed by the same viral polymerase. Such synthetic short nucleocapsids of the New Jersey and Indiana serotypes maintained the serotype specificity for transcription observed with the wild-type viruses. Nucleotides 21 to 50 appear to determine serotype specificity. Analysis of the essential regions within the 3' 20 nucleotides to define the VSV promoter will be discussed.

Phosphorylation States of the Phosphoprotein P of Vesicular Stomatitis Virus.

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The large polypeptide L and phosphoprotein P (NS) constitute the RNA dependent RNA polymerase activity of vesicular stomatitis virus (VSV), a rhabdovirus. In order to produce the phosphoprotein in large quantities amenable to detailed biochemical and structural analyses, we have cloned and expressed two serotypes of VSV P genes in *Escherichia coli*. P genes of New Jersey (Ogden) and Indiana (Mudd-Summers) serotypes were cloned into a T7 RNA polymerase-based *E. coli* expression vector (pET-3a) by using a PCR technique that employed overhanging primers with desired restriction sites. Synthesis of P message in these clones occur from a T7 phage promoter whereas translation is driven by the Shine-Dalgarno sequence of T7 gene 10 and its initiation codon AUG. The clones were then introduced into E. coli BL21 (DE3) in which T7 RNA polymerase is expressed from the lac promoter. Under optimal conditions of induction (IPTG), P proteins made in these clones comprise 5-15% of the total cell protein.

P protein expressed in bacteria ("bacterial P") was found to be unphosphorylated. It was transcriptionally active when reconstituted with viral L protein and N-RNA template in vitro. Bacterial P protein could be phosphorylated in vitro by BHK cell extracts as well as by protein kinase activities present in L and N-RNA preparations. Specific classes of phosphorylated P proteins produced by these activities were separated by ion-exchange chromatography and their transcriptional properties were studied in vitro. The sites of phosphorylation were determined by the use of mutant P proteins in which specific Ser/Thr residues were altered to Ala by in vitro mutagenesis. Such studies helped to define the exact role of the phosphate groups present in the various domains of the phosphoprotein.

TRANSCRIPTION MECHANISMS OF LYSSAVIRUSES

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Besides structural information, the molecular analysis of several rabies and rabies-related genomes has provided a powerful means to investigate the transcription mechanisms within the Lyssavirus genus. A detailed analysis of the rabies PV strain transcription was first performed by northern blotting and S1 nuclease mapping with single-stranded minus sense probes complementary to every messenger. These studies demonstrated that both G glycoprotein and M2 matrix protein genes can alternatively use two consecutive transcription stop signals, resulting in the production of either a short or a long messenger. In both cases, the extra length present in the long messenger shows no significant open reading frame. For example, the long G messenger corresponds to a G-Ψ bicistronic event where the rabies non-coding pseudogene Ψ is tandemly transcribed. This suggests that alternative termination is more likely to be of transcriptional importance. Assuming the sequential transcription of the rabies genome, we propose that it constitutes a means to regulate gene expression in modifiyng the distance separating the stop of the proximal gene from the start of the distal one.

This hypothesis is confirmed by extending analysis to most of the fixed rabies strains as well as some field rabies isolates. Using nucleotide sequencing of PCR amplified viral genes, we show that several strains had simply deleted the proximal G stop signal, resulting in the unique production of the large G-Ψ mRNA. Consistently, these strains show a substantially greater transcription efficiency of the L gene when compared to strains producing both G mRNA and G-Ψ mRNA.

In the strains maintaining the two stop signals, the recognition of the proximal G stop signal is the crutial point that determines the G/G-Ψ mRNA ratio. The different modulation of this ratio during the infection of fibroblastic or neuronal host cell cultures suggests that G stop recognition probably depends on viral or host-cell specific transcription factors. A typical 10-nucleotide long sequence located 10 nucleotides upstream from the G stop could represent the key point of this modulation, either by embedding the signal in its proper secondary structure, or by mediating the interaction with transcriptional factors. We are currently testing this hypothesis using single-stranded RNA probes covering the G stop signal in retardation and Northern-Western blotting experiments with extracts from uninfected and infected fibroblastic and neuronal cells.

Transcriptional analysis of the rabies-related Mokola virus has also been performed and shows a tendancy towards an M1-M2 bicistronic mRNA, in concert with other rabies-related viruses. In contrast with the G stop recognition, this phenomena is more probably related to the sequence of the M1 stop signal itself, which is divergent from cannonical ones.

The relevance of these results in terms of evolution of unsegmented negative stranded RNA virus genomes will be also discussed.

THIRD SESSION

P. COLLINS
J. A. MELERO
R. G. PATERSON
D. KOLAKOFSKY
M. A. BILLETER
A. K. BANERJEE
R. M. ELLIOTT
K. K. CONZELMANN
M. KRYSTAL

Molecular Studies of Human Respiratory Syncytial Virus.

