PICORNAVIRUS VARIATION

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INTRODUCTION

Variation of viruses has multiple theoretical and practical implications. Because mutations are stochastic events, the extensive variability of RNA viruses and retroviruses has manifested, perhaps more than ever before, the indetermination and unpredictability that may be inherent to biological phenomena. RNA virus populations do not have a precisely defined genome, but consist of indeterminate mixtures of related genomes termed *viral quasispecies* (Fig. 1), an application to viruses of a theoretical concept first proposed by M. Eigen and colleagues to describe replicons in early life on earth (Eigen and Schuster, 1979; Eigen and Biebricher, 1988; Domingo *et al.*, 1993). Initially, the quasispecies concept was ignored by many virologists as being purely "theoretical". However, as pointed out by Holland and colleagues (1992) in a recent volume which summarizes the state of the art in virus variation: "Quasispecies populations are not hypothetical abstractions. They exist in animals and humans infected by RNA viruses such as vesicular stomatitis virus, foot-and-mouth disease virus, poliovirus, human immunodeficiency virus, influenza viruses, etc.". Obviously, if comparative nucleotide sequencing of genomes from one population is restricted to a few hundred nucleotides several "identical genomes" may be found for simple statistical reasons (Fig. 1). The mutual influence between theoretical studies on quasispecies and experimental molecular virology has been determinant in providing the basis of what has been termed "RNA genetics", a number of distinctive features associated with the error-prone replication of RNA genomes (Holland *et al.*, 1982, 1992). Here we review evidence that picornaviruses are generally variable and that their populations are best described as quasispecies. The basic principles that influence picornavirus variability and evolution do not differ in any substantial way from the principles guiding variation of other RNA viruses and retroviruses. In spite of the extensive heterogeneity of the human immunodeficiency viruses (HIV) within and between infected individuals, a comparison of the studies carried out with HIV-1 with those for other RNA viruses (including picornaviruses)

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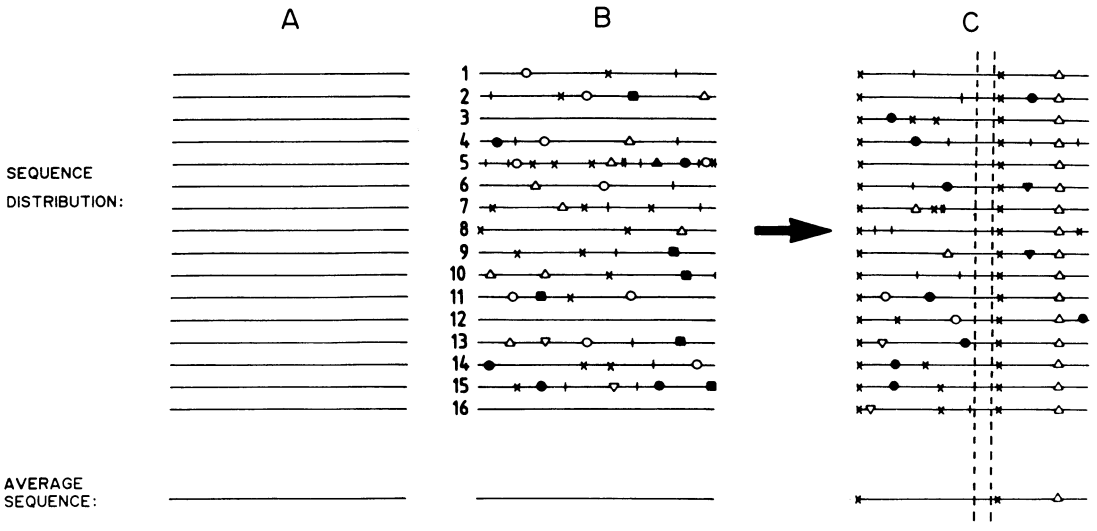

Fig. 1. A schematic representation of a picornavirus quasispecies. A depicts a homogeneous collection of picornavirus genomes, and B a heterogeneous distribution with an average of 4 mutations (symbols on the lines) per genome. In spite of being very heterogeneous, the average or consensus sequence of population B is identical to A. The replication of molecule 8 from the mutant spectrum of B yields a new quasispecies C with a new average sequence. Note that in spite of an extensive heterogeneity in distribution C, the discontinuous vertical lines delimit a genetically homogeneous stretch representing 8% of the genome length (about 600 nucleotides of the picornavirus genomic RNA). Thus, the absence of mutations after repetitive sequencing of a limited genomic region cannot be used as an argument against the quasispecies structure of a genome population. About 10^4 genomes are needed on average to find a mutation at any given nucleotide, but more than 10^{11} infectious genomes can be found in an infected host and about 10^5 in a single plaque on a cell monolayer (see text). Picornavirus populations (even single clones and natural isolates) are as B or C, not A. Mutant distributions are dynamic: they vary with time due to perturbations of equilibrium as the result of input of new variants and/or of changes in the environment. [Adapted from Domingo(1989), with permission from Birkhäuser Verlag, Basel].

led Temin (1989) to conclude that HIV" is not unique but merely different". We hope to persuade virologists that in addition to having important "theoretical" impact, quasispecies and picornavirus variation are intimately linked to "practical" problems, notably the control of picornaviral disease.

TWO APPROACHES TO PICORNAVIRUS VARIATION

Picornaviruses constitute a diverse group of human and animal pathogens which share with other RNA viruses the ability to vary rapidly in response to certain direct selective pressures, and to change more gradually and subtly when facing replication in modified environments. Indeed, picornaviruses able to escape neutralization by monoclonal antibodies (MAbs) or to resist inhibition by antiviral compounds are generally isolated with high frequencies, well above the reciprocal of the number of particles populating an infected host. Those picornaviruses which have been examined in some detail show remarkably high mutation and recombination rates and genetic heterogeneity *in vivo* and in cell culture. Such population microheterogeneity — the quasispecies genetic structure —

constitutes the raw material for long-term evolution and for rapid responses to environmental changes.

Two alternative — albeit complementary — approaches have been used to study RNA virus variation. One approach uses clasical procedures of molecular evolution such as sequence alignments and computations of genetic distances to derive phylogenetic relationships among viral genomes. This approach is very useful to relate different picornaviruses, to trace the possible origin of strains causing disease outbreaks, or to estimate rates of fixation of mutations with time, but it provides limited information on the molecular basis (or driving forces) of picornavirus evolution. The second approach consists on a detailed analysis of individual populations of viruses, including measurements of mutation rates during genome replication, mutant frequencies, and genetic heterogeneity in natural populations and in viruses passaged in a controlled environment. These analyses may alert of the potential of a virus to change in response to selective pressures. It cautions against indiscriminate use of certain antiviral agents, monoclonal antibodies or synthetic vaccines to control virus spread (Domingo and Holland, 1992), but it says little of the relationships among distantly related picornaviruses.

This review is mainly concerned with the general conclusions of these two approaches to study picornavirus variation and long-term evolution. For a broader introduction to picornavirus genetics the reader is referred to recent excellent reviews by Rueckert (1990), and Stanway (1990).

COMPARATIVE MUTATION RATES AND MUTANT FREQUENCIES FOR PICORNAVIRUSES

As it will become more apparent in the following paragraphs, it is important (and even necessary) to distinguish mutation rates from mutant frequencies. A mutation rate expresses the number of misincorporation events per nucleotide site and round of copying during genome replication, irrespective of the fate of the mutant molecules being generated. In contrast, a mutant frequency expresses the proportion of a mutant genome in a virus population. Thus, it is a population number (rather than a biochemical event expressed by the mutation rate), heavily dependent on the fate (increased or decreased stability, or ability to replicate) of the mutant genomes generated. For example, during picornavirus RNA copying into minus strands and subsequent amplification in the replication complex, the proportion of mutants unable to bind the virus-specific replicase would be very low even if they were generated at high rates. The reader is referred to reviews by Domingo and Holland (1988, 1993), Eigen and Biebricher (1988), Domingo (1989) and Domingo *et al.* (1993) for additional examples and comments on this important point.

