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Current Opinion in Virology

Adaptation by copy number variation in monopartite viruses

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Viruses evolve rapidly in response to host defenses and to exploit new niches. Gene amplification, a common adaptive mechanism in prokaryotes, archaea, and eukaryotes, has also contributed to viral evolution, especially of large DNA viruses. In experimental systems, gene amplification is one mechanism for rapidly overcoming selective pressures. Because the amplification generally incurs a fitness cost, emergence of adaptive point mutations within the amplified locus or elsewhere in the genome can enable collapse of the locus back to a single copy. Evidence of gene amplification followed by subfunctionalization or neofunctionalization of the copies is apparent by the presence of families of paralogous genes in many DNA viruses. These observations suggest that copy number variation has contributed broadly to virus evolution.

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Current Opinion in Virology 2018, **33**:7–12

This review comes from a themed issue on **Multicomponent viral systems**

Edited by **Stéphane Blanc** and **Yannis Michalakis**

https://doi.org/10.1016/j.coviro.2018.07.001

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Introduction

Viruses are extraordinarily diverse in their genomic architecture, gene content, and replication strategies. However, they share common challenges, including the need to exploit the host cell biosynthetic machinery and to evade host defense systems. Over time, evolutionary pressures select for viruses that are better able to replicate and spread to new hosts.

The genetic adaptations that confer fitness improvements can occur by changes as simple as point mutations in single genes. For most RNA viruses, point mutations are generated at a high frequency during genome replication because the replicases lack proof-reading function [1,2]. DNA virus polymerases have much higher fidelity so that point mutations arise less often [3]. This slower mutation rate is likely sufficiently rapid to keep pace with the slow evolution of host genes. However, there are situations, such as drug selection or infections of new and more resistant host species, where a more rapid mechanism of adaptation is beneficial.

In contrast to viruses with segmented genomes, this paper focuses on those in which the genome consists of one polymer of single or double-stranded nucleic acid. Acquisition of new genes by reassortment as occurs with segmented viruses is not possible for these monopartite viruses. However, they can adapt by acquiring large genomic segments by horizontal gene transfer from the host cell, recombination with other viruses, or amplification of endogenous regions of their own genomes. Because viral genome size is often strictly limited by packaging constraints, mechanisms that expand the genome size seem surprising. Nonetheless, considerable evidence exists for viruses having assimilated host genes [4–6] and, as we discuss below, experimental and observational data provide compelling support that copy number variation can and does occur and may play a prominent role in viral evolution.

Gene amplification in experimental systems

The first demonstration of experimental selection for viral gene amplification was described in bacteriophage T4 with an amber nonsense mutation in the essential gene 17 [7]. These phage replicated inefficiently in *Escherichia coli* containing an ochre suppressor. However, some progeny replicated well due to overexpression of gene 17, resulting from tandem amplification of up to 6 copies of a ~4 kb genomic segment containing genes 17 and 18.

Shortly after demonstration of gene amplification as an adaptive mechanism in phage, it was observed in the eukaryotic virus, vaccinia virus (VACV) [8]. Selection for resistance to hydroxyurea, an inhibitor of ribonucleotide reductase, yielded VACV mutants with multiple tandem copies of the viral ribonucleotide reductase gene. More recently, VACV selected for resistance to rifampin, which inhibits VACV virion assembly, yielded a virus with a duplication of a ~2.4 kb segment of the genome [9]. Among the genes in this segment, a truncated variant of A17 was found to account for the rifampin-resistant phenotype. A17 was considered the likely candidate

among the amplified genes since it was known to interact with the scaffold protein D13 during virion assembly. Prior to this study, all known rifampin-resistant mutations were in the D13 gene. Proof of A17's role came from finding that insertion of one extra copy of either the truncated or full length gene alone into VACV conferred rifampin-resistance. The observation that a truncation of A17 conferred rifampin-resistance underscores the fact that amplification does not need to involve a complete gene to provide a replication benefit. Intriguingly, this observation also suggests one mechanism for subfunctionalization, wherein a fragment of a gene encoding a single function may serve as the initiating event to permit separation of multiple functions.

