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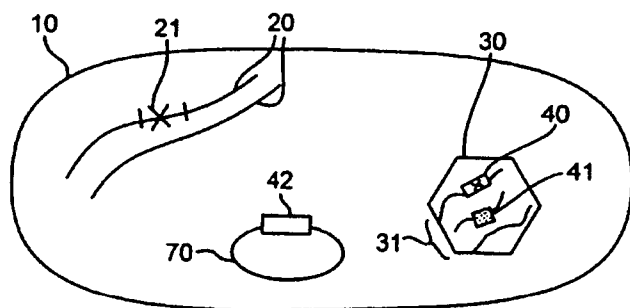


Figure 3

(57) Abstract: Bacterial packaging strains useful for generating recombinant double-stranded RNA nucleocapsids (rdsRNAs) are provided. The packaging strains are useful for the production of RNA encoding vaccine antigens, bioactive proteins, immunoregulatory proteins, antisense RNAs, and catalytic RNAs in eukaryotic cells or tissues. Recombinant ssRNA is introduced into the strains and packaged to form rdsRNAs *de novo*. The packaging strains and rdsRNAs may also comprise nucleic acid sequences that stabilize a closed loop eukaryotic translation complex; nucleic acid sequences encoding one or more proteins that interfere with a host cell type I interferon (IFN) response; as well as recombinant alphavirus replicons encoding a protein complex specific for plus strand RNA- dependent synthesis of minus strand RNA.

**BACTERIAL PACKAGING STRAINS USEFUL FOR
GENERATION AND PRODUCTION OF RECOMBINANT DOUBLE-STRANDED RNA
NUCLEOCAPSIDS AND USES THEREOF**

DESCRIPTION

Field of the invention

The present invention provides bacterial packaging strains useful for generating recombinant double-stranded RNA nucleocapsids (rdsRNAs) for the production of RNA encoding vaccine antigens, bioactive proteins, immunoregulatory proteins, antisense RNAs, and catalytic RNAs in eukaryotic cells or tissues. In particular, the invention provides bacterial packaging strains into which recombinant ssRNA is introduced and packaged to form rdsRNAs *de novo* that replicate within the packaging strains and in-turn produce RNA of interest.

Background

Viral nucleocapsids, the viral nucleoprotein core, possess numerous characteristics that may make them of value in the expression of heterologous gene sequences in biological systems. Lacking the outer membranes and adhesins of complete viruses, nucleocapsids are non-infectious particles consisting of the proteins and genetic material of the viral core that retain the capacity to encapsidate and replicate nucleic acid sequences. The risk of infection or environmental spread may thus be mitigated by the elimination of sequences encoding membranes, adhesins, proteases, and

other infective or cytolytic factors of the parent virus. RNA nucleocapsids further improve the safety of such gene expression systems by the selection of viral precursors that do not exhibit a DNA stage in their replicative cycle, and hence reduce the risk of incorporation of foreign nucleotide sequence into the genome of the cell or organism into which they are introduced. The inherent instability of RNA can be negated by the utilization of double-stranded RNA (herein “dsRNA”) viruses in the design of such nucleocapsid expression systems. Further, the elimination of non-nucleocapsid sequences and the typical segmentation of the genomes of dsRNA viruses make the design of artificial genomic segments replacing the deleted sequences and encoding heterologous RNA an attractive means by which to express genes of interest or deliver RNA of interest into biological systems. A recombinant nucleocapsid expression system could thus be designed such that it may contain sequences necessary to encode only the production of additional nucleocapsids, heterologous sequences of interest, and sequences necessary for their propagation and production in a cell.

The double-stranded RNA phage (herein “dsRP”) of the cystoviridae family are prototypical ds RNA viruses (Sinclair *et al.*, J Virol. 16:685; 1975); (Mgrew *et al.*, J Virol. 58:142; 1986); (Gottlieb *et al.*, Virology 163:183; 1988); (Mindich *et al.*, J Virol. 62:1180; 1988); (Mindich, Microbiol. Mol. Biol. Rev. 63, 149; 1999). The distinguishing attributes of cystoviridae dsRP are a genome comprised of three double-stranded RNA (herein “dsRNA”) segments (Mcgraw *et al.*, supra, 1986); (Gottlieb *et al.*, supra, 1988); (Mindich *et al.*, supra, 1988) designated segment-L, segment-M and segment-S, and a lipid-containing membrane coat (Sands and Lowlight, Can J Microbiol, 22:154; 1976); (Bamford, and Palva, Biochim Biophys Acta, 601:245; 1980). The genomic segments are contained within the nucleocapsid core, which is comprised of the proteins P1, P2, P4, and P7, and is produced by genes encoded on dsRNA segment-L (e.g. GenBank Accession # AF226851). Synthesis of positive-strand RNA (herein “mRNA”) occurs within the nucleocapsid and is carried out by RNA-dependent RNA polymerase encoded in part by gene-2 on segment-L (Mindich *et al.*, supra, 1988); (Van Etten *et al.*, J Virol, 12:464; 1973); gene-7 on segment-L also plays a role in mRNA synthesis (Mindich, *et al.*, supra, 1999).

DsRP phi-6, the archetype of this family of dsRNA phage, normally infects *Pseudomonas syringae* (Mindich, *et al.*, supra, 1999), however, more recently isolated dsRP phi-8, phi-11, phi-12 and phi-13 can infect and replicate to some extent in *Escherichia coli* strain JM109 (American type tissue culture collection (herein “ATCC”) # 53323) and O-antigen negative mutants of *Salmonella*

enterica serovar Typhimurium (herein designated “*S. typhimurium*”) (Mindich *et al.*, supra, 1999); (Mindich *et al.*, J. Bacteriol, 181:4505; 1999); (Hoogstraten *et al.*, Virology, 272: 218; 2000); (Qiao *et al.*, Virology 275: 218; 2000).

The life cycle of the archetype dsRP phi-6 in bacteria has been described (Mindich, Adv Virus Res, 35:137; 1988); (Mindich, *et al.*, supra, 1999). Phi-6 infects host cells by binding to the pilus allowing contact with the host cell membrane, thereby resulting in fusion and introduction of the nucleocapsid into the periplasm. The nucleocapsid then is transported into the cytoplasm, an event that requires the endopeptidase activity of protein P5 and the transporting property of protein P8. Interestingly, nucleocapsids that bear a complete P8 shell are capable of spontaneous entry into bacterial protoplasts, resulting in auto-transfection of the bacterial strain from which the protoplasts were prepared (Qiao *et al.*, Virology 227:103; 1997); (Olkkonen *et al.*, Proc. Natl. Acad. Sci. 87: 9173; 1990).

Upon entering the cytoplasm, P8 is shed and the remaining nucleocapsid, which contains the three dsRNA segments and possesses RNA-dependent RNA polymerase activity, begins to synthesize mRNA copies of the dsRNA segments L, M and S. The proteins produced by segment-L are mainly associated with procapsid production; segment-M is mainly dedicated to the synthesis of the attachment proteins and segment-S produces the procapsid shell protein (P8), the lytic endopeptidase (P5), and the proteins (P9 and P12) involved in the generation of the lipid envelope (Johnson and Mindich, J Bacteriol, 176: 4124; 1994). Packaging of the dsRNA segments occurs in sequential manner, whereby segment-S is recognized and taken up by empty procapsids; procapsids containing segment-S no longer bind this segment but now are capable of binding and taking up segment-M; procapsids that contain segments S and M no longer bind these segments but now are capable of binding and taking up segment-L, resulting in the generation of the nucleocapsid. Once the nucleocapsid contains all three single-stranded RNA (herein “ssRNA”) segments synthesis of the negative RNA strands begins to produce the dsRNA segments. The nucleocapsid then associates with proteins 5 and 8 and finally is encapsulated in the lipid membrane, resulting in the completion of phage assembly. Lysis of the host cell is thought to occur through the accumulation of the membrane disrupter protein P10, a product of segment-M, and requires the endopeptidase P5 (Mindich *et al.*, supra, 1999)

The assembly of and RNA polymerase activity in dsRP procapsids does not require host proteins, as procapsids purified from an *E. coli* JM109 derivative that expressed a cDNA copy of

segment-L are capable of packaging purified ssRNA segments L, M, and S (Mindich *et al.*, supra, 1999); (Qiao *et al.*, supra, 1997). Following uptake of the ssRNA segments in the above in vitro system, addition of ribonucleotides resulted in negative strand synthesis and the generation of the mature dsRNA segments. Furthermore, after the completion of dsRNA synthesis P8 associates with nucleocapsids and, as indicated above, the resulting product is capable of entering bacterial protoplasts and producing a productive infection; (Qiao *et al.*, supra, 1997).

Previous studies describe the generation of recombinant dsRP (herein referred to as "rdsRP") (Mindich, Adv Virus Res 53:341; 1999); (Onodera *et al.*, J Virol 66:190; 1992). A simple rdsRP was constructed by inserting the kanamycin-resistance allele into segment-M of dsRP phi-6. RdsRP harboring the recombinant segment were isolated by infecting JM109 carrying a plasmid that expressed the recombinant segment-M with wild-type dsRP phi-6. Through this approach a carrier state was established in host cells, in which infectious rdsRP were continuously produced by the carrier strain (Onodera *et al.*, supra, 1992); (Mindich, Adv Virus Res 53:341; 1999). The plaque-forming capacity of the phage produced by the carrier strains was maintained for three-five plate passages; however, after additional passages the nascent phage no longer formed plaques on the carrier strain, yet low-levels of infectious phage were still produced (Onodera *et al.*, supra, 1992). In some instances, a significant number of carrier strains lost the ability to produce infectious phage all together; the dsRNA from such bacterial strains displayed deletions in one or more of the segments (Onodera *et al.*, supra, 1992). In one instance a mutant phage lacking the segment-S was isolated from one such carrier strain that had lost the capacity to produce phage. In no instances were rdsRNs constructed with the express purpose of adapting the system to function in a eukaryotic cell or tissues. Thus, rdsRP produced by this method are inherently unstable, and are not useful for analysis of phage assembly and replication; the rdsRP provided by the prior art are not compatible with biotechnology applications and large-scale manufacturing.

It has been recently suggested that rdsRP could be developed that would be capable of expressing mRNA in eukaryotic cells, and that such rdsRP might be useful for the expression of vaccine antigens, bioactive proteins, immunoregulatory proteins, antisense RNAs, and catalytic RNAs in eukaryotic cells or tissues (U.S. patent application 20040132678 to Hone, which is herein incorporated by reference, hereafter 20040132678). 20040132678 provides extensive information on the usefulness of rdsRP, describes a model rdsRP and proposes methods for generating and using the same. However, 20040132678 provides no guidance on how to launch rdsRPs *de novo*, or how to

generate and isolate stable carrier strains that harbor and replicate the rdsRPs. In one example in 20040132678, it is proposed that batches of rdsRP can be generated by replicating a parent dsRP in the bacterial transformants that carry plasmids, which in turn express the recombinant segment of interest. It is unclear from this description as to whether such rdsRP harbor four dsRNA segments (i.e. the three wild-type segments and the recombinant segment) or whether such rdsRP harbor three dsRNA segments, two wild-type segments and the recombinant segment. In either instance, it is unclear how dependent the rdsRP are on the wild-type helper phage for propagation; it is also unclear how the rdsRP would be separated from the wild type dsRP. Furthermore, 20040132678 does not provide specific methods to stably incorporate recombinant segments into dsRP, and only provides scant attention to the specific methods for the subsequent replication and stable production of rdsRP. Moreover, 20040132678 does not provide stable rdsRP compositions lacking both wild-type segment-M and segment-S. Finally, 20040132678 also does not provide packaging strains that express segment-L and produce procapsids, and thus are capable of launching rdsRPs *de novo* and stably producing rdsRPs.

Hence, 20040132678 does not provide adequate information to enable those skilled in the art to generate packaging strains and stably produce rdsRP. Furthermore, 20040132678 does not discuss or suggest novel rdsRN compositions, or packaging strains, or methods to launch and stably produce and use rdsRNs, that are the subject of this invention.

SUMMARY OF THE INVENTION

According to the invention, a recombinant double-stranded RNA nucleocapsid (rdsRN) includes at least one dsRNA segment encoding functional double-stranded RNA viral or bacteriophage nucleocapsid proteins and one or more recombinant dsRNA segments that include at least one gene encoding a functional product that complements a selectable phenotypic mutation in a host (e.g. bacterial) cell, such as an auxotrophic mutation, cell wall synthesis mutation, or a mutation that prevents growth above freezing temperature. Preferably, the dsRN segments include RNA encoding a heterologous gene of interest such as an immunogen, with or without adjuvants, which would allow use of the invention in vaccines that elicit an immune response, although the function of the mRNA produced by such rdsRN's is not limited to this function. RNAs so produced could encode adjuvants, immunomodulatory proteins, therapeutic proteins, other bioactive proteins, or the RNA itself may function as siRNA or catalytic RNA. rdsRNs have advantages in terms of

stability and handling and safety, etc. relative to rdsRPs. The rdsRN is harbored in a bacterial packaging strain cell that includes the selectable phenotypic mutation, thereby allowing selection and maintenance of the rdsRNs within the bacterial packaging strain.

Exemplary embodiments of the invention are depicted schematically in Figures 1-3. In each of Figures 1-3, **10** represents a bacterial cell; **20** represents the genomic DNA of the bacterial cell **10**; **30** represents a nucleocapsid (comprised of proteins with packaging activity and RNA polymerase activity); and **31** (three wavy lines within nucleocapsid **30**) represents dsRNA contained within nucleocapsid **30**. Likewise, in each of Figures 1-3, **21** represents a selectable phenotypic mutation in genomic DNA **20**.

As can be seen in each of Figures 1-3, two elements, **40** and **41**, are consistently associated with dsRNA **31**. **40** represents a nucleic acid sequence encoding a gene product that complements selectable phenotypic mutation **21**, and **41** represents a nucleic acid sequence that encodes an RNA of interest.

A third element, **42**, is found in each of Figures 1-3, but its location varies. **42** represents nucleic acid sequences that encode genes necessary for nucleocapsid production (e.g. genes encoding proteins with packaging activity and RNA polymerase activity). Figure 1 illustrates an embodiment of the invention in which nucleic acid sequences **42** are located within dsRNA sequences **31** inside nucleocapsid **30**. In another embodiment of the invention, illustrated in Figure 2, nucleic acid sequences **42** are located within bacterial genomic DNA **20**. In yet another embodiment of the invention, illustrated in Figure 3, bacterial cell **10** also contains a plasmid (**70**), and nucleic acid sequences **42** are located on plasmid **70**.

An object of the present invention is to provide bacterial packaging strains, comprising sequences encoding dsRP procapsids in said strain and a mutation to enable selection and maintenance of the rdsRNs that express a functional gene that complements the mutation in said strain. In one embodiment, a bacterial strain for packaging, producing and/or delivering genes or RNA is provided, the strain comprising a) genomic DNA comprising at least one selectable phenotypic mutation; b) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity; c) dsRNA sequences contained within said one or more nucleocapsids, said RNA sequences encoding at least: i) a gene product that complements said at least one selectable phenotypic mutation, and ii) an RNA of interest operably linked to a eukaryotic

translation initiation sequence; and d) nucleic acid sequences encoding genes necessary for nucleocapsid production.

Another object of the present invention is to provide a method to generate rdsRNAs, wherein recombinant RNA segments are introduced into bacterial packaging strains, packaged to form a recombinant nucleocapsid containing a eukaryotic translation expression cassette, thereby launching the rdsRNAs *de novo*.

A further object of the present invention is to provide rdsRNAs capable of stably replicating in a bacterial strain. In one embodiment, a recombinant double-strand RNA nucleocapsid (rdsRNA) that comprises a) proteins with RNA packaging and RNA polymerase activity, and b) dsRNA sequences encoding at least: i) a gene product, and ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence.

Yet another object of the present invention is to provide bacterial strains that stably produce rdsRNAs that harbor one or more rdsRNA segments encoding a positive selection allele and functional eukaryotic translation expression cassettes.

A further object of this invention is to provide bacterial strains that stably produce rdsRNAs that carry alphavirus expression cassettes, such as but not restricted to the Semliki forest virus (Berglund *et al.*, Vaccine 17:497; 1999) or Venezuelan equine encephalitis (herein designated "VEE") virus (Davis *et al.*, J Virol 70:3781; 1996); (Caley *et al.*, J Virol 71:3031; 1997).

In yet another object of the current invention, methods are provided for the administration of rdsRNAs to eukaryotic cells and tissues, and the use of rdsRNAs to induce an immune response or to cause a biological effect in a target cell population.

Another object of the present invention is to provide live bacterial vectors that are capable of packaging rdsRNAs. Yet another object of the present invention is to provide live bacterial vectors that are capable of stably maintaining rdsRNAs. A further object of the present invention is to provide rdsRNAs capable of replicating in a bacterial vector strain. Still a further object of the current invention is to provide methods for the delivery of rdsRNAs to mammalian cells and tissues. Still a further object of the current invention is to provide methods for the use of said bacterial vectors carrying rdsRNAs to induce an immune response or to cause a biological effect in target cells or tissues. The selectable phenotypic mutations harbored by the host bacteria of the invention are, in a preferred embodiment, non-reverting selectable phenotypic mutations.

A further object of the invention is to provide an electroporation medium comprising said bacteria and/or said dsRNAs. In yet a further embodiment, various fluorinated RNAs which encode components of dsRNAs are provided.

The invention also provides bacterial strains for packaging, producing and/or delivering genes or RNA, which comprise a) genomic DNA comprising at least one selectable phenotypic mutation; b) nucleic acid sequences encoding genes necessary for nucleocapsid production; c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity; d) dsRNA sequences contained within said one or more nucleocapsids, said dsRNA sequences encoding at least: i) a gene product that complements said at least one selectable phenotypic mutation, and ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence; and e) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex. In one embodiment, the nucleic acid sequences that stabilize a closed loop eukaryotic translation complex comprise nucleic acid sequences that bind a mammalian polypyrimidine tract binding protein, and the nucleic acid sequences that bind a mammalian polypyrimidine tract binding protein may comprise a 3' non-translated region such as region X of hepatitis C virus.

In one embodiment of the invention, the nucleic acid sequences that stabilize a closed loop eukaryotic translation complex include nucleic acid sequences encoding alphavirus non-structural proteins 1, 2, 3, and 4. In another embodiment, alphavirus non-structural protein 2 is a mutant non-structural protein 2 that is devoid of proteolytic activity. In yet another embodiment, alphavirus non-structural proteins 1, 2, and 3 are translated together as a single polypeptide, and in some cases, alphavirus non-structural protein 4 is translated separately from alphavirus non-structural proteins 1, 2, and 3. In a preferred embodiment of the invention, a protein complex formed from the alphavirus non-structural proteins 1, 2, 3, and 4 is specific for plus strand RNA-dependent synthesis of minus strand RNA.

The invention also provides recombinant double-strand RNA nucleocapsids (rdsRNAs), comprising a) proteins with RNA packaging and RNA polymerase activity; b) dsRNA sequences encoding at least: i) a gene product, and ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence; and c) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex. In one embodiment, the nucleic acid sequences that stabilize a closed loop eukaryotic translation complex comprise nucleic acid sequences that bind a mammalian polypyrimidine tract binding protein, and the nucleic acid sequences that bind a mammalian

polypyrimidine tract binding protein may comprise a 3' non-translated region such as region X of hepatitis C virus.

In one embodiment of the invention, the nucleic acid sequences that stabilize a closed loop eukaryotic translation complex include nucleic acid sequences encoding alphavirus non-structural proteins 1, 2, 3, and 4. In another embodiment, alphavirus non-structural protein 2 is a mutant non-structural protein 2 that is devoid of proteolytic activity. In yet another embodiment, alphavirus non-structural proteins 1, 2, and 3 are translated together as a single polypeptide, and in some cases, alphavirus non-structural protein 4 is translated separately from alphavirus non-structural proteins 1, 2, and 3. In a preferred embodiment of the invention, a protein complex formed from the alphavirus non-structural proteins 1, 2, 3, and 4 is specific for plus strand RNA-dependent synthesis of minus strand RNA.

The invention further provides a vaccine preparation, comprising, bacterial cells, comprising a) genomic DNA comprising at least one selectable phenotypic mutation; b) nucleic acid sequences encoding genes necessary for nucleocapsid production c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity; d) dsRNA sequences contained within said nucleocapsid, said RNA sequences encoding at least: i) a gene product, and ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence; and e) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.