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We have completed the cloning and sequencing of the 15,222-nucleotide genomic RNA (vRNA) of human respiratory syncytial virus (RSV) strain A2. This provides a complete picture of the gene map and sequence relatedness of this atypical paramyxovirus and is a requisite step in efforts to develop methods of producing live RSV from cDNA. This latter capability would have obvious application in molecular studies and would make possible the characterization and manipulation of attenuated strains of RSV which are being developed for vaccines. At the present time, we are assembling full-length cDNAs of the A2 strain which will contain either (i) two nucleotide substitutions in intergenic regions which create two new restriction sites as markers, or (ii) the substitution of the F gene with its counterpart from a bovine strain. These cDNAs will be under the control of the phage T7 promotor for the synthesis of vRNAs containing the exact, authenic termini. It is hoped that conditions can be found which will render these synthetic vRNAs infectious. This might occur, for example, if the synthetic vRNAs are complemented intracellularly by helper proteins supplied by superinfecting standard A2 RSV. Under those conditions, the substitution of the A2 F gene by its bovine counterpart would provide a means for subsequently eliminating the strain A2 helper virus background using existing F-specific monoclonal antibodies which neutralize strain A2 but do not recognize bovine strains. We also are assembling cDNAs encoding truncated vRNAs which contain the correct vRNA termini but which contain the chloramphenical acetyl transferase gene or beta-galactosidase gene under the control of RSV transcriptive signals as markers for amplification and transcription. We will describe the progress of these and other studies of the structure, expression and functions of RSV RNAs and proteins.

GENETIC MECHANISMS INVOLVED IN THE GENERATION OF ANTIGENIC VARIANTS OF HUMAN RESPIRATORY SYNCYTIAL (RS) VIRUS

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The antigenic structure of the two RS virus external glycoproteins (G and F) was determined with panels of monoclonal antibodies (MAbs). The epitopes on the F molecule could be ascribed to five nonoverlapping antigenic sites; in contrast, the anti-G antibodies recognized unique epitopes, many of whose competition profiles overlapped extensively. The majority of the F protein epitopes were highly conserved among virus isolates whereas epitopes of the G protein showed extensive variation even among viruses of the same antigenic subgroup. To clarify the molecular basis of the antigenic and variability differences between the F and G proteins, escape mutants were selected with individual MAbs. Sequence analysis of the variants selected with anti-F antibodies revealed single amino acid substitutions which altered only the epitopes grouped in the same antigenic site. In contrast, the variants selected with anti-G antibodies contained any of the following three types of genetic changes: i) frame shifts caused by deletions or insertions of single adenosine residues, ii) premature in-frame stop codons generated by single nucleotide substitutions or iii) single amino acid changes caused by nucleotide substitutions. The first two types led to drastic alterations of the G protein C-terminal third and the loss of the binding sites for most anti-G MAbs. These results will be discussed in terms of the different mechanisms which generated the genetic changes selected in the escape mutants and their relevance for the natural history of RS virus.

RNA Editing in Paramyxoviruses

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We, and others, have recently demonstrated that an RNA editing event occurs in RNA transcripts derived from the P gene of paramyxoviruses. This phenomenon results in the synthesis of more than one mRNA species that differ from each other by the number of G residues at a specific insertion point in the RNA. One of the P gene-specific mRNAs is predicted to encode a cysteine-rich polypeptide. The cysteine-rich domain is reminiscent of a zinc finger motif. The polypeptide possessing the zinc finger-like domain (protein V) has been identified in cells infected with SV5, Sendai virus, mumps virus and human parainfluenza virus type 2. The SV5 protein V has been found to be phosphorylated, to bind RNA by Northwestern blot analysis and to be present in SV5 virions. The V protein overexpressed using the baculovirus expression system has been partially purified. We are in the process of examining the properties of site-directed mutants of protein V expressed in bacteria.

Sendai Virus Genome Replication via Cloned Viral Genes.
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The molecular genetics of the replication machinery of (-) RNA viruses has been limited by the inability to express functional components of the replicase complex *in vivo*. Much of our information about the replicase and the protein-protein interactions essential for its function, have therefore come from *in vitro* studies.

We have recently developed an *in vivo* system to study Sendai virus genome replication, in which a DI genome (which itself expresses no proteins) is amplified using cloned viral genes. Genes encoding proteins NP and L, as well as P/C, V/C, W/C, P without C, and the N and C-terminal halves of the P protein, were inserted into plasmids under the control of a T7 promoter. BHK cells infected with a vaccinia recombinant expressing T7 polymerase (vTF7-3) were transfected with different combinations of plasmids, and then superinfected with a DI stock in which the helper virus had been inactivated with UV light. DI genome replication was monitored by Northern blots, and the expression of the cloned genes by Western blots. From these experiments we have been able to make a number of observations:

1. proteins NP, P and L, as expected, are essential. Elimination of any one of the 3 reduces amplification to near background levels.
2. none of the nonstructural proteins of the P gene (C', C, Y1, Y2, V or W) is essential. Elimination of C', C, Y1, and Y2 expression has no effect. However, expression of V or W with NP, P and L has a negative effect.
3. the N and C-terminal portions of the P protein coexpressed from different plasmids will not substitute for P (the two domains of P will not function *in trans*).
4. a P mutant lacking the C-terminal 30 amino acids (and which therefore binds to nucleocapsids very poorly) functions at <10% the efficiency of P.

Measles viruses from recombinant cDNAs: a system allowing functional analyses and potentially suitable for vector applications

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Our system, developed to generate replicating measles virus (MV) from genomic cDNA cloned in plasmids, and based on microinjection of linearized DNA complexed with T3 or T7 RNA polymerase into a helper cell line (Ballart et al., EMBO J. 9, 379, 1990), is working reliably. Nevertheless, we are trying to improve the system, a) to increase the efficiency of virus production, b) to circumvent the laborious microinjections, and c) to substitute the currently used helper cell line, continuously replicating a defective MV genome, with cells expressing only those MV proteins necessary for RNA encapsidation, transcription and replication. The new system should be based on the expression of cloned genes, and thus be applicable to other nonsegmented negative strand RNA viruses.