In addition to examples of selection for drug resistant mutants, evolution by gene amplification has been shown to enable adaptation to overcome host cellular defenses. For example, VACV encodes two proteins, E3L and K3L, that block the host protein kinase R (PKR) antiviral pathway [10]. E3L is a double-stranded RNA-binding protein that is a potent inhibitor of PKR in many primate cells, while K3L acts as a pseudosubstrate inhibitor of PKR in some rodent cells but has limited efficacy in antagonizing primate PKRs. As a result, VACV containing K3L but lacking E3L (VACVΔE3L) replicates very inefficiently in human cells. Serial passage of VACVΔE3L through human cells selected for viruses with amplification of K3L and portions of the flanking genes [11••]. Consistent with a simple gene dosage mechanism, the viruses with K3L amplification expressed high levels of the K3L protein and knocking down K3L expression reversed the replication phenotype.

In a similarly designed set of experiments, Brennan *et al.* engineered a VACV recombinant lacking VACV PKR antagonists (E3L and K3L) and containing a cytomegalovirus PKR antagonist, rTRS1, that is active in some but not all Old World monkey cells [12•]. This virus replicated poorly in one African green monkey cell line and not at all in human and rhesus cells. However, after a few passages, mutant viruses emerged that replicated to ~10-fold higher titers as a result of amplification of the rTRS1 locus. Notably, these viruses replicated better than the parent virus not only in the African green monkey cells in which they were selected, but also in cells from other species. This result highlights the potential role of gene amplification in conferring rapid adaptation that could facilitate cross-species transmission.

A common feature in these experiments is the rapidity with which the amplifications arose. Erlandson, *et al.* isolated a virus with the A17 amplification after a single plaque purification in the presence of rifampin [9]. The emergence of adapted VACVs with amplifications of PKR antagonists took only 4–6 passages in multiple independent experiments [11••,12•]. It is unclear when the ribonucleotide reductase duplication arose following exposure to hydroxyurea, but resistance was noted between 3 and 6 passages, suggesting a similar time course [13]. One possible explanation for these kinetics is that random gene amplifications arise often during VACV replication. Though rare at any particular locus in the starting pool, a virus with an adaptive amplification will be enriched with each passage until it emerges as the dominant viral species after relatively few passages. Consistent with this model, Elde *et al.* detected a low frequency of gene duplications at several other loci in the VACV genome distant from the K3L amplification [11••]. Sequencing methods with higher fidelity than those used previously should help clarify the frequency, location, and size distribution of amplifications in unselected viral populations.

These studies reveal that the gene amplification mechanism is quite versatile. In the VACV experiments, the amplifications occurred at multiple loci throughout the genome, including both natural VACV sequences and those from heterologous sequences [8,9,11••,12•]. Multiple different 'breakpoints' were created by the amplification in different VACV experiments. Although no sequence similarities were recognized at these sites in the VACV experiments, most of the T4 phage with amplification of genes 17 and 18 shared the same breakpoint within a short region of homology (20 of 24 bases) in genes 16 and 19 [14]. Even though the mechanism accounting for the initial duplication is unclear and might vary in phage vs. eukaryotic viruses, once duplicated, expansion and contractions by a recombination mechanism appear to occur in a highly dynamic manner. For example, after plaque purification of VACVs with the amplified loci, the progeny virus genomes consistently contain a variable number of copies [11••,15]. In some cases, the amplifications increase the genome size by 10–20% [8,11••]. However, it is not known if these large genome variants are replication competent.

Although the amplification increases viral replication under selective conditions, it likely incurs a fitness cost. In support of this idea, the gene 17 and 18 locus amplification in the T4 phage with the amber mutation disappeared immediately when the selective pressure was removed by propagation in *E. coli* with an amber suppressor [14] (Figure 1a). Similarly, the amplification of the VACV A17 locus was lost when the virus was propagated in the absence of rifampin [9]. The K3L average gene copy number decreased when the VACVΔE3L having multiple K3L copies was passed in rodent cells, in which a single copy of K3L is sufficient for efficient replication [11••]. In the absence of pressure for high level expression of the amplified gene, viruses having the amplifications might be less fit because overexpression of the gene might be toxic to cellular processes or perturb the normal expression pattern that may otherwise be finely tuned for

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