In yet another embodiment, the invention provides a vaccine preparation, comprising, recombinant double-strand RNA nucleocapsids (rdsRNAs), comprising a) proteins with RNA packaging and RNA polymerase activity; b) dsRNA sequences encoding at least: i) a gene product that complements at least one selectable phenotypic mutation, and ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence; and iii) nucleic acid sequences encoding genes necessary for phage or virus nucleocapsid production; and c) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.

In a further embodiment, the invention provides a method of creating a recombinant bacterium for use as a bacterial packaging strain. The method comprises the steps of introducing at least one selectable phenotypic mutation into genomic DNA of a bacterium; genetically engineering said bacterium to contain DNA encoding functional double-stranded RNA phage nucleocapsid proteins; and inserting into said bacterium mRNA segments encoding

i. at least one gene encoding a functional product that complements said at least one selectable phenotypic mutation; ii. functional double-stranded RNA phage nucleocapsid proteins.

And iii) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.

In addition, the invention provides a bacterial strain for packaging, producing and/or delivering genes or RNA, comprising a) genomic DNA comprising at least one selectable phenotypic mutation; b) nucleic acid sequences encoding genes necessary for nucleocapsid production; c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity; d) dsRNA sequences contained within said one or more nucleocapsids, said dsRNA sequences encoding at least: i) a gene product that complements said at least one selectable phenotypic mutation, and ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence; and e) nucleic acid sequences encoding one or more proteins that interfere with a host cell type I interferon (IFN) response. In one embodiment, the one or more proteins binds to type I IRF-3 and blocks its activation. In some embodiments of the invention, the one or more proteins is NSP1 of rotavirus. In other embodiments, the one or more proteins binds and renders inactive IFN- α or IFN- β or both. In such embodiments, the one or more proteins may be a C12R IFN- α/β receptor from ectromelia virus.

The invention further provides a recombinant double-strand RNA nucleocapsid (rdsRN), comprising a) proteins with RNA packaging and RNA polymerase activity; b) dsRNA sequences encoding at least: i) a gene product, and ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence; and c) nucleic acid sequences encoding one or more proteins that interfere with a host cell type I interferon (IFN) response. In one embodiment, the one or more proteins binds to type I IRF-3 and blocks its activation. In some embodiments of the invention, the one or more proteins is NSP1 of rotavirus. In other embodiments, the one or more proteins binds and renders inactive IFN- α or IFN- β or both. In such embodiments, the one or more proteins may be a C12R IFN- α/β receptor from ectromelia virus.

The invention further provides a vaccine preparation, comprising, bacterial cells, comprising a) genomic DNA comprising at least one selectable phenotypic mutation; b) nucleic acid sequences encoding genes necessary for nucleocapsid production c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity; d) dsRNA sequences contained within said nucleocapsid, said RNA sequences encoding at least: i) a gene product, and ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence; and

e) nucleic acid sequences encoding one or more proteins that interfere with a host cell interferon (IFN) response.

In yet another embodiment, the invention provides a vaccine preparation, comprising, recombinant double-strand RNA nucleocapsids (rdsRNAs), comprising a) proteins with RNA packaging and RNA polymerase activity; b) dsRNA sequences encoding at least: i) a gene product that complements at least one selectable phenotypic mutation, and ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence; and iii) nucleic acid sequences encoding genes necessary for phage or virus nucleocapsid production; and c) nucleic acid sequences encoding one or more proteins that interfere with a host cell interferon (IFN) response.

The invention further provides a method of creating a recombinant bacterium for use as a bacterial packaging strain. The method comprises the steps of introducing at least one selectable phenotypic mutation into genomic DNA of a bacterium; genetically engineering said bacterium to contain DNA encoding functional double-stranded RNA phage nucleocapsid proteins; and inserting into said bacterium mRNA segments encoding i. at least one gene encoding a functional product that complements said at least one selectable phenotypic mutation; ii. functional double-stranded RNA phage nucleocapsid proteins; and iii) nucleic acid sequences encoding one or more proteins that interfere with a host cell interferon (IFN) response.

The invention further provides a recombinant alphavirus replicon, comprising nucleic acid sequences encoding alphavirus non-structural proteins 1, 2, 3, and 4, wherein alphavirus non-structural protein 2 is a mutant non-structural protein 2 that is devoid of proteolytic activity, and wherein alphavirus non-structural proteins 1, 2, and 3 are translated together, and non-structural protein 4 is translated separately. In some embodiments, the nucleic acid sequences are from an alphavirus selected from the group consisting of Sindbis virus and Venezuelan equine encephalitis. In one embodiment the nucleic acid sequences are from Venezuelan equine encephalitis. In one embodiment, a codon encoding cysteine at position 1012 in non-structural protein 2 is changed to encode an amino acid that is not cysteine (for example, glycine). In a further embodiment, a protein complex formed from the alphavirus non-structural proteins 1, 2, 3, and 4 is specific for plus strand RNA-dependent synthesis of minus strand RNA. The alphavirus replicon may further include an internal ribosome entry site (IRES) which directs independent translation of NSP4.

These and other objects of the present invention will be apparent from the detailed description of the invention provided herein. As an exemplary embodiment, bacterial packaging

strains, comprising sequences encoding segment-L of dsRP phi-8 that expresses procapsids in the strain and an *asd* mutation to enable selection and maintenance of the rdsRNAs that express a functional *asd* gene in said strain are described. Further, a prototype rdsRN encoding vaccine antigens and reporters is described and the ability of said rdsRN to effect the expression of encoded antigens and reporters in a mammalian context is demonstrated.

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic representation of a bacterial cell containing a nucleocapsid, in which nucleic acid sequences that encode genes necessary for nucleocapsid production are located within dsRNA sequences inside nucleocapsid.

Figure 2. Schematic representation of a bacterial cell containing a nucleocapsid, in which nucleic acid sequences that encode genes necessary for nucleocapsid production are located in the genomic DNA of the cell.

Figure 3. Schematic representation of a bacterial cell containing a nucleocapsid, in which nucleic acid sequences that encode genes necessary for nucleocapsid production are located on a plasmid.

Figure 4 shows the expression cassettes of various phi-8 recombinant segments -S and -M (rS, rS2 and rM, respectively). As configured in the Examples section below, the positive selection allele is the *asd* gene and the genes of interest encode candidate *Mycobacterium tuberculosis* antigens and the Hc-Red fluorescent protein. rS and rS2 were cloned into the *PstI* site of pT7/T3-18. rM was cloned as a *KpnI/PstI* fragment into the respective sites of pcDNA3.1_{ZEO}. All recombinant segments were placed under transcriptional control of the T7 promoter.

Figure 5 shows an rS expression cassette that includes an alphavirus (Semliki Forest Virus) self-amplifying replicon (nspl-4 and replicase binding sequence).

Figure 6 is a schematic of the development and function of a bacterial packaging strain.

Figure 7 provides the invasive characteristics of described packaging strains and parent strains.

Figure 8 is an immunoblot of whole cell lysates of *S. flexneri* MPC51pLM2653 before and after denaturation probed with procapsid-specific antisera demonstrating the in vivo assembly of procapsids.

Figure 9 is an electron micrograph of *S. flexneri* MPC51pLM2653 showing assembled procapsids.

Figure 10 is an RT-PCR of packaged *S. flexneri* MPC51pLM2653 bearing the rdsRN designated LSMtb4 demonstrating the presence of (-) strand and (+) cDNA's indicative of second strand synthesis.

Figure 11 is an electron micrograph of *S. flexneri* MPC51 bearing the self-replicating nucleocapsid LSMtb4.

Figure 12 is an electron micrograph of *Pseudomonas syringae* bearing wild-type bacteriophage phi-8 reconstituted by electroporation of said strain with RNA encoding wild-type segments -S, -M, and -L.

Figure 13 is a fluorescence micrograph of a HeLa cell 14 hours after invasion by *S. flexneri* MPC51 bearing rdsRN designated LSMtb4 probed with antigen85A specific antisera demonstrating the expression of antigen85A by a eukaryotic cell.

Figure 14 is a fluorescence micrograph of a HeLa cell 12 hours after invasion by *S. flexneri* MPC51 bearing rdsRN designated LSMHc-Red demonstrating the direct fluorescence of Hc-Red protein translated from LSMHc-Red produced mRNA within the eukaryotic cell.

Figure 15 is a fluorescence micrograph of a HeLa cell 12 hours after invasion by *S. flexneri* MPC51 bearing rdsRN designated LSMHc-Red probed with Hc-Red-specific antisera confirming the expression of the Hc-Red protein in the eukaryotic cell.

Figure 16A-B. Reduction of intracellular *Shigella flexneri* MPC51 rdsRN MSTBS3 load in HeLa and Caco-2 cells over time due to introduced attenuating mutations. A, HeLa cells; B, Caco-2 cells.

Figure 17. Western blot analysis of OptiPrep density gradient fractions.

Figure 18A-C. Antigen-specific cellular immune responses in mice vaccinated with rdsRN MSTBS3. A, antigen Ag85A; B, antigen Ag85B; C, antigen TB10.4.

Figure 19A-D. Antigen specific immune responses in rdsRN MSTBS3 vaccinated rhesus macaques. A, CD4 response to Ag85A/B; B, CD4 response to TB10.4; c, CD8 response to Ag85A/B; B, CD8 response to TB10.4.

Figure 20 A-B. RT-PCR analysis of total RNA from HeLa cells 20 hours post-invasion with *Shigella* packaging strains with and without rdsRN. Total RNA was extracted from HeLa cells 20 hours post-invasion with *S. flexneri* NCD, *S. flexneri* NCD carrying rdsRN 5TBC (A), *S. flexneri* NCD carrying rdsRN S4-GFP (B), and uninvaded (control). Upper panels show RT-PCR products obtained using primers specific for IFN- β , lower panels show RT-PCR products obtained using primers specific for the housekeeping gene GAPDH.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

1. Construction of bacterial packaging strains

The present invention provides bacterial packaging strains containing DNA sequences that encode and express functional double-stranded RNA phage/viral (dsRP) procapsid proteins in the strain, allowing the assembly of procapsids and packaging of dsRNA to form double-stranded RNA nucleocapsids (dsRNAs) within the packaging strains. In addition, the dsRNA may be genetically engineered to contain sequences that encode and express a functional gene of interest, e.g. a transgene. The dsRNA is thus recombinant dsRNA (rdsRNA) and the nucleocapsids are recombinant dsRN (rdsRN). The packaging strains also contain a genetic mutation that creates a selectable, lethal deficiency in the strains. The rdsRNAs are genetically engineered to encode and express a functional gene that complements the selectable deficiency created by the mutation, thereby enabling selection and maintenance of the rdsRNAs within the bacterial packaging strains.

The following elements of the dsRNA phage are included in the rdsRN: segment-L; a segment-S *pac* sequence; segment-S RNA-dependent RNA polymerase recognition sequence; segment-M *pac* sequence; and segment-M RNA-dependent RNA polymerase recognition sequence. Positive selection alleles may be genetically engineered into the phage as follows: a positive selection allele may be linked, for example, to the ribosome binding site of gene-8 on segment-S or the ribosome binding site of gene-10 on segment-M, or both. Additional genes of interest may be genetically engineered into the S and/or M segments by substituting regions of the S and M segments that are not necessary for the production of functional dsRNAs. Alternatively, the S and M segments may be eliminated entirely and substituted with sequences of interest. As used herein, "recombinant segments" refers to genetically engineered S and/or M segments, or the sequences of interest that replace the S and/or M segments.

While the system described herein would function using any double-stranded RNA phage or virus, the exemplary phi-8 rdsRN system described in the Examples section preferably utilizes the following elements of the cystoviridae genome(s) to function:

- (i) segment-L and all the genes thereon,
- (ii) *pac* sequences of segments- S and -M,

(iii) 3-prime terminal polymerase binding sequences of segments- S and -M.

(iv) gene 8 of segment-S

In the phi-8 example, all coding sequences on segments-S and -M are deleted, with the exception of gene 8, and are not required for the rdsRN system to function. In addition, other exogenous sequences necessary or desirable for expression of the genes of interest may be included in the recombinant segments, such as IRES elements, Kozak and Shine-Dalgarno sequences for translation initiation in eukaryotes and prokaryotes, respectively, polyadenylation sequences, promoter sequences, enhancers, transcription terminators, leader peptide sequences, and molecular tags for protein purification, such as His tag.

According to the practice of the present invention, segment-L may be introduced into the bacterial packaging strain either in an extrachromosomal expression vector or by integration into the bacterial chromosome, and the recombinant segments are introduced into the bacterial packaging strain via electroporation, as described in detail below.

The bacterial strains from which the packaging strain is derived in the present invention is not critical thereto and include, but are not limited to: *Campylobacter spp*, *Neisseria spp.*, *Haemophilus spp*, *Aeromonas spp*, *Francisella spp*, *Yersinia spp*, *Klebsiella spp*, *Bordetella spp*, *Legionella spp*, *Corynebacterium spp*, *Citrobacter spp*, *Chlamydia spp*, *Brucella spp*, *Pseudomonas spp*, *Helicobacter spp*, or *Vibrio spp*.

The particular *Campylobacter* strain employed is not critical to the present invention. Examples of *Campylobacter* strains that can be employed in the present invention include but are not limited to: *C. jejuni* (ATCC Nos. 43436, 43437, 43438), *C. hyointestinalis* (ATCC No. 35217), *C. fetus* (ATCC No. 19438) *C. fecalis* (ATCC No. 33709) *C. doylei* (ATCC No. 49349) and *C. coli* (ATCC Nos. 33559, 43133).

The particular *Yersinia* strain employed is not critical to the present invention. Examples of *Yersinia* strains which can be employed in the present invention include: *Y. enterocolitica* (ATCC No. 9610) or *Y. pestis* (ATCC No. 19428), *Y. enterocolitica* Ye03-R2 (al-Hendy *et al.*, Infect. Immun., 60:870; 1992) or *Y. enterocolitica aroA* (O'Gaora *et al.*, Micro. Path., 9:105; 1990).

The particular *Klebsiella* strain employed is not critical to the present invention. Examples of *Klebsiella* strains that can be employed in the present invention include *K. pneumoniae* (ATCC No. 13884).

The particular *Bordetella* strain employed is not critical to the present invention. Examples of *Bordetella* strains which can be employed in the present invention include *B. pertussis*, *B. bronchiseptica* (ATCC No. 19395).

The particular *Neisseria* strain employed is not critical to the present invention. Examples of *Neisseria* strains that can be employed in the present invention include *N. meningitidis* (ATCC No. 13077) and *N. gonorrhoeae* (ATCC No. 19424), *N. gonorrhoeae* MS11 *aro* mutant (Chamberlain *et al.*, Micro. Path., 15:51-63; 1993).

The particular *Aeromonas* strain employed is not critical to the present invention. Examples of *Aeromonas* strains that can be employed in the present invention include *A. salmonicida* (ATCC No. 33658), *A. schubertii* (ATCC No. 43700), *A. hydrophila*, *A. eucrenophila* (ATCC No. 23309).

The particular *Francisella* strain employed is not critical to the present invention. Examples of *Francisella* strains that can be employed in the present invention include *F. tularensis* (ATCC No. 15482).

The particular *Corynebacterium* strain employed is not critical to the present invention. Examples of *Corynebacterium* strains that can be employed in the present invention include *C. pseudotuberculosis* (ATCC No. 19410).

The particular *Citrobacter* strain employed is not critical to the present invention. Examples of *Citrobacter* strains that can be employed in the present invention include *C. freundii* (ATCC No. 8090).

The particular *Chlamydia* strain employed is not critical to the present invention. Examples of *Chlamydia* strains that can be employed in the present invention include *C. pneumoniae* (ATCC No. VR1310).

The particular *Haemophilus* strain employed is not critical to the present invention. Examples of *Haemophilus* strains that can be employed in the present invention include *H. influenzae* (Lee *et al.*, J. Biol. Chem. 270:27151; 1995), *H. somnus* (ATCC No. 43625).

The particular *Brucella* strain employed is not critical to the present invention. Examples of *Brucella* strains that can be employed in the present invention include *B. abortus* (ATCC No. 23448).

The particular *Legionella* strain employed is not critical to the present invention. Examples of *Legionella* strains that can be employed in the present invention include *L. pneumophila* (ATCC No. 33156), or a *L. pneumophila mip* mutant (Ott, FEMS Micro. Rev., 14:161; 1994).

The particular *Pseudomonas* strain employed is not critical to the present invention. Examples of *Pseudomonas* strains that can be employed in the present invention include *P. aeruginosa* (ATCC No. 23267).

The particular *Helicobacter* strain employed is not critical to the present invention. Examples of *Helicobacter* strains that can be employed in the present invention include *H. pylori* (ATCC No. 43504), *H. mustelae* (ATCC No. 43772).

The particular *Vibrio* strain employed is not critical to the present invention. Examples of *Vibrio* strains that can be employed in the present invention include *Vibrio cholerae* (ATCC No. 14035), *Vibrio cincinnatiensis* (ATCC No. 35912), *V. cholerae* RSI virulence mutant (Taylor *et al.*, J. Infect. Dis., 170:1518-1523; 1994) and *V. cholerae* *ctxA*, *ace*, *zot*, *cep* mutant (Waldor *J et al.*, Infect. Dis., 170:278-283; 1994).

In a preferred embodiment, the bacterial strain from which the packaging strain is developed in the present invention includes bacteria that possess the potential to serve both as packaging strain and as vaccine vectors, such as the *Enterobacteriaceae*, including but not limited to *Escherichia spp*, *Shigella spp*, and *Salmonella spp*. Gram-positive and acid-fast packaging and vector strains could similarly be constructed from *Listeria monocytogenes* or *Mycobacterium spp*.

The particular *Escherichia* strain employed is not critical to the present invention. Examples of *Escherichia* strains which can be employed in the present invention include *Escherichia coli* strains DH5 α , HB 101, HS-4, 4608-58, 1184-68, 53638-C-17, 13-80, and 6-81 (See, e.g. Sambrook *et al.*, *supra*; Grant *et al.*, *supra*; Sansonetti *et al.*, Ann. Microbiol. (Inst. Pasteur), 132A:351; 1982), enterotoxigenic *E. coli* (See, e.g. Evans *et al.*, Infect. Immun., 12:656; 1975), enteropathogenic *E. coli* (See, e.g. Donnenberg *et al.*, J. Infect. Dis., 169:831; 1994), enteroinvasive *E. coli* (See, e.g. Small *et al.*, Infect Immun., 55:1674; 1987) and enterohemorrhagic *E. coli* (See, e.g. McKee and O'Brien, Infect. Immun., 63:2070; 1995).

The particular *Salmonella* strain employed is not critical to the present invention. Examples of *Salmonella* strains that can be employed in the present invention include *S. typhi* (see, e.g. ATCC No. 7251), *S. typhimurium* (see, e.g. ATCC No. 13311), *Salmonella galinarum* (ATCC No. 9184), *Salmonella enteritidis* (see, e.g. ATCC No. 4931) and *Salmonella typhimurium* (see, e.g. ATCC No. 6994). *S. typhi* *aroC*, *aroD* double mutant (see, e.g. Hone *et al.*, Vacc., 9:810-816; 1991), *S. typhimurium aroA* mutant (see, e.g. Mastroeni *et al.*, Micro. Pathol., 13:477-491; 1992).

The particular *Shigella* strain employed is not critical to the present invention. Examples of *Shigella* strains that can be employed in the present invention include *Shigella flexneri* (see, e.g. ATCC No. 29903), *Shigella flexneri* CVD1203 (see, e.g. Noriega *et al.*, Infect. Immun. 62:5168; 1994), *Shigella flexneri* 15D (see, e.g. Sizemore *et al.*, Science 270:299; 1995), *Shigella sonnei* (see, e.g. ATCC No. 29930), and *Shigella dysenteriae* (see, e.g. ATCC No. 13313).

