Instituto Juan March
de Estudios e Investigaciones

59 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

RNA Viral Quasispecies

Organized by

S. Wain-Hobson, E. Domingo and
C. López Galíndez

C. K. Biebricher
J. M. Coffin
E. Domingo
M. Eigen
J. Holland
N. L. Letvin
L. A. Loeb
C. López Galíndez
B. W. J. Mahy
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A. Meyerhans
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The lectures summarized in this publication were presented by their authors at a workshop held on the 7th through the 9th of October 1996, at the Instituto Juan March.

Depósito legal: M. 41.206/1996
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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INTRODUCTION

Esteban Domingo

The workshop on RNA viral quasispecies gathered a number of experts on topics related to RNA virus evolution. It was indeed a broad scope of topics ranging from the molecular basis of copying fidelity of viral polymerases to the contribution of virus variation to pathogenesis and to the emergence of new viruses. As is often the case in a gathering of stout scientists with long personal histories in unique environments, agreement in some issues parelleled disagreement in many others.

Few virologists would now question that the quasispecies model of molecular evolution of macromolecules, proposed by M. Eigen a quarter of a century ago, is exerting a great influence in our current understanding of RNA viruses. Several examples were identified that document that specific mutations arising during viral replication are directly associated to new pathogenic potential of the evolved genomes. It is not yet possible to design experiments involving infections with viruses replicating with very high copying fidelity to test the effect of replication errors on virus pathogenesis. In spite of this, it is becoming increasingly clear that adaptability, measured as the ability to gain fitness, or the ability to cope with environmental changes (presence of antibodies, drugs) are directly related to the high mutation rates and quasispecies structure of viral populations.

There was much less agreement on the limits of applicability of the quasispecies concept. Are human populations quasispecies? And retrotransposons? Fortunately Eigen himself was there to clarify the origins of the concept, as also emphasized in several of his recent papers: Quasispecies implies a related set of simple replicons subjected to competitive selection. The main departure from previous models of population genetics is the consideration of the wild type as an ensemble of genomes instead of one genome with a defined nucleotide sequence. It is this mutant swarm - the preferred word of H. Temin- that offers sufficient plasticity for the ensemble to become an easy prey of selective forces and genetic drift.

The process of mutant generation, competition and selection can be analyzed in a controlled fashion in the replication of short-chained RNA species derived from bacteriophage Qβ. Several more complex model systems were discussed. The classical influenza viruses —which preceded other viral systems in defining concepts of structural and evolutionary virology— poliovirus, foot-and-mouth disease virus, vesicular stomatitis virus, hepatitis C virus, human respiratory syncytial virus, retrotransposons and,

of course, the human immunodeficiency viruses and their chimeric simian/human versions, among other animal and plant RNA viruses and genetic elements. Although several important concepts are emerging from these studies (limitations in the cloud of mutant swarms, multiple mutational pathways associated to escape from antibodies or to resistance to antiviral inhibitors, identification of mutations associated to deep fitness losses, the effect of viral population size on selective dominance of some classes of mutants, etc.) it was also quite clear that additional input from population genetics would be of great help to virology. Some connexions between classical population biology theory and quasispecies have already been established but more are needed to assess the value of viruses to approach evolutionary problems.

New possibilities of antiviral intervention based on the error prone replication of viruses were also discussed. Copying fidelity of reverse transcriptase can be modified by structural alterations of the enzyme and the manipulation of fidelity to drive viral replication into error catastrophe is no longer a dream. An elegant exploitation of error-prone replication is the generation of heavily substituted nucleic acids and proteins. *In vitro* hypermutagenesis constitutes an impressive tool to explore the functional space of enzymes, and a means to generate molecules with new biological properties.

In conclusion, the meeting offered a lot to most and, fortunately, there were sufficient points of disagreement to lend ourselves to believing that Fundación Juan March may consider a similar meeting some years from now.

VARIATION - MECHANISMS AND CONSEQUENCES

Chairman: Esteban Domingo

MULLER'S RATCHET AND VIRAL BOTTLENECKING.

John Holland, Department of Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116

More than a generation ago, Muller recognized that when genomic mutation rates are high in asexual organisms (and hence not subject to recombinational repair) , a ratchet-like accumulation of deleterious mutations might occur. The operation of Muller's ratchet was first convincingly demonstrated for a tripartite RNA virus (bacteriophage Ø6) by Lin Chao of the University of Maryland. This was confirmed for the animal viruses, vesicular stomatitis virus and foot-and-mouth disease virus. This presentation will outline general observations concerning Muller's ratchet carried out by our laboratory in collaboration with the laboratories of Esteban Domingo and Andrés Moya in Spain. Some major points are: (1) Muller's ratchet certainly must occur often in nature during virus transmission from host-to-host and from various foci infection within hosts. This is inevitable due to quasispecies mutant swarms generated by extreme mutation rates of RNA viruses. (2) Darwinian selection operates poorly during genetic bottlenecks but exerts strong effects upon large virus populations whether or not they are clonal populations. (3) Quantitative assays for RNA virus fitness regularly demonstrate selection for increasing RNA virus fitness during repeated transfers of large virus populations in a constant environment. (4) Effective sizes of genetic bottlenecks vary depending upon the fitness of starting virus. (5) Effects of genetic bottlenecks upon virus fitness are stochastic, hence unpredictable. (6) Subcloning of virus clones shows that clones are quasispecies not only at the genetic level but also at the phenotypic level of virus fitness. (7) RNA virus mutation rates are, in fact, poised near the error threshold because neither single site mutation rates nor biological adaptability can be greatly increased by chemical mutagenesis. (8) The competitive exclusion principal and Red Queen competitive behavior are readily observed during virus population competitions but remarkable nonlinear competition dynamics sometimes occur.

Other aspects of complex quasispecies behavior of RNA virus populations will be discussed.

Population Genetics of RNA viruses

Andrés Moya, Alma Bracho, Rosario Miralles, Eladio Barrio and Santiago Elena
Departament de Genètica, Facultat de Biologia, Universitat de València
c/ Dr. Moliner, 50. 46100 Burjassot, Valencia, Spain

RNA viruses are excellent experimental models for studying Evolution under the theoretical framework of Population Genetics. Population Genetics is a reductionistic research program of Evolutionary theory because it assumes that transformation laws within- and between- genotypes and phenotypes spaces are linear or, in other words, that genotype maps linearly onto phenotype. Among replicative entities, RNA viruses are those that better meet this assumption of the Population Genetics theory.

Muller's ratchet, Red Queen hypothesis and competitive exclusion principle are three relevant principles in Evolutionary theory that have been tested under appropriate experiments and they constitute part of an outstanding piece of work carried out by virologists and evolutionary biologists mostly dealing with vesicular stomatitis virus (VSV) and foot-and-mouth disease virus (FMDV) as model systems (see abstracts to this meeting by Holland, Domingo and Escarmis).

The objective of the present communication is to open the spectrum of evolutionary hypotheses that eventually can be properly tested with RNA viruses (in this case by using VSV as model system). Our group has focused its attention to three different topics: frequency-dependent selection, group selection and the neutral theory of molecular evolution:

(i) We have experimental evidence showing that fitness is frequency-dependent, and that when two given population are competing a stable equilibrium is reached making possible coexistence at least during periods where highly advantageous mutations do not appear.

(ii) The second topic is related to the underlying idea on viral evolution that the unit of selection is not the single particle but the entire "quasispecies". We have carried

out a set of evolutionary experiments where individual and group selection are acting in the same and/or opposite directions. Depending on the experimental regimes, the results are compatible with the hypothesis of stabilizing selection promoting attenuated virulence (here defined as first linear canonical component of fitness and growth rate decay) towards an average value, and consequently group selection acting against the natural tendency of viruses to increases, and disruptive selection where viral clones tend to disappear because the joint effect of Muller's ratchet and group selection is not overcome by individual selection.

(iii) The last topic studied is the fitness recovery of highly debilitated viral clones. Preliminary fitness experiments have shown that such clones have increased almost four orders of magnitude its initial and extremely low fitness values. Accordingly, we have carried out a systematic sequencing of two genes (coding for glycoprotein and phosphoprotein) in twenty clones for each gene randomly taken from the evolving population in three different culture points: 28 hr, 48hr of passage 1 and passage 40. We have not been able to detect any significant genetic differentiation among populations, and the three statistical tests of neutrality show either that the genetic variability observed do not depart from what it is expected from neutrality or some significant deviation that can be interpreted in terms of purifying selection and/or population size increase. By considering that fitness have increased towards neutrality we speculate that (a) darwinian selection is taking place at the very beginning of the process; (b) eventually such type of selection is not detected because the population do not meet the equilibrium assumptions, and (c) that sampling is not enough and biased.

HIV-1 VARIATION IN VITRO.

C. López Galíndez[1], E. Yuste[1], S. Sánchez Palomino[1]*, C. Casado[1], MJ Iglesias[1], I Olivares[1] and U Plikatt[2] and A Meyerhans[2].

1.Centro Nacional de Biología Celular y Retrovirus. Instituto de Salud Carlos III. Majadahonda. Madrid

2. Abteilung Virologie. Institut f Med Mikrobiologie u Hygiene. Freiburg

* Present address Centro de Biología Molecular Severo Ochoa. CSIC-UAM. Cantoblanco. Madrid

Genetic variation has been observed in many situations during HIV-1 infection either in vitro or in vivo. To help in the understanding of this variation we have undertake different classical in vitro experiments. To perform these studies, the first step was the obtainment of individual clonal populations of viruses. In HIV-1 this is a difficult process which was overcome by a plaque assay in MT4 cell line. With this method we obtained 10 biological clones derived from an Spanish isolate designated S61. Five of these clones were further purified by 5 serial plaque purification steps. These clones could be differentiated in a genetic screening by the RNase A mismatch method (RAMM). Five of these clones along with the global population were submitted to serial passages in MT-4 cells. These passages were performed at two multiplicities of infections 0.1 plaque forming unit (pfu) per cell and 0.001.pfu/cell. After the passages different changes were observed: appearance and disappearance of bands in the RAMM digestion pattern, point mutations detected by nucleotide sequencing and phenotypic alterations. In the V3 loop, the principal neutralizing epitope, aminoacid changes were noticed during the passages in the absence of immune pressure. One of these changes was associated with a phenotypic change from a non syncytial (NSI) to a syncytial (SI) virus. By in vitro mutagenesis we have identify an asparagine (in the SI virus) to isoleucine (in the NSI virus) amino acid change responsible of this phenotypic alteration. Taking in account these results we could deduced that most of the observed changes occurred in the passages performed in the lower multiplicity of infection, thus indicating that dilute passage conditions in vitro promotes the expression of variation in HIV-1 (1). This phenomenon could be related to the fact that less competing pressure is excerted by the global population in the lower multiplicity of infection.

We have compared by nucleotide sequencing initial and final viruses of the different clones. This analysis was performed in V1-V2 loop, V3 loop and fusion domain

in *env* gene and also in *nef* gene. These regions showed very different pattern of variation. In V1-V2 region most of the clones displayed deletions in the V1 loop. In the V3 loop region we detected point mutations giving rise to aminoacid changes. The fusion region was completely constant in every virus. In nef gene viruses tend to the loss of fragment of this genomic region but also many point mutations occurred. In summary, the viruses seems to be under very distinct constraints or selection pressures in different genomic regions. This results in the observation of different genomic changes in different regions. However, deletions seems to be over represented in these viruses after been submitted to serial passages in tissue culture particularly in some genomic regions.

Another important point which we wanted to test was the effect of Muller´s Ratchet in HIV-1 because the existence of two copies of the viral genome in the virion. We have used ten biological clones of virus S61, obtained by MT-4 plaque assay as previously mentioned, and we have subjected these viruses to plaque to plaque passages. From the 10 clones selected, 6 were able to perform 15 plaque to plaque passages. Of the 4 remaining, 2 were not able to surpass passage 7 or 8, and two other passage 13. To check the fitness of each individual clone, competition experiments were performed between a genetically marked clone and each of the clones. The genetic differentiation and the quantification of clones was performed by the Tracking Assay (TA) which is a modification of the Heteroduplex Mobility Assay. Taking advantage of this simple technique we have been able to recognize a high degree of heterogeneity in the fitness values of the different clones. In some of the competitions experiments unusual variants arose. We have analyze these variants by nucleotide sequencing part of *env* gene and a deletion of different length in the V1 loop has been observed.

All these experiment have allowed us to detect the existence in the in vitro situation of many different mutants within the same HIV-1 viral population. Depending of the experimental conditions, the sampling procedures or the selective forces acting different variants have arisen with different genotypic and phenotypic properties. In some experimental conditions, even very minor and/or low fitness variants were recovered. These results reflects the great possibilities that the quasispecies structure of HIV-1 viral population offers to viral evolution.

Reference: 1) Sánchez-Palomino et al. (1993). J Virol 67, 2938-2943.

HIV Population Dynamics and Genetic Variation.

John M. Coffin, Department of Molecular Biology, Tufts University, Boston, MA 02111

A number of recent studies have indicated that HIV infection in vivo is a highly dynamic but steady state process, with the large majority of infected cells dying soon after infection and from virus rapidly turning over in the blood. Current thinking is that about 99% of the virus seen in blood is derived from a single kinetic class of infected cells with a mean lifetime of about 1.5 days after infection, resulting in an average replication cycle time of about 2 days, or some 180 cycles a year. Thus, at the average time of progression to AIDS, the HIV population in an infected individual is about 1000 generations removed from the original infecting virus.

It is this extraordinary amount of replication (in terms of successive cycles, not population size or high mutation rate) that must be the source of the great genetic diversity observed with HIV and related viruses in vivo. Such viruses clearly exist as "semiquasispecies" within which considerable variation has developed, but are nowhere near mutational equilibrium, and still exhibit strong founder effects. The distribution of mutations in such a population is then a complex feature of time since infection, mutation rate, selective effects and genetic drift. The importance of the latter is itself dependent on effective population size and distribution.

An important goal of studying genetic diversity of HIV populations is to use the information to infer important features of the virus population structure and dynamics in vivo. Questions that might be appropriate include: How large is the replicating population? (in number of infected cells per round, not virus); How is it distributed (as a "well-stirred pot", or a number of independent populations which may seed the blood differentially); Is the population size steady or are there significant bottlenecks? For this purpose, a good mathematical model for mutation accumulation in virus populations is necessary. Purely stochastic (neutral) models are clearly unsatisfactory. Pure deterministic (Darwinian) models seem to work better, at least for simple drug resistance and for in vitro models we have studied, but looking at genetic diversity as a whole, a model which encompasses the broad range in between random drift and strict selection needs to be developed and applied. These issues will be discussed.

The appearance of drug-resistant HIV-1 variants in 3TC-treated patients:
the role of mutation and selection.

Nicole Back[1], Belinda Oude Essink[1], Wilco Keulen[2], Charles Boucher[2] and Ben Berkhout[1]. [1]Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, [2] Department of Virology, Academic Hospital, Utrecht University.

Human immunodeficiency virus type 1 (HIV-1) variants with resistance mutations in the reverse transcriptase (RT) gene appear during drug-therapy with nucleoside analogues. The drug 3TC (2',3'-dideoxy-3'-thiacytidine) leads to mutations within the catalytic core of the RT enzyme; a Met184Ile variant is observed initially in treated patients, followed by the appearance and eventual outgrowth of viruses with the Met184Val substitution. Here, we analyzed the forces responsible for this peculiar evolution pattern of 3TC-resistant HIV-1 variants. First, we measured the mutational frequencies from the wild-type codon (AUG) towards the Ile (AUA) or Val codon (GUG) in a limiting dilution drug-selection protocol. The frequency of the G to A mutation (towards Ile) was found to be approximately 5-fold higher than the frequency of the A to G mutation (towards Val). This result is fully consistent with the initial appearance of the 184Ile variant. Second, we compared the polymerase function of the wild-type and variant enzymes. A processivity-defect was scored for the two RT variants, but the Ile enzyme was affected most severely (RT activity: Met=wild-type > Val > Ile). Furthermore, the enzyme activity of the RT proteins correlated with the replication capacity of the corresponding virus mutants (Met > Val > Ile), consistent with the observation that 184Ile is outcompeted by 184Val. These combined results clarify the characteristic evolution pattern of 3TC-resistant viruses. Reduced fitness of the 3TC-resistant viruses is clinically relevant if it leads to diminished viral load, which may extend the asymptomatic period and postpone the development of AIDS.

