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Viral quasispecies

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Abstract

New generation sequencing is greatly expanding the capacity to examine the composition of mutant spectra of viral quasispecies in infected cells and host organisms. Here we review recent progress in the understanding of quasispecies dynamics, notably the occurrence of intra-mutant spectrum interactions, and implications of fitness landscapes for virus adaptation and de-adaptation. Complementation or interference can be established among components of the same mutant spectrum, dependent on the mutational status of the ensemble. Replicative fitness relates to an optimal mutant spectrum that provides the molecular basis for phenotypic flexibility, with implications for antiviral therapy. The biological impact of viral fitness renders particularly relevant the capacity of new generation sequencing to establish viral fitness landscapes. Progress with experimental model systems is becoming an important asset to understand virus behavior in the more complex environments faced during natural infections.

Keywords

quasispecies; evolution; virus; genome sequencing; adaptation; pathogenesis

Introduction

Viral quasispecies are the mutant distributions (also termed mutant swarms or mutant clouds) that are generated upon replication of RNA viruses, and some DNA viruses in infected cells and organisms. The quasispecies concept originated in a theoretical formulation of molecular evolution that emphasized error-prone replication of simple RNA or RNA-like replicons as an essential feature of self-organization and adaptability of primitive life forms (Eigen, 1971; Eigen, 2013; Eigen and Schuster, 1979). The mathematical equation of quasispecies relates to other formulations of Darwinian evolution,

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but its most distinctive feature is the emphasis placed on the occurrence of mutations during replication (Page and Nowak, 2002). Viral quasispecies are currently defined as collections of closely related viral genomes subjected to a continuous process of genetic variation, competition among the variants generated, and selection of the most fit distributions in a given environment (Domingo *et al.*, 2012). An important aspect of the quasispecies concept is that the large size of virus populations enables positive and negative interactions between individual viruses to establish a quasi-equilibrium of the variant proportions. Genetic variation is generated by the accumulation of mutations during replication and their re-arrangement by genetic recombination, and genome segment reassortment in the case of segmented genomes. Mutant swarms are established within single infected cells and cell clusters (Del Portillo et al., 2011; Jung et al., 2002; McWilliam Leitch and McLauchlan, 2013; Sala et al., 1994; Sobesky et al., 2007) and in infected organisms during disease episodes, outbreaks, epidemics and pandemics [(Gire et al., 2014; Martell et al., 1992), among many other studies that have been reviewed (Domingo et al., 2012)]. Basic features of quasispecies dynamics can be studied experimentally because mutant swarms are also generated upon replication of molecular or biological viral clones in cultured cells. The match between events at the cellular and epidemiological level lies in the error-prone replicative machinery that manifests in every environment in which viruses multiply. Cell culture systems are providing an impressive toolbox to explore consequences of quasispecies dynamics, and set the stage for an advanced understanding of more complex systems, notably quasispecies distributions in the patchy environments of differentiated organisms.

Here we review progress in three salient and interconnected features of viral quasispecies that have acquired increasing prominence in recent years: (i) the internal interactions of complementation and interference that are established within mutant spectra, that renders selection at a collective level possible; (ii) the relevance of mutant spectrum complexity and replicative fitness in virus adaptation and de-adaptation, and (iii) the dissection of mutational effects on fitness, as revealed by application of new generation sequencing (NGS) methodologies that are allowing unprecedented description of mutant spectra.

Formation of mutant spectra and origin of internal interactions

Two key parameters that explain the biological relevance of the rapid formation of mutant swarms during viral replication are the rate at which mutations are introduced during RNA genome copying, and the genome size. Mutation rates have been estimated by independent genetic and biochemical approaches to be in the range of 10^{-3} to 10^{-5} mutations introduced per nucleotide copied, almost a million-fold higher than standard mutation rates operating on cellular DNA. This difference led John J. Holland and colleagues to emphasize in a seminal paper a number of disease implications of a rapidly evolving RNA world immersed in a far more static cellular DNA world (Holland *et al.*, 1982). Most predictions of Holland and colleagues have been confirmed by findings made in the following decades that have linked rapid RNA genome evolution with RNA viral disease mechanisms [reviewed in (Domingo et al., 2012; Lauring and Andino, 2011)]. Since the size of RNA viral genomes lies between 3 Kb and 33 Kb, close to the inverse of the mutation rate, it is thought that it is unlikely to produce progeny RNAs identical to the parental genome even within the confined limits of a single replicative unit (each one of the replication complexes) within a single infected cell.