So far we have used the system to functionally test four MV matrix (M) genes cloned from cases of subacute sclerosing panencephalitis (SSPE) by substituting them for the M gene in the MV Edmonston strain genomic clone. One of the four constructs gave rise to virus reaching normal titers, indicating that abrogation of M gene function is not a strict requirement for the development of SSPE. By substituting fragments of the three nonfunctional SSPE M genes, the mutations responsible for functional inactivation of the corresponding proteins could be localized in the second half of the genes. By analogous substitution experiments the functionality of the short carboxy terminal part of the fusion (F) proteins is being tested; in all 10 SSPE cases analyzed, corresponding F gene regions have been found to be variously mutated, in contrast to the main portion of the genes. Furthermore, the phosphoprotein (P) gene editing region is replaced by constructed mutated regions to shed more light on the editing mechanism.

In principle, MV should also be applicable as a vector system, to express additional antigenic determinants. One approach, namely the creation of an additional transcription unit, is being tested: to supply the framework of a seventh gene, a synthetic DNA chip of 82 nucleotides, representing mainly the N/P gene boundary followed by some nucleotides specifying two restriction sites not present in our MV genomic plasmid, was inserted in the 3' terminal nontranslated regions of either the P or the hemagglutinin (H) genes. Both constructs gave rise to efficiently replicating viruses; analysis of RNA recovered from cells infected with these viruses, using retrotranscription followed by PCR amplification, showed that the MV genomes still contained the chip. Insertion of coding regions into the newly created restriction sites of the modified plasmids will show whether a) a larger insertion is still compatible with virus formation, b) the expected functional proteins are produced in cells infected with such recombinant viruses, and c) the burden of an additional gene can be transmitted intact over many cycles of virus replication.

In Vitro Transcription of Human Parainfluenza Virus Type 3: Role of Cellular Actin in mRNA Synthesis.

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The ribonucleoprotein complexes (RNP) of human parainfluenza virus type 3, although contained the viral polymerase subunits L and P, synthesized mRNAs very inefficiently in an in vitro transcription reaction. However, the mRNA synthesis was stimulated 7- to 10-fold upon addition of soluble cytoplasmic proteins from uninfected cells. In contrast to Sendai virus transcription that required cellular cytoskeletal protein tubulin, the in vitro mRNA synthesis from HPIV3 RNP was not stimulated significantly by purified tubulin. Moreover, cytoplasmic extract depleted of tubulin by immunoprecipitation stimulated HPIV3 transcription effectively, suggesting involvement of host protein(s) other than tubulin in the HPIV3 transcription process.

The transcription stimulatory factor was purified from uninfected cell extract by conventional chromatography, and the purified factor was characterized as actin on the basis of its following properties: i) it migrated in SDS-polyacrylamide gel as a 43 kD protein and reacted with anti-actin antibody; ii) it polymerized in presence of ATP, Mg^{++}, and KCl identical to cellular actin polymerization; iii) it bound strongly to blue-Sepharose and remained in the unbound fraction of heparin-Sepharose columns. Furthermore, when the cell extract was depleted of actin by immunoprecipitation with anti-actin antibody, the stimulatory activity was abolished indicating an involvement of actin the HPIV3 transcription stimulatory process. The stimulatory activity was also detected in the 43 kD protein in other cell lines, such as CV-1, HeLa, and BHK. Further characterization of the molecular form of actin responsible for this stimulation by using the monomer-binding protein DNaseI will be discussed.

EXPRESSION OF THE BUNYAMWERA VIRUS L PROTEIN USING RECOMBINANT VACCINIA VIRUSES

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The complete nucleotide sequence of the Bunyamwera virus L RNA segment was recently determined in this laboratory from cloned cDNAs (Elliott, 1989; Virology **173**, 426-436). The L segment is 6875 bases in length, and in the complementary sense RNA encodes the L protein (2238 amino acids, MWt 259,000) which is thought to be the virion associated transcriptase or RNA polymerase. We have made monospecific rabbit antisera to the amino- and carboxy-termini of the L protein by fusing appropriate cDNA sequences of the L segment to the bacterial ß-galactosidase gene using the pUEX series of plasmids (Bressan & Stanley, 1987; Nucl. Acids Res. **15**, 10056). These antisera recognise the L protein in Bunyamwera virus infected cells by both immunoprecipitation and Western blotting.

With the long term aim of defining the functional domains within the L protein we have expressed a full length cDNA using vaccinia virus vectors. Two systems have been used: firstly the "standard" system where the L segment cDNA has been cloned into a transfer vector (pSC11) under control of the vaccinia virus 7.5K promoter and recombined into the thymidine kinase (TK) locus in the vaccinia virus genome (Chakrabati *et al.*, 1985; Mol. Cell. Biol. **5**, 3403-3409); and secondly the "T7" system where the cDNA has been inserted into the TK locus but under the control of a bacteriophage T7 promoter - expression of the target gene in this case only occurs when T7 RNA polymerase, supplied by a second recombinant vaccinia virus (vT7-3), is synthesised in the same cell (Fuerst *et al.*, 1987; Mole. Cell. Biol. **7**, 2538-2544). Antigenically authentic L protein of apparently the same size as that of Bunyamwera virus was detected in cells infected with either type of vaccinia virus vector. More L protein was synthesised using the standard system than the T7 system.

We have demonstrated that the expressed protein is functional by a nucleocapsid rescue assay: Bunyamwera virus nucleocapsids were purified from infected cells by CsCl centrifugation, and then transfected into CV-1 cells which had previously been infected with the recombinant vaccinia viruses. Total cell RNA was extracted at 20hr post transfection and analysed by Northern blot hybridization. A dramatic amplification of the nucleocapsid RNA, as monitored by studying the level of the S RNA genome segment with an S segment specific probe, was observed in cells which expressed the L protein from the vaccinia viruses, whereas in control cells the S RNA was barely detectable. The level of amplification was higher using the standard rather than the T7 vaccinia virus system. We hope to exploit this methodology to investigate the functions of the bunyavirus L protein in fine detail.

