Review article
Virus-host symbiosis mediated by persistence

Luis P. Villarreal

Center for Virus Research, 3232 McGaugh Hall, University of California Irvine, Irvine, CA 92697, USA,
Tel. +1-949-824-9314, Fax. +1-949-824-9437, Email. lpvillar@uci.edu

Abstract

In the last several years, numerous reports have suggested that viruses of various types might have contributed to the evolution of important and basic host characteristics. This would represent a symbiotic viral involvement in host evolution. Here, I outline many of these suggestions. However, in order for viruses to succeed as symbionts, they must attain a stable or persisting relationship with their host. This review focuses on the relationship between persisting viruses and their host. In particular, mechanisms of persistence that involve addiction (toxin/antitoxin) strategies are presented with respect to viral defense. The potential role of persisting viruses in cyanobacterial and prokaryotic evolution is also presented. Popular models of symbiosis are then evaluated from the perspective of potential viral involvement. Human evolution in relationship to endogenous retroviruses is also mentioned, but this topic presented more thoroughly in a companion manuscript (F. Ryan). Finally, an example is presented using symbiotic dinoflagellates in which evaluating the possible role of persisting viruses is suggested.

1. Introduction

That viruses (genetic parasites) might contribute in constructive way to the establishment and maintenance of symbiosis is not a broadly held view. However, in the last several years numerous proposals have been published that suggest that viruses may symbiotically contribute to host evolution, including suggestions that DNA viruses and retroviruses were involved in the origin of the DNA replication apparatus for all three domains of life, (Forterre, 2005; Forterre et al., 2005; Forterre, 2006a,b; Koonin et al., 2006), that large DNA viruses (like Mimivirus) might constitute an ancient fourth (LUCA-like) domain of life (Raoult et al., 2004; Desjardins et al., 2005; Claverie et al., 2006), that cytoplasmic DNA viruses might have been the ancestors of the eukaryotic nucleus (Villarreal, 1999; Villarreal and DeFilippis, 2000; Bell 2001; Takemura, 2001; Bell, 2006), that RNA and retroviruses were ancestral to the RNAi innate immune system (Pickford and Cogoni, 2003; Bagasra and Prilliman, 2004; Vastenhouw and Plasterk, 2004; Schmidt, 2005), that retroviruses RNAse H fold or phage-like transposases were ancestral to the adaptive immune system (Dreyfus et al., 1999; Kapitonov and Jurka, 2005; Fugmann et al., 2006), that retroviruses were ancestral to the origin of flowering plants (Villarreal, 2005), and that endogenous retroviruses were ancestral to the development of the placental viviparous mammals (Villarreal, 1997; Harris, 1998; Blond et al., 2000; Mi et al., 2000; Stoye and Coffin, 2000; Blaise et al., 2003; Dupressoir et al., 2005). All of these situations represent potentially important examples of viral-host symbiosis that may have led to the biogenesis (symbiogenesis) of major new complex features of living systems. Although all these suggestions can be both argued for or against with the limited current information one thing seems clear: taken together, the role of viruses as symbionts for host evolution must now be considered more seriously (Koonin, 2005; Moreira and Lopez-Garcia, 2005; Iyer et al., 2006),

Viral based symbiosis will seem like a counter intuitive idea to most readers. Viruses are not organisms, they appear

not to establish stable long-term relationships with most hosts, and they lack metabolic sophistication. These obligate genetic parasites often kill or consume their host and seem to be too simple to contribute to host evolution in any meaningful and constructive way (see the companion article in this issue by F. Ryan for background and definitions concerning viral symbiosis). Thus historically, viruses have not factored into thinking concerning how symbiosis develops. However, in the last ten years, we have come to the realization that viruses are the dominant biological entities found in most habitats that support life in the world (both numerically and by genetic diversity). Various shotgun sequencing projects now inform us that the oceans, soil, and extreme environments such hydrothermal vents, are all composed predominantly of diverse viral agents (Paul et al., 2002; Rohwer, 2003; Breitbart et al., 2004; Edwards and Rohwer, 2005; Filee et al., 2005; Ghedin and Claverie, 2005; Hambly and Suttle, 2005; Comeau et al., 2006). Many (but not all) of these viruses appear to be tailed DNA viruses that infect prokaryotes. However, they are not all lytic as a substantial fraction of the sequenced genomes have clear genetic hallmarks (integrases and immunity regulators) of temperate or persistent viruses. Such viruses are expected to stably colonize their host.

