# Synthetic Viral Genomics: Risks and Benefits for Science and Society

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## I. Introduction

### A. Viruses and Biological Warfare

Viral disease outbreaks have long inspired fear in human populations. Highly pathogenic infectious disease has shaped world history, primarily by impacting the outcome of wars and other global conflicts and precipitating human movement. Historic accounts have documented the catastrophic consequences and human suffering associated with widespread viral outbreaks like smallpox virus, yellow fever virus, measles virus, human immunodeficiency virus (HIV), the severe acute respiratory syndrome coronavirus (SARS-CoV), the 1918 influenza virus and others (51). News accounts and film have reinforced the serious threat posed by the emergence of new viral diseases as well as the catastrophic consequences of intentional release of highly pathogenic viruses in human populations. As illustrated by the SARS epidemic and the continuing evolution of the H5N1 avian influenza, global and national infectious disease outbreaks can overwhelm disaster medical response networks and medical facilities, disrupt global economies, and paralyze health and medical services by targeting health care workers and medical staff (21). This review focuses on viruses of humans, animals and plants that are viewed as potential weapons of mass disruption to human populations, critical plant and animal food sources, and national economies; and will consider whether and how the availability of synthetic genomics technologies will change this landscape.

Biological warfare (BW) agents are microorganisms or toxins that are intended to kill, injure or incapacitate the enemy, elicit fear and devastate national economies. Because small amounts of microorganisms might cause high numbers of casualties, they are classified as weapons of mass destruction. A number of naturally occurring viruses have

potential uses as BW agents, although the availability of these agents is oftentimes limited. This report discusses the potential use of recombinant and synthetic DNAs to resurrect recombinant BW viruses de novo and the potential for altering the pathogenic properties of viruses for nefarious purposes. Examples of weaponized viruses include Variola major (Smallpox), Venezuelan equine encephalitis virus (VEE), and the filoviruses Marburg and Ebola viruses, with the classic example being the use of smallpox virus-contaminated blankets against indigenous North American Indian populations (76). It is now clear that many viruses possess properties consistent with applications in biological warfare and bioterrorism.

### B. Properties of Select BW Agents

Traditionally, biological warfare concerns have focused on a relatively limited, select group of naturally occurring pathogens viewed as having a set of desirable characteristics: 1) highly pathogenic, 2) readily available, 3) easily produced, 4) weaponizable, 5) stable, 6) infectious at a low dose, 7) easily transmissible, and 8) inspiring of fear (32). Viruses of concern include pathogens that replicate and produce serious morbidity and mortality in humans to pathogens that target farm animals and plants of economic importance. Historically, weaponization of agents has been constrained by availability, the biological characteristics specified within the genome of these organisms, the ability to replicate and produce large quantities of the material, and by the lack of appropriate associated technologies. Culture (growth) and containment conditions for most of the virus agents of concern have been solved and are readily available in the literature. Natural hosts and reservoirs of many viral agents have been identified, providing a means of readily acquiring these pathogens in nature, although this is not always the case. Most recently, full length genome sequences have been solved for many important human, animal and plant pathogens, providing a genetic template for understanding the molecular mechanisms of pathogenesis and replication. Structural studies have identified contact points between the virus and the host receptors needed for docking and entry, providing the means to humanize animal pathogens (42). With the advent of synthetic biology, recombinant DNA technology, reverse genetic approaches (i.e. the development of molecular clones of infectious genomes) and the identification of

virulence alleles, not only are new avenues available for obtaining these pathogens, but more ominously, tools exist for simultaneously modifying the genomes for increased virulence, immunogenicity, transmissibility, host range and pathogenesis (22, 59). Moreover, these approaches can be used to molecularly resurrect extinct human and animal pathogens, like the 1918 human influenza virus (81).

National biodefense strategies are focused on threats posed by this small group of plant, animal and human pathogens that occur in nature. However, counterterrorism think-tanks anticipate that these particular threats will ameliorate over the next decade because of medical countermeasures (e.g., drugs, vaccines, diagnostics), coupled with a limited set of pathogens that include all of the biological warfare characteristics. More important, the anticipated long-term threat in biological warfare is in recognizing and designing countermeasures to protect against genetically modified and designer pathogens, made possible by newly emerging technologies in recombinant DNA, synthetic biology, reverse genetics and directed evolution (59). How will synthetic genomics effect future biological weapons development? What are the risks and benefits of these new technologies and how serious a threat do they pose for human health and the global economy? This paper builds upon earlier work and seeks to review the methodologies in isolating recombinant viruses in vitro and the application of these methods globally to biological warfare and biodefense (27).

## II. Virus Classification and Reverse Genetic Approaches

### A. Overview of Virus Classification and Reverse Genetics

From the genome, all viruses must generate a positive strand mRNA that is translated into proteins essential for genome replication and the assembly and formation of progeny virions. Depending upon the nature of the genome, all viruses can be clustered into seven fundamentally different groups, which utilize different strategies to synthesize mRNA

from the input genome, a scheme called the Baltimore Classification (Figure 1).[1] Because virus infectivity is dependent upon the ability to transcribe mRNAs, reverse genetic strategies are designed to insure expression of critical viral mRNAs that encode essential replicase proteins needed to "boot" (initiate) genome infectivity and initiate genome replication.

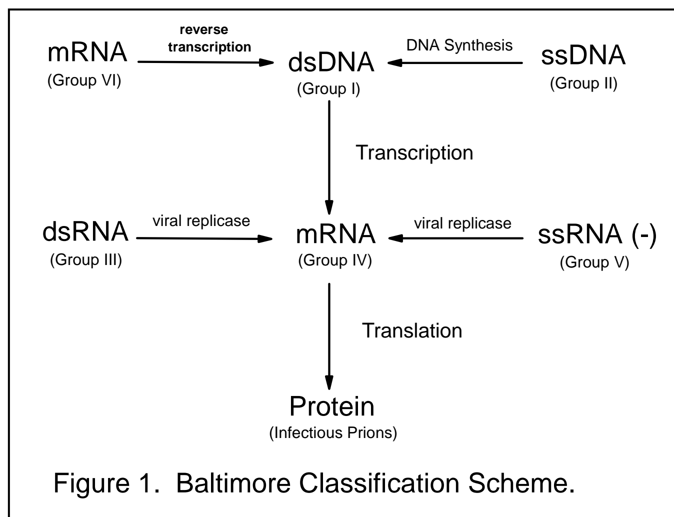


Figure 1. Baltimore Classification Scheme.

Group I viruses include the double-stranded DNA (dsDNA) viruses, like the Herpes viruses and Poxviruses which replicate in the nucleus or cytoplasm, respectively. The dsDNA viruses use cellular and/or virally-encoded transcriptase components to mediate expression of viral mRNAs. Poxviruses for instance require one or more viral proteins to initiate mRNA transcription and boot infectivity of the viral genome. Hence, smallpox virus genomes are not infectious unless the appropriate suite of viral proteins is provided in *trans* (in addition to the genome itself). In contrast, the Herpes virus genome is infectious in the absence of any viral proteins as cellular transcriptase machinery induces expression of early mRNAs and proteins that regulate expression of other viral genes and replication. Using vaccinia (poxvirus) as a model, an approach to successfully initiate/jump start and boot the infectivity of poxviruses has been developed, providing a template strategy for the family (11, 24). Herpes virus genomes are infectious in the absence of additional viral factors. Group II viruses encode single stranded DNA genomes which must be used as templates for the synthesis of a dsDNA before

[1] Named for the virologist David Baltimore, who proposed the system.

transcription and translation of mRNAs can occur within cells. At this time, group II BW agents have not been identified.

The Group III viruses contain double stranded RNA viruses, like reoviruses. Reovirus genomes consist of complementary positive and negative strands of RNA that are bound by hydrogen bonding, wrapped within a multistructured icosahedral core that is essential for virus transcription. The virion structure contains the necessary proteins required for initiating mRNA synthesis. Unlike many of the single-stranded RNA viruses, the dsRNA virus genomes are not infectious in isolation and the components necessary for booting genome infectivity remain unresolved.

Group IV viruses contain a single-stranded positive polarity RNA genome and include the flaviviruses, alphaviruses, picornaviruses (including poliovirus), coronaviruses (including the SARS virus), caliciviruses and others. Upon entry into cells, positive strand RNA genomes are immediately recognized by host translational machinery and the genome is translated into a suite of viral proteins, including the replicase proteins and RNA-dependent RNA polymerase which is necessary for initiating the viral replication cycle. Consequently, genome infectivity usually does require viral proteins or transcripts provided in trans to boot genome infectivity, although some exceptions have been reported (13). Group V viruses contain a single-stranded negative polarity RNA genome and include filoviruses (Ebola/Marburg), myxoviruses (influenza), and paramyxoviruses (Hendra). Group V genomes come in two different flavors, segmented (e.g., myxoviruses) or nonsegmented (e.g., paramyxoviruses and filoviruses). In either case, the genome is not infectious because it is complementary in sequence (anti-sense); it is the opposite of the positive strand that specifies amino acids and thus cannot be translated directly into any of the critical viral structural or replicase proteins needed for producing infectious virions. Negative strand RNA genomes are encapsidated into a complex ribonucleoprotein structure (RNP) usually composed of several virally encoded replicase proteins (e.g., polymerase complex proteins, support proteins, trans-acting proteins) that are incorporated into the virion during assembly. Together, these compose a functional replication complex. Upon entry, these RNP complexes immediately transcribe the genome negative strand RNA into mRNA that can be translated into the viral proteins.

Consequently, genome infectivity requires the presence of full length RNA and a set of virally encoded replicase proteins that function as a transcriptional complex to express mRNAs. If mRNAs encoding the transcripton complex are provided in trans, group V genomes become infectious and virus will be successfully recovered.

Group VI viruses, retroviruses (including HIV) and lentiviruses, encode single stranded positive polarity RNA genomes, but virions encode a reverse transcriptase enzyme to convert the mRNA genome into a complementary DNA (cDNA) which serves as template for dsDNA synthesis. Following the synthesis of dsDNA, group VI viruses use cellular transcriptional and translational machinery to express viral transcripts encoding structural and nonstructural proteins. At this time, the group VI viruses do not include any BW agents.

