Why genes overlap in viruses
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The genomes of most virus species have overlapping genes—two or more proteins coded for by the same nucleotide sequence. Several explanations have been proposed for the evolution of this phenomenon, and we test these by comparing the amount of gene overlap in all known virus species. We conclude that gene overlap is unlikely to have evolved as a way of compressing the genome in response to the harmful effect of mutation because RNA viruses, despite having generally higher mutation rates, have less gene overlap on average than DNA viruses of comparable genome length. However, we do find a negative relationship between overlap proportion and genome length among viruses with icosahedral capsids, but not among those with other capsid types that we consider easier to enlarge in size. Our interpretation is that a physical constraint on genome length by the capsid has led to gene overlap evolving as a mechanism for producing more proteins from the same genome length. We consider that these patterns cannot be explained by other factors, namely the possible roles of overlap in transcription regulation, generating more divergent proteins and the relationship between gene length and genome length.

Keywords: genome compression; mutation; evolution; capsid; virus

1. INTRODUCTION
Gene overlaps, which we define here as having nucleotides coding for more than one protein by being read in multiple reading frames, are a common feature of viruses. Proteins created by gene overlaps (sometimes called ‘overprinting’) are typically accessory proteins that play a role in viral pathogenicity or spread (Rancurel et al. 2009). These overlaps are typically assumed to be a form of genome compression, allowing the virus to increase its repertoire of proteins without increasing its genome length (Barrell et al. 1976; Scherbakov & Garber 2000; Lillo & Krakauer 2007; Chung et al. 2008).

Over the past several decades, many authors have suggested explanations for why gene overlap has arisen and become so common in viruses. In this study, we compare the amount of gene overlap across all known virus species to investigate the plausibility of these explanations. We find that the evidence is most consistent with the main effect being a physical constraint by the capsid (the protein capsule, into which the genome is packaged for transmission between host cells).

(a) Mutation rate
RNA viruses make up approximately one-half of the 2000 known species of virus. They have an extremely high mutation rate and several authors have suggested that this could explain the evolution of gene overlap. The causality might be indirect: because most mutations are harmful, the high mutation rate will limit genome length, and thus new genes or gene regions must come from overlapping (Holmes 2009). Alternatively, several studies have shown how gene overlap in theory might mitigate the detrimental effects of mutation: (i) a numerical simulation (Belshaw et al. 2007) shows a benefit of overlapping except in the case of synergistic epistasis between fitness traits, which is rarely observed in RNA viruses (Elena et al. 2006), (ii) an analytical model (Peleg et al. 2004) quantifies the increasing harm of mutations in overlapping genomes in terms of the information cost (Krakauer 2000) and comes to the same conclusion: a fitness advantage of overlap when mutation rate is high, and (iii) overlapping genes can be seen as one of many ‘antiredundant’ mechanisms that may lead, in the case of mutation, to the damage of distinct functions simultaneously, and which facilitates the removal of mutant genomes from the population (purging; Krakauer & Plotkin 2002). The authors show, through numerical simulations, that this kind of mechanism is likely to evolve in large populations, such as viruses. A more detailed study (Krakauer 2002) concludes that genome compression can increase the stability of the wild-type both by reducing mutation incidence (the advantage discussed above) and by reducing sequence redundancy.

This general argument predicts that DNA viruses, which make up the other half of the known virus species and which tend to have a lower mutation rate, will have less gene overlap.

(b) Capsid structure
Gene overlap might have evolved if genome length is physically limited by the size of the capsid. This was suggested over 30 years ago (Fiddes 1977) and has been invoked since to explain individual gene overlaps (Bransom et al. 1995). Some observations are consistent with capsid size constraining genome length: in most viruses studied, it is not possible to package an artificially enlarged genome (Cann 2001; Campbell 2007), and many studies on different virus groups have found virus genome length to be positively correlated with capsid
size (Belyi & Muthukumar 2006; Nurmemmvedov et al. 2007; Hu et al. 2008; Krupovic & Bamford 2008; Luque et al. 2009; Zandi & Van der Schoot 2009). Furthermore, the hypothesis is testable because, as we describe below, some capsid types might be expected to constrain genome length more than others (Cavalier-Smith 1983; Casjens 1985).