GENETIC LESIONS ASSOCIATED TO MULLER'S RATCHET IN AN RNA VIRUS

CRISTINA ESCARMÍS[1], MERCEDES DÁVILA[1], NATHALIE CHARPENTIER[1], ALMA BRACHO[2], ANDRÉS MOYA[2] AND ESTEBAN DOMINGO[1]*

[1]*Centro de Biología Molecular "Severo Ochoa", Madrid, Spain*

[2]*Department de Genética, Universitat de Valencia, Valencia, Spain.*

The molecular basis of Muller's ratchet has been investigated using the important animal pathogen foot-and-mouth disease virus (FMDV). Clones from two FMDV populations were subjected to serial plaque transfers (repeated bottleneck events) on host BHK-21 cells. Relative fitness losses were documented, in 11 out of 19 clones tested. Small fitness gains were observed in 3 clones. One viral clone attained an extremely low plating efficiency, suggesting that accumulation of deleterious mutations had driven the virus near a mutational meltdown and extinction. Nucleotide sequence analysis revealed unique genetic lesions in multiply transferred clones that had never been seen in FMDVs isolated in nature or subjected to massive infections in cell culture. In particular, a frequent internal polyadenylate extension has identified a mutational hot spot on the FMDV genome. Furthermore, amino acid substitutions in internal capsid sites which are severely restricted during FMDV evolution, amounted to half of capsid replacements in the transferred clones. In addition, a striking dominance of nonsynonymous replacements fixed upon large population infections of FMDV was not observed upon serial plaque transfers. The nucleotide sequence of the entire genome of a severely debilitated clone suggests taht very few muatations may be sufficient to drive FMDV near extinction. The results provide an account of the molecular basis of Muller's ratchet for an RNA virus, and insight into the types of genetic variants which populate the mutant spectra of FMDV quasispecies and that need not become dominant in viral populations.

MODEL SYSTEMS

Chairman: John Holland

RANDOM SEQUENCE MUTAGENESIS OF HIV REVERSE TRANSCRIPTASE: EFFECTS ON DRUG RESISTANCE AND FIDELITY OF DNA SYNTHESIS

Baek Kim and Lawrence A. Loeb
Joseph Gottstein Memorial Laboratory, Dept. of Pathology, University of Washington, Seattle, WA 98195, USA

Human immunodeficiency virus reverse transcriptase (HIV RT) can substitute for DNA polymerase I (pol I) in bacteria. Expression of HIV RT enables the *E. coli* mutant *polA12recA718* containing a temperature sensitive mutation in pol I to grow at non-permissive temperature. Expression of HIV RT also enables the same *E. coli* mutant to maintain the plasmid pBR322 at non-permissive temperature. Moreover, expression of HIV RT renders growth of the mutant sensitive to 3'-azido-3'-deoxythymidine (AZT), a commonly used anti-AIDS drug that targets HIV RT.

We are employing random mutagenesis coupled with genetic selection to generate a series of mutant HIV RTs. We targeted amino acids Asp-67 through Arg-78, which form part of the β3-β4 flexible loop and harbor many of the currently known mutations that confer resistance to nucleoside analogs. DNA sequencing of 109 selected mutants that complemented the Pol I^{ts} phenotype revealed substitutions at all 12 residues targeted, indicating that none of the wild-type amino acids is essential. However, single mutations were not observed at Trp-71, Arg-72, and Arg-78, consistent with evolutionary conservation of these positions among isolates from patients. The mutations we recovered included most of those known to be associated with drug resistance as well as previously unidentified mutations. Purification and assay of 14 mutant proteins revealed correlation between their DNA-dependent DNA polymerase activity *in vitro* and ability to complement the Pol I^{ts} phenotype. Activity of several mutants was resistant to AZT triphosphate.

Several of the highly active mutants exhibited alterations in the fidelity of DNA synthesis *in vitro*. Amino acid substitutions at Asp-67 yielded mutant enzymes that exhibited either higher or lower fidelity. The β3-β4 template binding domain is presumably involved in both analog specificity and replication accuracy. The technology of random sequence mutagenesis thus allows one to generate a vast array of new unnatural HIVs.

Human immunodeficiency virus reverse transcriptase substitutes for DNA polymerase I in *Escherichia coli*. B. Kim and L.A. Loeb. Proc. Natl. Acad. Sci. USA., 92: 684-688, 1995.

A screen in *Escherichia coli* for nucleoside analogs that target human immunodeficiency virus (HIV) reverse transcriptase: Coexpression of HIV reverse transcriptase and herpes simplex virus thymidine kinase. B. Kim and L.A. Loeb. J. Virol., 69: 6563-6566, 1995.

Human immunodeficiency virus reverse transcriptase. Functional mutants obtained by random mutagenesis coupled with genetic selection in *Escherichia coli*. B. Kim, T.R. Hathaway and L.A. Loeb. J. Biol. Chem., 271: 4872-4878, 1996.

Unnatural nucleotide sequences in biopharmaceutics. L.A. Loeb. Advan. Pharm., 35: 321-347, 1996.

FOOT-AND-MOUTH DISEASE VIRUS AS A MODEL SYSTEM

Esteban Domingo, Centro de Biologia Molecular "Severo Ochoa", Universidad Autónoma de Madrid, Cantoblanco, 28049, Madrid, Spain.

Foot-and-mouth disease virus (FMDV) is an important animal pathogen of the *Picornaviridae* family. Seven serological types and a spectrum of antigenic variants continuously evolving in nature renders this virus an excellent model to study the molecular basis of antigenic variation. These studies have been aided by: a) the elucidation by D. Stuart and colleagues of the three-dimensional structure of viral particles representative of several serotypes of FMDV; b) additional structural information for specific capsid domains; and c) sequences for FMDV variants subjected to a variety of selective influences, and even of minority components hidden in the low frequency range of genomes in quasispecies. The main conclusions of these studies, and the overall picture that they provide of the molecular evolution of FMDV, may be summarized as follows:

1) In spite of remarkable antigenic diversity with regard to interaction with polyclonal and monoclonal antibodies, antigenic heterogeneity of FMDV serotype C is mediated by very limited sequence variation at several antigenic sites (Mateu, 1995; Mateu et al., 1994). For FMDV, which replicates in cell culture, variation at antigenic sites can occur both in the presence and in the absence of antibody selection (Domingo et al., 1993; Sevilla et al., 1996; Holguín et al, submitted for publication).
2) Limitations in the numbers and types of amino acid substitutions in the FMD capsid appear to be imposed by structural and functional constraints (Lea et al., 1994). An example is the limitation in the number of substitutions fixed at internal capsid residues when competitive replication is allowed. This is in spite of the quasispecies being populated with variants harboring internal capsid substitutions (Escarmis et al., 1996; and this meeting).
3) Limitations to variation may be accentuated when a viral protein or protein domain performs multiple functions, as it is being recognized for an increasing number of viral proteins. Recent work by several groups has established that the G-H loop of capsid protein VP1 of FMDV includes an R-G-D motif which serves as a receptor-recognition site for the virus (Berinstein et al., 1995). Structural studies by I. Fita and colleagues suggest that the R-G-D residues are also direct points of interaction with some neutralizing antibodies raised against the virus (Verdaguer et al., 1995, 1996). Thus, an essential receptor recognition site need not be protected from immune attack. Interestingly, recent work with poliovirus (Harber et al., 1995) and rhinovirus (Mosser et al., 1996) suggests that there is significant overlap between the subset of amino acid residues involved in receptor recognition and those belonging to antigenic sites. These findings have obvious implications for coevolution of antigenic behavior and the host range of viruses.
4) The population size of FMDV evolving in cell culture may exert an important influence on the types of variants which become dominant in the evolving population. This is expected from the quasispecies structure of FMDV, and it has been recently shown by two groups of experiments. One involved a study of reversion of genetic and phenotypic traits of a persistent FMDV when subjected to serial cytolytic passage (Sevilla and Domingo, 1996). Both the extent of reversion of some phenotypic traits and some types of substitutions at the G-H loop of VP1 were associated with large population passage of the virus. The net result of the alternation between a persistent and a cytolytic infection was partial reversion of phenotypic traits accompanied with genetic diversification (Sevilla and Domingo, 1996).

In another group of experiments, it has recently been shown that passage of cloned FMDV in cell culture may render non-essential the "essential" R-G-D motif of VP1, and this was mediated by a few additional substitutions at neighboring capsid sites (Martinez et al., submitted for publication, and this meeting). It is remarkable that FMDV quasispecies replicating in a relatively constant environment have the potential to find alternative pathways for entry into host cells.

In summary, FMDV of serotype C evolving in the field or under defined cell culture conditions tolerates a very restricted number of mutations. Functional and structural constraints preserve its identity as an infectious agent with a durable spectrum of pathogenic features.

Tolerated changes are sufficient to permit adaptability and survival. It is tempting to speculate that different serotypes of FMDV resulted from rare jumps in sequence space, perhaps due to recombination or unusual hypermutation events. The fact that intermediate genotypes not assignable to a serotype have not been described, reinforces the view that only limited variations around any given serotype are compatible with FMDV viability and long-term survival.

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STUART T. NICHOL
Centers for Disease Control and Prevention
(CDC), Special Pathogens Branch, Divisions
of Viral and Rickettsial Diseases
1600 Clifton Rd.
ATLANTA, GA. 30333 (USA)

Title Changing landscapes and emerging RNA viruses

Due to high polymerase error rates, RNA viruses exist in the form of quasispecies and have tremendous potential for rapid evolution. We are in an era of rapidly changing global landscapes and local environments. The plasticity of RNA viruses allows them to quickly adapt to and exploit these altered environments. It comes as no surprise then that several prominent recent examples of emerging or re-emerging diseases are due to RNA viruses. However, a number of factors and their complex interplay can influence the emergence of disease. In addition to genetic variation (mutation, recombination, reassortment), "environmental" factors, defined in a broad sense (i.e. to include ecological, social, health care, behavior, and public health infrastructure influences) can all play important roles. Obvious examples of how a rapidly changing global landscape can promote emergence of RNA virus associated disease include 1) damming of rivers altering potential virus vector abundance and distribution, and 2) tropical deforestation bringing humans in close contact with these species-rich (hosts and their parasites) environments. Such factors coupled with enormous increases in the human population during the last 50 years and massive urbanization in many developing countries, have greatly increased the number of sampling events taking place in terms of testing the fitness of a wide variety of RNA virus microvariant swarms in different human cell backgrounds and potential transmission modes. This together with the tremendous increase in the speed and volume of global transportation, combines to create increased opportunity for emergence and re-emergence of diseases associated with RNA viruses.

Against this background, two prominent examples of emerging and re-emerging RNA virus diseases will be discussed; the "emergence" of hantavirus pulmonary syndrome (HPS) in the southwestern US in 1993 and the re-emergence of Ebola in Kikwit, Zaire in 1995. Hantaviruses are rodent-borne viruses of the *Bunyaviridae* family. Some of these viruses had previously been known to be associated with hemorrhagic fever with renal syndrome (HFRS) in humans, in various parts of Asia and Europe. It was with some surprise that a newly discovered hantavirus, Sin Nombre virus was found associated with a severe respiratory disease (approximately 50% mortality rate), HPS in humans in the southwestern US. Nucleotide sequence and phylogenetic analysis of these viruses, are most consistent with Sin Nombre virus not having recently emerged in the literal sense, but having been present in deer mice populations throughout North America for a long time. The recent HPS outbreak and discovery of the virus appear linked to ecological factors rather than virus genetic changes. El Nino effects had produced two consecutive mild spring seasons and abnormally high deer mice populations, which resulted in increased opportunity for virus transmission to humans and identification of a cluster of HPS cases. The hantavirus phylogenetic tree is most consistent with the co-evolution of hantaviruses with their specific rodent reservoirs. Such a scenario would

suggest that the current extensive of genetic diversity of these viruses has accumulated over a very long period of time, i.e. despite high polymerase error rates and the potential for RNA genome segment reassortment, these viruses have evolved slowly in nature over many hundreds of years. This relative genetic stability or stasis of an RNA virus will be revisited at the end of the presentation.

Ebola virus dramatically re-emerged, causing a large hemorrhagic fever outbreak in Kikwit, Zaire in 1995. Phylogenetic analysis of these and previously detected Ebola viruses, indicated that 4 distinct subtypes exist, Zaire, Sudan, Reston, and Ivory Coast. The 1995 Kikwit outbreak was related to the re-emergence of Ebola-Zaire, a virus that had not been seen since the large hemorrhagic fever outbreak in Yambuku, Zaire (approx. 600 miles from Kikwit) in 1976. Nucleotide sequence comparison of these geographically and temporally distant Ebola-Zaire strains showed they were virtually identical, with only 1.6% sequence divergence in their glycoprotein genes. As in the previous hantavirus example, it appears that Ebola viruses evolve slowly in nature despite their presumed high polymerase error rates and existence in the form of quasispecies. Again, it is likely that environmental factors rather that virus genetic changes are the predominant influence on the explosive re-emergences of outbreaks of disease caused by these high lethal viruses. As the natural hosts of Ebola viruses remain elusive, precise identification of these factors are still to be determined.

The genetic stasis of these (and several other) RNA viruses in nature, at first appears counter-intuitive and contrasts the commonly presented picture of rapid evolution as seen with viruses such as polio, human immunodeficiency and influenza A viruses in humans. Genetic stasis is compatible with high virus polymerase error rates and existence as quasispecies when RNA viruses are viewed from the standpoint of stability of virus environments and virus adaptive or fitness landscapes. The consistency or variation of the environmental conditions in which a virus replicates will determine the topology, and frequency and extent of distortion of the virus fitness landscapes. If conditions remain constant, the swarm of virus microvariants will form a cloud around a particular fitness peak on the potential fitness landscape, i.e. genetic stasis will prevail. Virus microvariants will continually be produced but most will be of lower fitness than the original virus. With alteration of environmental conditions (altered host, immune selection etc.) distortion of the virus fitness landscape may result in ridge-climbing to a nearby higher fitness peak, i.e. rapid evolution. Environmental conditions or the complex balance of selective forces will be a major force molding the diverse evolutionary patterns observed for RNA viruses. These are extremely complex systems, the behavior of which will be difficult, if not impossible, to predict.

Lessons Learned from the Recent Emergence and Re-emergence of RNA Virus Diseases

Brian W.J. Mahy (Director, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, CDC, USA)

Emerging and re-emerging virus diseases are defined as those which have newly appeared in the population, have reappeared or are rapidly increasing in incidence or geographic range, or are newly recognized.

Since 1988, more than forty new viruses associated with disease in humans have been recognized. These are either newly emerged or re-emerged viruses, and all except two of them (human herpesvirus 7 and human herpesvirus 8) have RNA genomes. During the same period, numerous new RNA viruses of veterinary importance have also emerged.

The factors contributing to the emergence of human virus disease are complex, but include human demographics and behavior, breakdown of public health measures, increase in the amount and speed of international travel, changes in technology and industry, economic development and land use, and virus evolution and adaptation (Lederberg, et al., 1992; Murphy and Nathanson, 1994). Although there are some examples of novel RNA genomes that clearly arose from a recent mutational event, most newly recognized viruses are found to have existed in the human or animal population for a long time, and to belong to families or genera that have already been characterized.