Table 1 lists a number of determinations of mutant frequencies for picornaviruses. The complexities of RNA genetics become apparent when trying to explain or reconcile the different values that have been reported for poliovirus. Parvin *et al.* (1986) determined the proportion of mutant genomes in progeny virions from a single plaque of poliovirus type 1 by repetitive sequencing of the VP1 gene. Since no mutations were detected in 95,688 nucleotides sequenced, the authors concluded that the mutation rate of poliovirus was less than 2.1×10^{-6} substitutions per nucleotide per infectious cycle. However, any mutant arising in the plaque and showing a slight selective disadvantage had a very good chance of being lost in the subsequent plaque isolations; in consequence it may never have been scored as a mutant

Table 1. Point mutation frequencies, and MAR mutant frequencies for picornaviruses

Virus	Procedure	Value[a]	Reference
Poliovirus	Repetitive nucleotide sequencing	$< 2 \times 10^{-6}$	Parvin *et al.*, 1986
Poliovirus	Reversion of amber mutant	2×10^{-6}	Sedivy *et al.*, 1987
Poliovirus	Reversion of guanidine dependence	2×10^{-4}	de la Torre *et al.*, 1990
Poliovirus	Chemical determination of error oligonucleotides [b]	3×10^{-3} - 5×10^{-3}	Ward and Flanegan, 1992
Poliovirus	Specific U → C transition	2×10^{-5}	de la Torre *et al.*, 1992
Poliovirus	MAR mutant frequency[c]	10^{-4} - 10^{-5}	Emini *et al.*, 1982; Minor *et al.*, 1983, 1986a
Mengovirus	MAR mutant frequency[c]	3×10^{-3} - 5×10^{-5}	Boege *et al.*, 1991
Foot-and-mouth disease virus	MAR mutant frequency[c]	10^{-5}	Martínez *et al.*, 1991a
Rhinovirus	MAR mutant frequency[c]	10^{-4} - 10^{-5}	Sherry *et al.*, 1986
Hepatitis A virus	MAR mutant frequency[c]	3×10^{-3}	Stapleton and Lemon, 1987

[a] Expressed generally as substitutions per nucleotide site. However, in some cases one specific base change was measured and in other cases any possible mutation at one or several sites was scored (see specific references for a precise interpretation of each value). MAR mutant frequency is the proportion of mutants resistant to neutralization by a MAb.

[b] Measured at many different sites of the viral genome.

[c] MAR mutant frequencies cannot be directly related to mutation rates or frequencies per site (see text).

— even in the case of its having been generated at a high rate! —. Thus, the value obtained by Parvin *et al.* (1986) was the frequency of neutral mutant genomes generated during early stages of a poliovirus plaque growth. The low value obtained may reflect the good adaptation to HeLa cells of the Mahoney poliovirus 1 used.

Sedivy *et al.* (1987) studied the reversion of an *amber* mutation introduced by site-directed mutagenesis in place of a serine codon at position 28 of the polymerase (3D) molecule of poliovirus. Each of the five revertants

that were sequenced had regained the serine codon, even though a single base change in the *amber* codon could have given rise to any of six different amino acids. The specific transversion A→C, which was required to revert the *amber* to a serine codon, occurred at a frecuency of $2.5x10^{-6}$ revertants per *amber* plaque forming unit. In the analysis, however, mutant virus was grown in a suppressor cell line, and well isolated, amber plaques were picked after 3, 4 and 5 days of incubation and analyzed for the presence of revertants. Thus, early-arising revertants (which should produce large plaques) could not have been scored as components of an *amber* plaque. Did this reversion frequency reflect the mutation rate *per site*? Nomoto and associates (Kuge *et al.*, 1989) found a strong bias towards transition mutations at an AUG codon located within a sequence inserted in the non-coding region of Sabin poliovirus type 1. This AUG decreased the plaque size of the virus and permitted identification of revertants originated from independent mutation events. The ratio of transitions to transversions approximated 50 at this deleterious AUG triplet. Thus, if the reversion of the amber mutation studied by Sedivy *et al.* (1987) was dependent on a single transversion (such as A→C, needed to regain the serine in wild type polymerase) and the bias seen by Kuge *et al.* (1989) applied also to codon 28 of the polymerase, the true mutation frequency *per site* would approximate 10^{-4} ($2.5x50x10^{-6}$) mutations per nucleotide (Kuge *et al.*, 1989). The difficulty of extending a mutation rate *per site* from a mutation rate for *a particular base substitution* (a given transition or transversion at one site) will be encountered whenever the reversion of a deleterious mutation is used in the measurement, as discussed in the early work on site directed-mutagenesis of the 3'-extracistronic region of bacteriophage Qß (Domingo *et al.*, 1976; Batschelet *et al.*, 1976; see also Holland *et al.*, 1992).

Not surprisingly, other determinations for poliovirus have yielded high mutant frequencies. Holland and colleagues (de la Torre *et al.*, 1990) measured the frequency of reversion of guanidine-dependent mutants of the Mahoney strain of poliovirus type 1 to guanidine independence. This phenotypic change is due to a single amino acid replacement at position 227 of viral polypeptide 2C. Using several clonal pools of virus they found that the mutation frequency (with all amino acid substitutions occurring at position 227) was $(6.5\pm6.3)x10^{-4}$, and the minimal corrected base substitution frequency per single nucleotide site in the codon analyzed was $(2.1\pm1.9)x10^{-4}$ (de la Torre *et al.*, 1990).

More recently, Ward and Flanegan (1992) have used the error oligonucleotide method developed by Steinhauer and Holland (1986) to measure mutant frequencies at eight G residues of the Mahoney strain of poliovirus type 1. The proportion of molecules devoid of a G residue present in the consensus wild type genome was consistently of $(2.0\pm1.2)x10^{-3}$ to $(4.1\pm0.6)x10^{-3}$ at each of the eight sites analyzed, irrespective of their extent of conservation among related poliovirus genomes isolated in the field. This chemical analysis measures the proportion of mutated residues in viable and non-viable genomes present in the virus population under study. For this reason such chemical methods may yield slightly higher mutant frequencies than procedures which measure mutant frequencies in viable genomes [such as MAb-resistant (MAR) mutant frequencies; see Table 1 and Holland *et al.* (1989)].

A very interesting example of co-substitution of several nucleotides in the poliovirus genome has been recently described by de la Torre *et al.* (1992) in studying the reversion of a *ts* mutant of poliovirus type 1. Using infectious cDNA clones they measured a frequency of $2x10^{-5}$ for a specific U→C transition within the 3AB coding region. However, several revertants had also reverted *four* silent substitutions introduced in the infectious cDNA to

provide restriction enzyme recognition sites. Thus, exceedingly high mutation rates may be attained during poliovirus replication, perhaps due to occasional, extremely mutagenic cellular environments or to the presence of error-prone polymerase subsets — polymerase molecules being also subjected to a potential high substitution level —. This finding with poliovirus has a precedent in several observations on hypermutability (and biased hypermutability, with a strong preference for one type of base substitution) made with defective-interfering RNA of vesicular stomatitis virus, measles virus variants, as well as with retroviruses (recent review in Holland *et al.*, 1992, and several chapters of the same book).

