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The works summarized in this publication were presented by their authors at a Course held on 20th to 24th June 1988 at the Fundación Juan March.

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Course on Genome Evolution

organized by E. Viñuela

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Watson et al (1987) *Molecular Biology of the Gene*, 4th Ed., Vol. II, Benjamin.

T. R. Cech (1986) *The Generality of Self-Splicing RNA: Relationship to Nuclear mRNA Splicing* Cell, 44, 207.

J. E. Darnell & W. F. Doolittle (1986) *Speculations on the Early Course of Evolution* Proc. Natl. Acad. Sci., USA. 83, 1271.

W. Gilbert, M. Marchionni & G. McKnight (1986) *On the Antiquity of Introns* Cell, 46, 151.

June 21st: *About Protein Sequences: Comparison & Alignment*

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R. F. Doolittle (1981) *Similar Amino Acid Sequences: Chance or Common Ancestry*. Science, 214, 149.

D.-F. Feng, M. S. Johnson & R. F. Doolittle (1985) *Aligning Amino Acid Sequences: Comparison of Commonly Used Methods*. J. Mol. Evol., 21, 112.

D.-F. Feng & R. F. Doolittle (1987) *Progressive Sequence Alignment as a Prerequisite to Correct Phylogenetic Trees*. J. Mol. Evol., 25, 351.

W. M. Fitch & E. Margoliash (1967) *Construction of Phylogenetic Trees*. Science, 15, 279.

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R. F. Doolittle, D.-F. Feng, M. S. Johnson & M. A. McClure (1986) *Relationships of Human Protein Sequences to Those of Other Organisms* Cold Spring Harbor Symp., 51, 445.

D. Yang, Y. Oyaizu, H. Oyaizu, G. J. Olsen & C. R. Woese (1985) *Mitochondrial Origins* Proc. Natl. Acad. Sci., USA 82, 4443.

J. A. Lake (1988) *Origin of the Eukaryotic Nucleus Determined by Rate-Invariant Analysis of rRNA Sequences*. Nature, 331, 184.

June 23rd: *Evolution of the Vertebrate Plasma Proteins*

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R. F. Doolittle (1985) *The Genealogy of Some Recently Evolved Vertebrate Proteins*. TIBS, 10, 233.

R. F. Doolittle (1987) *The Evolution of the Vertebrate Plasma Proteins*. The Biological Bulletin, 174, 269.

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M. A. McClure, M. S. Johnson, D.-F. Feng & R. F. Doolittle (1988) *Sequence Comparisons of Retroviral Proteins: Relative Rates of Change and General Phylogeny*. Proc. Natl. Acad. Sci., USA, 85, 2469.

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ABOUT THE PURPOSE OF THE COURSE

THE AFTERNOON LECTURES

R. F. DOOLITTLE
University of California, San Diego
(USA)

About the Purpose of the Course

Events of the last few decades in the study of biology and medicine have taken some remarkable turns. Especially in molecular biology, matters that not long ago seemed "unknowable" are now being revealed by almost routine laboratory procedures. DNA sequencing, for example, is providing so much information about ourselves that we are having difficulty assimilating it. One of the major truths that is emerging is that today's organisms are the result of three billion years of unbroken evolution, and much of that history is still recorded in our genes.

Thus, many of the most important proteins that occur in all human beings still bear marked resemblances to proteins that existed two billion years ago when animals and bacteria last shared a common ancestor. As an illustration, most living organisms on this earth, including man, have a battery of enzymes for metabolizing sugar into smaller waste products. If the amino acid sequence of any of these proteins is compared from man and a simple bacterium like *E. coli*, they are invariably found to be about 50% identical. Thus, of the approximately 300 amino acids that constitute one of these proteins, 150 are still the same as they were when bacteria and animals last shared an ancestor.

There is much we can learn about how present-day systems operate if we can appreciate how they came to be. We are already finding that the number of different kinds of protein is not unlimited. Rather, the same kinds of proteins are used over and over in different settings in slightly altered forms. There are thousands of kinases, proteases and protease inhibitors, for example, regulating biochemical events in a myriad of situations.

Most aspects of molecular evolution are relatively easy to understand, but without a little guidance, the unwary researcher can be badly misled. The major aim of this short course is to provide the typical biomedical researcher with enough fundamentals that he can put his own problem into a proper evolutionary perspective.

In this regard, the morning part of the course begins with a brief overview of current thinking about the origin of life on earth and the ancient RNA world. Some speculation will be offered on the nature of early proteins and on the invention of DNA. The controversy about when and how introns came to exist will be aired thoroughly.

On the second day, the course will get into more practical matters, particularly the use of computers for searching sequence data banks, making alignments, judging significance and constructing phylogenetic trees. Subsequent lessons will depend on using these tools for following both broad and narrow courses of evolution. The major divergence of the prokaryotic and eukaryotic worlds will be spotlighted as a problem of general interest, whereas the evolution of the vertebrate plasma proteins will be a much more specific topic, more relevant to human physiology and biomedicine. The course will conclude with a consideration of the evolution of retroviruses.