Despite the extreme rapidity of RNA virus evolution which ensures a constant potential for new viruses to be generated, replication cycles of many human viruses (e.g., polio, measles, mumps) depend on the presence of antigenic epitopes that cannot change significantly without compromising virus fitness. Such viruses are targets for eradication using existing vaccines. Other human viruses (e.g., influenza, hepatitis C and HIV) do not appear to have such constraints so that multiple antigenic variants will continue to emerge from the quasi-species population.

Many of the most recently emerged viruses are zoonoses, present in rodent or bat populations and infecting man when close contact occurs due to ecological or population changes. The reservoir from which Ebola virus has re-emerged several times in recent years is unknown, but it appears to involve a highly conserved ecological niche, since genome sequences of viruses isolated many years apart do not differ significantly (Sanchez, et al., 1996).

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Quasispecies in Wild Tula Hantavirus Populations

Alexander Plyusnin, Ying Cheng, Heikki Lehväslaiho and Antti Vaheri

Haartman Institute, Department of Virology,
FIN-00014 University of Helsinki, Finland.

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are worldwide distributed RNA viruses carried by rodent or insectivore hosts and transmitted to humans via inhalation of aerosols of animal excreta. Some of these viruses are etiologic agents of hemorrhagic fever with renal syndrome or hantavirus pulmonary syndrome, others are thought to be apathogenic for humans. The RNA genome of hantaviruses is of negative polarity and consists of three segments, named large (L), medium (M) and small (S), which encode a viral polymerase, two surface glycoproteins (G1 and G2) and a nucleocapsid protein (N), respectively. Accumulation of point mutations, as well as deletions or insertions, are the main sources of hantavirus genetic variation, but there is also evidence for reassortment of genomic RNA segments. Thus, both genetic drift and genetic shift seem to be involved in the evolution of hantaviruses, similarly to other members of the *Bunyaviridae* family. Sequence data indicate that the evolution of hantaviruses follows that of their natural carriers and therefore the persistently infected carrier rodents should be regarded as the evolutionary scene for these agents.

RT-PCR cloning and sequencing were used to study Tula hantavirus (TUL) S segment/N protein quasispecies in three wild TUL hantavirus strains that were in circulation within the same local rodent population. Within nucleotides (nt) 801-1500 from 117 individual cDNA clones, 74 nt mutations corresponding to 45 deduced amino acid (aa) substitutions were found. No identical mutations were observed in virus populations from different animals, but there were four sites with multiple substitutions. 70% of nt mutations observed were transitions; among them A -> G and U -> C occurred more frequently. Mutations were evenly distributed between positions in codons and no specific pattern in the deduced aa substitutions was found. Strong dominance of a master sequence was observed in all three mutant spectra. Most variants were represented by a single clone and up to 19% of mutants by two clones each. Six of the 74 nt substitutions were present both in the some mutant spectrum of one strain and in the master sequence of other TUL strains, indicating that they are either quasi neutral or well tolerated mutations. We conclude that fixation of quasi neutral substitutions may play a role in TUL evolution in persistently infected carrier rodents.

QUASISPECIES AND THE IMMUNE SYSTEM

Chairman: Simon Wain-Hobson

Evolution of the cellular immune response in HIV infection

Andreas Meyerhans
Department of Virology, University of Freiburg
D-79104 Freiburg

Human immunodeficiency virus (HIV) infection leads to a gradual loss of CD4-positive T lymphocytes and concomitant destruction of the immune system. As a consequence, microorganisms that are usually well controlled in their host, take the opportunity to expand and become life threatening.

Primary HIV infection is usually a clonal event. Initial viremia can attain levels of $>10^6$ virions per ml plasma. Virus expansion is transient and contracts to about 10^4/ml correlating well with the appearance of HIV-specific cellular immunity. However, HIV replication persists, the virus turnover being calculated as 10^{7-10} particles per day depending on disease stage. In light of this, the genetic and antigenic diversity of HIV within an infected individual is obvious. In contrast, the main forces driving the observed temporal changes in the HIV quasispecies and the importance of HIV variability for disease progression remain unclear.

In order to analyze factors that drive intrapatient HIV evolution, sequential changes of the HIV-1 nef quasispecies were established from a haemophilic 11, 25 and 41 months after clonal infection with a factor IX preparation. No significant difference was found between the expected and observed number of synonymous/non-synonymous substitutions. The increase of the maximal nef gene divergence was best presented by a linear relation. This suggested that randomly driven processes might have an important impact on HIV-1 nef gene evolution in vivo.

Analysis of the evolution of viral variants in HIV-1 Nef and the corresponding Nef-specific CTL response in 4 seropositives revealed the following features: 1. CTL-mediated recognition was oligoclonal i.e. several epitopes were recognized even within a single HIV protein. 2. The CTL response accommodated itself to novel virus variants. 3. Immunogenic variants of CTL epitopes persisted despite constant CTL-mediated recognition. Thus, although the HIV nef gene undergoes extensive temporal sequence changes, the oligoclonal nature of the CTL response seems to mediate a significant albeit incomplete restriction on virus growth and hinder the appearance of CTL escape variants.

Persistence of CTL-mediated recognition has also been shown for an immunodominant epitope in gag p24 that is restricted by HLA-B27.

However, mutagenesis of the respective gag region within the infectious molecular clone HIV-1 Lai demonstrated that antigenic variation of this epitope by a single purine-purine substitution was compatible with replication competence *in vitro*. As demonstrated by others, such a variant has appeared in a patient after 10 years of HIV-1 infection. This patient had a strong CTL response against the reference sequence. On the basis of the intense HIV turnover in vivo and the speed by which drug resistance develops, i.e. 2 weeks in the case of the RTase inhibitor nevirapine, it would seem that HIV-specific CTL do not play a major role in driving HIV-1 evolution in vivo.

Together these data are compatible with a model in which stochastic events play a substantial role in shaping intrapatient HIV variation.

Molecular and virologic correlates of SHIV pathogenicity in macaques. Keith A. Reimann[1], Andrew Watson[4], Jane Ranchalis[4], Wenyu Lin[1], Ronald Veazey[3], Joseph Sodroski[2] and Norman L. Letvin[1]. Beth Israel Hospital[1], Dana Farber Cancer Institute[2], New England Regional Primate Research Center[3], Harvard Medical School, Boston, MA, and Bristol-Myers Squibb, Seattle, WA[4].

To develop a better AIDS animal model, we have been explored the infection of rhesus monkeys with chimeric simian/human immunodeficiency viruses (SHIVs) composed of SIVmac239 expressing HIV-1 *env* and the associated auxiliary HIV-1 genes *tat*, *vpu* and *rev*. SHIV-89.6, constructed with the HIV-1 *env* of a cytopathic, macrophage-tropic clone of a patient isolate of HIV-1 (89.6), replicated to a high degree in rhesus monkeys during primary infection, but was not pathogenic. After two serial *in vivo* passages by intravenous blood inoculation of naive rhesus monkeys, this SHIV (SHIV-89.6P) induced CD4 lymphopenia and an AIDS-like disease with wasting and opportunistic infections. When inoculated into naive rhesus monkeys, SHIV-89.6P caused persistent infection and CD4 lymphopenia. The nucleotide sequence differences between the nonpathogenic SHIV-89.6 and the pathogenic SHIV-89.6P will be reviewed. Viral loads in rhesus monkeys infected with SHIV-89.6, SHIV-89.6P or with SIVmac251 were prospectively assessed by measuring plasma SIV p27, QC-PCR quantitation of plasma viral RNA and semi-quantitative provirus levels in PBL. The relative level of provirus in PBL was highest during primary infection in those animals that progressed to AIDS. However, 10 weeks post infection, PBL provirus levels were similar for all viruses. In contrast, monkeys infected with SHIV-89.6, SHIV-89.6P and SIVmac all showed high peak levels of plasma viral RNA during primary infection. Ten weeks post infection plasma levels of viral RNA remained measurable only in animals that progressed to AIDS. These results suggest that high provirus levels during primary infection and maintenance of virus replication are necessary for SHIV-induced disease progression.

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J.J. SKEHEL
MRC - National Institute for Medical Research
The Ridgeway, Mill Hill
London NW7 1AA (U.K.)

Antigenic variation in influenza

Both the haemagglutinin and neuraminidase glycoproteins of the influenza virus membrane vary antigenically but the HA, which is the target of infectivity neutralizing antibodies, is the more important. HA has two functions in virus replication, as the sialic acid receptor binding protein and as the virus membrane fusion protein. Inhibition of these processes provides pressures on replication that may be involved in variant selection.

Studies will be described of the sites to which neutralizing antibodies bind and of the receptor binding site which they encircle. The results lead to considerations of mechanisms for infectivity neutralization and emphasize the interrelationships between variation in receptor binding properties and antigenicity.

EVOLUTION OF HUMAN RESPIRATORY SYNCYTIAL VIRUS: FACTORS INFLUENCING THE EXTREME ANTIGENIC AND GENETIC VARIATION OF THE ATTACHMENT (G) GLYCOPROTEIN

José A. Melero

Centro Nacional de Biología Fundamental.- Instituto de Salud "Carlos III".- Majadahonda.- 28220 Madrid.- Spain

Human respiratory syncytial virus (HRSV) is the most common cause of lower respiratory tract infections in very young children (1). The virus is a member of the *Pneumovirus* genus of the *Paramixoviridae* family and its genome is a single strand of negative-sense RNA encoding, at least, 10 mRNA species. Early cross-neutralization data with hyperimmune sera indicated that HRSV isolates were antigenically heterogeneous (2). More recently, HRSV isolates have been classified into two antigenic groups (A and B) by their rectivity with panels of monoclonal antibodies (MAbs). Yearly epidemics of HRSV are caused by viruses of both antigenic groups, although group A viruses are more frequently isolated. The attachment (G) glycoprotein of the virus is less conserved than other gene products both between and within antigenic groups (3). Thus, our studies on HRSV evolution have focused on the G protein gene because of its capacity to differentiate strains that may be identical in other viral genes and because it is a major target of the neutralizing and protective humoral immune response (4).

The G molecule is a type II glycoprotein of 290-300 amino acids (depending on the viral strain) with an ectodomain extensively modified post-translationally by the addition of both O-linked and N-linked oligosacharides. Murine MAbs have identified three types of epitopes in the G molecule: i) strain-specific or variable epitopes, represented in a limited number of viruses of the same antigenic group, ii) group-specific epitopes, and iii) conserved epitopes, shared by viruses of groups A and B. The strain-specific epitopes have been mapped within the C-terminal hypervariable region of the G protein ectodomain. Epitopes of the other two types mapped in a central conserved region of the ectodomain that includes a four cysteine cluster (4).

Sequence analysis of a number os HRSV strains belonging to group A, isolated worldwide, demonstrated that closely related viruses can be isolated in distant places and in different years, indicating the capacity of the virus to diseminate that influences its mode of evolution. In addition, antigenic analysis of the G glycoprotein from HRSV isolates showed a high correlation with their phylogenetic relatedness. This result, together with a preferential accumulation of amino acid changes in antigenically relevant regions of the G glycoprotein, suggests that immune selection may be another factor influencing the mode of evolution of HRSV (5,6).

The isolation and sequence characterization of escape mutants with MAbs has provided insights into the genetic mechanisms that operate for the generation of antigenic variants of HRSV. Besides point mutations that introduce amino acid substitutions in critical residues of strain-specific epitopes, two other mechanisms are involved in the generation of variants with changes in those epitopes: i) point mutations that introduce premature stop codons that

shorten the polypeptide chain (7) and ii) insertions or deletions of a single adenosine in runs of 6 or 7 As leading to reading frameshifts (8). In addition, escape mutants selected with antibodies directed against either group-specific or conserved epitopes may contain multiple A-G substitutions generated by a hypermutation event (9). All these changes observed in *in vitro* selected mutants can be correlated with changes observed in the viral isolates, suggesting that the same genetic mechanisms may operate during the *in vivo* propagation of HRSV.

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30 years later - a new approach to Sol Spiegelmans *in vitro* evolutionary studies

Frank Oehlenschläger

Max-Planck-Institute for Biophysical Chemistry

Necessary conditions for evolution are amplification, mutagenesis and selection. Here we describe the evolutionary response of an *in vitro* replicating system to the selection pressure for fast growth and what happens to the amplified molecules within this replication system. In order to perform *in vitro* studies on the evolution of RNA molecules, a modified self-sustained-sequence replication (3SR) reaction was used. In the first step of 3SR reaction the RNA template molecule is reverse transcribed by HIV-1 reverse transcriptase, followed by second strand synthesis and transcription of the resulting ds DNA by T7 RNA polymerase. The selection pressure (fast growth) was achieved by applying the principle of serial transfer on this amplification system: At the end of the exponential growth phase of 3SR reaction, an aliquot of the reaction mixture was transfered into a new sample, containing only buffer, nucleotides and enzymes - RNA template molecules were provided only by transfer. The conditions in the exponential growth phase allow an amplification of RNA molecules independent from each other; the enzymes (HIV-1 reverse transcriptase and T7 RNA polymerase) and nucleotides are present in large excess, the amplified nucleic acid molecules need not to compete for them. Therefore, transfering immediately after the completion of the exponential growth phase is equivalent with a selection for fast growth; those molecules which can replicate faster will displace the others after several transfers. The experiments were performed using a serial transfer apparatus (STA), which allows to monitor the nucleic acid concentration *on line* by

measuring the laser induced fluorescence, caused by intercalation of thiazole orange monomers into the RNA/ DNA amplification products.

The serial transfer experiments were carried out with an RNA template (220b RNA), which represents a 220 bases segment of the HIV-1 genome and comprises the *in vivo* primer binding site (PBS) for HIV-1 reverse transcriptase. It could be shown, that even after two serial transfers two much shorter RNA species (EP1 and EP2) emerged. EP1 (48b) and EP2 (54b) were formed by deletion mutations within the original 220b RNA template in the very beginning of the serial transfer experiment; due to their higher replication rate (calculated from the growth curves derived *on line*) these two deletion mutants displaced the original 220b RNA template in the course of the following thirty transfers. We assume, that these two evolved RNA species might have developped independently from each other; their formation was probably induced by a strand transfer reaction of HIV-1 reverse transcriptase. Sequence analysis of these two evolution products seem to confirm this formation pathway.

30 years after Sol Spiegelmans extracellular darwinian experiment with Qß replicase, the study described here is another approach to Spiegelmans question: " What will happen to the RNA molecules if the only demand made on them is the Biblical injunction, *multiply*, with the biological proviso that they do so as rapidly as possible ? " The answer, derived from a modified 3SR amplification system (mimicing a part of the HIV-1 infection cycle *in vitro*), is just the same as thirty years ago: The RNA molecules will become smaller, their multiplication rate will increase.

STRUCTURE OF REPLICATING HCV QUASISPECIES IN THE LIVER MAY NOT BE REFLECTED BY ANALYSIS OF CIRCULATING HCV VIRIONS

Beatriz Cabot, Juan I. Esteban, María Martell, Joan Genescà, Victor Vargas, Rafael Esteban, Jaime Guardia & Jordi Gómez.

Liver Unit, Department of Medicine, Hospital General Universitari Vall d´Hebron, Universitat Autònoma de Barcelona

Hepatitis C virus is one of the most important causes of chronic liver disease worldwide. Although HCV replicates in hepatocytes and the dynamics of hepatitis C virus release from hepatic cells to blood is largely unknown, most studies on population structure parameters have focused on serum circulating particles. In this study, we have performed a detailed analysis of the HCV population structure of the well conserved 5´UTR and both genomic and antigenomic strands of a variable fragment encompassing the envelope 2-non structural region 2 junction (E2-NS2) in paired liver and serum samples from four patients (A, B, C and D) with chronic hepatitis C of different severity. In one patient (patient A), sequences from three different biopsy specimens from a liver explant were compared with each other. Results show that patients A and C had a more complex population sequence in the liver respect to the serum and a higher proportion of silent mutations. In the other two patients (B and D), the viral quasispecies was very similar in serum and in liver specimens. In patient A, sequences from the three liver specimens were very similar with regard to complexity and proportion of synonymous mutations.