Even though studies on variation of picornaviruses are still in their infancy the available evidence suggests that other picornaviruses share with poliovirus high mutation rates and mutant frequencies. In an early study, Eggers and Tamm (1965) estimated a frequency of 10^{-4} mutations per replication for the reversion of coxsackie A9 from dependence on HBB [2(α-hydroxybenzyl)-benzimidazole] to HBB independence. A number of antiviral compounds bind into a hydrophobic pocket in the picornaviral capsid inhibiting virus uncoating. Human rhinovirus 14 mutants with high resistance to one such compounds (WIN 52084) occurred at frequencies of $4x10^{-5}$, and those with low level resistance were found at. $1x10^{-3}$ to $4x10^{-4}$ resistant particles per wild type (Heinz *et al.*, 1989). The ease of selection of viruses resistant to antiviral compounds is shared with other RNA viruses and retroviruses and it represents one of the most important problems for effective antiviral therapy (reviewed by Domingo, 1989, and Domingo and Holland, 1992). In spite of the ease of deriving WIN-resistant rhinovirus variants, Andries *et al.* (1990) could cluster 100 rhinovirus serotypes into two groups, according to their sensitivity to some WIN compounds. Interestingly this grouping correlated not only with sequences lining the pocket at the drug binding site, but also with pathogenicity and with serological relationships (Cooney *et al.*, 1982). It is not clear why such disparate phenotypes correlate so closely in this highly variable virus group.

MAR mutants of picornaviruses can generally be isolated at frequencies of 10^{-3} to 10^{-5} mutants per wild type virus, both in natural isolates and in clonal populations in cell culture (Table 1). These frequencies are only indirectly related to mutation rates since they depend on the number of amino acid substitutions capable of conferring the MAR phenotype while preserving virus viability. Also, MAR mutant frequencies may be underestimated by phenotypic masking of mutant genomes in wild type envelopes or capsids (Valcarcel and Ortín, 1989; Holland *et al.*, 1989; Carrillo *et al.*, 1990) or by the lower fitness of the mutants relative to the parental populations from which they were isolated (Martínez *et al.*, 1991a; González *et al.*, 1991). In spite of these limitations, and since several different neutralizing MAbs have been tested for each picornavirus group, it may be significant that most MAR mutant frequencies are in the range of 10^{-3} to 10^{-5}, irrespectively of the extent of antigenic diversity attained by a picornavirus in the field. For example, no trend towars higher frequencies is observed for rhinoviruses, a group represented by about one hundred serotypes (Table 1). Recently, Domingo and Holland (1993) have reviewed mutation rates and mutant frequencies for several virus groups. The conclusion arising from these comparisons is that picornaviruses do not differ greatly from other RNA viruses and retroviruses in their average mutant frequencies. However, as expected, differences of intrinsic mutability and of tolerance to substitutions may influence mutant frequencies at particular sites on a viral genome.

In a comparative study involving poliovirus and vesicular stomatitis virus, Holland *et al.* (1990) showed that the spontaneous mutation

frequencies at defined genomic loci of the two viruses could not be increased by addition of chemical mutagens without loss of virus viability. In keeping with the concept of error threshold for error-prone replication (Eigen and Biebricher, 1988) the results with poliovirus suggest that it replicates with a fidelity very close to what may be compatible with maintaining meaningful genetic information. In other words, viral quasispecies are very adaptable because they replicate close to error catastrophe (Eigen and Biebricher, 1988).

QUASISPECIES. THE "POSITIVE-VERSUS-NEGATIVE SELECTION" GAME

With mutation rates averaging 10^{-3} to 10^{-5} substitutions per nucleotide site per round of copying, any picornavirus-infected cell must generate and contain multiple mutants. It has been estimated that an individual infected with an RNA virus may contain in the order of 10^9 to 10^{12} infectious particles at a given time point of an acute infection (Sellers, 1971; Halstead, 1980; Domingo *et al.*, 1985). Given a genomic complexity of 7kb to 9kb (which embraces picornaviruses) — combined with high population numbers and mutant frequencies (Table 1) — it can easily be calculated that all possible single and double mutants as well as decreasing proportions of triple, quadruple, etc. mutants are potentially present in most virus quasispecies (Domingo *et al.*, 1978, 1985; Temin, 1989). However, during replication, each mutant virus will be rated in competition with all other variants present and (continuously) arising in the population (Fig. 1). The proportion of each particular variant under defined environmental conditions will depend on the rate at which it arises but also on its competitive ability relative to other variants. The dynamic situation in which mutant swarms in continuous modification lead, nevertheless, to the same average or consensus genomic sequence has been termed population equilibrium (Holland *et al.*, 1982; Domingo and Holland, 1988). In general, most components of the mutant spectrum will show a decreased fitness relative to the parental average population (Domingo *et al.*, 1978; Eigen and Biebricher, 1988; Holland *et al.*, 1991; Martínez *et al.*, 1991a). A continuous process of *negative selection* will keep deleterious variants at low levels unless they can replicate by complementation of viral functions from standard genomes. Repeated plaque-to-plaque passage of virus often results in viruses with decreased fitness (Chao, 1990; Duarte *et al.*, 1992; Clarke *et al.*, 1993). This gradual accumulation of deleterious mutations (Muller's ratchet) is an additional proof of the quasispecies structure of RNA virus populations and it may be important to modulate virus virulence in nature (Duarte *et al.*, 1992).

In addition to the sieving effect of negative selection, virus evolution is strongly influenced by positive selection events such as that imposed by immune responses, by antiviral agents, etc. However, a virus variant may also increase in numbers irrespective of its competitive fitness, and without the intervention of positive selection. An example is the amplification of HIV-1 proviruses by antigenic stimulation of lymphoid cells (Wain-Hobson, 1992). It is not possible at present to assess the relative contributions of positive selection, negative selection and random sampling events in long-term evolution of picornaviruses. [This topic has been discussed by Domingo and Holland (1993) in relation to the neutralism-selectionism controversy].

Recombination frequencies can attain values of 10% to 20 % for the entire picornavirus genome (see King, 1988a, and Lai, 1992 for review). Recombinant molecules originate probably from polymerase jumping between genomes replicating in the same cell (Kirkegaard and Baltimore,

1986). The crossover sites do not seem to include a precise nucleotide sequence or a recognition signal. Recombination appears to be more frequent at conserved genomic stretches, and rare at capsid protein-coding regions (King 1988b). Recombination frequencies for FMDV are high in cell culture but only recently Krebs and Marquardt (1992) have provided the first evidence of recombination in nature by suggesting that FMDV O_1 Burgwedel / 1987 is a recombinant between the related O_1 Kaufbeuren and a C_1 strain.

Since the basic mechanisms involved in RNA recombination appear to be common to most positive strand RNA genomes, recent findings with other systems suggest important evolutionary implications of recombination in the case of picornaviruses. First, as in the case of point mutations, recombination events seem to occur largely at random (Banner and Lai, 1991), and thus, most resulting recombinant genomes must be subjected to very strong negative selection. Occasionally however, a recombinant genome may be sufficiently fit to initiate the exploration of a new ecological niche with the optimizing effects of point mutations generated during replication. If the new replicon is successful, a new virus has come into existence as a result of a large evolutionary jump. Since heterologous recombination may also occur involving disparate RNA molecules of cellular origin (Monroe and Schlesinger, 1983; Meyers *et al.*, 1991), recombination may actively promote reshuffling of viral and cellular domains to produce novel viable genomes (Zimmern, 1988) over a trial and error process lasting hundreds of thousands of years and myriads of recombination events. As will be elaborated further in the last section of this chapter, present day viruses can experience only limited variations and they represent "clouds" in sequence space (Eigen and Biebricher, 1988) which cannot expand beyond certain limits. This is why they possess an identity as viruses and we can classify them, although some times with difficulty. Recombination can be considered a means to rescue viable genomes from highly mutated or defective parental genomes (a conservative activity) but also a way to produce novel genomic combinations (a radical activity) that may mediate long-term evolutionary jumps of viruses.