2. Host Stability and Viral Persistence

It is well accepted that viruses can have a predator-prey like relationship with their host in which lytic viruses consume their host, as seen in most human epidemics. However, much more common (albeit less familiar) and much more stable are those unseen viruses that can colonize their host (or host genomes) in a persistent ways such that the host is unable to eliminate the virus, but does not succumb to an acute infection. All humans (or feral mice) for example, harbor such persisting but species-specific viral agents. Previously, my students K. Gottlieb, V. Defillippis and I defined the existence of two distinct life strategies for most viruses; acute and persistent (Villarreal et al., 2000).

Persistent infections are lifelong, usually in apparent infections of an individual host (inclusive of lysogeny). However, persistence is not simply a less efficient version of an acute infection. To be fit, a persistent virus must be temporally stable, often silent, even to the point of suppressing production of progeny virus replication. Furthermore, defective virus can clearly contribute to persistent infections, thus resembling 'selfish' or non-coding DNA. Persistence tends to be highly species-specific, less dependent on host population structure and co-evolve with their host, sometime showing almost clonal viral populations. On an evolutionary time scale, most successful viruses are persistent. To accomplish persistence, viruses require molecular strategies that allow for a generally silent, long-term residency in a specific host. Such strategies must be able to operate superimposed on all the innate and adaptive immune systems of their specific host. I have proposed that this strategy can be considered as a 'persistence module', which is also a version of an 'addiction module' (Villarreal, 2005). Thus, in order to evaluate if persisting viruses might have contributed to the evolution of symbiotic relationships with its host, it will be necessary to examine the likely mechanisms by which persistence is normally attained.

3. Persistence and Virus Addiction

As with bacteria, not until the 1950's did it become clear that in addition to lytic phage, persisting (temperate) phage infections were also prevalent. However, in the last several decades it has become also clear that in Archaea, the great majority of virus infections are persisting or chronic (Zillig et al., 1996). For excellent recent reviews see Prangishvili et al. (2006a,b). The viruses of Archaea show an unprecedented level of morphological and genetic diversity. All such known viruses have dsDNA genomes, many of which are circular and integrating (tRNA site adjacent) but also many which are linear and non-integrating (such as Ligamenvirales, TTV1). Interestingly, TTV1 production can be induced from stable persistence by cell stress (translational toxins, UV light), but induction is non-lytic. Thus, this whole domain of life has a mainly persistent relationship with its viruses. Although, some lytic viruses such as SH1 are known; it is not known how is this Archaeal extra-genomic virus stability is attained. Both viruses and hosts may use transcribed repeat elements as part of a defense system (clearly resembling RNAi system of Eukaryotes), but the mechanism is neither clear nor understood. Thus, the mechanism and details of viral persistence in Archaea are also not known.

Only in bacteria, have such mechanisms been worked out in considerable detail. For a non-integrating virus, the P1 phage of *E. coli* has been most examined and has lead to the addiction module idea. The concept of an addiction module was first proposed in 1993 to explain the ubiquitous but stable persistence of the episomal genome of the P1 phage of *E. coli* (Lehnherr et al., 1993). In order for this non-integrating phage to become a highly stable resident of its dividing host, it needs a molecular strategy that would compel the host to retain the phage that is beyond the primary need for viral self immunity control. It was observed that if *E. coli* underwent division and one daughter failed to retain the P1 phage (was cured), this daughter would die upon segregation (death on curing, or doc).

Molecular investigations into this situation established the existence of several 'addiction modules' that would kill

E. coli that had lost the P1 (Hazan et al., 2001). These modules consist of matched genes or functions in which a stable but toxic gene (such as a membrane pore protein of DNA restriction enzyme) was suppressed by the presence of an unstable but protective matching function (anti-pore protein, DNA modification enzyme, antisense-RNA, etc.). Loss of the P1 genome led to loss of the unstable protective function and death of the cell. In this case, one of the P1 addiction modules (Phd/doc) actually operated via a second addiction module (MazEF) which itself is a toxin/antitoxin (T/A) gene pair already present in most *E. coli* strains (Hazan et al., 2001; Kamada et al., 2003; Jensen and Gerdes, 1995; Hazan and Engelberg-Kulka, 2004). These addiction modules together would also compel P1 persistent cells to kill themselves if they became infected by other viruses (such as T4 and lambda, thereby creating a form of cellular or group immunity (Engelberg-Kulka et al., 1998).

