Rapid Evolution of RNA Genomes

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So far as is known, the entire present-day biosphere is based on DNA genomes, and autonomous RNA genomes are found only in viruses parasitic on cells with DNA chromosomes. Some RNA genomes such as the fungal viruses (*1*) are often referred to as RNA plasmids because they are transmitted only by cell fusion and cell division, and never as extracellular infectious virus particles. However, their ability to replicate their RNA genomes autonomously in the cytoplasm of cells places them generally among the RNA viruses.

Errors in the replication of DNA chromosomes vary considerably, but they average as low as 10^{-8} to 10^{-11} per incorporated nucleotide, in part because proofreading exonucleases remove misincorporated bases from newly synthesized DNA strands (*2*). The template strand is recognized as the correct coding partner because it is methylated at intervals.

In contrast, definitive studies of RNA bacteriophage Qβ by Weissmann and his collaborators showed that the error level per genome doubling at given base positions in the RNA genome is between 10^{-3} and 10^{-4}. Undoubtedly, this low fidelity of RNA genome replication is due to the absence of proofreading enzymes. No RNA proofreading exonucleases have yet been found associated with any RNA replicase. Despite this high rate of RNA genome mutation, Domingo, Weissmann, and their colleagues (*3*) demonstrated that wild-type Qβ phage predominates in equilibrium pools of virus because it replicates more rapidly than the mutants which arise (*3*). In this article, we discuss the data showing that RNA viruses generally exhibit a high mutation frequency. More important, we show that the rate of evolution (the rate at which viable mutations accumulate in the genome) is very much higher for RNA genomes than for DNA genomes, and that the concept of "wild type" often has only fleeting meaning for RNA genomes because of factors promoting genetic disequilibrium in RNA virus populations.

Summary. RNA viruses show high mutation frequencies partly because of a lack of the proofreading enzymes that assure fidelity of DNA replication. This high mutation frequency is coupled with high rates of replication reflected in rates of RNA genome evolution which can be more than a millionfold greater than the rates of the DNA chromosome evolution of their hosts. There are some disease implications for the DNA-based biosphere of this rapidly evolving RNA biosphere.

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Rates of nucleotide substitution in eukaryotic DNA genomes have been estimated for a variety of genes and pseudogenes, and there is general agreement that the rate of nucleotide substitution in functional genes averages only about 10^{-9} nucleotide substitution per site per year (*4*). The substitution rate is about four- to fivefold higher for third codon positions as compared to first and second codon positions and for pseudogenes as compared to functional genes (*4*), in support of theories of neutral evolution. Remarkably, the rates of nucleotide substitution per year in evolving eukaryotic virus RNA genomes can be millionsfold higher, as we show below. Thus, DNA chromosomes of eukaryotic host organisms generally require geologic time spans to evolve to the degree that their RNA viruses can achieve in a single human generation. The full implications of this fact are not readily grasped, but some important implications for general evolution and for diseases of plants, animals, and humans are discussed below.

Mutation Rates of RNA Viruses

The widely studied retroviruses replicate via DNA provirus copies of their RNA, and thus are outside the subject matter of this article. All other RNA viruses encode RNA replicase enzymes regardless of their different replication strategies (*5*).

Early genetics of both plant and animal RNA viruses indicated very high mutation rates. For example, shortly after plant viruses were proved to have infectious RNA genomes it became clear that mutation rates were quite high with (and without) nitrous acid mutagenesis (*6*). Granoff (*7*) reported 3 to 7 percent frequency of small plaque mutants of Newcastle disease virus in clonal pools, and these increased more than tenfold after mutagenesis. Fields and Joklik (*8*) showed that, after mutagenesis, reovirus ts (temperature sensitive) mutation rates increased from 0.3 to over 10 percent. Such phenotypes are a small percentage of the total mutants present.

Forward mutation and reversion frequencies are easily and reproducibly determined for DNA genes because the rates are rather low. But with the RNA viruses the rates are so high that it is usually difficult to determine whether one mutational event or several gave rise to a reasonably stable mutant phenotype. Normally, investigators choose not to select, nor to study, unstable mutants, so both forward mutation rates, and reversion rates of any phenotype tend to be underestimates. For example, a mutation frequency of poliovirus to guanidine resistance of about 3×10^{-5} (*9*) may represent the compounded rate of two or more independent mutations.

The genetics of vesicular stomatitis virus (VSV) is probably the best understood of all non-retrovirus RNA viruses, and the most extensive genetic characterization of VSV has been carried out by Pringle and his colleagues (*10, 11*). They have characterized hundreds of ts mutants of VSV Indiana and New Jersey serotypes, and of other rhabdoviruses, and have grouped them by complementation testing (*10, 11*). About 90 percent of spontaneous or induced mutants map in one gene (in complementation group I). This is not unexpected because group I mutants are encoded by about half of the 10-kilobase genome. This 5′ half of the virion RNA encodes the L protein, which provides the polymerase activity involved in viral transcription and replication (*12*). Pringle observed that the frequency of spontaneous VSV mutants selected at 39°C was about 1 percent.

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The frequency of ts mutants in some VSV strains could be increased to more than 40 percent by viral replication in the presence of the base analog 5-fluorouracil, but lower rates of induction occurred with other strains (*10*). Flamand (*13*) estimated spontaneous VSV mutation frequencies of 1.7 percent, and showed that the frequency of ts mutants went from 1 percent to about 2 percent when the nonpermissive temperature for detection of mutants was raised from 39° to 39.8°C. Obviously, more stringent assay conditions detect leaky mutants that, at lower temperatures, score as wild type. Others (*14*) have observed spontaneous mutation frequencies for rhabdoviruses ranging from about 1 to 5 percent for the ts mutant phenotype alone.

Significantly, Flamand (*13*) observed a transient increase in the spontaneous mutation frequency (up to 5 to 8.3 percent) when clones of VSV Indiana previously grown at 30°C were passaged at 39.3°C. Probably the L protein polymerase selected at the lower temperature generates more errors at higher temperatures for several cycles of growth until a mutant better adapted to the higher temperature is selected. The total number of mutations will always be significantly underestimated when ts assays alone are used. Also, a high rate of reversion, or second site pseudoreversion (*15*) can lead to underestimates, so the frequency of all genome mutations might often exceed 10 percent. In agreement with the above results in animal viruses, Bruenn and Brennan (*16*) have shown extreme and rapid divergence at the 3′ ends of yeast killer factor viral (or plasmid) RNA molecules, although mutation rates could not be calculated in their study.