The results show that there is no general correlation between liver and serum quasispecies and rises the question about the origin of the circulating virus in patients A and C. The overall parallelism between replicating quasispecies in three liver specimens, suggest that there is no anatomic compartmentalization of HCV replication in the liver, but the existence of hepatocytes with different kinetics of viral replication. In this regard, the high percentage of synonymous mutation and the high complexity of the replicating quasispecies in the liver might be indicative of a more ancient origin and a higher degree of adaptation of the hepatic quasispecies to the liver cells (representing the putative chronically infected pool). In contrast, the structure of circulating population in patients A and C with a narrow mutant spectrum and random distribution of silent mutations would indicate a more recent origin of most circulating virus (derived from the supposed actively replicating hepatic pool).

QUASISPECIES IN NATURE

Chairman: Robert G. Webster

Influenza: Variation in Nature

Robert G. Webster, Ph.D.

St. Jude Children's Research Hospital
Department of Virology/Molecular Biology
332 N. Lauderdale, P.O. Box 318
Memphis, TN 38101 USA

Each of the 15 known subtypes of influenza A viruses are perpetuated in the aquatic birds of the world where they cause no clinical disease signs and appear to be in evolutionary stasis (1). The influenza viruses in aquatic birds replicate predominantly in the intestinal tract and are transmitted through the water supply to avian and mammalian species. Phylogenetic analysis have revealed that there are two geographically separate clads of influenza viruses in the world; one in the Americas and one in Eurasia. The Eurasian avian influenza viruses have been recognized as the precursors of influenza virus genes that reassort with human strains to generate the Asian/57 (H2N2) and Hong Kong/68 (H3N2) pandemic strains.

Genetic variation in influenza virus occurs by genetic drift (mutations), genetic shift (reassortment or host range transmission) and less frequently may involve recombination. After transfer from the aquatic avian reservoir to other hosts, influenza viruses evolve rapidly. This is apparent in the avian influenza virus that transmitted to pigs in Europe in 1979 where the evolutionary rate was very high. It has been proposed that a mutator mutation in the polymerase complex may account for this genetic plasticity (2). In addition, these viruses have reassorted with human influenza viruses circulating in pigs in Europe and these reassortants have become dominant in pigs. Thus, the pig is considered the intermediate host in the genesis of human pandemic influenza viruses and recent studies show that pigs have receptors specific for both human and avian influenza viruses (Y. Kawaoka, personal communication).

Pandemics of influenza have continued to emerge in domestic mammalian and avian species often with disastrous consequences. Recent examples have been the emergence of highly pathogenic avian H5N2 influenza in Mexico and avian influenza virus in pigs in China. Antigenic and genetic analyses of influenza viruses isolated from pigs in Hong Kong in 1993 have established that two different groups of H1N1 viruses were co-circulating in pigs that originated in Southern China. One group belongs to the classic swine lineage and the other to the Eurasian avian lineage. Each of the eight gene segments in the avian-like strain was of avian origin. Phylogenetic analysis indicates that these genes form an Asian sublineage of the Eurasian avian lineage suggesting that these viruses are an independent introduction into pigs in Asia. The introduction of an avirulent H5N2 influenza virus from the aquatic bird reservoir into chickens in Mexico in 1993 provided an opportunity to study the evolution of an influenza virus in a field setting (3). In the course of less than one year, the hemagglutinin of the virus acquired insertions and substitutions at the connecting peptide region of the molecule and the virus became highly pathogenic. Thus, mutations and recombination may play a part in the stepwise acquisition of virulence by an avian influenza virus in nature.

Since people, pigs and aquatic birds are the principal variables associated with the interspecies transfer of influenza virus and the emergence of new human pandemic strains, it is important to understand the molecular changes involved in host range transmission. The detection of emerging and re-emerging influenza strains in pigs may serve as an early warning system for the appearance of the next pandemic of human influenza.

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Genetics of Poliovirus

Eckard Wimmer, Xuemei Cao, Rohit Duggal, Andrea Cuconati, Matthias Gomeier, and Aniko Paul. Dept. of Mol. Genet. and Microbiol., School of Medicine, State University of New York at Stony Brook, Stony Brook, NY, 11794, USA

The genetics of poliovirus (PV) are dominated by an average mutation rate per base pair per replication of 6.3×10^{-4}. The high error rate in genome replication may be the main reason for the small size of the genome and, hence, for a replicating system operating under conditions of genetic austerity. Variation of the PV genome is augmented by genetic recombination occurring with high frequencies ranging from 10^{-2} (intratypic) to 10^{-5} (between different serotypes).

PV expresses all of its translatable information in form of a polyprotein that is processed to functional polypeptides. Some precursor polypeptides in the processing cascade have functions distinct from that of end products, and several processing products have multiple distinct functions. This strategy provides a means of expanding the number of tools involved in virus replication. It also serves to explain why experiments of complementation have not led to discernible genetic complementation groups. We conclude that the PV genome is monocistronic.

Homologous recombination takes place by a mechanism of copy choice during minus strand synthesis, and it occurs between heterologous genomes as well as, preferentially, between sibling genomes. We have recently obtained evidence suggesting that genetic recombination can be achieved also *in vitro* by incubating viral RNA in extracts of uninfected HeLa cells, a system allowing cell-free, *de novo* synthesis of poliovirus.

Considering the genetic "plasticity" of the poliovirus genome, its restriction to only three serotypes is surprising. We have developed a hypothesis suggesting that the serotype restriction may be related to the specific manner in which PV interacts with its cellular receptor.

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ANTIVIRAL DRUG RESISTANCE IN HIV Douglas D. Richman, University of California San Diego and San Diego Veterans Affairs Medical Center

Antiviral drug resistance is proving to be a major obstacle to the highly promising progress in the field of antiretroviral chemotherapy. Investigation of drug resistance is also providing insights into viral genetics, enzymology, pathogenesis, evolution and population biology (1). Three aspects of HIV drug resistance will be discussed in this presentation. Drug resistant mutants of HIV pre-exist as minor subpopulations in untreated patients and emerge under the selective pressure of drug treatment (2). Independently evolving subpopulations (virodemes) are replicating in different tissues of the same individual, especially the brain and lymphoid tissue (3). Recombination can be readily shown to occur in a single replicative cycle in vitro to accelerate the generation of virus highly resistant to inhibitors of both reverse transcriptase and protease (4).

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Poliovirus Variation

P Minor

National Institute for Biological Standards and Control,
Blanche Lane, South Mimms, Potters Bar,
Hertfordshire, EN6 3QG, UK.

Following the transmission of poliovirus to primates by Landsteiner and Popper in 1909, the view developed that infection was exclusively neural. It was not until thirty years later that the current view was accepted that infection is primarily enteric with a brief phase of generalised infection outside the gut, and that neural infection and disease is a rare consequence of exposure. The live attenuated polio vaccine strains of Sabin whose molecular characteristics are well understood (Minor, 1992) were chosen specifically for their ability to grow in the gut but not in neural tissue, and most studies of the natural evolution of the virus are concerned with specimens shed from the gut in the faeces rather than virus from other possible sites of multiplication.

The genome of the virus is a positive sense RNA molecule of about 7.5 kb with a long 5' non coding region which acts as an internal ribosome initiation site for the single open reading frame in which the genes encoding the structural proteins of the virus precede those for the non structural proteins. The functional proteins are generated by proteolytic cleavage of the large precursor. Despite its intensively studied properties certain features of poliovirus remain of unknown function, including the highly variable hundred nucleotide sequence immediately preceding the initiation codon, and an alternative proteolytic cleavage site in the polymerase protein. This site is found in all polio isolates other than those related to the Sabin type 3 strain, as discussed below.

In epidemics or in prolonged excretion by hypogammaglobulinaemic individuals, the genome drifts at a rate of approximately 10 mutations per month. Specific mutations arise consistent with selection by the host, including the regeneration of the proteolytic cleavage site mentioned above. In vaccinees given a mixture of type 1, type 2 and type 3 vaccine, recombination between strains is a commonly detected event, and there is some basis for assuming that it is more common than mutation in some settings.

All three types of Sabin vaccine strains of poliovirus have mutations in the 5' non coding region which weaken its predicted secondary structure in a particular domain, and it has been shown that the mutations affect the efficiency of translation and can make virus growth temperature sensitive in appropriate cell lines in vitro. All of the strains revert to wild type in some or all vaccine recipients; the type 1 strain which appears to be under less selective pressure in this respect than the others can revert by second site mutations which strengthen the structure while the type 2 and 3 strains revert by direct back mutation. There is also evidence in vitro that the 5' non coding mutations can be suppressed by mutations in the 2A protease by mechanisms which are not fully understood (Macadam et al, 1994). It is interesting that the 2A suppressors do not appear to supress the attenuated phenotype. Other attenuating mutations occur in the structural proteins, of which that in the type 3 strain has been especially studied. It interferes with the assembly of the protomers to form pentameric subunits, substituting a large bulky residue for a small residue at the interface between adjacent units. Virus growth is thus temperature sensitive as a result. In vaccinees this phenotype is lost after about 10 days by the insertion of second site suppressors, whose effect is to tune the optimum growth temperature to 37°, that of the human gut. The suppressors can be at the interface between protomers, but also occur at the interface between adjacent pentamers, or at other regions of less obvious function, probably involved in structural transitions. Suppression may therefore act on a different stage to that of the suppressed mutation. This conclusion is borne out by studies on receptor variants which may have mutations in regions believed to be involved in receptor interactions or the same regions identified in the temperature sensitive studies, involved in transitions.

The flexibility and rapidly adapting character of poliovirus suggests that vaccine strains will revert unless the pathogenesis of the virus is understood and exploited, so that attenuation affects replication in sites where disease ensues but not in sites which result in the generation of protective immunity.

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QUASISPECIES IN RETROTRANSPOSONS; EVOLUTION OF THE TOBACCO RETROTRANSPOSON Tnt1 IN *NICOTIANA* SPECIES

Samantha Vernhettes[1,2], Marie-Angèle Grandbastien[2] and Josep M. Casacuberta[1]
Departament Genètica Molecular. CID (CSIC) Barcelona, Spain[1] and Lab. Biologie Cellulaire. INRA Versailles, France[2]

Retrotransposons are mobile genetic elements closely related to retroviruses. They transpose via an RNA intermediate which is reverse-transcribed in virus-like particles (VLP). Nevertheless, retrotransposons are supposedly non-infectious, their complete life cycle being intracellular. Retrotransposition, like retroviral replication, is a very error-prone process. We have recently shown that this high mutation rate generates, as in the case of RNA viruses, a high degree of genomic variability. We have shown that when the tobacco retrotransposon Tnt1 is expressed, its RNA is not a unique sequence but a population of different but closely related sequences. The structure displayed by Tnt1 RNA closely resembles to RNA viral quasispecies. We have shown that the expression of Tnt1 under different situations give rise to different populations of Tnt1 RNA molecules, suggesting that the quasispecies-like nature of Tnt1 RNA allows this element to evolve in face of an environmental change.

We have analysed the presence of Tnt1 elements in different *Nicotiana* species. Three different families of Tnt1 elements were found to be present in the genome of the seven Nicotiana species analysed. These three different families are characterised by having completely different U3 regions. Since the elements that control the expression of Tnt1 elements are located within the U3 region, these three different families of Tnt1 elements are supposed to be differently regulated. Our results show that these three families of Tnt1 elements were already present in the genome of the ancestor of all the *Nicotiana* species analysed. Nevertheless, the evolution of each one of the three families characterised has been very different within each one of the genomes analysed. Our results suggest that some of these families have conserved their activity in only a few number of genomes while other families are still active in others. This probably reflects that it exists a maximum number of active elements that a given genome can tolerate without compromising its viability, and could be an indication that competitive exclusion principle also applies for retrotransposons as it applies for RNA viruses.

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HIV-1 subtype B evolution is confined to sequence space with fixed distance to the subtype consensus

Prof Dr Jaap Goudsmit, Dr Vladimir V. Lukashov
Department of Human Retrovirology, AMC, UvA

The AIDS epidemic in Europe and the USA, as well as local epidemics in Southern America and Asia, are caused by a particular HIV-1 subtype designated HIV-1 subtype B (HIV-1 B). Once population-wide genetic variation of subtype B was shown to increase, it remained uncertain whether HIV-1 B is a phenotypic entity spreading as a distinct virus population. To examine this, we applied Eigen's concepts of sequence geometry and fitness topography to the analysis of inter- and intrahost evolution of the gp120 V3 domain of HIV-1 B in the course of the global AIDS epidemic. We observed that despite the high evolution rate of HIV-1, the non-synonymous distances to the subtype B consensus of sequences obtained in the beginning of the epidemic are similar to those obtained more than 10 years later. The evolution of the individual HIV-1 B sequences is continual, but those farthest from the consensus evolve towards the consensus. As a result, individual HIV-1 genomes fluctuate within a fixed sequence space around the consensus. Our findings demonstrate that the evolution of the V3 domain of HIV-1 B is confined to an area in sequence space within a fixed distance to the subtype B consensus. This in turn indicates that HIV-1 B is a distinct viral quasispecies that is well adapted to the present environment, able to maintain its identity in time and unlikely to merge with other HIV-1 subtypes during progression of the AIDS epidemic.

UNDERSTANDING AND ANALYZING QUASISPECIES

Chairman: Manfred Eigen

Playing with mutation rates of 0.1

Simon Wain-Hobson, Miguel Angel Martinez+, Valerie Pezo,
Monica Sala, Jean-Pierre Vartanian, Philippe Marlière,
Sylvie Pochet, Uwe Plikat* and
Andreas Meyerhans*

Institut Pasteur, Paris
+CSIC Universidad Autonoma de Madrid
*University of Freiburg

There is little need to belabour the fact that RNA viruses accommodate change faster than any other biological replicon. They reflect life close to the error catastrophe, so much so that chemical modification increases their mutation rate but slightly. Occasionally one sees hypermutated genomes with inordinate numbers of substitutions; such is the case for retroviruses, particularly the lentiviral subfamily. G→A hypermutants are remarkable in that up to 40-60% of all Gs may be monotonously substituted by A. Equally remarkably, the phenomenon may be spread throughout a 10 kb retroviral genome. Why? It has nothing to do with nucleic acid modification or mutant polymerases. It follows from reverse transcription of the RNA genome into DNA occurring in the presence of biased intracellular dNTP concentrations, notably when [dTTP]>>[dCTP]. The finding highlights the special features of the G:T base pair which is the most stable of all mismatches. G→A hypermutants may be reproduced in a simple in vitro reaction with an experimental best of 12/32 (38%) target Gs substituted. In cultured T cells the intracellular pools can be modified by the addition of deoxythymidine which generates a [dTTP]>[dCTP] bias. Such conditions are conducive to the production of hypermutants. If HIV replication is used as a probe of intracellular dNTP one can find hypermutants among 1-2% activated or resting peripheral blood mononuclear cells (PBMCs). This means that a the dNTP concentrations small proportion of PBMCs are potentially mutagenic for DNA replication or repair.

In vitro hypermutagenesis is not a fiefdom of lentiviral reverse transcriptase. A number of reverse transcriptases may be used but none is as efficient as HIV. Transposing the logic over to the converse reaction, DNA-dependent RNA polymerization, in vitro T3 transcription may be rendered hypermutagenic when the NTP concentrations are biased. Presumably the same will prove true of RNA-dependent RNA polymerization. Hence the general, albeit not new, idea that fluctuations in intracellular (d)NTP underlie mutation. However, RNA and retroviral replication constitute markers for single cells which brings a slightly new dimension to the problem.