Many viruses have capsids that are icosahedral (20 sided), varying in the number of protein units (capsomers) that form each side. In such viruses, an increase in capsid size is generally achieved through increases in the number rather than the size of these capsomers (Rossmann & Erickson 1985; Chapman & Liljas 2003; Shepherd & Reddy 2005; Krupovic & Bamford 2008). These increases in capsomer number are in discrete steps following a geometric pattern represented by the so-called T number series (Caspar & Klug 1962), which appears to be thermodynamically determined (Zandi et al. 2004). As the T values increase, the differences in volume (as a percentage) between adjacent T numbers become smaller, with the product of the capsid diameter and the reciprocal of the square root of T remaining constant (Walker & Anderson 1970; Rossmann & Erickson 1985; Hu et al. 2008). The actual pattern of historical transitions between different T numbers is unknown and probably determined by the type of fold found in the capsomer (Ahliquist 2005; Bamford et al. 2005; Krupovic & Bamford 2008). We discuss this in the electronic supplementary material. In figure 1, we illustrate the general principle using some DNA viruses, chosen because both their T number is known, and because of capsomer fold similarity, we can infer the likely next highest possible T number.

The critical point is that a small virus with an icosahedral capsid, unlike a large virus with an icosahedral capsid, cannot physically make relatively minor adjustments to the size of its capsid by increasing its T number. We speculate that such viruses are therefore more likely to acquire novel gene function via overlap. The situation is different among viruses with non-icosahedral capsids because in theory adjustments in capsid size, and hence genome length, are as easy to make for small viruses as large ones, e.g. individual capsomers can simply be added onto the end of a helical capsid, as shown for M13 phage (Cann 2001) and Tobacco Mosaic Virus (Dawson et al. 1989). We therefore predict that the negative relationship between gene overlap and genome length known at least for RNA viruses (Belshaw et al. 2007) will be stronger among viruses with icosahedral capsids than among viruses with non-icosahedral capsids.

(c) Gene length

The negative relationship between overlap proportion and genome length mentioned above could merely be an artefact of a relationship between gene length and genome length, and hence not require any biological explanation. The length of RNA virus genes with replicase functions increases with genome length (Belshaw et al. 2008); indeed, there may be a general tendency for genes to become larger in taxa with larger genomes, e.g. eukaryotes tend to have longer genes than prokaryotes (Rost 2002). It appears that most gene overlaps in RNA viruses started from two originally contiguous genes (Belshaw et al. 2007), so if mean gene length increases with genome length, then there will be fewer opportunities for overlaps to evolve in a given length of nucleotides. We investigate the importance of this relationship.

(d) Expression regulation

Some gene overlaps may have evolved to couple gene expression rather than to compress the genome (Normark et al. 1983; Krakauer 2000). This is thought to be common in bacteria (Johnson & Chisholm 2004; Lillo & Krakauer 2007), and there are a few possible examples in viruses (Scherbakov & Garber 2000), e.g. in the RNA phage MS2 the start of the lysis gene overlaps with the end of the coat gene; translation of the lysis gene requires coat protein synthesis termination followed by reinitiation (Berkhout et al. 1987), the frequency of which may be a mechanism to regulate relative protein levels. Suspected cases of gene regulation typically involve short terminal overlaps, so we explored the effect of excluding from our analyses all overlaps of length less than 60 bases, which is approximately the minimum size of a functional protein (Neidigh et al. 2002).

2. MATERIAL AND METHODS

(a) Virus genomes

We cannot use measures of overlap in individual species as data for statistical tests because many species will have the same overlap between homologous genes. Such overlaps will often have a common origin and hence individual species do not represent independent data. However, we can use virus families as independent data because there is very little homology across families and thus most gene overlaps are likely to have been independently acquired. For example, in RNA viruses the only putative homology across all families is a small region of their replicase (Zanotto et al. 1996). We therefore use family means throughout. We follow the taxonomy of the NCBI Virus Genome website (‘GenBank’; currently at http://www.ncbi.nlm.nih.gov/genomes/Genome- sHome.cgi?taxid=10239), and our analysis was based on a downloaded RefSeq flat file of July 2008. Unassigned genera and species were treated as additional independent data points. We follow NCBI’s placement of Hepadnaviridae and Caulimoviridae among the RNA viruses despite their mature virion containing DNA. This is sensible owing to
their possession of reverse transcriptase, which clearly allies them to reverse-transcribing RNA viruses.

