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Quasispecies and its impact on viral hepatitis

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Abstract

Quasispecies dynamics mediates adaptability of RNA viruses through a number of mechanisms reviewed in the present article, with emphasis on the medical implications for the hepatitis viruses. We discuss replicative and non-replicative molecular mechanisms of genome variation, modulating effects of mutant spectra, and several modes of viral evolution that can affect viral pathogenesis. Relevant evolutionary events include the generation of minority virus variants with altered functional properties, and alterations of mutant spectrum complexity that can affect disease progression or response to treatment. The widespread occurrence of resistance to antiviral drugs encourages new strategies to control hepatic viral disease such as combination therapies and lethal mutagenesis. In particular, ribavirin may be exerting in some cases its antiviral activity with participation of its mutagenic action. Despite many unanswered questions, here we document that quasispecies dynamics has provided an interpretation of the adaptability of the hepatitis viruses, with features conceptually similar to those observed with other RNA viruses, a reflection of the common underlying Darwinian principles.

Keywords: HAV; HBV; HCV; HDV; Interferon; Lamivudine; Viral load; Liver damage; Mutation; Recombination; Editing; Persistence; Immune escape; Lethal mutagenesis; Error catastrophe

1. General introduction: molecular basis and biological significance of viral genome variation

Genetic and phenotypic variation of viruses are intimately associated with mechanisms of viral pathogenesis. Genetic, biochemical, immunological, enzymological, and evolution studies applied to many different viruses have settled the conclusion that viruses which have RNA either as the genetic material or as a replicative intermediate share high mutation rates and quasispecies dynamics. Genetic and biochemical methods have contributed to measurements of mutation rates and frequencies, and have quantified differences in activity among variant forms of the same viral gene products. Immunology has defined phenotypic variation for relevant traits in virus–host interactions such as antigenic variation and the participation of virus escape from antibodies or cytotoxic T lymphocytes (CTLs) in viral persistence and in disease progression. Enzymological studies with virus-coded polymerases are providing insights into the molecular basis of error-prone replication. Studies on evolution have shown that high error rates are not a mere consequence of rapid RNA genome replication but have an adaptive value, are at the origin of quasispecies dynamics, and bear on our understanding of viral disease [for a recent overview of viral quasispecies, see the different chapters of (Domingo, 2006) and (Table 1)].

In this article we summarize mechanisms of viral genome variation, and quasispecies dynamics, with special reference to the hepatitis viruses, which display high mutation rates and quasispecies dynamics in agreement with their using RNA as genetic material or as a replicative intermediate. They include hepatitis A, C, D (or delta agent, a subviral pathogen that requires hepatitis B virus as helper virus), E and G viruses (the original GB virus isolated from tamarin monkeys that had been inoculated with a human serum; and later, clones GBV-A and GBV-B from passaged tamarin serum, and GBV-C or hepatitis G virus from human serum). These viruses are abbreviated as HAV, HCV, HDV, HEV, GBV-A, GBV-B, GBV-C or HGV, respectively. They replicate with the scheme RNA → RNA → RNA, with no DNA intermediate known. Hepatitis B virus (HBV) replicates with the scheme DNA → RNA → DNA, involving a pregenomic RNA as an obligatory intermediate in its replication (for a review on replication of the hepatitis viruses, see other articles

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Table 1
Some features of viral quasispecies

- A virus population is a spectrum of mutants.
- Mutant spectra may include phenotypic variants that contribute to virus adaptability. Subpopulations bearing biologically relevant, distinct mutations may coexist in the same infected individual host.
- Components of mutant spectra show different fitness levels.
- Fitness variations occur in viral populations depending on the passage regime (population size) and environmental changes. Fitness gradients guide movements in sequence space.
- In quasispecies dynamics, deleterious, neutral, advantageous (per se or directly compensatory) mutations can occur in components of the mutant spectrum. Their detrimental or advantageous nature is dependent on the environment in which replication takes place and on the sequence context (both at the level of the individual genome and the level of the surrounding mutant spectrum).
- A broad mutant spectrum can contribute to virus adaptability. Too narrow or too broad a spectrum may impair adaptability.
- Mutation rates above a critical error threshold result in loss of infectivity.
- The mutant spectrum can modulate virus behavior through positive interactions (complementation) and negative interactions (interference).
- Dominance of negative interactions can contribute to viral extinction.
- A high fitness mutant can be suppressed by a mutant spectrum of inferior fitness.
- A low fitness virus may be more resistant to further mutation than a higher fitness counterpart when the former lies on a flat fitness surface and the latter on a sharp fitness peak.

Based in (Domingo, 2006; Domingo, 2007; Eigen, 2002; Eigen and Biebricher, 1988) and references quoted in these articles.

in this volume). GBV-A, GBV-B and GBV-C are not universally regarded as active agents in hepatic disease, and they are covered only as interesting model systems in some evolutionary studies. Other agents occasionally associated with hepatic diseases (i.e. some enteroviruses, herpesviruses, etc.) are not included in this article.

1.1. Mutation and repair

The biochemical basis of error-prone replication is that most RNA-dependent RNA polymerases (RdRp) and RNA-dependent DNA polymerases (RdDp) (reverse transcriptases, RT) that have been characterized to date, lack a domain corresponding to a 3′ to 5′ exonucleolytic activity capable of excising incorrectly incorporated nucleotides – which produce a mismatch at the growing 3′-end – to allow incorporation of the correct nucleotide, base-paired with the template. This 3′ to 5′ exonucleolytic activity is termed proofreading-repair activity, and its absence in most RdRp and RdDp has been documented by structural and biochemical studies (Ferrer-Orta et al., 2006; Friedberg et al., 2006; Steinhauer et al., 1992; Steitz, 1999). Recently, a domain corresponding to a 3′ to 5′ exonuclease has been identified and characterized in some coronaviruses (Exo N in nsp14 of the SARS coronavirus). This exonucleolytic function plays an essential role in coronavirus RNA replication, but its possible contribution to RNA copying fidelity has not been demonstrated yet (Minskaia et al., 2006). Copying fidelity by RdRp and RdDp is determined by the relative rates of incorporation of the correct versus an incorrect nucleotide [recent studies with viral enzymes reviewed in (Arnold et al., 2005; Menéndez-Arias, 2002a)]. Average error rates for RNA viruses are in the range of 10^{-3} to 10^{-5} misincorporations per nucleotide copied (Batschelet et al., 1976; Drake and Holland, 1999). Mutant viral polymerases displaying either increased or decreased copying fidelity due to specific amino acid replacements have been characterized (Arnold et al., 2005; Mansky et al., 2003; Menéndez-Arias, 2002a; Pfeiffer and Kirkegaard, 2003; Vignuzzi et al., 2006). A consequence is that error-prone replication can itself affect the catalytic properties of viral polymerases, altering their error rates that can be subjected to selection (Arnold et al., 1999).

Most, but not all, cellular DNA-dependent DNA polymerases (DdDp), as well as DdDp encoded by some complex DNA viruses – such as poxviruses, herpesviruses and adenoviruses – include a proofreading-repair activity. The latter, together with a number of proteins present in the DNA replication complexes, can decrease the error rate to about 10^{-7} substitutions per nucleotide during cellular DNA synthesis (Friedberg et al., 2006). In addition to proofreading-repair, a number of post-replicative mismatch repair and DNA damage signaling pathways, can operate on double-stranded DNA, reducing the overall error rate to about 10^{-10} misincorporations per nucleotide copied during standard DNA replication (Friedberg et al., 2006). Post-replicative repair activities are either inactive or very inefficient on double-stranded RNA or on DNA–RNA hybrids found in replicative intermediates of riboviruses, retroviruses or hepadnaviruses.

A relevant, largely unanswered question, is whether proteins involved in repair functions are accessible to viral DNA genomes during their replication. Depending on the intracellular site of DNA genome replication, as well as the stoichiometry of available repair proteins in relation to the number of viral DNA genomes in a replication factory, postreplicative repair functions may be inefficient also on viral DNA. A possible limitation of postreplicative correction, together with the type of molecular mechanism involved in DNA replication – predominance of single-stranded or double-stranded DNA forms –, and whether a low fidelity or high fidelity DdDp catalyses nucleotide incorporation, will determine the error rate levels during viral DNA replication [reviewed in (Domingo, 2007)]. There is evidence that some DNA viruses with limited genetic complexity share with RNA viruses great genetic heterogeneity and potential for rapid adaptation, for example the animal parvoviruses (López-Bueno et al., 2003; López-Bueno et al., 2006) and the

plant geminiviruses (Isnard et al., 1998). To what extent simple and complex DNA viruses can exploit localized or generalized high mutation rates, and to what extent genetic variation affects their pathogenicity, are important questions requiring additional research.

1.2. Non-replicative mutation

There are other sources of mutation in viral genomes. Some cellular editing enzymes, such as the ADAR or APOBEC families of nucleic acid deaminases constitute part of the innate defense mechanisms against viral infections (Sheehy et al., 2002). The APOBEC cytidine deaminases act on single stranded DNA and lead to G → A and C → U hypermutation. The ADAR adenosine deaminases act on double stranded viral RNA and lead to A → G and U → C hypermutation [reviews in (Chiu and Greene, 2006; Valente and Nishikura, 2005)]. These cellular deaminase activities are involved in diversification of immunoglobulin genes (for example protein AID, a member of the APOBEC family) and in mRNA or tRNA editing, thus contributing to generate RNA and protein diversity (Schaub and Keller, 2002). In the face of a viral infection, they are part of the innate antiviral host response. Some viral proteins have evolved to counteract the hypermutagenic activity of cellular deaminases (Vif of HIV-1 binds APOBEC-3G and prevents the encapsidation of the deaminase into viral particles and the subsequent hypermutagenic activity on minus strand DNA).