Thus, formation of mutant spectra with an abundance of single point mutants (and gradually decreasing proportions of double, triple and multiple mutants) is a necessary consequence of the mutation rate-genome size relationship. Mutant swarm generation might be prevented only if negative (or purifying) selection eliminated the great majority of newly arising mutant genomes, which is not the case. Current evidence suggests that a considerable proportion of mutants might be eliminated by negative selection, but it is not clear whether all mutations considered lethal by standard measurements of viral production might not be in reality low fitness variants that can populate low frequency levels of mutant spectra, either because they can replicate minimally or because they are helped by complementation. Irrespective to the answer that NGS could provide to this question, the surviving portion of viral variants is sufficient to confer adaptability of RNA viruses.

A realization of recent years is that mutant swarms are not mere collections of mutants ranked by a mutation-selection balance that act independently of each other. Rather, very often the mutant ensemble acts as a unit of selection due to interactions among its members. Two types of interactions have been evidenced: negative interactions (also termed interfering interactions) by which some genomes affect negatively the replication of other genomes of the same ensemble, and positive interactions (akin to classical complementation) by which genomes reinforce each other to achieve a higher replication rate. The result of these interactions is that evolution operates at the level of the population, a process often called ‘group selection”. It is worth noting that these interactions occur among different genomes, so that the effects are different from those of positive or negative epistasis that are established between mutations within the same genome. Epistasis is also an important ingredient of virus evolution but we will not deal with its implications in the present review.

Suggestions that the behavior of a mutant ensemble did not match that of its individual component accumulated over several decades but it is only recently that a more conceptual generalization has been formulated. Evidences include observations with unperturbed mutant spectra and observations derived from artificial increases in mutant spectrum complexity such as those occurring during lethal mutagenesis of viruses (virus extinction by increases in mutation rate above an extinction threshold). Briefly, an unperturbed mutant spectrum could suppress replication of a high fitness variant (de la Torre and Holland, 1990). Populations of foot-and-mouth disease virus (FMDV) selected to be resistant to polyclonal antibodies displayed reduced fitness and the populations suppressed high fitness variants present in the low fitness populations (Borrego et al., 1993). Kirkegaard and colleagues showed that a poliovirus mutant spectrum could suppress the growth of inhibitor-resistant mutants (Crowder and Kirkegaard, 2005), and recently that the targeting of an oligomeric capsid protein with a drug gives rise to chimeric oligomers that render the drug-susceptible genomes dominant within the same replicative ensemble (Tanner et al., 2014). Counterparts *in vivo* of the types of suppressing effects documented in cell culture have been also described. A live-attenuated poliovirus vaccine suppressed a minority virulent virus and impeded disease in chimpanzees (Chumakov et al., 1991). A non-pathogenic lymphocytic choriomeningitis virus (LCMV) could suppress manifestation of a growth hormone deficiency syndrome induced in mice by a pathogenic variant of the same virus (Teng *et al.*, 1996).

Regarding suppressive effects by mutagenized virus, pre-extinction, low fitness populations of FMDV obtained by 5-fluorouracil mutagenesis interfered with replication of standard, infectious RNA (González-López et al., 2004). The interference was directly documented with specific capsid and replicase mutants that were competent in RNA replication (Perales et al., 2007). In this study it was proposed that *trans*-acting proteins that have acquired amino acid substitutions, and that function as complexes with viral or host proteins, may be responsible of the interfering activity because they may generate non-functional complexes. Also, a class of LCMV defective mutants generated by 5-fluorouracil mutagenesis interfered with replication of the standard virus, and contributed to virus extinction (Grande-Pérez et al., 2005). These types of defective mutants are termed defectors, and both experimental and theoretical evidence supports their participation in virus extinction by lethal mutagenesis [reviewed in (Domingo et al., 2010)]. Thus, a transition from complementation among genomes from a mutant spectrum at standard mutation rates towards a gradual dominance of interference when mutation rates increase can be proposed on the basis of current evidence (Figures 1 and 2).