And DNA-dependent DNA replication? Apart from retroviruses all DNA replication complexes contain 3' exonucleolytic activities. However a number of purified DNA polymerases do not, perhaps Taq DNA polymerase being the most widely used. Conditions can be found in which an overall mutation rate of 0.1 may be achieved. The mutation spectrum contains all four transitions and a sizeable proportion of transversions. Most importantly the mutation rates and spectrum may be tuned by playing with the dNTP concentrations. PCR hypermutagenesis derives its strength from multiple rounds of DNA replication. However, on a single cycle basis reverse transcription and transcription top the list. Taq DNA polymerase accepts a variety of substrates including deoxyimidazole 4-carboxamide triphosphate derivatives. These molecules represent ambiguous purine analogues and indeed gave rise to mutagenic PCR. Iterative PCR hypermutagenesis of a small gene pushed its sequence to the brink of recognition, analogous to many hundred million years of vertebrate evolution.

Such mutationpower can be used to explore protein evolution in vitro. Iterative hypermutagenesis through clonal intermediates allowed the substitution of 23% of residues in the small R67 dihydrofolate reductase conferring trimethoprim resistance. In fact this is tantamount to accelerating a Muller's ratchet experiment. After a number of cycles some sequences at the end of the lineage could hardly accept further mutation. Their growth characteristics were greatly reduced. However if iterative hypermutation was performed in liquid medium with competition between variants the parental R67 sequence was lost by 5 cycles. Up to 15% of amino acid residues could be changed comparable to the variation seen among naturally occurring R67 homologues. The proportion of synonymous mutation was greater than heretofore probably reflecting purifying selection.

In vitro hypermutagenesis enables us to explore the vastness of sequence space, albeit in an unnatural way. One could paraphrase Captain Kirk, to go where natural selection has not gone before. It could be fun.

Mutant distributions of RNA species replicated in vitro

Christof K. Biebricher
Max-Planck-Institute for Biophysical Chemistry, D-37077 Göttingen, Germany

RNA- and DNA-dependent RNA polymerases can be used to amplify certain RNA species in vitro [1-3]. The replicating enzymes are highly selective in replicating their templates and the selective rate value of a genotype can be determined precisely by its replication success under the prevailing conditions. During RNA replication, errors are produced at rates between 10^{-3} and 10^{-4}. Erroneous replication products (mutants) also replicate and compete with each other. After a sufficient number of replication rounds (generations), a stable mutant distribution or quasispeicies is formed. The mutant distributions of the short-chained RNA species MNV-11 (86 nt long) was investigated under different conditions by retrotranscribing the RNA into cDNA, cloning it into plasmids and sequencing the different clones. Rather homogeneous preparations of the mutants were obtained by transcription from the cDNA clones and investigated for their replication parameters.

When amplifying RNA clones with a small chain length, one should expect a rather homogeneous population of RNA molecules. However, the mutant spectra found were surprisingly broad: the consensus species never made up more than 40% of the total population and was accompanied by many mutants[4]. Multi-error mutants with several base exchanges, insertions and deletions predominated in the distribution. The mutants found had replication rates comparable to that of the wild-type and were thus enriched in the population by selection forces. When the growth conditions were changed, the mutant distribution was shifted. The wild type sequence of the species did not have the highest replication rate of the population, but was rather the best compromise between the various selection rate determinants: the rates of overall replication, enzyme binding and double strand formation.

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The Quasispecies Model

Manfred Eigen, Max-Planck-Institut für Biophysikalische Chemie, Göttingen

Past: The Quasispecies is object of Darwinian evolution.

Complexity of biological information is determined by length of the coding molecules (RNA, DNA, proteins) and their composition. In the case of nucleic acids, the number of all possible combinations is 4^{ν}. To arrange the resulting enormous number of different sequences, the concept of sequence space was introduced: Each sequence occupies one single point in this space, the distance between them represents their homology in sequence.
As a consequence, the sequence space is characterized by relative short mutual distances between any two points, a huge storage capacity and a tremendous connectivity. The quasi-species, a mutant clan having a defined master sequence, can be described as a „cloud" in this space wandering to higher fitness levels. We call this „cloud" quasispecies because it can be described mathematically by one normal mode which behaves like one defined species.
Experimental evidence for the quasispecies has been obtained for RNA-viruses e.g. the Qß-phage. In their hosts these viruses are present as huge mutant distributions generated by the error-prone replication of their enzymes (error rate of Qß-replicase $\approx 3 \times 10^{-4}$). Data have shown that Qß works exactly below its error-threshold, a critical value above which replication would lead to nonsense products (equivalent with a melting of the mutant distribution).
The best-adapted sequence (master sequence) usually constitutes only a minor fraction of the virus quasispecies distribution. Its fraction strongly depends on the error rate of the replicating enzyme: With increasing error rate it is replaced by error-mutants and near the error-threshold its fraction becomes quite small.

Present: It is possible to identify single molecules of a quasispecies distribution.

Fluorescence correlation spectroscopy offers the opportunity to detect single molecules if they are labeled with a fluorescent dye. In this technique a laser-beam is directed into the sample where it generates a volume element of $\approx 0.1 \cdot 10^{-15}$l volume. All molecules, which diffuse through this volume element are excited and their fluorescence intensity is detected in a time resolved manner. The autocorrelation of these intensity signals generate a correlation curve, which can be evaluated mathematically yielding the diffusion time and the fraction of the fluorescent molecules in the solution.
Combined with amplification by PCR or NASBA (nucleic acid sequence-based amplification) FCS enables the detection of RNA-/DNA-template species down to the level of single molecules: A third fluorescently labeled probe is integrated in the amplification reaction. Its extension during this reaction is monitored *online* by FCS. On the basis of this technique, a new highly sensitive test for HIV-1 in blood plasma was developed which extends down to few virus particles per ml.

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REDUCED FIDELITY OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 REVERSE TRANSCRIPTASES WITH AMINO ACID SUBSTITUTIONS AFFECTING Tyr-115

Ana M. Martín-Hernández, Mónica Gutiérrez-Rivas, Esteban Domingo and Luis Menéndez-Arias

Centro de Biología Molecular "Severo Ochoa", CSIC-Universidad Autónoma de Madrid, 28049 Cantoblanco (Madrid), Spain.

Human immunodeficiency virus (HIV) reverse transcriptase (RT) converts the viral genomic RNA to a double-stranded DNA intermediate which integrates into host cell DNA. The process of reverse transcription is error prone, contributing to the high genetic variability of the virus. HIV-1 RT is a DNA polymerase which lacks a proofreading function. Knowledge on the molecular basis of fidelity of DNA synthesis by RT is still scarce. We used site-directed mutagenesis to produce HIV-1 RT variants with substitutions at Tyr-115. This amino acid was sistematically replaced by Phe, Trp, Val, Ile, Met, Ala, Ser, Cys, Asn, His, Gly, Asp, Lys or Pro. Tyr-115 is located in the vicinity of polymerase catalytic site of HIV-1 RT and is conserved in other RNA-dependent polymerases and in DNA polymerases α. It is also known that the equivalent residue in human DNA polymerase α (Tyr-865) and in the bacteriophage φ29 polymerase (Tyr-254) plays a role in maintaining the fidelity properties of these enzymes. In HIV-1 RT, we observed that the substitution of Tyr-115 by Phe rendered a fully active polymerase, displaying similar kinetic parameters, processivity and misinsertion fidelity of DNA synthesis as the wild-type enzyme. In contrast, the replacement of Tyr by Asp, Lys or Pro produced enzymes with very low or undetectable polymerase activity, in assays done using poly(rA)$_{484}$ as template and oligo(dT)$_{20}$ as primer. The activity of the other variant enzymes was significantly reduced as a result of a dramatic increase in the K_m for dTTP. This effect was also detected using a DNA template mimicking a proviral HIV-1 *gag* sequence. In contrast, the observed differences in k_{cat} values were relatively small. Wild-type RT and the mutant Y115F displayed a similar processivity, but mutants Y115W, Y115A and Y115S were less processive. However, the dissociation equilibrium constant for DNA was similar for all mutants tested, and differences in the dissociation rate constant (k_{off}) during processive polymerization were relatively small. Misinsertion fidelity assays revealed that mutants Y115W, Y115A and Y115S had a misinsertion efficiency (f) which was two orders of magnitude higher than the wild-type RT. In our assay conditions, the f value for wild-type RT is 1.54×10^{-5}, while for the variant enzymes having Trp, Ala or Ser at position 115, f ranges from 1.0 to 9.1×10^{-3}. The effects of these substitutions on mispaired primer extension fidelity assays were less significant. The low fidelity of these mutants appears to be related to nucleotide recognition rather than altered DNA-DNA template-primer interactions. The effects observed on the steady state kinetic constants, processivity and fidelity of the mutated enzymes were mediated by the 66 kDa subunit, as demonstrated using chimeric heterodimers having the Y115A substitution in either p66 or p51.

POSTERS

THE ROLE OF A CONSERVED HAIRPIN MOTIF IN THE R-U5 REGION OF THE HIV-1 RNA GENOME

Atze T. Das, Bep Klaver, Bianca I.F. Klasens, Jeroen L.B. van Wamel and Ben Berkhout. Department of Human Retrovirology, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands.

The R-U5 region of human and simian immunodeficiency viruses contains certain structural features which are conserved despite the divergence in sequence. Besides the TAR hairpin, this region contains a hairpin in which the polyadenylation signal AAUAAA is positioned in the single-stranded loop. The fact that this polyA stem-loop structure and its thermodynamic stability are well conserved suggests a biological function for this structure motif. Consistent with this idea, we demonstrate that mutations that either stabilize or destabilize the stem region do severely inhibit the replication potential of the HIV-1 virus. Upon prolonged culturing of the transfected cells, revertant viruses were obtained. The mutant with a stabilized hairpin acquired additional mutations that disrupt basepairs. In contrast, the destabilized mutant reverted by acquisition of additional base changes that increased the stability of the polyA hairpin structure. In both cases, the thermodynamic stability of the reverted hairpins approaches the wild-type level. Destabilizing mutations in either the left- or right-hand side of the basepaired stem interfere with virus replication (mutants C and D). However, the double-mutant CD, which combines the two mutated segments that were designed such that they form new basepairs, did replicate more rapidly when compared to the two individual virus mutants.

The polyadenylation capacity of the RNA structure mutants was assayed by inserting the wild-type and mutant hairpin fragments upstream of the SV40 polyadenylation signal in the pSV2CAT vector. Usage of the HIV-1 (upstream) or SV-40 (downstream) polyA sites was analyzed by Northern blotting. The wild-type HIV-1 polyA site is efficiently used in this system. Destabilization of the hairpin did not affect HIV-1 polyA site usage, indicating that the stem-loop structure is not essential for polyadenylation. In contrast, stabilization of the hairpin reduced HIV-1 polyA site usage, possibly because binding of polyadenylation factors to the AAUAAA is hindered. This polyadenylation defect of the stabilized hairpin explains the replication defect of the corresponding mutant virus. Although destabilization of the hairpin does not affect polyadenylation, replication of the corresponding viruses is reduced. This suggests that the RNA structure plays another role in virus replication. Transient transfection experiments demonstrated that transcription of the proviral genomes, translation of the viral mRNAs and packaging of viral genomes are not affected by the polyA hairpin mutations. Surprisingly, preliminary results indicate that this structured RNA motif plays a role in the mechanism of reverse transcription.

INFLUENCE OF SERIAL PASSAGES IN HETEROLOGOUS HOSTS ON THE QUASISPECIES STRUCTURE AND FIXATION OF MUTATIONS IN THE NUCLEOPROTEIN AND GLYCOPROTEIN GENES AND IN TWO NONCODING REGIONS OF THE RABIES VIRUS GENOME

Kissi B. (1), Badrane H. (2), Audry , L. (1), Brahimi M. (3), Tordo, N. (2) and Bourhy H.(1). (1) Rabies Unit, (2) Lyssavirus Laboratory, Institut Pasteur, Paris, France; (3) Institut Pasteur d'Algérie, Alger, Algeria

To understand mutations and genetic rearrangments that allow rabies infections of new hosts and adaptation in nature, the consensus sequences and the quasispecies structures of the nucleoprotein and glycoprotein genes as well as two noncoding sequences of a rabies virus genome were determined. These sequences were obtained from the brain and from the salivary gland of the original host, a European fox, and after serial passages in cell culture, in mice by the intracerebral and intramuscular routes, in dogs and in cats. For each analysis, 10 to 23 clones were sequenced. A relative genetic stasis of the consensus sequences confirmed previous results about the stability of rabies virus. However, the shape of the quasispecies structure was dramatically affected by some types of heterologous passages. for example, only six passages in mice by the intramuscular route lead to an increase of the proportion of mutant clones in the nucleoprotein from 15% (original specimen) to 61%. The ratio nonsynonymous/synonymous substitutions was high: 4.3. At this stage a mutation in the glycoprotein was already fixed. This study allow us to compare the rate of mutations and fixation of some of these mutations (synonymous, nonsynonymous, transitions and transversions), the detection of deletions and insertions according to the different genomic areas and to the different hosts.

Analysis of human antibody responses to epitopes in a variable region of the attachment (G) protein of respiratory syncytial virus.

Patricia A. Cane, Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, UK.

The attachment (G) protein of respiratory syncytial virus (RSV) shows considerable genetic and antigenic variability. It has been suggested that this protein may be evolving under selective pressure since nonsynonymous mutations outnumber synonymous mutations and there appears to be progressive accumulation of amino acid changes with time (1). However, many viruses exhibit antigenic variation and there is conflicting evidence as to whether such variation is due to direct selection of variants by the immune response or whether there are selective forces unrelated to the immune response which can favour amino acid changes that coincidentally result in a change of antigenic specificity. Alternatively, mutants with a selective disadvantage will be kept at minimal levels by negative selection and since antigenic sites are usually located on the outer edges of proteins with possibly greater tolerance of amino acid substitutions, such sites may appear more susceptible to change (2).

A problem with the suggestion that the RSV G protein is evolving under selective pressure is that it has not previously been shown that there is a human immune response to the variable regions of the protein. The data reported here using recombinant fusion proteins representing different parts of the G protein together with synthetic peptides, show that there are sites recognised by the human antibody response to this protein during primary infection that map in the carboxy terminal region of the protein. This is one of the most variable parts of the G protein and it appears that the human antibody response is highly specific to the infecting strain of virus and can discriminate between peptides based on naturally occurring variants with single amino acid changes capable of abrogating antibody recognition. Four antigenic sites were detected which include amino acids 232-240, 250-258, 265-273 and 283-291: all these sites include potential N-glycosylation sites in at least some isolates. No serum reacted with more than two of the sites, and most reacted with only one, with the site recognised being related to the infecting genotype of virus.

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Ulrich Kettling

In vivo evolution studies on phages using a continuous culture system

In contrast to any other experimental system with the aim to follow bacteriophage evolution the two-step system first described by Y. Husimi and coworkers (Husimi et al., 1982) and termed cellstat by analogy to the chemostat principle for the first time gave rise to the possibility of watching an evolving phage population under completely constant and controllable conditions. By seperating the host from the phage culture a system was devised and built up where the host cells play the role of a nutrient which is continuously supplied into the phage culture vessel. This system has been used to serve different purposes: Analysing reversion kinetics starting with a recombinant phage, producing deletion phages by culturing on a complementing host, or studying growth kinetics of phages in comparison with theoretical models (Husimi, 1989; Kong & Yin, 1995; Schwienhorst et al., 1996). Moreover by using phages as surface expression vectors (Smith, 1985; Cesareni, 1992) and combining the selection value of phages with the function of an inserted protein a continuous optimization of this protein might be possible. Finally this system should in principle also be usable for propagation of eukaryotic viruses. Here we present typical reversion kinetics obtained through the culture of an insert carrying fd phage; the emergence of a wild type analogous deletion mutant was observed and kinetics of this take-over process were analysed.

