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The Role of Defective RNAs in Citrus Tristeza Virus Diseases

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	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings		1		1
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Cooperation. Cooperation between teams was complementary and supportive. It was complementary since we worked on two unusually different CTV isolates and developed different reagents, with the Florida group concentrating on the infectious CTV clone and the Israeli group on the infectious dRNA. It was synergistic because the final stage of infecting citrus plants was accomplished through the joint capabilities of our teams. Eight publications that were jointly co-authored provide testimony to this statement.

Abstract

This program focused on citrus tristeza virus (CTV), the largest and one of the most complex RNA-plant-viruses. The economic importance of this virus to the US and Israeli citrus industries, its uniqueness among RNA viruses and the possibility to tame the virus and eventually turn it into a useful tool for the protection and genetic improvement of citrus trees justify these continued efforts.

Although the overall goal of this project was to study the role(s) of CTV associated defective (d)-RNAs in CTV-induced diseases, considerable research efforts had to be devoted to the engineering of the helper virus which provides the machinery to allow dRNA replication. Considerable progress was made through three main lines of complementary studies.

For the first time, the generation of an engineered CTV genetic system that is capable of infecting citrus plants with *in vitro* modified virus was achieved. Considering that this RNA virus consists of a 20 kb genome, much larger than any other previously developed similar genetic system, completing this goal was an extremely difficult task that was accomplished by the effective collaboration and complementarity of both partners. Other full-length genomic CTV isolates were sequenced and populations examined, resulting in a new level of understanding of population complexities and dynamics in the US and Israel. In addition, this project has now considerably advanced our understanding and ability to manipulate dRNAs, a new class of genetic elements of closteroviruses, which were first found in the Israeli VT isolate and later shown to be omnipresent in CTV populations. We have characterized additional natural dRNAs and have shown that production of subgenomic mRNAs can be involved in the generation of dRNAs. We have molecularly cloned natural dRNAs and directly inoculated citrus plants with 35S-cDNA constructs and have shown that specific dRNAs are correlated with specific disease symptoms. Systems to examine dRNA replication in protoplasts were developed and the requirements for dRNA replication were defined. Several artificial dRNAs that replicate efficiently with a helper virus were created from infectious full-genomic cDNAs. Elements that allow the specific replication of dRNAs by heterologous helper viruses also were defined. The T36-derived dRNAs were replicated efficiently by a range of different wild CTV isolates and hybrid dRNAs with heterologous termini are efficiently replicated with T36 as helper.

In addition we found: 1) All CTV genes except of the p6 gene product from the conserved signature block of the *Closteroviridae* are obligate for assembly, infectivity, and serial protoplast passage; 2) The p20 protein is a major component of the amorphous inclusion bodies of infected cells; and 3) Novel 5'-Co-terminal RNAs in CTV infected cells were characterized.

These results have considerably advanced our basic understanding of the molecular biology of CTV and CTV-dRNAs and form the platform for the future manipulation of this complicated virus. As a result of these developments, the way is now open to turn constructs of this viral plant pathogen into new tools for protecting citrus against severe CTV forms and development of virus-based expression vectors for other citrus improvement needs.

In conclusion, this research program has accomplished two main interconnected missions, the collection of basic information on the molecular and biological characteristics of the virus and its associated dRNAs toward development of management strategies against severe diseases caused by the virus and building of novel research tools to improve citrus varieties. Reaching these goals will allow us to advance this project to a new phase of turning the virus from a pathogen to an ally.

Achievements

Genomic analyses of citrus tristeza virus (CTV) isolates originating from our laboratories and from Spain and California suggested that differences in defective RNA (dRNA) populations and/or genomic differences might be involved in major biological variations among CTV isolates. Consequently, it became clear that functional characterization of CTV components involved in disease production would depend on the availability of genetic systems that allow *in vitro* manipulation of genomic and dRNAs that are capable of infecting citrus plants. The development of these systems and their use for CTV research was a major challenge since CTV comprises the largest plant RNA genome, and the ability to infect with RNA is inversely correlated with the size of the genome. The development of this system allowed the assignment of viral assembly functions to the two coat proteins plus HSP70h and p61. Infectivity studies progeny recombinant (r) virus in citrus indicator plants demonstrated that the cloned virus maintained all its essential biological functions of the parental population including host reactions and vector transmission. Viral determinants of pathogenicity host range, and vector transmission can now be elucidated using this genetic system. We have molecularly cloned natural and chimeric dRNAs and directly inoculated citrus plants with 35S-cDNA constructs. However the efficiency of dRNA replication was initially low and only detected by sensitive PCR diagnosis. Considerable advancements were recently achieved using the *Nicotiana benthamiana* protoplast system. These studies provided information on cis-acting elements and other components required for dRNA replication and are expected to facilitate the future application of these elements as tools for the possible transfer of informational molecules into citrus plants.