Several APOBEC-3 proteins can associate with the capsid of HBV and mediate hypermutagenesis of the viral genome. Application of a differential DNA denaturation PCR technique (Suspene et al., 2005b) to HBV genomes rescued after co-electroporation of cells with DNA expressing APOBEC proteins, revealed genomes with G → A hypermutation mediated by APOBEC -3B, -3C, -3F and -3G, and with C → T hypermutation mediated by APOBEC -3B, -3F and -3G, resulting in inhibition of HBV replication (Bonvin et al., 2006; Suspene et al., 2005a). Hypermutated genomes have been detected in some (but not all) sera from patients with acute or chronic HBV infection (Noguchi et al., 2005; Suspene et al., 2005a; Suspene et al., 2005b).

With some exceptions, hypermutated genomes are generally defective, and hypermutation has been regarded as an unregulated defense response that uses enzymes which play key physiological roles in the host cells. However, the constitutively expressed ADAR 1-S and the interferon-α-induced ADAR 1-L contribute to the editing of a specific nucleotide of the HDV antigenome RNA. This editing event allows the expression of delta antigen S and L from a single open-reading frame, and it is essential to maintain HDV infection (Hartwig et al., 2006; Sato et al., 2001). Secondary structures on the target RNA appear to play an important role in directing the editing activity to some specific RNA residues (Dawson et al., 2004).

Mutation may also result from chemical damage to viral genomes (deamination, depurination, depyrimidination, reactions with oxygen radicals), direct and indirect effects of ionizing radiation, photochemical reactions, and so on (Friedberg et al., 2006; Naegeli, 1997). Together with the mutational activities of cellular deaminases, these different mechanisms are sometimes referred to as non-replicative mechanisms of viral genome mutation. The available evidence supports error-prone replication as the major source of mutations in viral genomes, including the hepatitis viruses.

1.3. Replicative and non-replicative recombination

A second, widespread mechanism of viral genome variation is recombination. Several forms of molecular recombination have been recognized: homologous, nonhomologous, replicative, and non-replicative [reviewed in (Chetverin et al., 2005; Domingo, 2007; Gmyl et al., 2003; Nagy and Simon, 1997)]. In homologous recombination there is extensive nucleotide sequence identity between the two parental genomes around the cross-over site. Non-homologous recombination is not associated with substantial nucleotide sequence identity. Replicative recombination requires viral genome replication to occur. Non-replicative recombination has been discovered with some RNA viruses, upon cotransfection of cells with viral RNA fragments which cannot replicate by themselves (Gallei et al., 2004; Gmyl et al., 2003). A common form of homologous, replicative recombination that probably operates in DNA and RNA viruses is copy choice or template switching; it involves detachment from a template of the polymerase complex with a nascent product, and continuation of the copying process at the same position of another template molecule.

Recombination can be intermolecular, involving two different template molecules – such as in the copy choice mechanism – or intramolecular, leading to internal deletions and synthesis of defective genomes including the defective interfering (DI) RNAs that can be encapsidated into DI particles (Roux et al., 1991). Defective genomes are increasingly recognized as active participants in the evolutionary behaviour of viral populations [reviewed in (Domingo, 2007)]. Different viruses exploit recombination to different extents, either as an exploratory activity to produce new genomic combinations, or as a means to rescue viable genomes from parental genomes with debilitating mutations. Host proteins such as endoribonucleases and exoribonucleases may modulate the recombination frequencies inherent to each polymerization machinery (Cheng et al., 2006; Serviene et al., 2005). Recombination has been characterized in several hepatitis viruses (Bollyky et al., 1996; Costa-Mattioli et al., 2003; Cristina and Colina, 2006; Gauss-Muller and Kusov, 2002; Kalinina et al., 2002; Noppornpanth et al., 2006; Wang and Chao, 2005; Yang et al., 2006), therefore adding recombination to mutation as a mechanism of genome variation in this important group of pathogens. For viruses with segmented genomes (which is not the case for the hepatitis viruses described so far), segment reassortment provides an additional mechanism of genetic and phenotypic variation.

Mutation universally, and recombination in many cases, constitute the basic variation mechanisms that precede intra-host and inter-host evolutionary events, also in the case of the hepatitis viruses.

2. Mutation rates, mutation frequencies, and rates of viral evolution

An understanding of quasispecies dynamics and how it affects intra-host adaptability of RNA viruses in general, and the hepatitis viruses in particular, requires that mutation rates be distinguished from mutation frequencies. As stated by Mayr (1994), the outcome of selection is a two step process: mutation and selection, which can be expressed as *mutation rate* and *mutation frequency*. The term *mutation rate* quantifies the biochemical event of misincorporation (incorporation of one or any incorrect nucleotide) during copying of a viral nucleic acid template. A mutation rate is independent of the fate – increase or decrease in proportion – of the mutated progeny produced, relative to the non-mutated version. It generally refers to a specific nucleotide site, and it may describe a specific misincorporation (one type of incorrect nucleotide) or any misincorporation (any type of incorrect nucleotide) at that site. A mutation rate can be averaged over multiple sites, and expressed as number of substitutions per nucleotide copied.

In contrast to mutation rate, a *mutation frequency* is a quantification of the proportion of mutant genomes in a viral genome population. Accordingly, it is expressed as substitutions per nucleotide. Mutation frequency may refer to the proportion of a specific mutant type (often termed the *mutant frequency*) or to any type of mutant (termed the *mutation frequency*). Mutation and mutant frequencies are influenced by the capacity of any mutant to produce progeny, relative to the capacity of the non-mutated class or of other mutants produced in the same replicative ensemble. A specific mutation may be generated at a high rate (because of biochemical reasons such as the sequence context of the template) but may be found at low frequency because it inflicts a fitness cost (imposes a selective disadvantage) upon the genome(s) harbouring it. Yet, of note regarding viral pathogenesis, a mutation may inflict a fitness cost to a virus while replicating in an organ of an infected host but not while replicating in another organ (or tissue, or cell type). The advantageous, neutral, deleterious and lethal nature of any mutation and, thus, the conservation and variation of genomic nucleotides, are relative to a given physical and biological environment (Table 1).

A striking and illustrative example, extensively studied by Escarmís and colleagues is the frequent generation of an internal oligoadenylate tract in the genome of the picornavirus foot-and-mouth disease virus (FMDV) (which belongs to the same family as HAV) when the virus is subjected to plaque-to-plaque transfers, despite entailing a significant fitness cost for the virus. The reason why the FMDV RdRp appears to be biochemically "instructed" to generate an internal oligoadenylate (not described in any natural isolate of an animal virus, but found is some plant viruses) is probably a propensity towards slippage mutagenesis (also termed polymerase "stuttering") at four successive adenylated residues (or their complementary uridylate residues in the minus strand RNA) that precede the second functional AUG codon. Current evidence suggests that during plaque-to-plaque transfers, negative selection (elimination of unfit genomes) is less intense than during large population passages which involve a more pronounced competition among genomes. In the course of repeated plaque-to-plaque transfers, biochemical instruction prevails over selection [for a review of this aspect of RNA genetics, see (Escarmís et al., 2006)].

A *mutation frequency* is a population number which may be actually close to the corresponding *mutation rate* when the replication capacity of the mutant generated does not differ substantially from that of the ensemble, and the number of rounds of replication between the occurrence of the mutation and its detection in a population of genomes is limited. The technical details and calculations used to derive a mutation rate or frequency must be examined with care. Some published values used to claim low mutation rates for some RNA viruses were in reality based on an extrapolation of the frequency of a subset of advantageous or neutral mutations (for example, scored in individual viral plaques), excluding deleterious mutations which are often the most abundant during virus replication. In examining claims of unusually low mutation rates and high polymerase copying fidelity for an RNA virus, based on a mutation frequency, the reader should consider that an outstanding low value ($<10^{-6}$ substitutions per nucleotide) may mean either: (a) a selective disadvantage of the genome that harbors the mutation on which the calculation is based, or (b) the requirement of two or more mutations when the alteration of a phenotypic trait is measured. (The frequency of occurrence of n mutations in the same genome is obtained by multiplying the frequency of occurrence of each individual mutation; thus, if the average frequency for one mutation is 10^{-4} a triple mutant is expected at a frequency of about 10^{-12}), (c) a general or site-specific high polymerase fidelity, which should arise a very definite interest with regard to its molecular basis (Holland et al., 1992). Conversely, a high mutation frequency for a DNA virus whose replication is catalyzed by a high fidelity DdDp main mean that either (a) repair activities are not functional, or (b) the mutant overgrows the wild type (due to a selective constraint, fitness advantage, or other) prior to the measurement of its frequency. Some mathematical treatments to calculate mutation rates taking into account reversion of a low fitness mutant and its competition with wild type have been published, most of them derived from the original equations proposed by (Batschelet et al., 1976).

These are not just academic considerations (as important as the latter are), but relevant concepts to appreciate to its full extent the biological relevance of high mutation rates and quasispecies dynamics as a feature of RNA viruses in general and of the hepatitis viruses in particular, as they replicate in their hosts and contribute to disease. Despite difficulties, limitations, and nuances in deriving and interpreting mutation rates and frequencies, independent genetic and biochemical methods support mutation frequencies for RNA viruses in the range of 10^{-3} to 10^{-5} substitutions per nucleotide. The average of a total of 19 values for riboviruses, retroviruses and hepadnaviruses, obtained in 15 different laboratories was $(2.6 \pm 6.6) \times 10^{-4}$ (Domingo, 2007). A much broader range is obtained for eukaryotic and prokaryotic DNA viruses, with values around 10^{-8} for some complex bacteriophages and about 10^{-4} for parvoviruses (Domingo, 2007; López-Bueno et al., 2006).