The Afternoon Lectures

The afternoon program of the short course is composed of a series of ten one-hour lectures by eleven eminent scientists from around the world. The series will be initiated with a lecture by Nancy Maizels and Allan Weiner, both of Yale University, who have identified what appears to be a remarkable "molecular fossil" dating back to the RNA world. The other lecture on Day I will be by Gabriel Dover of Cambridge University. Dover is the originator of the concept of "molecular drive", a provocative concept that views DNA as something more than a passive container of information. On the second day James Lake (UCLA) will lecture on the origins of the major groups of living organisms as determined by comparisons of ribosomal RNA sequences. Lake has devised a new scheme of rate-invariant analysis that is re-defining previously drawn taxonomies. The second Tuesday lecture will be by John Walker (Cambridge University). Walker was the first person to realize that certain properties of proteins, such as binding a small molecule like ATP, can be detected by searching for a simple consensus sequence involving (almost) essential amino acids. His work has led to the unearthing of nucleotide-binding domains in many different proteins.

On Wednesday, the lectures will take two quite different tacks. Jaap Beintema (Groningen) will discuss the evolution of the vertebrate digestive enzyme ribonuclease. Beintema has determined the structure of this protein from scores of different animals, collecting data that is of interest to systematic biology and enzymology alike. The second talk will be by Adrian Gibbs (Canberra), an authority on the evolution of togaviruses. Gibbs is also well known for having invented the "dot matrix" approach to sequence comparison.

On Thursday, the theme of virus evolution is continued. Walter Fitch (Univ. Southern California), one of the pioneers of computer-assisted sequence analysis, will report on a computer study of influenza virus sequences. He will be followed by Peter Palese (New York), one of the world's most notable authorities on the flu virus.

On Friday, Georgio Bernardi (Paris) will lecture on genomic analyses of vertebrates and especially trends in the variation of base compositions and codon usage. The final lecture of the week will be delivered by Jerrold Lowenstein (Univ. Calif., San Francisco). Lowenstein has been able to perform immunochemical analyses on fossils that are tens of millions of years old. This remarkable approach allows direct access to an ancient world that our computers can only infer and never prove.

It is evident that the 10 afternoon lectures cover a broad spectrum of molecular evolutionary topics at the very forefront of modern research. We hope they will prove interesting and stimulating to an equally broad spectrum of biomedical researchers in the Madrid community.

THE GENOMIC TAG MODEL FOR THE ORIGIN OF PROTEIN SYNTHESIS

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(USA)

A central problem in envisioning the evolution of modern protein synthesis has been that none of the individual components -- ribosomal RNAs and proteins, initiation and elongation factors, tRNAs and tRNA synthetases -- appears to be useful individually, yet the molecular apparatus for translation must have evolved stepwise. We propose that modern tRNAs derive from 3' terminal tRNA-like structures that tagged genomes for replication in the RNA world. In early genomes these tRNA-like structures would have provided an initiation site for the replicase, and would also have functioned as primitive telomeres to ensure that terminal nucleotides were not lost during replication. Such structures persist at the termini of modern viruses, including RNA phages and plant viruses, and as punctuation in the rRNA operons of E. coli.

This picture suggests that the CCA-adding activity was originally an RNA enzyme, that modern DNA telomeres with the repetitive structure C_mA_n descend from the CCA terminus of tRNA, and that the ancient precursor of the modern enzyme RNase P functioned to convert genomic into functional RNA molecules by removing the 3'-terminal tag. Such contemporary molecules may be considered "molecular fossils", since their structures or activities reflect evolutionary history as well as the requirements of function.

We propose, furthermore, that an early tRNA synthetase may have derived from an aberrant activity of the RNA replicase. If this is the case then tRNA synthetases would first have evolved as RNA enzymes, which may account for the remarkable diversity in structure of modern synthetases. Finally, we speculate that if early ribosomes evolved to facilitate polymerization of homopolymers of basic amino acids, then homopolymer synthesis by peptide-specific ribosomes may have provided a pathway for the origin of a rudimentary genetic code prior to the evolution of the first external templates, or mRNAs.

MOLECULAR DRIVE: THE CONSEQUENCES OF DNA TURNOVER MECHANISMS ON THE BIOLOGY AND EVOLUTION OF MULTIGENE FAMILIES AND INTERNALLY REPETITIOUS GENES

G. A. DOVER
University of Cambridge
(England)

Mechanisms of DNA turnover (gene conversion; unequal crossingover; transposition; slippage and several RNA-mediated exchanges) affect the longterm genetic composition of populations. This is because they induce small but persistent non-Mendelian fluctuations in the copy-number of DNA sequences, leading sometimes to the gradual spread of a variant sequence through a gene family (or repetitious gene), and eventually through a sexual population. This process is known as molecular drive, and is operationally distinct from natural selection and genetic drift (1). It can lead to distinct species-specific genomes and phenotypes (1-3, 6). Examples are known where the internally driven changes have induced molecular coevolutionary changes in other genes whose products functionally interact with the gene family or repetitious sequence (3,5). The dynamics of population change under molecular drive, and the subsequent interaction with selection and drift are complex (4). They are affected by the rates of turnover relative to the mutation rate, and also the units, biases and polarities of turnover relative to the gene (8). In principle, the genetic cohesion of a population can be maintained at any given generation during a period of homogenisation for a new variant sequence (4). This could ensure a gradual and collective exploitation of the environment leading to novel species-specific functions which did not arise through the process of Darwinian adaptation, in the first instance (6). Finally, the DNA turnover mechanisms influence the rate and direction of DNA divergence (the molecular clock) and throw doubt on the use of such a clock for deciding species relationships (7,8).