A significant subclass of defective genomes are the defective interfering (DI) particles able to modulate replication of their standard infectious parental viruses (reviewed by Roux *et al.*, 1991). DI RNAs have been described for a variety of picornaviruses (Cole and Baltimore, 1973; Nomoto *et al.*, 1979; McClure *et al.*, 1980; Radloff and Young, 1983). However, generation of DIs during picornaviral infection is not a frequent event perhaps due to poor complementation of viral functions in systems which use a number of cis-acting activities. The possible role of DIs in natural picornaviral infections remains largely unexplored.

PICORNAVIRUSES VARY WITHIN INFECTED INDIVIDUALS AND DURING DISEASE EPIDEMICS

Poliovirus and foot-and-mouth disease virus (FMDV) have been the picornaviruses most extensively analyzed with regard to genetic heterogeneity and variation *in vivo* and in cell culture. Some interesting points can be made by comparing the two viruses which are quite different regarding host range and the infection process. Humans are the sole natural host of poliovirus, and most (>99%) infections are subclinical. Even for poliovirus type 1— responsible of about 90% of poliomyelitis cases — disease is seen in only about 0.5% of infections by this virus serotype (Minor,

1989). In an outbreak in Finland (1984 - 1985) caused by poliovirus type 3 it was estimated that about 10^5 individuals were infected but only 10 were ill (Hovi *et al.*, 1986). In contrast to the restricted host-range of poliovirus, many different animal species belonging to the artiodactyla are natural hosts for FMDV. Even during one disease outbreak more than one animal species may be afflicted with acute systemic infection; subclinical infections appear to be rare in the common hosts, but a carrier state may be established in ruminants as a sequel of an acute infection. In spite of encountering widely different environments, poliovirus and FMDV show a similar extent of genetic change in nature. By sequencing a VP1/2A-coding region, it was estimated that the genetic heterogeneity during a poliomyelitis epidemic was 1% to 2% of nucleotides (Kew *et al.*, 1990). This is similar to the estimated 0.7% to 2.2% nucleotide heterogeneity over the entire FMDV genome for viruses isolated during an episode of FMD (Domingo *et al.*, 1980). An animal infected with FMDV includes multiple variants (Domingo *et al.*, 1980; Carrillo *et al.*, 1990). Likewise, poliovirus type 2 and 3 vaccine strains can undergo more than 100 mutations during replication in only one or two individual humans (Kew *et al.*, 1981). Thus, not surprisingly, contemporary poliovirus or FMDV virus isolates are also genetically heterogeneous (Sobrino *et al.*, 1986; Rico-Hesse *et al.*, 1987; Villaverde *et al.*, 1988; Kinnunen *et al.*, 1991). Comparison of sequential isolates during an epidemic outbreak suggests a rate of fixation of mutations of 6.9×10^{-3} to, 1.4×10^{-2} substitutions per nucleotide per year for the poliovirus genome (Kew *et al.*, 1990) and of 6.5×10^{-3} for FMDV (Villaverde *et al.*, 1988, 1991). (Table 2).

Examination of poliovirus genomes excreted by primary vaccinees revealed rapid genetic variation by mutation and recombination (Nottay *et al.*, 1981; Kew *et al.*, 1981; Kew and Nottay, 1984; Jameson *et al.*, 1985). A number of mutations have been associated with attenuation of poliovirus for humans. A pyrimidine transition disrupts a base pair at the 5'-untranslated region (5'-UTR) resulting in reduced translation efficiency. In addition, attenuated strains include an amino acid substitution in capsid protein VP1 and VP3 (Westrop *et al.*, 1989; Macadam *et al.*, 1989; Weeks-Levy *et al.*, 1991), the latter being responsible of the *ts* character of the Sabin type 3 vaccine strain. These three mutations are frequently lost within days of replication of poliovirus in vaccinees (Evans *et al.*, 1985; Minor *et al.*, 1986b; Dunn *et al.*, 1990). Reversion of the *ts* character of Sabin type 3 poliovirus occurred by second site mutations (Minor *et al.*, 1989). Mutant and recombinant polioviruses have also been isolated from rare cases of vaccine-associated poliomyelitis. Thus, the efficacy of live attenuated poliovirus vaccines is not due to inability of the virus to change genetically (or even antigenically, at least at some sites), since poliovirus behaves as a quasispecies capable of rapid variation in humans.

Multiple genomic mutations at different loci distinguish attenuated FMDV from their virulent counterparts and no conclusive evidence has been obtained to permit assigning the altered phenotype to a genetic change (Parisi *et al.*, 1985; Cao *et al.*, 1991).

In addition to poliovirus and FMDV, extensive genetic heterogeneity and variations during natural infections have been documented for all picornaviruses examined, including rhinoviruses (Tyrrell, 1990), enterovirus 70 (Takeda *et al.*, 1984), coxsackie A10 virus (Kamahora *et al.*, 1985), coxsackie B4 virus (Prabhakar *et al.*, 1985), hepatitis A virus (HAV) (Siegl *et al.*, 1984; Weitz and Siegl, 1985; Ticehurst *et al.*, 1989; Robertson *et al.*, 1992) and the insect picornavirus Drosophila C virus (Clewley *et al.*, 1983). As complete or partial nucleotide sequences of sufficient length are determined, it becomes clear that each picornavirus isolate is genetically unique (reviews in Domingo and Holland, 1988, 1993; Palmenberg, 1989; Stanway, 1990).

Phenotypic changes can be the result of one or a limited number of mutations in a genome, and variants with few mutations have a good chance of being represented in the quasispecies mutant spectra, provided they do not adversely alter the ability of the virus to replicate. (Domingo *et al.*, 1978, 1985; Domingo and Holland, 1988, 1992, 1993). Thus, it is expected that frequent changes in biological properties occur with picornaviruses within relatively short time periods. Among other examples, a Coxsackie B3 virus with altered receptor apecificity was selected upon passage in rhabdomyosarcoma cells (Reagan *et al.*, 1984). A limited number of mutations distinguish diabetogenic from non-diabetogenic encephalomyocarditis virus (EMCV) (Ray *et al.*, 1983), or fast-growing variants of HAV from slow growing strains (Venuti *et al.*, 1985; review in Ticehurst *et al.*, 1989). Neurological complications have been described for diseases caused by a number of picornaviruses — other than poliovirus — such as enterovirus 70 (Bharucha and Mondkar, 1972; Kono *et al.*, 1973) or FMDV, even though in this case neurotropism is very rare (reviewed by Domingo *et al.*, 1990). It is obvious that a large number of mutations occurring in picornaviruses during natural infections may be without apparent biological relevance. This is unavoidable since mutant spectra must provide a reservoir of variants capable of exploring (and perhaps adapting) to a wide range of environments. Such potential adaptability may require an "excess of variation" relative to what would be strictly optimal for a single lineage completing an evolutionary cycle in a defined environment. An interesting possibility is that a virus replicating in a very specialized site within a restricted number of host species could perform its life cycles with no need of exploring sequence space beyond the one optimized to replicate and assemble infectious and transmissible virions. Such a viral system would not need high mutation rates and its replication machinery could have evolved to possess (or be associated with) some proofreading — repair activity (Holland *et al.*, 1992). However, such a potential virus has yet to be identified. It was suspected that HAV, because of its highly specialized hepatotropic character — and also because of its striking antigenic stability suggested by early results — might be an example of a well adapted, relatively invariant virus. As additional HAV sequences are determined, however, it appears that HAV shows also considerable genetic variability (Ticehurst *et al.*, 1989; Robertson *et al.*, 1992; Brown *et al.*, 1991; see also MAR mutant frequencies in Table 1). Other RNA viruses such as rabies and measles were also considered relatively invariant. However, recent data suggest that they may actually be more heterogeneous and variable than previously suspected (Benmansour *et al.*, 1992; Rota *et al.*, 1992).