A further development that took place as a consequence of the recognition of intra mutant spectrum interactions affected by the interplay between mutagenic agents and inhibitors is the proposal of sequential treatments based on lethal mutagenesis. It is well accepted that combination therapies with two or more inhibitors are the choice to prevent or delay selection of inhibitor-resistant mutants. However, when mutagenic agents and inhibitors are given as therapeutic agents, a sequential inhibitor-mutagen administration may have an advantage over the corresponding combination in producing reductions of viral load and virus extinction. One of the reasons for this advantage is that the interplay by which the presence of a mutagen can increase the frequency of inhibitor-escape mutants is avoided [reviewed in (Perales et al., 2012)].

Regarding complementation within mutant spectra, an initial observation was that fitness of biological viral clones was on average lower than fitness of the complete populations from which the clones were derived (Domingo et al., 1978; Duarte et al., 1994), indicating that the mutant ensemble had some replicative advantage over its individual components. The importance of an ample mutant spectrum was unambiguously documented by comparing biological properties of a high fidelity PV mutant selected by its resistance to the purine nucleoside analogue ribavirin (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). The high fidelity mutant produced a restricted mutant spectrum and was unable to reach the brain of susceptible mice. However, either when complemented by a complete mutant spectrum or when the mutant spectrum produced by the fidelity mutant was broadened by mutagenesis, the variant made its way to the central nervous system of mice. Since these studies, additional fidelity mutants have been tested, and the results support that biased mutant compositions can be a mechanism of virus attenuation with implications for vaccine design [(Coffey *et al.*, 2011; Korboukh *et al.*, 2014; Meng and Kwang, 2014; Vignuzzi *et al.*, 2008) among other studies].

Some extreme cases of complementation between variants generated during quasispecies evolution that can greatly alter viral population structure or function have been described. A recombinant non-fusogenic measles virus (MV) mutant evolved in cell culture to regain

membrane fusion activity. A mutant with a wild type and a mutant genome in the same particle was competent in membrane fusion, while MV with the two genome types individually did not display fusion activity (Shirogane *et al.*, 2012). Long-term passage of FMDV in cell culture led to viral genome segmentation, consisting of genomic forms with internal deletions that were infectious by complementation (García-Arriaza et al., 2004; Moreno et al., 2014). The selective advantage of the segmented viral form was triggered by point mutations accumulated during virus passage that made possible a remarkable change in genome structure. The coexistence of two surface protein variants of hepatitis B virus enhanced viral replication and cellular and antibody immune responses (Cao et al., 2014).

There are additional observations that support the view that mutant spectra are true actors of evolutionary outcomes. Viral quasispecies can be endowed with a molecular memory consisting of minority genomes present at higher than basal frequency levels because the genomes had been dominant in a prior phase of evolution of the same lineage (Briones and Domingo, 2008; Domingo, 2000; Ruiz-Jarabo et al., 2000). This allows a viral population to respond more effectively to selective constraints that had already been experienced by the same virus lineage. The evidence just summarized is consistent with the idea that viral quasispecies can act as a unit of selection, group selection, one of the features of complex systems in the sense that the properties of the "whole" cannot be directly inferred from the properties of the "individuals". Viral quasispecies have approached virology to the growing field of biological complexity.

Mutant spectrum complexity and fitness in virus adaptation and de-adaptation

Fitness, defined as the overall capacity of a virus to produce infectious progeny has long been recognized as a critical parameter that reflects virus adaptation to a specific environment (Domingo and Holland, 1997). Unperturbed replication of large viral populations in a constant environment results in fitness gain, adaptation, while repeated bottlenecks that result in accumulation of mutations result in fitness decrease, de-adaptation. Recently, the direct connection between fitness and viral load has been related to disease progression and response to antiviral treatments [reviewed in (Domingo et al., 2012)]. Farci and colleagues were the first to document an effect of hepatitis C virus (HCV) quasispecies complexity in disease outcome and the response to treatment (Farci, 2011; Farci et al., 2000). The availability of a cell culture system for HCV has permitted the preparation of HCV displaying different fitness levels as a result of hundred serial passages in human hepatoma cells (Perales et al., 2013; Sheldon et al., 2014). One of the conclusions of these studies is that replicative fitness can be an independent predictor of the response of HCV to inhibitors used in therapy. It is known that viruses never exposed to inhibitors can nevertheless include in their mutant spectra mutations that confer resistance to the inhibitors [(Nájera et al., 1995) and several other studies]. Therefore, in the studies with HCV it was important to exclude that inhibitor resistance mutations had not been acquired as a consequence of fitness gain. This possibility was excluded not only by NGS but also by the multiplicity of infection (MOI)-independent kinetics of virus production in the presence of inhibitors, and the maintenance of the resistance phenotype in biological clones of the