We measured gene overlap only as the overlap between the reading frames of different genes, i.e. we ignored regulatory regions (which are often unknown) or overlaps in the same frame. We also only included genomes that are classified by NCBI as either reviewed or validated (see below for exceptions). In the case of segmented viruses, we only included species where all segments were thus classified. The taxa included and excluded in our analysis, along with mean overlap proportion and genome length, are listed in the electronic supplementary material, table S1. We also added the recently discovered overlaps in Potyviridae (Chung et al. 2008), Reoviridae (Firth 2008; Firth & Atkins 2008b) and Sequiviridae (Firth & Atkins 2008a), and the overlap in Schizochytrium single-stranded RNA virus (Takao et al. 2006). We were thus able to calculate means of gene overlap for 36 RNA and 26 DNA virus families (or unassigned genera or species). Exclusion of the less well-annotated species is important, e.g. their inclusion obscures the underlying relationship between overlap proportion and genome length among double-stranded (ds) DNA viruses (electronic supplementary material, figure S1). Excluded data represent seven families plus 10 unassigned genera or species of DNA virus, and 10 families plus 21 unassigned genera or species of RNA virus.

(b) Statistics
For analysing the trends in overlap proportion between families, we excluded families that have no gene overlap and then used natural logarithms of the overlap proportion and genome length to obtain approximate linear relationships. This exclusion is necessary for logarithmic transformation and should not affect our findings: only one DNA virus family, the Nanoviridae, has no overlap (as discussed above, we are only considering families represented by at least one validated or reviewed RefSeq entry). This family is unusual in having multiple small circular segments each coding for a single protein. Eighty RNA virus families are also excluded because they appear to have no gene overlap. Some of these probably do have some gene overlap: some Bunyaviridae provisional RefSeq entries have gene overlap, and an overlapping gene has been reported in a member of the Picornaviridae—Theiler's murine encephalomyelitis virus (Theilovirus; Van Eyll & Michiels 2000) and in a member of the Dicistroviridae (Sabath et al. 2009). In order to compare the amount of gene overlap in RNA and DNA viruses, we calculated the mean of the family means, both including and excluding the small number of families that lacked overlap.

(c) Mutation rate
We included all the estimated mutation rates that we could find in the literature (electronic supplementary material, table S3). Rates are typically expressed as the number of substitutions per base per round of genome copying. A few studies give the rate per round of cell infection, which will be higher but not misleadingly so, especially given the likely error margins on these values. Despite an extensive search, we could find estimates for only six DNA viruses, only two of which have genome lengths within the range of RNA viruses.

(d) Capsid type
Using a standard reference work (Van Regenmortel et al. 2000), we classified virus families (and unassigned genera and species) as having either icosahedral or flexible capsid types. We treat as flexible all non-icosahedral types, e.g. capsids described as spherical, filamentous, helical or rod-shaped, or where there is no well-defined capsid (electronic supplementary material, table S1).

(c) Relationship between changes in capsid volume and genome length
The discrete changes in the size of icosahedral capsids are relatively larger for viruses with small genomes than for viruses with large genomes. We illustrate this principle using some DNA viruses whose $T$ number is known and where the next highest $T$ number can be predicted from other viruses that share the same capsomer fold (see electronic supplementary material, Supplementary Methods and table S2). These include eight tailed, icosahedral dsDNA bacteriophages with the HK97 fold and similar capsid molecular weight (around 40 kDa). To expand the range of $T$ values, we included four single-stranded (ss) DNA viruses with $T = 1$ (no dsDNA viruses with this property are known). These viruses have a different fold, β-barrel fold, but their coat protein is of similar weight. The only transition that is hypothetical is from $T = 16$ (SPO1 and Syn9) to $T = 19$, i.e. no dsDNA phage with $T = 19$ is known, and we predicted this transition from the ‘$3n + 1$ rule’ (Thuman-Commike et al. 1999). For these calculations, the capsid has been approximated to a sphere (Purohit et al. 2005) and the relative volume increase has been calculated assuming the product of the radius and the reciprocal of the square root of $T$ to be constant (Walker & Anderson 1970; Rossmann & Erickson 1985; Hu et al. 2008).