It might be argued that phenotypically expressed mutation frequencies approaching or exceeding 10 percent are somehow artifacts of the screening assays, and that such frequencies in nature would be lethal too often and the lethality would lead to RNA genome extinction. Presumably there is a high lethality due to mutations at many sites, but this is compensated by small genome size, by high replication rates, and by high yields of RNA viruses. An independent estimate of RNA genome mutation rates can be calculated from the rate of appearance of antigenic mutants in RNA viruses, and this roughly agrees with those obtained by ts assays. Monoclonal antibody directed against a single viral antigenic determinant allows rapid selection of viral mutants resistant to that antibody (*17*). Portner *et al.* (*18*) compared the rates of mutation to monoclonal antibody resistance for three unrelated negative-strand RNA viruses, namely, influenza, VSV, and Sendai. The rates of mutation were the same for all three, that is, about $10^{-4.5}$ mutations per replication. The structure of hemagglutinin membrane glycoprotein (HA) of influenza virus and the sequence of a number of mutants have been determined (*19*). About 60 percent of the amino acids in HA are conserved and the others are variable. However, only about 37 amino acid sites are involved in antigenic changes in nature and far fewer are targets for laboratory selection with monoclonal or polyvalent antibody. Different monoclonal antibodies frequently select mutants at the same single amino acid position, and only nine amino acid positions undergo substitution as a result of antibody selection in the laboratory (*19*). The genome target for mutant selection by a single monoclonal antibody is probably most often one codon and is probably never more than ten nucleotides. Therefore, if a 10-kilobase RNA virus genome averages $10^{-4.5}$ mutations per replication and we assume the genome targets to comprise about ten bases, then the overall rate of mutation of the entire 10,000 bases (assuming equal probability of base substitutions at all positions) would average about $10^{-1.5}$, even if we ignore silent mutations due to redundancy in the genetic code. Reversion rates of 10^{-4} to 10^{-5} for RNA virus mutants (*10, 11*) if they represent the probability of change at a single base out of 10,000 bases would also indicate overall genome mutation frequencies in excess of approximately 10 percent. Thus, several independent methods provide extremely high estimates of the mutation frequencies of animal virus RNA genomes. This is of the order of 10^4- to 10^6-fold greater than the mutation rates of stable DNA genomes of similar complexity.

Accumulation of RNA Genome Mutations Depends on Disequilibrium

In order to discuss rapid evolution of RNA genomes (which requires continuing buildup of viable genome alterations) the concept of population equilibrium must be considered. Domingo *et al.* (*3*) examined the nucleotide sequence heterogeneity of a Qβ RNA phage population by T1 ribonuclease oligonucleotide mapping and deduced from clonal analysis that the nucleotide sequence of each viable clone differs in one to two positions from the average sequence in a wild-type parental population. They concluded that "the genome of Qβ phage cannot be described as a defined unique structure, but rather as a weighted average of a large number of different individual sequences." Competition experiments between mutant and wild-type phages showed that the faster growing wild type outgrew mutants exhibiting a relative replication rate of 0.8 to 0.9, and the population came to a mathematically predictable equilibrium with wild type predominating. They proposed that mutants arose at a high rate on one hand, and were strongly selected against on the other, such that an RNA virus population achieved dynamic equilibrium. They pointed out that ordinary nucleic acid sequencing methods would detect RNA heterogeneity only if the growth rate of a mutant were very close to that of wild type (within 0.01 to 0.1 percent). In sequencing MS2 phage RNA, Min Jou *et al.* actually observed equimolar amounts of MS2 RNA genomes differing in at least one nucleotide position (*20*). Semler and Holland (*21*) observed variability at several positions with VSV DI particle RNA. Bruenn and Brennan (*16*) showed yeast killer factor viral (or plasmid) RNA to be heterogeneous at multiple positions. Donis-Keller *et al.* (*22*) showed substantial and reproducible sequence heterogeneity in satellite tobacco necrosis virus RNA, providing direct chemical proof for the enormous mutability of RNA genomes.

All of the above evidence suggests that every RNA virus pool containing 10^9 or more infectious particles per milliliter is always a mixture of countless variants even if it was prepared from an isolated clone and given the appellation "wild type." Nevertheless, certain RNA genomic species can exhibit such strong selective fitness that under appropriate conditions they will consistently dominate the equilibrium population. Clewley *et al.* (*23*) observed identical wild-type T1 oligonucleotide maps in VSV laboratory strains passaged repeatedly in different locations for more than a decade, whereas wild-type VSV Indiana strains from different outbreaks of cattle disease in the United States exhibited quite different oligonucleotide maps. Palese and colleagues (*24, 25*) and Nakajima *et al.* (*26*) showed that influenza strains from localized outbreaks were identical or very similar. Nottay *et al.* (*27*) showed that the reference Mahoney strain of type 1 poliovirus is similar to the LSc2ab vaccine mutant strain derived from it, and to two other substrains of Mahoney with very different passage histories in

other laboratories. We have observed that wild-type VSV Indiana passaged dilute hundreds of times in cell culture retained a T1 oligonucleotide map identical to starting virus (*28*). In contrast to these equilibrium-stability conditions there are other conditions leading to rapid evolution of RNA genomes as discussed below.

Holland *et al.* (*29, 30*) demonstrated that persistent VSV Indiana infection of BHK_{21} cells initiated with repeatedly cloned virus leads to rapid and continuous evolution of the RNA genome as measured by T1 oligonucleotide mapping of virus clones isolated at intervals over 5 years of persistent infection. A continuous progression of evolutionary change was observed, and the original virus was never detected after the early months of persistence. Youngner and his colleagues had originally showed that ts mutants arose and were selected over wild-type virus (*31, 32*) very early in persistent infection. During more than 7 years of VSV persistence we have observed a never-ending progression of genome sequence alterations from that of the original clone. Interestingly, we observed that these mutants with altered genomes often exhibit stability upon repeated dilute passages of virus in acute infections of cultured cells, or in mouse brain passages (*28–30*) in vivo.