The particular *Mycobacterium* strain employed is not critical to the present invention. Examples of *Mycobacterium* strains that can be employed in the present invention include *M. tuberculosis* CDC1551 strain (See, e.g. Griffith *et al.*, Am. J. Respir. Crit. Care Med. Aug;152(2):808; 1995), *M. tuberculosis* Beijing strain (Soolingen *et al.*, 1995) H37Rv strain (ATCC#:25618), *M. tuberculosis* pantothenate auxotroph strain (Sambandamurthy, Nat. Med. 2002 8(10):1171; 2002), *M. tuberculosis* *rpoV* mutant strain (Collins *et al.*, Proc Natl Acad Sci USA. 92(17):8036; 1995), *M. tuberculosis* leucine auxotroph strain (Hondalus *et al.*, Infect. Immun. 68(5):2888; 2000), BCG Danish strain (ATCC # 35733), BCG Japanese strain (ATCC # 35737), BCG, Chicago strain (ATCC # 27289), BCG Copenhagen strain (ATCC #: 27290), BCG Pasteur strain (ATCC #: 35734), BCG Glaxo strain (ATCC #: 35741), BCG Connaught strain (ATCC # 35745), BCG Montreal (ATCC # 35746).

The particular *Listeria monocytogenes* strain employed is not critical to the present invention. Examples of *Listeria monocytogenes* strains which can be employed in the present invention include *L. monocytogenes* strain 10403S (e.g. Stevens *et al.*, J. Virol 78:8210-8218; 2004) or mutant *L. monocytogenes* strains such as (i) *actA plcB* double mutant (Peters *et al.*, FEMS Immunology and Medical Microbiology 35: 243-253; 2003); (Angelakopoulous *et al.*, Infect and Immunity 70: 3592-3601; 2002); (ii) *dal dat* double mutant for alanine racemase gene and D-amino acid aminotransferase gene (Thompson *et al.*, Infect and Immunity 66: 3552-3561; 1998).

Having selected a strain that will serve as parent of the packaging strain, the strain is then genetically manipulated to introduce DNA sequences that express dsRP procapsids into said strain and introduce a mutation to enable selection and maintenance of the rdsRNAs that express a functional gene that complements the mutation in said strain.

In general, the genes in segment-L encode the proteins necessary to generate fully functional procapsids, including the shell-encoding protein gene 8. However, for the phi-8 phage, gene 8 is located on segment S, and while the gene 8 product is not essential for assembly of a phi-8 procapsid, efficiently self-replicating phi-8 nuclisocapsids require a functional gene 8 product. Thus,

when phi-8 phage is used in the practice of the invention, gene 8 of segment S is preferably included in the construct. For other phage, gene 8 activity is encoded on segment L, and thus the capacity to express segment-L mRNA is sufficient to produce functional nucleocapsids. The particular dsRP from which segment-L is obtained is not critical to the present invention and includes, but is not restricted to, one of Phi-6 Segment-L (Genbank accession no. M17461), Phi-13 Segment-L (Genbank accession no. AF261668), or Phi-8 Segment-L (Genbank accession no. AF226851), and are available from Dr. L. Mindich at Department of Microbiology, Public Health Research Institute, NY, NY.

Alternatively, cDNA sequences encoding segment-L can be generated synthetically using an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA 94404 U.S.A.) and procedures provided by the manufacturer. To synthesize the cDNA copies of segments- L and the recombinant segments- S and/or -M, a series of partial segments of the full-length sequence are generated by PCR and ligated together to form the full-length segment using procedures well known in the art (Ausubel *et al.*, supra, 1990). Briefly, synthetic oligonucleotides 100-200 nucleotides in length (i.e. preferably with sequences at the 5'- and 3'ends that match at the 5' and 3' ends of the oligonucleotides that encodes the adjacent sequence) are produced using an automated DNA synthesizer (e.g. Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA 94404 U.S.A.)). Using the same approach, the complement oligonucleotides are synthesized and annealed with the complementary partners to form double stranded oligonucleotides. Pairs of double stranded oligonucleotides (i.e. those that encode adjacent sequences) are joined by ligation to form a larger fragment. These larger fragments are purified by agarose gel electrophoresis and isolated using a gel purification kit (E.g. The QIAEX® II Gel Extraction System, from Qiagen, Santa Cruz, CA, Cat. No.12385). This procedure is repeated until the full-length DNA molecule is created. After each round of ligation, the fragments can be amplified by PCR to increase the yield. Procedures for *de novo* synthetic gene construction are well known in the art, and are described elsewhere (Andre *et al.*, supra, 1998); (Haas *et al.*, supra, 1996); alternatively, synthetic genes can be purchased commercially, e.g. from the Midland Certified Reagent Co. (Midland, TX).

Although the present invention specifies the use of unaltered segment-L sequences, it will be apparent to those skilled in the art that modifications resulting in truncated or mutant derivatives of

said sequences, but that do not prevent the formation of functional procapsids, can also be used without deviating substantively from the intent of the invention described herein.

The particular promoter used to express segment-L is not important to the present invention and can be any promoter that functions in the target strain, such as but not restricted to inducible promoters such P_{BAD} (Genbank Accession No. X81838) and P_{pagC} (Genbank Accession No. M55546) or constitutive promoters such as P_{lpp} (Genbank Accession No. V00302) and P_{ompA} (Genbank Accession No. X02006).

Segment-L can be introduced into the packaging strain in an expression vector, such as pT7/T3-18 (Ambion, Austin, TX, Cat. No. 7201), or integrated into the chromosome by allelic exchange using methods known to those skilled in the art (E.g. PCR, DNA purification, restriction endonuclease digestion, agarose gel electrophoresis, ligation). The location of chromosomal integration is not important to the present invention, although in a preferred embodiment DNA encoding the segment-L expression cassette is integrated into the chromosome so as to inactivate a gene and generate a phenotype selectable under defined culture conditions (e.g. *aroA* (Genbank Accession No. X00557), *aroC* (Genbank Accession No. AY142231), *leuD* (Genbank Accession No. L06666) *asd* (Genbank Accession No. V00262), *murI* (Genbank Accession No. AY520970) *kdsA* (Genbank Accession No. AY174101), and *htrB* (Genbank Accession No. AF401529). Procedures for chromosomal integration and methods for culturing said mutants are well documented (Hamilton *et al.*, J. Bacteriol. 171: 4617; 1989); (Blomfield *et al.*, Mol. Microbiol. 5: 1447; 1991).

The particular mutation that is introduced into the packaging strain is not important to the present invention and can be any mutation that is capable of generating a selectable phenotype under defined culture conditions, such as but not restricted auxotrophic mutations such as *aroA* (Genbank Accession No. X00557), *aroC* (Genbank Accession No. AY142231), *leuD* (Genbank Accession No. L06666) or mutations in genes essential to cell wall synthesis such as *asd* (Genbank Accession No. V00262) or *murI* (Genbank Accession No. AY520970), or mutations that prevent growth at temperatures above 32°C, such as *htrB* (Genbank Accession No. AF401529).

Mutations can be introduced into the bacteria using any well-known genetic technique. These include but are not restricted to non-specific mutagenesis, using chemical agents such as N-methyl-N'-nitro-N-nitrosoguanidine, acridine orange, ethidium bromide, or non-lethal exposure to ultraviolet light (Miller (Ed), 1991, In: *A short course in bacterial genetics*, Cold Spring Harbor Press, Cold Spring Harbor, NY). Alternatively, the mutations can be introduced using standard

genetic techniques, such as Tn10 mutagenesis, bacteriophage-mediated transduction, lambda phage-mediated allelic exchange, or conjugational transfer (Miller (Ed), *supra*, 1991); (Hone, *et al.*, J. Infect. Dis. 156:167; 1987); (Noriega, *et al.*, Infect. Immun., 62:5168; 1994); (Hone, *et al.*, Vaccine, 9:810; 1991); (Chatfield, *et al.*, Vaccine 10:53; 1992); (Pickard, *et al.*, Infect. Immun., 62:3984; 1994); (Odegard, *et al.*, J Biol Chem 272:19688; 1997); (Lee, *et al.*, J. Biol. Chem., 270:27151; 1995); (Garrett, *et al.*, J. Biol. Chem., 273:12457; 1998). The mutations can be a point mutation, a codon substitution, or, preferably, a non-reverting deletion mutation. The deletion mutations can be single base mutation up to deletion of the entire coding sequence. Deletion mutations have advantages in large scale mutations as such mutations impart greater stability to the product.

The mutations can be either constitutively expressed or under the control of inducible promoters, such as the temperature sensitive heat shock family of promoters, or the anaerobically-induced *nirB* promoter (Harborne *et al.*, Mol. Micro., 6:2805; 1992) or repressible promoters, such as *uapA* (Gorfinkiel *et al.*, J. Biol. Chem., 268:23376; 1993) or *gcv* (Stauffer *et al.*, J. Bact., 176:6159; 1994). Selection of the appropriate methodology will depend on the target strain and will be well understood to those skilled in the art.

The specific culture conditions for the growth of the bacterial vector strains that stably harbor rdsRNAs are not critical to the present invention. For illustrative purposes, the mutants can be grown in a liquid medium such as TS medium (Difco, Detroit, MI, Cat. No. 244620), Nutrient broth (Difco, Detroit, MI, Cat. No. 233000), Tryptic Soy broth (Difco, Detroit, MI, Cat. No. 211822), using conventional culture techniques that are appropriate for the bacterial strain being grown (Miller, *supra*, 1991). As an alternative, the bacteria can be cultured on solid media such as Nutrient agar (Difco, Detroit, MI, Cat. No. 212000), Tryptic Soy agar (Difco, Detroit, MI, Cat. No. 236920), or M9 minimal agar (Difco, Detroit, MI, Cat. No. 248510).

Mycobacterium vaccine vector strains are cultured in liquid media, such as Middlebrook 7H9 (Difco, Detroit, MI, Cat. No. 271310) or Sauton Synthetic Medium, preferably at 37°C. The strains can be maintained as static or agitated cultures. In addition, the growth rate of *Mycobacterium* can be enhanced by the addition of oleic acid (0.06% v/v; Research Diagnostics Cat. No. 01257) and detergents such as Tyloxapol (0.05% v/v; Research Diagnostics Cat. No. 70400). The purity of *Mycobacterium* cultures can be evaluated by evenly spreading 100 µl aliquots of the *Mycobacterium* culture serially diluted (E.g. 10-fold steps from Neat – 10⁻⁸) in phosphate buffered saline (herein

referred to PBS) onto 3.5 inch plates containing 25-30 ml of solid media, such as Middlebrook 7H10 (BD Microbiology, Cockeysville, MD, Cat. No. 221174).

The optical density at 600nm at which the bacteria are harvested is not critical thereto, and can range from 0.1 to 5.0 and will be dependent on the specific strain, media and culture conditions employed.

2. Construction of rdsRNA segments

As emphasized earlier, U.S. 20040132678 relies on helper phage (i.e. a wild type dsRP) to launch the production of rdsRPs. Under these conditions the methods provided in 20040132678 produce rdsRP that contain either wild-type segment-S or wild-type segment-M. Given that the lytic functions of dsRP are contained in segments -S and -M, it is not clear whether such configurations will be stable in large-scale production. It is also not clear how this methodology will separate the wild type dsRP from the rdsRP. Furthermore, 20040132678 provides no guidance on how to launch rdsRNs *de novo*, and how to generate and isolate stable carrier strains that harbor and replicate the rdsRNs. In contrast, the present invention pertains to the stable production of rdsRNs.

Typically, viral genomic size variations of up to about 10 percent are tolerated, which enables a degree of flexibility in the size of the genome in recombinant viral vectors (Domingo and Holland, *Annu. Rev. Microbiol.* 51:151;1997). In the practice of the present invention, the size of the rdsRNA segment in the rdsRNs is equal to the sum of segments-S -M and -L plus or minus approximately 10%. To illustrate this point, phi-8 may be used as an example. The genome size of phi-8 is 14,984 bp, accordingly, to generate stable rdsRNs in packaging strains expressing segment-L of phi-8, the size of the recombinant segment(s) in such rdsRNs would be approximately 7933 ± 1500 bp.

As will be shown below, this rule does not strictly apply, as it is possible to obtain rdsRNs derived from phi-8 that harbor only segment-L and a 4.5 kb rdsRNA segment-S (Sun *et al.*, *Virology*, 308: 354; 2003). This suggests that rdsRNs are capable of a surprising degree of genomic flexibility. In the above described example, the recombinant segment-S was composed of sequences derived from both the wild-type segment-S and the wild-type segment-M. In this particular literature example, “derived from” (e.g. “derived from wild-type segment-S”) describes sequences present in this recombinant rdsRNA construct that originate from the genomic sequence of the wild-type phage and are rearranged from their wild-type gene or coding feature order. Such sequences

may be amplified from wild-type phage by RT-PCR and cloned, or chemically synthesized based on known sequences that occur in the wild type phage.

It is also possible to provide compositions that contain a genomic size that approaches the wild type genomic size. In one approach, a recombinant segment-S is generated that has a size of 7933 ± 1500 bp. Another approach utilizes two rdsRNA segments, one containing segment-S packaging sequence and the other containing segment-M packaging sequence, wherein the total size of the recombinant segments is 7933 ± 1500 bp (Figure 4). In both approaches, at least one segment contains a positive selection allele and a single or both recombinant segments carry eukaryotic expression cassettes.

In a preferred embodiment, the components of rdsRNA segments can be assembled, for example, by joining the following cDNA and DNA sequences (Figure 4):

rdsRNA segment-S:

1. The ϕ -8 segment-S *pac* sequence and gene 8 (Hoogstraten *et al.*, supra, 2000).
2. A positive selection allele linked to the ribosome binding site of gene 8 or of the wild-type segment-S. Gene 8 encodes a membrane protein which may remain associated with the nucleocapsid and is the first open reading frame of wild-type segment-S.
3. An IRES sequence with *HpaI*, *EcoRI*, *Sall* and *NotI* restriction endonuclease (RE) sites located 3-prime to the IRES sequence so that the *HpaI* (a blunt-end RE) provides an ATG start codon that is functionally linked to the IRES
4. The bovine poly-adenylation sequence (obtained from pcDNA3.1 (Invitrogen, Carlsbad, CA, Cat. No. V860-20).
5. The ϕ -8 segment-S RNA-dependent RNA polymerase recognition sequence (Hoogstraten *et al.*, supra, 2000).

rdsRNA segment-M:

6. The ϕ -8 segment-M *pac* sequence (Hoogstraten *et al.*, supra, 2000).
7. A positive selection allele linked to the ribosome binding site of gene-10. Gene 10 is the first open reading frame of the wild-type segment-M and encodes a membrane protein of ϕ -8.
8. An IRES sequence with *HpaI*, *EcoRI*, *Sall* and *NotI* restriction endonuclease (RE) sites located 3-prime to the IRES sequence so that the *HpaI* (a blunt-end RE) provides an ATG start codon that is functionally linked to the IRES;

9. The bovine poly-adenylation sequence (obtained from pcDNA3.1 (Invitrogen, Carlsbad, CA, Cat. No. V860-20).
10. The ϕ -8 segment-M RNA-dependent RNA polymerase recognition sequence (Hoogstraten *et al.*, supra, 2000).

Although not wishing to be limited in scope by theory, it is likely that when the size of the rdsRNA segment-S is greater than 7900 $\pm$ 1500 bp, the rdsRNA segment-M will no longer be packed, since a rdsRNA segment-S greater than 7900 $\pm$ 1500 bp will induce a conformation change in the procapsid to enable recognition and uptake of segment-L, thereby generating a rdsRN with a genomic size $\pm$ 10% that of the wild-type genome.

The cDNA sequences encoding the rdsRNA segments can be generated synthetically using an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA) and procedures provided by the manufacturer. To synthesize the cDNA copies of segments- L and the recombinant segments, a series of segments of the full-length sequence are generated by PCR and ligated together to form the full-length segment using procedures well know in the art (Ausubel *et al.*, supra, 1990). Briefly, synthetic oligonucleotides 100-200 nucleotides in length (i.e. preferably with sequences at the 5'- and 3'ends that match at the 5' and 3' ends of the oligonucleotides that encodes the adjacent sequence) are produced using an automated DNA synthesizer (e.g. Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA). Using the same approach, the complement oligonucleotides are synthesized and annealed with the complementary partners to form double stranded oligonucleotides. Pairs of double stranded oligonucleotides (i.e. those that encode adjacent sequences) are joined by ligation to form a larger fragment. These larger fragments are purified by agarose gel electrophoresis and isolated using a gel purification kit (E.g. The QIAEX® II Gel Extraction System, from Qiagen, Santa Cruz, CA, Cat. No. 12385). This procedure is repeated until the full-length DNA molecule is created. After each round of ligation, the fragments can be amplified by PCR to increase the yield. Procedures for *de novo* synthetic gene construction are well known in the art, and are described elsewhere (Andre *et al.*, supra, 1998); (Haas *et al.*, supra, 1996); alternatively synthetic genes can be purchased commercially, e.g. from the Midland Certified Reagent Co. (Midland, TX).

Positive selection alleles

The particular positive selection allele that is incorporated into the rdsRNA segment is not important to the present invention, and can be any allele that is capable of restoring a dominant

negative mutation in the packaging strain, such as but not restricted to genes that complement auxotrophic mutations such as *aroA* (Genbank Accession No. X00557), *aroC* (Genbank Accession No. AY142231), *leuD* (Genbank Accession No. L06666), or genes that complement mutations in genes essential to cell wall synthesis such as *asd* (Genbank Accession No. V00262), or *murI* (Genbank Accession No. AY520970), or genes that complement mutations in genes essential to cell division such as *ftsZ* (Genbank Accession No. AF221946).

Source of IRES sequences

mRNA molecules lacking a 5' cap modifier, which is normally added in the nucleus to nuclear mRNA transcripts and enhances ribosome recognition, are poorly translated in eukaryotic cells unless an IRES sequence is present upstream of the gene of interest. The particular IRES employed in the present invention is not critical and can be selected from any of the commercially available vectors that contain IRES sequences or from any of the unencumbered sequences available. Thus, IRES sequences are widely available and can be obtained commercially from plasmid pIRES2-EGFP (Clontech, Palo Alto, CA, Cat. No. 63206) by PCR using primers specific for the 5' and 3' ends of the IRES located at nucleotides 665-1251 in pIRES2-EGFP. The sequences in plasmid pIRES-EGFP can be obtained from the manufacturer (see www.clontech.com). A similar IRES can also be obtained from plasmid pCITE4a (Novagen, Madison, WI, Cat. No. 69913; see also U.S. Patent number 4,937,190) by PCR using primers specific for the 5' and 3' ends of the CITE from nucleotides 16 to 518 in plasmid pCITE4a (the complete sequence of pCITE4a is available at the website located at novagen.com/docs/NDIS/69913-000.HTM); on plasmids pCITE4a-c; (U.S. Patent number 4,937,190); pSLIRES11 (Accession: AF171227); pPV (Accession # Y07702); pSVIRES-N (Accession #: AJ000156); (Creancier *et al.*, J. Cell Biol., 10: 275-281; 2000); (Ramos and Martinez-Sala, RNA, 10:1374-1383; 1999); (Morgan *et al.*, Nucleic Acids Res., 20:1293-1299; 1992); (Tsukiyama-Kohara *et al.*, J. Virol., 66: 1476-1483; 1992); (Jang and Wimmer *et al.*, Genes Dev., 4: 1560-1572; 1990), or on the dicistronic retroviral vector (Accession #: D88622); or found in eukaryotic cells such as the fibroblast growth factor 2 IRES for stringent tissue-specific regulation (Creancier, *et al.*, supra, 2000) or the Internal-ribosome-entry-site of the 3'-untranslated region of the mRNA for the beta subunit of mitochondrial H⁺-ATP synthase (Izquierdo and Cuezva, Biochem. J., 346:849; 2000). As there is no IP on the HCV IRES, plasmid pIRES-G (Hobbs, S.M. CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Block F, 15, Cotswold Road,

Belmont, Sutton, Surrey SM2 5NG, UK) may serve as the source of IRES and the sequence of this plasmid is available (Genebank accession no. Y11034).

Furthermore, an Internet search using an NCBI nucleotide database located at ncbi.nlm.nih.gov and using the search parameter "*IRES not patent*" yields 140 files containing IRES sequences. Finally, IRES cDNA can be made synthetically using an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA), using procedures provided by the manufacturer. To synthesize large IRES sequences such as the 502 bp IRES in pCITE4a, a series of segments are generated by PCR and ligated together to form the full-length sequence using procedures well known in the art (Ausubel *et al.*, supra, 1990). Smaller IRES sequences such as the 53 bp IRES in hepatitis C virus (Genebank accession no. 1KH6A) can be made synthetically in a single round using an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA) and procedures provided by the manufacturer.