Some review papers

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ORIGIN OF THE EUKARYOTIC NUCLEUS DETERMINED BY RATE-INVARIANT ANALYSIS OF rRNA SEQUENCES

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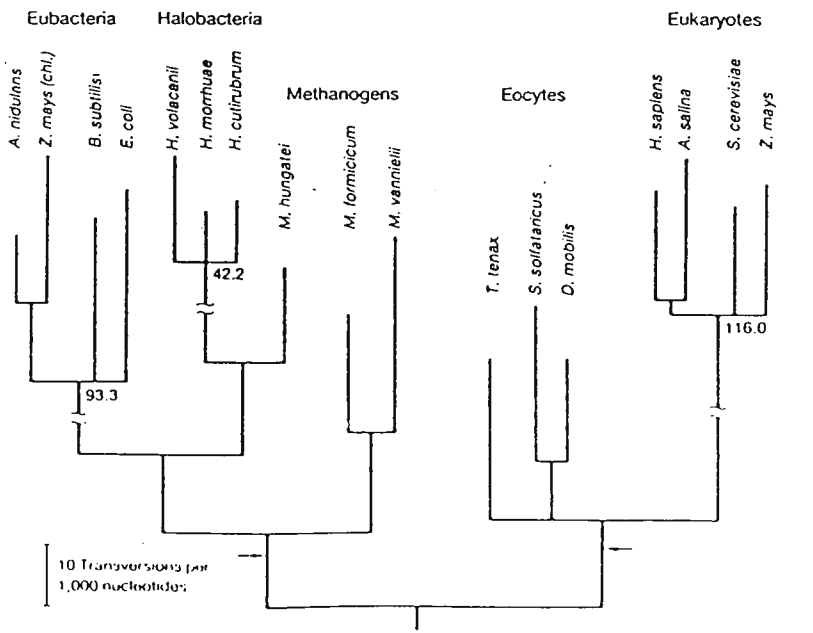
University of California, Los Angeles
(USA)

The origin of the eukaryotic nucleus is difficult to reconstruct. Eukaryotic organelles (chloroplast, mitochondrion) are eubacterial[1,2] endosymbionts[3], but the source of nuclear genes has been obscured by multiple nucleotide substitutions. Using evolutionary parsimony[4], a newly developed rate-invariant treeing algorithm, the eukaryotic ribosomal rRNA genes are shown to have evolved from the eocytes[5], a group of extremely thermophilic, sulphur-metabolizing, anucleate cells. The deepest bifurcation yet found separates the reconstructed tree into two taxonomic divisions. These are a proto-eukaryotic group (karyotes) and an essentially bacterial one (parkaryotes). Within the precision of the rooting procedure, the tree is not consistent with either the prokaryotic–eukaryotic or the archaebacterial–eubacterial–eukaryotic groupings. It implies that the last common ancestor of extant life, and the early ancestors of eukaryotes, probably lacked nuclei, metabolized sulphur and lived at near-boiling temperatures.



EVOLUTION OF MITOCHONDRIAL ATP SYNTHASE AND OF METABOLITE TRANSPORTERS

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Medical Research Council, Cambridge
(England)

The multi-subunit enzyme ATP synthase is found in eubacteria, mitochondria and chloroplasts. Studies of its subunits and genes are helping to show how it has evolved. The enzymes from these sources are similar in structure and contain a basic core of eight different polypeptides; extra subunits are associated with the mitochondrial enzyme. Genes for bacterial complexes are found in clusters. In *E. coli* the eight genes are arranged in the *unc* operon. The operon divides into two subclusters that correspond to the extrinsic membrane sector, F_0 and the intrinsic membrane sector F_1. The gene order persists in other bacteria although the clusters are not always co-transcribed. Two members of the *Rhodospirillaceae* have a cluster containing F_1 genes only. In a blue green alga gene orders are most closely related to those found in chloroplast DNA where six of the eight polypeptides of ATP synthase are encoded. This provides further evidence of common ancestry between cyanobacteria and chloroplasts. Most of the subunits of the mitochondrial enzyme are nuclear encoded. They are synthesised as precursors that are able to enter the mitochondrion. One subunit, the proteolipid, has two different precursors encoded in two separate genes in bovine DNA. Their functions are not known at present. In addition to the two expressed genes two pseudogenes for this subunit also have been characterised. Further study of these nuclear genes should help to explain their evolution and may provide evidence for or against a symbiotic origin of mitochondra.