Obijeski and his colleagues (*27*) have shown the epidemiological utility of oligonucleotide mapping of RNA genomes in comparing poliovirus isolates obtained during a recent poliomyelitis epidemic that spread through unvaccinated communities. The virus apparently spread from the Netherlands to British Columbia, Alberta, and Ontario, then from Ontario to Pennsylvania Amish communities, and finally to Amish communities in the midwestern United States over the course of a year. Consecutive case isolates exhibited similar oligonucleotide maps, but the viral RNA genome changed slowly and progressively so that the United States isolate of June 1979 differed from the original Netherlands isolate of May 1978 by 15 T1 oligonucleotides. Thus about 1 to 2 percent of the poliovirus genome bases had been substituted during the 1-year course of the epidemic (*27*). The most similar maps were between isolates from members of the same family, and these two differed by only one spot (about ten base changes), suggesting that the genome usually changes slightly as it spreads among individuals. Conclusions from such studies are less certain because the epidemic was not initiated by a clone, a number of different variants may spread from one person to a contact, and the dates of spread are always uncertain. However, this work does indicate that the poliovirus RNA genome underwent a steady progression of base substitutions and that many genome permutations were viable in nature. More recently, Kew *et al.* have shown that poliovirus vaccine strains can sometimes undergo more than 100 mutations during replication in only one or two individual humans (*33*), and Domingo *et al.* (*34*) found high mutation levels (mutations at 0.7 to 2.2 percent of RNA genome sites) during foot-and-mouth disease outbreaks in cattle. The above studies exhibit a rate of base substitutions per average site per year in genome RNA in excess of 10^{-2} to 10^{-3}, which exceeds by a million times the average rate of evolution of the chromosomal DNA of their eukaryotic hosts.

Similar types of RNA genome plasticity have been observed during epidemics of influenza A viruses, and oligonucleotide mapping and sequencing have provided evidence for sequential mutations in influenza virus RNA's in nature (*19, 24–26, 35*). In general, viruses isolated at about the same time from different persons within any local outbreak have identical or very similar oligonucleotide maps, whereas viruses from earlier or later outbreaks exhibit increasing divergence. Young *et al.* were able to construct a likely evolutionary tree relating gradual mutational changes of the genome of H1N1 isolates in 1977 (*24, 25*). The sudden emergence of the influenza viruses H2N2 in 1956 and of H3N2 in 1968 ("genetic shift") was probably due to reassortment of genome segments from a number of circulating strains that had undergone extensive "genetic drift" due to many mutations, but the "reemergence" of H1N1 virus after 20 years "dormancy" remains unexplained. Sequencing of the genes for HA1 suggest that antigenic drift in the hemagglutinin of human H3 influenza subtypes can proceed as rapidly as 1 percent amino acid residue changes per year (*35*). Sequence conservation at certain sites (*19, 36*) is undoubtedly due to strong selective pressure.

Young and Palese (*24*) provided evidence that the H1N1 influenza serotype variants circulating in winter 1978–1979 were "recombinants" of the H1N1 1977 strains with H3N2 serotype segments. Nakajima *et al.* (*26*) showed by oligonucleotide mapping that there were at least four distinct kinds of H1N1 serotype genomes circulating in Japan in 1978–1979. It appears that rapid, independent mutational genetic drift of each of the eight segments of influenza A, together with reassortment of segments can explain the genetic plasticity of influenza virus. All eight gene segments exhibit rapid evolution, not just those segments encoding viral surface antigens (*37*).

Recombination of RNA Genomes

One of the important biological insights of the last decade has been the recognition that insertons, transposons, splicing, and other illegitimate (and legitimate) recombination mechanisms contribute greatly to genetic variation, regulation, differentiation, oncogenesis, and evolution (*38*). It is important to know whether true recombination (that is, covalently linked genome rearrangement) plays a significant role in the evolution of RNA genomes as well. The recombination of segmented genome RNA viruses, such as influenza and reoviruses, represents reassortment of segments in doubly infected cells, and probably no covalent joining is present. The only studies suggesting that true recombination occurs between RNA genomes are the genetic data of Cooper (*39*) and others (*40*) with poliovirus. However, this genetic data has not yet been confirmed by RNA sequencing or mapping analysis of the recombinant genomes. In one of the earliest attempts to detect recombination between mutants of a nonsegmented RNA virus (the negative strand Newcastle disease virus of fowl), Granoff (*41*) was unable to observe recombination between two mutant genotypes at a level in excess of the mutation rate. Pringle and Wunner (*42*) could detect no recombinants on careful analysis of crosses between mutants of VSV.

We have attempted a sensitive screening procedure to search for intertypic recombinants between VSV Indiana and VSV New Jersey serotypes (*43*). We used a ts, small-plaque mutant of VSV Indiana; this mutant is slowly replicating and nonreverting as a result of hundreds of mutations accumulated over 7 years of persistent infection (*29, 30*). We crossed this with large-plaque wild-type VSV New Jersey. If a precise double crossover substituted the surface glycoprotein gene of VSV Indiana into the New Jersey genome, large-plaque recombinants resistant to antiserum to the New Jersey type might result. However, we have been unable to detect any recombinants to date, suggesting that such precise crossovers between these genomes are either very rare or nonexistent.

RNA recombination may be common but unobservable because of the extremely high background of mutational change in RNA genomes. Alternatively, true intergenomic RNA recombination might be rare because the high mutation rate renders them redundant. Unsegmented RNA viral genomes are always small, averaging about 2 to 4 megadaltons, and therefore viral genes devoted to recombination could be not only unnecessary, but burdensome. Independent modular evolution and modular exchange of subgenomic segments may not be such a widespread means of evolutionary change for unsegmented RNA viruses as it is for segmented RNA viruses (*24, 25*) and for DNA genes in general (*44*). Finally, there is no convincing evidence (*45*) that nonretrovirus RNA viruses normally replicate or recombine in cells via DNA copies of their genomes, and hence rapid mutational divergence may be their only major evolutionary mechanism.