WHAT MAKES *ZEA* RETROTRANSPOSON *GRANDE1* DIFFERENT?

Carlos M. Vicient, Javier Fanlo and José Antonio Martínez-Izquierdo.
Departamento de Genética Molecular, CID-CSIC. Jordi Girona, 18, 08034 Barcelona. Tel 34-3-4006127. Fax 34-3-2046851. E-mail: jamgmj@cid.csic.es

Grande1 is a new type of retrotransposon from the genus *Zea* which has all the main features of Ty3/gypsy retrotransposons as well as some interesting distinctive characteristics. A full sequenced copy of the retrotransposon, from the teosinte *Zea diploperennis*, has 13769 bp including the two LTRs of 631 and 626 bp respectively. Grande1 is present besides in *Z. diploperennis*, in modern maize (*Z. mays mays*) in around 1500 copies per haploid genome and it shows an interspersed pattern of distribution by *in situ* hybridization on maize chromosomes (Aledo *et al.*, 1995).

The full sequenced copy, Grande1-4, has all the protein domains characteristics of retrotransposons and retroviruses in the first half of the element, except the *env* gene. There is a relatively good level of similarity among the amino acid motives of Grande1-4 reverse transcriptase, and those conserved in retroviruses and retrotransposons. However, some changes in the reading frame have to be made to align the protein motives, suggesting that Grande1-4 is not a functional copy of the retrotransposon. Surprisingly, the codon usage of Grande1 reverse transcriptase greatly differs from the codon usage tabulated for maize genes. Fact, that will be discussed later.

The second half of Grande1 (around 7 bk) has besides some ORFs two arrays of unrelated tandem repeats separated by 3.5 kb. Both tandems are present in the majority of Grande1 elements as can be seen in Southern analysis, with different number of individual copies of 89 bp for tándemA and 81 bp for tándemB respectively. The individual tandem sequences are very conserved intra- and inter-elements and they present important secondary structure, probably forming relatively stable long stem-loop structures (Monfort *et al.*, 1995).

So far, no full length transcript corresponding to Grande1 genomic RNA has been found. This fact implicate that either we are not able to find the tissues or conditions (like certain genomic stresses), for Grande1 activity or Grande1 is not functional. However, Grande1 as a whole, is not transcriptionally inactive, because a transcript of around 900 b hybridize with the sequence corresponding to an open reading frame close to tandemB (ORF1). ORF1 transcript is antisense in relation to the coding sequence of reverse transcriptase. ORF1 is flanked by all the regulatory sequences typical of eucariotic genes. In fact, the promoter region fused to the *uidA* reporter gene is able to drive the expresion of GUS in maize suspension cells trasnformed by bombarding with microprojectiles. On the other hand, mRNAs from sorghum, where Grande1 is absent, hybridized with ORF1, suggesting that *Gene1* corresponds to a cellular gene. The codon usage of ORF1 fits perfectly with the tabulated for monocot genes or particularly for maize genes.

The ORF1 deduced protein (P1) is rich in glycine, serine and acid amino acids (pI =3.8). In spite of the absence of significant similarity between ORF1-encoded protein and known proteins, there is some resemblance between P1 and DNA binding proteins, in particular with non-histones HMGs.

In summary, the great size of Grande1 is due to the second half of the element which has as singular features: two arrays of tandem repeats and an ORF (ORF1) which is trascribed. Transcripts corresponding to ORF1 are also present in sorghum were Grande1 is absent. The promoter of *Gene1* is functional. The codon usage of ORF 1 fits very well with the tabulated for maize genes in contrast of the Grande1 reverse transcriptase that greatly differs from both. Taking in account all these data the following hypothesis is proposed: the ancestor of Grande1 could be transmitted horizontally after the divergence between *Sorghum* and *Zea* and *Tripsacum* genera, capturing then a cellular gene corresponding to ORF1 and then being amplified until the current situation.

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Evolution subverting essentiality: dispensability of the Arg-GlyAsp in multiply passaged foot-and-mouth disease virus

Miguel A. Martínez and Esteban Domingo
Centro de Biología Molecular "Severo Ochoa". CSIC-UAM.
Cantoblanco, Madrid, Spain

RNA viruses replicate with replication errors thousands to millions fold greater than DNA-based organism. The outcome of the highly mutable viral genomes is that RNA viral populations form complex distributions of mutant genomes termed viral quasispecies. The evolutionary potential of such systems is largely unexplored. DNA genetics has imposed the notion of nucleotide sequence conservation for genomic regions encoding functionally essential proteins and for regulatory sequences. This is often extended to RNA genetics and statements correlating sequence conservation with functional importance of a protein domain encoded by an RNA genome abound in the literature of RNA viruses. But even this rule very rooted in DNA genetics may fail for RNA viruses, as documented in the present report. Work with the important animal pathogen foot-and-mouth disease virus (FMDV) by several groups over the last decade has permitted identification of an essential Arg-Gly-Asp domain on capsid protein VP1(residues 141-143) which is involved in recognition of the cellular receptor. Structural work further indicated that the Arg-Gly-Asp motif is not only part of the receptor recognition site but it also interacts directly with anti-viral neutralizing antibodies.

Monoclonal antibody SD6, which recognizes a continuous epitope located within amino acids 136 to 147 of VP1 of FMDV C-S8c1 was used to isolate resistant mutants. Viruses C-S8c1 and C-S8c1p100, resulting from one hundred serial cytolytic infections in BHK-21 cells, were incubated with mAb SD6 and resistant mutants were selected. To identify the mutations responsible for the resistance phenotype, the sequence of the VP1-coding region was determined. Sequences of 26 mutants isolated from FMDV C-S8c1 and 31 isolated from FMDV C-S8c1p100 were compared with an additional 56 mutants previously isolated from FMDV C-S8c1. The amino acid substitutions found in mutants derived from FMDV C-S8c1 affected only VP1 residues, Ala 138, Ser 139 and His 146. Immunochemical and structural results have shown that the highly conserved VP1 amino acids 141-145 were involved in recognition by mAb SD6. The finding of 86 antigenic variants substituted only in three different residues out the 10 that make contact with the paratope clearly demonstrates the restrictions to variation that must be operating to keep residues 141-145 of FMDV C-S8c1, and the Arg-Gly-Asp invariant. In contrast, the sequences of 31 SD6-resistant mutants isolated from FMDV C-S8c1p100, revealed a complete different repertoire of antigenic variants with substitutions affecting the highly conserved residues 141 to 145. Thus the restrictions to variation in this antigenic region were largely lost after serial passages of FMDV C-S8c1 in BHK-21 cells.

From the above results we conclude that the evolutionary potential of FMDV embraces the possibility that upon extensive replication of the virus in a constant environment, even conservation of the Arg-Gly-Asp motif may become dispensable.

Application of HMMs to the Multiple Alignment of Gene and Protein Sequences

Marcella A. McClure
Department of Biological Sciences
University of Nevada, Las Vegas, USA
mars@parvati.lv-whi.nevada.edu

Multiple sequence alignment of distantly related viral proteins remains a challenge to all currently available alignment methods. In the last few years a plethora of Markov modeling and Expectation-Maximization algorithms have been applied to a variety of molecular computational biology problems. Hidden Markov modeling (HMM) methods, based on a human speech recognition approach, have been applied to multiple sequence alignment, motif identification, and database searching. These methods essentially create a stochastic production model representing the sequences used to train the model. In its simplest form a model is initialized *a prior* for; 1) the transition into a match, deletion or insertion state, and 2) the occurrence of a given amino acid (or nucleotide) in a match or insert state. Using the initial model, and all training sequences, all possible paths for each sequence through the model are evaluated to obtain new estimates of the parameters that will increase the likelihood of the model. This process is repeated until the model converges. A multiple alignment is generated by computing the negative logarithm of the probability of the single most likely path through the model for a particular sequence given all the possible paths generated by the training sequences.

The HMM approach has a variety of advantages over more classical multiple alignment methods: 1) it is grounded in probability theory; 2) knowledge of phylogenetic history or pairwise ordering is not required; 3) matches and insertions/deletions (indels) are treated probabilistically, in a variable, position dependent manner; 4) experimentally derived information can be incorporated into the model *a priori*; 5) the model can provide information regarding both the stochastic and selected features of a protein family; and 6) the computational cost of aligning a set of sequences to an HMM is directly proportional to the number of sequences to be aligned. In most other existing multiple alignment methods the computational cost increases exponentially with the number of sequences to be aligned. To date there are no other methods with the flexibility of design allowed by the HMM approach.

Using parameterization constraints inferred from my two earlier studies on HMM approaches to aligning distantly related protein sequences the work presented here will concentrate on determining the lower limits of both protein and nucleic acid sequence divergence that can be tolerated and still produce an adequate multiple alignment before resulting to additional parameter constraints, manual refinement or the need to provide *a prior* information to the model.

Models will be initially trained with all available reverse transcriptase/ribonuclease H, aspartic acid protease and integrase gene and protein sequences of the primate lentiviruses (three nucleic acid and three protein sequence models). Progressive addition of the non-primate lentivirus and retrovirus sequences should lead to a destabilization of alignment quality. Two extremes of alignment instability should be easily observable: 1) the early, progressive loss of sub-lineage sequence identity not common to all members of the sequence family, and 2) the late loss of correct identification of the ordered series of motifs conferring protein function common to the sequence family. This study will provide a guide in the use of HMMs for various aspects of sequence analysis and should be useful in the analysis of various stages of quasi-species divergence.

The underlying assumption of the HMM approach is that each sequence is an independent realization of a single, unknown, underlying process (evolution), i.e., the model assumes a star-like phylogeny. The current implementations of the HMM approach are ideal for the study of a quasi-species given that it is hypothesized to initially derive from one or a few sequences. At what level does the quasi-species begin to exhibit more classical tree-like behavior? At what level of divergence would one need a more complex HMM architecture so that multifurcating lineages of a mutant cloud can be adequately modeled? To what extent is the lack of independent quasi-species sampling hindering our understanding of quasi-species evolution? Or is the temporal/spatial nature of a quasi-species such that our sequencing efforts rarely capture the early, non-tree like evolutionary behavior of this phenomena?

EVOLUTION OF BVD VIRUS: STASIS IN PERSISTENT INFECTION AND MINOR CHANGES DURING INTRAUTERINE TRANSMISSION

HP. Stalder and E. Peterhans

Institute of Veterinary Virology, University of Berne, Switzerland

Due to the high error rate and lack of correction mechanisms of the polymerase RNA viruses have a high capacity for genetic change. This is further accentuated by the high number of progeny virus. We have previously shown that BVD viruses are genetically highly heterogeneous (Hertig et al., Gene *153*, 191-195, 1995).

Persistent infection in immunotolerant animals represents a unique situation because immunotolerance can be expected to eliminate virus with altered antigenic properties. This contrasts sharply to the situation of acute infection where the immune system could favor antigenic variants. Confirming and extending the observations of Paton and coworkers (Br. Vet. J. *150*, 603, 1994), we did not see changes over a period of 1 year in the E2 and NS3 regions of BVD virus taken from a persistently infected heifer. Further analysis of 22 individual clones showed that the quasispecies distribution changed over this time period while the consensus sequence remained unaltered.

Of interest for the evolution of BVD virus is also the effect of transmission to the fetus early in gestation which extends persistent infection to the next generation. We have analyzed this situation in two cows, one of which gave birth to two persistently infected calves over a period of two years. BVD viruses of the first cow and its calf were identical in a 247bp fragment of the 5'UTR and in a 262bp fragment of the NS3 regions. In contrast, virus from the calf differed in 5 positions from that of its mother in a 1000bp fragment of the E1/E2 regions. All substitutions led to a mutation of an amino acid. Viruses of the second cow and its two calves were identical in the 5'UTR fragment but one calf differed from the mother in 1 position in NS3. In a 412bp region in E2 containing all 5 substitutions in the first mother-calf pair, virus from one of the two calves had two substitutions, one of which led to a mutation in the amino acid sequence. Virus of the other calf differed from that of its mother in one position.

In summary, our observations indicate that BVD virus in persistently infected animals is highly conserved but not in complete evolutionary stasis. Whether the changes associated with vertical transmission are due to a genetic bottleneck or can be explained by selection remains to be investigated.

PASSAGE IN CULTURED CELLS EXERTS EVOLUTIONARY PRESSURE SELECTING MINOR POPULATIONS OF BVD VIRUS (BVDV)

P. Meier, B. Adler[1], H. Adler[1], HP. Stalder and E. Peterhans

Institute of Veterinary Virology, University of Berne, Switzerland, [1]current address: Dana-Farber Cancer Institute, Boston, MA 02115, USA

In vivo, mononuclear phagocytes and lymphocytes are major host cells of BVDV. In contrast, cell culture isolation and propagation of BVDV is carried out mainly with epithelial cells or fibroblasts. We have investigated if adaptation to such cells is associated with mutations in viral proteins. The noncytopathic BVDV strain SD-1, isolated from a persistently infected animal, was used in this study because the entire nucleotide sequence of this strain has previously been determined *ex vivo*, i.e., prior to adaptation to cultured cells (Deng and Brock, *Virology* 191, 867-879, 1992).

We studied the gene encoding E2, the major surface glycoprotein, because this gene is known to be quite heterogeneous between different BVDV strains. Sequencing of 30 clones derived from virus not grown in cultured cells showed a quasispecies distribution with one main population. Cells were then inoculated with virus-containing serum of the persistently infected animal and 10 further passages were carried out. RNA was prepared from the cells and DNA was amplified by PCR after reverse transcription.

Direct sequencing of PCR fragments of virus grown in lamb synovial membrane cells revealed 6 amino acid changes in the E2 region compared to the main population of the initial virus. In Madin-Darby bovine kidney cells (MDBK) 2 changes could be observed but no changes were seen after cultivation in embryonic bovine turbinate cells or bovine bone marrow-derived macrophages. Analysis of the 5'-UTR where differences in the nucleotide sequence between isolates from cattle, sheep or pigs have been reported revealed no mutations.

No antigenic differences between the initial and virus grown in lamb synovial membrane cells were found in studies using a panel of monoclonal antibodies, contrasting with observations made in *in vivo* (Gunn et al., *Proceedings of The Second Symposium On Pestiviruses, pp*:163-166, 1992). Virus grown in lamb synovial membrane cells was subsequently passaged in bovine embryonic turbinate cells. After 7 passages all changes seen in lamb synovial cells were no longer detected. The virus grown in lamb synovial cells was biologically cloned and is currently being passaged in bovine embryonic turbinate cells.

Our results suggest that adaptation of BVDV to certain cell types represents an evolutionary pressure that leads to selection of minor populations.

Multiple means towards hypermutation: outmutating a parental gene

Valerie Pezo, Monica Sala, Miguel Angel Martinez*, Sylvie Pochet, Simon Wain-Hobson

Institut Pasteur, Paris
*CSIC Universidad Autonoma de Madrid

By employing biased dNTP pools it is possible to render reverse transcription hypermutagenic in vitro confirming the proposal that retroviral G→A hypermutation in vivo results from fluctuations in intracellular dNTP pools. Transposing the logic over to the converse reaction, DNA-dependent RNA polymerization, in vitro T3 transcription may be rendered hypermutagenic when the NTP concentrations are biased. Presumably the same will prove true of RNA-dependent RNA polymerization. Hence the general, albeit not new, idea that fluctuations in intracellular (d)NTP underlie mutation. Due to the lack of proof reading mechanisms, the impact of such fluctuations is far more considerable for retroviruses and RNA viruses than on the host cell.