Examples of heterologous genes of interest that can be inserted in rdsRNAs

In the present invention, the gene of interest introduced on a eukaryotic translation expression cassette into the rdsRN may encode an immunogen, and the rdsRN may thus function as a vaccine for eliciting an immune response against the immunogen. The immunogen may be either a foreign immunogen from viral, bacterial and parasitic pathogens, or an endogenous immunogen, such as but not limited to an autoimmune antigen or a tumor antigen. The immunogens may be the full-length native protein, chimeric fusions between the foreign immunogen and an endogenous protein or mimetic, a fragment or fragments thereof of an immunogen that originates from viral, bacterial and parasitic pathogens.

As used herein, "foreign immunogen" means a protein or fragment thereof, which is not normally expressed in the recipient animal cell or tissue, such as, but not limited to, viral proteins, bacterial proteins, parasite proteins, cytokines, chemokines, immunoregulatory agents, or therapeutic agents.

An "endogenous immunogen" means a protein or part thereof that is naturally present in the recipient animal cell or tissue, such as, but not limited to, an endogenous cellular protein, an immunoregulatory agent, or a therapeutic agent.

Apoptosis is programmed cell death and differs dramatically from necrotic cell death in terms of its induction and consequences. Apoptosis of cells containing foreign antigens is a

powerful known stimulus of cellular immunity against such antigens. The process by which apoptosis of antigen containing cells leads to cellular immunity has sometimes been called cross-priming (Heath, W.R., *et al.*, Immunol Rev 199:9; 2004, Gallucci, S. M *et al.*, Nature Biotechnology. 5:1249; 1999, Albert, M.L. *et al.*, Nature 392:86; 1988). There are several mechanisms for induction of apoptosis which lead to increased antigen specific cell mediated immunity. Caspase 8 mediated apoptosis leads to antigen specific cellular immune protection (Heath, W.R., *et al.*, Immunol Rev 199:9; 2004). Expression of Caspase 8 by rdsRNAs in the cytoplasm will be a powerful method for inducing programmed cell death in the context of foreign antigens expressed by rdsRNAs leading to high levels of antigen specific cellular immunity. Death receptor-5 (DR-5) also known as TRAIL-R2 (TRAIL receptor 2) or TNFR-SF-10B (Tumor Necrosis Factor-Superfamily member 10B) also mediates caspase 8 mediated apoptosis (Sheridan, J.P., *et al.*, *Science* 277:818; 1997). Reovirus induced apoptosis is mediated by TRAIL- DR5 leading to subsequent clearance of the virus (Clarke, P. S. *et al.*, J. Virol; 2000). Expression of DR-5 by rdsRNAs should provide a potent adjuvant effect for induction of antigen specific cellular immunity against rdsRNAs expressed antigens. Antigen expressing cells can also be induced to undergo apoptosis through Fas ligation, which is a strong stimulus for induction of antigen specific cellular immune responses (Chattergoon, M.A. *et al.*, Nat Biotechnology 18:974; 2000). rdsRNAs expressing Fas or Fas cytoplasmic domain/CD4 extracellular domain fusion protein will induce apoptosis and antigen specific cellular immune responses against antigens expressed by rdsRNAs.

The enhancement of cellular immunity by rdsRNAs mediated apoptosis described above is not limited to antigens specifically coded for by the rdsRNAs itself but includes any antigen in the cell where the rdsRNAs express specific mediators of apoptosis. As an example, if rdsRNAs are delivered to tumor cells where apoptosis is induced then cellular immunity against important tumor antigens will be induced with elimination, reduction or prevention of the tumor and/or metastasis.

In a further embodiment of this invention if rdsRNAs, with or without the capacity to induce apoptosis and with the ability to code for and produce foreign antigens against which strong cellular immune responses will be mounted, are delivered inside tumor or other cells strong cellular responses against those cells will be produced. These cellular responses will lead to immune mediated tumor cell destruction, further cross priming and induction of cellular immunity against tumor or other important antigens with subsequent elimination, reduction or prevention of the tumor

and/or metastasis. An example of such a foreign antigen is an HLA antigen different from the host cell HLA against which a strong heterologous cellular response will be mounted.

Recombinant rdsRNAs capable of inducing apoptosis and delivering specific tumor antigens will induce strong antigen specific cellular responses against these tumor antigens, including breaking of some tolerance for these antigens leading to elimination, reduction or prevention of tumors and/or metastasis without the need for direct delivery of the rdsRNAs into the tumor itself.

Apoptosis following DNA damage or caspase 9 induces tolerance to certain antigens. (Hugues, S. E., *et al.*, Immunity 16:169; 2002). Induction of tolerance is important in controlling or preventing autoimmune diseases such as but not limited to diabetes, rheumatoid arthritis, Crohns disease, inflammatory bowel disease and multiple sclerosis. Production of caspase 9 or other apoptosis mediated tolerance inducing proteins by rdsRNAs in cells such as but not limited to β pancreatic cells, colorectal and nerve cells will produce limited apoptosis which will induce tolerance against the antigen targets of autoimmunity in those cells thereby treating or preventing the autoimmune disease condition. Identification of specific antigens involved in autoimmune reactions will allow induction of tolerance against these autoimmune target antigens through rdsRNAs production of these antigens and Caspase 9 or other molecules capable of inducing apoptotic mediated tolerance that will lead to treatment and/or prevention of these autoimmune diseases.

Another embodiment of the present invention, therefore, provides rdsRN which encode at least one gene which expresses a protein that promotes apoptosis, such as but not limited to expression of *Salmonella* SopE (Genbank accession no. AAD54239, AAB51429 or AAC02071), *Shigella* IpaB (Genbank accession no. AAM89553 or AAM89536), caspase-8 (Genbank accession no. AAD24962 or AAH06737), etc., in the cytoplasm of host cells and imparts a powerful method for inducing programmed cell death in the context of antigens expressed by said rdsRN, thereby invoking high-level T cell-mediated immunity to the target antigens. Alternatively, rdsRN can be produced which encode at least one gene which expresses DR-5, such as human DR-5 (Genbank accession # BAA33723), herpesvirus-6 (HHV-6) DR-5 homologue (Genbank accession # CAA58423) etc., thereby providing a potent adjuvant effect for induction of antigen-specific cellular immunity against the target antigens.

Alternatively or additionally, the immunogen may be encoded by a synthetic gene and may be constructed using conventional recombinant DNA methods (See above).

The foreign immunogen can be any molecule that is expressed by any viral, bacterial, or parasitic pathogen prior to or during entry into, colonization of, or replication in their animal host; the rdsRN may express immunogens or parts thereof that originate from viral, bacterial and parasitic pathogens. These pathogens can be infectious in humans, domestic animals or wild animal hosts.

The viral pathogens, from which the viral antigens are derived, include, but are not limited to, Orthomyxoviruses, such as influenza virus (Taxonomy ID: 59771; Retroviruses, such as RSV, HTLV-1 (Taxonomy ID: 39015), and HTLV-II (Taxonomy ID: 11909), Papillomaviridae such as HPV (Taxonomy ID: 337043), Herpesviruses such as EBV Taxonomy ID: 10295); CMV (Taxonomy ID: 10358) or herpes simplex virus (ATCC #: VR-1487); Lentiviruses, such as HIV-1 (Taxonomy ID: 12721) and HIV-2 Taxonomy ID: 11709); Rhabdoviruses, such as rabies; Picornaviruses, such as Poliovirus (Taxonomy ID: 12080); Poxviruses, such as vaccinia (Taxonomy ID: 10245); Rotavirus (Taxonomy ID: 10912); and Parvoviruses, such as adeno-associated virus 1 (Taxonomy ID: 85106).

Examples of viral antigens can be found in the group including but not limited to the human immunodeficiency virus antigens Nef (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 183; Genbank accession # AF238278), Gag, Env (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2433; Genbank accession # U39362), Tat (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 827; Genbank accession # M13137), mutant derivatives of Tat, such as Tat- Δ 31-45 (Agwale *et al.*, Proc. Natl. Acad. Sci. USA 99:10037; 2002), Rev (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 2088; Genbank accession # L14572), and Pol (National Institute of Allergy and Infectious Disease HIV Repository Cat. # 238; Genbank accession # AJ237568) and T and B cell epitopes of gp120 (Hanke and McMichael, AIDS Immunol Lett., 66:177; 1999); (Hanke, *et al.*, Vaccine, 17:589; 1999); (Palker *et al.*, J. Immunol., 142:3612-3619; 1989) chimeric derivatives of HIV-1 Env and gp120, such as but not restricted to fusion between gp120 and CD4 (Fouts *et al.*, J. Virol. 2000, 74:11427-11436; 2000); truncated or modified derivatives of HIV-1 env, such as but not restricted to gp140 (Stamatos *et al.*, J Virol, 72:9656-9667; 1998) or derivatives of HIV-1 Env and/or gp140 thereof (Binley, *et al.*, J Virol, 76:2606-2616; 2002); (Sanders, *et al.*, J Virol, 74:5091-5100 (2000); (Binley, *et al.* J Virol, 74:627-643; 2000), the hepatitis B surface antigen (Genbank accession # AF043578); (Wu *et al.*, Proc. Natl. Acad. Sci., USA, 86:4726-4730; 1989); rotavirus antigens, such as VP4 (Genbank accession # AJ293721); (Mackow *et al.*, Proc. Natl. Acad. Sci., USA, 87:518-522; 1990) and VP7 (GenBank

accession # AY003871); (Green *et al.*, J. Virol., 62:1819-1823; 1988), influenza virus antigens such as hemagglutinin or (GenBank accession # AJ404627); (Pertmer and Robinson, Virology, 257:406; 1999); nucleoprotein (GenBank accession # AJ289872); (Lin *et al.*, Proc. Natl. Acad. Sci., 97: 9654-9658; 2000) herpes simplex virus antigens such as thymidine kinase (Genbank accession # AB047378; (Whitley *et al.*, In: New Generation Vaccines, pages 825-854).

The bacterial pathogens, from which the bacterial antigens are derived, include but are not limited to: *Mycobacterium spp.*, *Helicobacter pylori*, *Salmonella spp.*, *Shigella spp.*, *E. coli*, *Rickettsia spp.*, *Listeria spp.*, *Legionella pneumoniae*, *Pseudomonas spp.*, *Vibrio spp.*, *Bacillus anthracis* and *Borellia burgdorferi*.

Examples of protective antigens of bacterial pathogens include the somatic antigens of enterotoxigenic *E. coli*, such as the CFA/I fimbrial antigen (Yamamoto *et al.*, Infect. Immun., 50:925-928; 1985) and the nontoxic B-subunit of the heat-labile toxin (Klipstein *et al.*, Infect. Immun., 40:888-893; 1983); pertactin of *Bordetella pertussis* (Roberts *et al.*, Vacc., 10:43-48; 1992), adenylate cyclase-hemolysin of *B. pertussis* (Guiso *et al.*, Micro. Path., 11:423-431; 1991), fragment C of tetanus toxin of *Clostridium tetani* (Fairweather *et al.*, Infect. Immun., 58:1323-1326; 1990), OspA of *Borellia burgdorferi* (Sikand *et al.*, Pediatrics, 108:123-128; 2001); (Wallich *et al.*, Infect Immun, 69:2130-2136; 2001), protective paracrystalline-surface-layer proteins of *Rickettsia prowazekii* and *Rickettsia typhi* (Carl *et al.*, Proc Natl Acad Sci U S A, 87:8237-8241; 1990), the listeriolysin (also known as "Llo" and "Hly") and/or the superoxide dismutase (also know as "SOD" and "p60") of *Listeria monocytogenes* (Hess, J., *et al.*, Infect. Immun. 65:1286-92; 1997); Hess, J., *et al.*, Proc. Natl. Acad. Sci. 93:1458-1463; 1996); (Bouwer *et al.*, J. Exp. Med. 175:1467-71; 1992), the urease of *Helicobacter pylori* (Gomez-Duarte *et al.*, Vaccine 16, 460-71; 1998); (Corthesy-Theulaz, *et al.*, Infection & Immunity 66, 581-6; 1998), and the *Bacillus anthracis* protective antigen and lethal factor receptor-binding domain (Price, *et al.*, Infect. Immun. 69, 4509-4515; 2001).

The parasitic pathogens, from which the parasitic antigens are derived, include but are not limited to: *Plasmodium spp.*, such as *Plasmodium falciparum* (ATCC#: 30145); *Trypanosome spp.*, such as *Trypanosoma cruzi* (ATCC#: 50797); *Giardia spp.*, such as *Giardia intestinalis* (ATCC#: 30888D); *Boophilus spp.*, *Babesia spp.*, such as *Babesia microti* (ATCC#: 30221); *Entamoeba spp.*, such as *Entamoeba histolytica* (ATCC#: 30015); *Eimeria spp.*, such as

Eimeria maxima (ATCC# 40357); *Leishmania* spp. (Taxonomy ID: 38568); *Schistosoma* spp., *Brugia* spp., *Fasciola* spp., *Dirofilaria* spp., *Wuchereria* spp., and *Onchocerca* spp.

Examples of protective antigens of parasitic pathogens include the circumsporozoite antigens of *Plasmodium* spp. (Sadoff *et al.*, Science, 240:336-337; 1988), such as the circumsporozoite antigen of *P. bergerii* or the circumsporozoite antigen of *P. falciparum*; the merozoite surface antigen of *Plasmodium* spp. (Spetzler *et al.*, Int. J. Pept. Prot. Res., 43:351-358; 1994); the galactose specific lectin of *Entamoeba histolytica* (Mann *et al.*, Proc. Natl. Acad. Sci., USA, 88:3248-3252; 1991), gp63 of *Leishmania* spp. (Russell *et al.*, J. Immunol., 140:1274-1278; 1988); (Xu and Liew, Immunol., 84: 173-176; 1995), gp46 of *Leishmania major* (Handman *et al.*, Vaccine, 18:3011-3017; 2000) paramyosin of *Brugia malayi* (Li *et al.*, Mol. Biochem. Parasitol., 49:315-323; 1991), the triose-phosphate isomerase of *Schistosoma mansoni* (Shoemaker *et al.*, Proc. Natl. Acad. Sci., USA, 89:1842-1846; 1992); the secreted globin-like protein of *Trichostrongylus colubriformis* (Frenkel *et al.*, Mol. Biochem. Parasitol., 50:27-36; 1992); the glutathione-S-transferase's of *Frasciola hepatica* (Hillyer *et al.*, Exp. Parasitol., 75:176-186; 1992), *Schistosoma bovis* and *S. japonicum* (Bashir *et al.*, Trop. Geog. Med., 46:255-258; 1994); and KLH of *Schistosoma bovis* and *S. japonicum* (Bashir *et al.*, supra, 1994).

As mentioned earlier, the rdsRN vaccine may encode an endogenous immunogen, which may be any cellular protein, immunoregulatory agent, or therapeutic agent, or parts thereof, that may be expressed in the recipient cell, including but not limited to tumor, transplantation, and autoimmune immunogens, or fragments and derivatives of tumor, transplantation, and autoimmune immunogens thereof. Thus, in the present invention, dsRP may encode tumor, transplant, or autoimmune immunogens, or parts or derivatives thereof. Alternatively, the dsRP may encode synthetic genes (made as described above), which encode tumor-specific, transplant, or autoimmune antigens or parts thereof.

Examples of tumor specific antigens include prostate specific antigen (Gattuso *et al.*, Human Pathol., 26:123-126; 1995), TAG-72 and CEA (Guadagni *et al.*, Int. J. Biol. Markers, 9:53-60; 1994), MAGE-1 and tyrosinase (Coulie *et al.*, J. Immunothera., 14:104-109; 1993). Recently it has been shown in mice that immunization with non-malignant cells expressing a tumor antigen provides a vaccine effect, and also helps the animal mount an immune response to clear malignant tumor cells displaying the same antigen (Koeppen *et al.*, Anal. N.Y. Acad. Sci., 690:244-255; 1993).

Examples of transplant antigens include the CD3 molecule on T cells (Alegre *et al.*, Digest. Dis. Sci., 40:58-64; 1995). Treatment with an antibody to CD3 receptor has been shown to rapidly clear circulating T cells and reverse cell-mediated transplant rejection (Alegre *et al.*, supra, 1995).

Examples of autoimmune antigens include IAS β chain (Topham *et al.*, Proc. Natl. Acad. Sci., USA, 91:8005-8009; 1994). Vaccination of mice with an 18 amino acid peptide from IAS β chain has been demonstrated to provide protection and treatment to mice with experimental autoimmune encephalomyelitis (Topham *et al.*, supra, 1994).

In addition, rdsRNA segments can be constructed that encode an adjuvant, and can be used to increase host immune responses to immunogens. The particular adjuvant encoded by the rdsRNA is not critical to the present invention and may be the A subunit of cholera toxin (i.e. CtxA; GenBank accession no. X00171, AF175708, D30053, D30052), or parts and/or mutant derivatives thereof (e.g. the A1 domain of the A subunit of Ctx (i.e. CtxA1; GenBank accession no. K02679), from any classical *Vibrio cholerae* (e.g. *V. cholerae* strain 395, ATCC # 39541) or El Tor *V. cholerae* (E.g. *V. cholerae* strain 2125, ATCC # 39050) strain. Alternatively, any bacterial toxin that is a member of the family of bacterial adenosine diphosphate-ribosylating exotoxins (Krueger and Barbier, Clin. Microbiol. Rev., 8:34; 1995), may be used in place of CtxA; for example, the A subunit of heat-labile toxin (referred to herein as EltA) of enterotoxigenic *Escherichia coli* (GenBank accession # M35581), pertussis toxin S1 subunit (E.g. *ptxS1*, GenBank accession # AJ007364, AJ007363, AJ006159, AJ006157, etc.); as a further alternative, the adjuvant may be one of the adenylate cyclase-hemolysins of *Bordetella pertussis* (ATCC # 8467), *Bordetella bronchiseptica* (ATCC # 7773) or *Bordetella parapertussis* (ATCC # 15237), e.g. the *cyaA* genes of *B. pertussis* (GenBank accession no. X14199), *B. parapertussis* (GenBank accession no. AJ249835) or *B. bronchiseptica* (GenBank accession no. Z37112).

Cytokine encoding rdsRNA segments can also be constructed. The particular cytokine encoded by the rdsRNA is not critical to the present invention includes, but not limited to, interleukin-4 (herein referred to as "IL-4"; Genbank accession no. AF352783 (Murine IL-4) or NM_000589 (Human IL-4), IL-5 (Genbank accession no. NM_010558 (Murine IL-5) or NM_000879 (Human IL-5), IL-6 (Genbank accession no. M20572 (Murine IL-6) or M29150 (Human IL-6), IL-10 (Genbank accession no. NM_010548 (Murine IL-10) or AF418271 (Human IL-10), IL-12_{p40} (Genbank accession no. NM_008352 (Murine IL-12 p40) or AY008847 (Human IL-12 p40), IL-

12_{p70} (Genbank accession no. NM_008351/NM_008352 (Murine IL-12 p35/40) or AF093065/AY008847 (Human IL-12 p35/40), TGF β (Genbank accession no. NM_011577 (Murine TGF β 1) or M60316 (Human TGF β 1), and TNF α Genbank accession no. X02611 (Murine TNF α) or M26331 (Human TNF α).

Furthermore, small inhibitory RNA's or antisense RNA's may also be encoded in rdsRNA segments for regulation of protein expression in targeted tissues.

Recombinant DNA and RNA procedures for the introduction of functional expression cassettes to generate rdsRNA capable of expressing an immunoregulatory agent in eukaryotic cells or tissues are described above.

As exemplary vaccine constructs to be encoded in eukaryotic expression cassettes, virus-like particles (Herein "VLP") can be constructed to induce produce protective immune responses against viral pathogens. Influenza VLP's have been shown to self assemble following plasmid expression of gene sequences encoding the hemagglutinin (HA), neuraminidase (NA), and the matrix proteins (M1 and M2) (Latham *et al.*, J. Virol, 75:6154-6165; 2001). VLP's so constructed are further capable of membrane fusion and budding to further potentiate antibody-producing immune responses and protective immunity in animal models (Pushko *et al.*, Vaccine. 2005 Sep 2; [Epub ahead of print]). HIV VLP's can be similarly assembled from minimal sequences encoding amino acids 146-231 of the capsid protein, a six amino acid myristylation sequence, the sequence encoding the P2 peptide, a GCN4 leucine zipper domain, and the gp160 envelope precursor (Accola *et al.*, J. Virol, 74:5395-5402; 2000). The major protein L1 of HPV has been shown to self-assemble into VLP's a variety of cell lines and produces humoral and cellular immunity, making the gene encoding this protein an attractive immunogen (Shi *et al.*, J Virol., 75(21): 10139–10148;2001).