Thus, addiction modules can operate via T/A (toxin/antitoxin) gene sets to compel the stable maintenance of the virus and the cell (symbiotically). If this addiction state is violated, cells die rather than tolerate loss of addiction, resulting in a form of molecular group identity. In bacteria, toxin-antitoxin modules are now known to be very common, especially in long-lived species (Engelberg-Kulka and Glaser, 1999; Engelberg-Kulka et al., 2006). These modules often respond to cell stress and kill the individual host cell (clearly resembling apoptosis of eukaryotes). In so doing, it is thought that T/A genes provide defense against the spread of virus, but the T/A genes themselves resemble virus derived genes. Thus, new addiction modules can be stably acquired from successful new persisting genetic parasites, not cellular ancestors. This can create a situation that leads to cumulative acquisition of genetic complexity in the host from external sources. Since these very same persistence systems also provide a new host immune system, with altruistic (suicidal) characteristics (i.e. killing individuals whose addiction state is disrupted), a viral-based origin of host immunity and complexity now becomes apparent via persistent mechanisms. Therefore, a viral-mediated host 'altruism' can result from addiction without the need for concept of kin selection.

In my book (Villarreal, 2005), I have generalized this argument and suggest that that most persistent viral infections employ some form of addiction strategy (although not necessarily a T/A gene pair). This includes a state of 'virus addiction'. Here, a persistently infected host will use the pathogenic potential of its specific virus to kill other hosts that it encounters that are not also persistently infected (and protected). In virus 'addiction', killing works on a non-colonized population, not an individual cell level, and depends on the pathogenic capacity of the virus to harm non-infected populations. It is now clear that species-specific viral agents, which are harmful to non-persistent populations, persistently infect most natural populations of prokaryotes and animals. This concept is equivalent to that of 'aggressive viral symbiosis' as proposed by F. Ryan in an accompanying manuscript of this journal and originally proposed nearly ten years ago (Ryan, 1997). In bacteria, there are many examples in which bacterial genes are harmful to other related bacterial populations. Bacteriocins (*E. coli*) and pyocins (*P. aeuruginosa*) are prevalent toxins and have the capacity to kill related host that lack the immunity to specific bacteriocins (Riley, 1998; Michel-Briand and Baysse, 2002). As suggested above, it is very interesting to note that most of these bacteriocins are themselves evolved either from phage structural proteins (base plates, tails, capsids) or from phage encoded pore toxin or endonucleases (the soluble type) proteins that have persisted in host genomes (Nakayama et al., 2000).

4. Cyanobacterial Evolution

Cyanobacteria are of special interest for the study of symbiosis since they are considered to have originated from the photosystem II form of photosynthesis, in which H_2O is the electron acceptor and which had such an enormous consequence to the evolution of complex life forms. Their fossils (stromatolites) represent one of the earliest examples of ancient cellular communities on earth. Cyanobacteria are also considered to have been the symbiotic progenitors of chloroplasts of algae and of plants and transformed our view of the role symbiosis plays in evolution (Margulis and Bermudes, 1985). Also, they are observed to still form many symbiotic relationships with other organisms, such as with lichen and sponges. In modern earth, they still hold a special position and are one of the few examples of a vast biological community visible from space, in that blooms of cyanobacteria can be observed from the NASA space shuttle.

What does virus have to do with any of this? It has been proposed that the termination of some of these toxic blooms can be due to the action of lytic cyanophage, although this observation is not uniformly made (Chen and Lu, 2002; Yoshida et al., 2006). Two major classes of phage are known to infect cyanobacteria (prochlorococcus, synechococcus) T7-like and T4-like phage (Sullivan et al., 2005). Recently, however, it was very surprising to learn that both of these lineages of phage encode their own distinct version of photosystem II core proteins (Lindell et al., 2004; Sullivan et al., 2006). It had not previously been thought that phage could encode and maintain such a basic metabolic function, thought to be highly specific to the host (Mann et al., 2005). Furthermore, these phage proteins were more resistant to photodynamic inactivation than those of the host, indicating they are distinct phage-specific versions.