passaged populations (Sheldon et al., 2014). Translated into clinical practice, it means that the evolutionary history of HCV in infected patients may influence the response to treatments. In particular, long-term replication of HCV during prolonged chronicity may increase viral fitness to the detriment of treatment efficacy, as observed in clinical practice. These observations render essential to develop methods to define fitness values *in vivo* and to explore the fitness landscape in viral populations.

Virus population mutation composition and fitness landscape

Characterizing the genetic structure of virus populations remains a fundamental challenge. High-throughput sequencing technologies are capable of generating very large datasets, but distinguishing true genetic variation from sequencing error remains a central problem due to the high average sequencing error rates for NGS (Dohm *et al.*, 2008; Shendure and Ji, 2008) and given that even small error probabilities result in a significant number of sequencing errors. To overcome this limitation an experimental approach was developed that dramatically reduces sequencing errors (Acevedo and Andino, 2014; Acevedo *et al.*, 2014).The basic premise of this method, called Circular Sequencing (or CirSeq), is to create tandem repeats templated using circularized genomic RNA fragments (Figure 3). The physical linkage of the repeats provides sequence redundancy over a genomic fragment derived from a single individual within the population. A simple error detection and correction approach derived from information and coding theory allows for rapid and highly accurate discrimination of amplification and sequencing errors. A consensus generated from a three-repeat tandem reduces the theoretical minimum error probability associated with current Illumina sequencing platform by up to 8 orders of magnitude, from 10^{-3} to 10^{-11} per base, allowing capture of a near-complete distribution of mutant frequencies of RNA virus populations generated within a cell or an organism. Using this approach, we are now able to estimate changes in mutation frequency of variants that exist in the population at very low levels. This opens an opportunity to determine fitness landscape at single nucleotide resolution.

This technology was recently used to assess the genetic composition of populations of poliovirus replicating in human cells in culture. Starting from a single laboratory-derived viral clone, poliovirus populations were obtained following serial passage in HeLa S3 cells. A total of 7 serial passages were carried out transferring 10^6 plaque forming units (p.f.u.) at low multiplicity of infection (m.o.i. ~ 0.1) for a single replication cycle (8 hr) at 37°C. Under these infection conditions, genetic complementation and drift are minimized and the change in allele frequency is primarily determined by mutation and selection. The mutation frequency dataset obtained from serial passaging of a viral population, allowed determining the relative frequency for all single nucleotide variants in the viral genome under these defined conditions. Measuring changes in the frequency of those variants over time, was then used to estimate the fitness effect of a given mutation within the population, and then the mutation can be mapped in protein or RNA structure to link fitness to virus mechanisms (Figure 3B). While this experiment only defines forces shapping quasispecies structure in a relative stable environment, this type of technology open the door to examine the forces shaping evolution in dynamic environments, like the infected individual.

Mutational robustness

The focus on mutation as a driving force in viral evolution has tended to overlook the tremendous cost of low replicative fidelity. Most mutations have deleterious effects on viral fitness (Fernandez *et al.*, 2007; Sanjuan *et al.*, 2004). Tolerance to mutational load determines the nature and extent of genetic diversity that can be maintained in the population. Thus, viral population diversity results from both the generation of mutations and the tolerance to them; these two factors together drive adaptation and virus evolution. Robustness is the invariance of phenotypes in the face of genetic or environmental perturbations. Genetic or mutational robustness describes situations in which the perturbation, mutation, is heritable. RNA viruses have tremendous reproductive capacity, generating thousands of progeny per genome. Despite frequent bottleneck events, a total population of millions of viruses in an infected host is not uncommon. The efficiency of purifying (or negative) selection is the product of effective population size (N_e) and the average mutational fitness effect(s). In large populations, strong selection will quickly purge mutants with lower fitness. Larger population sizes may also result in higher multiplicities of infection, where a given cell supports replication of at least two different genomes. Here, genetic complementation can increase the robustness of the viral population.