In addition to enzymes involved in oxidative phosphorylation, mitochondrial membranes contain about twelve different metabolite transport proteins. It is now clear that at least four them have related structures and constitute a multigene family. One of them, the ADP/ATP translocase has multi expressed genes in mammals, and as is the ATPase proteolipid, they are expressed differently in various tissues. The evolution of this gene family will be discussed.

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STRUCTURE, PROPERTIES AND MOLECULAR EVOLUTION OF MEMBERS OF THE RIBONUCLEASE SUPERFAMILY

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(The Netherlands)

Pancreatic ribonucleases form a group of homologous proteins found in considerable quantities in the pancreas of a number of mammalian taxa and a few reptiles. The ribonuclease content varies greatly in different species. Large quantities are found in ruminants and species that have a ruminant-like digestion, and in a number of species with cecal digestion. Probably an elevated level of pancreatic ribonuclease is the response to the necessity of digesting large amounts of ribonucleic acid derived from the microflora of the stomach of ruminants.

Amino acid sequences of 39 mammalian and snapping turtle ribonucleases have been used to construct trees by the maximum parsimony procedure. These trees are in fairly good agreement with the biological classification of the species involved.

In mammalian tissues other than pancreas, ribonucleases have been found that are similar to the pancreatic ribonucleases in several but not all respects. Several ribonuclease components have been isolated from human urine. The main component is identical to the pancreatic enzyme, except in glycosylation characteristics. The amino acid sequence of another component, called non-secretory ribonuclease was determined. Sequence information indicates that ribonucleases of human liver and spleen and an eosinophil-derived neurotoxin are identical or very closely related gene products. The sequence is identical at about 30% of the amino acid positions to all of the secreted mammalian ribonucleases for which information is available. Identical residues include active-site residues histidine 12, histidine 119, and lysine 41, other residues known to be important for substrate binding and catalytic activity, and all eight half-cystine residues common to these enzymes. Major differences include a deletion of six residues in the (so-called) S-peptide loop, insertions of two, two, and nine residues, respectively, in three other external loops of the molecule, and an addition of three residues at the amino-terminus (Fig. 1). The sequence shows the human non-secretory ribonuclease to belong to the same ribonuclease superfamily as the mammalian secretory ribonucleases, turtle pancreatic ribouclease, and human angiogenin (Fig. 1). Sequence data suggest that a gene duplication occurred in an ancient vertebrate ancestor; one branch led to the non-secretory ribonuclease, while the other branch led to a second duplication, with one line leading to the secretory ribonucleases (in mammals) and the second line leading to pancreatic ribonuclease in turtle and an angiogenic factor in mammals (human angiogenin). The non-secretory ribonuclease has five short carbohydrate chains attached via asparagine residues at the surface of the molecule; these chains may have been shortened by exoglycosidase action. This characteristic plus the resistance of the protein to denaturation and proteolytic degradation suggests that it originated in lysosomes and escaped digestion by lysosomal proteinases due to its high net positive charge and compact structure.

In order to determine the primary structure of the pancreatic ribonuclease from a species, it is sometimes difficult to obtain sufficient tissue. To circumvent this problem we started to use recombinant DNA-techniques. Total RNA was isolated from mouse pancreas using the quanidinium-thiocyanate method. After isolating poly (A^+) RNA, cDNA was synthesized and cloned. After screening the library with a rat ribonuclease cDNA probe the positive clones were isolated and sequenced. There were no differences with the previously determined protein sequence. The mRNA codes for a preribonuclease of 149 amino acid residues including a signal peptide of 25 amino acids. The 3' noncoding region has 260 bp and the total mRNA length is about 900 bp. Comparison with the rat pancreatic ribonuclease sequence showed a high rate of nucleotide substitution without a significant difference between the coding and the 3' noncoding regions (both about 6×10^{-9} nucleotide substitutions per site, per year; values corrected according to the two parameter method of Kimura. Within the coding region non-silent and silent substitution rates are 4.4×10^{-9} and 12.9×10^{-9} nucleotide substitutions per site, per year, respectively (uncorrected values). The latter value is one of the highest rates observed in molecular evolution of eukaryotes. The signal peptide region shows a lower evolutionary rate with very similar values for the non-silent and silent rates. Nucleic acids coding for pancreatic ribonucleases of other rodent species are under investigation.