3. RESULTS
We find that 75 per cent of the approximately 2000 known virus species have at least some gene overlap. The negative relationship between overlap proportion and genome length in RNA viruses (figure 2; linear regression $r^2 = 0.23$, $p = 0.006$) reported previously (Belshaw et al. 2007) also exists among DNA viruses (figure 3; linear regression, $r^2 = 0.38$, $p = 0.002$). This relationship is found within all the constituent virus groups, e.g. ssDNA and dsDNA viruses (electronic supplementary material, figures S2 and S3), and within the two types of overlap: internal overlaps, where one gene is completely overlapped by a larger second, and terminal overlaps, where two genes overlap for part of their lengths—one upstream and one downstream (electronic supplementary material, figures S4 and S5).

(a) Mutation rate
As summarized in figure 4 (electronic supplementary material, table S3), the mutation rates of RNA viruses are higher than those of DNA viruses. An important caveat here is that some DNA viruses have a longer genome than any RNA virus (compare figures 2 and 3) and are thus not comparable. There are estimates for only two DNA viruses of similar genome length to RNA viruses (Inoviridae and Microviridae). Nevertheless, these two values are lower than the mutation rate of any known RNA virus (we have found two lower mutation rate estimates for RNA viruses, in MLV and FLUBV.
but these are both questionable because there are much higher estimates for the same or related viruses.

Mutation rate appears to be a poor explanation of gene overlap in viruses because RNA viruses, despite their higher mutation rate, tend to have less genome overlap than DNA viruses of similar genome length—the opposite of the expectation if mutation causes overlap. The mean number of nucleotides in an overlap as a percentage of the genome length is 4.6 per cent for RNA viruses and 6.6 per cent for DNA viruses whose genome length is within the upper limit for RNA viruses (and 4.3% for DNA viruses if we include all genome lengths). Excluding families that lack overlap from these calculations does not change this result: the value is unchanged for small DNA viruses but increases to 6.1 per cent for RNA viruses and to 4.5 per cent for DNA viruses of all genome lengths. A lower value for overlap proportion in RNA viruses obtained using—a rather than a logarithmic—an arcsin transformation, which linearizes less effectively but allows inclusion of zero overlap values (n = 15).

(b) Capsid structure

Separating virus families into ones with icosahedral capsids and those with flexible capsids (figures 2 and 3) shows that this negative relationship between overlap and genome length is actually derived only from the former group. Combining RNA and DNA viruses into one regression analysis, the relationship between overlap proportion and genome length is strong among families with icosahedral capsids (linear regression, $r^2 = 0.27$, $p = 0.001$, $n = 35$) but there is no evidence for it among families with flexible capsids (linear regression, $r^2 = 0.02$, $p = 0.58$, $n = 19$). We find the same result treating RNA and DNA viruses separately: in RNA viruses with icosahedral capsids $r^2 = 0.26$ and $p = 0.02$, while in RNA viruses with flexible capsids $r^2 = 0.14$ and $p = 0.21$; in DNA viruses with icosahedral capsids $r^2 = 0.81$ and $p < 0.001$, while in DNA viruses with flexible capsids $r^2 = 0.08$ and $p = 0.60$.

This finding is not caused by the sample size differences. For both virus types, we sampled families with
icosahedral capsids to give us the same number as families with flexible capsids and repeated the analysis 100 000 times: in only 9 per cent (RNA viruses) or 0.1 per cent (DNA viruses) of the replicates did the relationship decline to the same level as among families with flexible capsids, as measured by their $p$-value (excluding the outlying value for Plasmaviridae raises the DNA virus result only to 0.5%). As the data for RNA and DNA viruses are independent, these two probabilities (9% and 0.1%) can simply be multiplied.

(c) Gene length
Most of the decrease in overlap proportion with increasing genome length is caused by overlaps becoming rarer rather than shorter. The evidence for this is that there is a strong negative relationship between the number of overlaps per unit length and genome length (linear regression, $r^2 = 0.37$ and 0.38 in RNA and DNA viruses, respectively; electronic supplementary material, figure S6). By contrast, there is a much weaker negative relationship between mean overlap length and genome length (linear regression, $r^2 = 0.02$ and 0.16 in RNA and DNA viruses, respectively; electronic supplementary material, figure S7).