## B. Infectious Genomes, Molecular Clones and Reverse Genetics

The basic concepts central to understanding virus reverse genetics and molecular clones are summarized in Figures 1 and 2. The central idea is that the virion is an extracellular vehicle that transfers the viral genome (e.g., RNA or DNA genomes) between susceptible cells and protects the nucleic acid genome from degradation in the environment (Figure 2, Part A). Following entry, the viral genome is programmed to initiate a series of events that result in the production of a replicase complex that transcribes mRNA and replicates the genome. As discussed in the previous section, nucleic acid structure and organization determines the pathway of events needed to express mRNA and initiate virus gene expression and infection. Not all viruses, however, require virion attachment and entry to mediate a productive infection. In these cases, viral genomes can be isolated from virions and transfected directly into susceptible host's cells. If the genome is infectious, viral RNAs and proteins will be expressed allowing for the production and release of progeny virions (Figure 2, Part B). Classic examples of viruses with "infectious genomes" include the herpes viruses, polioviruses, alphaviruses, polyomaviruses, and flaviviruses which are classified among the Group I, II or IV viruses. However, not all viral genomes are infectious upon delivery into cells. Viruses with Group III or V genomes have never

been demonstrated to be infectious upon genome delivery into susceptible cells. Some Group I (poxviruses) and group IV virus genomes (e.g., norovirus, a causative agent of non-bacterial gastroenteritis, or "cruise ship disease" and the coronavirus infectious bronchitis virus) are not infectious upon delivery into susceptible cells (13). In these instances, genome infectivity requires the presence of specific cofactors to initiate viral replication. These cofactors typically represent one or more proteins that encode essential replicase proteins or encapsidate the genome into an RNP structure necessary for initiating transcription of mRNA from the genome. In this example, infectious bronchitis virus genome infectivity requires the nucleocapsid protein in trans while the components needed to boot norovirus genome infectivity remain unknown (13).

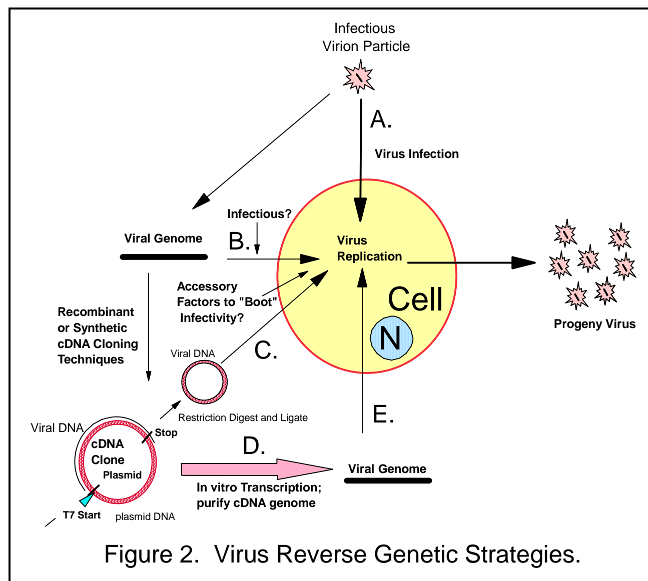



Figure 2. Virus Reverse Genetic Strategies.

In the late 1970's, a simple observation altered the course of virology research globally. Using a small dsDNA virus genome as a model (the Group I polyomavirus SV40) researchers cloned the viral genome into a bacterial plasmid and propagated the viral genome in bacteria. Upon isolation of the plasmid DNA from bacteria, restriction enzymes were used to excise the dsDNA viral genome, re-ligate the genome in vitro into a circular dsDNA and rescue virus following transfection of the genome into susceptible cells (Figure 2, Part C)(28). (Many advances in biotechnology have been, and continue to be, dependent upon this restrict-isolate-ligate technique, or variations of it.) Shortly

thereafter, full length cDNAs of positive strand RNA genomes were isolated following reverse transcription, the cDNAs cloned and propagated in bacterial plasmids, and following introduction of full length DNA into eukaryotic cells, recombinant viruses were rescued from the transfected cultures, although very inefficiently. The major problems with this approach were the difficulty in generating the appropriate termini, accurate genome sequence, problems in nuclear transport of the full length RNA genome, and splicing of the viral genomic RNA. To rectify the efficiency problems, bacteriophage promoters (T7, SP6, T3) were introduced upstream of the cloned viral cDNAs, allowing in vitro transcription of full length RNA copies of the viral genome using the appropriate phage RNA polymerase, nucleotide triphosphates, and other constituents (Figure 2, Part D). The full length RNAs, near exact replicas of the viral genome, were highly infectious upon transfection of susceptible host cells (Figure 2, Part E)(2, 65, 66). The ability to clone full length copies of viral genomes allowed for ease of manipulation of the genome and the introduction of specific mutations. Recovered viruses contained the introduced mutations that were encoded within the full length cDNA clones, providing a ready means of performing detailed genetic analyses of virus replication and pathogenesis.

As noted earlier not all viral genomes are infectious, complicating the development of full length cDNAs and the recovery of recombinant viruses. Isolated dsRNA genomes from Group V negative sense RNA viruses are not infectious because the genome sequence cannot be translated directly into a functional replicase complex needed to transcribe the incoming genomic RNA. As Group V virions contain a replicase protein complex essential for transcription, genome infectivity requires that cells be co-transfected with plasmids that express the genomic RNA and plasmids expressing transcripts that encode the replicase protein complex are needed for genome infectivity (Figure 3a). For most group V viruses, both genome negative and positive sense RNA infectivity can be booted using this approach with most investigators expressing full length plus (coding) strands from the initial transcript. The plus strands are transcribed to full length negative strands, which are used to express the appropriate set of mRNA encoding the full component of positive and negative strand RNAs. Using this approach

Schnell et al. successfully recovered the first recombinant negative stranded RNA virus, rabies virus, from a cloned cDNA, ushering in an era of Group V virus reverse genetics (68, 82). These findings were rapidly extended to other linear negative stranded RNAs like paramyxoviruses and then to segmented negative strand RNA viruses like influenza and other myxoviruses, and then select bunyaviruses and arenaviruses (20). Reverse genetic strategies for group V viruses with segmented genomes are most complex as multiple plasmids expressing copies of each genome segment must be simultaneously delivered to a cell along with the support plasmids encoding the transcriptase complex.

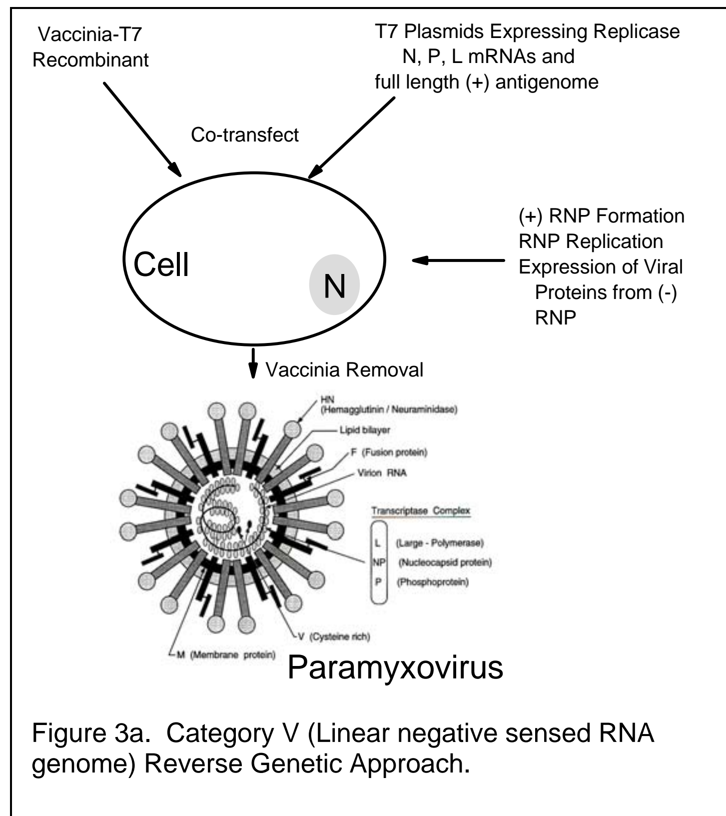



Figure 3a. Category V (Linear negative sensed RNA genome) Reverse Genetic Approach.

Most of the RNA viruses have relatively small genomes (under approximately 20,000 bases or base-pairs). Viruses with extremely large genomes (over 100,000 base-pairs, e.g., herpes viruses, poxviruses, or ~20,000-30,000 base pairs, e.g., coronaviruses, filoviruses) have presented additional obstacles in the development of stable molecular clones. Generation of infectious clones for viruses encoding large RNA or DNA genomes is complicated by the need for sequence accuracy (e.g., incorrect sequences usually contain lethal mutations), the lack of suitable cloning vectors that stably maintain large DNA inserts, large genome size, and that the genomes oftentimes encode regions that are toxic or unstable in bacteria. In poxviruses for example, the ~200 kilobase pair (kbp) genome has covalently closed hairpin ends (structures formed by the DNA itself) that are required for genome replication and virion encoded products are also essential for booting genome infectivity (24).

Herpes virus genomes are ~150 kbp in size. One solution was to stably clone large viral genomes as bacterial artificial chromosome (BAC) vectors. BAC vectors are based on the replication of F factor in E.coli, which is tightly controlled and allows stable maintenance of large, complex DNA fragments up to 600 kbp and both herpesvirus and poxvirus genomes can be stably maintained in BAC vectors (17, 24). For Herpes viruses, BAC shuttle vector sequences encoding a marker are inserted by homologous recombination into the genome. Circular viral DNA, which is generated during the Herpes virus replication cycle, is purified from infected cells (so-called Hirt prep) and introduced in bacterial cells, which essentially generates a large plasmid containing the Herpes virus genome (49). As herpesvirus genomes are infectious, the BAC DNA sequences are rapidly lost after delivery to a suitable host cell, along with some surrounding viral sequences, because they are dispensable for viral DNA replication (71). Using the Cre/lox system (another basic tool of molecular biology), a self-recombining full length

pseudorabies virus BAC was developed where the full length genome is automatically removed from the BAC sequences by the expression of Cre recombinase after transfection, reducing the potential for random deletions of viral sequences (72) (Figure 3b). Recombinant Herpes virus genomes that have been successfully cloned include mouse cytomegalovirus, herpes simplex virus 1, human cytomegalovirus, pseudorabies virus, and Kaposi's Sarcoma virus (11, 24, 49).