Another appropriate distinction must be made between mutation rate and the *rate of evolution*, also termed the *rate of*

accumulation (or *fixation*) of mutations. The latter is generally calculated by comparing the consensus sequence of sequential viral isolates, and it includes a time factor, so that it is commonly expressed as substitutions per nucleotide and year. A compilation of 56 different measurements for RNA viruses show that most values fall in the range of 10^{-3} to 10^{-4} substitutions per nucleotide and year (s/n/y), with a few outstandingly high (10^{-1}–10^{-2} s/n/y) or low (10^{-7}–10^{-8} s/n/y) values (Domingo, 2007). The rates of evolution estimated for HCV are in the range of 8×10^{-4} to 2×10^{-3} s/n/y (Abe et al., 1992; Lu et al., 2001; Ogata et al., 1991; Okamoto et al., 1992; Smith et al., 1997), and those for HGV are of 3×10^{-4} to 2×10^{-3} s/n/y (Giménez-Barcons et al., 1998; Nakao et al., 1997). A broader range of 6×10^{-4} to 3×10^{-2} s/n/y has been determined for the delta antigen (Imazeki et al., 1990; Lee et al., 1992). These values for HDV show that a defective virus can evolve at rates comparable to non-defective, standard viruses, as documented also by the rapid evolution of other subviral replicons (Domingo et al., 2001).

Unfortunately, some times the rate of evolution is referred to as the mutation rate. This confusing terminology is partly derived from classical population genetics which did not consider the key distinction between the mutation rate in its biochemical meaning and the more readily observable rate of evolution. Overlooking such a distinction obscures important properties of viral populations, particularly the implications of a dynamic genetic heterogeneity in viral disease, irrespective of the intra-host or inter-host rate of evolution as measured with consensus genomic sequences.

Selection and random drift acting on essentially heterogeneous populations, pushed by transmission events, lead to diversification of "consensus" sequences that have been grouped into types, subtypes and variants. Because diversification is a dynamic process for any epidemiologically active virus, the number of types and subtypes (either genetic or antigenic) increases with time. Currently, HCV isolates of a different genotype correspond to those showing an average nucleotide sequence similarity in their genomes in the range of 65–69%; those of a different subtype show a nucleotide sequence similarity of 77–80%, and 91–99.9% for components of the mutant spectrum of the same quasispecies (Clementi, 2003). These boundaries are rather arbitrary, may require periodic updating, and are not applicable to other viruses. In particular, a viral quasispecies may produce divergent outliers in their exploration of sequence space. The extent of divergence varies for different genomic regions. In HCV, the glycoprotein E1 – and E2 – coding regions may differ in up to 50% of nucleotides among independent isolates, and even more when some protein subdomains are analyzed (Clementi, 2003).

Procedures to determine the mutant spectrum complexity of viral quasispecies have been previously reviewed (Domingo et al., 2006). The three most common parameters calculated are: (i) Mutation frequency, defined as the proportion of mutant nucleotides in a genome distribution, relative to the consensus sequence. (ii) Shannon entropy, defined as the proportion of different genomes in a mutant distribution. (iii) Genetic distance (also termed Hamming distance), defined as the number of mutations that distinguish any two sequences of the distribution; the average of all possible pairs reflects the genetic complexity. These three parameters can be calculated for nucleotide sequences derived from molecular clones or biological clones (when the virus produces plaques or foci in cell culture). Directions for the calculations, as well as precautions to avoid biases in the values of complexity, have been discussed (Domingo et al., 2006).

The methodology originally used to define and analyse the quasispecies structure of RNA genetic elements was the direct sampling of nucleotide sequences by RNA fingerprinting. In the case of hepatitis viruses, and many animal viruses, the genomic RNA cannot be examined directly because its concentration in serum and other biological samples is often very low.

Alternative, rapid sampling procedures have been used to compare relative genetic heterogeneities of viral populations. These include restriction enzyme site polymorphisms, heteroduplex mobility assays, ribonuclease A mismatch cleavage, oligonuclotide mapping, and microarray techniques. These independent methods have confirmed the extensive heterogeneity of RNA virus populations, particularly in populations of HCV in infected patients.

3. Mutation rate and the adaptive value of mutant spectrum complexity

When the first error rates and frequencies for RNA viruses were obtained, they were met with skepticism due to their high values [for a historical account, see (Holland, 2006)]. Although the generality of high mutation rates is widely accepted today, some have argued that due to the detrimental nature of most mutations, the adaptive value of the RNA virus extreme mutation rate has to be carefully reconsidered, suggesting the existence of a trade-off between replication efficiency and copying fidelity. In their view, high mutation rates are a consequence of rapid RNA replication, and an increase in copying fidelity would come at a cost, resulting in a lower replication rate. Studies with a high fidelity mutant of poliovirus (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006) have documented that the mutant replicated at a slightly lower rate than wild type virus in cell culture, but that it had a strong selective disadvantage regarding invasion of the brain of susceptible mice. Control experiments showed that the impediment to cause neuropathology was due to the limited complexity of the mutant spectrum as the virus replicated in mice. Thus, in this case, high mutation rates and a broad mutant spectrum were essential for virus adaptability to a complex environment such as that provided by host organisms.

The requirements of the internal population structure of a viral quasispecies to adapt to a new or changing environment are expected to depend on the nature of the selective constraint confronted by the viral population. Adaptation to a single antiviral inhibitor, which very often represents a highly specific selective pressure directed to a defined viral target, may be compatible with a range of mutation rates and mutant spectrum complexities similar or even below the levels commonly seen in viral populations (Keulen et al., 1999). A lower than standard (typical of wild type virus) mutation rate may be sufficient when

one (or very few) mutation(s) can cause a decrease in the sensitivity of the virus to the inhibitor. [Incidentally, the ease of deriving resistance to antiviral inhibitors, in particular nucleoside analogues (which is often associated with a single amino acid substitution in the relevant enzyme), may have an ancient evolutionary basis, in the need of primitive replication systems to cope with inhibitory metabolites.] This possibility was discussed previously in connection with quasispecies and drug resistance [see (Domingo, 2003), and previous versions of that article].

In contrast to successful adaptive responses achieved with limited genetic variation, adaptation to complex environments – such as those that may be needed to invade a new organ in the course of intra-host replication – may require any of a number of constellations of multiple mutations. In that case, a high mutation rate and a complex mutant spectrum may not only confer a selective advantage to the virus but may become imperative for long-term survival. Remarkably, increasing evidence suggests that constellations of mutations required for adaptability may be located either in the same genomic molecule or in a number of different genomes which may complement each other, again requiring a rich, broad and complex mutant spectrum (Moreno et al., 1997; Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). Standard mutation rate values, as those recorded for many RNA viruses (Domingo, 2007), may have been inherited from primitive protein-mediated replication systems or may have been the outcome of adaptation after extended episodes of co-evolution with their hosts in complex and variable environments. Although additional experiments are needed to generalize the possible adaptive advantage of complex mutant spectra, current experimental evidence supports an adaptive value for high mutation rates of RNA viruses, independent of the rate of viral RNA synthesis. Obviously, this does not exclude that high or low fidelity mutants could be found that manifest also a substantial alteration of the rate of RNA synthesis. Most likely, however, copying fidelity will be the result of multiple factors, and additional studies are necessary to assess to what extent the replication rate is one of them.

Knowledge acquired on the life cycle of several viruses has taught us that rapid replication is not a trait universally selected among RNA viruses. Viruses have evolved capacities to produce acute, chronic, latent, persistent, symptomatic or asymptomatic infections, with widely different time courses. Borna disease virus usually produces a net yield of less than one progeny genome per infected cell *in vivo*; HAV is slow replicating despite having a genomic organization similar to some of the fastest replicating picornaviruses, and HAV exploits rare codons to modulate its translation rate so as to limit its overall replication rate for better biological efficiency (Sánchez et al., 2003b). Again, it is important to dispel the notion that rapid replication evolved as a universal necessity for viruses, and that high mutation rates were its unavoidable and perhaps unneeded consequence.

4. Main features of quasispecies dynamics

The mutation rates and frequencies for RNA viruses (Section 2) mean that the occurrence of mutations is not occasional but continuous and unavoidable in the case of RNA genome replication. This, we believe, is one of the major changes of emphasis in the last decades, regarding our understanding of viruses at the population level. When a consensus viral genome sequence remains invariant it is not because mutations do not occur but because the methodology of sequence determination identifies a weighted average of multitudes of related sequences and overlooks intra-population heterogeneity. For RNA viruses between 3 and 33 Kb genomes, an average of 0.1 to 1 mutations introduced for *each* template copied is expected. In addition, since mutations can affect viral polymerases, and several polymerase residues may exert an effect on template copying fidelity (Section 1), mutation rates are themselves a substrate for selection. Therefore, mutation rates are modulable, as are other viral functions (Earl and Deem, 2004).

High mutation rates must be added to a rapid turnover of viral particles *in vivo*, notably in the case of HIV-1, HCV, and HBV [reviewed in (Pawlotsky, 2006)]. For example, in an infected adult, an average of 10^{12} new HCV virions per day is produced (Neumann et al., 1998), and these reflect probably a minority subset of all HCV replicative events occurring each day in one infected host!