Defective Interfering Particles

Despite the lack of evidence for intermolecular RNA recombination between infectious genomes of most RNA viruses (other than poliovirus), all or nearly all RNA viruses generate recombinant subgenomic deletion particles designated defective interfering particles (DI particles) (*46, 47*). These consist of shortened viral genomes packaged in virions containing normal viral proteins. These shortened genome fragments retain one or both termini of the genome RNA, since the 3′ RNA ends are obligatory replicase attachment sites. Although the recombinational mode generating shorter DI particle RNA genomes from viral RNA is not established, most investigators favor models involving strand-switching by viral replicase during replication (*48, 49*). Perrault (*50*) has reviewed the structures of DI particle genome RNA's and proposed a related general model for their generation. These schemes are basically an elaboration of the old "copy-choice" model for DNA recombination.

Regardless of the mode of generation of DI particles, they are always defective and replicate by means of enzymes and structural proteins encoded by infectious helper virus; also they specifically interfere only with closely related viruses able to act as helpers (*51*). Interference appears to occur mainly at the level of virus replication (*52*) and can be extremely potent or very slight, depending upon the virus (*53*) and the host cell type (*54*). The DI particles are most efficiently generated under conditions in which many infectious virus genomes simultaneously infect the same cell (*55*), and the rate of generation varies with different virus-host cell systems (*53, 54, 56*). In a rather typical system (VSV in BHK_{21} cells) the rate of generation of DI particles is about 10^{-7} per standard virus replication (*56*).

Prevention of RNA Virus Lethality

In 1973 we observed that VSV DI particles prevented standard infectious VSV from killing 100 percent of BHK_{21} cells. A long-term infection of BHK_{21} cells then ensued (*30, 57*) in which the genomes of standard virus and DI particles have multiplied slowly in the cytoplasm of these cells for more than 8 years. Figure 1 demonstrates fluorescent antibody visualization of VSV antigens in these persistently infected cells. During the last 8 years these "carrier cells" have multiplied at normal or near-normal rates despite the presence of intracellular viral genomes. Throughout this period new kinds of viral mutants and DI particles have constantly arisen, competed for a short time, and then have been displaced (*30, 45, 57*). Many investigators have established similar carrier states with other RNA viruses, and in some systems the early appearance of ts mutants, interferon, and other factors appears to be more important than DI particles in establishment of persistence (*32, 47, 58*).

Once persistent infections by RNA viruses become well established, yields of infectious virus are often extremely low (*28, 45, 59*), and very often no mature virus can be recovered. For example, VSV Indiana, which normally produces more than 10^4 infectious particles in each BHK_{21} cell during acute infections (in less than 12 hours), has after 2½ to more than 8 years of persistent infection often yielded no mature virus for days, weeks, or even months. It usually yields less than 10^{-5} to 10^{-6} infectious particles per carrier cell per day when virus is detectable. This is partly due to the accumulation of debilitating mutations in the viral genomes, but some mutant virus clones after recovery from persistently infected cells will produce more than 10^3 infectious particles per cell upon reinfection of normal BHK_{21} cells. Other virus clones exhibit very poor replicative capability (*43*), and it is likely that most of the RNA genomes within cells after years of persistence are more like RNA plasmids than RNA viruses since most of them never form mature infectious virus particles. Careful analysis of the state of intracellular virus

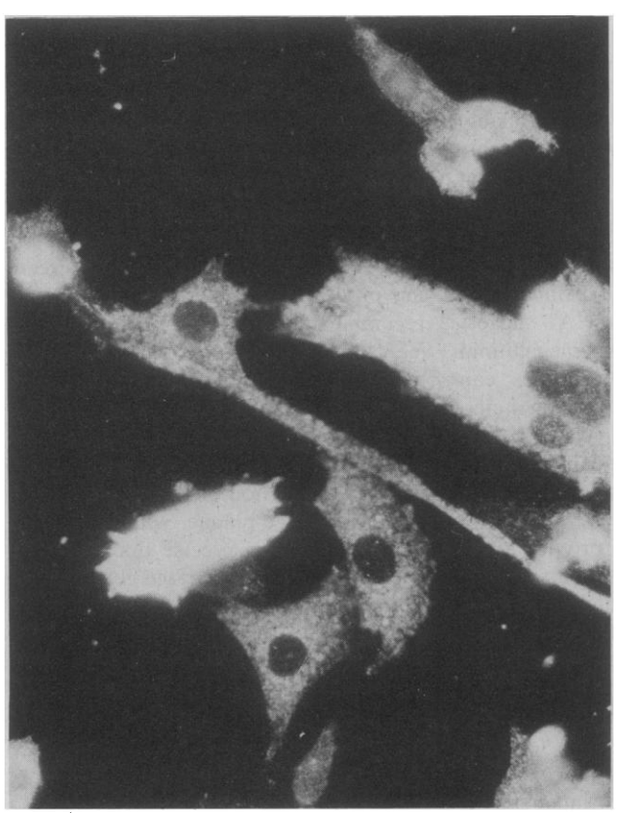

Fig. 1. Detection of intracellular viral antigens in BHK_{21} cells persistently infected by VSV. After 8 years of persistent infection there is little or no production of mature infectious virus because of accumulated viral mutations, and because of strong interference due to intracellular DI genomes.

in a persistent Sendai virus (parainfluenza type 1) infection of BHK_{21} cells shows that another major reason for low virus yields is the presence of a large excess of DI particle genomes over infectious virus genomes (from 15- to more than 100-fold excess) in the carrier cell cytoplasm (*60*). A similar situation exists within VSV Indiana carrier cells and rabies carrier cells (*61*), causing them to be specifically resistant to challenge by homologous virus (*30, 57*). Thus, DI particles are amplifiable, subviral, genome recombinants that exert specific regulatory effects on the viral genomes which generated them, thereby enabling the viral RNA genomes to hide, replicate slowly, and undergo extensive evolution within cells that would otherwise have quickly been killed.