Recombination

Many RNA viruses undergo recombination or reassortment during replication. In cells infected at high multiplicity, for example, this exchange of genetic information can increase genetic diversity by combining previously unique mutations into the same genome. While the impact of viral recombination in evolution remain somewhat obscure, it is clear that these processes can also repair mutated genomes by purging mutations even in cells infected at low multiplicity (Domingo, 2000). Thus, these “repairs” maintain viral fitness, and they may serve to increase the mutational tolerance of viral populations. Given the complex relationship among complementation, recombination, and robustness, the effective multiplicity of infection within infected individuals is likely to be a critical factor influencing virus evolution. To critically test the role of recombination in virus evolution it will be necessary to manipulate recombination rates and determine if recombination rate remodels the genetic composition of the population and the ability of the virus to accumulate and maintain diversity and adapt to changes.

Understanding RNA recombination is also important due to its potential to produce new hybrid strains, which may have novel properties and enhanced pathogenicity. Many recombinant RNA virus strains provide ample indication that recombination does exist in nature to generate new variation that enhances virus fitness. In some viruses this new variation is achieved by borrowing genetic material from their hosts. For example, influenza A virus has been observed to recombine with cellular RNA, resulting in increased pathogenicity for the hybrid viruses (Khatchikian *et al.*, 1989). Importantly, the pathogenic consequences of recombination need to be carefully considered whenever multivalent live-attenuated vaccines are used. For example, recombination of poliovirus vaccine strains leads to the frequent recovery of recombinant pathogenic poliovirus (Georgescu *et al.*, 1994; Kew and Nottay, 1984) similarly, this is also observed for infectious bronchitis virus (Jia *et al.*,

1995; Kusters *et al.*, 1990; Wang *et al.*, 1994). It is hypothesized that pathogenic poliovirus strains can be generated by recombination with non-symptomatic enteroviruses of species C (Liu *et al.*, 2003) in vaccinated individuals (Arita *et al.*, 2005; Jegouic *et al.*, 2009; Jiang *et al.*, 2007; Kew *et al.*, 2002; Shimizu *et al.*, 2004). It is thus possible to extend the concept of quasispecies to include viral strains that co-circulate and that, by coinfecting cells in the infected indivuals, can recombine increasing the sequence space that the virus can explore in search for evolutionary innovation.

Concluding remarks

Quasispecies theory has encouraged viewing and examining viruses as complex mutant spectra, and not merely as simple genetic entities that can be described with a defined nucleotide sequence, as believed only a few decades ago. Recent procedures to reliably penetrate into the mutant spectrum composition of evolving viral populations should dive us into the intricacies of selection episodes and bottleneck events with unprecedented accuracy. The influence of quasispecies extends also to the design of new antiviral approaches now under active investigation with the use of virus-specific mutagenic agents to induce deterioration of viral functions through an excess of mutations. The expectation is that progress in experimental and theoretical quasispecies will continue providing new insights into virus behavior at the population level, as a clear demonstration of the power of basic science to produce practical applications in often unpredictable ways.

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- Viral quasispecies are collections of closely related viral genomes under selection
- Genetic variation is generated mutations accumulated during replication and recombination
- Positive and negative interactions among individual virus determine the mutant spectra
- New generation sequencing enables unprecedented description of the population dynamics