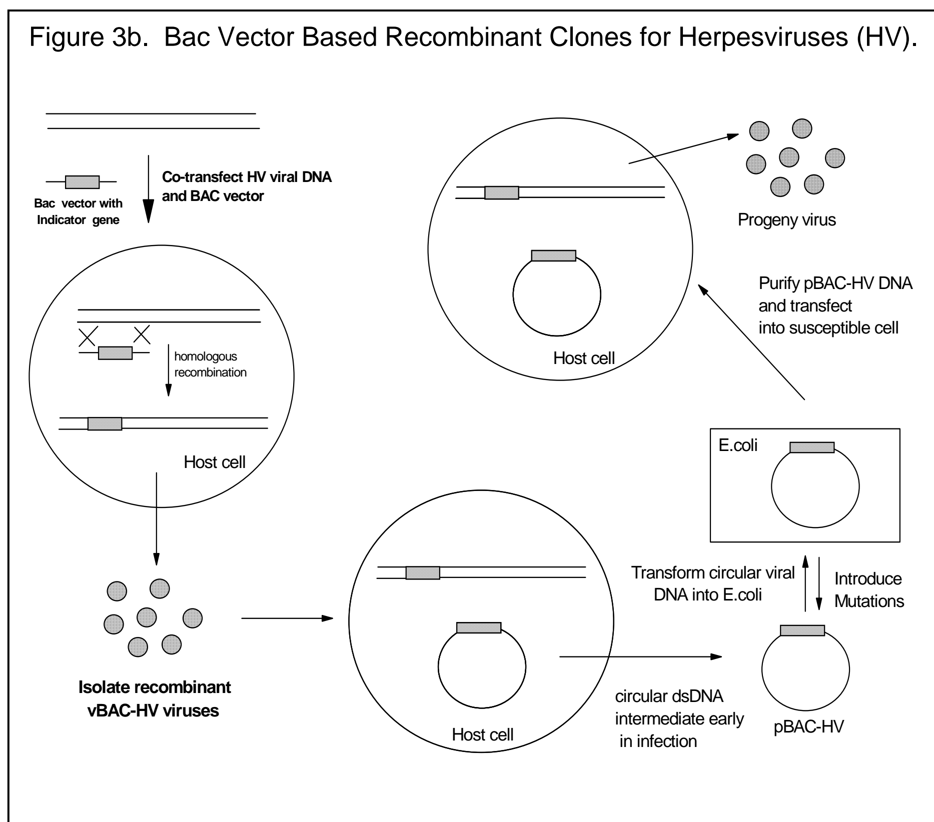



Figure 3b. Bac Vector Based Recombinant Clones for Herpesviruses (HV).

Poxvirus genome structure and replication modes make the development of an infectious poxvirus molecular clone an order of magnitude more difficult than generation of the Herpes virus molecular clone. Poxvirus genomes replicate in the cytoplasm and require several viral proteins to mediate mRNA transcription and a unique DNA-dependent RNA polymerase that are normally contained within the virion to initiate virus infection.

Consequently, purified poxvirus DNA is not infectious. In addition, the linear dsDNA genome has closed hairpins at each end of the genome that are essential for DNA replication. How were these problems solved? As described with Herpes viruses, a mini BAC encoding a marker called green fluorescent protein (GFP) was recombined into the thymidine kinase gene encoded in the vaccinia genome (a model for smallpox). Recombinant viruses harboring the BAC cassette were identified by GFP expression. However, transformation of Vaccinia BAC vectors into E.coli required conversion of the linear genome with covalently closed ends into a closed circular DNA. To accomplish this, Domi and Moss blocked late viral gene expression knowing that this favored additional recombination events that allowed head to tail concatamers of full length genome from which monomeric recombinant genome in a covalently closed circle would result, a favored genome orientation for insertion into E.coli. Transfection of VAC-BAC DNA into mammalian cells, previously infected with a helper fowl pox virus whose replication is defective in mammalian cells, allowed recovery of recombinant vaccinia virus (23, 24).

Although BACs are remarkably stable, both poxviruses and herpesvirus genomes contain repetitive sequence elements and other sequences that might be unstable with passage as no biological selective pressure exists to maintain virus genome sequence fidelity in E. coli. Because the large genome size makes it impractical to sequence the entire genome, in vivo pathogenesis studies have been used to demonstrate equivalent levels of pathogenicity and virulence between wildtype and recombinant herpes viruses, further supporting the hypothesis that BAC recombinant genomes are highly stable in E.coli (12). The availability of large dsDNA genomes in BACs provides two major opportunities for future research, the construction of expression vectors for treatment of human diseases and the mutagenesis of the viral genome for understanding gene function, virus replication and pathogenesis.

A second solution to large genome instability was developed using coronaviruses as models. Seven contiguous cDNA clones that spanned the 31.5 kilobase (kb) coronavirus genome (e.g., mouse hepatitis virus [MHV] or SARS-CoV) were amplified, isolated and ligated into standard polymerase chain reaction (PCR) cloning vectors (PCR is one

technique used to amplify sequences that are rare and/or not available in large quantities, to provide enough material for subsequent experiments). The ends of the cDNAs were engineered with unique junctions, generated by class IIS restriction endonucleases like BglI or Esp3I. These enzymes leave asymmetric ends, which are designed to seamlessly reproduce the exact virus sequence, allow directional assembly of adjacent cDNA subclones, and direct the production of an intact full length cDNA construct of ~31.5 Kb in length. With enzymes like Esp3I, interconnecting restriction site junctions can be located at the ends of each cDNA and systematically removed during the assembly of the complete full-length cDNA product (Figure 4a). The availability of a contiguous set of DNAs containing unique interconnecting junctions provides for the systematic assembly of large DNA molecules greater than 1,000,000 base pairs by in vitro ligation (85). In the case of coronaviruses (Figure 4b), full length cDNAs are assembled that contain a T7 transcription site at the 5' end of the genome. RNA transcripts driven from the full length cDNA were infectious upon delivery into susceptible cells (85, 87). Alternatively, coronavirus genomes can be stably cloned into BAC vectors. T7 or eukaryotic promoters encoded upstream of the viral sequences allow for the synthesis of full length RNA genome sequences, which are infectious upon introduction into cells (1).

Seamless assembly (also called No See'm Sites (85)) cascades have been used to assemble full length cDNAs of the coronaviruses mouse hepatitis virus, transmissible gastroenteritis virus, infectious bronchitis virus and the SARS-CoV (85,86,87). Because certain type IIS restriction endonucleases (e.g., Esp3I, AarI, Sap1) recognize asymmetric binding sites and leave asymmetric ends, these enzymes can be used to create the unique interconnecting junctions, which can be subsequently removed from the final assembly product allowing for the seamless reconstruction of an exact sequence (Figure 4b). This approach avoids the introduction of nucleotide changes that are normally associated with building a full-length cDNA product of a viral genome. These non-palindrome restriction sites will also provide other novel recombinant DNA applications. For example, by PCR

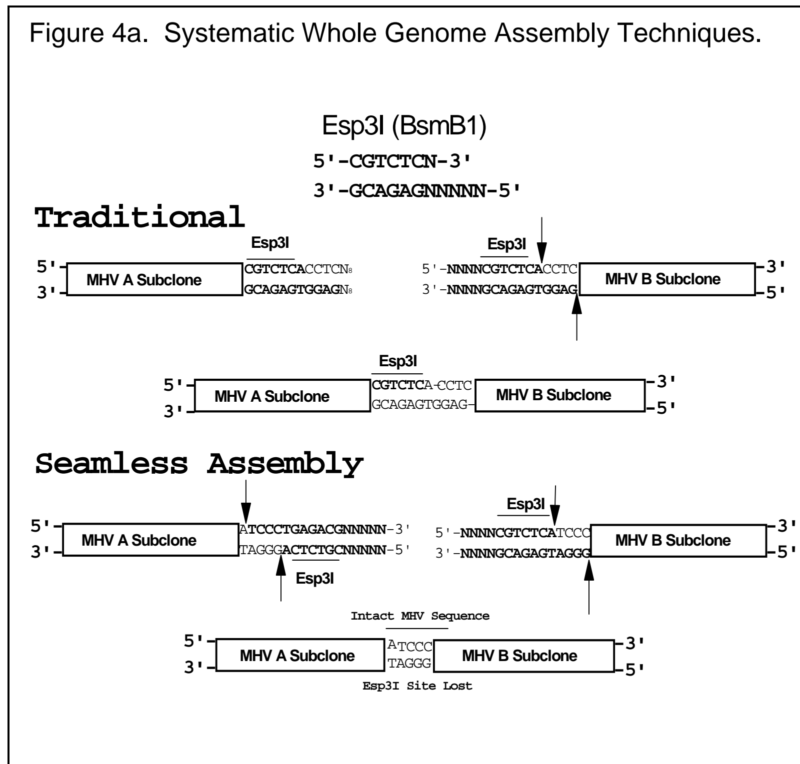



Figure 4a. Systematic Whole Genome Assembly Techniques.

it will be possible to insert Esp3I or a related non-palindromic restriction site at any given nucleotide in a viral genome and use the variable domain for simple and rapid site-specific mutagenesis. By orientating the restriction sites as "No See'm", the sites are removed during reassembly, leaving only the desired mutation in the final DNA product. The dual properties of strand specificity and a variable end overhang that can be tailored to match any sequence allow for Esp3I sites to be engineered as "universal connectors" that can be joined with any other four nucleotide restriction site overhangs (e.g. EcoRI, PstX1, BamH1). Alternatively, "No See'm" sites can be used to insert foreign genes into viral, eukaryotic, or microbial genome or vector, simultaneously removing all evidence of the restriction sites that were used in the recombinant DNA manipulation.

Finally, these restriction sites allow for the rapid assembly of small synthetically produced cDNAs into progressively larger cDNAs. For example, enzymes like AarI recognize a 7 nucleotide recognition sequence and leave a four nucleotide asymmetric end (usually). In a random DNA sequence, this site occurs every 8,000 base pairs or so.

Using a recursive assembly cascade $2^{\sim 256}$ different 8Kb cDNAs can be assembled into extremely large >1,000,000 bp DNAs designed in BACs for stable maintenance in bacteria (85-87).