Rapid Evolution of RNA Genomes During Repeated Passage

The rapid rate of evolution of the VSV genome during persistent infection might appear to conflict with its relative stability during repeated passages in cells (*23, 27, 28*) and with the data (*3*) showing that Qβ RNA phage populations tend to reach equilibrium. However, we have found that there are conditions under which VSV genomes replicate extensively while maintaining genome equilibrium, and other conditions in which they are driven to disequilibrium and rapid evolution. Repeated dilute passage of VSV (that is, more than 200 low-input multiplicity passages) usually does not lead to progressive accumulation of genome map changes; however, serial undiluted (high multiplicity) passages in BHK_{21} cells does (*28, 59*). The mutation rate is probably high in both modes of passage, but the rate of accumulation of genome mutations is quite different. Ahmed *et al.* (*62*) have also observed considerable genetic variation of reovirus after undiluted serial passages, and Brand and Palese (*63*) showed that plaque-to-plaque (dilute) passage of VSV caused little variation in genome maps. Youngner *et al.* (*64*) observed considerable accumulation of ts mutants (more than 70 percent) within nine serial undiluted passages of wild-type VSV in mouse L cells or in BHK_{21} cells (if the latter were first treated with actinomycin D).

It seems clear that either persistence or high input short-term infections can provide conditions in which the inherent high mutation rates of RNA viruses are reflected in high rates of genome change. One of the many possible reasons for this is that DI particles accumulate during persistent infections and serial undiluted passages (*55*) and these can lead to disequilibrium of RNA virus populations (see below). Another possible reason is the fact that high virus inputs introduce a number of different variants into each cell, and these can complement each other (intergenomic complementation) and influence each other's replication and evolution. Even lethal mutations can be complemented and propagated until further mutations suppress their lethality.

Brand and Palese (*63*), however, observed rapid genome change of influenza virus under dilute passage conditions (where DI particle effects should have been minimal) in which VSV did not show rapid change. Youngner *et al.* (*64*) observed accumulation of ts mutants in BHK_{21} cells treated with actinomycin D to reduce DI particle replication. It appears that certain cell-virus interactions can promote rapid RNA genome evolution under conditions which in other systems would lead to maintenance of a rather stable equilibrium. In fact, we have observed rapid genome change during dilute passages of certain mutants of VSV but not with most other mutants (*28*). These might be viewed as "mutator gene" variants since these differences are observed among cloned mutant virus pools, each of which was derived by mutation from an original clone of VSV.

It is to be expected that certain mutations in the viral replicase might increase the frequency of mutation beyond the already high rates alluded to earlier. Such mutants will be difficult to characterize phenotypically and genotypically since they are likely to exert pleiotropic effects on other genes (both intra- and intergenome effects), and they should be so unstable that populations of such mutator viruses will be extremely heterogeneous. In fact, Pringle and his colleagues (*11, 65*) have characterized a trans-acting mutant of VSV New Jersey (ts D1) which is unstable and which appears to exert pleiotropic effects on viral gene products (that is, abnormal electrophoretic mobility of viral proteins, with different proteins affected in different subclones of ts D1). Mutator gene mutants of varying potency might occur regularly in virus populations because of the overall high rate of mutation, and in turn contribute to the high rate. This is another potential explanation for the fact that undiluted (high input) passages generate more rapid RNA genome evolution.

Promotion of Disequilibrium in RNA Genome Populations

After persistent rabies virus infection was established in BHK_{21} cells, Kawai *et al.* (*66*) observed that small-plaque mutants of infectious virus appeared and that these were quantitatively resistant to the original DI particles used to help establish persistence. However, DI particles that were newly generated by these small-plaque mutants were able to interfere effectively. Horodyski and Holland (*67*) observed the same phenomenon during VSV persistence, and during repeated passages with high virus input. Furthermore, they observed that this phenomenon continues during persistence because the small-plaque mutants can further mutate to escape quantitative interference by the D1 particles, which they generated (Fig. 2). This demonstrates how DI particles can promote viral evolution by repeatedly displacing previously more fit virus variants. In general, the situation is complex because there may be many viral mutants and various DI particles that also compete among themselves.

Viral mutants are likely to escape DI particle interference by mutation of viral polymerase proteins. However, in VSV the newly arisen DI particles interfere with the virus which generated them because they share similar replicase initiation sites at the 3′ ends of the plus and the minus strands of the DI particle RNA, and at the 3′ end of viral plus strands. This arises because all of these termini are complementary to the 5′ termini of virion minus strand (*46, 47*). Semler and Holland observed that the sequence of the 5′ end of VSV virion RNA (3′ end of DI particles) diverges much more rapidly during persistent infection than does the 3′ end (*21*). Therefore, some forms of coevolution of viral replicase and of viral and DI particle RNA probably occur. However, once the equilibrium of viral variant populations is upset (by DI particles or by any other means) all variants present in the viral population are affected in their competitive abilities. Therefore, evolutionary change can be promoted at any genome site where competitive, viable mutations are possible. Virus variants that spread to other cells often will not encounter (nor quickly generate) DI particles, and thus DI particle selection is not continuous. Since alphaviruses (*68*) and arenaviruses (*68*) also can mutate to become resistant to DI particles during persistence, this phenomenon is probably common among RNA viruses, and

may often be a major factor in generating diversity of RNA genomes.

This DI selection effect is a characteristic intracellular selection exerted directly on immature viral nucleocapsids by viral subgenomic mutants. Numerous other factors in infection of higher plants and animals tend to promote disequilibrium and rapid evolution. Among these are varying growth rates of virus mutants in different host species or differentiated cell types, selective effects of insect vectors on genomes of plant or animal viruses, interferon effects, cellular immune mechanisms, and the like. Immunological promotion of the antigenic drifts and shifts of influenza viruses are widely recognized, but the less obvious quantitative changes in antigenicity of other RNA viruses are not. These changes are less obvious because serotypes of most viruses do not change as rapidly as occurs among influenza viruses. It is generally believed that there is only one serotype of measles, or mumps, or of rabies virus worldwide, and in fact all isolates of any of these can induce antibodies which cross-neutralize all other strains of the same virus.

In general, the neutralization-reactive surface components of viruses mutate and diverge more rapidly than internal components, suggesting that immune selective forces drive their evolution preferentially. For example, poliovirus capsid protein changes occurred with high frequency in the epidemic that spread from the Netherlands to Canada and then to the United States (*27*). The antigenic cross-reactivity of internal components of different serotypes and the rapid generation of diversity within serotypes of RNA viruses suggest that one serotype may mutate to another more often than is believed.