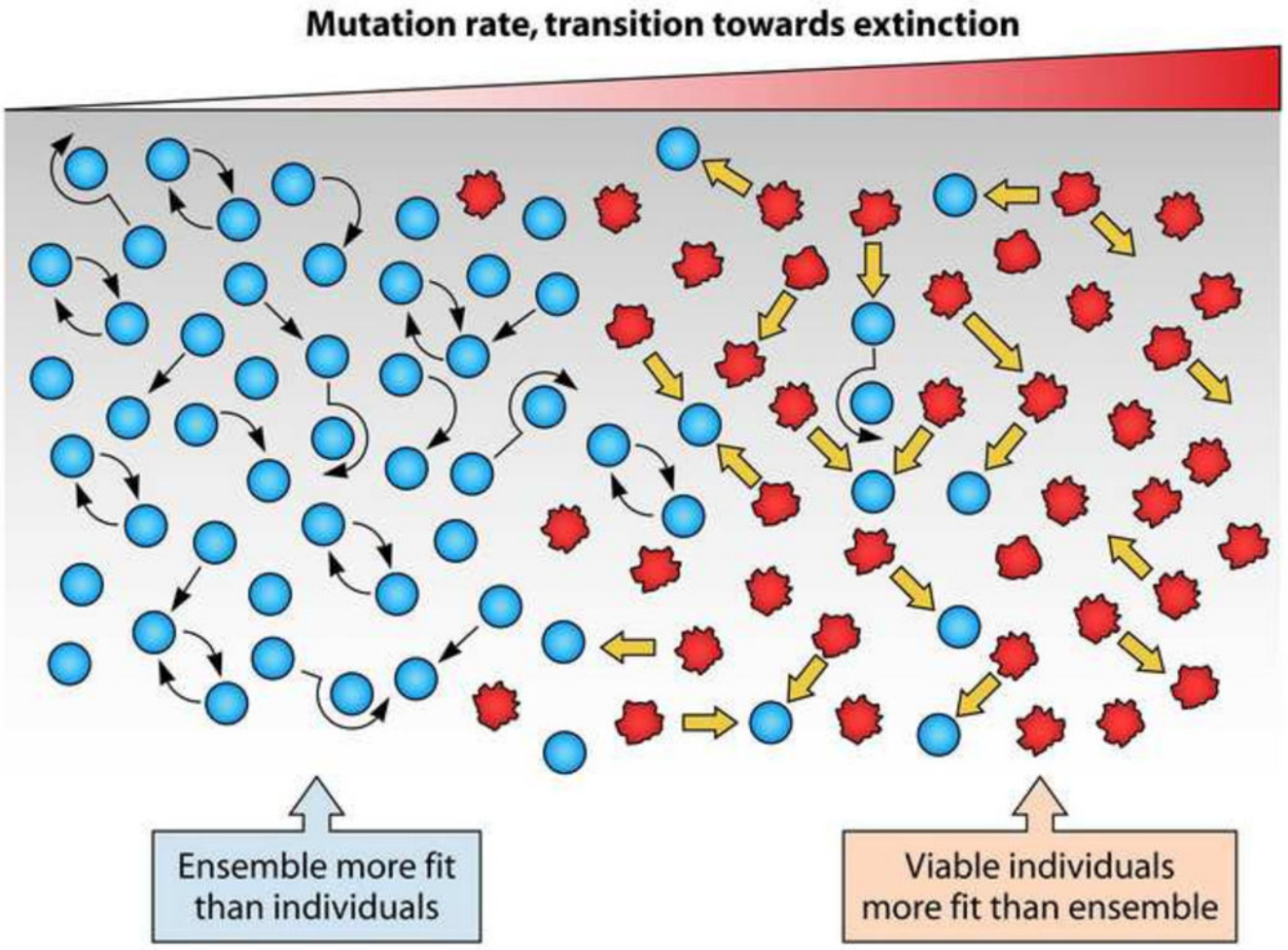

Figure 1.

Schematic representation of a transition from a population in which complementation prevails into a population dominated by interference produced by defector genomes whose proportion increases with the mutation rate. See text for references. [The figure is reproduced from (Domingo et al., 2012), with permission from the American Society for Virology, Washington DC, USA].