Given that very frequently, in each infected cell, multiple (often in the thousands) viral genomes (or their complementary strands) are copied as templates, replicative ensembles consist of dynamic distributions of related sequences subjected to continuous genetic variation, competition and selection. Such dynamic distributions are termed *viral quasispecies*. (Fig. 1). Quasispecies was initially developed as a deterministic theory of molecular evolution by Manfred Eigen and Peter Schuster to explain self-organization and early evolution of life on earth (Eigen and Schuster, 1979). The theory was extended to finite populations in changing environments (Eigen, 1987; Eigen, 2000; Saakian and Hu, 2006; Wilke et al., 2001), and including recombination as a mechanism of genetic variation (Boerlijst et al., 1996). (Generally, deterministic models are formulated to place a problem in the most general context and in solvable mathematical terms, and then stochastic extensions allow a closer approximation to reality). Models have included quasispecies evolution in the face of the host immune response (Kamp, 2003). Quasispecies is one of several possible theoretical formulations of Darwinian evolutionary dynamics (Page and Nowak, 2002), adequate to interpret the behaviour of replication systems displaying high mutation rates, and with no conflict with alternative models of classical population genetics (Wilke, 2005). Some features of quasispecies dynamics are summarized in Table 1. The reader can find updated reviews of the theoretical concept as applied to viruses in (Biebricher and Eigen, 2005; Biebricher and Eigen, 2006), and of the biological implications of quasispecies in (Domingo et al., 2001, 2006; Figlerowicz et al., 2003).

Multiple viral quasispecies coexist in infected hosts at different replication sites and at successive time points, offering a rich substrate for internal interactions within each quasispecies, for occasional interactions between quasispecies, and for selection and random events to act upon. Infected organisms represent true examples of biological complexity with all its inherent emergent

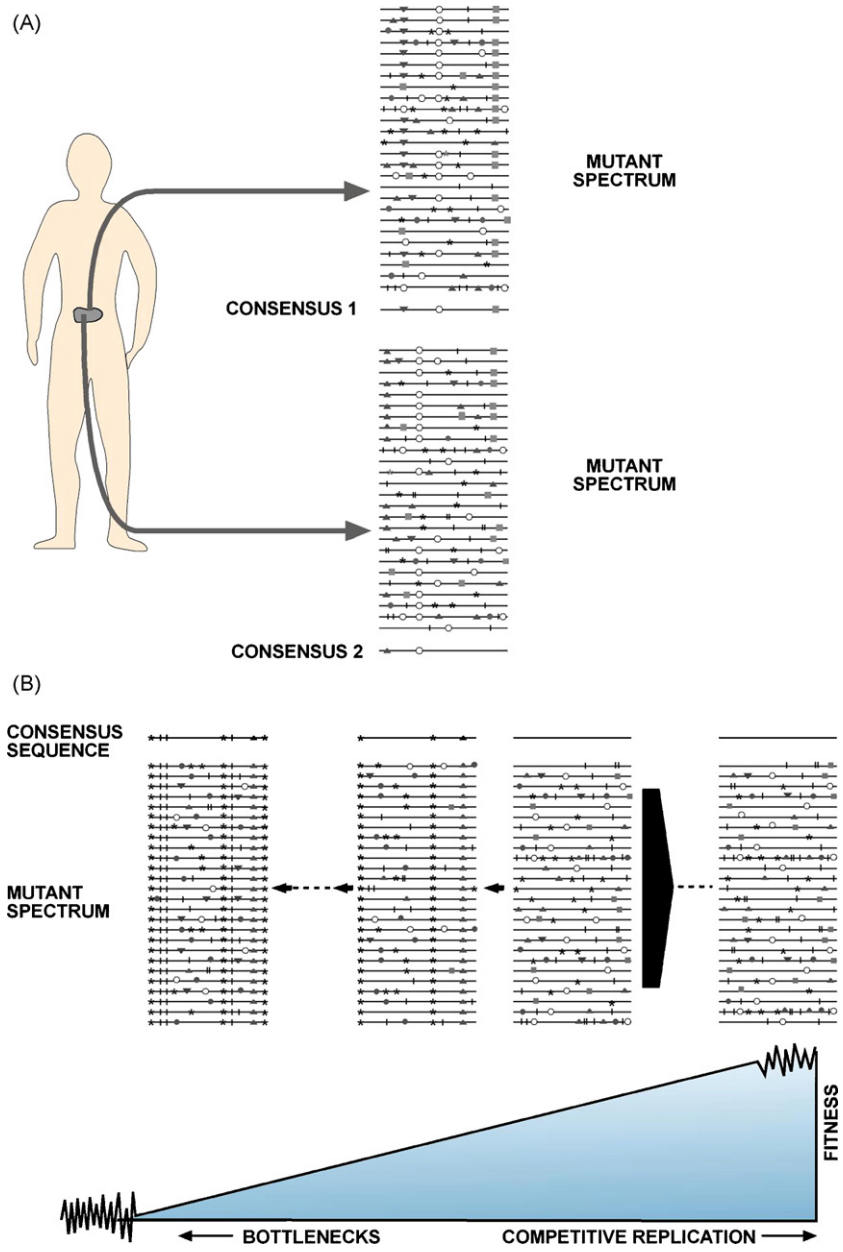

Fig. 1. Schematic representation of viral quasispecies and fitness variations. (A) An infected individual contains multiple, replicating viral quasispecies, even within the same organ. Here two mutant spectra are depicted with their consensus sequences. Each horizontal line represents a genome, and each symbol on a line represents a type of mutation. Real viral quasispecies can contain thousands of continuously mutating genomes that conform huge mutant clouds. (B) The passage regime of the virus can affect virus population fitness. Small arrows represent repeated bottleneck passages which result in fitness decrease. The large arrow represents large population passages that generally result in fitness gain. Fluctuations in fitness shown at high and low fitness values (at the two small angles of the fitness triangle) have been observed experimentally and interpreted as the stochastic effect of mutations on fitness when fitness gain is limited by population size or when fitness is very low. [Based in (Escarmís et al., 2006; Novella, 2003; Novella et al., 1999); figure modified from (Domingo et al., 2006), with permission].

behaviors and unpredictabilities [recent reviews in (Domingo, 2006; Domingo, 2007)].

4.1. Suppression in viral quasispecies

One of the predictions of quasispecies theory is that a mutant could dominate a fitter one by virtue of being surrounded by a more favourable (more closely related) mutant spectrum (Eigen and Biebricher, 1988; Swetina and Schuster, 1982). Suppression of a mutant by a mutant spectrum [that has been even interpreted as the triumph of the "mean" over the fittest (Gomez and Cacho, 2001)!] was shown experimentally for the first time with vesicular stomatitis virus (VSV) in cell culture (de la Torre and Holland, 1990). The mutant had to be present at a minimum, expression threshold level, to overcome suppression by the mutant spectrum. Such suppressive effects have been documented with several virus–host systems both in cell culture and *in vivo* (Domingo, 2006; Domingo, 2007). Expression threshold levels have not been systematically measured but they are expected to be environment-dependent,

being the surrounding mutant spectrum a key feature of the environment.

Suppressive or interfering effects of mutant spectra appear to increase with the mutation rate in viral quasispecies, as suggested by a strong suppressive effect of infectivity by viral populations which are close to extinction driven by enhanced mutagenesis (González-López et al., 2004; Grande-Pérez et al., 2005). Interference by a class of defective genomes termed "defectors" has been proposed to contribute to viral extinction through enhanced mutagenesis (virus entry into error catastrophe and lethal mutagenesis, reviewed in Section 7). These effects necessitate of coinfection of the same cell by multiple virus variants or that the latter be generated within an infected cell. For some viruses, there is evidence that multiple particles infect each susceptible cell *in vivo*, and that some cell subsets may be prone to multiple infections (Cicin-Sain et al., 2005; Chohan et al., 2005).

Suppressive effects together with their opposite, complementation potential, among components of a mutant spectrum are another demonstration that quasispecies behavior cannot be understood merely as the sum of behaviors of its individual components considered in isolation. Thus, quasispecies are often defined as dynamic genomic distributions subjected to variation, competition and selection, *and that act as a unit of selection*.

Quasispecies theory, adapted to the study of viruses as populations, has implications for both long-term evolution of viruses, and for viral pathogenesis. It has implications for virus evolution because the "substrate" for positive selection, negative selection and random drift, is not a defined genome type but a mutant cloud. It has implications for viral pathogenesis because specific types of variants can either permit survival of the virus in different compartments of the infected host, or establish interactions with host components, that may lead to disease. In fact, the connections between viral evolution and viral disease are even more complex, and some of their many facets are examined next.

5. At least three modes of virus evolution that can affect viral pathogenesis and persistence

The relevance of quasispecies to virus adaptability and survival stems from two distinct features: the rapid generation of diversity with the supply of phenotypic variants such as escape mutants, and the effects of mutant spectra *per se* that may endow viral populations with properties that are not attributable to specific variants.

Interestingly, most of the implications of quasispecies have been also observed at play with the hepatitis viruses. Even populations of HAV, despite their remarkable antigenic invariance, contain spectra of mutants that can be a substrate for selection and genetic diversification (Sánchez et al., 2003a). However, the most extensive studies on quasispecies *in vivo* have been carried out with HCV. HCV quasispecies were first detected and described in infected patients fifteen years ago (Martell et al., 1992). Since then, they have been reported in patients with acute and chronic infection (Farci et al., 2000; Honda et al., 1994; Koizumi et al., 1995; Kurosaki et al., 1995; Yuki et al., 1997), blood donor-to-recipient (Esteban et al., 1997; Hohne et al., 1994; Kojima et al., 1994; Mas et al., 2004) and mother-to-baby pairs (Farci et al., 2006; Weiner et al., 1995), as well as in a variety of biological samples, including serum, peripheral blood mononuclear cells, and liver specimens (Cabot et al., 2000; Pal et al., 2006). In addition, they have been found in genomic and antigenomic RNA strands. All together, the presence of HCV quasispecies in all tissues where the virus is found at different stages of the infection, and in all clinical situations examined, renders the quasispecies structure of HCV an inherent property of the virus. This property, and the manner in which the virus takes advantage of its potential, has been studied from two perspectives: (a) in relation to the clinical features of the infection (transmission, persistence, and liver damage) and to the response to treatment [reviewed in (Gomez et al., 1999; Pawlotsky, 2000)], and (b) in the context of studies that either attempt to decipher key points in the biological life cycle of the virus as it confronts the immune system, or that are directed towards developing new therapeutic strategies.