3. Construction of rdsRNA segments that carry alphavirus expression cassettes

As noted above, rdsRNAs can harbor a mammalian translation expression cassette comprised of the semliki forest virus (herein referred to as "SFV") self-amplifying replicon from plasmid pSFV1 (Invitrogen Inc., Carlsbad, CA) functionally linked to a gene of interest. Genes encoding SFV non-structural protein 1-4 (herein referred to as "nsp1-4") and the replicase recognition site in pSFV1 are amplified by PCR and inserted by blunt-end ligation into the *HpaI* site immediately downstream and functionally linked to the IRES in rSeg-S resulting in rSeg-S::SFV1 (Figure 5). A *SmaI* RE site in plasmid rSeg-S::SFV1 can serve as an insertion site for any foreign or endogenous gene of interest, such as those outlined above.

Note that in rdsRNAs that harbor rdsRNA segment-S containing a positive selection allele and an alphavirus nsP1-4 and amplicon about 8100 bp, the uptake of segment-L will be impeded when the gene of interest exceeds 800 bp (i.e. the genome size is more than 10 percent greater than the wild-type genome). Note that in all circumstances, rdsRNAs that harbor rdsRNA segment-S containing a positive selection allele and an alphavirus nsP1-4 and amplicon do not need a rdsRNA segment-M.

This limitation in capacity can be solved by generating packaging strains that express modified derivatives of segment-L that lack the 5-prime *pac* sequence. Such sequences will express the proteins necessary for procapsid production but will not be packaged in the nucleocapsid, thereby providing an additional 7000 bp of capacity in the rdsRN generated in said strain.

The modified segment-L in such constructs can be introduced into the packaging strain in an expression vector, such as pT7/T3-18 (Ambion, Austin, TX, Cat. No. 7201) or integrated into the chromosome by allelic exchange using methods known to those skilled in the art (Hamilton *et al.*, supra, 1989); (Blomfield *et al.*, supra, 1991). The location of chromosomal integration is not important to the present invention, although in a preferred embodiment DNA encoding the segment-L expression cassette is integrated into the chromosome so as to inactivate a gene and generate a phenotype selectable under defined culture conditions, such as *aroA* (Genbank Accession No. X00557), *aroC* (Genbank Accession No. AY142231), *leuD* (Genbank Accession No. L06666) *asd* (Genbank Accession No. V00262), *murI* (Genbank Accession No. AY520970) *kdsA* (Genbank Accession No. AY174101), and *htrB* (Genbank Accession No. AF401529). Procedures for chromosomal integration and methods for culturing said mutants are well documented (Hamilton *et al.*, J. Bacteriol. 171: 4617; 1989); (Blomfield *et al.*, Mol. Microbiol. 5: 1447; 1991).

4. Methods to generate rdsRNAs *de novo*

The rdsRNAs are produced in packaging strains by introducing RNA encoding all of the information necessary to produce the rdsRNA following uptake into the procapsid. The rRNA may be directly introduced or may be encoded on non-replicating plasmids, which may be co-introduced into the packaging strain. The genes encoding segment-L and hence the procapsid may be present in the packaging strain on a plasmid or be integrated into the chromosome of the packaging strain. As a further option, the positive strand RNA encoding segment-L may be introduced into the packaging strain in concert with positive strand RNA encoding recombinant segments –S and –M. Once the procapsid incorporates the recombinant ssRNA's (herein referred to as ssRNA) of segments- S and

-M, which must be of sufficient size and display the appropriate packaging sequences to produce a signal for the uptake of segment-L mRNA, the latter is then incorporated and all packaged ssRNA is converted to dsRNA, resulting in the generation of a rdsRN. At this point, the rdsRN is capable of generating recombinant segments -S and -M mRNA and segment-L mRNA; the latter expresses the proteins that constitute the procapsid, which uptake incorporate the recombinant segment and segment-L mRNA, then converted to dsRNA thereby generating additional rdsRNs (Figure 6).

In vitro synthesized recombinant segment mRNA is introduced into packaging strains by electroporation. Preferably, the RNA is *in vitro* transcribed from linear DNA templates using fluorinated rNTP's (Durascribe, Epicentre, Madison, WI) to produce fluorinated RNA, which is nuclease-resistant RNA. Fluorinated dUTP and dCTP are incorporated into the reaction mixture at a final concentration of 5 mM each. While fNTP's are preferred, any modified rNTP, which imparts nuclease resistance, such as thiol or aminohexyl substituted rNTP's, is useful in the present invention. Thus, those skilled in the art will be able to substitute any modified NTP which imparts nuclease resistance in place of fluorinated NTP's.

The RNA or preferably the fluorinated RNA encodes at least a gene product that complements said at least one selectable phenotypic mutation and an RNA of interest operably linked to a eukaryotic translation initiation sequence. In a preferred embodiment, the fluorinated RNA encodes at least a gene product that complements said at least one selectable phenotypic mutation, gene-8 (SEQ ID 7) for stabilization of nucleocapsid production; and an RNA of interest operably linked to a eukaryotic translation initiation sequence.

To launch the rdsRN in said packaging strain, an electroporation medium is generated, composed of

- i) An electrocompetent bacterial strain, at a density of $10^8 - 10^{11}$ cfu/ml for packaging, launching and producing rdsRN, comprising
 - a) genomic DNA comprising at least one non-reverting selectable phenotypic mutation;
 - b) nucleic acid sequences encoding genes necessary for procapsid production; and
 - c) one or more procapsids comprising proteins with RNA packaging and RNA polymerase activity.

- ii) 1 ng – 1 mg, preferably 1 mcg – 100 mcg, more preferably 5 mcg – 40 mcg RNA encoding at least a gene product that complements said at least one selectable phenotypic mutation and an RNA of interest operably linked to a eukaryotic translation initiation sequence.

In a preferred embodiment, the rdsRN are launched in said packaging strain, using an electroporation medium composed of

- i) An electrocompetent bacterial strain, at a density of $10^8 - 10^{11}$ cfu/ml for packaging, launching and producing rdsRN, comprising
 - a) genomic DNA comprising at least one non-reverting selectable phenotypic mutation;
 - b) nucleic acid sequences encoding genes necessary for procapsid production; and
 - c) one or more procapsids comprising proteins with RNA packaging and RNA polymerase activity.
- ii) 1 ng – 1 mg, preferably 1 mcg – 100 mcg, more preferably 5 mcg – 40 mcg RNA encoding at least a gene product that complements said at least one selectable phenotypic mutation, gene-8 (SEQ ID 7) for stabilization of nucleocapsid production; and an RNA of interest operably linked to a eukaryotic translation initiation sequence.

Alternatively, the rdsRN are launched in said packaging strain, using an electroporation medium composed of

- i) An electrocompetent bacterial strain, at a density of $10^8 - 10^{11}$ cfu/ml for packaging, launching and producing rdsRN, comprising
 - a) genomic DNA comprising at least one non-reverting selectable phenotypic mutation;
- 1 ng – 1 mg, preferably 1 mcg – 100 mcg, more preferably 5 mcg – 40 mcg RNA encoding at least a gene product that complements said at least one selectable phenotypic mutation, nucleic acid sequences encoding genes necessary for procapsid production, gene-8 (SEQ ID 7) for stabilization of nucleocapsid production; and an RNA of interest operably linked to a eukaryotic translation initiation sequence.

In a further preferred embodiment, the rdsRN are launched in said packaging strain, using an electroporation medium composed of

- i) An electrocompetent bacterial strain, at a density of $10^8 - 10^{11}$ cfu/ml for packaging, launching and producing rdsRN, comprising
 - a) genomic DNA comprising at least one non-reverting selectable phenotypic mutation;
 - b) nucleic acid sequences encoding genes necessary for procapsid production; and
 - c) one or more procapsids comprising proteins with RNA packaging and RNA polymerase activity.
- ii) 1 ng – 1 mg, preferably 1 mcg – 100 mcg, more preferably 5 mcg – 40 mcg fluorinated RNA encoding at least a gene product that complements said at least one selectable phenotypic mutation, gene-8 (SEQ ID 7) for stabilization of nucleocapsid production; and an RNA of interest operably linked to a eukaryotic translation initiation sequence.

In yet a further preferred embodiment, the rdsRN are launched in said packaging strain, using an electroporation medium composed of

- i) An electrocompetent bacterial strain, at a density of $10^8 - 10^{11}$ cfu/ml for packaging, launching and producing rdsRN, comprising
 - a) genomic DNA comprising at least one non-reverting selectable phenotypic mutation;
- ii) 1 ng – 1 mg, preferably 1 mcg – 100 mcg, more preferably 5 mcg – 40 mcg fluorinated RNA encoding at least a gene product that complements said at least one selectable phenotypic mutation, nucleic acid sequences encoding genes necessary for procapsid production, gene-8 (SEQ ID NO: 7) for stabilization of nucleocapsid production; and an RNA of interest operably linked to a eukaryotic translation initiation sequence.

The method used to electroporate said RNA or preferably, fluorinated RNA into said packaging strain is not important to the present invention and can be achieved using standard procedures well known to the art (Ausubel *et al.*, supra, 1990; Sambrook, supra). Following electroporation the

electroporation medium is admixed with recovery medium, as described (Ausubel *et al.*, supra, 1990; Sambrook, supra) and incubated at 37°C for 30 min – 4 hr, preferably 2 hr.

Electrotransformants are isolated on solid media under conditions that only permit the growth of strains that harbor and express the positive selection allele in the recombinant segment (e.g. Trypticated Soy agar (herein referred to as TSA), Difco, Detroit, MI).

Bacterial isolates containing rdsRNAs are cultured at temperatures that range from 25°C to 44°C for 16 to 96 hrs; however, it is preferable to culture the transformants at initially at 28°C for 48 hr. Colonies that grow on the selective solid media are subsequently isolated and purified by standard methods (Ausubel *et al.*, supra, 1990); (Sambrook, supra). To verify that the isolates selected are carrying the functional rdsRNA of interest, individual isolates are screened by RT-PCR using primers designed to specifically amplify positive and negative (second) strand RNA sequences of, but not limited to, the strand-specific packaging sequences, the positive selection allele, the IRES, and the gene of interest. Methods of RNA preparation for analysis are well known to those skilled in the art, such as the following. Individual isolates may be cultured in liquid media (e.g. Trypticated Soy broth (herein referred to as “TSB”), Difco MO) and the resultant cultures harvested after reaching an optical density at 600 nm (OD₆₀₀) of 0.001 to 4.0, relative to the OD₆₀₀ of a sterile TSB control. The nucleocapsids are isolated from such cultures using methods reported elsewhere and well known to those skilled in the art (Gottlieb *et al.*, J. Bacteriol 172:5774; 1990); (Sun *et al.*, supra, 2003). The PCR primers for such an analysis are designed using Clone Manager[®] software version 4.1 (Scientific and Educational Software Inc., Durham, NC) or OLIGO 4.0 primer analysis software (copyright Wojciech Rychlik). This software enables the design of PCR primers that are compatible with the specific DNA fragments being manipulated. RT-PCRs are conducted in a Bio Rad iCycler, (Hercules, CA) and primer annealing, elongation and denaturation times in the RT-PCRs are set according to standard procedures (Ausubel *et al.*, supra). The RT-PCR products are subsequently analyzed by agarose gel electrophoresis using standard procedures (Ausubel *et al.*, supra, 1990); (Sambrook, supra). A positive clone is defined as one that displays the appropriate RT-PCR pattern that indicates that the rdsRNA segment has been stably maintained in the strain. The RT-PCR products can be further evaluated using standard DNA sequencing procedures, as described below.

Having identified the desired transformants, individual strains are stored in a storage media, which is TS containing 10-30% (v/v) glycerol. Bacterial isolates are harvested from solid media using

a sterile cotton wool swab and suspended in storage media at a density of 10^8 - 10^9 cfu/ml, and the suspensions are stored at -80°C.

Batches of purified rdsRNAs are purified from said carrier strains using methods well known in the art and published extensively in detail elsewhere (Mindich, *et al.*, J Virol 66, 2605-10; 1992); (Mindich, *et al.*, Virology 212:213-217; 1995); (Mindich, *et al.*, J Bacteriol 181:4505-4508; 1999); (Qiao, *et al.*, Virology 275:218-224; 2000); (Qiao, *et al.*, Virology 227:103-110; 1997); (Olkkonen, *et al.*, Proc Natl Acad Sci U S A 87:9173-9177; 1990); (Onodera, *et al.*, J Virol 66, 190-196; 1992).

5. Use of rdsRNAs to induce to cause a biological effect *in vivo*

The specific method used to formulate the novel rdsRP expression systems described herein is not critical to the present invention and can be selected from a physiological buffer (Felgner *et al.*, U.S. Patent # 5589466 (1996); aluminum phosphate or aluminum hydroxyphosphate (e.g. Ulmer *et al.*, Vaccine, 18:18; 2000), monophosphoryl-lipid A (also referred to as MPL or MPLA; Schneerson *et al.*, J. Immunol., 147: 2136-2140; 1991); (e.g. Sasaki *et al.*, Inf. Immunol., 65: 3520-3528; 1997); (Lodmell *et al.*, Vaccine, 18:1059-1066; 2000), QS-21 saponin (e.g. Sasaki, *et al.*, J. Virol., 72:4931; 1998); dexamethasone (e.g. Malone, *et al.*, J. Biol. Chem. 269:29903; 1994); CpG DNA sequences (Davis *et al.*, J. Immunol., 15:870; 1998); or lipopolysaccharide (LPS) antagonist (Hone *et al.*, *supra* 1997).

The rdsRNA can be administered directly into eukaryotic cells, animal tissues, or human tissues by intravenous, intramuscular, intradermal, intraperitoneally, intranasal and oral inoculation routes. The specific method used to introduce the rdsRNA constructs described herein into the target cell or tissue is not critical to the present invention and can be selected from previously described vaccination procedures (Wolff, *et al.*, Biotechniques 11:474-85; 1991); (Johnston and Tang, Methods Cell Biol 43:353-365; 1994); (Yang and Sun, Nat Med 1:481-483; 1995); (Qiu, *et al.*, Gene Ther. 3:262-8; 1996); (Larsen, *et al.*, J. Virol. 72:1704-8; 1998); (Shata and Hone, J. Virol. 75:9665-9670; 2001); (Shata, *et al.*, Vaccine 20:623-629; 2001); (Ogra, *et al.*, J Virol 71:3031-3038; 1997); (Buge, *et al.*, J. Virol. 71:8531-8541; 1997); (Belyakov, *et al.*, Nat. Med. 7, 1320-1326; 2001); (Lambert, *et al.*, Vaccine 19:3033-3042; 2001); (Kaneko, *et al.*, Virology 267: 8-16; 2000); (Belyakov, *et al.*, Proc Natl Acad Sci USA 96:4512-4517; 1999).

The specific biological effects covered by the invention described herein include, but are not limited to, protective or modulatory immune responses, therapeutic responses, and downregulation

of expression (eg siRNA) or upregulation of expression (eg cytokine expression) of host proteins. Initially, the rdsRNAs are administered at dose of 10^2 - 10^9 nucleocapsid particles, and are administered by an appropriate route, such as orally, intranasally, subcutaneously, intramuscularly, or via invasive bacterial vectors (Sizemore et al, Science. 1995 Oct 13;270(5234):299-302). The number of doses varies depending on the potency of the individual rdsRNAs and can be a single-, two- or three-dose regimen spaced by 2- to 4-week intervals. Each expression study includes a negative control rdsRN that does not contain the gene of interest, and a DNA vaccine vector that encodes the gene of interest can serve as a positive control.

Methods for measuring biological effects of rdsRN encoded gene expression in biological systems are well known to those skilled in the art. As an example, to measure serum IgG and IgA responses to gp120, sera are collected before and 10, 20, 30, 40, 50, 60, 70, and 80 days after vaccination. About 400-500 μ l of blood is collected into individual tubes from the tail vein of each mouse and allowed to clot by incubating for 4 hr on ice. After centrifugation in a microfuge for 5 min, the sera are transferred to fresh tubes and stored at -80°C . Mucosal IgG and IgA responses to antigens expressed by the genes of interest are determined using fecal pellets and vaginal washes that will be harvested before and at regular intervals after vaccination (Srinivasan *et al.*, Biol. Reprod. 53: 462; 1995); (Staats *et al.*, J. Immunol. 157: 462; 1996). Standard ELISAs are used to quantitate the IgG and IgA responses to gp120 in the sera and mucosal samples (Abacioglu *et al.*, AIDS Res. Hum. Retrovir. 10: 371; 1994); (Pincus *et al.*, AIDS Res. Hum. Retrovir. 12: 1041;1996). Ovalbumin can be included in each ELISA as a negative control antigen. In addition, each ELISA can include a positive control serum, fecal pellet or vaginal wash sample, as appropriate. The positive control samples are harvested from animals vaccinated intranasally with 10 μ g of the antigen expressed by the gene of interest mixed with 10 μ g cholera toxin, as described (Yamamoto *et al.*, Proc. Natl. Acad. Sci. 94: 5267; 1997). The end-point titers are calculated by taking the inverse of the last serum dilution that produced an increase in the absorbance at 490 nm that is greater than the mean of the negative control row plus three standard error values.

To measure cellular immunity, cell suspensions of enriched CD4⁺ and CD8⁺ T cells from lymphoid tissues are used to measure antigen-specific T cell responses by cytokine-specific ELISPOT assay (Wu *et al.*, AIDS Res. Hum. Retrovir. 13:1187; 1997). Such assays can assess the numbers of antigen-specific T cells that secrete IL-2, IL-4, IL-5, IL-6, IL-10 and IFN- γ . All

ELISPOT assays are conducted using commercially-available capture and detection mAbs (R&D Systems and Pharmingen), as described (Wu *et al.*, Infect. Immun. 63:4933; 1995) and used previously (Xu-Amano *et al.*, J. Exp. Med. 178:1309; 1993); (Okahashi *et al.*, Infect. Immun. 64: 1516; 1996). Each assay includes mitogen (Con A) and ovalbumin controls.

6. Use of bacterial vectors carrying rdsRNAs to induce an immune response or to cause a biological effect in target cells or tissues.

Delivery of the rdsRNA to and expression of the encoded sequences in a targeted eukaryotic cell, tissue, or organism may be accomplished by inoculation with rdsRNAs carried in a non-pathogenic or attenuated bacterial vaccine vector. Biological responses of interest include, but are not limited to, protective or modulatory immune responses, therapeutic responses, and downregulation of expression (eg siRNA) or upregulation of expression (eg cytokine expression) of host proteins.

The bacterial species from which the bacterial vaccine vector is derived in the present invention is not critical thereto and include, but are not limited to: *Campylobacter spp.*, *Neisseria spp.*, *Haemophilus spp.*, *Aeromonas spp.*, *Francisella spp.*, *Yersinia spp.*, *Klebsiella spp.*, *Bordetella spp.*, *Legionella spp.*, *Corynebacterium spp.*, *Citrobacter spp.*, *Chlamydia spp.*, *Brucella spp.*, *Pseudomonas spp.*, *Helicobacter spp.*, or *Vibrio spp.*

The particular *Campylobacter* strain employed is not critical to the present invention. Examples of *Campylobacter* strains that can be employed in the present invention include but are not limited to: *C. jejuni* (ATCC Nos. 43436, 43437, 43438), *C. hyointestinalis* (ATCC No. 35217), *C. fetus* (ATCC No. 19438) *C. fecalis* (ATCC No. 33709) *C. doylei* (ATCC No. 49349) and *C. coli* (ATCC Nos. 33559, 43133).

The particular *Yersinia* strain employed is not critical to the present invention. Examples of *Yersinia* strains which can be employed in the present invention include *Y. enterocolitica* (ATCC No. 9610) or *Y. pestis* (ATCC No. 19428), *Y. enterocolitica* Ye03-R2 (al-Hendy *et al.*, Infect. Immun., 60:870; 1992) or *Y. enterocolitica aroA* (O'Gaora *et al.*, Micro. Path., 9:105; 1990).

The particular *Klebsiella* strain employed is not critical to the present invention. Examples of *Klebsiella* strains that can be employed in the present invention include *K. pneumoniae* (ATCC No. 13884).

The particular *Bordetella* strain employed is not critical to the present invention. Examples of *Bordetella* strains that can be employed in the present invention include *B. pertussis* and *B. bronchiseptica* (ATCC No. 19395).