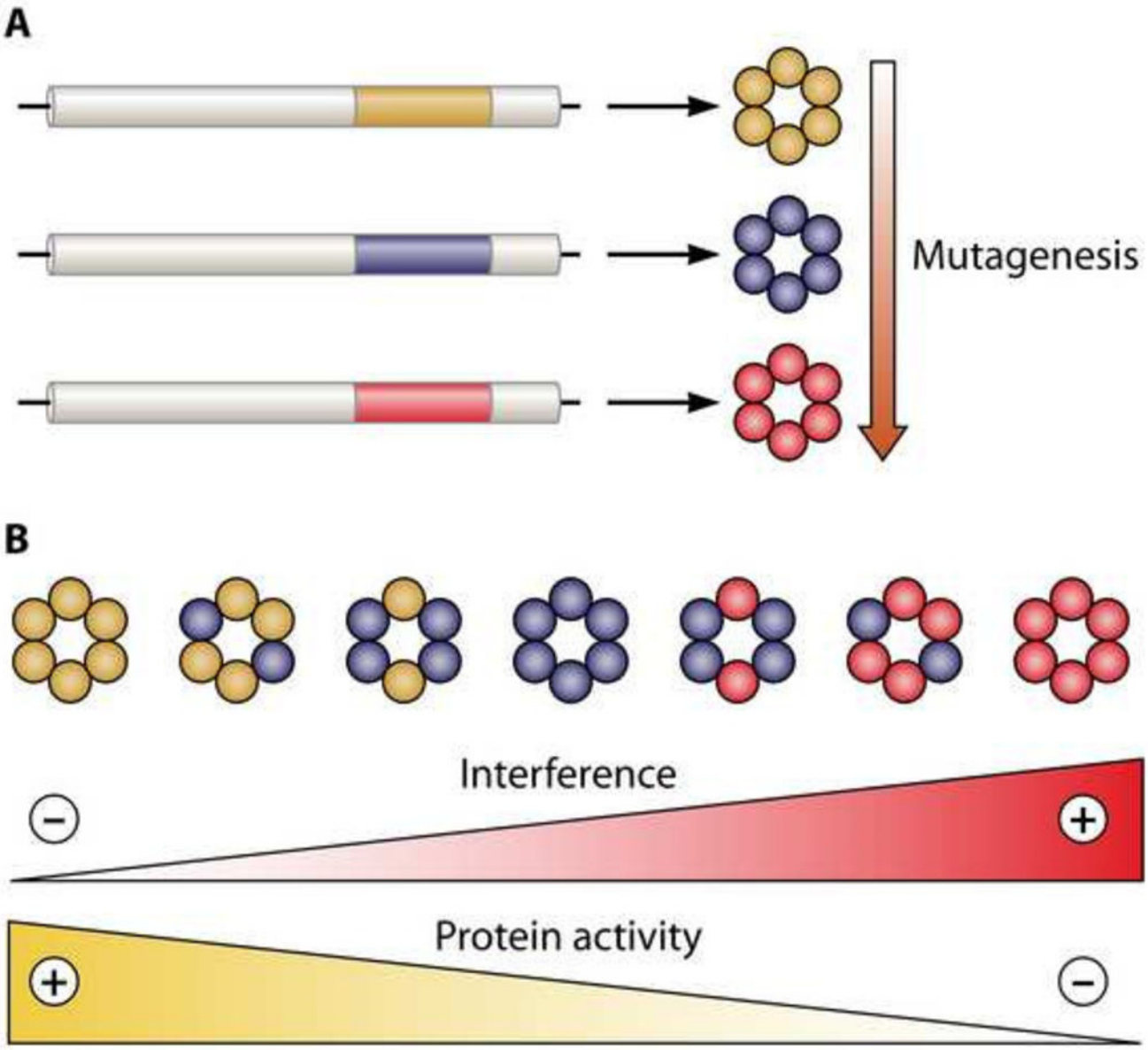

Figure 2.

Illustration of a lethal defection mechanism mediated by a trans-acting protein which is functional as a hexamer. Yellow, blue and red colors represent a gene and encoded protein with increasing numbers of amino acid substitutions. Substituted monomers display interfering potential by virtue of forming chimeric hexamers with reduced activity. [The figure is reproduced from (Domingo et al., 2012), with permission from the American Society for Virology, Washington DC, USA].