Complementation of L (Polymerase) Deficient Rabies Virus DI Particles by Heterologous Helper Viruses and by Rabies Virus L expressed in Cells.

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Two internal deletion type rabies virus defective interfering particles (DIs) have been isolated and characterized (SAD DI-1 and PV DI-1). Both DI genomes possess an internal deletion spanning large parts of the L gene and both transcribe mRNAs corresponding in size to standard virus N, NS, M, and G mRNAs. After coinfections with various heterologous helper viruses the DI genomes are replicated and transcribed correctly by the helper virus L proteins. The L deficiency of the DI genomes is also complemented by cells transfected with cDNA encoding the SAD B19 rabies virus L protein. As a consequence infectious DI particles are produced. Thus both SAD DI-1 and PV DI-1 encode their own functional N, NS, M and G proteins and can be propagated without helper virus. This in vivo replication and transcription system will be used for mutational analysis of the rabies virus L protein.

RESCUE OF A FOREIGN GENE BY SENDAI VIRUS

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ABSTRACT. A simple protocol for the rescue of a synthetic genome into paramyxovirus has been developed. First, a synthetic Sendai virus-like RNA, containing the antisense coding region of the chloramphenicol acetyltransferase (CAT) gene replacing the coding region of the Sendai virus genome, was transcribed off a cDNA. When introduced into cells which are infected with Sendai virus, this RNA construct was transcribed, replicated, and packaged into infectious virions. The addition of infected cell extract to the RNA prior to transfection markedly enhanced levels of CAT expression and rescue. However, this enhancement is not due to encapsidation of the RNA into nucleocapsids as the RNA remains nuclease sensitive. Uninfected cell extract also enhances expression and rescue efficiency, implying involvement of a cellular factor(s) with the synthetic viral-like RNA construct which allows for enhanced polymerase recognition. This system will now allow for the dissection of the various *cis*-acting RNA signals within the paramyxovirus genome.

POSTER SESSIONS

PRODUCTION OF MONOCLONAL ANTIBODIES SPECIFIC FOR THE INFLUENZA VIRUS PA PROTEIN

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Transcription and replication of influenza virus are mediated by three polymerases (P) proteins PA, PB1, PB2 in association with the Nucleoprotein. The P proteins have been shown to exist as a complex both in virions and in the infected cell. PB2 and PB1 proteins, while forming part of this complex, carry out the recognition and binding to the cap-1 structure of the host-cell messenger RNAs, and the addition of nucleotides to the growing viral mRNA chains, respectively. No specific role has yet been assigned to the PA protein.
In order to gain information about the role(s) of the PA protein during the replication-transcription of the viral genome and to identify functional domains on the protein we have prepared monoclonal antibodies (mabs) to the PA protein of influenza virus strain A/Victoria/3/75.
We used complete PA protein, overproduced in E. coli using a T7-derived expression vector, as an antigen source. The protein, recovered as an insoluble pellet from induced bacteria and further purified from PAGE-SDS gels, was used to inmunize Balb/c mice. We obtained a panel of mabs that are being presently characterized in detail. Preliminary characterization shows that: 1) they react, in Western blot, to the PA protein of influenza purified virus; 2) some of the mabs recognize the PA protein in the nuclei of MDCK infected cells; 3) some of them inmunoprecipitate the PA protein from S^{35}-Met labelled extracts of cells infected with influenza virus.

IN VITRO TRANSCRIPTION OF GERMISTON VIRUS (BUNYAVIRUS)

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Transcription of the bunyavirus Germiston has been studied in an in vitro system containing purified virus, reticulocyte lysate and the four ribonucleoside triphosphates, under salt conditions (Mg^{++}, K^{+}) where transcription and translation were coupled . The virion transcriptase synthesized RNA molecules which are similar, if not identical, to mRNA isolated from infected cells. We showed that the transcripts possess 5' non viral coded extensions which characterize the authentic mRNAs. We demonstrated that these extra sequences are capped and contain the 5' uppermost region (12-13 nucleotides) of alpha and beta globin mRNA present in rabbit reticulocyte lysate. However, complete mRNA molecules were synthesized only when translation was carried out efficiently .

VACCINIA RECOMBINANTS WHICH EXPRESS MUTATED FORMS OF HUMAN RESPIRATORY SYNCYTIAL (RS) VIRUS F GLYCOPROTEIN, INTRACELLULAR PROCESSING AND HUMORAL IMMUNE RESPONSE IN MICE. J. A. López, C. Albo, J. A. Melero and A. Portela. Servicio de Biología Molecular. Centro Nacional de Microbiología. Instituto de Salud "Carlos III". Majadahonda. 28220-Madrid. Spain.

There is experimental evidence, from animal models, to indicate that the F glycoprotein of human RS virus plays an important role in the humoral and protective immune response to the virus. Recently, we have mapped an epitope (47F) of the F glycoprotein in the amino terminal third of the F_1 subunit and identified either of amino acids 262 or 268 as essential for the epitope integrity.

To evaluate the contribution of epitope 47F to the global antigenicity and immunogenicity of the F molecule, three vaccinia recombinants have been obtained which express the following proteins: i) VA-F encodes the wild type F molecule, ii) VA-FT encodes the F protein mutated in amino acid 262, and iii) VA-FR47 encodes the F protein with the amino acid substitution at residue 262 and 5 additional amino acid changes (Asn_{67} to Tyr, Asn_{120} to Tyr of the F_2 subunit and Phe_{223} to Leu, Phe_{237} to Ser, Asn_{262} to Tyr and Ala_{442} to Val of the F_1 subunit).