The particular *Neisseria* strain employed is not critical to the present invention. Examples of *Neisseria* strains that can be employed in the present invention include *N. meningitidis* (ATCC No. 13077) and *N. gonorrhoeae* (ATCC No. 19424), *N. gonorrhoeae* MS11 *aro* mutant (Chamberlain *et al.*, Micro. Path., 15:51-63; 1993).

The particular *Aeromonas* strain employed is not critical to the present invention. Examples of *Aeromonas* strains that can be employed in the present invention include *A. salmonicida* (ATCC No. 33658), *A. schubertii* (ATCC No. 43700), *A. hydrophila*, *A. eucrenophila* (ATCC No. 23309).

The particular *Francisella* strain employed is not critical to the present invention. Examples of *Francisella* strains that can be employed in the present invention include *F. tularensis* (ATCC No. 15482).

The particular *Corynebacterium* strain employed is not critical to the present invention. Examples of *Corynebacterium* strains that can be employed in the present invention include *C. pseudotuberculosis* (ATCC No. 19410).

The particular *Citrobacter* strain employed is not critical to the present invention. Examples of *Citrobacter* strains that can be employed in the present invention include *C. freundii* (ATCC No. 8090).

The particular *Chlamydia* strain employed is not critical to the present invention. Examples of *Chlamydia* strains that can be employed in the present invention include *C. pneumoniae* (ATCC No. VR1310).

The particular *Haemophilus* strain employed is not critical to the present invention. Examples of *Haemophilus* strains that can be employed in the present invention include *H. influenzae* (Lee *et al.*, supra), *H. somnus* (ATCC No. 43625).

The particular *Brucella* strain employed is not critical to the present invention. Examples of *Brucella* strains that can be employed in the present invention include *B. abortus* (ATCC No. 23448).

The particular *Legionella* strain employed is not critical to the present invention. Examples of *Legionella* strains that can be employed in the present invention include *L. pneumophila* (ATCC No. 33156), or a *L. pneumophila mip* mutant (Ott, FEMS Micro. Rev., 14:161; 1994).

The particular *Pseudomonas* strain employed is not critical to the present invention. Examples of *Pseudomonas* strains that can be employed in the present invention include *P. aeruginosa* (ATCC No. 23267).

The particular *Helicobacter* strain employed is not critical to the present invention. Examples of *Helicobacter* strains that can be employed in the present invention include *pylori* (ATCC No. 43504), *H. mustelae* (ATCC No. 43772).

The particular *Vibrio* strain employed is not critical to the present invention. Examples of *Vibrio* strains that can be employed in the present invention include *Vibrio cholerae* (ATCC No. 14035), *Vibrio cincinnatiensis* (ATCC No. 35912), *V. cholerae* RSI virulence mutant (Taylor *et al.*, J. Infect. Dis., 170:1518-1523; 1994) and *V. cholerae* *ctxA*, *ace*, *zot*, *cep* mutant (Waldor *et al.*, J. Infect. Dis., 170:278-283; 1994).

In a preferred embodiment, the bacterial species from which the bacterial vaccine vector is derived in the present invention includes attenuated derivatives of bacteria previously shown to possess the potential to serve as vaccine vectors, such as the *Enterobacteriaceae*, including but not limited to *Escherichia spp*, *Shigella spp*, and *Salmonella spp*. Gram-positive and acid-fast packaging and vector strains could similarly be constructed from *Listeria monocytogenes* or *Mycobacterium spp*.

The particular *Escherichia* strain employed is not critical to the present invention. Examples of *Escherichia* strains which can be employed in the present invention include *Escherichia coli* strains DH5 α , HB 101, HS-4, 4608-58, 1184-68, 53638-C-17, 13-80, and 6-81 (Sambrook *et al.*, *supra*, 2001); (Sansonetti *et al.*, Ann. Microbiol. (Inst. Pasteur), 132A:351; 1982), enterotoxigenic *E. coli* (Evans *et al.*, Infect. Immun., 12:656; 1975), enteropathogenic *E. coli* (Donnenberg *et al.*, J. Infect. Dis., 169:831; 1994) and enterohemorrhagic *E. coli* (McKee and O'Brien, Infect. Immun., 63:2070; 1995).

The particular *Salmonella* strain employed is not critical to the present invention. Examples of *Salmonella* strains that can be employed in the present invention include *S. typhi* (ATCC No. 7251), *S. typhimurium* (ATCC No. 13311), *Salmonella galinarum* (ATCC No. 9184), *Salmonella enteritidis* (ATCC No. 4931) and *Salmonella typhimurium* (ATCC No. 6994). *S. typhi* *aroC*, *aroD* double mutant (Hone *et al.*, Vacc., 9:810-816; 1991), *S. typhimurium* *aroA* mutant (Mastroeni *et al.*, Micro. Pathol., 13:477-491; 1992).

The particular *Shigella* strain employed is not critical to the present invention. Examples of *Shigella* strains that can be employed in the present invention include *Shigella flexneri* (ATCC No. 29903), *Shigella flexneri* CVD1203 (Noriega *et al.*, Infect Immun. 62:5168; 1994), *Shigella*

flexneri 15D (Vecino *et al.*, Immunol Lett. 82:197; 2002), *Shigella sonnei* (ATCC No. 29930), and *Shigella dysenteriae* (ATCC No. 13313).

The particular *Mycobacterium* strain employed is not critical to the present invention. Examples of *Mycobacterium* strains that can be employed in the present invention include *M. tuberculosis* CDC1551 strain (Griffith *et al.*, Am. J. Respir. Crit. Care Med. Aug;152(2):808; 1995), *M. tuberculosis* Beijing strain (Soolingen *et al.*, 1995) H37Rv strain (ATCC#:25618), *M. tuberculosis* pantothenate auxotroph strain (Sambandamurthy, Nat. Med. 2002 8(10):1171; 2002), *M. tuberculosis* *rpoV* mutant strain (Collins *et al.*, Proc Natl Acad Sci USA. 92(17):8036; 1995), *M. tuberculosis* leucine auxotroph strain (Hondalus *et al.*, Infect. Immun. 68(5):2888; 2000), BCG Danish strain (ATCC # 35733), BCG Japanese strain (ATCC # 35737), BCG, Chicago strain (ATCC # 27289), BCG Copenhagen strain (ATCC #: 27290), BCG Pasteur strain (ATCC #: 35734), BCG Glaxo strain (ATCC #: 35741), BCG Connaught strain (ATCC # 35745), BCG Montreal (ATCC # 35746).

The particular *Listeria monocytogenes* strain employed is not critical to the present invention. Examples of *Listeria monocytogenes* strains which can be employed in the present invention include *L. monocytogenes* strain 10403S (e.g. Stevens *et al.*, J. Virol 78: 8210-8218; 2004) or mutant *L. monocytogenes* strains such as (i) *actA plcB* double mutant (Peters *et al.*, FEMS Immunology and Medical Microbiology 35: 243-253; 2003); (Angelakopoulous *et al.*, Infect and Immunity 70: 3592-3601; 2002); (ii) *dal dat* double mutant for alanine racemase gene and D-amino acid aminotransferase gene (Thompson *et al.*, Infect and Immunity 66: 3552-3561; 1998).

Methods to attenuate *E. coli*, Salmonella, Mycobacteria, Shigella, and Listeria are not important to the present invention and are well known to those skilled in the art (Evans *et al.*, supra, 1975); (Noriega *et al.*, supra, 1994); (Hone *et al.*, supra, 1991).

Once a non-pathogenic or attenuated bacterial vaccine vector strain has been selected, said strain is modified to serve as an rdsRN packaging strain. This is accomplished using the strategies described in detail above that entail introducing segment-L sequences that expresses dsRP procapsids in said strain and a mutation to enable selection and maintenance of the rdsRNs that express a functional gene that complements the deficiency created by the mutation in said strain.

To generate strains that package and stably maintain the desired rdsRN, *in vitro* synthesized recombinant segment RNA(s) are introduced into packaging strains by electroporation and transformants are isolated in solid media under conditions that only permit the growth of strains that

harbor and express the positive selection allele in the recombinant segment (e.g. Trypticated Soy agar will only permit the growth of *asd* and *murI* mutants when the wild-type gene complements that genomic defect, Difco, Detroit, MI, Cat. No. 244520). The methods for generating rdsRNA segments, in vitro mRNA synthesis and electroporation are all provided above. To verify that the isolates are carrying the rdsRN of interest, individual isolates are cultured in liquid media (e.g. TS, Difco, Detroit, MI, Cat. No. 244620) and nucleocapsids are isolated from said cultures using methods reported elsewhere and well known to those skilled in the art (Gottlieb *et al.*, supra, 1990); (Sun *et al.*, supra, 2003). DsRNA is isolated from the nucleocapsids using commercially available RNA extraction kits and screened by RT-PCR using primers that amplify defined fragments within the recombinant segments, including but not limited to PCR primers that amplify the positive selection allele, the IRES and the gene of interest, as discussed in detail above. A positive clone is defined as one that displays the appropriate RT-PCR pattern that indicates that the rdsRNA segment has been stably maintained in the strain. The RT-PCR products can be further evaluated using standard DNA sequencing procedures, as described below.

The specific culture conditions for the growth of said bacterial vaccine vector strains that stably harbor rdsRNAs are not critical to the present invention. For illustrative purposes, the said mutants can be grown in a liquid medium such a LB medium (Difco, Detroit, MI, Cat. No. 244620), Nutrient broth (Difco, Detroit, MI, Cat. No. 233000), or Tryptic Soy broth (Difco, Detroit, MI, Cat. No. 211822), using conventional culture techniques that are appropriate for the bacterial strain being grown (Miller, supra, 1991). As an alternative the bacteria can be cultured on solid media such as Nutrient agar (Difco, Detroit, MI, Cat. No. 212000), Tryptic Soy agar (Difco, Detroit, MI, Cat. No. 236920), or M9 minimal agar (Difco, Detroit, MI, Cat. No. 248510).

Mycobacterium vaccine vector strains are cultured in liquid media, such as Middlebrook 7H9 (Difco, Detroit, MI, Cat. No. 271310) or Saulton Synthetic Medium, preferably at 37°C. The strains can be maintained as static or agitated cultures. In addition, the growth rate of *Mycobacterium* can be enhanced by the addition of oleic acid (0.06% v/v; Research Diagnostics Cat. No. 01257) and detergents such as Tyloxapol (0.05% v/v; Research Diagnostics Cat. No.70400). The purity of *Mycobacterium* cultures can be evaluated by evenly spreading 100 µl aliquots of the *Mycobacterium* culture serially diluted (e.g. 10-fold steps from Neat – 10⁻⁸) in phosphate buffered saline (herein referred to PBS) onto 3.5 inch plates containing 25-30 ml of solid media, such as Middlebrook 7H10 (BD Microbiology, Cockeysville, MD, Cat. No. 221174).

The amount of the bacterial vaccine vector to be administered with the rdsRN of the present invention will vary depending on the species of the subject, as well as the disease or condition that is being treated. Generally, the dosage employed will be about 10^3 to 10^{11} viable organisms, preferably about 10^3 to 10^9 viable organisms.

The bacterial vector harboring the rdsRN is generally administered along with a pharmaceutically acceptable carrier or diluent. The particular pharmaceutically acceptable carrier or diluent employed is not critical to the present invention. Examples of diluents include a phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine *et al.*, J. Clin. Invest., 79:888-902; 1987); (Black *et al.*, J. Infect. Dis., 155:1260-1265; 1987), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine *et al.*, Lancet, II: 467-470; 1988). Examples of carriers include proteins, e.g., as found in skim milk, sugars, e.g., sucrose, or polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-90% (w/v) but preferably at a range of 1-10% (w/v).

The biological activity of vector strains is assessed in an appropriate animal model (e.g. BATS/cJ mice, rabbits, guinea pigs or Rhesus macaques). Initially, the rdsRN vector strains are administered at doses of 10^2 - 10^9 cfu, and are administered by an appropriate route (e.g. *E. coli*, *Salmonella* and *Shigella* can be given intragastrically or intranasally, whereas rBCG vectors are injected subcutaneously). The number of doses will vary, depending on the potency of the individual vector strain, and the valency of the encoded recombinant product of interest.

Methods of measurement of immune and other biological responses to rdsRN encoded products are well known to those skilled in the art. To measure serum IgG and IgA responses to gp120, sera are collected before and 10, 20, 30, 40, 50, 60, 70, and 80 days after vaccination. About 400-500 μ l of blood is collected into individual tubes from the tail vein of each mouse and allowed to clot by incubating for 4 hr on ice. After centrifugation in a microfuge for five minutes, the sera are transferred to fresh tubes and stored at -80°C. Mucosal IgG and IgA responses to antigens expressed by the genes of interest are determined using fecal pellets and vaginal washes that will be harvested before and at regular intervals after vaccination (Srinivasan *et al.*, Biol. Reprod. 53: 462; 1995); (Staats *et al.*, J. Immunol. 157: 462; 1996). Standard ELISAs are used to quantitate the IgG and IgA responses to gp120 in the sera and mucosal samples (Abacioglu *et al.*, AIDS Res. Hum.

Retrovir. 10: 371; 1994); (Pincus *et al.*, AIDS Res. Hum. Retrovir. 12: 1041; 1996). Ovalbumin can be included in each ELISA as a negative control antigen. In addition, each ELISA can include a positive control serum, fecal pellet or vaginal wash sample, as appropriate. The positive control samples are harvested from animals vaccinated intranasally with 10µg of the antigen expressed by the gene of interest mixed with 10 µg cholera toxin, as described (Yamamoto *et al.*, Proc. Natl. Acad. Sci. 94: 5267; 1997). The end-point titers are calculated by taking the inverse of the last serum dilution that produced an increase in the absorbance at 490 nm that is greater than the mean of the negative control row plus three standard error values.

Cellular immunity may be measured by intracellular cytokine staining (also referred to as intracellular cytokine cytometry) or by ELISPOT (Letsch A. *et al.*, Methods 31:143-49; 2003). Both methods allow the quantitation of antigen-specific immune responses, although ICS also adds the simultaneous capacity to phenotypically characterize antigen-specific CD4+ and CD8+ T-cells. Such assays can assess the numbers of antigen-specific T cells that secrete IL-2, IL-4, IL-5, IL-6, IL-10 and IFN- (Wu *et al.*, AIDS Res. Hum. Retrovir. 13: 1187; 1997). ELISPOT assays are conducted using commercially-available capture and detection mAbs (R&D Systems and Pharmingen), as described (Wu *et al.*, Infect. Immun. 63:4933; 1995) and used previously (Xu-Amano *et al.*, J. Exp. Med. 178:1309; 1993); (Okahashi *et al.*, Infect. Immun. 64: 1516; 1996). Each assay includes mitogen (Con A) and ovalbumin controls.

7. Recombinant DNA techniques

The recombinant DNA procedures used in the construction of the packaging strains, bacterial vectors and rdsRNAs, including, but not limited to, PCR, restriction endonuclease (herein referred to as "RE") digestions, DNA ligation, agarose gel electrophoresis, DNA purification, and dideoxynucleotide sequencing, are described elsewhere (Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 1992); (Bothwell *et al.*, supra); and (Ausubel *et al.*, supra), bacteriophage-mediated transduction (de Boer, supra); (Miller, supra, 1992) and (Ausubel *et al.*, supra), or chemical (Bothwell *et al.*, supra); (Ausubel *et al.*, supra); (Felgner *et al.*, supra); and Farhood, supra), electroporation (Bothwell *et al.*, supra); (Ausubel *et al.*, supra); (Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 1992) and physical transformation techniques (Johnston *et al.*, supra); (Bothwell *et al.*, supra). The genes can be incorporated on phage (de Boer *et al.*, Cell, 56:641-649;

1989), plasmids vectors (Curtiss *et al.*, supra) or spliced into the chromosome (Hone *et al.*, supra) of the target strain.

Gene sequences can be made synthetically using an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA 94404 U.S.A.) and procedures provided by the manufacturer. To synthesize large sequences i.e greater than 200 bp, a series of segments of the full-length sequence are generated by PCR and ligated together to form the full-length sequence using procedures well know in the art. However, smaller sequences, i.e. those smaller than 200 bp, can be made synthetically in a single round using an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA) and procedures provided by the manufacturer.

Recombinant plasmids are introduced into bacterial strains by electroporation using a BioRad Gene-Pulser® set at 200Ω, 25 μF and 2.5 kV (BioRad Laboratories, Hercules, CA) [38]. Nucleotide sequencing to verify cDNA sequences is accomplished by standard automated sequencing techniques (Applied Biosystems automated sequencer, model 373A). DNA primers for DNA sequencing and polymerase chain reaction (herein referred to as “PCR”) are synthesized using an Applied Biosystems ABI™ 3900 High-Throughput DNA Synthesizer (Foster City, CA 94404).

The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.

EXAMPLES

Example 1: Recombinant DNA procedures

Restriction endonucleases (herein “RE”); New England Biolabs, Beverly, MA), T4 DNA ligase (New England Biolabs, Beverly, MA) and Taq polymerase (Life Technologies, Gaithersburg, MD) were used according to the manufacturers’ protocols; Plasmid DNA was prepared using small-scale (Qiagen Miniprep^R kit, Santa Clarita, CA) or large-scale (Qiagen Midiprep^R kit, Santa Clarita, CA) plasmid DNA purification kits according to the manufacturer’s protocols (Qiagen, Santa Clarita, CA); Nuclease-free, molecular biology grade deionized water, Tris-HCl (pH 7.5), EDTA pH 8.0, 1M MgCl₂, 100% (v/v) ethanol, ultra-pure agarose, and agarose gel electrophoresis buffer were purchased from Life Technologies, Gaithersburg, MD. RE digestions, PCRs, DNA ligation reactions and agarose gel electrophoresis were conducted according to well-known procedures (Sambrook, *et al.*, supra, 1989); (Ausubel, *et al.*, supra, 1990). Nucleotide sequencing to verify the

DNA sequence of each recombinant plasmid described in the following examples was accomplished by conventional automated DNA sequencing techniques using an Applied Biosystems automated sequencer, model 373A.

PCR primers were purchased from the Integrated DNA Technologies (Coralville, IA) or the University of Maryland Biopolymer Facility (Baltimore, MD) and were synthesized using an Applied Biosystems DNA synthesizer (model 373A). PCR primers were used at a concentration of 200 μ M and annealing temperatures for the PCR reactions were determined using Clone manager software version 4.1 (Scientific and Educational Software Inc., Durham, NC) or OLIGO primer analysis software version 4.0. PCRs were conducted in a Bio Rad iCycler, (Hercules, CA). The PCR primers for the amplifications are designed using Clone Manager[®] software version 4.1 (Scientific and Educational Software Inc., Durham, NC) OLIGO primer analysis software version 4.0. This software enables the design of PCR primers and identifies RE sites that are compatible with the specific DNA fragments being manipulated. PCRs were conducted in a Bio Rad iCycler, (Hercules, CA) and primer annealing, elongation and denaturation times in the PCRs were set according to standard procedures (Ausubel *et al.*, supra). The RE digestions and the PCRs were subsequently analyzed by agarose gel electrophoresis using standard procedures (Ausubel *et al.*, supra); (Sambrook, *supra*). A positive clone was defined as one that displays the appropriate RE pattern and/or PCR pattern. Plasmids identified through this procedure can be further evaluated using standard DNA sequencing procedures, as described above.

Escherichia coli strains Top10 and DH5 α were purchased from Invitrogen (Carlsbad, CA) and strain SCS110 was purchased from Stratagene (La Jolla, CA) These served as hosts of the recombinant plasmids described in the examples below. Recombinant plasmids were introduced into *E. coli* by electroporation using a Gene Pulser (BioRad Laboratories, Hercules, CA) set at 200 Ω , 25 μ F and 1.8 kV or chemical transformation, as described (Ausubel *et al.*, supra).

Bacterial strains were grown on tryptic soy agar (Difco, Detroit, MI) or in tryptic soy broth (Difco, Detroit, MI), unless otherwise stated, at an appropriate temperature. Media were supplemented with 100 μ g/ml ampicillin, 50 μ g/ml kanamycin, and/or chloramphenicol 20 μ g/ml (Sigma, St. Louis, MO) as needed. Bacterial strains were stored at -80°C suspended in tryptic soy broth (Difco) containing 30% (v/v) glycerol (Sigma, St Louis, MO) at *ca.* 10⁹ colony-forming units (herein referred to as “cfu”) per ml.