Although the connections between virus evolution and disease mechanisms are multiple and exceedingly complex (beyond our expertise and the scope of this article), we can tentatively identify (with some unavoidable simplifications) at least three evolutionary events that have an impact in viral pathogenesis: (a) the generation of mutants that can escape selective pressures derived either from the host immune response or from external interventions; (b) evolution towards increased or decreased virulence associated with specific genetic modifications in the viral genome; and (c) disease potential modified by the composition and complexity of the mutant spectrum.

In the next sections we review some of the general implications of specific mutants and population complexity, with special reference to the hepatitis viruses [as previous reviews, see (Clementi, 2003; Esteban et al., 1999; Pawlotsky, 2006)].

5.1. Escape mutants

Some phenotypic changes of viruses are mediated by a single nucleotide or amino acid substitution. To be of adaptive value, such a minimal genetic change should not only be produced by the genome copying machinery, but it should not entail a fitness cost of such an intensity that it decreases the proportion of the mutant below its minimum expression threshold level (see Section 4.1). When these requirements are fulfilled, the mutant will be represented in the quasispecies at a frequency such that the phenotypic traits associated with the mutant will be exposed to selection events. Frequent viral mutants of this type are antibody-, cytotoxic T lymphocyte (CTL)-, or inhibitor-escape mutants.

5.1.1. Immune evasion

In HCV, variation of hypervariable region 1 (HVR-1) of glycoprotein E2, presumably due to antibody (Ab) selection, has been associated with the development of HCV persistence in humans (Farci et al., 2000). The availability of cell culture systems for HCV has opened the possibility of studies on the capacity of Abs to neutralize HCV and to approach the dynam-

ics of selection of escape mutants, as previously documented with other viruses.

Variation of the "a" antigenic determinant has been associated with some cases of anti-HBV vaccine failure [reviewed in (Esteban et al., 1999)]. Amino acid substitutions in the S protein are frequent among human carriers who are seropositive for anti-s antibodies (Yamamoto et al., 1994). The relevance of HBV s antigen mutants at the epidemiological level is suggested by an increase in the prevalence of s mutants following anti-HB vaccination of children (Hsu et al., 1999). A clonal analysis of viral sequences from chronic HBV carriers documented that the coexistence of hepatitis B surface (HBs) antigen and anti-HBs antibodies was associated with an increase of "a" determinant heterogeneity, with substitutions that had been described as immune escape variants (Lada et al., 2006). The analyses suggest selection of immune-escape HBV variants during some chronic HBV infections, and constitute an example of biologically relevant coexistence of phenotypic variants in the same host individuals (Table 1). Chronically infected patients often include substitutions at CTL epitopes of HBV (Tai et al., 1997).

CTL-escape mutants of HCV, and their implication in viral persistence, have been described during infection of chimpanzees. These studies suggest that a CD8+ T response focused to few epitopes, and emergence of escape mutants, promoted viral persistence, whereas an early and broad CD8+ response favored viral clearance (Cooper et al., 1999; Erickson et al., 2001; Weiner et al., 1995). There is increasing evidence of the involvement of CTL-escape mutants in HCV persistence in humans (Chang et al., 1997; Timm et al., 2004); [review in (Bowen and Walker, 2005)]. Similar observations have been made regarding clonality of the immune response to HBV, and selection of escape mutants (Margeridon et al., 2005). The contribution of mutants that evade recognition by helper (CD4+) T lymphocytes and cytotoxic (CD8+) T lymphocytes to viral persistence has been described for several virus–host systems (Ciurea et al., 2001; Goulder et al., 1997; Kawada et al., 2006; Koenig et al., 1995; Quiñones-Mateu and Arts, 2006). A number of mechanisms of CTL-escape have been identified [reviewed by Bowen and Walker (2005)]: (a) amino acid substitutions that diminish or abolish binding of the epitope to MHC class I molecules, so that MHC-peptide complexes are not expressed or cannot be recognized by T cell receptors (TCRs); (b) amino acid replacements that alter the intracellular processing of viral proteins, leading to peptides that do not bind efficiently to MHC class I molecules; (c) the presence of substituted epitopes that may antagonize responses to the wild type epitope.

The antagonistic effects of variant epitopic peptides raises a broader question of possible modulating effects of non-identical antigenic structures expressed by mutant spectra of viral quasispecies. An extreme case is represented by reinfection of humans by different Dengue virus serotypes, that may result in biased cytokine responses that can contribute to severe Dengue virus-associated pathology (Mongkolsapaya et al., 2003; Mongkolsapaya et al., 2006). In chronic infections in which multiple, related viral protein sequences, arising in a highly dynamic way, when some of them reach a sufficient level, modulating effects that affect humoral or cellular immune responses become possible; however, their occurrence, required threshold levels, and potential biological consequences remain largely unexplored.

The kinetics of viral replication in relation to the timing of the host immune response may be crucial for virus clearance. HCV can replicate very rapidly in infected hosts, often preceding by days or weeks the evoked immune response. This means that generation of HCV antigenic variants and competition among them, continues, and that the capacity of specific variants to become dominant (in a process such as that diagrammatically represented by the large arrow in Fig. 1) may be subtly dependent on the timing of the generation in relation to the intensity of the selection imposed by CD4+ and CD8+ T cell responses. The outcome may be either a permanent or transient clearance despite a vigorous intrahepatic CD4+ and CD8+ T cell response (Thimme et al., 2002). In this process, the paradoxical nature of liver immunobiology (regarding immune tolerance versus immune surveillance) must be taken into account (Bowen et al., 2005).

The presence and maintenance of Ab- or CTL-escape mutants in viral populations can be influenced by viral fitness in at least two ways: (a) the fitness cost entailed by the relevant mutation and corresponding amino acid substitution, and (b) the different fitness levels attained in the mutant spectrum by minority variant viruses endowed with the same antigenic profile. Mechanism (a) has been suggested by reversion of epitope mutations in HIV-1, either at later stages of a persistent infection, when a specific CTL pressure wanes, or upon transmission of the virus to individuals who express a different MHC allele repertoire (Borrow et al., 1997; Friedrich et al., 2004; Leslie et al., 2004; Quiñones-Mateu and Arts, 2006). Mechanism (b) has not been explored *in vivo*, but it has been documented in model studies in cell culture (Martin et al., 2006). The experiments show that, independently of possible fitness costs entailed by an Ab-escape mutation, the different fitness levels that can be attained by variants with the same antigenic profile, affect the mutant repertoire selected by a neutralizing monoclonal Ab (Martin et al., 2006).

The stoichiometry of viral particles infecting a target cell may influence the recognition and killing of infected cells by specific CTLs. When a CTL-escape virus mutant coinfects a cell with a wild type (non-mutated epitope) virus, epitope-specific CTLs can recognize this infected cell; cell killing should prevent any replicating, non-encapsidated virus to reach the external environment, rendering biologically void the presence of the CTL-escape mutant. This possibility has been suggested for HIV-1 given the multiple viral particles that often infect target cells (Gratton et al., 2000).

Given the multiple implications of Ab- and CTL-escape for viral pathogens, it is justified to hypothesize that "the error-prone nature of HCV replication is intrinsic to its persistence, allowing for rapid evolution to adapt to immune selective pressure exerted by the host" (Bowen and Walker, 2005). There is little question that additional studies will be carried out to test this hypothesis, and even if, as it appears, the genetics of viral variation plays a role, it must be always recognized that persistence of a pathogen *in vivo* is the result of a complex interplay between viral and host influences.

5.1.2. Drug resistance

Mutants with decreased sensitivity to antiviral inhibitors have been isolated nearly systematically with any RNA virus and many DNA viruses [reviews in (Domingo, 2003) and previous versions of the same article)]. The most extensively studied case, due to its dramatic consequences for medical practice, is resistance of HIV-1 to the antiretroviral agents used in therapy, selected in infected patients subjected to antiretroviral therapy (Menéndez-Arias, 2002b; Quiñones-Mateu and Arts, 2006). The fitness costs of a drug-resistance mutation will influence its permanence in the infected organisms in the presence or absence of the drug. Maintenance of genomes bearing a debilitating drug-resistance mutation can be achieved through compensatory mutations (Nijhuis et al., 1999). Emergence of HBV mutants resistant to antiviral inhibitors generally results in progression of liver disease (Bartholomeusz and Locarnini, 2006b). Subtitution M204 I/V in the viral reverse transcriptase (RT) affects the catalytic domain (YMDD) and is selected by lamivudine and L-nucleosides. Resistance to adefovir is associated with RT substitutions N236T and A181V. The key substitutions conferring lamivudine resistance in HBV located within domain C of its polymerase, entail a decrease in replication efficiency which can be compensated by substitutions located in domain B of the polymerase (Allen et al., 1998; Delaney et al., 2003; Fu and Cheng, 1998).

Since the HBV RT gene overlaps with the HBs antigen gene, drug-resistant mutations can impact s function. The medical implications of such drug-mediated s mutants is largely unexplored (Bartholomeusz and Locarnini, 2006a).

A key issue concerns the isolation of HCV mutants with decreased sensitivity to the nucleoside analogue ribavirin, used in anti-HCV therapy (Section 6). Replacement Phe 415 Tyr in the HCV RdRp (NS5B), isolated form patients treated with ribavirin, decreased the sensitivity of the virus to the nucleoside analogue; a fitness cost of this mutation is suggested by the reemergence of viruses with Phe415 in some patients after ribavirin treatment was discontinued (Young et al., 2003). Resistance to ribavirin is a very special case because it is possible that part of the anti-HCV activity of this nucleoside analogue is exerted via its mutagenic activity (lethal mutagenesis). The isolation of viruses resistant to ribavirin implies that viruses have the potential to escape extinction by lethal mutagenesis (this is discussed in Section 7).