The availability of monoclonal antibodies now allows detailed study of virus cross-reactions at specific antigenic determinants, and some rather unexpected results have recently emerged. Koprowski and his colleagues (*69*) observed that isolates of rabies virus can differ considerably in their antigenicity and can be grouped into a number of related subserotypes according to their degree of cross-reaction with a battery of monoclonal antibodies. Disconcertingly, immunization of mice with a vaccine strain of rabies provided solid protection against challenge with a street virus of the same rabies group, but only partial protection against rabies virus of another group (40 percent of the immunized mice died). Crick *et al.* (*70*) also pointed out the problem of rabies virus antigenic variation for vaccine potency. Even in practical terms of vaccine production, rabies virus cannot be viewed as a single virus worldwide, but as a spectrum of closely related and evolving viruses which cause that specific disease. In fact, of the 16 antigenic determinants

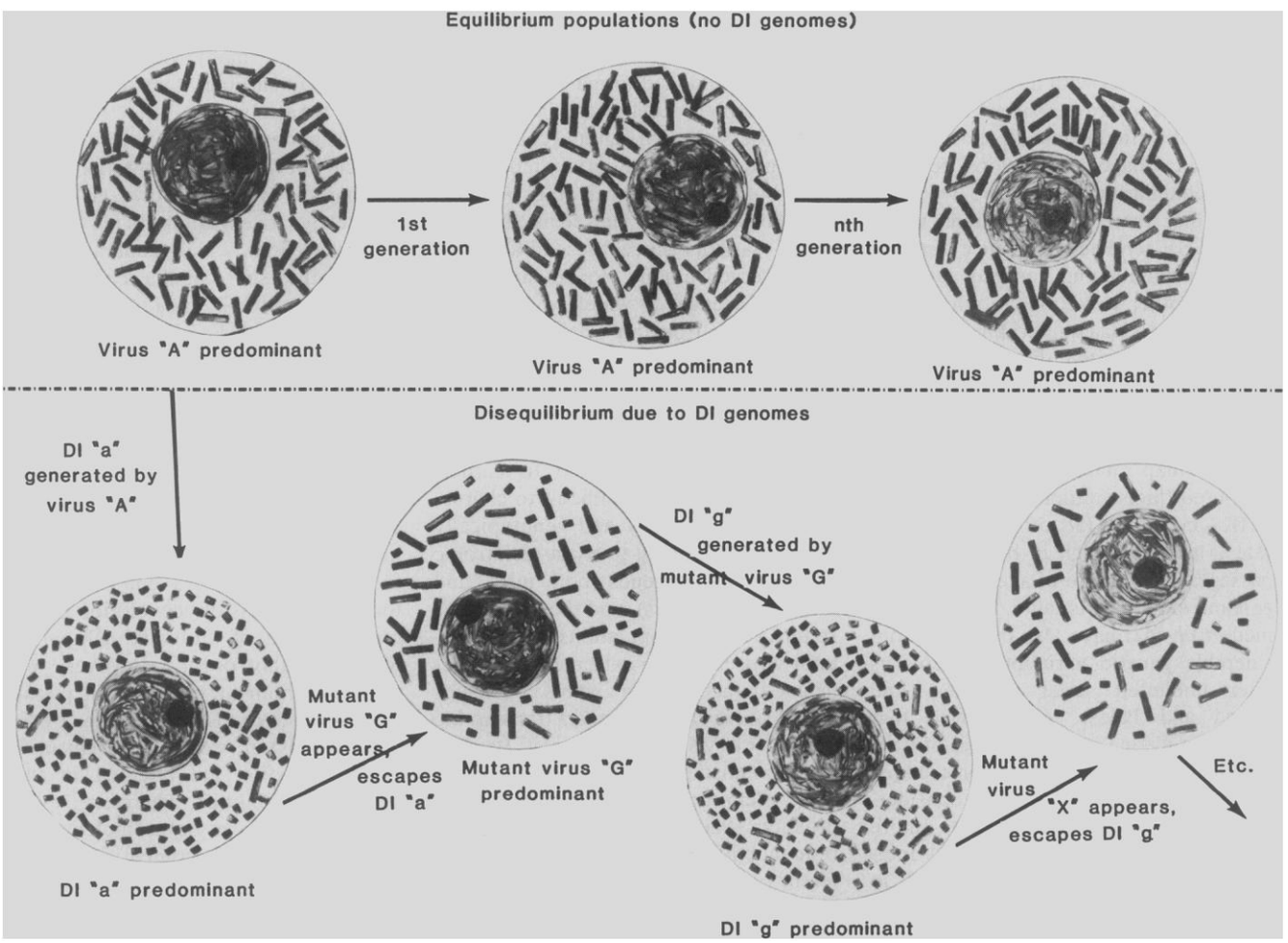

Fig. 2. Simplified scheme illustrating how DI particle genomes can promote viral evolution intracellularly by repeatedly displacing more fit variants. Population equilibrium is favored during dilute passages of virus because intracellular DI particle genomes are rare or absent, and one fast-growing virus mutant can often outgrow all competing virus mutants for long periods of time. Equilibrium is disrupted during persistent infection or undiluted passaging of virus because DI particle genomes arise continuously and strongly interfere with replication of their parental virus mutants (which might otherwise have outgrown all competing virus mutants for prolonged periods). Mutant viruses resistant to these preexisting DI are selected and dominate the population until they themselves are inhibited by their own newly generated DI, and are in turn displaced by newly arising virus mutants.

identified on the CVS rabies virus strain, nine are shared with Duvenhage virus, six with Lagos bat virus, and five with Mokola virus (these are rabies-related rhabdoviruses) (*69*). These results are in accord with our observation (*59*) that two strains of rabies virus have completely different T1 oligonucleotide maps (that is, more than 5 percent sequence divergence, or more than 500 base substitution differences in their genomes). Birrer *et al.* (*71*) have shown that there is a high frequency of antigenic variation of measles virus.