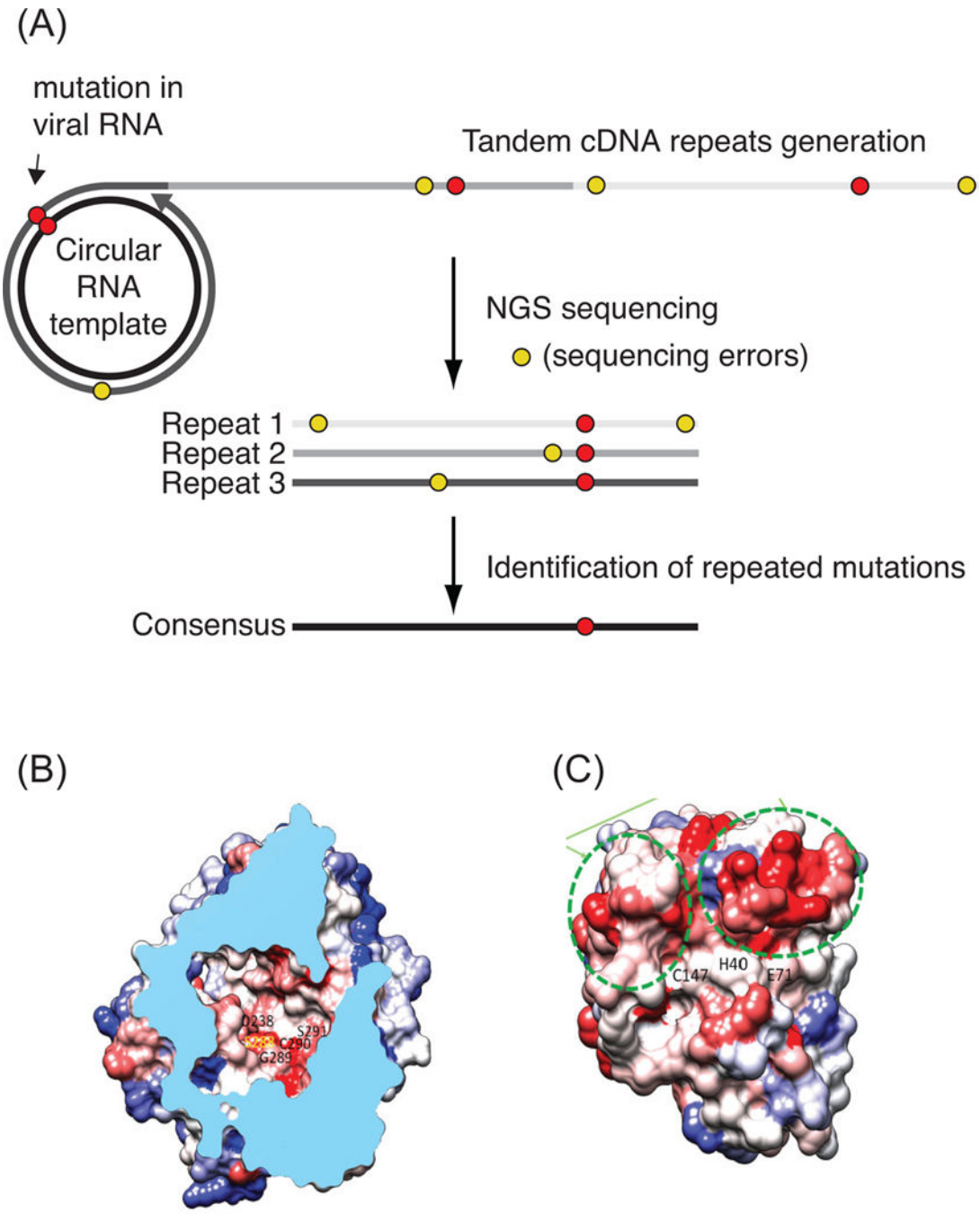

Figure 3.

(A) Schematic representation of CirSeq procedure. Short viral genomic fragments (~90b) are circularized to serve as templates for rolling-circle cDNA synthesis. The resulting tandem repeats are sequenced as a single read. The repeats can be subsequently identified and aligned. The red symbol represents true genetic variation, which, because are present in the viral genome RNA template, appear in each repeat. The yellow symbols represent sequencing errors. Sequencing errors are unlikely to occur in each of the three repeats, and thus can be identified and excluded from the consensus sequence. (B and C) Fitness values

shown on a scale of red (lethal) to white (neutral) to blue (highly beneficial) on poliovirus polymerase and protease. (B) A split view of the active core reveals strong negative selection in regions associated with essential polymerase functions: RNA binding and catalysis. (C) The front view of the poliovirus protease shows the active core where a number of residues are under strong negative selection essential protease functions.