The advantage of combination therapy (with multiple inhibitors targeting different viral functions) to control pathogenic viruses that display quasispecies dynamics was stated almost two decades ago (Domingo, 1989), and it is currently advocated to control infection by the hepatitis viruses (Bartholomeusz and Locarnini, 2006a, 2006b; Neyts, 2006; Qureshi, 2006). Some early recognized guidelines, in addition to the use of combination therapy, included the temporary shelving of drugs to try to minimize circulation of drug-resistant virus variants, and limitation of the time of treatment (Domingo and Holland, 1992). Similar concerns are expressed in the face of possible emerging and reemerging viral infections (Regoes and Bonhoeffer, 2006). Obviously, the time required to develop resistance to antiviral inhibitors may vary for different viruses because of differences in the cycles of intra-host replication and transmission. Selection of resistant viral mutants is a direct consequence of the statistical consequences of quasispecies dynamics (see the calculation of the approximate frequency of a triple mutant mentioned in Section 2), supported both experimentally and by theoretical considerations (Bonhoeffer et al., 1997; Coffin, 1995; Nájera et al., 1995)].

The realization of the need of combination therapy illustrates the impact that an understanding of quasispecies (intra-host population structure, independently of evolutionary rates of consensus sequences) can have in applied virology. In clinical practice, the benefits of combination therapy have been evidenced by sustained low viral loads in many patients infected with HIV-1, subjected to highly active antiretroviral therapy in its various forms. Unfortunately, the adaptive potential of HIV-1 quasispecies with its multiple intra-host reservoirs is unceasing, and the development of inhibitor-resistant virus variants follows its pace. The isolation of HBV mutants resistant to lamivudine (Allen et al., 1998; Pallier et al., 2006) and the isolation of ribavirin-resistant mutants of HCV (Young et al., 2003) predict that, unless the lessons from HIV-1 are taken into account, the next decades will witness rounds of designs of anti-hepatitis virus inhibitors, and subsequent circulation of virus variants with decreased sensitivity to the inhibitors.

Similar concepts apply to immunotherapy. Treatment with a single neutralizing monoclonal antibody (mAb) should be avoided because of the likely selection of neutralization escape mutants of viruses. A combination of two neutralizing anti-HCV mAbs that recognize different conformational epitopes of E2 has been suggested to prevent reinfection by HCV in liver transplant patients (Eren et al., 2006). Their efficacy in prophylactic treatment is expected to be influenced by the number of mutations in the E2-coding region needed to escape neutralization by each mAb, the fitness cost of mutations and the viral load involved in the reinfection.

5.2. Evolution towards modification of virulence

Mutant viruses may display altered virulence, as a trait associated with a defined genomic sequence, despite the presence of a mutant spectrum. The literature describes many examples, including mutations which mediate virulence of poliovirus and other picornaviruses (Agol, 2006; Tracy et al., 2006). A revealing example was the demonstration that molecular clones of SIV copied from the genome of viruses isolated from macaques displaying different degrees of AIDS-like disease reproduced the disease stage from the original animals when inoculated into naïve macaques (Kimata et al., 1999). Thus, there are complex phenotypic traits imprinted in defined genomic sequences.

In an experiment of adaptation of a swine FMDV to the guinea pig, an amino acid substitution in a non-structural protein was directly responsible of adaptation to the new host and the ensuing pathology. This was independent of the presence of a mutant spectrum, as shown by reproducing the disease by administration of a virus expressed from an infectious clone which included the relevant mutation (Núñez et al., 2001). Thus, adaptive mutations can mediate emergence and reemergence of viral disease,

and alteration of virulence, independently of mutant spectrum complexity [additional examples in (Domingo, 2007; Parrish and Kawaoka, 2005)].

Different HCV genotypes and subtypes have accumulated genetic signatures that render, for example, genotype 1 less susceptible to interferon IFN-α plus ribavirin therapy than genotypes 2 and 3 (Martinot-Peignoux et al., 1995; Poynard et al., 1998). This effect of genotype is distinct from the effects of mutant spectrum complexity to be discussed in Section 5.3.

Variation in HCV can have functional consequences other than those mentioned in Section 5.1. Some may affect cell tropism, notably facilitating extrahepatic virus replication. Variation in the IRES may affect the translational properties of the viral genome in different cells and tissues [reviewed by Pawlotsky (2006)]. There is evidence that HCV infection may be associated with some cardiac disorders (Matsumori et al., 2006; Matsumori et al., 2000). This reinforces the concept put forward decades ago that mutant forms of RNA viruses can be associated with atypical forms of disease (Holland et al., 1982). It is worth recalling a statement by J. J. Holland and his colleagues: "There is an unspoken assumption among many physicians and scientists that a particular RNA virus will generally cause a particular disease. This assumption may be true in a very broad practical sense, but it is important to understand that it can never be true in a formal scientific sense. Because a particular RNA virus simply does not exist, a particular RNA virus disease does not exist either. The science of infections diseases is still in its infancy because we still understand so little of the fine details of host-pathogen interactions" (Holland et al., 1992).

5.3. Mutant spectrum complexity in disease outcome and response to treatment

The mutant spectrum of viral quasispecies may hide mutants whose presence may nevertheless affect viral pathogenesis. Individual components of VSV quasispecies displayed either a higher or lower capacity to induce interferon than the average population in natural viral isolates (Marcus et al., 1998). An early example of predictive value of quasispecies complexity was that the number of different sequences found in the mutant spectrum of the coronavirus mouse hepatitis virus correlated with its pathogenic potential for mice (Rowe et al., 1997).

The results of infection of mice with a high fidelity PV mutant (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006) (see also Section 3) demonstrate that mutant spectrum complexity may *per se* be a virulence determinant. In HCV, the complexity of the mutant spectrum may affect the outcome of an acute infection towards chronification and the response to treatment, as discussed in the next sections.

6. Medical implications of HCV quasispecies

6.1. Quasispecies complexity and outcome of the infection

Acute HCV infection progresses to chronic infection in 85% of patients, and subsequently, chronic infection leads to hepatitis and some degree of liver damage. There is a transition in the first months of the infection from acute to chronic disease followed by longstanding disease. In transfusion recipients, Farci and colleagues demonstrated a correlation during this transition between the evolutionary dynamics of the virus and clinical outcome (Farci et al., 2000). Patients infected by viruses that underwent little evolution during the acute stage of infection recovered completely, whereas patients in which the virus evolved rapidly, developed chronic infection. The authors concluded that the diversity of the viral population depends on the host immune response. If the response is effective, the diversity of the HCV quasispecies declines until the final variant is cleared. These findings have prognostic value for hepatitis C acquired by transfusion.

The composition of the quasispecies following massive infection in a transfusion recipient closely matches that of the infecting blood unit in 70% of the cases (Laskus et al., 2004). In other words, the recipient is infected with a "well structured" quasispecies. But this may be not the case after infection by other transmission routes. If the size of the viral inoculum is very small, mutations can accumulate without a possibility of effective selection and, ultimately, the viral spectrum will be more and more heterogeneous until extinction. In this case a fittest virus may never be found (Mas et al., 2004). This observation was interpreted according to the error catastrophe theory (Solé et al., 2006). As discussed in Section 7, any replicating system can only accept a maximum average error rate without losing its encoded information. Mutation frequencies for RNA viruses suggest that they might replicate near their error threshold, and on occasions they might briefly exceed it (Eigen and Biebricher, 1988; Holland, 2006). The proposal of Solé and colleagues could provide an explanation for the spontaneous resolution of the infection in non-transfused patients exposed to HCV. In fact, recovery appears to be more common among patients exposed to small inocula such as those produced by accidental needle sticks, intravenous drug use, and sexual transmission, than among those infected by a large virus dose. The low risk of persistence after exposure and seroconversion in sexual transmission has been associated with the small number of viral particles carrying a limited combination of mutations (unstructured quasispecies) which may decrease the potential for overcoming the immune response of the exposed host (Quer et al., 2005). Although the precise factors influencing viral clearance or persistence are not well understood, a significant association has been found between a broad and sustained HCV-specific T cell response and viral clearance in acute hepatitis C infection. In addition, the mode of transmission may be one of the factors that determine the transition from acute to persistent infection.

Progression of liver disease in patients with chronic HCV infection has been reported to be more likely in patients in whom the duration of the infection is longer, and in men, older patients, those with HIV and HBV coinfection, and consumers of alcohol. Among the virus-related factors, neither viral concentration nor viral genotype are indicators of likely progression. It should be pointed out that HCV-RNA levels are very stable in most HCV chronic carriers, with only a small proportion of patients having significant viral load fluctuations (Yeo et al., 2001).