In the case of prochlorococcus, it was known that the T7-like phage are distinct from those of *E. coli*, as these

cyanophage are temperate (integrating & persisting) but have tight associations with the host (high light, low light adaptation). Prochlorococcus is also of special interest from a general evolutionary perspective since its plant-like photosystem II is thought to represent the basal version of this system in evolution. In fact, current analysis of genomic sequence indicates that the Prochlorococcus photosystem II has been rapidly evolving due to large-scale ongoing lateral transfer of these genes. The origin of such genes, however, has been mysterious. However, together these observations make several clear points: viruses have invented core proteins of photosystem II, viruses colonize their host, and host photosystem II is rapidly evolving due to ongoing genetic colonization.

One implication from the above results is that viruses may be the source of photosynthetic host genes. Such an implication, however, is at odds with the widely held view that a viral role in the lateral gene transfer only uses viruses as a vector, like a football lateral, to transfer genes from one host lineage to another. Viruses don't invent such genes; they merely truck them between hosts. Recent, metagenomic studies of oceanic DNA viruses, however, indicates that viruses are creating genes in vast numbers. Viral persistence, would allow some of these viral genes to directly affect host survival. For example, Prochlorococcus is known to dwell in specific oceanic habitats and each of these habitats appears to harbor its own version of this cyanobacteria (called an ecotype). Recently, six such ecotypes were characterized and sequenced (Bouman et al., 2006; Coleman et al., 2006). Since the overall genome sequence is highly conserved (over 99%), it was possible to identify all the genetic differences between these ecotypes.

The differences were due to about 5 multigene regions of 'phage islands' in which a phage-mediated integration event had clearly occurred (adjacent to tRNA sites). These islands consisted of sequences that were roughly 80% phage identifiable genes (structural proteins, immunity modules, integrases, etc.), the core photosynthesis genes plus about 20% of unique, mostly light responsive genes of unknown function. Although the persistence or addiction systems of these phage are not yet characterized, persisting plasmids are also common in cyanobacteria (Lau et al., 1980). An implication of this observation is that dynamic persistent phage colonization is highly associated with local fitness differences in these ecotypes. Furthermore, the presence of such phage-like islands appears to be a general situation for all prokaryotes.

For example, in the ECOR collection, the 72 sequenced *E. coli* genomes differ mainly by colonization patterns of prophage, cryptic and defective prophage and some plasmids. Since these islands are also mainly adjacent to tRNA sites, they are clearly products of virus-mediated integration (Hurtado and Rodriguez-Valera, 1999; Mazel et al., 2000; Nilsson et al., 2004). When the *E. coli* genome was initially sequenced and compared to the *B. subtillis*

genome, the two genomes were found to mostly differ by several hundred multigene chromosomal domains that were also mostly adjacent to tRNA integration sites (Sonenshein et al., 2002).

All of this supports the concept that persisting (genomic) viruses are major forces in cyanobacteria (and prokaryote) evolution. However, the usual view is that when viral genes resemble host genes, they must necessarily arise from host sources (Clokie et al., 2006) or are stolen from them (Moreira and Lopez-Garcia, 2005). Given the enormously greater evolutionary capacity of viruses, which can assemble viral genes by modular, mosaic evolution relative to host; viruses must now be considered as a possible primary source of host related genes.

5. Viruses as Gene Factories

With the completed sequencing of many viral genomes, we have now evaluated entire lineages of related DNA viruses to understand how they generally evolve as new genes. The first such entire lineage evaluated was for the baculoviruses (large DNA viruses of insects and invertebrates), and a clear and consistent picture has emerged (Herniou et al., 2001). Most DNA viral evolution is by gene acquisition onto a conserved core gene set (with some gene loss) and these newly acquired viral genes are unique, not host derived. On average, from nearly 2 million viral sequences, about 80% of the new viral genes have no match in the GenBank database habitats (Breitbart et al., 2004; Breitbart and Rohwer, 2005). Those viral genes that are similar to host genes are most often basal to host genes (Claverie et al., 2006).