At this time, well developed molecular clones have been constructed with representative viruses in most of the known virus families; specifically, the Groups I-IV genomes, thus providing a systematic approach for generating molecular clones of many Categories I, III, and IV BW agents. In addition, recent advances in synthetic biology provides promise for reconstructing microbial genomes de novo (15), as has been elegantly demonstrated with the recovery of recombinant poliovirus and ΦX174 viruses (14, 73) from synthetically derived genomes. In these instances, accurate sequences were available for

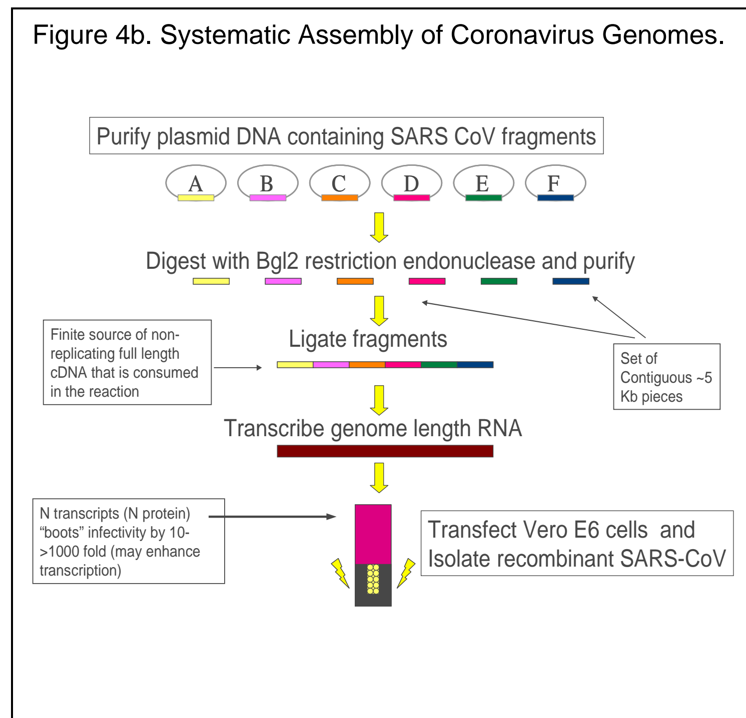



Figure 4b. Systematic Assembly of Coronavirus Genomes.

de novo synthesis, as functional molecular clones had existed for both viruses for many years. Consequently, the combination of proof of principle, available templates for genome construction and sequence information make it likely that any virus genome

could be synthetically reconstructed from sequence databases, assuming that the sequence is correct (18, 36).

### C. Review of Controlled Viruses

The United States Department of Health and Human Services (HHS), the Centers for Disease Control and Prevention (CDC), and the United States Department of Agriculture (USDA) have identified bacteria, viruses, toxins, rickettsia, and fungi that pose a potential threat to public health or welfare. Some of these organisms are considered Select Agents and High Consequence Livestock Pathogens and all research laboratories with access to these agents must submits names and fingerprints of all individuals listed as working with Select Agents to the Department of Justice. Every person who enters a laboratory containing registered Select Agents must have FBI security clearance or be accompanied and monitored by such a cleared person. This includes visitors and employees performing routine cleaning, maintenance, and repairs. The CDC oversees and regulates all laboratories that possess or use select agents and the transfer of select agents and toxins that may be used to threaten the overall public health and safety as published in the Federal Register on March 18, 2005 (42 C.F.R. Part 73, 7 C.F.R. Part 331, and 9 C.F.R. Part 121) (Appendix 1). In addition, the Department of Commerce regulates the transport of many pathogenic agents deemed important for maintaining the public health or that could impact the economic vitality of the US. Many, but not all, overlap with the Select Agent List and the USDA High Consequence Livestock Pathogens. Finally, the National Institutes of Health has assembled a list of high priority agents for biodefense research, and provides special funding for basic science, vaccines and therapeutics. Select agents are typically grouped among category A agents that pose the most serious perceived risk to national security while category B agents include many important food and waterborne agents that are easy to disseminate. The category C agents are emerging pathogens of special concern or pathogens that could be engineered for mass dissemination.

All work with microbes that might be harmful to workers or to the environment is conducted according to a variety of regulations directed to the general area of "biosafety

and containment". What is important here is that biosafety and containment are accomplished through a suite of institutional and worker actions and these activities are referred to by the level of containment achieved. "Biosafety Level 1" (BSL-1) is the least stringent containment; BSL-4 the most stringent (used for the deadliest pathogens for which there are no treatments).

Priority viruses will be discussed according to the Baltimore Classification Scheme. The key columns in these tables are the last three, *Nature*, *Laboratory*, and *Synthetic*. A "yes" in *Nature* indicates that the virus can be found in nature (thus, all viruses on the list except smallpox, 1918 H1N1 and 1957 H2N2 influenza, and the 2002-2003 strain of SARS CoV). A "yes" under *Laboratory* means that the virus can be found in some kind of lab, be it a research laboratory, a reference laboratory (e.g., the American Type Culture Collection), a commercial laboratory, etc. This is virtually all viruses on the list (smallpox is closely guarded, and the recently resurrected 1918 influenza virus, at least for now, is in a limited number of known laboratories). *Synthetic* captures two characteristics. First, is it possible to synthetically construct a virus of a specific family? These are indicated in bold, and takes into account both whether a synthetic DNA construct can supply the appropriate nucleic acid, and if enough is known about the other aspects of booting the system that it is imaginable that a synthetic approach would be taken. Second, for the individual viruses on the list, the range of possibility takes into account both whether it is possible to construct, and whether this would be an attractive possibility compared to finding it in nature, or trying to steal it from a laboratory (in the case of a bioterrorist). So for example, even though foot-and-mouth disease virus is easy to find in nature and highly contagious, it is also easy enough to synthesize that bioterrorists hoping to hide their tracks may prefer the synthetic route.

The Group I agents include the dsDNA viruses contained among the Herpes viruses, Poxviruses and Asfarviruses (Figure 5). Herpes viruses contain linear dsDNA genomes of about 150,000 base pairs and include Herpes B virus (primate) and Malignant catarrhal fever viruses (swine), both of which are readily available in nature and for which culture conditions have been detailed in the literature. Herpes virus genomes are infectious; full length molecular clones and recombinant viruses have been described for several human

and animal herpes viruses (72). Although molecular clones for Herpes B virus and Malignant catarrhal fever virus have not been described, a significant body of literature provides a theoretical template and guide for the development of similar constructs with a high probability of success.

Poxvirus genomes range in size from 150,000 to 196,000 base pairs in length and the genomes are not infectious upon introduction into susceptible cells. However, poxvirus genome infectivity can be booted by coinfection with an avian poxvirus that has an abortive infection in mammalian cell lines, but provides essential proteins for transcribing the poxvirus genome. A molecular clone has been described for vaccinia virus, providing a theoretical template for guiding similar technology with other members in the family (23, 24). Poxviruses like Variola major and Variola minor (smallpox) and monkey pox

Figure 5. Category I Restricted Agents.

| Family | Virus | Genome Size | Infectious/ Boot Infectivity | HMS-CDC | NIH A-C | Commerce | USDA | Nature | Laboratory | Synthetic |
|---|---|---|---|---|---|---|---|---|---|---|
| **Category I** | dsDNA Genome | Linear | Mixed/yes | | | | | | | Yes but Difficult |
| **Herpesviruses** | | | Yes/Yes | | | | | | | |
| | Herpes B Virus | 156,789 | | Y | | | | Yes | Yes | Unlikely |
| | Malignant catarrhal fever virus | 156,789 | | | | | Y | Yes | Yes | Unlikely |
| **Poxviruses** | | | No/Yes* | | | | | | | Yes, but Difficult |
| | Variola Major | 186,103-185,578 | No/No | Y | A | Y | | No | No* (Limited) | Plausible but difficult |
| | Variola Minor | 186,986 | No/No | Y | A | Y | | No | No* (Limited) | Plausible, but difficult |
| | Monkey pox | 196,858 | No/No | Y | A | Y | | Yes | Yes | Unlikely |
| | White pox | | | | A | Y | | Yes | Yes | Unlikely |
| | Goat pox | 149,999 | No/No | | A | | Y | Yes | Yes | Unlikely |
| | Sheep pox virus | 149,955 | No/No | | A | | Y | Yes | Yes | Unlikely |
| | Camel pox | | No/No | | A | | Y | Yes | Yes | Unlikely |
| | Lumpy skin disease virus | 150,773 | No/No | | | | Y | Yes | Yes | Unlikely |
| **Asfarvirus** | African swine fever virus | 170,101 | No/No | | | | Y | Yes | Yes | Possible |

*Variola samples are maintained in two laboratories worldwide.

viruses are select agents. Although most poxviruses can be readily found in nature and/or are maintained in laboratory settings, Variola major and minor are notable exceptions that are thought extinct in the wild. These two viruses are maintained in high security facilities in the US and Russia and it is very unlikely that these agents can be recovered from natural settings.

Group III priority agents include the reoviruses African horse sickness and exotic bluetongue strains, which primarily infect domesticated animals (Figure 6). Reovirus genomes contain ten segments of double stranded RNA and these genomes are not infectious in isolation. Reproducible schemes to boot reovirus genome infectivity have recently been developed by the Dermody laboratory. Although these viruses are available in nature and in laboratory settings, the inability to initiate genome infectivity had hampered the successful development of reverse genetic approaches and molecular clones. Consequently, the use of natural or laboratory acquired strains represented the

| Family Category III | Virus | Genome | Infectious/ Boot Infectivity | HM S/C DC | NIH A-C | Commerce | USDA | Nature | Laboratory | Synthetic |
|---|---|---|---|---|---|---|---|---|---|---|
| **REOVIRUS** | **dsRNA Segmented Genome (10)** | **Linear, dsRNA** | **No, Yes*,** | | | | | | | **Not Possible** |
| **Reovirus** | African horse sickness virus | 1-3965; 6-1566<br>2-3203; 7-1179<br>3-2792; 8-1166<br>4-1978; 9-1169<br>5-1566; 10-798 | **No, No** | | | | **Y** | **Yes** | **Yes** | Unlikely |
| | Bluetongue virus (exotic) | 1-3944; 6-1658<br>2-2953; 7-1156<br>3-2772; 8-1125<br>4-1981; 9-1049<br>5-1769; 10-822 | **No, No** | | | | **Y** | **Yes** | **Yes** | Unlikely |

**Figure 6.** Category III Priority Viruses.

most likely approach to acquiring these agents for bioterrorism purposes, although the reovirus reverse genetic system should be an appropriate template for developing molecular clones to other reoviruses..

Group IV viruses contain single stranded positive polarity RNA genomes and include agents in the calicivirus, potyvirus, picornavirus, alphavirus, flavivirus and coronavirus families (Figure 7). These viruses have dramatically different virion structures, genome organizations, and transmission modes between hosts; they target different tissues, display different virulence and pathogenic determinants and use different replication strategies upon entry into susceptible cells. Common features, however, include an infectious positive sense RNA genome and relatively straightforward and well developed approaches for obtaining full length cDNA clones from which recombinant viruses can be easily isolated in culture. In most cases these viruses replicate efficiently in culture, and animal models of disease exist, allowing for easy cultivation, maintenance, and testing in a laboratory setting. A general rule of thumb is that the BSL2 positive single stranded RNA (e.g., human noroviruses) pathogens are more readily accessible than the BSL3 pathogens (e.g., SARS-CoV, VEE, etc.) in laboratory settings. BSL4 pathogens are the least accessible. Poliovirus, which is targeted for eradication, is not included among any of the high priority pathogen lists but has been synthetically reconstructed by the Wimmer laboratory. Wild poliovirus is eradicated from the North and South American continents and Europe, but is still prevalent in Africa and parts of Asia. The virus has been present in many laboratories throughout the world, although current efforts are aimed at limiting the availability of wildtype stocks to a few locations in the US. Should eradication efforts prove successful, poliovirus should almost certainly be listed as a high priority agent. In the future, poliovirus might represent a likely candidate for synthetic reconstruction efforts because whole genome sequence is available, genome size is small and could be purchased for about $10,000 US dollars, and synthetic polioviruses have been reconstructed in the laboratory. This possibility, however, may be several decades away and is also dependent upon an end to global vaccination efforts.