DNA replication and PCR amplification may also be rendered error prone by employing biased dNTP pools. Modified bases have long been used as mutagens. Deoxyimidazole-4-carboxamide triphosphate derivatives represent stripped down purines analogues which, due to rotation about the carboxamide bond, can mimic either adenosine or guanosine. Although poor substrates for PCR they gave rise to mutagenic PCR.

Such mutationpower can be used to explore protein evolution in vitro. Iterative hypermutagenesis through clonal intermediates allowed the substitution of 23% of residues in the small R67 dihydrofolate reductase conferring trimethoprim resistance. In fact this is tantamount to accelerating a Muller's Ratchet experiment. After a number of cycles some sequences at the end of the lineage could hardly accept further mutation. Their growth characteristics were greatly reduced. However if iterative hypermutation was performed followed by liquid selection with competition between variants the parental R67 sequence was lost by 5 cycles. Up to 15% of amino acid residues could be changed comparable to the variation seen among naturally occurring R67 homologues. The proportion of synonymous mutation was greater than heretofore probably reflecting purifying selection.

JOSEP QUER
Recerca Medicina Interna II, Area d'Investigació B
Hospital Vall d'Hebron, Pag. Vall d'Hebron 119-129
08035 BARCELONA (Spain)

POPULATION DYNAMICS DURING COMPETITION BETWEEN VIRAL QUASISPECIES

ABSTRACT

We report here the first experimental observation of a highly predictable nonlinear behavior in two competing RNA virus populations and, particularly, the presence of critical points, defined as points from which viral competitions may follow different trajectories. Critical points were reached after nearly constant periods of time. The dynamics of relative fitness values of both competing viral populations were calculated. Despite the noise observed in relative fitness values, produced by the continuous and stochastic generation of mutant variants, it appears that RNA virus competition (i.e. natural selection) provides a low pass filter, thus inducing a highly reproducible evolutionary behavior.

SEQUENCE VARIABILITY OF HIV-1 FROM VENEZUELA. A PHYLOGENETIC ANALYSIS USING THREE NON-CONTINUOUS GENOMIC REGIONS, REVEALED A PUTATIVE "RECOMBINANT" ISOLATE

Miguel E. Quiñones-Mateu and Esteban Domingo

Centro de Biologia Molecular "Severo Ochoa" (CSIC-UAM), 28049 Madrid. Spain

Objective: To analyze two groups of HIV-1 isolates from Venezuela (1991 and 1994/95) by HMA subtyping and to characterize sequence evolution for three different genomic regions in recent years.

Methods: Twenty-eight blood samples from HIV-1 positive Venezuelan patients were obtained in two periods: Mar-Oct'91 and Aug'94-May'95. The HIV-1 serostatus and clinical profile of each subject was established. Proviral DNA was obtained and three genomic regions were amplified and sequenced for each sample: LTR region, the first exon of *tat* gene, and the C2V3-coding region of *env*. PCR products of an internal fragment spanning the V1-V5-coding region of gp120 were genetically compared with 14 previously characterized HIV-1 strains (subytpes A-H) by a heteroduplex mobility assay. Phylogenetic trees for the three regions were constructed using the neighbor-joining method and the statistical robustness was tested by boostrap resampling (1000 datasets). Genetic distances and mutant frequencies were determined for each studied region.

Results: The analysis of heteroduplexes of the *env* PCR products of the Venezuelan samples and the reference sequences showed unequivocal electrophoretic patterns, corresponding to subtype B. These qualitative results were confirmed by phylogenetic analysis based on nucleotide sequences. In each tree derived from LTR, *tat*, or C2V3-coding region, Venezuelan isolates clustered with subtype B viruses. Sub-clustering of the Venezuelan isolates was not seen in LTR- or C2V3-tree, but in *tat*-tree a Venezuelan sub-cluster was detected. Pairwise comparisons of nucleotide sequences showed an average intraclade divergence of 7% (2 to 11%), 8% (0 to 15%), and 10% (2 to 14%) for LTR, *tat*, and C2V3, respectively. Not differences were observed among genetic distances of both groups. Average point mutant frequencies, relative to the consensus defined by the samples under study, were 4.0×10^{-2}, 4.7×10^{-2}, and 5.2×10^{-2} substitutions per nucleotide for LTR, *tat*, and C2V3, respectively. The results indicate a high variability for the studied regions.

Conclusions: Circulating HIV-1 identified in Venezuela belong to subtype B. Genetic distances for the three regions of both time periods were very similar. Nucleotide diversity of C2V3-coding region was comparable to that described for HIV-1 isolates from countries where the epidemic had started many years earlier. It is the first study that includes a phylogenetic analysis using LTR and *tat* regions. Mutant frequencies were similar to LTR, *tat*, and C2V3 sequences confirming the high variability for all HIV-1 genomic regions. A putative recombinant isolate was described and its possible implications will be discussed.

Establishment and Maintenance of the Quasispecies Cloud Size of Cucumber Mosaic Virus Satellite RNAs is Dependent on the Helper Virus.

M.J. Roossinck The S. R. Noble Foundation, PO Box 2180, Ardmore OK 73402, USA

Cucumber mosaic virus (CMV) is the type member of the cucumovirus genus of plant viruses. CMV can harbor a small molecular parasite (*ca.* 335 nucleotides), known as a satellite RNA (sat RNA). The sat RNAs of CMV exist as numerous related strains with 85% to 98% sequence similarity. The CMV sat RNAs are supported by both subgroups of CMV as well as the related tomato aspermy cucumovirus (TAV). The generation of variation in CMV sat RNAs has been monitored by using cDNA clones of sat RNAs that are capable of producing infectious transcripts *in vitro*. After serial passage through a tobacco host, the progeny satellite RNAs are analyzed by cDNA cloning and sequencing. Twenty clones of each population have been analyzed to assess quasispecies cloud size. In addition, a population from plants infected for an equal time without passages was assessed. Different helper viruses vary in the time required for sat RNA variation to occur, in the amount of variation from the parental sat RNA, and in the size of the sat RNA quasispecies cloud tolerated by the helper virus.

A Sendai virus vector leading to the efficient expression of mutant M proteins interfering with virus particle budding.

Geneviève Mottet, Andreas Mühlemann, Caroline Tapparel, Frédéric Hoffmann and Laurent Roux* Department of Genetics and Microbiology, University of Geneva Medical School, CMU, 9 avenue de Champel, 1211 Geneva 4

A Sendai virus expression vector in the form of a transcribing copy-back defective interfering RNA was constructed and shown to efficiently express a tagged matrix protein in the only context of a Sendai virus infection. In attempt to identify relevant M protein domains involved in viral assembly and budding, a series of deletion mutants were tested for their ability to bind to cellular membrane fractions. The deletion of a region spanning amino acids 105-137 significantly decreased this binding when the protein was expressed in a system driven by the T7 RNA polymerase away from any other viral proteins. Plus or minus charges were introduced in the hydrophobic portion of a predicted amphiphilic helix in this region, at aa position Thr112 or Val113. M proteins with altered membrane binding properties were produced in this way. The genes encoding these mutant M proteins were then inserted in the Sendai virus vector, and shown to be expressed at levels similar to that of the endogenous wild type M protein. At first, the presence of a negative charge in the hydrophobic region of the putative amphiphilic helix prevented the incorporation of the mutant protein into virus particles and appeared to decrease the efficiency of virus particle budding. In contrast, the introduction of a positive charge appeared to increase the M mutant uptake into virions. On further examination, it turned out that it is the presence of Val113 which represents a critical feature for M protein function, since whenever the Val was substituted with a charged or an uncharged residue, M was not incorporated into virus particles. It is noteworthy that all the mutant M that did not assemble into virus particles exhibited a single band migration profile on PAGE, that corresponds, by analogy with the wild type M protein, to the phosphorylated form. It is then postulated that the substitution of Val113 induces the phosphorylation of all the M proteins. This would then explain the absence of M in virus particles, since the phosphorylated form of M has been shown to poorly bud (Lamb, R.A. and Choppin, P.W. 1977. The synthesis of Sendai virus polypeptides in infected cells III. Phosphorylation of polypeptides. *Virology* 81:382-397; Kamata, M.,Hiraki, A., Ama, M. and Kim, J. 1996. Xth International Congress of Virology). The use a Sendai virus vector has therefore been shown instrumental in the identification of mutant M proteins interfering with the viral assembly-budding process. In the end, repeated passages of the viral stocks expressing the mutant M proteins along with the wild type M, is being performed to eventually assess the stability of a redundant function either neutral or with detrimental effect on viral production.

Phenotypic study of viral isolates of HIV-1 and its variations. Correlation of SI/NSI with clinical progression

Clotet B,** Ruiz, Lidia,* Cabrera C,* Ibáñez A,* Puig T,* Sirera G,**. *Retrovirology Laboratory IRSI-Caixa; **HIV Unit, Hospital Universitari Germans Trias y Pujol, Badalona, Barcelona, Spain

Hydroxyurea (HU) has shown to potenciate the anti-HIV-1 effect of didanosine (ddI) in vitro. We report 24 weeks follow-up of the anti-HIV activity of the ddI+HU combination administered to patients with CD4 mean value below 200/mm3.

Our first objective was to assess the anti-HIV activity through variations in viral load and in CD4+ cell count. Also, we evaluated the potential side-effects of this drug combination in advanced HIV-1 positive patients.

Seventeen HIV-1 seropositive patients with 145 CD4+/mm^3 mean value at baseline (range: 1-430/mm^3) received ddI+HU for 24 weeks: 200 mg of ddI/12h and 500mg of HU/12h. Eleven patients were naive for ddI and 6 had already received this treatment (>3 months).

Over the 24 week-period, the ddI+HU combination was associated with a mean decrease in HIV-1 RNA levels of 1.44 $\log_{10}$. In 7 of these patients (41%) the decrease was greater than 1.5 $\log_{10}$. The mean increase in absolute CD4+ count was 60 cells/mm3 at week 24.

Additionally, we studied the syncytium inducing (SI) capacity at baseline and at the end of the study, to evaluate if there were differences in treatment efficacy with respect to the NSI/SI phenotype. The decrease in viral load in patients with SI phenotype was not significant.

The tolerance was good although grade II-III alopecia appeared in 3 patients with very low CD4 cell count (<50/mm^3). Also there were no significant variations in biological parameters associated with renal, hepatic, and pancreatic functions. Hydroxyurea associated to ddI is a promising combination that deserves a controlled trial to confirm its efficacy.

C. Terzian, I. Laprevotte, S. Brouillet and A. Henaut. Phylogenetic and Host Genomic Signatures: Tracing The Origin Of Retroelements At The Nucleotide Level.

Abstract
We investigate the nucleotide sequences of 23 retroelements (4 mammal retroviruses, one human -, 3 yeast -, 2 plant - and 13 invertebrate retrotransposons), in terms of their oligonucleotide composition in order to address the problem of the relationship between retrotransposons and retroviruses, and the coadaptation of these retroelements to their host genomes. We have identified by computer analysis over-represented 3- through 6-mers in each sequence. Our results indicate retrotransposons are heterogeneous in contrast to retroviruses, suggesting different modes of evolution by slippage-like mechanisms. Moreover, we have calculated the Observed number/Expected number ratio for each of the 256 tetramers and analysed the data using a multivariate approach. The tetramer composition of retroelement sequences appears to be influenced by host genomic factors like methylase activity.

Genetic Variation and Quasispecies in Puumala Hantaviruses

Antti Vaheri[1], Olli Vapalahti[1], Åke Lundkvist[2], Jan Hörling[2], Ying Cheng[1], Heikki Lehväslaiho[1] , Irina Gavrilovskaya[3], Heikki Henttonen[4] and Alexander Plyusnin[1]

[1]*Haartman Institute, Department of Virology, FIN-00014 University of Helsinki, Finland;* [2]*Swedish Institute for Infectious Disease Control, Stockholm, Sweden;* [3]*Institute of Poliomyelitis and Encephalitides, Moscow, Russia;* [4]*Finnish Forest Research Institute, Vantaa, Finland*

Hantaviruses (family *Bunyaviridae*) are negative-strand RNA viruses with a tripartite genome which consists of a (L)arge, (M)edium and (S)mall segment encoding, respectively, an L protein (which acts as a replicase, transcriptase and endonuclease), a glycoprotein precursor processed to yield two surface glycoproteins G1 and G2, and a nucleocapsid protein (N). Hantaviruses have adapted themselves in their natural hosts (rodent or insectivore) during co-evolution in which they produce chronic infection with no apparent harm. As is often the case, transmission of a virus to a new host may result in severe diseases, such as hemorrhagic fever with renal syndrome or hantavirus pulmonary syndrome in human hantavirus infection. The nature of the host should be considered one of the most important factors which determine and influence the pathogenicity of hantaviruses.

RT-PCR sequencing of the S and the M segment nucleotides was used to study phylogenetic relationships between four groups of strains, which originated from Finland, Sweden and European Russia, and to determine the range of genetic variability in European Puumala virus (PUU) strains. The geographical clustering of PUU genetic variants that we observed was consistent with the proposed scenario of recolonization of Northern Europe during the last postglacial period by bank voles (*Clethrionomys glareolus*), natural host for PUU. While all PUU strains shared a common ancient ancestor, the more recent ancestors were different for the four branches of PUU connected, most probably, to separate glacial refugia of bank voles. Different levels of S gene/N protein diversity of PUU were revealed; the range of genetic diversity within this serotype was decreasing from the level of geographically separated strains to the level of a local rodent population. Most amino acid substitutions were located to antigenically important sites. Populations of wild-type PUU within an individual rodent were shown to be represented by quasispecies suggesting a potential for rapid evolution. The ratio of nonsilent to silent nucleotide mutations registered in the S genes/N proteins of PUU quasispecies was considerably higher than that in PUU strains, resulting in a more wide range of quasispecies N protein sequence diversity. Most notably, during adaptation to a new host, variants carrying substitutions in the regulatory regions of the S segment were observed in the mutant spectrum of wild-type PUU.

Information contained in populations of natural protein sequences. Correlated mutations in protein interfaces.

Florencio Pazos[1], Osvaldo Olmea [1,2] and Alfonso Valencia[1].

[1]Protein Design Group. CNB-CSIC. Madrid and [2] CIGB, Cuba.
e-mail "valencia@samba.cnb.uam.es"

Evolution has modelled proteins to achieve particular functions. Protein sequences may contain traces of this process of adaptation. These forces involved have very different origins from phylogenetic relations to constrains derived from protein structure and function.
Compensating changes detected as correlated mutations between sequence positions contain part of the information about this process. We discuss the implications of correlated mutations for protein evolution and structure.
We show to practical applications of this information:
a) Residue-residue contacts. On large test set it is possible to predict a small number of three-dimensional contacts. Unfortunately, this information is insufficient for predicting protein structure but it can be efficiently combined with other predictive tools.
b) Similar methods can be use to predict the best docking solution between proteins or protein-domains. This is a specially relevant problem in biology since for many molecular complexes only the sequence and not the structure of components is known. Different cases illustrate the possibilities of the method. First, the information about sequence correlation is enough to distinguish the right inter-domain docking solution among many wrong alternatives for two domain proteins. Second, the case of Haemoglobin shows that the same principles can be applied to multimers. Finally, we perform a blind prediction for the domain-domain contact in the heat-shock protein Hsc70. Even if geometry-based docking methods do have good performance but are applicable only to proteins of known structure, sequence based method are less precise but more general since it can be applied without knowledge of the structures.

HIV genetic variation is directed and restricted by DNA precursor availability.