These recombinants are currently being used to evaluate the immunogenicity of the wild type and mutant proteins. Preliminary results indicate that the F protein encoded by the recombinant VA-FR47 induces an abnormal humoral response, which might be related to a defect on the intracellular processing of the mutated molecule. In contrast, the maturation process and the humoral immune response induced by the F protein encoded by VA-FT is undistinguishable from wild type.

Respiratory syncytial virus variability during an epidemic.

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Forty-two isolates of respiratory syncytial (RS) virus from a single epidemic (Autumn 1989) were analysed to determine the extent of variability of the virus. Parts of the genes coding for the nucleocapsid (N) protein and the small hydrophobic (SH) protein were amplified using the polymerase chain reaction (PCR) and the amplified regions further analysed by restriction mapping or nucleotide sequencing. It was found that at least six different lineages (4 subgroup A and 2 subgroup B) of RS virus were circulating at the same time and in the same area. Analysis of the nucleotide sequences of the attachment (G) protein genes of a limited number of the isolates confirmed the distinctness of the lineages. The amino acid sequence variability of this major virion glycoprotein could contribute to the ability of this virus to repeatedly reinfect individuals.

CHARACTERIZATION OF "IN VITRO" MADE RNA-NUCLEOPROTEIN COMPLEXES OF INFLUENZA VIRUS.

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We have studied the "in vitro" interaction of influenza virus nucleoprotein (NP) with the vRNA corresponding to the hemagglutinin segment of the A/Puerto Rico/8/34 strain. The vRNA was synthesized "in vitro" from a cDNA clone with T7 RNA polymerase. The NP was purified from infected cells following a modification of the procedure described by Honda et al (J. Biochem., 104, 1021-1026, 1988).

The RNA-NP complexes were resistant to RNase A digestion, indicating that the RNA was covered in its full length by NP molecules. Assays made by nitrocellulose retention method with varying RNA:NP ratios and different size RNA molecules suggest a cooperative binding of NP to RNA. These studies represent a first step towards the "in vitro" preparation of functional RNPs. This will allow an in-depth analysis of the influenza RNA replication process and the rescue of genetic markers after transfection with "in vitro" made RNPs of virus infected cells.

COMPLETE SEQUENCE OF THE FIRST FILOVIRUS L-PROTEIN AND THE RELATIONSHIP TO OTHER NONSEGMENTED NEGATIVE STRAND RNA VIRUSES

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Marburg virus (MBG), Ebola virus (EBO), and the recently isolated Reston and Pennsylvania viruses are members of the third family of nonsegmented negative-strand RNA viruses, the Filoviridae. MBG and EBO cause a severe hemorrhagic disease in humans with a high mortality rate whereas Reston and Pennsylvania seem to be less pathogenic.

To analyze the genome structure of MBG and its genetic relationship to other nonsegmented negative-strand RNA viruses, i. e., Paramyxoviridae and Rhabdoviridae, we started to clone and sequence the genome of the Musoke strain of MBG. The genomic RNA is 19,3 kb in length containing 7 genes in the following order: 3' NP-VP35-VP40-GP-VP30?-VP24?-L.

The L gene is 7741 b in length and contains an ORF of 6990 nucleotides. The deduced amino acid sequence (2330 amino acids) has a calculated molecular weight of 267 k. Computer assisted comparison of the MBG L-protein with those of other nonsegmented negative strand RNA viruses (Rabies, VSV, HPIV3, Sendai, NDV, Measles) revealed a higher degree of similarity to the paramyxovirus than to the rhabdovirus L-proteins. Barik et al. (Virology 175, 1990) and other authors have shown the presence of 4 blocks of conserved amino acids in the L-proteins of paramyxo- and rhabdoviruses (box A-D), which probably represent various functional sites of the viral polymerases. We could identify at least box A, B, and C in the aminoterminal part of the MBG L-protein. Further we found the following conserved oligopeptide motifs which might be implicated in polymerase function of different L-proteins of nonsegmented RNA viruses: a LDDD motif and the pentapeptide (M)GDNQ, which both may be considered as variants of the GDD motif (Kramer and Argos, Nucleic Acids Res., 1984), several potential ATP binding sites, and 3 basic domains, which could serve as RNA binding domains.

THE POLYADENYLATION SIGNAL OF INFLUENZA VIRUS RNA CONSISTS OF A STRETCH OF URIDINES FOLLOWED BY THE RNA DUPLEX OF THE PANHANDLE STRUCTURE

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In order to understand the signals involved in polyadenylation of the influenza virus mRNAs, we used a ribonucleoprotein transfection system in which a plasmid-derived RNA containing the reporter gene chloramphenicol acetyltransferase (CAT), flanked by the noncoding sequences of the NS segment of influenza A/WSN/33 virus, is transcribed and amplified after transfection into cells. Using this system, we introduced mutations into both the 5' and 3' ends of the synthetic RNA and measured the levels of RNA transcription and CAT expression of the mutants. The levels of cRNA and mRNA were detected by RNase protection assay in RNP-transfected cells. The results reveal that polyadenylation of mRNAs requires a stretch of uridine residues approximately 17 to 22 nucleotides from the 5' end of vRNAs and an RNA duplex (panhandle) juxtaposed to the uridine stretch. The data suggest that the mechanism of polyadenylation involves the slippage (stuttering) of the viral polymerase on this RNA structure.