The Group IV viruses are also very abundant in nature and many are present in laboratories. The main exception is the human 2002-3 SARS-CoV epidemic strain that is likely extinct in the wild, but is present in many laboratories throughout the world. Globally, most SARS-CoV isolates were late phase epidemic strains because many early and zoonotic (animal) isolates were never successfully cultured and not distributed outside of China (19, 41). Molecular clones have been described for prototype animal caliciviruses, picoronaviruses, potyviruses, alphaviruses, flaviviruses and coronaviruses, including many, but not all of the agents of interest in Figure 7. At this time, molecular clones for human noroviruses have not been successfully developed.

Group V viruses contain a single stranded negative polarity RNA genome and include members of the bunyavirus, arenavirus, filovirus, paramyxovirus, rhabdovirus, and influenza virus families (Figure 8, below). As with the group IV viruses, these viruses differ dramatically in virion structure, genome organization, transmission modes, human disease severity, virulence and pathogenesis. In general, negative stranded RNA genomes are either nonsegmented and linear (e.g., paramyxovirus, filoviruses, rhabdovirus) or segmented and linear (e.g., bunyavirus, arenavirus, myxoviruses). These viruses are readily found in nature either in human and animal hosts or vectors; all of which have been well described in the literature. Most are easily cultured in laboratory settings. Again, laboratory availability diminishes with increased BSL ratings, so that BSL3 (e.g., 1918 influenza, Rift Valley Fever) and BSL4 (e.g., Ebola, Marburg, Lassa Fever, etc.) are the least available. The exceptions include the 1918 Spanish influenza virus and H2N2 (1957 pandemic) Asian influenza viruses which are likely extinct in the wild. The 1918 Spanish influenza was resurrected from a molecular clone and is only available in a few laboratories worldwide, but the H2N2 strain is more prevalent in laboratory settings (81). Both viruses are likely capable of producing pandemic disease,

Figure 7. Category IV Priority Viruses.

| Family | Virus | Genome | Infectivity/Boot Infection | HMS/CDC | NIH A-C | Commerce | USDA | Nature | Laboratory | Synthetic |
|---|---|---|---|---|---|---|---|---|---|---|
| **Category IV** | **Positive Polarity RNA Genomes** | **Linear** | **Yes/Yes** | | | | | | | |
| **Calicivirus** | | **Linear** | **Yes/Yes** | | | | | | | **Possible** |
| | Human Norovirus | 7,654 | No/No | | B | | | Yes | Yes | Not yet |
| | Vesicular exanthema virus | 8284 | ?/No | | | | Y | Yes | Yes | Plausible |
| | Rabbit Hemorrhagic virus | 7467 | ?/No | | | | Y | Yes | Yes | Unlikely |
| **Picornavirus** | | | **Yes/Yes** | | | | | | | **Yes** |
| | HAV | 7,478 | Yes/Yes | | B | | | Yes | Yes | Unlikely |
| | Foot&Mouth Virus | 8,161 | Yes/Yes | | | | Y | Yes | Yes | Plausible |
| | Poliovirus* | 7,440 | Yes/Yes | | | | | Yes | Yes | Done |
| | Swine vesicular disease virus | 7,401 | Yes/Yes | | | | Y | Yes | Yes | Plausible |
| **Potyvirus** | **ssRNA + polarity** | | | | | | | | | **Yes** |
| | Plum Pox Virus | 9741 | Yes/Yes | Yes | | Yes | | Yes | Yes | Unlikely |
| **Alphavirus** | | | **Yes/Yes** | | | | | | | **Yes** |
| | VEE | 11,444 | Yes,Yes | Y | B | Y | Y | Yes | Yes | Plausible |
| | EEE | 11,675 | Yes,Yes | Y | B | Y | Y | Yes | Yes | Unlikely |
| | WEE | 11,484 | Yes,Yes | | B | Y | | Yes | Yes | Unlikely |
| | Chikungunya virus | 11,826 | Yes | | | Y | | Yes | Yes | Unlikely |
| **Flavivirus** | | | **Yes/Yes** | | | | | | | **Yes** |
| | Dengue | 10,735 | Yes/Yes | | A | Y | | Yes | Yes | Unlikely |
| | West Nile | 10,962 | Yes/Yes | | B | | | Yes | Yes | Unlikely |
| | Yellow Fever | 100,862 | Yes/Yes | | C | Y | | Yes | Yes | Unlikely |
| | Wesselsbron disease virus | NA | Yes/No | | | | Y | Yes | Yes | Unlikely |
| | Japanese Encephalitis Virus | 10,976 | Yes/Yes | | B | | Y | Yes | Yes | Unlikely |
| | Central European TB-encephalitis | 10,978-10,871 | Yes/Yes | Y | C | ? | | Yes | Yes | Unlikely |
| | Far Eastern TB encephalitis virus | NA | Yes/Yes | Y | C | ? | | Yes | Yes | Unlikely |
| | Louping ill virus | 10,871 | No/No | | | | Y | Yes | Yes | **Unlikely** |
| | Kyasanur Forest virus | Incomplete | Yes/No | Y | B | ? | | Yes | Yes | Unlikely |
| | Omsk HF Virus | 10,787 | Yes/No | Y | C | ? | | Yes | Yes | Unlikely |
| | Russian Spring/Summer Encephalitis virus | | Yes/No | Y | C | Y | | Yes | Yes | Unlikely |
| | Classical swine fever virus | 12,301 | Yes/ | | | | Y | Yes | Yes | Unlikely |
| **Coronavirus** | SARS-CoV | 29,751 | **Yes/Yes** | | C | | | No[1] | Yes | **Yes** |

**[1]The 2002-2003 epidemic strain is likely extinct in the wild; many zoonotic forms exist; *poliovirus is not included in any priority pathogen lists.**

as the Spanish Flu H1N1 and Asian H2N2 strains have not circulated in human populations for over 90 and 50 years, respectively. Reverse genetics systems for prototypic members of each virus family have been reported in the literature although success is more rare with arenaviruses and bunyaviruses. In contrast, well documented reverse genetic systems have been described for paramyxoviruses, rhabdoviruses, myxoviruses, and filoviruses providing clear templates for reconstruction of synthetic viruses.

Although many Category I-V agents are available in laboratory settings, serial passage of virus in cell culture oftentimes selects for "culture adapted" variants that display altered or reduced pathogenicity in the original host. In fact, serial passage in cell culture or alternative animal model has been used to attenuate virus pathogenesis and was used as a method to develop live attenuated poliovirus and measles virus vaccines. Consequently, laboratory strains may not reproduce wildtype virus pathogenicity and virulence when reintroduced into the natural host and may not represent the preferred source of starting material for bioterrorism applications.

## III. Barriers to Synthesizing and Resurrecting Viruses by Synthetic Biology and Reverse Genetics

Genetic engineering of viruses requires the development of infectious clones from which recombinant viruses can be isolated. Two basic strategies exist to develop and molecularly clone a viral genome: classic recombinant DNA approaches or synthetic biology. Although the basic methodology is different, the outcome is the same, a full length DNA copy of the viral genome is constructed which is infectious upon delivery to a permissive host cell. Classic recombinant DNA approaches require the availability of viral nucleic acid, which is normally isolated from infected tissues or cells and used as template for cloning and sequence analysis. For RNA viruses, the approach includes using reverse transcriptase and polymerase chain reaction to clone overlapping pieces of the viral genome and then whole genome assembly and sequence validation before successful recovery of recombinant viruses (10). Virus genome availability is an important issue and until recently, a major bottleneck in constructing a molecular clone to

any BW virus. Most, though not all, viral BW agents are not readily available except in high containment BSL3 and BSL4 laboratories throughout the world. The few sites and lack of funding support historically limited access to a small number of researchers, although increased support for BW research has greatly increased the distribution and availability of these agents throughout the world (31). Most viruses are also available in zoonotic reservoirs although successful isolation may require an outbreak or knowledgeable individuals carrying out systematic sampling of hosts in endemic areas. Then, containment facilities for replicating virus are necessary. Some exceptions to this general availability of controlled viruses include early 20$^{th}$ century influenza viruses like the 1918 H1N1 (Spanish flu), the 1957 H2N2 (Asian Flu), smallpox viruses (extinct 1977) and perhaps the 2002-2003 epidemic SARS-CoV strains, all of which are likely extinct in the wild given the lack of recent human disease. With the molecular resurrection of the 1918 H1N1 strain using recombinant DNA techniques (81), these viruses only exist in select laboratories distributed throughout the world.

Two general approaches exist for synthetic reconstruction of microbial genomes from published sequence databases: de novo DNA synthesis and polymerase cycling assembly (PCA). Roughly 50 commercial suppliers worldwide provide synthetic DNAs using either approach, mostly in the range of <5.0Kb, although at this time only a few companies can assemble DNAs >30Kb. For example, Blue Heron's GeneMaker™ is a proprietary, high-throughput gene synthesis platform with a ~3-4 week turnaround time and is reported to be able to synthesize any gene, DNA sequence, mutation or variant-including SNPs, insertions, deletions and domain-swaps with perfect accuracy regardless of sequence or size (http://www.blueheronbio.com/). Most commercial suppliers, however, use polymerase cycling assembly (PCA), a variation on PCR. Using published sequence, sequential ~42 nucleotide oligomers are synthesized and oriented in both the top and bottom strand, as pioneered for ΦX174 (73) (Figure 9). Top and bottom strand oligomers overlap by ~22 bp. The PCA approach involves: 1) phosphorylation of high purity 42-mers (oligonucleotide strands of DNA) in the top and bottom strand, respectively, 2) annealing of the primers under high stringency conditions and ligation with the Taq ligase at 55$^{o}$C, 3) assembly by polymerase cycling assembly (PCA) using

the HF polymerase mixture from Clontech (N-terminal deletion mutant of Taq DNA polymerase lacking 5'-exonuclease activity and Deep Vent$_R$ polymerase [NEB] with 3' exonuclease proofreading activity), 4) PCR amplification and cloning of full length amplicons (Figure 9). The key issue is to use HPLC to maximize oligomer purity and to minimize the numbers of prematurely truncated oligmers used in assemblages. As PCR is an error prone process, the PCA approach is also error prone and it requires sequence verification to ensure accurate sequence. PCA is also limited to DNAs of 5-10 Kb in length which is well within the genome sizes of many viral genomes, although improvements in PCR technologies could extend this limitation. Both approaches, coupled with systematic genome assembly techniques shown in Figure 4, will allow assembly of extremely large viral genomes, including poxviruses and herpes viruses.