Reagent list

KpnI (New England Biolabs, Beverly, MA, Cat. Nos. R0142S), *PstI* (New England Biolabs, Beverly, MA, Cat. No. R0140S), Tryptic Soy broth (Difco, Detroit, MI, Cat. No. 211822), Tryptic Soy agar (Difco, Detroit, MI, Cat. No. 236920), Miniprep[®] plasmid DNA purification kit (Qiagen, Valencia, CA, Cat. No. 27106), glycerol (Sigma, St. Louis, MO, Cat. No. G5516), *HpaI* (New England Biolabs, Beverly, MA, Cat. No. R0105S), Calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA, Cat. No. M0290S), Vent_R[®] DNA polymerase (New England Biolabs, Cat. No. M0254S), QIAquick PCR purification kit (Qiagen, Cat. No. 28106, Valencia, CA), diaminopimelic acid (Sigma-Aldrich, St. Louis, MO, Cat. No. D1377), *BglIII* (New England Biolabs, Beverly, MA, Cat. No. R0144S), IPTG (Invitrogen, Carlsbad, CA, Cat. No. 15529-019), Cell culture lysis reagent (Promega, Madison, WI, Cat. No. E1531), lysozyme (Sigma, St. Louis, MO, Cat. No. L6876), potassium phosphate (Sigma, St. Louis, MO, Cat. No. P5379), magnesium chloride (Sigma, St. Louis, MO, Cat. No. M1028) *DraIII* (New England Biolabs, Beverly, MA, Cat. No. R0510S), *PsiI* (New England Biolabs, Beverly, MA, Cat. No. V0279S), Proteinase K (Ambion, Austin, TX, Cat. No. 2542-2548), Durascribe T7 transcription kit (Epicentre, Madison, WI), Durascribe SP6 transcription kit (Epicentre, Madison, WI), MEGAscript[®] T7 transcription kit (Ambion, Austin, TX, Cat. No. 1334), MEGAscript[®] SP6 transcription kit (Ambion, Austin, TX, Cat. No. 1330), MEGAclear columns (Ambion, Austin, TX, Cat. No. 1908), BrightStar biotinylated RNA millennium marker (Ambion, Austin, TX, Cat. No. 7170), BrightStar nylon membrane (Ambion, Austin, TX, Cat. No. 10102), BrightStar Biodetect kit (Ambion, Austin, TX, Cat. No. 1930), Tris-HCl buffer (Quality Biological, Gaithersburg, MD, Cat. No. 351-007-100), magnesium chloride (Sigma-Aldrich, St. Louis, MO, Cat. No. M1787), ammonium acetate (Sigma-Aldrich, St. Louis, MO, Cat. No. A2706), sodium chloride (Sigma-Aldrich, St. Louis, MO, Cat. No. S7653), potassium chloride (Sigma-Aldrich, St. Louis, MO, Cat. No. P3911), dithiothreitol (Sigma-Aldrich, St. Louis, MO, Cat. No. D9779), EDTA (Sigma-Aldrich, St. Louis, MO, Cat. No. E8008), polyethylene glycol 4000 (Fluka, Buchs, Switzerland, Cat. No. 95904), SUPERase RNase inhibitor (Ambion, Austin, TX, cat. No 2694), biotin-14-CTP (Invitrogen, Carlsbad, CA, Cat. No. 19519-016), RNase ONE ribonuclease (Promega, Madison, WI, Cat. No. M4261).

Example 2: Construction of rdsRNA segments that complement an *asd* mutation and express fluorescent reporters and *Mycobacterium tuberculosis* antigens and LCMV antigens

The goal of the study was to develop recombinant segments that can be incorporated into a prototype rdsRN based on the dsRNA genome of phi-8 (Mindich *et al.*, J. Bacteriol, 181: 4505; 1999); (Mindich, Microbiol. Mol. Biol. Rev, 63: 149; 1999); (Hoogstraten *et al.*, Virology, 272: 218; 2000); (Sun *et al.*, Virology, 308: 354; 2003). As discussed above, the phi-8 genome consists of three segments: S, M, and L. A prototype rdsRN was constructed such that the RNA-dependent RNA polymerase encoded by wild-type segment-L (herein referred to as “wtL”) expresses passenger genes cloned into recombinant segments- M and -S (herein referred to as “rM” and “rS”, respectively). Both rM and rS encode a wild-type aspartic semialdehyde dehydrogenase gene (herein referred to as “*asd*,” GenBank # V00262) linked to the bacterial ribosomal binding site of gene 10 and gene 8, respectively (see Figure 4) and a gene of interest (i.e. the fluorescent protein HcRed and the mycobacterial antigen TBS) functionally linked to the IRES of hepatitis C virus. Notice that no phage structural genes on segments- M and -S were initially incorporated into rM or rS although the *asd* allele of rS was replaced with gene 8 of the wild type segment-S in a later incarnation of the invention to stabilize the rdsRN's. The *asd* allele and the IRES::HcRed and Mtb antigen encoding sequences are flanked by the 5-prime and 3-prime untranslated sequences that encode the *pac* and the negative-strand RNA synthesis initiation sequences, respectively. In other words, the reported genes on rM are flanked by the 5-prime *pac* sequence of segment-M and the 3-prime terminal sequence of segment-M. Similarly, rS consists of the reported genes flanked by the 5-prime *pac* sequence of segment-S and the 3-prime terminal sequence of segment-S genes (Figure 5). Note that this configuration only permits the production of rdsRNs and that neither phage nor rdsRP particles are formed.

Construction of recombinant segments was accomplished using synthetic DNA and standard recombinant DNA techniques, such as PCR, RT-PCR, site-directed mutagenesis, restriction enzyme digests, gel electrophoresis, ligation, dideoxynucleotide sequencing, and bacterial transformation, as described in Example 1.

Recombinant segment-S (rS) was synthesized by Midland Certified Reagent Co., (Midland, TX). The 5- and 3-prime sequences were derived from a cDNA copy of phi-8 segment-S (kindly provided by Dr. Leonard Mindich, GenBank accession no. AF226853) with the modifications

described below. In 5-prime to 3-prime orientation, rS (SEQ ID NO: 1) consists of the following fused components (Figure 4):

- (i) *S pac* sequence and ribosomal binding site (herein referred to as “RBS”) of gene 8 is the first 187 nucleotides of segment-S (GenBank accession no. AF226853) and is required for uptake by procapsid (Hoogstraten *et al.*, supra,; 2000). The RBS is required for initiation of translation in prokaryotic cells.
- (ii) *asd* gene functionally linked to the RBS above for positive selection in Δasd *E. coli* strain X6212. The *asd* sequence was obtained from bases 240-1343 of GenBank accession no. V00262.
- (iii) The hepatitis C virus internal ribosomal entry site for initiation of translation in eukaryotic cells. The sequence spans bases 36-341 of GenBank accession no. AJ242651.
- (iv) Multiple cloning site (MCS) for insertion of passenger gene.
- (v) Semliki Forest Virus 3' untranslated region for polyadenylation of rS mRNA; nucleotides 1-261 of GenBank accession no. V01398.
- (vi) The phi-8 segment-S 3-prime terminal sequence, i.e. bases 3081-3192 of segment-S (GenBank accession no. AF226853), which is required for RNA stability and for phi-8 polymerase binding prior to initiation of negative strand RNA synthesis.

A synthetic DNA fragment comprised of the above components (SEQ ID NO:1) was joined to *Pst*I-digested pT7/T3-18 DNA (Cat. No. 7201, Ambion, Austin, TX) using the T4 DNA ligase as described in Example 1. Following ligation, the DNA was introduced into *E. coli* strain DH5 α -E (Invitrogen, Carlsbad, CA, Cat. No. 11319-019) using electroporation (Example 1), and transformants were isolated by culturing on TSA supplemented with ampicillin (100 μ g/ml) at 37°C for 16-24 hr. Successful ligation products were identified by isolating super-coiled DNA from 2 ml cultures that were inoculated by stabbing the resulting single colonies with a sterile toothpick and placing the toothpick into sterile TSB supplemented with ampicillin (100 μ g/ml); the cultures were incubated with agitation (200 rpm) at 37°C for 16-24 hr. After centrifugation the liquid supernatant was discarded and the bacterial pellets were resuspended in 100 μ l of solution P1 of the Miniprep[®] plasmid DNA purification kit (Qiagen, Valencia, CA, Cat. No. 27106). Plasmid DNA was then extracted and purified by following the instructions of the manufacturer (See Qiagen, Valencia, CA, Cat. no. 27106 instruction manual). The purified plasmid DNA was digested with the restriction

endonuclease *PstI* (New England Biolabs, Beverly, MA, Cat no. R0140S) according to the manufacturer's instructions and using buffers provided by the manufacturer and incubated at 37°C for 1 hr. The resulting DNA fragments were fractionated by agarose gel electrophoresis as described (See Example 1) and plasmids displaying the appropriate pattern were further characterized by dideoxynucleotide sequencing (Example 1). This procedure identified four independent isolates that carried plasmid pT7/T3-18 carrying DNA encoding rS. The plasmids in these isolates was designated AF1 and the four isolates harboring this plasmid were streaked onto TSA supplemented with ampicillin (100 µg/ml) and incubated at 37°C for 16-24 hr. The bacteria were subsequently harvested using a sterile cotton wool swab (Puritan, Guilford, ME, Cat. No. 25-8061WC) and suspended in TSB containing 30% (v/v) glycerol (Sigma, St. Louis, MO, Cat. No. G5516) at a density of about 10⁹ cfu/ml and stored in 1 ml aliquots at -80°C.

A second rS (rS2) was constructed by PCR amplification of bp 1-1294 of the phi-8 wt segment-S sequence (GenBank accession no. AF226853) linked by a *BglII* site by to the Hepatitis C IRES and downstream sequence of rS (bp1301-2020) as described above (rS2, SEQ ID NO:3). This construct was ligated into the *PstI* site of pT7T3-18 and transformed into *E. coli* Top10. Transformants were analyzed as described above and six correct isolates were designated pAF1S2.

Recombinant segment-M (rM, SEQ ID NO:2) is similar to rS, except that the 5-prime *pac* and 3-prime terminal sequences were derived from wt segment-M, nucleotides 1-262 and 4677-4741, respectively, of GenBank accession no. AF226852. Thus, like rS, rM consists of exogenous sequences flanked by phi-8 5-prime *pac* and 3-prime terminal sequence (see Figure 4). The 2060 bp rM (SEQ ID NO: 2) was cloned into the *KpnI* and *PstI* sites (New England Biolabs, Beverly, MA, Cat. Nos. R0142S and R0140S, respectively) of the plasmid pcDNA3.1_{zeo}(+) (Invitrogen, Cat. No. V860-20, Carlsbad, CA). Recombinant plasmids harboring the appropriate inserts were identified using the procedure employed for rS and the novel plasmid was designated pAF19.

To construct a eukaryotic expression cassette, pAF1 was digested with *HpaI* and *NotI* (New England Biolabs, Beverly, MA, Cat. No. R0105S). This RE digest resulted in a directional cloning site within the MCS such that, once ligated, the Hc-Red gene and mycobacterial antigen package are immediately downstream of, and functionally linked to, the HCV IRES. Following digestion, the ends of the linearized plasmid were dephosphorylated with Calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA, Cat. No. M0290S) to prevent recircularization. The

dephosphorylated plasmid was then purified by electrophoresis in a 0.8% agarose gel followed by gel extraction.

The 2.0 kb mycobacterial antigen fusion sequence (TBS) was PCR amplified from the plasmid pAdApt35.Bsu.TB.S (Crucell) using Accuprime DNA polymerase (Invitrogen, Carlsbad, CA) and primers including *HpaI* and *NotI* RE sites. The size of the amplified sequence was verified by agarose gel electrophoresis, and was purified using a QIAquick PCR purification kit by following manufacturer's instructions (Qiagen, Cat. No. 28106, Valencia, CA).

The fragment encoding TB.S was ligated into dephosphorylated pAF1 using T4 DNA ligase (New England Biolabs, Cat. No. M0202S) and the resulting plasmid was designated pSTB2.

The TBS antigen fusion sequence and HC-Red coding sequence were similarly inserted into the *HpaI* site in rM carried on pAF19 (Figure 4) using recombinant DNA procedures as above. The resulting plasmids were designated pMTB7 and pMHc-Red.

Plasmids pSTB2 and pLM2775 (encoding the wild-type segment-S) were linearized with RE's *SphI* and *PsiI* and the resultant linearized fragments were purified by agarose gel electrophoresis and extraction. Plasmids pMTB7 and pMHc-Red were linearized similarly with *SphI* and *PsiI*. The linearized DNA sequences from each of the four RE digests were used as templates in Durascribe T7 (Epicentre, Madison, WI) *in vitro* transcription reactions according to the manufacturer's instructions to produce fluorinated RNA transcripts of wtS, rS encoding antigens TBS, rM encoding encoding antigens TBS, and rM encoding Hc-Red.

A third set of rdsRNA segments were similarly constructed to express the glycoprotein antigens GP-1 and GP-2 of lymphocytic choriomenengitis virus (herein "LCMV"). A 1511 bp fragment was PCR-amplified from plasmid pCMV-GP encoding the GP polyprotein precursor located on the large chromosome of the LCMV genome. The sequence was amplified using Accuprime DNA polymerase (Invitrogen, Carlsbad, CA) and primers including *HpaI* and *NotI* RE sites. The size of the amplified sequence was verified by agarose gel electrophoresis, and was purified using a QIAquick PCR purification kit by following manufacturer's instructions (Qiagen, Cat. No. 28106, Valencia, CA). Plasmids pAF1S2 and pAF19 were linearized with RE's *HpaI* and *NotI* (New England Biolabs, Beverly, MA, Cat. No. R0105S) and dephosphorylated using calf intestinal phosphatase (New England Biolabs, Beverly, MA, Cat. No. M0290S). The GP encoding sequence was similarly digested with *HpaI* and *NotI* and ligated into the linearized pAF1S2 and

AF19 plasmids resulting in plasmids pSGP1 and pMGP2. These plasmids thus encode the GP polyprotein precursor gene functionally linked to the HCV IRES of rS2 and rM, respectively.

pSGP1 and pMGP2 were digested with RE's *KpnI* and *PsiI*, respectively, to serve as *in vitro* transcription templates. Fluorinated RNA transcripts were generated from each plasmid using the Durascribe T7 (Epicentre, Madison, WI) *in vitro* transcription kit according to the manufacturers instructions. The resultant transcripts thus encoded the GP-1 and GP-2 antigen sequences in both rS2 and rM.

Example 3: Construction of a prototype packaging and delivery strain

The objective of this study was to create a prototype bacterial packaging strain. *Shigella flexneri* 15D possesses a non-reverting chromosomal *asd* marker insertion deletion mutation resulting in a defect in the production of aspartate semialdehyde dehydrogenase (herein referred to as "ASD") and hence the lacks the ability to synthesize the cell wall component diaminopimelic acid (herein referred to as "DAP") (Sizemore *et al*, Vaccine. 1997 Jun;15(8):804-7). Growth, in the absence of genetic complementation, requires the supplementation of culture media with 50 µg/ml DAP (Sigma-Aldrich, St. Louis, MO, Cat. No. D1377).

While *Shigella* was chosen only as an example, its invasive characteristics and natural tropism for mucosal immune cells also make it an ideal delivery vector. As the *asd* mutation is to be complemented by an rdsRN encoded *asd* allele, it was also necessary to create a second chromosomal lesion to attenuate the strain so as to cause it to lyse after entry into a mammalian cell and release the rdsRN's. Approximately 1 kb regions upstream and downstream of the *murI* gene (encoding glutamate racemase) were amplified by PCR, joined by ligation of PCR primer encoded *NheI* sites and ligated into pCVD442 (ref X) at primer encoded *SstI* and *XbaI* sites. The resulting plasmid was transferred by conjugation to *S. flexneri* 15D. Cointegrates were identified by antibiotic resistance, sucrose sensitivity, and PCR analysis, and resolved by means well known to those skilled in the art to produce the *asd*, *murI* strain MPC51. The *murI* mutation renders the cell unable to synthesize the peptidoglycan component D-glutamate and requires supplementation of M9 minimal growth media with 50 µg/ml D-glutamate in order to attain normal growth. Further, HeLa cell invasion assays revealed the strain to be invasive but incapable of prolonged intracellular survival (Figure 7).

To determine if this auxotrophic requirement can be complemented *in trans* through expression of *asd* encoded in an rdsRN, *S. flexneri* MPC51 was transformed with pLM2653, a

plasmid that expresses wtL mRNA under the control of SP6 promoter and produces the phi-8 proteins necessary and sufficient to assemble a procapsid (Sun *et al.*, Virology, 308: 354; 2003). Plasmid pLM2653 was introduced by electroporation and selected for by addition of 100 µg/ml ampicillin.

Procapsid assembly in MPC51pLM2653 was assessed by differential filtration of native and SDS-denatured cell lysates, which were analyzed by immunoblotting with antisera specific for procapsid proteins (Figure 8). Briefly, as the expected MW of each procapsid is 15 MDa, it was shown that procapsid proteins (87 kDa and 34 Da,) failed to pass through a 100 kDa cutoff membrane unless the lysate was treated with 10% SDS, indicating assembly (Figure 8). Furthermore, transmission electronmicrographs of thin-sectioned MPC51pLM2653 clearly revealed large numbers of approximately 60 nM procapsid particles (Figure 9).

Example 4: Introduction of ssRNA encoding rdsRNA segments into a prototype packaging strain and launching functional self-replicating rdsRN's in said strain

The goal of the studies in this example was to develop an approach to launching and maintaining rdsRN's in a bacterial packaging strain. The strategy selected involves transforming a packaging strain, *S. flexneri* MPC51pLM2653 (Example 3) with *in vitro* synthesized ssRNA (+) encoding rM and rS constructs described in Example 2. Following entry into MPC51pLM2653, the ssRNA(+) is packaged into the procapsid and negative-strand synthesis is completed, thereby creating the rdsRN's (Figure 6). The rdsRN then synthesizes mRNA encoding wtL, rM, and rS mRNA (i.e. ssRNA(+)) that is passively secreted from the rdsRN into the cytoplasm of the carrier strain(Figure 6). These transcripts produce more procapsids via expression of wtL, rM and rS, which both carry a functional *asd* gene, complement the *asd* mutation in MPC51, thereby eliminating the DAP requirement for growth. The wtL, rM, and rS mRNA are also packaged into procapsids, thus forming additional rdsRN's.

Electrocompetent cells of MPC51pLM2653 were prepared using standard techniques (Ausubel *et al.*, supra). Briefly, single clones were grown at 37°C in M9 media supplemented with Ampicillin (100 µg/ml), Kanamycin (50 µg/ml) and 50 µg/ml DAP (Sigma-Aldrich, St. Louis, MO, Cat. No. D1377). Fifty ml cultures were started at OD₆₀₀ <0.1 and cells were harvested during exponential growth, between OD₆₀₀ 0.3 and 0.6. Cells were washed once with ice cold 5 mM EDTA, 10% glycerol (v/v) and then washed three times with ice-cold 10% (v/v) glycerol, each

wash was followed by centrifugation at 4000 x g. After the final wash and spin, the cells were resuspended in 10% (v/v) glycerol, dispensed into 200 µl aliquots, and stored at -80°C.

The ssRNA(+) of rS and rM employed in electroporating MPC51 pLM2653 were synthesized *in vitro* using linearized pSTB2 and pMTB7 as DNA templates, respectively, as described in Example 2. MPC51 pLM2653 cells were electroporated with 2 µg ssRNA(+) (1.0 µg rS + 1.0 µg rM). Electroporation was conducted using a Gene-Pulser set at 200Ω, 25 mcF and 1.8 kV (BioRad, Hercules, CA). The cells were allowed to recover for 2 hr at 28°C in SOC medium (cat #15544-034, Invitrogen, Carlsbad, CA) supplemented with 50 µg/ml DAP and no antibiotic. Subsequently, the cells were spread on M9 agar with the appropriate antibiotics, supplemented with 50 µg/ml D-glutamate and 0.1 µg/ml DAP, which is below the minimum concentration of DAP required to support growth of MPC51 pLM2653. The cells were allowed to grow at 25-27°C, following which they were transferred to M9 Ap/Kn/D-glutamate and DAP was supplemented to 0.01 µg/ml or withdrawn. The cells were cultured at 22°C-25°C, the resulting colonies were ampicillin resistant due to pLM2653 and were DAP-independent, due to expression of the *asd* genes on rS and rM.