Our capabilities of performing CTV research had been transformed from a biological and empirical phase to one that now can be advanced using the most advanced molecular engineering tools. It is now possible to elucidate the molecular and biological functions of the CTV genes. In addition, these will allow examination of other fundamental questions with considerable practical implication such as the molecular basis and mechanism of recombination within populations of CTV isolates and their associated dRNAs. These tools will allow analysis of biologically active virus particles constructed from different CTV populations. Supplementing or

subtracting dRNA molecules or dRNA components from these well defined genetic systems will allow the rational analysis of the complex nature of this large virus and the possible causes of its ill defined pathogenic nature. The potential for application of these new tools is immense. Further characterization of the CTV molecules and the associated dRNAs will eventually lead to construction of a new set of tools for protecting citrus against severe CTV isolates. CTV based vectors will find even a greater application for the functional analysis of homologous and heterologous genetic material in fruit bearing experimental citrus plants. Developing these systems will considerably reduce the testing time for mature citrus plants in which fruit bearing time and loss of juvenile characteristics following transformation might be as long as 5 to 10 years. The addition of these capabilities to citrus breeding could revolutionize the industry.

Cooperation, The considerable progress made on this project is due to the effective cooperation of the Israeli and US laboratories. There was joint design of experiments and continuous exchange of information and reagents. A BARD supported postdoctoral study and a sabbatical further fostered our long-standing cooperation. Eight publications that were jointly co-authored provide testimony to this statement.

Background

1. Construction of an infectious CTV cDNA clone. CTV (genus *Closterovirus* family *Closteroviridae*)(1,2) is one the most important virus of citrus. Decline isolates of CTV rapidly kill citrus trees and stem-pitting isolates make citrus production non-economical due to poor tree vigor and small fruit. During the last century, CTV has destroyed entire citrus industries, yet in other cases CTV epidemics have had little impact on production. The virions are encapsidated by two coat proteins, one covering about 95% of the genome and the other encapsidates the opposite terminus. The single-stranded RNA genome of c. 20 kb is divided into twelve open reading frames (ORFs). ORF 1a encodes a 349-kDa polyprotein containing two papain-like protease domains plus methyltransferase-like and helicase-like domains. Translation of the polyprotein is thought to occasionally continue through the polymerase-like domain (ORF 1b) by a +1 frameshift. Ten 3'

ORFs are expressed by 3' co-terminal subgenomic (sg) mRNAs that are promoted internally. Strains of CTV that belong to the same virus species have unusual characteristics. First, the sequence variation among the different genotypes is large, with as little as 50-80% nucleotide identity in much of the genome. Also, while the sequence divergence between some isolates is uniform throughout the genome, the divergence of other isolates is asymmetrical and progressively increases toward the 5' terminus to as little as 42% identity within the 5' non-translated region (NTR) (3,4,7). Secondly, CTV isolates have multiple dRNAs of various sizes and abundance (5-7).

CTV isolates consist of unusually complex populations of distinct genotypes and dRNAs. There is limited information concerning how these populations arise and are maintained. Do diverse genotypes and dRNAs share replication machinery or are there mechanisms to segregate the processes? This program led to construction of a full-length cDNA clone of the CTV genome from which *in vitro* transcripts that infect plant protoplasts was developed. However, the large size of this viral RNA and the complex ligation strategies required for cloning the full length cDNA, were near the limit of our capabilities. For routine replication experiments in protoplasts, it was imperative to create, by deletion of approximately 40% of the genome, an autonomous CTV RNA replicon that provides a much simpler genetic system. Using this system, we examined the ability of the CTV replicase complex to recognize and replicate CTV RNAs with termini representing a wide range of sequence variation.