Only a few examples of viruses acquiring genes from hosts are seen (i.e., viral genes at the tips of host gene dendrograms). In the case of the bacterial phage, it has become clear that the long held hypothesis that new phage genes are being assembled from mosaic domains acquired mainly via recombination with other temperate and lytic phage is correct (Hendrix, 2003; Hendrix et al., 2003; Saren et al., 2005; Casjens, 2005). The most extensive evidence for this view has come from the ongoing 50-year study of phage that affect fermentation of the dairy industry (Brussow et al., 1998; Brussow, 2001). Lytic phage, which acquire new mosaic genes, can evolve from temperate phage that have lost their immunity control (Tuohimaa, Riipinen et al., 2006). This extensive mosaicism has led some to propose a new virus taxonomy based on single structural genes (Proux et al., 2002). Furthermore, phage, plasmids and transposable elements all appear to also evolve by the same mosaic process, which suggests an essentially seamless continuum between viral and plasmid evolution (Osborn and Boltner, 2002).

More recently, the very large (1.2 MB) Mimivirus of amoebae has been characterized, with 911 ORFs including

translational genes, plus many unique genes (Claverie et al., 2006). This very large virus itself appears to evolve by genetic colonization of other bacteria-like genetic parasites, but the eukaryotic-like genes it contains are mostly basal to those found in eukaryotic host (Filee et al., 2006). The host persistence of such a complex virus would likely have an enormous impact on host evolution. Various shotgun sequencing projects of ocean water have confirmed the vast genetic novelty of viruses present in natural habitats, especially the oceans (Ghedin and Claverie, 2005; Breitbart et al., 2004; Breitbart and Rohwer, 2005). Some have proposed that this enormous viral population can provide a source of genetic novelty that preassembles compatible genes into gene sets and could be considered as the biological "big bang" of evolution (Filee et al., 2006).

6. Virus Footprints in Models of Symbiosis

For the most part, it has not been determined if viruses or their addiction modules contribute in any observable way to current models of symbiosis. Yet there are numerous observations that suggest viral footprints are everywhere in symbiosis and that resulting addiction states may be an enabling strategy for symbiosis. For example, although mitochondria evolved from alpha-proteobacteria, but many organelle proteins, such as DNA polymerase, DNA primase and RNA polymerase have been displaced by T7/T3 related phage genes like those of cyanobacterial (Filee and Forterre, 2005; Forterre, 2006). T7 is a lytic *E. coli* phage family that has become integrating and temperate in cyanobacteria. Why were such crucial plastid genes stably replaced by these viral versions? Perhaps T7 related proteins have promoted stable colonization, somehow compelling a symbiotic relationship.

In higher plants, chloroplast transcription is controlled by two RNA polymerase: a plastid encoded eubacterial-like and a nuclear encoded T7-like polymerase (Kanamaru and Tanaka, 2004). The nuclear localization of the T7-like polymerase gene is curious and could suggest some role in linking the chloroplast to the nucleus. Since photosynthesis produces molecular O_2 which in turn produces ROS (reactive oxygen species, the most toxic of cellular molecules), it seems possible that photosynthesis could have provided the T part of a TA pair in which the symbiotic cellular host needed to provide a strongly reducing antitoxin environment. Some paramecium and hydra that support symbiotic photosynthetic green algae are clearly providing a cellular environment that protects the algae from highly prevalent and lytic phycodna virus (Van Etten et al., 1982; Van Etten, 2003). All these species seem to have their own specific version of phycodna virus that can lyse their resident unicellular chlorella-like algae.

This relationship could identify a state of 'virus addiction' that compels the algae to remain within their host for protection. The photosynthetic sea slug (*E. chlorotica*) is able to host and use chloroplast as from algae eaten early in its development. During sexual reproduction, after extended symbiosis, all adult slugs start producing a very unusual endogenous retrovirus and release gametes but die soon thereafter (Pierce et al., 1999). It remains to be evaluated if this retrovirus (absent from nonsymbiotic species) is involved in the symbiosis and the horizontal transfer of photosynthetic genes (Pierce et al., 2003).

With Wolbachia, we have seen the presence of species-specific persisting WO phage highly expressed as viral capsid structures in the calyx that surrounds the host wasp egg (Duron et al., 2006; Wright et al., 1978). Since egg survival is being manipulated by Wolbachia, the WO persistence may well play a role in this symbiosis (Moran et al., 2005; Bordenstein et al., 2006). These WO capsid proteins may be providing some type of TA function (bacteriocin-like) to stabilize the symbiosis. *Buchnera aphidicola* is a primary symbiont of pea aphid, but there is also a secondary highly protective symbiotic bacteria (*H. defensa*) that produces a podoviral phage encoded distending toxin (Moran et al., 2005).