CONVERSION OF VSV NS1 TO NS2 PROTEIN SPECIES BY A SPECIFIC VIRION CORE-ASSOCIATED PROTEIN KINASE AND UNCOUPLING FROM THE VIRUS TRANSCRIPTION PROCESS, Jacques Perrault and J. David Beckes, Biology Department and Molecular Biology Institute, San Diego State University, San Diego, CA 92182

Near stoichiometric phosphorylation of the vesicular stomatitis virus (VSV) NS protein takes place during the transcription process carried out by detergent-disrupted virions in vitro. We have shown that at least two distinct protein kinase associated with transcription-competent virion cores can phosphorylate NS in vitro (Beckes and Perrault, submitted). Using reaction conditions which differentiate between these two activities, we show here that one of the kinases (VSVK1) gives rise to the phosphorylated NS1 species while the second (VSVK2) converts NS1 to faster migrating species, collectively referred to here as NS2. While both types of phosphorylated NS species accumulate concomitantly with transcription, NS2 is the major product. NS1 to NS2 conversion, however, is specifically inhibited by pre-treatment of virion cores with 5'-p-fluorosulfonylbenzoyl adenosine (FSBA) with no effect on the transcription process itself. Likewise, addition of cell extracts also inhibits the appearance of NS2 without affecting transcription. The latter effect is probably due to cellular phosphatases since the same extracts added subsequent to transcription specifically remove phosphates from NS2. We conclude that although a phosphorylated form of NS may be required for VSV transcription, NS1 to NS2 conversion is not coupled to this process.

THE MODE OF ANTIVIRAL ACTION OF HUMAN AND MURINE Mx PROTEINS

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Most interferon-induced Mx proteins possess intrinsic antiviral activities, including murine Mx1 and human MxA. Mx1 and MxA proteins accumulate in different subcellular compartments: Mx1 is located in the nucleus, whereas MxA is cytoplasmic. Constitutive expression of elther Mx1 or MxA protein in transfedted 3T3 cells conferred resistance to influenza virus. Cell lines expressing MxA also acquired resistance to vesicular stomatitis virus (VSV). Mutations affecting the nuclear transport signal located near the carboxy terminus destroyed the antiviral potential of Mx1 protein, suggesting that Mx1 inhibits a multiplication step of influenza virus taking place in the cell nucleus. MxA variants with mutations affecting the GTP-binding consensus motif failed to confer resistance to either VSV or influenza virus, indicating that this conserved domain is indeed necessary for Mx protein function. Mutating a single amino acid near the carboxy terminus destroyed the anti-VSV activity of MxA protein but did not affect the anti-influenza virus activity.

Our efforts to define the viral replication steps blocked by Mx proteins revealed that Mx1 inhibited transcription of the parental influenza viral genome, whereas MxA blockerd a later step of the influenza virus multiplication cycle. Inhibition of VSV by MxA was due to strongly reduced transcription of the parental genome.

We believe that Mx proteins physically interact with viral proteins, thereby interfering with their normal functions.

INMUNODOMINANCE OF THE C-TERMINAL END OF THE HUMAN RESPIRATORY SYNCYTIAL VIRUS G GLYCOPROTEIN

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Escape mutants selected with anti-G monoclonal antibodies contained any of the three following genetic changes: i) frame shifts which altered the C-terminal third of the G molecule, ii) premature in-frame stop codons which removed a variable number of amino acids at the C-terminal end or iii) single amino acid substitutios. The first two type of changes predicted drastic alterations on the G protein structure which were reflected in the loss of binding sites for many anti-G antibodies. Single amino acid substitutions in the last five residues of the G protein or premature stop codons which removed only the carboxy terminal amino acid caused also loss of numerous antibody binding sites. In contrast, amino acid substitutions more internal in the G molecule had only limited influence in the antigenic structure of the G molecule. These results emphasize the importance of the G protein C-terminal end for a large number of overlapping epitopes, which can be distinguished otherwise by their presence in natural isolates and their reactivity with anti-idiotypic antibodies.

STRUCTURE AND EXPRESSION OF THE TWO SUBSEGMENTAL mRNAs TRANSCRIBED FROM THE S RNA OF UUKUNIEMI VIRUS.

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Uukuniemi virus (UUK), the prototype of the Uukuvirus genus within the Bunyaviridae family, has a tripartite negative-strand RNA genome. The small (S) RNA segment codes for the nucleocapsid protein N and a non-structural protein NSs in an ambisense mode, i.e. the N mRNA is transcribed from the 3' end of the viral RNA (vRNA) whereas the NSs mRNA is transcribed from the 3' end of the viral complementary RNA (vcRNA). Between the two ORFs there is an untranslated intergenic region of 74 nucleotides.

We have examined the 5' and 3' ends of the N and NSs mRNAs in order to get a better understanding of the mechanisms of transcription initiation and termination. We have also investigated the expression and localization of the NSs protein, the function of which is unknown, but which may be a component of the replication or packaging machinery.

To study whether UUK mRNAs contain nonviral host-derived sequences at their 5' ends, as has been shown for some other bunyaviruses, as well as for incluenza and arenaviruses, a representative number of 5' ends of both N and NSs mRNAs were cloned and sequenced. Nonviral 5' ends ranging in size from 7 to 25 nucleotides were found with 12 being the average as determined by primer extension. Comparison of the 5' end sequences revealed that one third were missing the most 5' virus-encoded A residue, which, with one exception, was replaced by a G residue.