2. Replication of CTV dRNAs Most CTV isolates are associated with multiple defective RNAs (dRNAs) which vary greatly in abundance and in size, ranging from smaller than 2 kb to near full-length (5-7). Sequence analysis suggests that the reported all dRNAs could have been formed by a single internal deletion within the genomic RNA (5-7), but with different lengths of 5' and 3' termini, thus resulting in non-conserved junction sites. Most isolates have one or two dRNAs in major abundance with additional minor species. In such heterologous pools of genomic RNAs and dRNAs, it is not known how population structures of dRNAs are formed or maintained. Are heterologous dRNAs replicated by the majority replicase complex? Development of biologically active cDNA clones of dRNAs that allow manipulation and mutagenesis and a defined system that supports dRNA replication

were needed for examining interactions and specificity's between dRNA and helper. A chimeric cDNA constructed from naturally occurring defective RNA of 2379 nt (D2.3) of the VT strain was found to infect citrus plants but at a very low efficiency. The infectivity of dRNAs was considerably improved with the *N.benthamiana* protoplast system.

3. Replicase compatibility and the effects of dRNA structural components on replication. Viral genomic RNAs contain, in addition to ORFs, sequences essential for gene regulation, replication, and genome packaging. dRNAs retain conserved *cis*-acting replication elements necessary for recognition by the replicase complex, but depend on the parental helper virus for the viral replicase and other *trans*-acting factors for replication. Some dRNAs associated with CTV consist of relatively small 5'- and 3'- termini, suggesting that the replication signals of CTV reside primarily in these terminal regions. In addition to replicase recognition signals, other factors have been shown to influence dRNA fitness. One factor is size of the dRNA molecule. For example, larger DI RNAs of certain viruses accumulate to higher levels than smaller molecules, but the opposite was found for DI RNAs of other. However, the fitness of *Tobacco mosaic virus* (TMV) dRNAs was not strictly correlated with size (9), suggesting that the juxtaposition of structure affected the ability to be replicated. The sizes of the CTV dRNA species range from small (~1.6 kb) to nearly genomic full-length (6-8). The effects of the overall size and the lengths of termini on fitness of CTV dRNAs were unknown. Translation of an ORF through much of the dRNA can be another factor involved in efficient amplification of dRNAs. This could be due to the production of a necessary *cis*-acting protein or to the translation process which previously was suggested to be necessary for efficient accumulation *in vivo* (White *et al.*, 1992). In contrast, accumulation of some TMV dRNAs was facilitated by expression of a functional *cis*-encoded viral. Sequence analysis of naturally occurring CTV dRNAs demonstrated that most had a large 5' ORF but some did not contain a significant 5' ORF. We found that CTV dRNA replication required translatability of the 5' ORF. We also found that 3' heterologous sequences had only a little effect on the levels of replication in protoplasts, whereas the more divergent 5' heterologous sequences caused greater reductions.

Methodologies and Materials

Virus isolates most of the studies in Florida and Israel were conducted with CTV isolates T36 and VT, respectively (3,4). Cloning, hybridizations and other molecular, immunological and biological technologies used through these studies were presented throughout the publications listed in this report.

Results

The overall goal of this project was to study the role(s) of CTV associated dRNAs in CTV-induced diseases. Progress made through three main lines of complementary studies is summarized bellow.

1. Cloning of infectious CTV constructs. A full-length cDNA clone of CTV was generated from which RNA transcripts were capable of replication in protoplasts. Deletion of ten 3' genes resulted in an autonomous replicon that was a much easier system to study structural and/or sequence requirements of cis-acting elements necessary for genomic RNA replication. **This was the first report of an infectious cDNA molecule derived from the genomic CTV RNA.** To investigate the origin and maintenance of the genotypes in CTV populations, we tested the CTV replicase for its acceptance of divergent sequences by creating chimeric replicons with heterologous termini and examining their ability to replicate. Exchange of the similar 3' termini resulted in efficient replication, while substitution of the divergent (up to 58% difference in sequence) 5' termini resulted in reduced but significant replication, generally in proportion to the extent of sequence divergence. Using trace amounts of virions from protoplasts infected with RNA transcripts for serial infection of *N. benthamiana*, protoplasts resulted in a considerable amplification of virus titers. Mechanical (slash) inoculation with virion extracts from the 4th-6th cycled protoplasts enabled the regular infection of recombinant virions. Macroscopic and microscopic disease symptoms typical to infection with wild type T36 virions were observed on CTV indicator plants inoculated with recombinant virions.