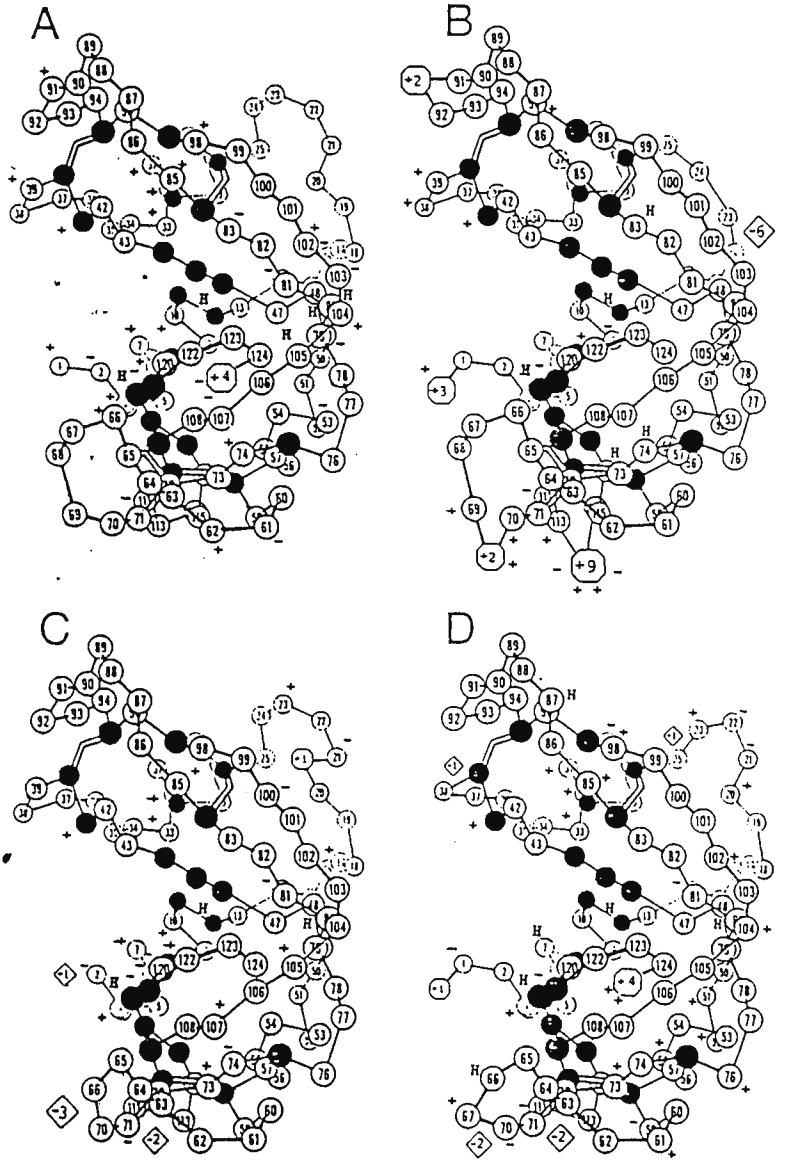

FIGURE 1: Location of insertions and deletions in the amino acid sequences presented in Figure 2 in the three-dimensional structure of bovine pancreatic ribonuclease A. Positions are numbered according to the residue number of bovine ribonuclease. Black circles indicate identical residues in the sequences presented in Figure 2 and all other known sequences of mammalian secretory ribonucleases (Beintema & Van der Laan, 1986). Numbers of deleted residues are presented in diamonds [with a (−) sign] next to the locations of these deletions. Numbers of inserted residues are indicated in polygons [with a (+) sign] inserted in the main chain. Positions with lysine or arginines, with histidines, and with aspartic or glutamic acids are indicated with (+), (H), and (−), respectively. (A) Human pancreatic ribonuclease; (B) human nonsecretory ribonuclease; (C) turtle pancreatic ribonuclease; (D) human angiogenin. Figure adapted from Dickerson & Geis (1969).

VIRUS EVOLUTION: A MAELSTROM OF MODULES

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The sequencing of viral genomes is yielding a goldmine of information. The resulting data have mostly confirmed that the virus groupings made using traditional taxonomic criteria (particle morphology and serological tests). However nucleotide sequences, and encoded amino acid sequences, have a greater information content and a longer memory than most other characters, and are providing evidence for distant relationships which traditional taxonomy could never have predicted. For example, these comparisons have shown gene sequence similarities between viruses with icosahedrally and helically-constructed particles, between animal and plant viruses, between viruses with DNA and RNA genomes, and even between some genes of viruses, prokaryotes and eukaryotes; an indication of their common ancestry (Gibbs, 1987; Goldbach, 1986,1987; Zimmern, 1987).

These studies have also shown that a virus may share one or more genes with a second virus, and others with a third (see, for example, Domier *et al.*, 1987). Thus many seem to have originated as a combination of modules drawn from a viral gene pool (Botstein, 1980). For example, the *gag* and *pol* proteins of retroviruses share sequence similarities with those of a wide range of viruses and virus-like transposable elements, all of which transfer their genetic information alternately through complete DNA and RNA transcripts (Mason, Taylor & Hull, 1987). Different members of this group of viruses and elements have different additional genes, which adapt them to their particular life-style, for example, the transmissible viruses have *env* or other particle protein genes, whereas the congenitally transmitted ones do not, similarly the episome-like viruses (hepadnaviruses and caulimoviruses) have circular genomes, whereas those that insert into the host genome have terminal repeats. The modular genes within those groups also recombine, and the extent of this mixing within the retroviruses has recently been revealed by work of McClure *et al.* (1988). Two other sets of gene modules will be discussed, firstly those of the picornavirus, comovirus and potyvirus groups, and secondly those of the tymovirus, tricornavirus, tobamovirus, tobravirus and alphavirus groups.