Brown and his co-workers (*72*) have demonstrated that swine vesicular disease virus and Coxsackie B5 viruses of humans are closely related, but distinct, viruses with about 50 percent sequence homology. Since swine vesicular disease was never reported before 1966, this may represent a human Coxsackie B5 variant that adapted to swine as it underwent antigenic drift. If so, it will soon (or already does) qualify as a new virus. Antigenic drift of foot-and-mouth disease virus to generate antigenically distinct serotypes is widespread (*34, 73*). Where antigenic drift has been analyzed, it has been found to be due to accumulation of point mutations. This occurs in influenza viruses in nature (*17, 24, 35*), and in persistent infections by Visna virus (a retrovirus) (*74*). We have even observed changes in antibody reactivity of VSV mutants recovered after years of persistent infections in cell culture in the absence of antibody (*59*), an indication that immune selective pressure is not essential for antigenic drift. Specific recognition of virus antigens by antibody and by T lymphocytes is not the only selective force acting on cell surface components of virus-infected cells. Interferon-mobilized natural killer (NK) cells regularly eliminate tumor cells persistently infected by, for example, VSV, measles, or mumps injected into nude mice (but not control uninfected tumor cells) (*75*). However, we were able to select VSV mutants that were not susceptible to NK cells (*75*). Tumor cells persistently infected by these mutant (VSV-P) virions grew rapidly and metastasized in nude mice.

We have subjected these NK-resistant VSV-P mutants to selective pressure in cell culture by passaging 20 times under rabbit antibody to VSV. A clone of these mutants can undergo rapid antigenic drift and be neutralized poorly by antibody to the original cloned virus from which it was derived (Fig. 3). We intend to continue this selection by antibody for years and determine whether new serotypes arise as the original antigenic reactivity

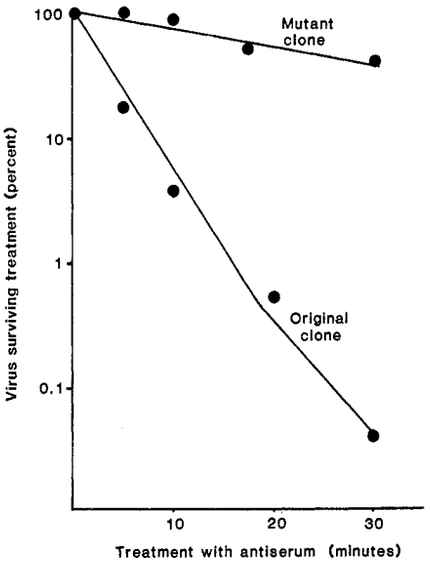

Fig. 3. Kinetic analysis of inactivation of an original (tsG31) and mutant clone of VSV Indiana, at 37°C, by a 1/300 final dilution of mouse antiserum to the original tsG31 virus. The mutant clone was derived from the tsG31 clone after it had persistently infected BHK_{21} cells; these cells had been grown as tumors in nude mice for nearly a year before virus was isolated and subjected to 20 passages of acute infection in BHK_{21} cells in the presence of rabbit antibody to another strain of VSV Indiana. The mutant clone is neutralized more slowly by antibody to the original virus.

is lost by mutational drift. Again it should be emphasized that immune selection by, for example—antibody, NK cells, T cells, and the like—can promote evolution at any site on RNA genomes where viable competitive mutations are possible by promoting disequilibrium of virus populations. Clearly such rapid evolution of RNA viruses could pose severe challenges to immune systems despite their own diversity.

Implications for Persistent RNA Virus in Degenerative Human Disease

During the past decade there has been increasing interest (*76, 77*) in the possible involvement of slow, persistent, inapparent and latent virus infections in a wide variety of human autoimmune and degenerative diseases that do not on the surface appear to be of infectious origin. Diseases such as rheumatoid arthritis, juvenile sudden onset diabetes, lupus erythematosis, Paget disease of bone, multiple sclerosis, Parkinsonism, presenile and senile dementia, amyotrophic lateral sclerosis, and many others are potential candidates for viral involvement.

There are many possible mechanisms including (i) long-term low-level genome replication within certain differentiated cell types and tissues causing direct cell damage; (ii) virus antigens exposed at the surface of cells triggering immune system damage to cells; (iii) persistent shedding of virus antigens leading to immune complex disease; (iv) depression (or hyperreactivity) of immune responses due to infection of immunocytes by viruses; (v) generation of "anti-self" immune responses as a result of viral protein interactions with "self" antigens on cell surfaces, and the like. The presence of long-term or latent virus infection often may be extremely difficult to detect whenever mature infectious virus is not produced (because mutations, host cell factors, and DI particles prevent virus maturation), or whenever only small amounts of mutationally debilitated, slowly replicating virus are produced (*59*). Measles virus involvement in the slowly progressing, fatal brain disease subacute sclerosing panencephalitis (SSPE) was proved by virus isolation only long after an association with measles was apparent. Isolation of measles virus from SSPE is difficult and sometimes impossible because mature virus is not present in the brain (despite the presence of intracellular RNA genomes). Only after prolonged cultivation of living infected brain cells with susceptible indicator cells in culture does mature infectious measles virus appear (*78*). Rubella virus is also a cause of some SSPE (*78*). Measles virus and other RNA viruses have been implicated in multiple sclerosis (*79, 80*), but virus involvement still remains unproved for this important disease despite promising early results with interferon treatment (*81*) and the detection of measles virus nucleic acid sequences in one of four multiple sclerosis patients (*79*).

Paget disease is a slowly progressive, painful, deformity inducing disease of bone that afflicts up to 3 percent of adults worldwide. Years ago, electron microscopy of the abnormal Paget disease osteoclasts showed massive accumulation of particles resembling helical nucleocapsids of negative-strand RNA viruses (*82*). Nevertheless, the decades-long slow progression of this disease, and its subacute nature led most clinicians and scientists to view RNA virus etiology as unlikely. Recently, however, well-controlled fluorescent antibody studies showed the presence of respiratory syncitial virus antigens in the abnormal bone osteoclasts from each of 12 Paget disease patients tested, and not from control patients with other bone diseases (*82*). Mature virus has not yet been isolated, and it would not be surprising if other

RNA viruses or viral products will also be found in association with some other cases of Paget disease. Respiratory syncitial virus (RSV) is an RNA virus that causes croup and other respiratory diseases of infancy, childhood, and adulthood. Since 95 percent of children develop antibodies to this virus before 5 years of age, many older persons with Paget disease could have had persistent RSV infection for decades, and extensive RNA genome evolution could have occurred during this time in one individual. Since RNA viruses can mutate to escape immune responses while persisting within cells, it would not be surprising if SSPE, Paget disease, and many other degenerative diseases often involved specific immune system "blindspots" in individuals, that is, immunocyte failure to control certain specific mutant virus types out of countless mutants arising during persistence.