- 1.2 CTV assembly and infectivity The resulting genetic system allowed the examination of genes involved in the production of infectious virus particles. Serial passages of virions in protoplasts and serologically specific electron microscope (SSEM) showed that both coat proteins plus the HSP70h and p61 are needed for efficient virion formation. Thus, all but the p6 of the conserved signature gene block of the *Closteroviridae* appear to be involved in assembly of CTV.

1.2.2 Accumulation of the P20 gene product in amorphous inclusion bodies The functions of most of the CTV 3' ORFs were unknown. The highest produced subgenomic RNAs are those of the coat protein genes and the 3' most genes, p20 and p23. The proteins from these ORFs were examined in homologous and heterologous combinations in the yeast two-hybrid assay. The p20 protein exhibited a high affinity for itself, suggesting that it might aggregate in infected cells. The cytopathology of CTV includes characteristic paracrystalline and amorphous inclusions in the phloem elements of infected citrus. Polyclonal antiserum raised against the bacterially expressed p20 gene product detected a protein of approximately 22-23 kDa accumulating to relatively high levels in infected citrus but not in healthy citrus. Immunogold localization using antibodies to p20 showed strong and specific labeling of the amorphous inclusion bodies present in infected cells. Mesophyll protoplasts of *N. benthamiana* transfected with CTV containing GFP-ORF fused in-frame to the 3' end of p20 ORF expressed high levels of GFP. The fusion protein was concentrated in one specific area in the cytoplasm and lacked an organized shape. The accumulation of high levels of p20 protein in infected tissues, specific localization of the p20-GFP fusion protein, immunolocalization of p20 into inclusions, and strong homologous p20-p20 interactions in the yeast-two hybrid assay suggest that the p20 protein of CTV is a major component of the characteristic amorphous inclusion bodies

2.1. Characterization of a 2.3 kb dRNA and its use for constructing an infectious cDNA clone: A naturally occurring dRNA of 2379 nt (D2.3) from the VT strain of CTV was characterized by cloning and sequencing. The D2.3 RNA was found to result from the fusion of 2 regions of 1521 and 858 nt from the 5' and 3' ends, respectively, of the CTV-VT genome. A cDNA clone was synthesized from the D2.3 RNA template and the construct was tagged initially with the Bar gene. The recombinant (r) cDNA was inserted downstream to the cauliflower mosaic virus 35S promoter and used to superinfect stem bark tissue from CTV-infected plants by bombardment. The bombarded stem bark was grafted on virus-free citrus plants and 4-12 weeks after inoculation the new flush was collected and tested for the presence of single and double stranded r-dRNA. We were able to demonstrate using RT-PCR, the presence of the r-dRNA in systemically infected leaves. Sequencing the RT-PCR

products synthesized from double-stranded RNA confirmed the presence of the chimeric segment used for tagging. **This was the first report of an infectious cDNA molecule derived from CTV D-RNA.**

2.2 Creation of infectious CTV-dRNAs and testing CTV replicase compatibility with heterologous 3' and 5' NTR sequences within the genomic RNA We found that 3' heterologous sequences had a little effect on the levels of replication in protoplasts, whereas the more divergent 5' heterologous sequences caused greater reductions. A series of dRNA like molecules, each with a single large internal deletion, were created from an infectious cDNA clone of the CTV T36 isolate and examined for amplification in *N. benthamiana* protoplasts using a deletion mutant as the helper virus. Of twelve synthetic dRNAs, only three with sizes of 3650, 3819, and 4460 nucleotides were efficiently replicated. dRNA replication did not appreciably affect levels of accumulation of the genomic or the subgenomic RNAs of the helper virus. To investigate the maintenance of dRNAs in CTV populations, we examined heterologous interactions between dRNAs and helper viruses. Wild type populations of heterologous strains T68 and T3, as well as the homologous T36, supported replication of synthetic T36 dRNAs. Replacement in the T36 dRNA of the 5' region, which is most variable among CTV strains, with the corresponding sequences from VT, T68, T3, or T30 resulted in chimeric dRNAs that failed to be replicated by the T36 helpers, but were replicated to detectable levels by the T68 helper.