Structural studies have also indicated that, during evolution, three-dimensional molecular structures are conserved longer than primary gene sequence homologies (Rossmann & Argos, 1981). Such studies have shown that all the coat proteins of the isometric virions of viruses with small ssRNA genomes contain a similar characteristic structure, which is an eight-stranded anti-parallel β-barrel (Swiss roll), that has probably evolved from one

ancestral gene (Rossmann, Arnold, Erickson, Frankenberger, Griffith, Hecht, Johnson, Kamer, Luo, Mosser, Rueckert, Sherry & Vriend, 1985). Much less is known of the structure of proteins from helically constructed particles. Many may share a common origin with TMV virion protein whose main structural domain consists of four α-helical rods.

Recent work has shown that all the processes that result in evolutionary change in other organisms, such as mutation, deletion, recombination and pseudo-recombination (reassortment) occur with all groups of viruses. The genomes of quite closely related viruses may differ greatly. For example, the genomes of individual herpesviruses and poxviruses differ in size over a two-fold range and there is considerable variation in their sequence arrangement, especially that of their terminal regions (Davison & McGeoch, 1986). Similar genome variability is also found in viruses with small RNA genomes. It is now becoming clear that the nucleotide sequences of viral genomes are exposed to a great range of selective pressures, and some of these will be discussed citing recent work on the tymoviruses.

This genetic *maelstrom* of mutation and module mixing results in the variability and change observed in populations of some viruses (Holland *et al.*, 1982; Steinhauer & Holland, 1987; Smith & Inglis, 1987), notably those of orthomyxoviruses. Nonetheless other viruses maintain stable populations. How or why do they do this?

The seminal paper of this subject is that of Domingo, Sabo, Taniguchi & Weissmann (1978). They studied a stock of Qβ levivirus, a virus with a RNA genome, which had been repeatedly passaged at high multiplicity for more than 50 generations over several years and had always given "reproducible fingerprints". Nonetheless, when they examined individual clones from that stock, they found that, on average, every viable genome in the stock differed from the 'average' population genomic sequence at 1.6 sites. In competition experiments with the parental stock, five variants all disappeared after 10-20 generations. Thus they concluded that the parental population, though heterogeneous, appeared stable because it was "in a dynamic equilibrium, with viable mutants arising at a high rate on the one hand, and being strongly selected against on the other". The stability of some virus populations and the changes in others reflect the extent and type of selection that each population is experiencing. Probably most virus populations are 'quasi-species' (Eigen *et al.*, 1981).

Sequence homologies have also been used to estimate the relative or absolute time of separation of members of groups, namely to assess their phylogenies. Such calculations assume that, for each set of related sequences, there is a relationship between the estimated differences between sequences and their times of separation, namely that there is a 'molecular clock' that functions consistently in all branches of each phylogeny. However there is some evidence, which will be discussed, that viral gene 'clocks' are not stable (Gibbs, 1987).

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EVOLUTION OF HUMAN INFLUENZA VIRUSES

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There are three types of human influenza viruses, A, B and C, in order of decreasing virulence. At the invitation of Dr. Palese, in whose laboratory was performed the hardwork of sequencing many of the genes from influenza viruses isolated from 1934 to 1986 (see references 1, 2 and 3 for names of other crucial participants), I undertook to establish the evolutionary relationships of these genes.

The first analysis was of the NS (non-structural) gene from A type viruses. The first result was that the genealogical tree was not of the usual variety but of a previously unobserved type that had a greater resemblance to a cactus than to a tree, one where the central trunk is the only surviving lineage. The average age of a branch was 7.4 years from the time it left the central trunk. We wondered whether the strange shape could be the result of positive Darwinian evolution. Evidence supporting a plausible rationale for this supposition will be presented that hinges upon concepts from Kim Atwood et al's experiments showing periodic selection in E. coli (4).

More specifically, we suggest that the presence of only a single surviving lineage world-wide after five to ten years would be the consequence if favorable amino acid replacements occur that permit the virus to survive a little longer (and reproduce a few more times) before immune surveillance caught up with it.

Unfortunately, the NS gene is not known to be under immune surveillance. However, that problem is handled by noting that the NS gene, although on a separate RNA fragment, is "linked" to the hemagglutinin gene (HA, which is under immune surveillance) by virtue of the virus genome being haploid. Thus reassortment can only occur in individuals who are infected by more than one virus at the same time. Thus what happens to HA will in some degree, happen to NS as well and it could then be that we see selection-like kinetics on a gene subject to no positive selection itself. Does the same anomalous picture then appear upon examining the HA gene (H3 in our case)? It does but more so. It evolves even faster and the average age of the isolates is only 1.2 years since leaving the main surviving lineage, indicating very strong selection.

A similar analysis of the least virulent C type virus shows it to have the more common tree-like structure with multiple lineages and a much slower rate of evolution. When the same analysis was performed on the B type virus, of intermediate virulence, an intermediate result was obtained.

The evolutionary rate of the HA gene in the A type virus is 7 substitutions per year or 7×10^{-3} substitutions per site per year, the fastest known evolutionary rate of change.