To identify possible transcription termination sequences, the 3' ends of the N and NSs mRNAs were mapped either by nuclease protection assays or by hybridization with probes located at different positions relative to the presumed 3' ends. The 3' end of the N mRNA was shown to be located just a few nucleotides downstream of the ORF for the NSs protein, whereas the N mRNA estended into the coding-region (last 9 amino acids) of the N protein. This suggested that the two mRNAs overlap each other by some 100 nucleotides and thus would be self-complementary. An RNase resistent product about 100 nucleotides long was indeed obtained after self-annealing. No common structural motifs could be found close to the 3' ends. However, some 35 (N mRNA) and 40 (NSs mRNA) nucleotides upstream from the 3' ends, a 24 nucleotide long almost perfect palindromic structure was found. This could be base-paired into a 9 residue long stem (with one mismatch) and a 6 reidue loop structure.

To study the function and localization of the NSs protein we first expressed the protein in a baculovirus expression system and raised antisera against it. In parallel we also expressed the N protein. The rabbit antisera obtained were shown to identify NSs in UUK infected cells both by immunoprecipitation and immunofluorescence. Localization of NSs in infected cells by immunofluorescence showed a typical cytoplasmic staining with no detectable nuclear staining, in contrast to results obtained for Rift Valley Fever virus, which exhibits a clear nuclear staining. The cytoplasmic localization was also confirmed by analysis of cytoplasmic and nuclear fractions after solubilization of cells in TX-100. The possibility of finding NSs in virus particles has also been considered but preliminary attempts to demonstrate this have been negative. To further characterize NSs, we looked for phosphorylation of the protein. We have, however, obtained no evidence that NSs is phophorylasted. During infection NSs first appears at about 6 h p.i., reaches a maximum rate of synthesis at 12 h p.i. and is still expressed at 24 h p.i. at a level comparable to that at 8 h p.i.. This was comparable to the kinetics of the N protein. The half-life for NSs was estimated to be about 1.5 h. In summary, our results have not yet elucidated the function of NSs in iunfected cells.

TOWARDS RECONSTITUTION OF THE TRANSCRIPTIVE AND REPLICATIVE COMPLEX OF NEWCASTLE DISEASE VIRUS IN-VITRO

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Newcastle disease virus (NDV), the prototype virus of the paramyxovirus genus, is responsible for the devastating disease of poultry commonly known as fowl pest.

Viral penetration into the host cells is mediated by the haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins. Once within the cell, viral transcription and replication are brought about by the combined action of three virally encoded proteins, the nucleocapsid (NP), phospho- (P), and large (I.)proteins. In order to study the respective roles of each of these proteins at a molecular level, we set out to clone each gene into suitable expression vectors, and to purify the overexpressed proteins from E. coli.

A genebank of cDNA clones to the genome of NDV strain Ulster 2C was constructed using random primers and reverse transcriptase. Extensive colony hybridization, nucleotide sequence analysis and restriction mapping identified certain clones as corresponding to specific NDV genes. NP was represented by a single clone covering the entire open reading frame, P required two clones, and L, five. The NP gene was cloned directly into the prokaryotic expression vector pKK223-3 (Pharmacia), and several manipulations were performed to reduce the Shine-Dalgarno-ATG spacing to an optimum distance. The gene was also cloned into the cloning vector pBluescript KS+, in phase with the lacZ alpha peptide. Western blots of both these constructs showed NP protein to be present in the crude cell lysate.

The P gene was constructed in pBluescript from two overlapping clones around a central NsiI. The final clone was inserted into pKK233-3 in the same fashion as for NP. Western blotting with polyclonal and monoclonal antibodies detected expression of P as both native and lacZ-fusion proteins.

The five clones covering the NDV L gene were assembed within pBluescript KS+, in a complex multi-stage procedure. Site-specific mutagenesis was used to created an NcoI site at the start codon, and a linker containing the same enzyme site was inserted at the 3' end of the full length clone. This will now be cloned into the vector pKK233-2, and L protein production will be monitored.

The aim of the work is to reconstitute in-vitro the NDV transcriptive complex from cloned proteins so that the contributions of the individual proteins can be analysed. By utilizing this approach, techniques such as site-directed mutagenesis can be used to study the importance of proposed key regions of any of the proteins. This method also ensures that the proteins can be obtained free of contamination with other viral proteins, potentially a problem with studies carried out on proteins obtained directly from the virus.

HETEROGENEITY OF THE MUTATION RATE OF INFLUENZA A VIRUSES

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In order to analyse the genes of influenza A viruses involved in the generation of their high variability, we are searching for differences in the mutation rate among several virus strains and clones. We have measured , by using monoclonal antibodies specific for H3 subtype hemagglutinin, the mutation rate to viable monoclonal antibody-resistant (*mar*) genotype of A/Victoria/3/75 (H3N2) (VIC) strain and another two strains (A/PR/8/34 (H1N1) and A/chicken/"N" Germany/49 (H10N7)), in which we had introduced the H3 gene through recombination. Our results did not show significant differences in the mutation rate among these three strains. However, we have found that several clones from the VIC strain have different mutation rates to the *mar* genotype. These results suggest that the mutation rate of an influenza virus reflects a weighted average of the mutational contributions from an heterogeneous population.

Based on these results, we have isolated three presumptive mutator mutants by screening from a non-mutagenized virus population, and at present we are trying to characterize them.

CHARACTERIZATION OF A PROTEIN KINASE ACTIVITY THAT PHOSPHORYLATES HUMAN RESPIRATORY SYNCYTIAL (RS) VIRUS P PROTEIN.

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Human respiratory syncytial (RS) virus,a Pneumovirus that belongs to Paramyxovirus family, is the main causative agent of lower respiratory tract disease in infants (1),and it has been classified by the Health World Organization as a pathogen with preferential attention for the development of specific treatment.