2.3 Fitness of Defective RNAs is affected by the lengths of their 5'- and 3'-termini and coding capacity To examine factors affecting fitness of dRNAs, we investigated a series of *in vitro*-constructed dRNAs for their ability to be amplified in protoplasts by an efficiently replicating CTV deletion mutant. The minimal sequences required for accumulation of the dRNAs were within the genomic 5' proximal ~1 kb and the 3' 270 nucleotides. However, other factors were involved, because a dRNA with only the minimal sequences failed to be replicated. Rescue of a non-viable dRNA by insertion of non-viral sequences between the termini suggested that "spacing" between terminal *cis*-acting signals influenced fitness. A continuous ORF through most of the sequences derived from the 5' of the genome was a requirement for dRNA amplification. In general, insertions, deletions, or nucleotide substitutions were tolerated in the dRNAs as long as an ORF was retained, whereas, dRNAs with

mutations that prematurely terminated the ORF were not viable. To discriminate between a requirement for an essential protein and ribosomal travel, perhaps to present replication signals to the replicase complex, mutations were made to modify the potential protein but still maintain an ORF. Deletions, insertions of non-viral sequences, or switching of reading frames that altered the amino acid sequence of the protein, except the N-terminal 161 amino acids, did not reduce the fitness of the dRNAs. Yet, termination of the ORF in the middle of non-viral sequences did end the ability of the dRNAs to be amplified. These results suggest that although a continuous ORF was needed for fitness, its protein product did not affect the amplification of the dRNAs.

3. The involvement of a subgenomic messenger (sg) RNA in the generation of 3' variable populations of dRNA molecules: The fusion sites between the termini of naturally occurring dRNAs from three CTV isolates were sequenced. Seven of the eight clones showed a common 3' terminus of 940-nt fused to 5' termini of different sizes. An extra cytosine nucleotide was found at the junction site of the majority of the common 3' dRNAs. Molecular analysis of the plus and minus strands of the 0.9 kbp double-stranded RNA, corresponding to the CTV ORF 11 sgRNA, showed that they were identical in length and sequence to the common 3' sequence of the dRNAs. These results imply that viral sgRNA messengers also function as building components for genomic rearrangement and exchange of complete viral genes.

3. 1. Subtypes of the VT strain of CTV with different dRNAs show differential Seedling Yellows Reactions Infection of sour orange plants with the VT strain of CTV has been reported to cause SY symptoms. Later passages resulted in inconsistent SY reactions. Eleven VT subtypes selected from chronically infected Alemow plants were found to cause a consistent pattern of either SY or non-SY (nSY) reactions. Eight of the subtypes, which caused the SY reaction, harboured dRNAs of about 2.4 or 2.7 kb, whereas all three nSY subtypes harboured a different dRNA of about 4.5 kb. Considerably higher ELISA titres were found in sour orange plants with nSY than in those with SY symptoms. Plants infected with a VT subtype, which harboured the 2.7-kb D-RNA, were protected for at least 41 weeks from superinfection with subtype VT12 with the 4.5 kb dRNA. Partial or no protection was observed after 18 weeks and 41 weeks, respectively with the reciprocal

combination. The possible involvement of dRNAs in the long-standing enigma of the CTV-SY phenomenon is implicated. The 3' ends of both subtypes showed at least three different utmost sequences, whereas the upstream-untranslated regions were completely similar. This result indicates that sequence conservation in the end-most region is less critical than remaining 3' end UTR of this virus.