But estimates of evolutionary rates usually assume that all sites may evolve. In fact, some nucleotide positions may be invariable. An essential methionine cannot allow any of the three nucleotides in its codon to vary. If many sites cannot change, then the rate of change in the changeable sites could be considerably greater than the above rate, large as it already is. If the substitutions occurred randomly over the whole sequence, then there is an expected distribution for the number of sites that have changed once, twice, thrice, etc. The observed distribution is significantly different from this so we know the substitutions are not random over the sequence. But if we assume that some of the sites are invariable, we can ask if there is a number of variable sites that would give the observed distribution. It happens there is a very good fit if only 37% of the positions are variable. Thus the evolutionary rate of the A type, H_3 hemagglutinin is 18 X 10^{-3} substitutions per variable site per year (10^{-2}), a truly astounding rate.

We believe such a high rate may be required if one needs to change more than one epitope at a time in order to escape immune surveillance.

(1) Buonagurio, D.A., Nakada, S., Parvin, J.D., Krystal, M., Palese, P. and Fitch, W.M., Evolution of Influenza A Viruses over 50 years: Rapid, Uniform Rate of Change in NS Gene. Science, 232, 980-982 (1986).

(2) Buonagurio, D.A., Nakada, S., Fitch, W.M. and Palese, P., Epidemiology of Influenza C Virus in Man; Multiple Evolutionary Lineages and Low Rate of Change. Virology, 153,12-21 (1986).

(3) Yamashita, M., Krystal, M., Fitch, W.M. and Palese, P., Influenza B Virus Evolution: Co-circulating Lineages and Comparison of Evolutionary Pattern with Those of Influenza A and C Viruses. Virology, 163, 112 - 122 (1988).

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VARIATION OF INFLUENZA VIRUSES: EVOLUTIONARY CHANGES IN NATURE AND MEASUREMENT OF MUTATION RATE

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Perhaps the most important factor in the continuing threat of influenza A virus infection in man is that of virus variation. Effective vaccination has been developed against small pox, hepatitis B, measles, mumps, rubella, yellow fever and poliomyelitis. In all of these cases, however, the virus appears to be stable in nature and only one (or a few) distinct serotype(s) circulate in the human population. In contrast, influenza A viruses undergo dramatic changes with time in both surface proteins and nonsurface proteins. This necessitates the development of new vaccine strains at regular 2-3 year intervals. Many laboratories have made efforts to study these changes on a molecular level and much information on the variation, phylogeny and evolution of influenza viruses in nature has been forthcoming.

Molecular epidemiology of influenza A viruses.

Using molecular techniques, we have attempted to analyze epidemiologically important influenza A virus strains in order to learn more about the genomic changes of these isolates in nature. The most extensive study concerned the sequence analysis of the NS genes of human influenza A viruses. In collaboration with Dr. Walter Fitch (USC), we established that, in general, the NS genes of influenza A viruses isolated over a 50-year period follow a direct evolutionary lineage. This finding allowed us to calculate an evolutionary rate for the NS genes of human influenza A viruses ($\sim 2 \times 10^{-3}$ substitutions/site/year) which is approximately 10^6- fold faster than the estimated evolutionary rate of mammalian genes. Thus, influenza A viruses represent a unique evolutionary system in which specimens for different time points are available and for which the time span of 50 years is sufficient to observe rapid genetic changes.

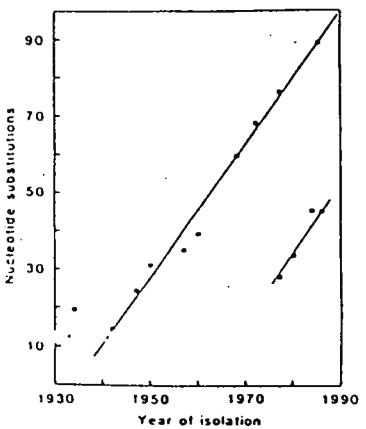

Fig. Linearity with time of number of substitutions in the NS genes of influenza A viruses. The abscissa represents the year of isolation of the influenza A viruses used in the analysis. The ordinate indicates the number of substitutions observed in the NS genes of strains isolated beteen 1933 and 1985. The NS genes of the four new H1N1 viruses are also represented in this graph (filled squares).

- Buonagurio, D.A., Nakada, S., Parvin, J.D., Krystal, M., Palese, P. and Fitch, W.M. Evolution of humanh influenza A viruses over 50 years: Rapid, uniform rate of change in NS gene. Science 232, 980-982, 1986.

Epidemiological pattern of influenza C viruses.

Another major effort was directed at the analysis of evolutionary changes in the genomes of influenza C viruses. Our approach was to sequence the genes coding for the surface protein (HE) and for the nonstructural proteins (NS1 and NS2). Briefly, the following conclusions were obtained: (a) Influenza C virus strains isolated over a 40-year period do not belong to a single direct evolutionary lineage. Instead, different (but closely related) evolutionary lines coexist at any given time. (b) The evolutionary rate of change must be slower than that of influenza A viruses since some C virus genes of strains isolated 30 years apart have very few nucleotide changes. (c) Finally, evidence was provided that reassortment of influenza C viruses occurs in the human population and, that as a consequence, a pool of closely related influenza C virus genes is present in strains which circulate in the human population.