In spite of a similar behaviour at the genetic level, poliovirus and FMDV show differences regarding the ability to induce widely protective responses within a serotype. Minor (1990) compared the antigenic structure of representative picornavirus groups. Poliovirus vaccines (live attenuated Sabin vaccines or formalin inactivated Salk vaccines, each of them containing a strain of each of the three poliovirus serotypes) have been effective over many decades (Minor, 1989). In contrast, anti-FMD vaccines must be periodically updated, and tailored according to the predominant viral types and subtypes in a given geographical area (Bachrach, 1968; Brown, 1989; Domingo *et al.*, 1990). A different long-term antigenic stability may result, among other possibilities, of one or several of the following effects. A large proportion of the nucleotide substitutions which are fixed during the evolution of the poliovirus genome in nature is silent (Kew *et al.*, 1990) whereas both silent and non-silent replacements are found during FMDV genome evolution, and, in some cases, non-silent mutations are dominant (Díez *et al.*, 1990a ; Martínez *et al.*, 1992). In spite of a similar overall three-dimensional structure of poliovirus (Hogle *et al.*, 1985) and

FMDV (Acharya *et al.*, 1989) antigenic determinants are located on surface loops which differ in length, composition and flexibility between the two viruses. Some immunodominant antigenic loops may tolerate more (and perhaps more drastic) substitutions in FMDV than in poliovirus. For example, the immunodominat antigenic site 1 of poliovirus type 3 (VP1 residues 89 to 100) — also present but less dominant in type 1 (Minor *et al.*, 1986a) — appears to be more conserved among independent poliovirus isolates (from different years and locations) than the immunodominant site A (VP1 residues 140 to 160) of FMDV [compare Table 2 in Minor (1989) with Fig. 1 in Domingo *et al.* (1992)]. Unlike FMDV in which all antigenic sites are among the most variable capsid regions in primary sequence, some poliovirus sites appear to bear considerable restrictions for variation. No change in antigenic site 1 of poliovirus types 1 or 3 could be evidenced by testing neutralizing MAbs against strains isolated from vaccinees (Minor *et al.*, 1986b, 1987; review in Minor, 1989). Site 3a of poliovirus type 3 appears also as very conserved among recent isolates (Yang *et al.*, 1992). If indeed surface loops of poliovirus are more limited in their tolerance for variation, certain testable predictions can be made. The number of different amino acid substitutions able to confer the MAR phenotype might be, on average, lower for poliovirus than for FMDV. In addition, or alternatively, the relative fitness of the MAR mutants to their parental wild type virus should also be lower for poliovirus than for FMDV. However, no such differences are obvious when comparing MAR mutant frequencies (Table 1). Only a limited number of measurements of relative replication ability have been carried out for picornavirus MAR mutants (Martínez *et al.*, 1991a; González *et al.*, 1991) or for drug-resistant rhinovirus mutants (Ahmad *et al.*, 1987; review in Domingo, 1989), and many more measurements are needed to reveal, in a statistically significant manner, possible differences of tolerance to substitutions among virus groups. Large, flexible loops, structurally independent of other domains, may be prone to variation and capable of inducing large repertoires of antibodies. Some amino acid substitutions at the main antigenic site A (VP1 residues 138 to 150) of FMDV of serotype C abolish many (or all) of the multiple, overlapping epitopes that have been mapped within this site (Mateu *et al.*, 1989, 1990; Martínez *et al.*, 1991b). A critical replacement led to the generation of a subtype-specific epitope in an FMDV of a different subtype (Hernández *et al.*, 1992). Drastic effects of this sort have been described for poliovirus MAR mutants but not for field isolates (Minor *et al.*, 1983). In FMDV the impact of such critical replacements on antigenic specifity may be compensated by additional, non-critical replacements within site A (Mateu *et al.*, 1992). The molecular basis of such compensatory effects is not known. During long-term evolution of FMDV multiple antigenic subtypes and variants are frequently generated (Domingo *et al.*, 1990, 1992; Martínez *et al.*, 1992), whereas intratypic poliovirus variation is generally of limited epidemiological significance in spite of the frequent selection of antigenic variants in cell culture (Table 1) or in the gut (Minor, 1990). HAV shows also considerable conservation of antigenic sites (Lemon and Binn, 1983; Lemon and Ping, 1989) in spite of its genetic diversification (Lemon *et al.*, 1987; Stapleton and Lemon, 1987). Indeed, the nucleotide sequence differences among HAV strains and variants range between 0.3% to 8.5% of nucleotide sites (Ticehurst *et al.*, 1989) values comparable to those for poliovirus or FMDV isolated during disease episodes. In conclusion, picornaviruses share elevated MAR mutant frequencies at most antigenic sites examined (Table 1) but they vary in the extent of long-term antigenic diversification that they can attain (or have attained) in nature. This may be due to different tolerances of antigenic sites to amino acid substitutions, and to the effects of substitutions (and groups of substitutions) on antigenic specificity. Depending on the primary amino acid

sequences — which are very divergent among antigenic sites of different picornaviruses — predominance of additive or compensatory effects upon accumulation of amino acid substitutions may result in either substantial or limited antigenic diversification.

PERSISTENT INFECTIONS BY PICORNAVIRUSES AND THE ROLE OF VIRUS VARIATION

The prototype picornavirus capable of establishing a persistent infection in mice is Theiler's murine encephalomyelitis virus (TMEV) — isolated from the central nervous system (CNS) of a paralytic mouse (Theiler, 1934) —. Several variants of TMEV have been described and divided into virulent TMEV (strains GDVII, FA) and persistent TMEV (strains To, BeAn 8386, DA, WW) (reviews in Rodríguez *et al.*, 1987; Welsh *et al.*, 1989; Brahic *et al.*, 1991). Members of the virulent group replicate actively in neurons and cause acute fatal encephalitis. Members of the persistent group (also termed TO for Theiler's original) replicate less efficiently in neuronal cells, and cause an early acute poliomyelitis followed by a persistent infection of the white matter which leads to chronic, demyelinating disease. For this reason, TMEV serves as an adequate model for multiple sclerosis, a human neurological condition of suspected viral etiology with clinical and histological manifestations reminiscent of those caused by TMEV. Pairwise sequence comparisons between TMEV strains (Pevear *et al.*, 1988, and references therein) show about 90% identity at the nucleotide level and 95% at the amino acid level between virulent and persistent strains. Studies using chimaeric viruses constructed with genomic segments originating either from the persistent or the virulent group have led to the conclusion that the capsid polypeptides are the main determinants of efficient viral replication *in vivo*. A virus including the VP1 coding region of GDVII inserted into the genetic context of strain DA was unable to persist in spite of not killing mice. The reciprocal construct, — that is, the VP1 coding region of DA into the genome of GDVII — produced a virus equally attenuated but able to persist and cause demyelination. For the DA strain to regain nearly full virulence it was necessary to include the entire capsid - protein coding region of GDVII. Furthermore, a MAR mutant of TMEV DA with a replacement in protein VP1 showed decreased replication in the CNS and mild demyelination as compared with its parental DA (Zurbriggen *et al.*, 1991). These results (reviewed by Brahic *et al.*, 1991) illustrate how substitutions in the capsid proteins or their corresponding coding RNAs can affect viral multiplication and pathogenesis. The three-dimensional structure of the DA (Grant *et al.*, 1992) and Be An (Luo *et al.*, 1992) particles — both belonging to the persistent group — shows that most differences between the capsids of the two groups map on the surface of a star - shaped plateau, perhaps reflecting differences in receptor recognition or in antigenic behaviour. Genetic variations during persistence of TMEV *in vivo* and in L929 cells persistently infected with the DA strain were detected by Roos *et al.* (1982). In this *in vitro* cell culture system, interferon seems to play a role in persistence, but no extensive phenotypic variations in the virus nor the presence of defective-interfering particles could be shown. Persistence *in vivo* may be favored by the genetic make-up of the virus and host factors which may lead to inefficient clearing by the host immune response. The H-2D genomic region of mice (probably in combination with other genetic factors), contributes to the resistance to TMEV by controling the CTL response to the virus (Rodríguez and David, 1985; Brahic *et al.*, 1991).