This phage toxin is active against the larvae of the parasitoid wasp, which is the major predator of the pea aphid. This phage toxin would clearly have the toxic half of a TA module, but its role in symbiosis is not clear. Intriguingly, hymenoptera parasitoid wasps must themselves produce an endogenous symbiotic DNA virus (polydnavirus) in the calyx that surrounds the egg for oviposition (Beckage, 1998; Turnbull and Webb, 2002; Whitfield, 2002; Federici and Bigot, 2003; Stoltz and Makkay, 2003). This polydnavirus is essential for the parasitoid larvae to suppress host defenses and allow the wasp to develop (Beckage, 1998). Here, viral symbiogenesis is clearly established in a highly successful insect species.

7. Viruses as Human Symbiotic Ancestors

The human genome, in particular, is colonized by numerous human specific endogenous retroviruses (HERV K, HERV W, HERV FRD). The details of this relationship are developed and presented in the companion manuscript of F. Ryan in this journal. Since it is now well established that at least some of these retroviruses are involved in basic placental function, it is clear that retroviruses contributed to the evolution of humans, and possible most placental mammals. Humans also harbor many non-genomic persisting viruses that have co-evolved with us, such as 8 human specific herpes viruses. The consequences of such colonization for human evolution, however, are not understood but there are many reasons to suspect that persisting viruses have had a major effect on human evolution.

8. An Exercise in Considering Symbiosis from the Perspective of a Persistent Virus

The possibility that persisting, latent, viruses acting via addiction strategies might contribute to stable symbiotic relationships should now be adopted as an experimental approach. Let us examine one specific example to see how this might affect experimental evaluation. Dinoflagellates have chloroplasts and are often symbiotic with many marine invertebrates, such as corals, sea anemones and giant clams, which provide photosynthetic products. Dinoflagellates also have virus (Soyer, 1978). Coral bleaching correlates with loss of symbiotic dinoflagellates (zooxanthellae) and is of major ecological concern. This bleaching can also be induced by cellular stress, such as elevated temperatures. Such stress is also associated with the induction of latent virus, suggesting a disrupted addiction module.

For example, the sea anemone *Anemonia viridis* will induce the production of viral like particles (VLPs) following heat stress and these VLPs are able to lyse free living zooxanthella (Wilson et al., 2001). However, latently infected zooxanthella are immune to VLP mediated lysis. These observations are usually considered from the perspective of viral mediated lysis of the dinoflagellate symbiont and the latent virus has been referred to as the 'enemy within" (Wilson et al., 2005). I would suggest this situation needs to be evaluated from the alternative perspective that this specific latent virus may be providing an addiction strategy that stabilizes the symbiosis, and that the temperature stress has destabilized the toxin/antitoxin system.

Most dinoflagellates are photosynthetic, free-living, motile   single cells, many of which make toxins. Dinoflagellates are unusual eukaryotes in that they have a closed (non-s-phase dissolving) nuclei that lacks histones in which the chromosomes are in a condensed liquid crystal state and transcriptionally active DNA domains project out of the nucleus into the cytoplasm. All known DNA viruses of dinoflagellates are cytoplasmic and the great majority are lytic (resembling chlorella viruses) (Nagasaki et al., 2003). Red tide blooms of dinoflagellates have been reported to terminate by such DNA virus infections (Onji et al., 2003).

This suggests that the latent viruses of zooxanthella could be cytoplasmic and extrachromosomal as well, but would be highly host specific. Stability of such a latent virus (like episomal P1 phage mentioned above) would likely require some type addiction module that both assures cellular immunity and provides stability that preserves the host dinoflagellate in the environment of the sea anemone. If such a state exists, I would suggest that it could be involved in preserving the specific symbiotic relationship and resisting other genetic parasites. Such a proposal would be amenable to experimental evaluation since free-living versions of zooxanthella can be grown apparently without

latent virus. However, if we continue to dismiss the possible importance of viral persistence and fail to evaluate it experimentally, we may inhibit our understanding of the genetic and selective forces that promote and enable symbiosis.

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