Consequently, knowledgeable experts can theoretically reconstruct full length synthetic genomes for any of the high priority virus pathogens, although technical concerns may limit the robustness of these approaches. It is conceivable that a bioterrorist could order

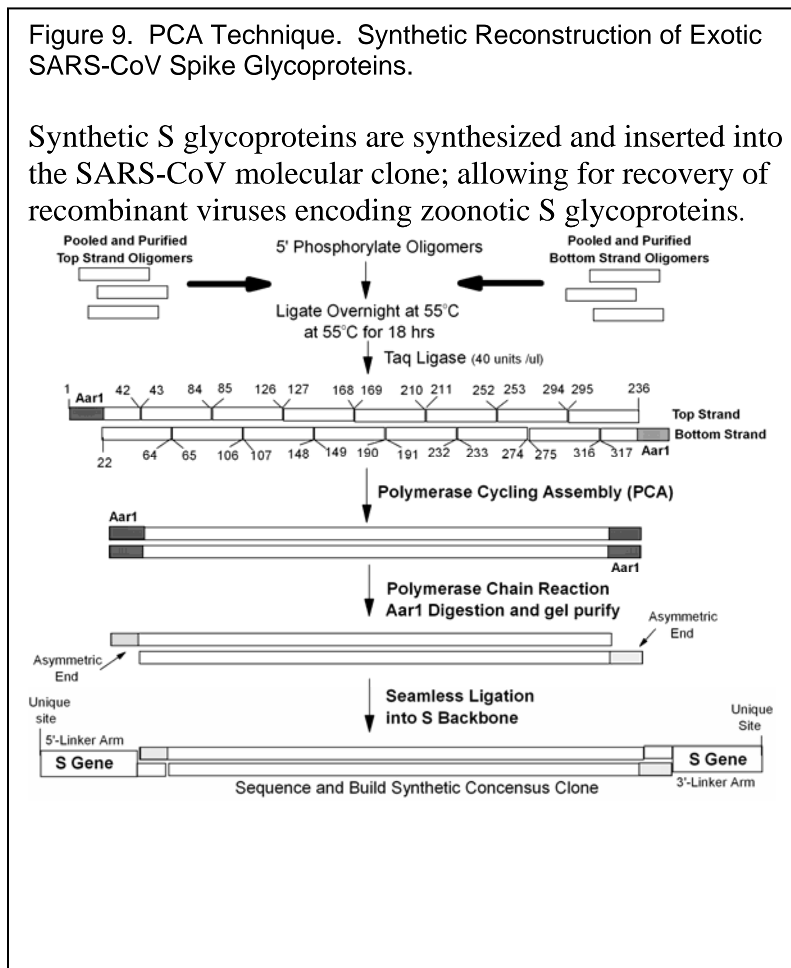



Figure 9. PCA Technique. Synthetic Reconstruction of Exotic SARS-CoV Spike Glycoproteins.

Synthetic S glycoproteins are synthesized and inserted into the SARS-CoV molecular clone; allowing for recovery of recombinant viruses encoding zoonotic S glycoproteins.

genome portions from various synthesis facilities distributed in different countries throughout the world and then assemble an infectious genome without ever having access to the virus. To our knowledge, no international regulatory group reviews the body of synthetic DNAs ordered globally to determine if a highly pathogenic recombinant virus genome is being constructed.

What, then, are the technical barriers to the reconstruction of viral genomes? Three major issues are generally recognized: sequence accuracy, genome size and stability, and expertise. They are discussed in this order below.

Sequence databases record submissions from research facilities throughout the world. However, they have limited ability to review the accuracy of the sequence submission. Consequently, these databases are littered with mistakes ranging from 1 in 500 to 1 in 10,000 base pairs. In general, large sequencing centers are more accurate than independent research laboratories (18, 36). Accurate sequence is absolutely essential for rescuing recombinant viruses that are fully pathogenic (7, 10, 30, 85, 86) as even a single nucleotide change can result in viable virus that are completely attenuated in vivo (74). Sequence accuracy represents a significant barrier to the synthetic reconstruction of these highly pathogenic viruses. RNA viruses exist in heterogeneous "swarms" of "microspecies," thus requiring the identification of a "master sequence;" i.e., the predominant sequence identified after sequencing the genome numerous times. Consequently, full length sequence information may have been reported, but the published sequence may actually not be infectious. Problems with sequence accuracy are proportional to genome size, as reported sequence for large viral genomes will more likely include a higher number of mutations than small genomes. In many instances, sequence errors will reside at the ends of viral genomes because the ends are oftentimes more difficult to clone and sequence.

Using state of the art facilities, the smallpox genome from a Bangladesh 1975 strain was sequenced (47). However, an error rate of 1:10,000 would result in about 19-20 mistakes and 10-14 amino acid changes in the recombinant genome. Should these mistakes occur within essential viral proteins or occur in virulence alleles, recovery of highly pathogenic

recombinant viruses might be impossible. More recently, another genome sequence of Variola major (India 1967) has been reported in the literature (Bangladesh 75, and India 67; Accession # X69198 and L22579). These full length genomes differ in size by 525 base pairs, contain ~1500 other allelic changes scattered throughout the genomes, and also differ in size and sequence with the Variola minor genome (Figure 5). Although roughly 99.1% identical, which of these reported sequences are correct? Will pathogenic virus be recovered from a putative molecular clone of either, both or neither? If neither is infectious, which changes are responsible for the lethal phenotype? In the absence of documentation of the infectivity of a reported sequence, it becomes difficult to accurately predict the correct sequence that will allow for the recovery of infectious virus. At best, a combination of bioinformatics, evolutionary genetic and phylogenetic comparisons among family members may identify likely codon and nucleotide inconsistencies, simultaneously suggesting the appropriate nucleotide/codon at a given position. In the case of poxviruses, only two full length sequences of Variola major have been reported, hampering such sequence comparisons. Ultimately this approach only allows informed guesses that may not result in the production of recombinant virus. Obviously, reported full length genomic sequences that have been demonstrated to generate infectious viral progeny provide an exact sequence design for synthetic resurrection of a recombinant virus, greatly increasing the probability of success. In the absence of this data, multiple full length submissions are needed to enhance the probability of success.

Another problem hampering the development of synthetic DNA genomes for genetic manipulation are genome size and sequence stability in microbial vectors. Many viral full-length cDNAs, including coronavirus genomes and certain flavivirus genomes like yellow fever virus are unstable in microbial vectors (10). Low copy BAC vectors and stable cloning plasmids oftentimes reduce the scope of this problem although instability has been reported with large inserts following passage (1, 85). Plasmid instability might be caused by sequence toxicity associated with the expression of viral gene products in microbial cells or the primary sequence might simply be unstable in microbial vectors, especially sequences that are A:T rich. To circumvent this problem, plasmid vectors have been developed that contain poly-cloning regions flanked by several transcriptional

and translational stops to attenuate potential expression of toxic products (86). The development of wide host range, low copy vectors that can be used in Gram positive or lactic acid bacteria may also allow amplification of sequences that are unstable in E. coli hosts. Alternatively, theta-replicating plasmids that are structurally more stable and that accommodate larger inserts than plasmids that replicate by rolling circle models may alleviate these concerns in the future (3, 35, 58). Poxvirus vectors also provide an alternative approach for stably incorporating large viral genome inserts, although long-term stability of these vectors is unknown (1, 77).

The technical skill needed to develop full length infectious cDNAs of viruses is not simple and requires a great deal of expertise and support: technically trained staff, the availability of state of the art research facilities, and funding. Theoretically, the ability to purchase a full length DNA of many viral biodefense pathogens is now possible, especially for those virus genomes that are less than 10 kb in length. In addition, defined infectious sequences are documented and methods have been reported in the literature. Infectious genomes of many Class IV viruses could be purchased and the need for trained staff becomes minimized. Today, a picornavirus or flavivirus genome could be purchased for as little as $15,000, a coronavirus genome for less than $40,000. It is much more difficult to reconstruct large viral genomes, meaning that trained staff and state of the art facilities become very essential to the process.

However, it is conceivable that technical advances over the next decade may even render large viral genomes commercially available for use by legitimate researchers, but perhaps also by bioterrorists.

## IV. Risk and Benefits of Synthetic Organisms

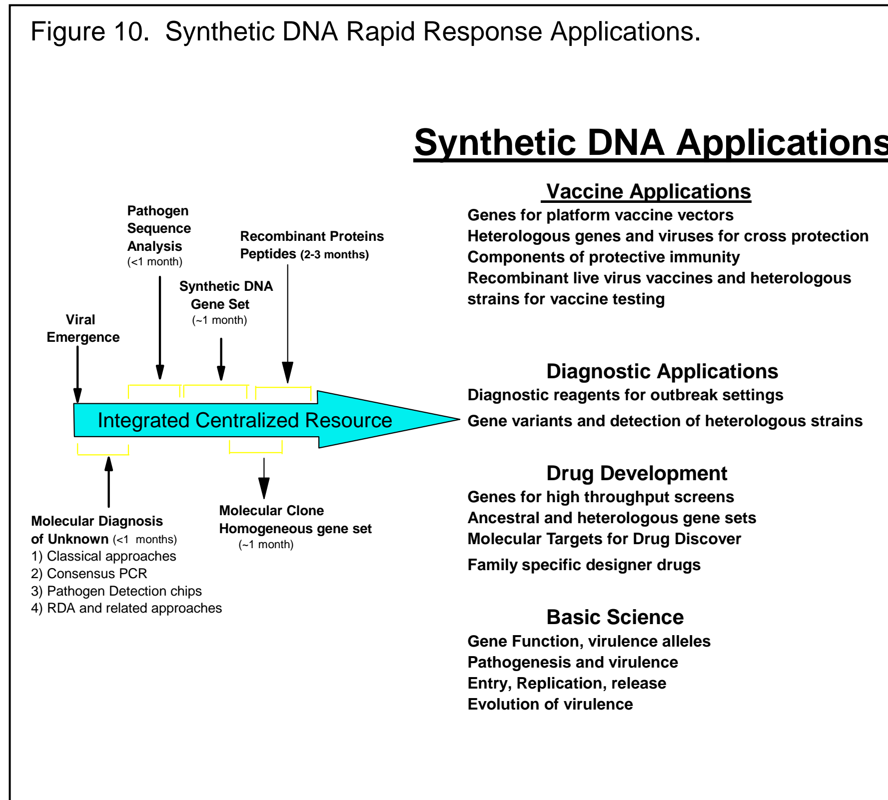
### A. Benefits to Society

The benefits of recombinant DNA have been heavily reviewed in the literature and include the development of safe and effective virus platform technologies for vaccine design and gene therapy, the production of large quantities of drugs and other human and animal medicines, and agricultural and other products key to robust national economies. Genetic engineering of bacteria and plants may allow for the production of large quantities of clean burning fuels, produce complex drugs, design highly stable biomolecules with new functions, and develop organisms that rapidly degrade complex pollutants (52, 56, 64, 78). Comparative genomics also provides numerous insights into the biology of disease-causing agents and is allowing for the development of new diagnostic approaches, new drugs and vaccines (27). Synthetic biology enhances all of the opportunities provided by recombinant DNA research. The main advantages of synthetic genomics over classic recombinant DNA approaches are speed and a mutagenesis capacity that allow for whole genome design in a cost effective manner (6). How will synthetic biology protect the overall public health?