HAV can cause subclinical infections mainly in children; chronic disease however, appears to be very rare (Hollinger and Ticehurst, 1990). In cell culture HAV often establishes persistent infections with little perturbation of the host cell metabolism (Provost and Hilleman, 1979; Gauss-Muller and Deinhardt, 1984; Vallbracht *et al.*, 1984). Variants cytolytic for some cell lines but able to persist in other cells have been described (Ticehurst *et al.*, 1989). As is the case also for other picornaviruses, some RNA stretches within the 5'-extracistronic region are prone to variation, and point mutations and deletions often accumulate upon passage of HAV in cell culture (Nüesch *et al.*, 1988; Ticehurst *et al.*, 1989). No detailed analysis of genetic or antigenic variations undergone by HAV during inapparent infections *in vivo* have been reported.

Perhaps the system in which genomic modifications during persistent infections have been examined in greater detail is FMDV. This aphthovirus is able to persist in the esophagous and throat of ruminants either as outcome of an acute infection with incomplete virus clearing or as a result of immunization with some live-attenuated vaccines. This *in vivo* carrier state for FMDV was first described by van Bekkum *et al.* (1959) and it is considered an important reservoir of FMDV in areas where the disease is enzootic (reviewed in Domingo *et al.*, 1990). Persistent infections in cattle established with plaque-purified FMDV have revealed that the virus underwent genetic and antigenic changes in the animals (Costa Giomi *et al.*, 1984, 1988; Gebauer *et al.*, 1988). The rate of accumulation of point substitutions in the VP1 coding region was 9.0×10^{-3} to 7.4×10^{-2} substitutions per nucleotide per year, one of the largest rates of evolution recorded among RNA viruses (Table 2). In contrast, values for several cellular genes are 10^{-8} to 10^{-9} substitutions per nucleotide per year. This million-fold difference was first emphasized by Holland *et al.* (1982) as a hallmark of RNA genetics. The higher rate of fixation of subtitutions in carrier animals as compared with an FMD epizootics (Table 2) can be interpreted as a result of the continuous replication of viral genomes in carriers and the alternation of periods of active replication with others without replication during extended disease episodes (Beck and Strohmaier, 1987; Martínez *et al.*, 1992). Some of the substitutions fixed in carrier cattle were antigenically relevant since they altered FMDV reactivity with MAbs (Gebauer *et al.*, 1988).

Several early experiments (Dinter *et al.*, 1959; Seibold *et al.*, 1964) suggested that FMDV could establish persistent infections in cell culture, but the limited stability of the carrier cells that were derived did not allow detailed studies. A persistent infection with plaque purified FMDV type C was readily established by growing the cloned BHK-21 cells which survived an initial cytolytic infection (de la Torre *et al.*, 1985). As the carrier cells were passaged, genetic and phenotypic changes were observed in the cells and in the resident virus. The virus became increasingly virulent for the parental BHK-21 cells (used to establish persistence) and the cells, in turn, became increasingly resistant to the virus (de la Torre *et al.*, 1988). Such cell resistance was not due to absence or limitation in host cell receptors since a block for virus replication was observed upon transfection with infectious FMDV RNA. Also, cell fusion experiments suggested that the resistance to FMDV was mediated by trans-acting inhibitors rather than by the absence of a factor needed for viral replication (de la Torre *et al.*, 1989a). Surprisingly, the evolution of the cell occurred via extremely heterogeneous cell populations in which many clones — freed of FMDV by treatment with ribavirin (de la Torre *et al.*, 1987) — differed in the degree of cellular transformation, and in resistance to FMDV (de la Torre *et al.*, 1989b). By repetitive nucleotide sequencing of the VP1 gene segment of nine viral clones from passage 59 of the carrier cells the virus population was found to be

Table 2. Rate of accumulation of mutations (evolution rates) of picornaviruses, as compared to immunodeficiency viruses and cellular genes

Virus	Genomic segment	Value[a]	Reference
Foot-and-mouth disease virus	VP1[b]	9.0×10^{-3} - 7.4×10^{-2}	Gebauer *et al.*, 1988
	VP1	6.5×10^{-3}	Villaverde *et al.*, 1991
	VP1	1.4×10^{-3}	Martínez *et al.*, 1992
Poliovirus	Average over genome	6.9×10^{-3} - 1.4×10^{-2}	Kew *et al.*, 1990
Human immunodeficiency virus	env	3.2×10^{-3} - 1.6×10^{-2}	Hahn *et al.*, 1986
	gag	3.7×10^{-4} - 1.8×10^{-3}	Hahn *et al.*, 1986
Simian immunodeficiency virus	gp120[c]	8.5×10^{-3}	Burns and Desrosiers, 1991
Cellular genes		10^{-8} - 10^{-9}	Britten, 1986; Weissmann and Weber, 1986

[a] Expressed as substitutions per nucleotide per year. Except where indicated (b, c) calculations are based on sequence comparisons of isolates during one or multiple epidemic outbreaks of the indicated virus.

[b] Cattle persistently infected with plaque-purified foot-and-mouth disease virus.

[c] Infections established with a molecular clone of simian immunodeficiency virus.

genetically heterogeneous at a level of 5×10^{-4} mutations per nucleotide screened (de la Torre *et al.*, 1988). Thus, persistently infected cell cultures consist of complex distributions of variant cells and viruses. It is likely that this dual heterogeneity endows the system with unique genetic flexibility allowing a coevolution of the cells and the resident virus which permits prolonged persistence.

By passage 58 of the carrier cells, the consensus nucleotide sequence of the resident FMDV had diverged from the parental cloned virus in about 0.3% of genomic nucleotides (de la Torre *et al.*, 1985), and by passage 100 such divergence had attained 1%, with point mutations fairly uniformly distributed along the 5' UTR and the P1 region (Díez *et al.*, 1990a; Escarmís *et al.*, 1992). One of the most striking features of the latter virus (termed R100) is the absence of a cysteine (in position 7 of VP3) involved in disulfide bridges which delimit the narrower part of a pore at the virion five-fold axis (Acharya *et al.*, 1989). This structurally critical cysteine is conserved in all aphthoviruses sequenced to date, and its absence in R100 suggests that even highly conserved residues can be substituted when virus replication takes place in a modified environment (in this case a persistent versus an acute infection) (Díez *et al.*, 1990a).