We are confident that this rarity of gene overlaps in larger viruses is not an artefact caused by larger viruses having longer genes, and hence fewer genes per unit length. This is because there is a very highly significant negative relationship between (i) the ratio of overlap number to gene number and (ii) genome length (linear regression, $r^2 = 0.21$ and $p = 0.007$ in RNA viruses, $r^2 = 0.42$ and $p < 0.001$ in DNA viruses; electronic supplementary material, figure S8).

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Figure 3. Relationship between overlap proportion and total genome length for DNA virus families. See figure 2 legend for explanation of symbols. Linear regression $r^2 = 0.39$, $p < 0.001$. (1) Adenoviridae ($n = 12$); (2) Anellovirus ($n = 1$); (3) Bacillus phage GIL16c ($n = 1$); (4) Baculoviridae ($n = 1$); (5) Circoviridae ($n = 3$); (6) Corticovirus ($n = 1$); (7) Fuselloviridae ($n = 3$); (8) Geminiviridae ($n = 82$); (9) Geobacillus phage GBSV1 ($n = 1$); (10) Herpesviridae ($n = 26$); (11) Inoviridae ($n = 18$); (12) Iridoviridae ($n = 1$); (13) Lipothrixviridae ($n = 2$); (14) Microviridae ($n = 13$); (15) Myoviridae ($n = 35$); (16) Papillomaviridae ($n = 13$); (17) Parvoviridae ($n = 8$); (18) Phycodnaviridae ($n = 1$); (19) Plasmaviridae ($n = 1$); (20) Podoviridae ($n = 32$); (21) Polyomaviridae ($n = 2$); (22) Poxviridae ($n = 7$); (23) Salmonella phage ST64B ($n = 1$); (24) Siphoviridae ($n = 106$); (25) Tectiviridae ($n = 1$); (26) Xanthomonas phage OP2 ($n = 1$). The outlier represents the four base pair overlap in Acholeplasma phage L2 (RefSeq NC_001447).

Figure 4. Relationship between overlap proportion and mutation rate. Open circles are family means for DNA viruses, closed circles are family means for RNA viruses. The negative infinity value represents families without overlap (all values are logarithmically transformed).
4. DISCUSSION

Our findings are consistent with the inflexibility of icosahedral capsids constraining virus genome length, and gene overlap being a mechanism for acquiring new gene functions under this constraint.

We do not find evidence for gene overlap having evolved as a response to the deleterious effects of mutation. However, we clearly need mutation rate estimates for more small DNA viruses, especially dsDNA viruses and those ssDNA viruses that are known to have a high rate of evolution (substitutions per site per year; Duffy et al. 2008). Also, the expectation of more overlap among RNA compared with DNA viruses, owing to their higher mutation rates, could be masked by RNA viruses having a secondary structure (Yoffe et al. 2008) and this further constraining overlap at synonymous sites (Krakauer 2000).

The hypothesis that gene overlap evolved in response to length constraint by the capsid assumes that there is a fitness cost to the virus in enlarging its capsid. We think this is a reasonable assumption because capsid enlargement can be expected to reduce the virus’s reproductive output, which we can measure as the burst size or growth rate. Many studies have shown burst size/growth rate to be limited by the host cell’s resources (Eigen et al. 1991; Hadas et al. 1997; You et al. 2002; Kim & Yin 2004) and producing a larger capsid will require more of these resources.

We think it very unlikely that the inevitable misannotations in some RefSeq entries will have affected our conclusions. We recognize that detecting gene overlaps is difficult, with new overlaps recently being discovered using specially designed computer programs (Shibuya & Rigoutsos 2002; Firth & Brown 2006; Sabath et al. 2008). However, the two critical points for our study are, first, that the trends we report are also found among small genomes (RNA viruses and the smaller DNA viruses) as well as among the larger DNA viruses, and it is among the latter where we expect most misannotations (because many overlaps will not have been confirmed experimentally); second, misannotation will be a source of random error that will obscure trends rather than create them, e.g. in our study the inclusion of provisional RefSeq genomes obscures completely the relationship between overlap proportion and genome length in dsDNA viruses (electronic supplementary material, figure S1); indeed, our study is a good example of the importance of data quality in comparative genomic studies. Similarly, uncertainty over the location of stop codons might mean that short terminal overlaps are more likely to be misannotations than other overlaps; however, exclusion of these only strengthens the support for our findings (electronic supplementary material, figure S9), and we find the same relationship between overlap proportion and genome length when we consider only internal overlaps (electronic supplementary material, figures S4 and S5), which are generally unlikely to be misannotations because on average they are much larger (Belshaw et al. 2007).