Jean-Pierre Vartanian[1], Uwe Plikat[4], Michel Henry[1], Renaud Mahieux[3], Laurent Guillemot[2], Françoise Barré-Sinoussi[2], Andreas Meyerhans[4] and Simon Wain-Hobson[1]

[1, 2, 3] Institut Pasteur Paris, France

[4] Universität Freiburg

Among viruses, retroviruses are uniquely sensitive to fluctuations in intracellular dNTP concentrations. Indeed, HIV replication may be enhanced or restricted by modulation of intracellular dNTP pools. Addition of thymidine to established cell lines, resulted in a dramatic reduction of virus production, and a catastrophic loss of dCTP via the allosteric regulation of the ribonucleotide reductase enzyme (RR). This is probably the cause of G→ A hypermutation, a remarkable phenomenon, where up to 30% of all G residues were found to be substituted by A, in a complete HIV genome, with a strong preference within the 5' GpA dinucleotide contexte. The correlation with the dNTP pool imbalances and the fidelity of HIV replication was investigated in a detergent permeabilized virions and biased dNTP pool as well as by infection of thymidine treated U937 cells, resting or activated peripheral blood mononuclear cells (PBMCs). Different types of hypermutants during reverse transcription, were readily produced demonstrating that the host cell may control HIV replication errors and restrict the mutant spectrum.

QUANTITATION OF FITNESS DIFFERENCES IN CLONAL POPULATIONS OF HIV-1

E. Yuste[1], S. Sánchez-Palomino[1#], C. López-Galíndez[1]

1- Centro Nacional de Biología Celular y Retrovirus. Instituto de Salud Carlos III, Madrid, Spain.
Present address: Centro de Biología Molecular "Severo Ochoa"(CSIC),UAM, Madrid, Spain.

The objective of this study was to quantify the relative fitness of 10 biological clones of an HIV-1 isolate and to compare the competitive ability of each clone along with the parental population.

Parental viral population, s61, was obtained from a 4-year-old child by standard coculture procedures. Biological clones were recovered from the global population using a MT4 plaque assay.

To obtain a fitness estimation each clone was mixed with a genetically marked one from the same viral population. These two virus populations were allowed to replicate in direct competition during five dilute passages in cell culture, and the ratio of the two viral population was determined at each passage. In competition assays each biological clone was mixed with the marked virus at three diferent fixed rates (1:9, 1:1, 9:1).

To distinguish viral clones, we have taken advantage of the ability of the Heteroduplex Tracking Assay (HTA) to detect genetic differencies in sequence. These genetic alterations do not need to be related to a phenotypic feature. We have selected the V1-V2 hipervariable regions of the ENV gene to diferentiate clones in the HTA analysis.

Some previous work was done to asses the sensibility of our assay and we confirmed that we were able to distinguish all the biological clones from the genetically marked one. Finally, as variation rate in V1-V2 region was very high, we wanted to know if there were any changes in the heteroduplex mobility pattern during five passages in cell culture. The conditions used were the same than those used in competition assays and we did not find any change in the HTA pattern.

At the end of the passages, the slopes of fitness vectors were obtained and compared. A wide range of clone fitness was found. These results show that one HIV-1 viral population consists of a mixture of lower and higher fitness clones. Our results, once more, show the high biological heterogeneity and adaptability of HIV-1 populations.

List of Invited Speakers

Workshop on

RNA VIRAL QUASISPECIES

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Christof K. Biebricher	Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen (Germany). Tel.: 49 551 201 14 42 / 14 41 Fax : 49 551 201 14 35 E-mail: Biebricher@daniel.dnet.gwdg.de
John M. Coffin	Department of Molecular Biology and Microbiology, Tufts University, 136 Harrison Avenue, Boston, MA. 02111 (USA). Tel.: 1 617 636 65 28 Fax : 1 617 636 80 86
Esteban Domingo	Centro de Biología Molecular «Severo Ochoa», Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 84 85 Fax : 34 1 397 47 99
Manfred Eigen	Max-Plank-Institut für Biophysikalische Chemie, Karl-Friedrich-Bonhoeffer-Institut, Am Fassberg, Postfach 2841, D-37077 Göttingen-Nikoausberg (Germany). Tel.: 49 551 201 16 82 Fax : 49 551 201 14 35
John Holland	Department of Biology, 0116, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA. 92093-0116 (USA). Tel.: 1 619 534 25 20 Fax : 1 619 534 71 08
Norman L. Letvin	Beth Israel Hospital, Harvard Medical School, 330 Brookline Avenue, Boston, MA. 02215 (USA). Tel.: 1 617 667 20 42 Fax : 1 617 667 82 10
Lawrence A. Loeb	Joseph Gottstein Memorial Laboratory, Department of Pathology, Box 357705, School of Medicine, University of Washington, Seattle, WA. 98195-7705 (USA) Tel.: 1 206 543 93 60 Fax : 1 206 543 39 67 E-mail: laloeb@u.washington.edu

Cecilio López Galíndez	Centro Nacional de Biología Celular y Retrovirus, Instituto de Salud Carlos III, Crta. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda, Madrid (Spain). Tel.: 34 1 639 06 40 Fax : 34 1 638 82 06 E-mail: clopez@iscii.es
Brian W.J. Mahy	Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, (CDC), 1600 Clifton Rd. N.E., Atlanta, GA. 30333 (USA). Tel.: 1 404 639 35 74 Fax . 1 404 639 31 63
José A. Melero	Centro Nacional de Biología Fundamental, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda, Madrid (Spain). Tel.: 34 1 509 79 41 / 509 79 07 Fax : 34 1 509 79 19 / 509 79 18
Andreas Meyerhans	Department of Virology, University of Freiburg, Hermann-Herderstr. 11, D-79104 Freiburg (Germany). Tel.: 49 761 203 66 14 Fax : 49 761 203 66 39 E-mail: Andreas@sun1.ukl.uni-freiburg.de
Philip D. Minor	National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG (U.K.). Tel.: 44 1707 65 47 53 Fax : 44 1707 64 67 30
Andrés Moya	Departamento de Genética, Facultad de Biología, Universidad de Valencia, c/Dr. Moliner 50, 46100 Burjassot, Valencia (Spain). Tel.: 34 6 386 43 79 Fax : 34 6 386 43 72
Stuart T. Nichol	Centers for Disease Control and Prevention, (CDC), Special Pathogens Branch, Division of Viral and Rickettsial Diseases, 1600 Clifton Rd. N.E., Atlanta, GA. 30333 (USA). Tel.: 1 404 639 11 15 Fax : 1 404 639 11 18 E-mail: stn1@ciddvd1.em.cdc.gov
Douglas D. Richman	University of California, San Diego, Dpts. of Pathology & Medicine, 0679, 9500 Gilman Drive, and San Diego Veterans Affairs Medical Center, La Jolla, CA. 92093-0679 (USA). Tel.: 1 619 552 74 39 Fax : 1 619 552 74 45

John J. Skehel

MRC, National Institute for Medical Research, Division of Virology, The Ridgeway, Mill Hill, London NW7 1AA (U.K.).
Tel.: 44 181 959 36 66 Ext. 25 25
Fax : 44 181 913 85 25

Simon Wain-Hobson

Institut Pasteur, Unité de Rétrovirologie Moléculaire, 28 rue du Dr. Roux, 75724 Paris (France).
Te.l: 33 1 45 68 88 21
Fax : 33 1 45 68 88 74
E-mail: simon@pasteur.fr

Robert G. Webster

St. Jude Children's Research Hospital, Department of Virology/Molecular Biology, 332N. Lauderdale, P.O. Box 318, Memphis, TN. 38101 (USA).
Tel.: 1 901 495 34 00
Fax : 1 901 523 26 22

Eckard Wimmer

Department of Molecular Genetics and Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, N.Y. 11794-5222 (USA).
Tel.: 1 516 632 87 87
Fax : 1 516 632 88 91
E-mail: wimmer@asterix.bio.sunysb.edu

List of Participants

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List of Participants

Ben Berkhout	Department of Human Retrovirology, University of Amsterdam, Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam (The Netherlands). Tel.: 31 20 566 48 22 Fax : 31 20 691 65 31 E-mail: B.Berkhout@amc.uva.nl
Hervé Bourhy	Rabies Unit, Institut Pasteur, 28 rue Dr. Roux, 75724 Paris (France). Tel.: 33 1 45 68 87 50 Fax : 33 1 40 61 30 20 E-mail: hbourhy@pasteur.fr
Patricia A. Cane	Department of Biological Sciences, University of Warwick, Coventry CV4 7AL (U.K.) Tel.: 44 1203 52 35 23 Fax : 44 1203 52 35 68 E-mail: PY@DNA.BIO.WARWICK.AC.UK
Josep M. Casacuberta	Departament Genètica Molecular, CID (CSIC), c/Jordi Girona 18-26, 08034 Barcelona (Spain). Tel.: 34 3 400 61 00 Ext. 249 Fax : 34 3 204 59 04 E-amil: jcsgmp@cid.csic.es
Concepción Casado	Centro Nacional de Biología Celular y Retrovirus, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda, Madrid (Spain). Tel.: 34 1 639 06 40 Fax : 34 1 509 79 18
Cristina Escarmís	Centro de Biología Molecular «Severo Ochoa», CSIC, Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 50 70 Fax : 34 1 397 47 99
Fernando García-Arenal	Departamento de Biotecnología, E.T.S.I. Agrónomos, Universidad Politécnica, Ciudad Universitaria, 28040 Madrid (Spain). Tel.: 34 1 544 48 07 Ext. 258 Fax : 34 1 336 57 57

Jordi Gómez	Liver Unit, Department of Medicine, Hospital General Universitari Vall d'Hebron, Universitat Autònoma, Passeig Vall d'Hebron s/n, 08025 Barcelona (Spain). Tel.: 34 3 428 26 37 Fax . 34 3 428 33 83
Jaap Goudsmit	Department of Human Retrovirology, AMC, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam (The Netherlands). Tel.: 31 20 566 48 53 Fax : 31 20 691 65 31
Ulrich Kettling	Max-Planck-Institute for Biophysical Chemistry, Department 081, Am Faßberg 11, D-37077 Göttingen (Germany). Tel.: 49 551 201 16 82 Fax . 49 551 201 14 35 E-mail: ukettli@gwdg.de
Pedro R. Lowenstein	Department of Medicine, Molecular Medicine Unit, University of Manchester, Oxford Road, Manchester M13 9PT (U.K.). Tel. and Fax : 44 161 275 56 72 E-mail: lowenstein@man.ac.uk
José Antonio Martínez-Izquierdo	Departamento de Genética Molecular, CID-CSIC, Jordi Girona 18, 08034 Barcelona (Spain). Tel.: 34 3 400 61 27 Fax : 34 3 204 68 51 E-mail: jamgmj@cid.csic.es
Miguel A. Martínez de la Sierra	Centro de Biología Molecular «Severo Ochoa», CSIC, Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 84 77 Fax : 34 1 397 47 99 E-mail: MAMARTINEZ@mvax.cbm.uam.es
Marcella A. McClure	Department of Biological Sciences, University of Nevada, 4505 Maryland Parkway, Box 454004, Las Vegas, NV. 89154-4004 (USA) Tel.: 1 702 895 33 99 Fax : 1 702 895 39 56 E-mail: mars@parvati.lv-whi.nevada.edu
Luis Menéndez-Arias	Centro de Biología Molecular «Severo Ochoa», CSIC, Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 84 77 Fax : 34 1 397 47 99

Frank Oehlenschläger	Max-Planck-Institute for Biophysical Chemistry, Department 081, Am Faßberg 11, D-37077 Göttingen (Germany). Tel.: 49 551 201 16 82 Fax . 49 551 201 14 35 E-mail: foehlen@gwdg.de
Ernst Peterhans	Institute of Veterinary Virology, University of Berne, Länggass-Straße 122, CH-3001 Berne(Switzerland). Tel.: 41 31 631 24 13 Fax : 41 31 631 25 34 E-mail: peterhans@ivv.unibe.ch
Valérie Pezo	Institut Pasteur, Unité de Rétrovirologie Moléculaire, 28 rue du Dr. Roux, 75724 Paris (France). Tel.: 33 1 45 68 88 21 Fax : 33 1 45 68 88 74 E-mail: vpezo@pasteur.fr
Alexander Plyusnin	Haartman Institute, Department of Virology, P.O. Box 21, (Haartmaninkatu 3), FIN-00014 University of Helsinki (Finland). Tel.: 358 0 434 64 86 Fax : 358 0 434 64 91 E-mail: Alexander.Plysnin@Helsinki.FI
Josep Quer	Recerca Medicina Interna II, Area d'Investigació B, Hospital Vall d'Hebron, Pg. Vall d'Hebron 119-129, 08035 Barcelona (Spain). Tel.: 34 3 427 20 22 Ext. 4388 Fax . 34 3 428 43 01 E-mail: j-quer@ar.vhebron.es
Miguel E. Quiñones-Mateu	Centro de Biología Molecular «Severo Ochoa», CSIC, Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 84 77 Fax : 34 1 397 47 99 E-mail: mquinone@mvax.cbm.uam.es
Marilyn J. Roossinck	The S.R. Noble Foundation, PO Box 2180, 2510 Sam Noble Parkway, Ardmore, OK. 73402 (USA). Tel.: 1 405 223 58 10 Fax : 1 405 221 73 80 E-mail: mroossinck@aardvark.ucs.uoknor.edu
Laurent Roux	Department of Genetics and Microbiology, University of Geneva Medical School, CMU, 9 avenue de Champel, 1211 Genève 4 (Switzerland). Tel.: 41 22 702 56 83 Fax : 41 22 702 57 02 E-mail: roux@cmu.unige.ch

Lidia Ruiz Tabuenca	Laboratorio de Retrovirología «irsiCaixa» y Unidad de SIDA, Hospital Universitario Germans Trias i Pujol, Badalona, Barcelona (Spain). Tel.: 34 3 465 63 74 Fax : 34 3 465 76 02 E-mail: irsi.caixa@bcn.servicom.es
Christophe Terzian	Centre de Génétique Moleculaire, CNRS, 91198 Gif sur Yvette (France). Tel.: 33 1 69 82 31 46 Fax : 33 1 69 82 43 86 E-mail: terzian@cgm.cnrs-gif.fr
Noël Tordo	Institut Pasteur, Laboratoire des Lyssavirus, 25 rue du Dr. Roux, 75724 Paris (France). Tel.: 33 1 40 61 31 34 Fax : 33 1 40 61 32 56 E-mail: ntordo@pasteur.fr
Antti Vaheri	Haartman Institute, Department of Virology, P.O. Box 21, (Haartmaninkatu 3), FIN-00014 University of Helsinki (Finland). Tel.: 358 0 434 64 90 Fax : 358 0 434 64 91 E-mail: Antti.Vaheri@helsinki.fi
Alfonso Valencia	Protein Design Group, Centro Nacional de Biotecnología, CSIC, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 585 45 70 Fax : 34 1 585 45 06 E-mail: valencia@samba.cnb.uam.es
Jean-Pierre Vartanian	Unité de Rétrovirologie Moléculaire, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris (France). Tel.: 33 1 45 68 88 65 Fax : 33 1 45 68 88 74 E-mail: jpvart@pasteur.fr
Zhibing Yun	Division of Clinical Virology, Karolinska Institute at Huddinge, University Hospital, F68, Huddinge (Sweden). Tel.: 46 8 746 53 15 Fax : 46 8 746 53 60 E-mail: zhyu@vird01.hs.sll.se
Eloisa Yuste	Centro Nacional de Biología Celular y Retrovirus, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda, Madrid (Spain). Tel.: 34 1 639 06 40 Fax : 34 1 638 82 06

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Instituto Juan March de Estudios e Investigaciones
Castelló, 77 • Teléfono 34 - 1 - 435 42 40 • Fax 576 34 20
28006 Madrid (España)

The lectures summarized in this publication were presented by their authors at a workshop held on the 7th through the 9th of October 1996, at the Instituto Juan March.

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