A second, unexpected feature was the presence in the genome of R100 of the longest polyribocytidylate (poly C) tract so far described, to our knowledge, for a picornavirus (Escarmís *et al.*, 1992). The poly C — whose function in picornaviruses is unknown — of R100 is about 420 residues long and it includes a few uridine residues interspersed at fairly regular intervals at the 3' end region of the homopolymeric tract. It is about 145 residues longer than the poly C present in the genome of the parental virus used to initiate persistence. In spite of its hypervirulence for BHK-21 cells, R100 is highly attenuated for cattle and mice (Díez *et al.*, 1990b). Since attenuated cardiovirus was obtained by engineering a short poly C tract (Duke *et al.*, 1990), the presence of a long homopolymeric tract in R100 was unexpected. It is clear that a long poly C is compatible with attenuation of FMDV, at least for cattle and mice.

It is suspected that persistence of poliovirus *in vivo* may be involved in some degenerative diseases of motor neurons of the brain and spinal cord—such as amyotrophic lateral sclerosis—which may occur many years after acute poliomyelitis (Campbell *et al.*, 1969; Mulder *et al.*, 1972; Dalakas, 1986; Sharief *et al.*, 1991). Early evidence of persistence of poliovirus in cell cultures was provided by Ackermann and Kurtz (1955), and by Vogt and Dulbecco (1958). In the latter study morfologically altered HeLa cells with increased resistance to poliovirus were described. During persistence in lymphoblastoid cell lines, *ts* mutants were identified (Carp, 1981). By cotransfecting poliovirus genomic RNA with a subgenomic RNA —with most of the capsid-coding region deleted— Kaplan *et al.* (1989) established HeLa cell lines persistently infected with poliovirus, which evolved to produce cells highly resistant to poliovirus. In some cells resistance was due to absence of the cellular receptor, and in other cell clones there was an intracellular block to poliovirus replication.

A neuroblastoma cell line persistently infected with poliovirus has been studied by Colbère-Garapin and her colleagues (Colbère-Garapin *et al.*, 1989; Pelletier *et al.*, 1991; Borzakian *et al.*, 1992). Variant polioviruses with altered host range were isolated from these neuroblastoma cells that could establish persistence in nonneuronal cells. In particular, such virus variants, but not wild type or attenuated polioviruses, persisted in HEp-2c cells. Borzakian *et al.* (1992) have shown that establishment of persistence in HEp-2c depends on the virus strain, whereas maintenance of persistence involved selection of variant cells in which multiplication of poliovirus was restricted, a situation parallel to that found during persistence of FMDV in cell culture (see above). A reduced expression of the poliovirus receptor was seen in some cell clones but not in others, and cell fusion experiments suggested that the restriction to poliovirus infection was a dominant trait (Borzakian *et al.*, 1992). These authors suggested the existence of an equilibrium between an abortive and a lytic infectious cycle subjected to alteration by environmental changes. This is a very attractive model — in line with the dynamic nature of picornaviral populations — which deserves further attention when analyzing persistent infections by other highly variable RNA viruses.

Gibson and Righthand (1985) established cloned human WISH cells persistently infected with echovirus 6, a system in which the presence of highly defective virus populations appears to play an important role. In particular, during persistence VPO was present suggesting a defect in its processing to yield VP2 and VP4 in viruses unable to attach to susceptible cells (Righthand and Blackburn, 1989). A persistent infection of HeLa cells with a *ts* rhinovirus type 2 has also been described, in which cell subpopulations resistant to homologous rhinovirus were selected (Gercel *et al.*, 1985).

Thus, virtually for any picornavirus persistent infections have been described *in vivo* or in cell culture, and in many cases virus as well as cell variations appear as important elements in sustaining this type of infection.

PHYLOGENETIC RELATIONHIPS AMONG PICORNAVIRUSES. VIRUSES AS CLOUDS IN A SEQUENCE SPACE

Picornaviruses were initially classified on the basis of biological and physical properties. Rueckert (1990) and Stanway (1990) divided the family Picornaviridae into four genera: *enteroviruses, cardioviruses, rhinoviruses* and *aphthoviruses*. Nucleotide and amino acid sequence comparisons revealed important problems in early classifications. For example, the enteroviruses included not only the related polioviruses and coxsackieviruses, but also hepatitis A virus which shows less than 15% amino acid identity with poliovirus in the capsid proteins. Palmenberg (1989) carried out the first systematic, comparative alignment of picornaviral sequences, and established phylogenetic relationships among picornaviruses. Based on the protein alignments, the corresponding nucleotide sequences were analyzed by application of a maximum parsimony algorithm. For P1 sequences, the percent identities [defined as one hundred times the number of identical nucleotides in a pairwise alignment, divided by the length of the compared sequences (excluding gaps)] ranged from greater than 70% (for different serotypes of the same picornavirus) to less than 30% (for HAV as compared with other picornaviruses). It must be noted however that overall sequence identities averaged over an entire genome (or a specific gene) may be quite at variance with identities for defined genomic segments or the encoded proteins. For example, nucleotide sequence identity for the polymerase (3D) gene is 65% between HRV-14 and poliovirus, and 55% between HRV-14 and HRV-2 (Skern *et al.*, 1984). This difference fades when the capsid protein VP1-coding region is compared, the identities being in the range of 42% to 44% (Andries *et al.*, 1990). Based on comparisons of the P1 genome segment, Palmenberg (1989) proposed four main groups of picornaviruses: I (FMDV), II (Mengo and TMEV), III (HAV), and IV (poliovirus, rhinovirus, coxsackievirus, and bovine enterovirus). Major departures from previous classifications are the recognition that hepatitis A virus constitute a distinct group and that the Theiler's murine encephalomyelitis viruses are related to the cardioviruses. More recently, the ICTV Study Group on picornaviruses has proposed a further division of the Picornaviridae family into five genera: *enterovirus, rhinovirus, hepatovirus, cardiovirus* and *aphthovirus* (Minor *et al.*, submitted 1993). A number of viruses (equine rhinoviruses, Drosophila C virus, etc.) have not been assigned to any genus.

Phylogenetic relationships have also been derived for different serological subtypes and for different isolates within a picornavirus genus. They include, among others, studies with the three serotypes of poliovirus (Rico-Hesse *et al.*, 1987; Kew *et al.*, 1990; Kinnunen *et al.*, 1992), the three European serotypes of FMDV (Dopazo *et al.*, 1988) and isolates from different subtypes of FMDV type C (Martínez *et al.*, 1992). Extremely ramified trees are generally produced reflecting that genetically heterogeneous virus lineages cocirculate during a disease outbreak and that even a single infected individual harbors mixtures of variants (Domingo *et al.*, 1980; Sobrino *et al.*, 1986; Gebauer *et al.*, 1988; Villaverde *et al.*, 1988, 1991; Kinnunen *et al.*, 1991). Viral heterogeneity within infected organisms has generally been disregarded in epidemiological models of virus spread and

disease control, and yet it is one of the most relevant parameters to account for vaccine failures and incomplete protection. Furthermore, such heterogeneity is an expected consequence of the quasispecies structure of RNA virus population, and it may define a transient molecular clock when frequent host-to-host transmission occur (Villaverde *et al.*, 1991). A recent model for the development of AIDS places emphasis on antigenic diversity of HIV-1 as the cause rather than a consequence of immunodeficiency (Nowak *et al.*, 1991). Above an antigenic diversity threshold the virus induces, according to this model, a collapse of the host lymphocyte population. For poliovirus type 3, the extent of genetic variation in one infected individual was at times as significant as variation among independent isolates (Kinnunen *et al.*, 1991).