A major advantage is in the development of rapid response networks to prevent the spread of new emerging diseases. Platform technologies allow for rapid detection and sequencing of new emerging pathogens. The SARS-CoV was rapidly identified as a new coronavirus by gene discovery arrays and whole genome sequencing techniques within a month after spread outside of China (37, 46, 83, 84). Similar advances were also made in the identification of highly pathogenic avian H5N1 influenza strains, hendra virus and in other outbreaks. Sequence information allowed for immediate synthesis of SARS and H5N1 structural genes for vaccines and diagnosis and the rapid development of candidate vaccines and diagnostic tools within a few months of discovery. Classic recombinant DNA approaches requires template nucleic acid from infected cells and tissues (limited supply), followed by more tedious cloning and sequence analysis in independent labs throughout the world. As access to viral nucleic acids historically limited response

efforts to only a few groups globally, research productivity was stifled. Synthetic biology results in a true paradigm shift in virus vaccine, therapeutic and diagnostic discovery, resulting in the near simultaneously engagement of multiple laboratories as genome sequence becomes available (Figure 10).

Figure 10. Synthetic DNA Rapid Response Applications.




Genome sequence provides for rapid incorporation of synthetic genes into platform technologies that allow for rapid diagnosis and epidemiologic characterization of the incidence, prevalence and distribution of new pathogens in human and animal hosts. Synthetic genes can be immediately incorporated into recombinant virus or bacterial vaccine platforms and tested in animal models and/or humans. Synthetic genes and proteins become essentially immediately available for structural studies, for high throughput identification of small molecule inhibitors and for the rational design of

drugs. Synthetic full length molecular clones become available for genetic analysis of virus pathogenicity and replication, construction of heterotypic strains for vaccine and drug testing, rapid development of recombinant viruses containing indicator genes for high-throughput screens and for the development of live attenuated viruses as vaccines or seed stocks for killed vaccines.

Thus, the availability of synthetic genes and genomes provides for rapid development of candidate drugs and vaccines, although significant bureaucratic hurdles must be overcome to allow for rapid use in vulnerable human populations. We note that highly pathogenic respiratory viruses can be rapidly distributed worldwide, providing only limited opportunities and time for the prevention of global pandemics and the preservation of the overall public health.

## B. Risks to Society

### 1. Bioterrorism

The historical record clearly shows that many nations have had biological weapons programs (of varying degrees of development) throughout the 20th century including many European nations, the USSR and the United States, Japan and Iraq. From relatively unscientific programs early in the 20th century, progressively more sophisticated scientific programs developed during WWI and the Cold War. There is little doubt that the genomics revolution could stimulate a new generation of potential program development (27, 76). It is also well established that the biological revolution, coupled with advances in biotechnology could be used to enhance the offensive biological properties of viruses simply by altering resistance to antiviral agents (e.g., herpes viruses, poxviruses, influenza), modifying antigenic properties (e.g, T cell epitopes or neutralizing epitopes), modifying tissue tropism, pathogenesis and transmissibility, "humanizing" zoonotic viruses, and creating designer super pathogens (27, 59). These bioweapons could be targeted to humans, domesticated animals or crops, causing a devastating impact on human civilization. Moreover, applications of these approaches are certainly not limited to the list of pathogens recorded throughout this report—well developed

engineering tools have been developed for only a few BW agents, making them relatively poor substrates for biodesign. A clever bioterrorist might start with a relatively benign, easily obtainable virus (BSL2) and obtain an existing molecular clone by simply requesting it from the scientists who work with these agents. Then, using the expanding database of genomic sequences and identified virulence genes, the benign viral genome could be modified into more lethal combinations for nefarious use.

As recombinant DNA approaches, infectious DNA clones and the general methods needed to bioengineer RNA and DNA viruses have been available since the 1980-1990's, what new capabilities does synthetic biology bring to a biowarrior's arsenal? Clearly, recombinant viral genomes and bioweapon design can be accomplished using either or a combination of both approaches, suggesting that synthetic biology will have little impact on the overall capabilities of bioweapons research. However, synthetic biology provides several attractive advantages as compared with standard recombinant DNA approaches; specifically 1) speed, 2) mutagenic superiority, 3) ease of genome construction and 4) low cost. The main paradigm shift may be that the approach is less technically demanding and more design-based, requiring only limited technical expertise because the genome can be synthesized and purchased from commercial vendors, government sponsored facilities, or from rogue basement operations (e.g., bioterrorist sponsored or private entrepreneur). Main technical support might include a competent research technician and minimal equipment to isolate recombinant pathogens from the recombinant DNAs.

Standard recombinant DNA techniques are hands-on, laborious and slow, requiring multiple rounds of mutagenesis and sequence validation of the final product. At the end of this effort, there is no guarantee that the designer or synthetic genome will function as intended (see other sections), dictating the need for high throughput strategies. Synthetic genomes can be devised fairly rapidly using a variety of bioinformatics tools and purchased fairly cheaply ($1.10/base at current rates), allowing for rapid production of numerous candidate bioweapons that can be simultaneously released (e.g., survival of the fittest approach) or lab tested and then the best candidate used for nefarious purposes. The latter approach assumes that an organization has funded the development of a secure

facility, has provided trained personnel and is willing to test the agents and/or passage them in humans, as animal models may be unreliable predictors of human pathogenesis. Assuming the technology continues to advance and spread globally, synthetic biology will allow for rapid synthesis of large designer genomes (e.g., ~30 Kb genome in less than a couple of weeks); larger genomes become technically more demanding. It seems likely that a standard approach could be designed for recovering each synthetic virus, further minimizing the need for highly trained personnel.

Will synthetic or recombinant bioweapons be developed for BW use? If the main purpose is to kill and inspire fear in human populations, natural source pathogens likely provide a more reliable source of starting material. Stealing the BW agent from a laboratory or obtaining the pathogen from natural outbreak conditions is still easier than the synthetic reconstruction of a pathogenic virus. These conditions, however, change as $1^{st}$ and $2^{nd}$ generation candidate vaccines and drugs are developed against this select list of pathogens, limiting future attempts to newly emerged viruses. If notoriety, fear and directing foreign government policies are principle objectives, then the release and subsequent discovery of a synthetically derived virus bioweapon will certainly garner tremendous media coverage, inspire fear and terrorize human populations and direct severe pressure on government officials to respond in predicted ways.

### 2. Prospects for Designer Super Pathogens

Advances in genomics may provide new approaches for mixing and matching genetic traits encoded from different viral pathogens, as over 1532 genome length sequences are available in Genbank. A large number of recombinant viruses have been assembled using reverse genetic approaches including chimeric flaviviruses, chimeric enteroviruses and coronaviruses, HIV, lentiviruses and others usually for the purposes of generating vaccines or dissecting basic questions about, e.g., viral metabolism (29, 34, 39, 40, 50). Importantly, recombinant viruses are actively being designed with programmed pathogenic traits as a means of controlling certain insect and animal pests, providing both theoretical and practical strategies for conducting effective biowarfare (53, 69). More importantly, the identification of numerous virus virulence genes that target the innate

immune response (e.g., interferons, tumor necrosis factors, interleukins, complement, chemokines, etc.), apoptosis (programmed cell death) and other host signaling pathways provides a gene repository that can be used to potentially manage virus virulence (5, 8, 9, 26, 70). Poxviruses and Herpes viruses, for example, encode a suite of immune evasion genes and pro-apoptotic genes (48, 54). More recently, virus encoded microRNAs were identified in Epstein Barr Virus (EBV) and other herpes viruses, which function to silence specific cellular mRNAs or repress translation of host genes that function in cell proliferation, apoptosis, transcription regulators and components of signal transduction pathways (62). Although the function of many viral micro-RNAs are unknown, it is likely that they regulate protein coding gene expression in animals and influence pathogenesis (61). Moreover, microRNAs could also be designed and targeted to downregulate specific human signaling pathways.

The identification of virulence alleles is traditionally a first step to attenuating virus virulence. However, highly virulent murine pox virus (ectromelia) were recovered after the host IL-4 gene was incorporated into the genome. IL-4 expression altered the host Th1/Th2 immune response leading to severe immunosuppression of cellular immune responses, high viremias, and increased pathogenesis following infection. The recombinant virus was lethal in both control and in immunized or therapeutically treated mice (33, 67). More troubling was the belated recognition that this outcome could have been predicted based on our understanding of pox molecular virology and pathogenesis, suggesting that increased virulence can be rationally modeled into existing pathogens (55) and subsequent extension of these findings to other, but not all animal poxviruses (75). Many key questions remain unanswered regarding the ability to translate results with inbred mouse strains and murine poxviruses to outcome responses in outbred human populations infected with recombinant human poxviruses. Today, these outcomes cannot be predicted. Is it possible to enhance virulence by recombinant DNA approaches in other virus families and animal models? The influenza NS1 gene (an interferon antagonist gene) also enhances the replication efficiency of avian Newcastle disease virus in human cells (57), although the in vivo pathogenesis of these isolates has not been evaluated.

More recently the SARS-CoV ORF6, but not the ORF3a group specific antigens (specific proteins of the virus) were shown to enhance mouse hepatitis virus virulence in inbred mice strains. The mechanism by which the SARS-CoV ORF6 product enhances MHV virulence is not known at this time (60). Finally, viral gene discovery and sequence recovery using DNA microarrays will greatly increase the electronic availability of sequences from many novel human, animal and insect viruses (83, 84). This revolution in pathogen detection, coupled with rapid genome sequencing, provides a rich parts list for designing novel features into the genome of viruses.