The relationship between overlap proportion and genome length is much weaker among icosahedral RNA compared with icosahedral DNA viruses ($r^2$ values of 0.26 and 0.81, respectively). We suggest the following explanation. There is some evidence among RNA viruses that the genome length can physically influence the size of the capsid (Schneemann 2006; Hu et al. 2008), perhaps because RNA virus genomes tend to be involved in capsid formation (Hohn 1976; Prasad & Prevelige 2003; Rao 2006); by contrast, the genome of DNA viruses enters the already formed (pro)capsid (Fane & Prevelige 2003; Prasad & Prevelige 2003). RNA viruses might thereby have a little more flexibility in expanding their genome length without overlap.

This putative lower level of constraint among RNA viruses is consistent with some other observations. First, polyploidy (the packaging of more than one genome or genome segment copy) is known among RNA but not DNA viruses. Three of these examples are in families with flexible capsids (Orthomyxoviridae, Paramyxoviridae and Rhabdoviridae) and one is in a family with icosahedral capsids, Birnaviridae (Rager et al. 2002; Luque et al. 2009). Second, most of the known examples of species being polymorphic for capsid size (so-called $T$ number modulation) are among RNA viruses (Krol et al. 1999; Rao 2006; Schneemann 2006; Zandi & Van der Schoot 2009) rather than DNA viruses (Baker et al. 1999).

Another observation is consistent with capsid size influencing genome architecture. Many RNA virus species have segmented genomes (analogous to chromosomes), and a few of these package these genomic segments into separate capsids. It has been suggested (Agranovsky 1996) that this phenomenon evolved in order to relieve packaging constraints and the need for overlap. For example, one can compare some members of the Comoviridae and Bromoviridae, which package genomic segments into separate icosahedral capsids and in which there is little or no overlap, with Tymoviridae, which have single icosahedral capsids and make extensive use of overlap. These all plant viruses whose capsid size might be severely constrained by the need to pass through the plasmodesmata (Lucas & Gilbertson 1994; Rao 2006). Genome segmentation is rare among DNA viruses, but two of the three families in which it occurs, Nanovirus and Geminiviridae, are also icosahedral plant viruses in which at least some members have genomic segments that are packaged separately (the third family is the non-icosahedral Polydnaviridae).

There are of course other factors involved in the evolution of gene overlaps in viruses, e.g. there are overlaps in non-icosahedral viruses and in other organisms: (i) selection for faster replication could lead to gene overlap: if the small genome of some viruses is the result of selection for faster replication (small genomes being quicker to copy), then we might expect small viruses to have more overlap because overlap allows more proteins to be coded for by a given genome length. There is a
more general evolutionary model to explain genome compression which incorporates mutation rate, population size and replication rate (Kraakauer 2002), but our comparative data cannot test this model. Selection for replication speed has also been proposed as an explanation for the high mutation rate of RNA viruses, via a possible trade-off between copying speed and copying fidelity (Elena & Sanjuán 2005; Belshaw et al. 2008), and this area deserves more attention, (ii) proteins and protein regions created de novo by overlapping may have novel chemical and physical properties. This is suggested both by contemplating the effects of a resulting shift in codon usage bias (Keese & Gibbs 1992) and by observing that such genes tend to have unusual protein structure and composition (Rancurel et al. 2009). Nevertheless, we find it difficult to explain the striking relationship between overlap and genome length if overlaps evolved primarily in order to create proteins with novel chemical or physical properties—why should this be more important for small viruses than for large viruses? Also, we do not know the relative importance of gene overlap in creating the genomes of today’s RNA and DNA viruses. Large dsDNA viruses have acquired many genes via gene duplication and horizontal transfer from the host (Shackelton & Holmes 2004), while there are very few examples of these processes known among RNA viruses (Holloway 2009); however, the comparison needs to be between RNA viruses and DNA of comparable viruses (Holmes 2009); however, the comparison needs to be between RNA viruses and DNA of comparable genomes, and this has yet to be done, and (iii) some overlaps may be selectively neutral. It is likely that overlaps evolved from the translation of novel or extended open reading frames (ORFs) created by the mutational gain or loss of start and stop codons, respectively, although open reading frames (ORFs) created by the mutational 

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