Only in a few cases, the degree of significance of the tree nodes and the standard deviations of branch lengths have been assessed in phylogenetic trees by application of statistical procedures. This practice is particularly important when attempting to define relationships among isolates of a highly variable RNA virus. Martínez *et al.* (1992) applied the bootstrap resampling method (Felsestein, 1985) to assess the significance of a tree obtained by the least-squares method, and which related the VP1-coding region of type C FMDVs isolated in Europe, South America and The Philippines. Six highly significant lineages — one of them corresponding to a virus group which is now probably extinct — were defined, but several sublines were shown to be of questionable significance upon application of the bootstrap resampling method. Obviously, such statistical treatments do not evaluate or correct for problems derived from the sampling of virus genomes which are entered into the tree derivations, and which are due to the fact that the available sequences represent a minority subset of the total population. Interestingly, for FMDV type C, quantification of those mutations that led to an amino acid replacement along well established lineages showed that there was no significant accumulation of amino acid substitutions with time. In contrast, net accumulation of silent replacements at a fairly constant rate was observed. Antigenic variation of FMDV serotype C over six decades was essentially due to combinations among a very limited number (often two) of amino acids per heterogeneous position (Martínez *et al.*, 1992). It will be interesting to extend the type of study of Martínez *et al.* (1992) to other picornaviruses for which representative evolutionary lineages with isolates extending over many decades may be available.

Constraints for variation at the RNA level are also suggested by a number of comparisons of the secondary structure at the extracistronic regions. The internal ribosome entry site (IRES) has a defined secondary structure very similar for cardioviruses and aphtoviruses, but distinct from the corresponding structure for enteroviruses and rhinoviruses (Pilipenko *et al.*, 1989a, b). Perturbation of some key domains of the IRES results in decreased initiation of translation, and natural variations in primary structure often consist of groups of compensatory mutations to ensure conservation of secondary (and probably higher order) structure. A tight secondary structure is also predicted for 5'UTR stretches other than the IRES such as the S fragment (at the 5' side of the poly C) of FMDV (Clarke *et al.*, 1987; Escarmís *et al.*, 1992). In FMDV, block deletions of around 40 or 80 residues are often observed even when comparing viruses of the same serotype. Such deletions (or insertions) correspond to the loss (or gain) of one or two predicted pseudoknot structures (Clarke *et al.*, 1987; Escarmís *et al.*, 1992). In other systems pseudoknots have been implicated in regulation of translation or RNA splicing, but their role in FMDV is not understood. Deletions of this type may be frequent because they involve the complete loss

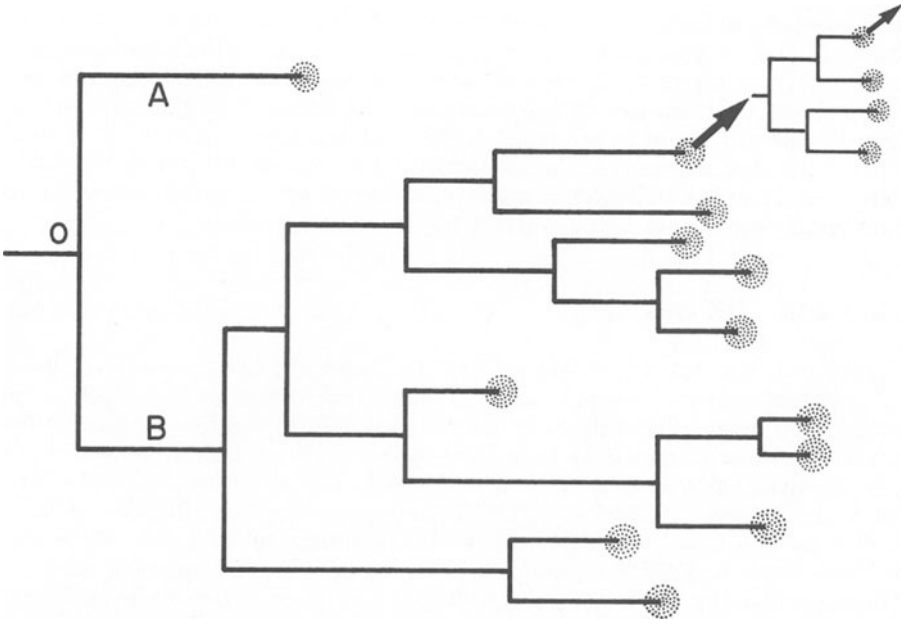

Fig. 2. A schematic phylogenetic tree of the type that has been derived for picornaviruses and other RNA viruses. The tree has been rooted on a theoretical origin O. From O two main branches A and B originate. B gives rise to additional branchings, whereas A represents an evolutionary dead-end. At the tip of each branch a cloud represents the quasispecies distributions of genomes corresponding to each isolate. The arrow at the top right suggests that for a single isolate multiplying in a few hosts, an additional branching and further diversification would be found. The process would repeat in a single host or even in different cells of an infected organ, with decreasing genetic distances. In spite of each quasispecies being restricted to a cloud, diversification in sequence space may be rapid, and large evolutionary jumps may be mediated by homologous and heterologous recombination with optimization by point mutations (see text).

of a non-essential RNA stretch. It has been suggested that RNA insertions or deletions in regulatory regions may affect biological features of picorna viruses (Stanway *et al.*, 1984).

Primary sequence similarity may extend to other virus groups particularly in non-structural proteins such as viral polymerases, helicases, or proteases (Franssen *et al.*, 1984; Haseloff *et al.*, 1984; Kamer and Argos, 1984; Argos *et al.*, 1984; Argos, 1988; Gorbalenya and Koonin, 1989; Koonin *et al.*, 1991; Bruenn, 1991; review in Zimmern, 1988). Such similarities often correspond to catalytic domains of viral enzymes and they could reflect either common ancestry or convergent evolution of viral quasispecies required to use and interact with cellular components. Tenuous relationships can also been identified among structural proteins of viruses as different as FMDV and Sindbis (Fuller and Argos, 1987) or Mengo and hepatitis B (Argos and Fuller, 1988).

RNA viruses, as other more complex life forms, seem to exist under two apparently contradictory forces: variation to ensure adaptability and conservation to ensure functionality. Fig. 2 shows an imaginary phylogenetic tree in which virus isolates —the tips of the branches— are depicted as spherical clouds. Each cloud is the domain of genetic distances in which a given virus with a genetic make-up is allowed to exist. It cannot expand beyond those limits without loosing functionality. The need of certain biochemical functions dictate common features (primary sequences or higher order structures) among members of distant clouds. The tree could

represent relationships among different picornaviruses or among representatives of one picornavirus. The arrow in Fig. 2 suggests that if viruses within a narrower space-time coordinates are examined (such as during a disease outbreak, or even within an infected individual) multiple lineages would be found albeit related by shorter genetic distances (branch lengths). Thus, quasispecies is an important step in the molecular evolution of RNA viruses since it is the raw material on which positive selection and random sampling events can act (Domingo and Holland, 1993).

CONCLUDING REMARKS

The field of RNA virus evolution has progressed dramatically during the last decade. Many biological implications of RNA virus variation are becoming apparent. The design of new antiviral compounds and synthetic vaccines and their successful use greatly depend on an understanding of the dynamics of viral populations in infected hosts. The very successful antiviral vaccines (poliomyelitis, measles, mumps) may be the exception rather than the rule, and mankind may have to face other epidemics like AIDS (Temin, 1989; Holland *et al.*, 1992).

Quasispecies is not a mere description of complexity at the population level. Quasispecies represents above all the recognition of the dynamics of mutant generation and competition among variants, a process that cannot be dissociated from genome replication. This creates an important operational difference from mutant generation (and their fixation) in higher organisms. For this reason, quasispecies is conceptually distinct from genetic polymorphisms (Domingo and Holland, 1993). Picornaviruses which have been instrumental in many developments of molecular biology may be so also in the oncoming studies on molecular evolution of RNA viruses.

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