In oder to develop prophylactic measures, it must know the viral epitopes that elicit an effective immune response and the function of those proteins relevant in the virus growth cycle.

In our laboratory we are studying the function of the viral P protein.This is a structural phosphoprotein that,presumably, contributes to the inner viral nucleocapsid.In the Long strain its deduced amino acids sequence is known (2) and its phosphorylated serine residues have been mapped (3).P protein phosphorylation is not required either for the synthesis and accumulation of viral RNA(with both plus and minus polarities) or for the nucleocapsid formation (4).

In a recombinant vaccinia virus system the synthetized P protein is phosphorylated with a similar pattern to that produced during RS viral infection.This suggests that the P protein postranslational modification is due,at least in part, to a cellular kinase(s).The characterization of such protein kinase(s) is the topic of this poster presentation.

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CONCLUSIONS AND PERSPECTIVES

Negative Strand RNA Viruses Come of Age

Until very recently, it has not been possible to engineer changes in (-)RNA virus genomes because, unlike (+)RNA viruses, neither RNA nor DNA copies of the genomes were infectious. Because of this, as Juan Ortin has pointed out in the introduction, progress in our understanding of this separate group of viruses has been severely limited. The problem is relatively straightforward, but not trivial. Both the genomic and antigenomic RNAs of these viruses function as polymerase templates only when assembled as nucleocapsids. Starting from DNA, ways must then be found to encapsidate the RNA transcripts, and if the entire genome is not contained in the DNA, to provide the missing viral functions *in trans*.

The reason that this was a particularly appropriate time for this Juan March Foundation workshop on (-)RNA virus transcription and replication is that within a relatively brief timespan, three systems have been reported in which changes can be made at the level of DNA and their consequences examined *in vivo*:

1) A simple system, but one restricted to examining the structure-function relationships of the viral gene products, was reported by Wertz's lab in Alabama. A DI genome is introduced into a cell naturally but without helper virus. The viral proteins are then provided *in trans* from transfected plasmids containing T7 promoters, and T7 polymerase is provided via a recombinant vaccinia virus. The DI genome can thus be replicated, and also

appear in infectious particles. This approach will be invaluable in delineating protein domains, and determining the corresponding domains with which they interact.

2) The most spectacular system is that of Billeter's lab in Zurich, who use a cell line (derived from the brain of a SSPE patient) persistently infected with measles virus, but which remarkably does not bud out any infectious virus. A full length (15.8 kB!) clone under the control of a T7 promoter is then transcribed in vitro with only two NTPs just to start the chain, and the whole complex is then microinjected into the helper cells. The endogenous viral activity of the helper cell is required only to prime the pump; once the recombinant genome is assembled, it should itself be infectious. Very few virus plaques come out of this operation, but those that do grow normally and can be characterized. Viable constructs then lead to new engineered viruses. So far, this system has been applicable only to measles virus, because a suitable helper cell line is not yet available for other viruses. However, it should soon be possible to also create a helper cell by recombinant means for other viruses.

3) Palese and Krystal (Mt. Sinai, New York) have constructed an arteficial influenza virus segment with a T7 promoter upstream and a class II restriction site downstream, such that an in vitro (-)sense transcript can be made whose 5' and 3'ends are precisely those of the natural segment. A CAT gene (or another gene) in the

antisense orientation is then placed in between. The T7 transcript is then assembled into nucleocapsids also *in vitro*, and these are transfected into cells superinfected with natural helper virus. CAT expression here requires both genome replication and transcription from the arteficial genome, and the CAT segment is also present in the progeny virions. Engineered segmented viruses have thus been created.

The use of these systems has opened a new vista for research with this important group of human and animal pathogens. A large range of new viruses can now be engineered with this group as with positive strand viruses; only the manipulations will be more complex. It is now also possible to cleanly investigate the nature of the cis-acting sequences which control viral RNA synthesis, as well as the function of each viral protein. For the nonsegmented viruses where classical genetics has been both difficult and mostly inconclusive, this form of reverse genetics or "punk" genetics has rescued the field from creeping obscurity. The ability to manipulate these viral genes as well as the viral genomes as DNA represents a quantum jump for for future research. Negative strand RNA viruses have come of age in the recombinant DNA world.

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TRANSCRIPTION AND REPLICATION OF
NEGATIVE STRAND RNA VIRUSES

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240 **Genetic Strategies in Development.**
Symposium in honour of Antonio García Bellido. Lectures by S. Ochoa, S. Brenner, G. S. Stent, E. B. Lewis, D. S. Hogness, E. H. Davidson, J. B. Gurdon and F. Jacob.

244 **Course on Genome Evolution.**
Organized by E. Viñuelas. Lectures by R. F. Doolittle, A. M. Weiner/N. Maizels, G. A. Dover, J. A. Lake, J. E. Walker, J. J. Beintema, A. J. Gibbs, W. M. Fitch, P. Palese, G. Bernardi and J. M. Lowenstein.

246 **Workshop on Tolerance: Mechanisms and implications.**
Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco and P. Marrack.

247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

249 **Workshop on Molecular Diagnosis of Cancer.**
Organized by M. Perucho and P. García Barreno. Lectures by F. McCormick, A. Pellicer, J. L. Bos, M. Perucho, R. A. Weinberg, E. Harlow, E. R. Fearon, M. Schwab, F. W. Alt, R. Dalla Favera, P. E. Reddy, E. M. de Villiers, D. Slamon, I. B. Roninson, J. Groffen and M. Barbacid.

251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening,

J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

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.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P. Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorín, M. Beato and A. A. Travers. Lectures by F. Azorín, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

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