Another approach might be to "humanize" zoonotic viruses by inserting mutations into virus attachment proteins or constructing chimeric proteins that regulate virus species specificity (viral attachment proteins bind receptors, mediating virus docking and entry into cells). For example, the mouse hepatitis virus (MHV) attachment protein, the S glycoprotein, typically targets murine cells and is highly species specific. Recombinant viruses contain chimeric S glycoproteins that are composed of the ecto-domain of a feline coronaviruses fused with the c-terminal domain of MHV S glycoproteins targets feline, not murine cells for infection. The pathogenicity of these chimeric coronaviruses is unknown (39). As information regarding the structure and interactions between virus attachment proteins and their receptors accumulate, data will provide detailed predictions regarding easy approaches to humanize zoonotic strains by retargeting the attachment proteins to recognize human, not the animal receptors (43-45). Conversely, it is not clear whether species retargeting mutations will result in viruses that produce clinical disease in the human host.

Synthetic DNAs and systematic assembly approaches also provide unparalleled power for building genomes of any given sequence, simultaneously providing novel capabilities for nefarious use. For example, genome sequences represent fingerprints that allow geographic mapping of the likely origin of a given virus. Recombinant viruses generated from classic recombinant DNA techniques will carry the signature of the parental virus used in the process as well as novel restriction sites that were engineered into the genome during the cloning process. In contrast, synthetic viral genomes can be designed to be

identical with exact virus strains circulating in any given location from any year. This powerful technique provides the bioterrorist with a "scapegoat" option; leaving a sequence signature that misdirects efforts at tracking the true originators of the crime. Even better, the approach could be used to build mistrust and/or precipitate open warfare between nations. A simple example might involve the use of the picornavirus foot and mouth disease virus, which is not present on the North American continent, yet is endemic in Africa, Asia, the Middle East and South America. North American herds are not vaccinated against this pathogen, the virus is highly contagious, and the disease is subject to international quarantine. Geographically distinct FMDV strains contain unique sequence signatures allowing ready determination of origin. A North American outbreak of an infectious "synthetic" FMDV virus containing signature sequences reminiscent of strains found in select Middle East or Asian nations that are viewed as terrorist states by the US government would inflame worsening tensions and could provide a ready excuse for military retaliation. Project costs would likely be less than $50K, including synthesis, recovery and distribution. Another possibility may be to optimize replication efficiency by optimizing for human codon use, especially useful in "humanizing" zoonotic viruses although to our knowledge codon optimization has never been linked to increased replication or pathogenesis. In both examples, standard recombinant DNA approaches would be difficult and tedious, while synthetically derived genomes could be readily manufactured within weeks.

Virus pathogenesis is a complex phenotype governed by multiple genes and is heavily influenced by the host genetic background. Virus genes influence virus-receptor interaction, tissue tropism, virus-host interactions within cells, spread throughout the host, virion stability and transmission between hosts. Colonization of hosts is influenced by ecologic factors including herd immunity, cross immunity and host susceptibility alleles. In general, the rules governing virulence shifts are hard to predict because of the lack of research and ethical concerns that have historically limited this type of research. In fact, the research itself promotes an emerging conundrum as to the limits of biodefense research: the need to know to protect the overall public health versus the development of models to elucidate the fundamental principles of pathogen design (4). Synthetic biology

and recombinant DNA approaches provide numerous opportunities to construct designer pathogens encoding a repertoire of virulence genes from other pathogens, while simultaneously providing a rapid response network for preventing the emergence and spread of new human and animal diseases. The state of knowledge prevents accurate predictions regarding the pathogenic potential of designer viruses; most likely, replication and pathogenesis would be attenuated. As a principle goal of bioterrorism is to inspire fear, highly pathogenic outcomes may not be necessary as large scale panic would likely result after the release of designer pathogens in US cities. Given the reported findings and the large repertoire of host, viral and microbial virulence genes identified in the literature, the most robust defense against the development of designer viral pathogens for malicious use is basic research into the mechanisms by which viral pathogenesis might be manipulated and applied counter measures that ameliorate these pathogenic mechanisms. This justification, however, blurs the distinction between fundamental academic research and bio-weapon development.

### 3. Ancient Pathogen Resurrection

Paleomicrobiology is an emerging field dedicated to identifying and characterizing ancient microorganisms in fossilized remains (25). Mega-genomic high throughput large scale sequencing of DNA isolated from mammoths preserved in the permafrost not only identified over 13 million base pairs of mammoth DNA sequence, but also identified novel bacterial and 278 viral sequences that could be assigned to dsDNA viruses, retroviruses and ssRNA viruses (63). Although DNA genomes can survive for almost 20,000 years (25), RNA virus fossil records do not exist beyond a ~90-100 year window, making it difficult to understand the evolution of virulence, molecular evolution, and the function of modern day viral genes. Among RNA viruses, the current record is the molecular resurrection of the highly pathogenic 1918 influenza virus, which required almost 10 years of intensive effort using standard recombinant DNA approaches from many laboratories (81). Obviously, synthetic reconstruction of ancient viral genomes may provide a rapid alternative as sequence database grow more robust over the next few decades. How pathogenic are these ancient pathogens? Will vaccines and anti-virals protect humans from ancient virus diseases?

Moreover, alternative approaches also exist to regenerate ancient viral sequences. Ancestral gene resurrection using bioinformatics approaches offers a powerful approach to experimentally test hypotheses about the function of genes from the deep evolutionary past (79). Using phylogenetic methods (38), ancestral sequences can be inferred but the approach suffers from the lack of empirical data to refute or corroborate the robustness of the method. More recently, the sequence of ancestral genes was accurately predicted as evidenced by the synthetic reconstruction of a functional ancestral steroid receptor, Archosaur visual pigment and other genes (15, 16, 79, 80). To our knowledge, phylogenic reconstruction of ancient virus sequences has not been tested empirically but it may be possible to construct replacement viruses encoding ancient structural genes from inferred sequence. Such viruses would have unpredictable pathogenicity, but would likely be highly resistant to vaccines and therapeutics targeted to modern day strains.

## 4. Summary

Chemical synthesis of viral genomes will become less tedious over the coming years. Costs will likely decrease as synthesis capabilities increase. Moreover, the technology to synthesize DNA and reconstruct whole viral genomes is spreading across the globe with dozens of commercial outfits providing synthetic DNAs for research purposes. DNA synthesizers can be purchased through on-line sites such as eBay. It is likely that engineering design improvements will allow for simple construction of larger genomes. The technology to synthetically reconstruct genomes is fairly straightforward and will be used, if not by the United States, then by other Nations throughout the world. It is also likely that synthetic genes and synthetic life forms will be constructed for improving the human condition and they will be released into the environment. As with most technology, synthetic biology contains risks and benefits ranging from a network to protect the public health from new emerging diseases to the development of designer pathogens. Synthetic genome technology will certainly allow for greater access to rare viral pathogens and allow for the opportunity to attempt rationale design of super pathogens. It is likely that the threat grows over time, as technology and information provide for more rational genome design. The most robust defense against the development of designer viral pathogens for malicious use may be basic research into the

mechanisms by which viral pathogenesis might be manipulated so that applied countermeasures can be developed.

*Addendum* (November 2007): Since the writing of this initial report, recent studies have demonstrated the availability of a reverse genetic systems for reovirus, a group III dsRNA virus (Kobayashi T, Antar AA, Boehme KW, Danthi P, Eby EA, Guglielmi KM, Holm GH, Johnson EM, Maginnis MS, Naik S, Skelton WB, Wetzel JD, Wilson GJ, Chappell JD, Dermody TS. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe. 2007 Apr 19;1(2):147-57) and for additional group V single stranded negative polarity RNA viruses like Rift Valley Fever Virus (Ikegami T, Won S, Peters CJ, Makino S. Rescue of infectious rift valley fever virus entirely from cDNA, analysis of virus lacking the NS gene, and expression of a foreign gene. J Virol. 2006 Mar;80(6):2933-40.)

## Appendix 1. EHS/CDC Select Agent List (Viruses)

1. African horse sickness virus [1]
2. African swine fever virus 1
3. Akabane virus 1
4. Avian influenza virus (highly pathogenic) 1
5. Blue tongue virus (exotic) 1
6. Camel pox virus 1
7. Cercopithecine herpes virus (Herpes B virus) 3
8. Classical swine fever virus 1
9. Crimean-Congo haemorrhagic fever virus 3
10. Eastern equine encephalitis virus 2
11. Ebola viruses 3
12. Foot and mouth disease virus 1
13. Goat pox virus1
14. Japanese encephalitis virus 1
15. Lassa fever virus 3
16. Lumpy skin disease virus 1
17. Malignant catarrhal fever 1
18. Marburg virus 3
19. Menangle virus 1
20. Monkey pox virus 1
21. Newcastle disease virus (exotic) 1
22. Nipah and Hendra complex viruses 2
23. Peste des petits ruminants 1
24. Plum pox potyvirus4
25. Rift Valley fever virus 2
26. Rinderpest virus 1
27. Sheep pox 1
28. South American haemorrhagic fever viruses (Junin, Machupo, Sabia, Flexal, Guanarito) 3
29. Swine vesicular disease virus 1
30. Tick-borne encephalitis complex (flavi) viruses (Central European Tick-borne encephalitis, Far Eastern Tick-borne encephalitis, Russian Spring and Summer encephalitis, Kyasanur Forest disease, Omsk Hemorrhagic Fever) 3
31. Variola major virus (Smallpox virus) and Variola minor (Alastrim) 3
32. Venezuelan equine encephalitis virus 2
33. Vesicular stomatitis virus (exotic) 1
34. Reconstructed replication competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (Reconstructed 1918 Influenza virus)

[1]USDA High Consequence Livestock Pathogens or Toxin

[2]USDA/HSS Overlap Agent

[3]HHS Select Infectious Agent

[4]APHIS Plant Pathogens (Animal and Plant Health Inspection Service, a division of USDA)

**Appendix I, cont.**

### Prions

1.Bovine spongiform encephalopathy agent (USDA High Consequence Livestock Pathogens or Toxin)

## Appendix II. US Department of Commerce-Pathogen and Zoonotic virus list

**a. Viruses:**

a.1. Chikungunya virus;
a.2. Congo-Crimean haemorrhagic fever virus;
a.3. Dengue fever virus;
a.4. Eastern equine encephalitis virus;
a.5. Ebola virus;
a.6. Hantaan virus;
a.7. Japanese encephalitis virus;
a.8. Junin virus;
a.9. Lassa fever virus
a.10. Lymphocytic choriomeningitis virus;
a.11. Machupo virus;
a.12. Marburg virus;
a.13. Monkey pox virus;
a.14. Rift Valley fever virus;
a.15. Tick-borne encephalitis virus (Russian Spring-Summer encephalitis virus);
a.16. Variola virus;
a.17. Venezuelan equine encephalitis virus;
a.18. Western equine encephalitis virus;
a.19. White pox; or
a.20. Yellow fever virus.

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