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Epidemiological pattern of influenza B viruses is intermediate between that of human influenza A viruses and that of influenza C viruses.

Recently, we have established--again in collaboration with Dr. Fitch--an evolutionary model for influenza B viruses. Sequence analyis of the HA and NS genes of influenza B virus strains isolated between 1940 and 1987 suggests that mutations do not always accumulate with time. It appears that several evolutionary lineages of influenza B viruses co-exist at any one time. Analysis of the data also suggests that the overall rate of evolutionary change is slower than that of human influenza A viruses and faster than that of influenza C viruses. The following diagram illustrates these points.

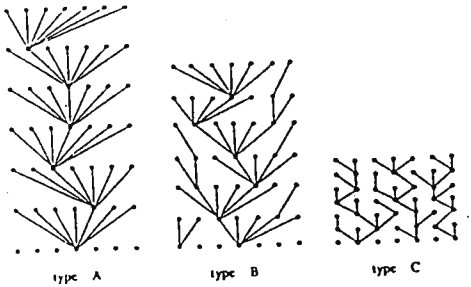

Fig. Evolutionary model for influenza A, B and C viruses in man. Dots lying on a horizontal plane represent influenza virus variants arising in the same season (year). The branch lengths indicate average relative change from the preceding season. The model of type A influenza virus shows variants arising from--in general--only one lineage because of the dominating effects of (favorable) variants, whereas the models of type B and C viruses show the co-circulation of multiple lineages. Variation of influenza B viruses appears to be slower than that of influenza A viruses and faster than that of C viruses. This is illustrated by the intermediate length of the evolutionary branches of the B virus pattern. For all influenza A, B and C viruses an arbitrary number of seven seasonal cycles is shown on the diagram.

- Yamashita, M., Krystal, M., Fitch, W.M. and Palese, P. Influenza B virus evolution: Co-circulating lineages and comparison of evolutionary pattern with those of influenza A and C viruses. Virology 163, 112-122, 1988.

Comparison of the mutation rate of animal viruses: Influenza A virus, poliovirus type I and Rous sarcoma virus.

Attempts were made to measure mutation rates of RNA viruses replicating in tissue culture. It was postulated that this parameter is an important characteristic of an RNA virus and that it may correlate with the variability of the virus observed in nature. The dramatic outcome of the study was that there are significant (several log) differences in the mutation rates of RNA viruses. The rates were determined by collecting progeny-virus derived from a single virion after one replication cycle. In the virion case of influenza A and poliovirus type 1, one gene each from 100 progeny viruses were sequenced. In 91,708 nucleotidees analysed, seven point mutations were observed in the NS genes of influenza viruses. A total of 105 VP1 genes of poliovirus were sequenced and in the 95,688 nucleotides analyzed, no mutations were observed. With respect to Rous sarcoma virus seven different sequences of the genome were analyzed. In all, 65,250 nucleotides were screened by denaturing gradient gel electrophoresis and nine mutations were detected. We then calulated mutation rates of 1.4×10^{-4}, 1.5×10^{-5} and less than 2.1×10^{-6} mutations/ nucleotide/replication cycle for Rous sarcoma virus, influenza A virus and poliovirus type 1, respectively. This suggests that mutation rates of RNA viruses may vary by a factor of 100.

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TWO MODES IN GENOME EVOLUTION

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We have analyse the compositional patterns of vertebrate genomes, namely the distributions of GC levels of DNA molecules (in the 50-100 KB size range), of coding sequences and of individual codon positions. These analyses have shown that genome evolution proceeds according to two modes, long periods of conservation of compositional patterns being broken by shifts from the existing patterns into new ones. Compositional patterns are conserved essentially because the mutational process itself is conservative as far as GC levels are concerned. Selection also plays a role, however. In contrast, shifts of compositional patterns appear to occur under the influence of changes in environmental conditions and to be mainly due to positive selection.

PHYLOGENIES OF LIVING AND EXTINCT SPECIES BY RADIOIMMUNOASSAY

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Radioimmunoasssay (RIA) is an extremely sensitive method for measuring small amounts of proteins and for assessing the relationships between proteins of different species. In close relationships, RIA can distinguish between protein subspecies, and in distant relationships, it can measure immunological distances between groups such as reptiles, amphibians, fish, invertebrates and algae that diverged hundreds of millions of years ago. RIA phylogenies have been developed for these diverse taxa.

RIA has also been applied to detecting fossil proteins and to investigating their degree of relatedness with the proteins of living species. This method has been used to resolve taxonomic disputes surrounding such extinct groups as mammoths and mastodons, Steller's sea cow, the Tasmanian wolf, and the quagga. Recent applications of RIA include detection of species-specific albumin in the urine of packrats and hyraxes thousands of years old and identification of the species of bloodstains on ancient tools and weapons.

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