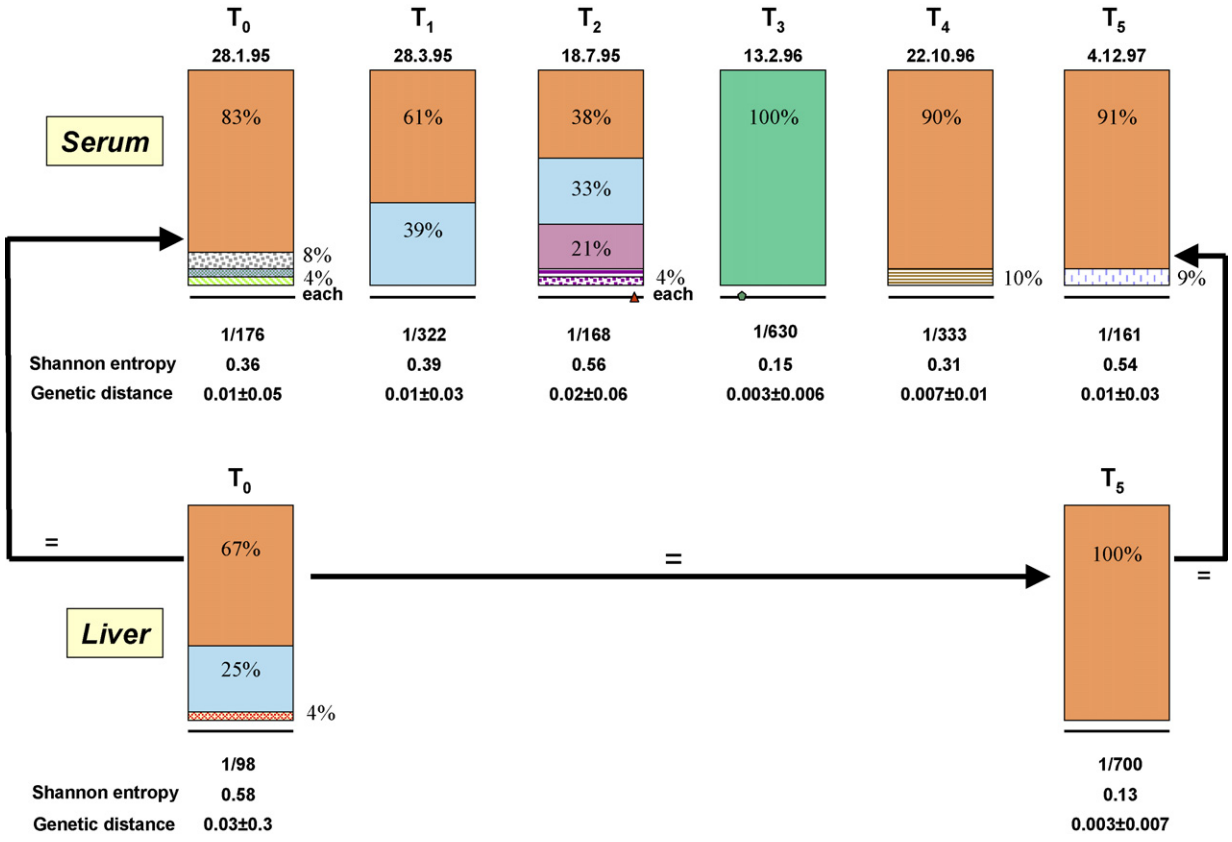

Fig. 2. Diversity of viral sequences and shift in the HCV quasispecies in serum and liver over time, in a non-progressive patient chronically infected with HCV. Amino acid sequences at the coding junction region E2-p7-NS2 quasispecies in serum (S0 to S5) and liver (L0 and L5), were grouped in clusters represented by histograms. Groups were made according to similarity, so that any amino acid sequence in any subgroup shared one or more amino acid replacements which were not present together in the same amino acid sequence subset in the same or in other samples. That is, inter-group amino acid sequences consisted in one or two substitutions, and intra-group amino acid sequences differed in single point substitutions. The same colors and patterns identify identical subgroups. Mutations in the consensus sequences of the viral quasispecies over time are shown at the bottom of each histogram (each symbol represents one mutation). Shannon entropy and genetic distance are shown below the respective histogram. Based in (Cabot et al., 2001).

Several studies have tried to correlate the complexity of the circulating quasispecies with the degree of liver damage (Cabot et al., 2000; Gonzalez-Peralta et al., 1996; Hayashi et al., 1997; Koizumi et al., 1995; Lopez-Labrador et al., 1999; Naito et al., 1994; Qin et al., 2005; Yuki et al., 1997). Although some reports suggest a higher rate of progression among patients infected with more complex quasispecies, the latter is probably a reflection of disease duration. This observation may fit the theoretical models of viral diversification in which antigenic variants are not completely replaced by emergent ones, and the continuous accumulation of variants could reflect a history of immune evasion and cell destruction (Nowak and Schuster, 1989; Nowak et al., 1991). In a study involving a large cohort of patients, complexity of the quasispecies correlated with ALT levels, which could mean that the complexity could be related with immune-mediated liver injury (Rothman et al., 2005).

Several in depth studies have depicted a very complex situation that does not encourage the expectations to reach a good correlation between quasispecies complexity and degree of liver damage. First, the composition of the circulating viral population does not always reflect the composition of the hepatic population (Cabot et al., 2000; Jang et al., 1999; Maggi et al., 1997; Roque-Afonso et al., 2005). Second, in some patients extrahepatic replication may represent an important contribution to the complexity of circulating quasispecies (Deforges et al., 2004; Forton et al., 2004; Pal et al., 2006). Third, continuous reorganisation of the quasispecies structure, with periods of relative stability in some patients and sporadic variations in major and minor variants in others, seems to be frequent (Cabot et al., 2001; Franco et al., 2003) (Fig. 2). Fourth, the re-appearance of memory variants that respond to "déjà vu" external stimuli (Ruiz-Jarabo et al., 2000) may induce quick shifts in the composition of the population, as shown in the case of HIV-1 (Briones et al., 2003). Re-inoculation studies in chronically HCV-infected chimpanzees in which pre-existing, slower replicating quasispecies outlived faster-replicating variants in the second inoculation, may be a good example of the memory property of HCV (Wyatt et al., 1998). More indirect evidence of involvement of memory genomes is found in longitudinal evaluation of replicating quasispecies in chronically infected patients (Cabot et al., 2001; Franco et al., 2003).

Thus, even though quasispecies evolution may be associated with progression from acute to chronic hepatitis C, there is no conclusive evidence that quasispecies evolution or diversification has a causative role in disease progression to cirrhosis once chronic hepatitis C has developed. Nevertheless, it should be

noted that the vast majority of studies aiming at defining the role of the quasispecies structure in the pathogenesis of chronic HCV infections have focused on hypervariable region 1 (HVR1) of the viral E2 gene.

Again, a better understanding of virus-host interactions is crucial. The capacity of HCV for rapid error-prone replication, its mechanisms of inhibition of chemokine production, relative induction of type I IFN versus IFN-γ, mechanisms of HCV resistance to type I IFN, as well as a number of induced host genes, are intertwoven to lead either to clearance or chronification of virus (Rehermann and Nascimbeni, 2005).

6.2. Quasispecies complexity and response to treatment

Interferon as a single agent, and combinations of interferon with the nucleoside analogue ribavirin have both been used for treating chronic hepatitis C infections. Ribavirin is ineffective or causes a transient decrease in viral load when used alone, but in combination with interferon (IFN) it at least doubles the decrease in viral load reached with a comparable duration of IFN monotherapy. Better clinical results have been achieved with the introduction of polyethylenglycol (PEG)-modified IFN-alfa plus ribavirin, such that 42% of patients with genotype 1, and 82% with genotypes 2 or 3, reach a sustained virological response (absence of detectable HCV RNA in serum) (Pawlotsky, 2003).

IFN-alfa has at least two types of antiviral effects: first, an immunomodulatory action that enhances the antiviral immune response, and, second, the induction of an antiviral state in the infected cell through various pathways. One of the most extensively investigated pathways is the induction of double-stranded RNA-activated protein kinase R (PKR) which leads to inhibition of cellular protein synthesis through phosphorylation of translation initiation factor, eIF2-alfa. The action of ribavirin may be related either to its capacity to modulate the immune response or to its mutagenic activity during viral RNA synthesis (Crotty et al., 2001; Crotty et al., 2000; Graci and Cameron, 2006; Parker, 2005) (next section).

Sustained responses to IFN-alfa plus ribavirin treatment are more likely in patients with shorter duration of the disease, those who are younger and those with milder liver histological damage. Among the virological factors, a low viral load and a HCV genotype other than 1 are optimal conditions for a sustained response. With respect to the influence of viral quasispecies in a sustained response, several studies have focused on the complexity (number of variants) and diversity (mean genetic distance between variants) of the viral population at baseline. Other studies have addressed the dynamics of the quasispecies after initiation of treatment, or the variations and evolution at specific genomic regions with a potential to mediate drug resistance. These viral parameters have also been analysed in combination with parameters that define the host immune response. Next we review some of these approaches.

Okada et al. (1992) first reported that the degree of variability of the HVR1 region of the E2 gene correlated with interferon responsiveness, and several studies have confirmed the correlation between quasispecies complexity and response to interferon therapy. Patients with more complex HVR1 populations (Enomoto et al., 1994; Kanazawa et al., 1994; Koizumi et al., 1995; Le Guen et al., 1997), or with a greater degree of intrapopulation divergence (Polyak et al., 1997) respond less well or do not respond to treatment. The availability of the combination of Peg-IFN and ribavirin, which provides a more effective antiviral response, has led to re-evaluation of the issue of quasispecies as a predictive factor for response to treatment. Farci et al. (2002) questioned whether pre-treatment viral diversity could predict the final response, but found an interesting association between the reduction in quasispecies diversity once treatment is started and viral clearance; such a reduction had prognostic value. These results again contrast with the more varied pattern of quasispecies evolution in responders found by Chambers et al. (2005). A study with a large cohort of patients showed that increased quasispecies diversity, together with active and specific anti-HCV immune status before starting therapy, was associated with a poor virologic response (Morishima et al., 2006). This is consistent with the idea that the immune response drives quasispecies diversity *in vivo*, and also may provide some light to interpret different results in other studies which involved a lower number of patients.