3.2 Recovery from CTV-Seedling Yellows associated with a change in the population of dRNAs Sour orange plants with severe seedling yellows (SY) symptoms caused by the Mor-T isolate of CTV were found after 2 years to show a partial recovery as indicated by the appearance of twigs with larger leaves and with increased ELISA titers. Tissue passages from SY recovered (SYr) twigs to sour orange seedlings gave nSY reactions and did not cause the typical quick decline in sweet orange grafted on sour orange. Alemow plants infected for 6-8 months with SY and SYr tissues showed differences in the population of the associated dRNAs. Plants infected with SY showed the presence of a population of dRNAs of 2.634 and 2.825 nt, whereas the SYr plants contained a 5127 nt dRNA, with >4000 nts from the 5' which include the coding region of the two putative papain-like proteases (P-PRO) and the N terminal part of the methyltransferase domain (MT), previously also implicated in the nSY reaction of the VT12 subtype. The sequenced parts from the RFs and dRNAs of the SY and SYr MOR subtypes were closely similar both among themselves and with the VT isolate. These results further implicate the involvement of 5' sequence regions from nSY associated dRNAs in the suppression of the apoptotic-like SY symptomatology.

3.3 Novel 5'-Co-terminal subgenomic RNAs in CTV infected cells We report the presence of two additional populations of 5' co-terminal plus-strand sgRNAs of c. 0.8 kb and 10 kb, designated as low and large molecular tristeza (LMT and LaMT) RNAs respectively, in infected citrus plants and *N. benthamiana* protoplasts. Sequence characterization of 27 cDNA clones derived from polyA tailed LMTs showed seven and eight molecules that terminated at CTV-VT genomic positions 744-746 and 842-854 respectively. The 3' termini of eight LaMT clones were positioned between 10768 to 10797 of CTV genome. The absence of 3' products corresponding to an expected cleavage process of full-length genomic RNA favors.

the suggestion that both LMT and LaMT result from termination during positive-stranded RNA synthesis.

3.4 The 3'-nts of CTV RNAs are considerable more variable than the upstream (150-nt) non translated regions: Sequence analysis of the 3' terminal nucleotides of cDNAs obtained from the single- and double-stranded RNAs of the VT isolate, revealed the presence of a heterologous population of molecules with equal ratios of CT, CC or CCAT- 3'. Contrary to this variability, the upstream 150 nts representing part of the 3' end untranslated region (UTR) of the CTV genome were highly homogenous (99% similarity). Two other CTV strains HT and MT, respectively were found to consist of molecules with only one (CCAT) or two (CT and CCAT) types of 3' ends. These results demonstrate that CTV isolates consist of mixtures of molecules with different 3' nucleotides and suggest that the conservation of the terminal nucleotides is less critical than the rest of the 3' NTR.

Discussion and scientific implications

Within the framework of this program, we made substantial progress towards the characterization and taming of the CTV genome and its associated dRNAs. We characterized more natural dRNAs and showed that production of subgenomic mRNAs can be involved in the generation of dRNAs. We molecularly cloned natural dRNAs and directly inoculated citrus plants with 35S-cDNA constructs. We showed that specific dRNAs are correlated with specific disease symptoms and implicated certain dRNA in the seedling yellows recovery phenomena. We developed systems to examine dRNA replication in protoplasts and are defining the requirements for dRNA replication. We created several artificial dRNAs from infectious full-genomic cDNAs that replicate efficiently with a helper virus. We also are defining the specificity of replication of dRNAs by heterologous helper viruses. The T36-derived dRNAs are replicated efficiently by a range of different wild CTV isolates and hybrid dRNAs with heterologous termini are efficiently replicated with T36 as helper. The finding of a hot spot for recombination consisting of the 5' end of CTV suggests that messenger RNA synthesis is involved in recombination of the *Closteroviridae*.

The main achievement however was the development *in vitro* genetic systems for CTV. Using this system, questions related to virion assembly could be addressed.

This system was further improved through the deletion of ten 3' genes resulting in an efficient RNA replicon that was easy to manipulate. To investigate the origin and maintenance of the genotypes in CTV populations, we tested the CTV replicase for its acceptance of divergent sequences by creating chimeric replicons with heterologous termini and examining their ability to replicate. Exchange of the similar 3' termini resulted in efficient replication, while substitution of the divergent (up to 58% difference in sequence) 5' termini resulted in reduced but significant replication, generally in proportion to the extent of sequence divergence. Questions related to the structural and sequence requirements of cis-acting elements involved in genomic RNA replication can now be addressed. Serial passages also allowed the generation of sufficient recombinant virions that enabled the regular infection of citrus indicator plants with recombinant virus. Symptoms on plants infected with recombinant virion were similar to those of wild type, thus opening the way to future studies aimed to assign biological function to different virus genes and genomic sequences.

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