Fujinami and Oldstone (*83*) found no evidence for generalized immune system deficits in SSPE and suggested that antibody modulation of viral antigens from cell surfaces may be important. Choppin *et al.* (*84*) find that there is very little expression of the measles matrix protein in SSPE and little antibody response to it. Much more work is needed to determine why persistent virus infection leads to serious or fatal disease in certain individuals; but viral mutation is likely to play a role. For example, Hughes and Johnson (*85*) find very early appearance of VSV mutants in the brains of infected mice; these mutants cause a characteristic central nervous system disease, and Fields *et al.* demonstrated the role of virus mutations in reovirus virulence (*86*).

Implications of RNA Genomes for General Evolution

RNA viruses greatly outnumber DNA viruses in eukaryotic hosts (*87*). This multitude of RNA viruses may represent a wide variety of rapidly evolving and diverging representatives of a rather small number of RNA genome structural classes. In any case, most plant, animal, and human virus disease agents have RNA genomes (*87*). How these ubiquitous, diverse, and highly variable RNA genomes fit into the overall scheme of evolution is not immediately obvious. RNA viruses might sometimes transfer genetic information from host to host, but this is hardly a major function since retroviruses and DNA viruses are much better equipped for gene conversion and genetic transduction. RNA genomes might exert important effects on cellular systems (such as RNA splicing systems), but such effects remain undiscovered.

The major evolutionary role of RNA genomes is probably their most obvious and widely shared characteristic, the ability to kill cells and organisms. From the standpoint of an infected individual, disease and death are negative; but population control and selection for fitness are positive contributions at the population level. Moreover, viral disease and death contribute to evolutionary diversity by upsetting population equilibria in plants and animals, particularly in situations where a relatively few species might otherwise establish dominance. The disease-inducing and killing capacity of RNA viruses must have global impact on evolution. Each year, countless aphids and other insect vectors spread numerous RNA plant viruses whose subtle effects (*88*) on plant competition inevitably affect evolution. Likewise, mosquitoes, biting flies, and the like spread hundreds of arthropod-borne RNA virus diseases to mammals from the arctic regions to equatorial jungles, and all the while insects themselves become infected by RNA viruses that act to influence the flux of insect populations. Even fish are subject to numerous RNA virus diseases.

Several specific examples illustrate how small RNA genomes can differentially regulate plant and animal host populations. Cucumber mosaic virus (CMV), which exists as many strains and under many names, is a segmented genome RNA virus found in all areas of the world. It can cause devastating crop losses affecting a wide variety of agricultural plants. Waterworth and colleagues (*89*) found that a low-molecular-weight RNA satellite genome (CARNA5) associates with, is supported by, and encapsidated by CMV, and can greatly alter CMV disease in crop plants. The presence of CARNA5 greatly reduced CMV disease symptoms in squash, tobacco, pepper, and Bantam sweet corn, whereas it greatly increased disease symptoms in tomatoes, leading to lethal necrotic symptoms. The origin of this tiny regulatory RNA molecule is unknown, but it appears after CMV is passaged through tobacco plants (*88*), and it obviously could profoundly affect the relative competitive abilities of plants.

Rabies is a small RNA rhabdovirus able to infect nearly all mammals. The many different strains of rabies virus are almost diabolical in their ability (*90*) to travel slowly up nerve axons from a peripheral bite site to reach the brain and salivary glands without exciting adequate immune responses. Rabies virus then induces salivary shedding of virus and concurrent behavioral changes likely to increase transmission to other animals. It has long been known that this highly fatal disease regulates populations of predators such as foxes. Anderson *et al.* have devised a simple mathematical model for the dynamics of rabies interaction with, and regulation of, fox populations (*91*). This model gives excellent fit to the observed rabies epidemiological patterns of 3- to 5-year oscillations in both fox population density and rabies prevalence. The period of these cycles is determined mainly by the growth rate of these populations, and rabies virus acts as a time-delayed density-dependent regulator of fox population size. Clearly the small RNA genome of rabies virus is cyclicly affecting not only the evolutionary patterns of foxes but those of their prey, their prey's plants, and insect food supplies. Remarkably, the oscillatory interactions between fox levels and rabies incidence closely resemble the dynamics of cycling interactions (*92*) between RNA viruses and their DI particles.

In this era of antibiotics, it may seem unlikely that infectious agents still exert such major regulatory effects upon human populations. However, we have yet to rule out virus involvement in human malignancy, atherosclerosis (*93*), diabetes (*94*), and other major contributors to human mortality.

It is possible that RNA genomes were the first life forms and constituted the first "biosphere" on earth (*95*), that they were supplanted by DNA genomes because of the greater stability of DNA, and that RNA genomes have persisted and exerted important regulatory and diversifying influences on DNA-based organisms thereafter. Among those regulated DNA-based organisms are humans, who regard the regulatory and diversity-generating effects of RNA genomes as "diseases."

Note added in proof: Air (*96*) recently reported extreme nucleotide variation and extensive amino acid variation (20 to 74 percent) among hemagglutinin gene 3′ ends of the 12 subtypes of type A influenza virus. Only cysteine and a few other amino acids are totally conserved among all subtypes. Genetic drift within subtypes showed 0 to 9 percent amino acid variation and many more "silent" nucleotide substitutions. No greater rate of sequence change occurred in the antigenically selected hemagglutinin gene than in unselected genes. King *et al.* (*97*) presented biochemical evidence for recombination between ts mutants of foot-and-mouse disease virus and Lazzarini *et al.* (*98*) have reviewed structures and likely origins of DI particle RNA's.

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