The majority of studies analysing the implications of HCV quasispecies in the response to antiviral treatment have focused on HVR1 of the E2 gene. In 1995, Enomoto et al. suggested that the genetic heterogeneity at a specific domain of the non-structural 5A (NS5A) region of HCV, known as the IFN sensitivity-determining region (ISDR), was closely related to the response to treatment in Japanese patients with HCV genotype 1b infection (Enomoto et al., 1995). This issue raised some controversy because in some studies from Western countries (Chambers et al., 2005; Squadrito et al., 1997), but not all (Puig-Basagoiti et al., 2001; Saiz et al., 1998), most HCV-1 infected patients with a sustained virological response showed no or only a few mutations within the ISDR region. The sequence adjacent to the ISDR domain may be involved in PKR binding and thus linked to IFN resistance, but once more, even though this region may play a role in HCV resistance to naturally-secreted IFN, it is not confirmed that the region contains determinants for blocking administered IFN-alfa (Nousbaum et al., 2000). In another study, Taylor et al. (1999) demonstrated that envelope protein 2 (E2) of HCV genotype 1a/1b contains a 12-amino-acid sequence that is similar to the PKR autophosphorylation site and to the phosphorylation site of the translation initiation factor eIF2-alfa, the latter being a target for PKR. This has been named the PePHD domain. The authors showed that E2 from genotype 1 HCV, but not type 2 or 3 isolates, can interact with, and inhibit the kinase activity of PKR in cell cell-free systems. Nevertheless, it does not seem that the mutational pattern in this region influences the response to IFN-alfa (Quer et al., 2004), nor could the emergence of mutations in this region be correlated with treatment outcome. Thus, the sequence of this region before or during treatment cannot be used to predict the success of treatment (Gaudy et al., 2005).

Because the antiviral effects of INF-alfa and ribavirin do not act directly on the viral protein or RNA, but instead have an indirect action through a variety of cellular antiviral activities and immune response stimulation, it is likely that these drugs do

not exert selection pressure on particular viral sequences. If this were the case, it would be difficult to establish a defined pattern of mutations in specific viral regions that can be associated with treatment failure, as in HIV patients treated with HAART.

7. Error catastrophe as an antiviral strategy. Possible impact for treatment of viral hepatitis

One of the consequences of quasispecies dynamics is the existence of an error threshold relationship for maintenance of genetic information [recent reviews in (Biebricher and Eigen, 2005; Biebricher and Eigen, 2006)]. When error rates exceed a tolerable limit – that depends on genome size, relative viral fitness and population size – there is a collapse of the entire quasispecies distribution, and nucleotide sequences lose their informative content. Such a transition is termed entry into error catastrophe, and its application to virus extinction through enhanced mutagenesis is termed lethal mutagenesis [reviews in (Anderson et al., 2004; Domingo, 2005; Eigen, 2002)].

Guided by extensive theoretical work, the first demonstration of an adverse effect of enhanced mutagenesis on viral replication was obtained by Holland et al. (1989) working with poliovirus and VSV, using mutagenic agents that acted through different mechanisms, including base and nucleoside analogues. They showed that mutation frequencies at a defined nucleotide site of the viral genomes could be increased only very modestly without severe losses of infectivity. These results were then extended to HIV-1 (Loeb et al., 1999) and to a number of RNA viruses displaying different replication mechanisms [reviews in (Anderson et al., 2004; Domingo, 2005)].

Two relevant advances in this area of research have boosted the interest in lethal mutagenesis as an antiviral strategy. One is the discovery that ribavirin, which is extensively used in anti-HCV therapy (Section 6), acts as mutagenic agent for some viruses (Crotty et al., 2000; Graci and Cameron, 2006). Since ribavirin is a licensed drug that has been amply used to treat several viral infections, it is possible that, in some cases, its antiviral activity has been exerted through (or with the contribution of) mutagenesis. A key question is whether ribavirin may exert its anti-HCV activity with the participation of lethal mutagenesis. There is evidence that ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes (Lanford et al., 2001). Mycophenolic acid, which as ribavirin, is an inhibitor of inosine monophosphate dehydrogenase (resulting in reduced intracellular GTP pools) had no antiviral effect, and virions from ribavirin-treated cultures showed dramatic reductions of specific infectivity. Inhibition of replication and increases in mutation frequency were quantitated in full-length and subgenomic HCV replicons (Contreras et al., 2002; Kanda et al., 2004; Zhou et al., 2003).

The HCV polymerase (NS5B) can catalyze the incorporation of ribavirin monophosphate opposite cytidine and uridine *in vitro* (Maag et al., 2001). Templates containing ribavirin can direct the incorporation of CMP and UMP with equal efficiency, and also cause significant block of RNA elongation. These results with purified NS5B suggest that the HCV replication machinery has the ingredients for ribavirin triphosphate to act as inhibitor and mutagen during HCV RNA synthesis. (Maag et al., 2001; Vo et al., 2003). In favor of a direct action of ribavirin-nucleotides on NS5B *in vivo* is the isolation of NS5B mutants with increased resistance to ribavirin, with specific amino acid replacements in the enzyme that probably implied a fitness cost (Young et al., 2003) (see also Section 5.1).

The second relevant advance was that the mutagenic base analogue 5-fluorouracil prevented the establishment of a persistent LCMV infection in mice (Ruiz-Jarabo et al., 2003). This proof-of-principle of the feasibility of an antiviral approach *in vivo* based on the concept of error catastrophe, and the recognition of ribavirin as a mutagenic agent for some viruses, have encouraged additional experimental studies on lethal mutagenesis. Some studies have provided evidence of increased mutation frequencies of HCV in patients treated with ribavirin (Asahina et al., 2005) while other studies have not found significant increases. Relevant considerations are the effective ribavirin-nucleotide concentrations that can be attained *in vivo* at the sites of HCV replication, relative to the concentrations found effective in cell culture, possible intervention of host factors that may alter the behavior of NS5B, and others (Airaksinen et al., 2003; Hong, 2003). Studies with HIV populations from additional patients, with more extensive HCV quasispecies analysis, are needed to clarify this important point.

In parallel, a number of studies with other RNA viruses have explored the molecular mechanism underlying lethal mutagenesis. The main conclusions from these studies are: (a) the transition towards extinction occurs with a decrease of specific infectivity (ratio of infectious to total genomes), without modification of the consensus sequence, and variable increases in mutant spectrum complexity. (b) In the cases in which a comparative study has been carried out, virus extinction was dependent on a mutagenic action, not merely on inhibitory activity manifested by the mutagen. This was documented by the incapacity of inhibitory combinations to extinguish the virus despite reaching the same degree of inhibition than attained in the presence of the mutagenic agent and that mediated extinction (Pariente et al., 2003). (c) Viral extinction is facilitated by low viral fitness and low viral load. (d) Preextinction viral RNA (from mutagenized viral populations, prior to extinction) can interfere with infectivity. These observation [reviewed in (Anderson et al., 2004; Domingo, 2007; Domingo, 2005)] support the view that mutagenesis, and not solely inhibition of viral replication, mediates viral extinction in accord with the parameters (effect of viral load and viral fitness) included in the error threshold relationship (Biebricher and Eigen, 2005; Eigen, 2002).

A study on the extinction of lymphocytic choriomeningitis virus (LCMV) from persistently infected BHK-21 cells by 5-fluorouracil suggested that the presence of defective LCMV genomes could contribute to the elimination of the virus (Grande-Pérez et al., 2005). The experimental results, based on a decrease of virus infectivity preceding a decrease of virus replication, were supported by *in silico* modeling studies using realistic parameters. In this view, viral extinction by lethal mutagenesis would be facilitated by both lethal mutations and debilitating mutations that could exert a deleterious effect on the quasispecies as a whole. The negative effect

of defective genomes can be viewed as an extreme case of suppressive effects of the mutant spectrum, discussed in Section 4.1 (González-López et al., 2004; Grande-Pérez et al., 2005).

Lethal mutagenesis with nucleoside analogues has opened an interesting connection between antiviral therapy and cancer chemotherapy. A clinical trial with a nucleoside analogue is current under way with AIDS patients (Harris et al., 2005). Given the multiple therapeutic options opened to control HCV (Neyts, 2006; Qureshi, 2006) and the general observation of the benefits of combination therapy, the possibility to combine classical approaches with lethal mutagenesis is, in principle, appealing. Much will depend on the finding of virus-specific agents with limited cell toxicity and a deeper understanding of the host components involved in the response to infection by the hepatitis viruses.

8. Concluding remarks

Quasispecies dynamics has an impact in the biology of the hepatitis viruses, as reflected in this review. To what extent, however, escape variants contribute to viral persistence, or how disease progression or treatment effectiveness are affected by the nature and complexity of mutant spectra, are open questions. In approaching these important problems what has been learned with other viruses should be taken into consideration. The dynamic behavior of mutant spectra in their interaction with host organisms is basically presided by universal Darwinian principles applied to any system of simple replicons displaying error-prone replication.

Many important questions have to be addressed that can contribute to a better understanding of the hepatitis viruses, and to plan control strategies. At the level of basic replication mechanism, it would be important to define the fidelity properties of polymerases that catalyze hepatitis virus replication, definition of replication complexes and the involvement of host factors in replication. This would open the way to the design of new antiviral inhibitors – for example to interfere with critical polymerase-host factor interactions – or drugs capable of decreasing the copying fidelity of the replication machinery to favor the transition into error catastrophe. Obviously the clarification of the mechanism of action of ribavirin when administered together with IFN derivatives (and other drugs) may also provide new insights into treatment strategies.

The contribution of the cellular and antibody immune responses to virus clearance and the viral and host factors that prevent clearance will certainly be further addressed. Findings in this area will be essential to design preventive and therapeutic vaccines, and to plan combination treatments that may involve guidance of immune response together with suppression of viral replication. All this necessitates a lot of basic research in a number of interconnected areas. Quasispecies has provided an interpretation of the capacity of the hepatitis viruses to adapt to the changing environment of the living host, but has opened a wealth of new questions on how the viral response towards adaptation may either succeed or fail with regard to virus maintenance.

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