In order to improve growth characteristics, MPC51pLM2653 was electroporated with *in vitro* transcribed ssRNA from pMTB7 and the wild-type segment-S encoding plasmid pLM2775 as a source of gene 8. This procedure and subsequent growth steps were performed as described above and resulted in a DAP-independent MPC51 strain bearing an rdsRN designated LSMtb4 (Figure 10)

Similarly, MPC51pLM2653 was electroporated with *in vitro* transcribed ssRNA from the wild-type segment-S encoding plasmid pLM2775 as a source of gene 8 and from pMHc-Red, which encodes the Hc-Red protein functionally linked to the IRES of rM. This procedure and subsequent growth steps were performed as described above and resulted in a DAP-independent MPC51 strain bearing an rdsRP designated LSMHc-Red.

In a third example, ssRNA *in vitro* transcribed from the rS2 (includes gene-8) construct pSGP1 encoding the LCMV GP antigen was employed to alleviate the need for gene-8 sequence from the wtS. *S. flexneri* MPC51pLM2653 was electroporated with *in vitro* transcribed RNA from pSGP1 and pMGP2 as in the above described examples. Subsequent growth steps were carried out as described above, resulting in a DAP-independent MPC51 strain bearing an rdsRN designated LSgpMgp. The presence of the rdsRN was confirmed by immunoblotting of whole cell lysates with nucleocapsid-specific antisera and RT-PCR with primers specific for both (+) and (-) strands of rS2 and rM.

It is noteworthy that electroporation of MPC51 with any combination of rS and rM *in vitro* transcribed ssRNA's resulted in no transformants unless accompanied by wtL ssRNA or a plasmid encoding wtL, or the target strain was previously transformed with a plasmid encoding wtL. Furthermore, treatment of *in vitro* transcribed ssRNA's with RNase A prior to electroporation of MPC51pLM2653 results in the recovery of no transformants. As an additional example demonstrating the efficiency and efficacy of RNA electroporation as a means of creating rdsRN's, *in vitro* transcripts of all three wild-type segments were electroporated into *Pseudomonas syringae* (the natural host of phi-8) by methods identical to those described above. This resulted in the recreation of wild-type lytic bacteriophage phi-8 as evidenced by plaque formation in bacterial lawns derived from the electroporation and transmission electron microscopy visualization of wild-type phage particles within *P. syringae* cells derived from the electroporation (Figure 12).

DAP-independence appears to result from amplification of the *asd*⁺ genes on rS and/or rM RNA encoded by the respective rdsRN. Indeed, RT-PCR analysis of total RNA from strains carrying the above described rdsRN's using primers specific for the amplification of either minus or plus strand RNA results in recovery of cDNA's of rS and rM. Recall that minus strand synthesis in the phage occurs only after the uptake of all three genomic segments. *S. flexneri* MPC51 carrying rdsRN's LSMtb4 and LSMHc-Red have remained DAP-independent for over 3 months in continuous culture at the time of this submission. Finally, these constructs continue to produce capsid proteins detectable by immunoblotting and assembled nucleocapsids visible by transmission electron microscopy (Figure 11).

In sum, therefore, these results demonstrate that the complementation of the *asd* deletion in MPC51pLM2653 is the result of RNA uptake and the packaging and self-replicating function of a rdsNC within the packaging strain. These findings further demonstrate that these procedures produce rdsRN's that are maintained in the resulting strains.

Example 5: Expression of rdsRN encoded sequences in a mammalian cell

While the scope of the invention does not limit delivery of rdsRN's to mammalian tissue or organisms by the use of a bacterial packaging strain, it was decided for purposes of illustration to utilize the attenuated invasive bacterium *Shigella flexneri* MPC51 for this purpose (Examples 3 and 4) as it is naturally invasive in many tissue culture cell lines and animal models. The strain was engineered as described in Example 3 to cause lysis of the bacterial cells after invasion of

eukaryotic cells and escape of the endocytic vesicle in order to release the rdsRN's into the eukaryotic cell cytoplasm.

As a first line of evidence, MPC51 bearing rdsRN LSMtb4 was chosen. This rdsRN construct encodes a *M. tuberculosis* antigen package designated TBS. The precise composition of this package is unimportant to the invention described herein, however for explanatory purposes a component of this antigen package is a sequence encoding the protein antigen 85A. As described in Example 2, the antigen package was ligated to the HCV IRES sequence and is under the translational control of this sequence. The absence of expression of the TBS construct within the bacterial strain carrying this rdsRN (MPC51+LSMtb4) was confirmed by immunoblot of whole cell lysates probed for antigen 85A with a polyclonal antisera (Abcam, Cambridge, UK).

HeLa cells (Invitrogen, Carlsbad, CA) were grown in DMEM + 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA) + 1% Antibiotic/Antimycotic solution (Invitrogen, Carlsbad, CA) to at least 60% confluency on cover slips in 6 well tissue culture flasks at 37°C, 5% CO₂. Concurrently, *S. flexneri* MPC51+LSMtb4 was grown in 50 ml M9 at 28°C with shaking to an OD₆₀₀ of at least 0.6. Twenty-four hours prior to invasion assays, media was removed from HeLa cells and replaced with DMEM + 10% FBS. Two hours prior to invasion HeLa cell media was replaced with DMEM and bacterial cultures were diluted and allowed to grow statically at 37°C. The O₂ concentration in the tissue culture incubator was then reduced to 1% with N₂, and a PBS washed suspension of MPC51+LSMtb4 in DMEM was added to HeLa cultures at a multiplicity of infection (MOI) of 100 and allowed to incubate for 1 hour. Simultaneously, bacterial suspensions of *S. flexneri* 15D (*asd*) and MPC51pLM2653 similarly prepared were added to wells of HeLa cells in the same manner.

After a one hour invasion incubation, HeLa cells were washed twice with PBS, and culture media (DMEM+FBS) was replaced and supplemented with 150 µg/ml gentamicin sulfate (Sigma, St. Louis, MO) for one hour to kill any remaining bacterial cells that had not invaded and hence were not protected by HeLa cells. Gentamicin is broadly antibacterial at this concentration, but does not cross eukaryotic cell membranes. After 1 hour, culture media was replaced with fresh DMEM+FBS and tissue culture cells were left to incubate at 37°C, 5% CO₂ for 14 hours.

Cover slips with HeLa cells were then removed, fixed with 2% paraformaldehyde in PBS (pH 7.4), permeabilized with .1% Triton X-100 in PBS (pH 7.4), blocked for 2 hours with 3% BSA, 5% Normal Goat Serum, 0.05% Sodium Azide in PBS (pH 7.4), and probed with antigen85A

specific antisera (IgY) (Abcam, Cambridge, MA) at a dilution of 1:100 in 1% BSA, 3% NGS, 0.05% Sodium Azide in PBS (pH7.4). Cells were then counter-probed with a FITC-conjugated rabbit anti-IgY (Abcam, Cambridge, MA) at a dilution of 1:100 in 1% BSA, 3% NGS, 0.05% Sodium Azide in PBS (pH7.4) and examined by fluorescence microscopy. Control HeLa cell groups which were exposed to either no bacteria, *S. flexneri* 15D, or MPC51pLM2653 exhibited no fluorescence indicative of antigen 85A expression. HeLa cells exposed to invasive MPC51+LSMt4 fluoresced brightly indicative of antigen 85A expression in the cell as part of the TBS antigen fusion protein (Figure 13). It is important to note again that antigen85A expression was not detectable in the *Shigella* packaging/carrier strain and no live *Shigella* were recovered after the 14 hour incubation. This is clear evidence of mammalian translation of rdsRN produced recombinant segment mRNA.

In a manner similar to that described above, HeLa cell invasions were performed with MPC51+LSMHc-Red, which has the sequence of the directly fluorescent Hc-Red gene encoded on it's rM segments. Twelve hours after the invasion exposure, HeLa cell cover slips were fixed and observed by fluorescence microscopy. *S. flexneri* 15D and MPC51pLM2653 control groups exhibited no Hc-Red fluorescence; while MPC51+LSMHc-Red exposed HeLa cells were directly fluorescent (Figure 14). As further proof that this fluorescence was due to Hc-Red expression, HeLa cells were fixed and probed with an antibody specific for Hc-Red and counterprobed with a secondary antibody conjugate. Again, only cells exposed to MPC51+LSMHc-Red were fluorescent (Figure 15). Further, Hc-Red expression could not be detected by immunoblot or direct fluorescent microscopic analysis of MPC51+LSMHc-Red alone. These combined results provide evidence by direct observation of fluorescence and immuno-detection of proteins that rdsRN encoded RNA has been translated into protein in a mammalian cell. Finally, as no or only few live *Shigella* can be recovered at this time point, naked RNA has a finite half-life, and only (+) strand or mRNA can be translated into encoded proteins, it seems clear that the source of the translated message is mRNA produced by the rdsRN's after lysis of the packaging/delivery strain inside the HeLa cell.

Example 6. Exemplary sequences for recombinant segment-S and recombinant segment-S2

In reference to Figure 4, the following are exemplary sequences that have been used in the practice of the invention for construction of recombinant segment-S (rS).

*Pst*I:

ctgcag

Bacteriophage phi-8 segment-S *pac* and ribosomal binding site of gene-8 187 (bases 1-187 of segment-S, GenBank accession no. AF226853):

gaaattttca aatcttttga ctatttcgct ggcatagctc ttggagtgga agccttcctt gaaaggcgcg aagggtcccca ccagctcggg
gtgattcgtg acatttcctg ggatctcgga gtcagctttg tctctaggag actgagcgtt cgggtctcagg tttaaactga gattgaggat
aaagaca (SEQ ID NO: 1) → connect to the *asd* gene

E. coli asd (bases 240-1343 of GenBank accession no. V00262):

atgaaaaatgt tggttttatc ggctggcgcg gtatggcggg ctccgttctc atgcaacgca tggttgaaga ggcgcacttc gacgccattc
gccctgtctt ctttttact tctcagcttg gccaggtgc gccgtctttt ggcggaacca ctggcacact tcaggatgcc ttgatctgg
aggcgctaaa ggccctcgat atcattgtga cctgtcaggg cggcgattat accaacgaaa tctatccaaa gtttcgtgaa agcggatggc
aaggttactg gattgacgca gcatcgtctc tgcgcatgaa agatgacgcc atcatcattc ttgaccccggt caatcaggac gtcattaccg
acggattaaa taatggcatc aggacttttg ttggcggtaa ctgtaccgta agcctgatgt tgatgtcgtt ggggtggtta ttgccaatg
atcttgttga ttgggtgtcc gttgcaacct accaggccgc ttccggcggg ggtgcgcgac atatgcgtga gttattaacc cagatgggccc
atctgtatgg ccatgtggca gatgaactcg cgaccccgtc ctctgtattt ctgatatcg aacgcaaagt cacaacctta acccgtagcg
gtgagctgcc ggtggataac ttggcggtgc cgctggcggg tagcctgatt ccgtggatcg acaaacagct cgataacggt
cagagccgcg aagagtggaa agggcaggcg gaaaccaaca agatctcaa cacatcttc gtaattccgg tagatggtt
atgtgtcgtg gtcggggcat tgcgctgcca cagccaggca ttactatta aattgaaaaa agatgtgtct attccgaccg tggaagaact
gctggctgcg cacaatccgt gggcgaaagt cgttccgaac gatcgggaaa tcaactatgcg tgagctaacc ccagctgccg
ttaccggcac gctgaccagc ccggtaggcc gcctgcgtaa gctgaatatg ggaccagagt tctgtcagc cttaccgtg
ggcgaccagc tgctgtgggg ggccgaggag ccgtgcgtc ggatgcttcg tcaactggcg taa (SEQ ID NO: 2)

→ connect to the *IRES*

Hepatitis C virus-IRES (bases 36-341 of GenBank accession no. AJ242651):

atactcccc tgtgaggaa tactgtcttc acgcagaaag cgcctagcca tggcgtagtatgagtgctg tgcagcctcc aggaccccc
ctccggggag agccatagtg gtctgcggaaccggtgagta caccggaatt gccaggacga ccgggtcctt tcttgatca
accgctcaatgcctggaga ttgggcgtg cccccgccag actgctagcc gagtagtgtt gggtcgcgaaaggccttggtg gtactgcctg
atagggtgct tgcgagtgc ccgggaggtc tcgtagaccgtgcacatg (SEQ ID NO: 3) → connect to multiple
cloning site

Multiple cloning site and termination codons:

atg gtt aac gcg gcc gct taa tta ata aat aaa taa (SEQ ID NO: 4)

→ *connect to SFV-3' untranslated*

Semliki forest virus-3-prime untranslated region (bases 1-262 of GenBank accession no. V01398):

gttagggta ggcaatggca ttgatatagc aagaaaattg aaaacagaaa aagttagggt aagcaatggc atataacat aactgtataa
cttgtaacaa agcgcaacaa gacctgcgca attggccccg tggccgcct cacggaaact cggggcaact catattgaca cattaattgg
caataattgg aagcttacat aagcttaatt cgacgaataa ttggattttt atttatttt gcaattgggt ttaatatatt cc (SEQ ID NO: 5)

→ *connect to ϕ 8 segment-S 3' RNA pol binding site*

Phi-8 segment-S 3-prime polymerase binding site (bases 3081-3192 of GenBank accession no. AF226853):

gcttagcggc aatcgaaccc tccg xcataagg aggtttagca aatccgcggc tcttatgagc tgtccgaaag gacaacccga
aagggggagc gaggacttcg gtcctccgct cc (SEQ ID NO: 6)

PstI:

ctgcag

In reference to Figure 4, the following are exemplary sequences that have been used in the practice of the invention for construction of recombinant segment-S2 encoding gene 8 of the wild-type phage phi-8 (rS2).

PstI:

ctgcag

Bacteriophage phi-8 segment-S *pac* (bp 1-187 GenBank accession no. AF226853):

gaaattttcaaatcttttgactatttcgctggcatagctcttcggagtgaagccttcctgaaaggcgcgaagggtccccaccagctcggggtgattc
gtgacatttcctgggatctcggagtcagctttgtctctaggagactgagcgttcggtctcagggtttaactgagattgaggataaagaca (SEQ
ID NO:1)

Bacteriophage phi-8 gene 8 of segment-S (bp188-1291 GenBank accession no. AF226853):

atgggtagaatctttcaactgttgatgcgcttaggcgttaaacagggtgcagcaagtgttgtaaagccgggatc gatgctgtagcaagcgatt
gctccagcagatcatgtccaaagacgggtgctattcagctgtctaaggcactcggtttcaccgctgtggagcagatgtcgagtgaagtgtcgaag
cgtatctctatgagatcgttgagcatcttctgctcgtcgacgaggccacgttgccgatgcgcttatggcgtgtatcaccgatgcaggtgatatcgc
cattgagcgtctgcttccctccgtagaggatgtcgacaaaggcgaggcgcttgccgccacgctgactgtcgtcttggctctcttctc gatgaacaa
agaacaagctgaagagcttaaacgttcgatggcatcgaaaggcttgagtccggaccgggttacctcggaggacagaccctgttgaccgtcaa
gtccactgggtactggcctgacagagtatgacgctcaaggcaagaatggcgtccctcgcgggatgtctgtaacaagcgtactgcattgttctcgt
gctgtacacagtgatcagtaacttctggccgtatcactatggtagaggttaaagctggctcgcacgaggcgagctacctccagtgctga
tcgtgttgaattcggggccccgggtcctccgtaagtgcgatcgagcgtgagacacaacgcgcactgcaagaagaacagccgcgtgcattgcc
ttcgggcagccgcaccgcggaacgggttgctgggcccagcaggggtgatgtcccgtgtcacacctccgccaggctcattcaccttcaccg
gtgagggcgaccatgtcccgttgcacaaactcgtcgcgcagaacgacactgatggcgttggtgcggatcattgaactggatgcattccagat
gcaaggaaaaatattagtcgatggtagcatgactacttctggacgccgctcaacagcgcgtcgtgccgatatcggggatcgcggagtcag
taggtcgattcgtgctctgtagccagtatcatcaacgcgaaggagaagcgttcgtgatgc (SEQ ID NO:7)

BglIII:

agatct

→ connect to the IRES

Hepatitis C virus-IRES (bases 36-341 of GenBank accession no. AJ242651):

atcactcccc tgtgaggaac tactgtcttc acgcagaaag cgcctagcca tggcgttagtatgagtgtcg tgcagcctcc aggaccccc
ctcccgggag agccatagtg gtctgcggaaccgggtgagta caccggaatt gccaggacga ccgggtcctt tcttgatca
acccgctcaatgcctggaga ttgggcgtg cccccgccag actgctagcc gagtagtgtt gggtcgcgaaaggccttggtg gtactgcctg
ataggggtgct tgcgagtgcc ccgggaggtc tcgtagaccgtgcaccatg (SEQ ID NO: 3) → connect to multiple
cloning site

Multiple cloning site and termination codons:

atg gtt aac gcg gcc gct taa tta ata aat aaa taa (SEQ ID NO: 4)

→ connect to SFV-3' untranslated

Semliki forest virus-3-prime untranslated region (bases 1-262 of GenBank accession no. V01398):
 gttagggtta ggcaatggca ttgatatagc aagaaaattg aaaacagaaa aagttagggt aagcaatggc atataacat aactgtataa
 cttgtaaaa agcgcaaaa gacctgcgca attggccccg tggccgcct cacggaaact cggggcaact catattgaca cattaattgg
 caataattgg aagcttcat aagcttaatt cgacgaataa ttggattttt atttatttt gcaattggtt ttaatatatt cc (SEQ ID NO: 5)
 → connect to $\phi 8$ segment-S 3' RNA pol binding site

Phi-8 segment-S 3-prime polymerase binding site (bases 3081-3192 of GenBank accession no. AF226853):
 gcttagcggc aatcgaacc tccg xcataagg aggttagca aatccgcggc tcttatgagc tgtccgaaag gacaaccga
 aagggggagc gaggacttcg gtctccgct cc (SEQ ID NO: 6)

PstI:

ctgcag

Example 7. Exemplary sequences for Recombinant segment-M

With reference to Figure 4, the following are exemplary sequences that may be used in the practice of the invention for construction of Recombinant segment-M.

KpnI:

ggtacc → connect to segment M pac

Segment M pac sequence and ribosomal binding site of gene 10 (bases 1-262 of GenBank accession no. AF226852)

gaaattttcaaagctttcggcaataagggtggaaatttcaaagagggtcgagccgacgaacctctgtagaaccgggaagtcctgtcttacttg
 cgagagcaattgaactagggcagc
 accgggggtcgataagcgcagaagtgaggcgcggggattgaagcaaatcacctaagcgtaaacgacggacctcgagggtggcggagtcta
 cataggatcccctagctactagacagaaac
 cattctaacaaggagatgcac (SEQ ID NO: 8) → connect to *Bgl II*

BglIII:

agatct → connect to the *asd* gene

E. coli asd (bases 240-1343 of GenBank accession no. V00262):

atgaaaaatgt tggttttatc ggctggcgcg gtatggtcgg ctccgttctc atgcaacgca tggttgaaga gcgcgacttc gacgccattc
 gccctgtctt cttttctact tctcagcttg gccaggtgc gccgtctttt ggcggaacca ctggcacact tcaggatgcc ttgatctgg
 aggcgctaaa ggccctcgat atcattgtga cctgtcaggg cggcgattat accaacgaaa tctatccaaa gttcgtgaa agcggatggc
 aaggttactg gattgacgca gcatcgtctc tgcgcatgaa agatgacgcc atcatcattc ttgaccccgat caatcaggac gtcattaccg
 acggattaaa taatggcatc aggacttttg ttggcggtaa ctgtaccgta agcctgatgt tgatgtcgtt gggtggttta ttgccaatg
 atcttgttga ttgggtgtcc gttgcaacct accaggccgc ttccggcggt ggtgcgcgac atatgcgtga gttattaacc cagatgggcc
 atctgtatgg ccatgtggca gatgaactcg cgaccccgtc ctctgctatt ctcgatatcg aacgcaaagt cacaacctta acccgtagcg
 gtgagctgcc ggtggataac ttggcggtgc cgctggcggg tagcctgatt ccgtggatcg acaaacagct cgataacggt
 cagagcccgcg aagagtggaa agggcaggcg gaaaccaaca agatcctcaa cacatcttcc gtaattccgg tagatggttt
 atgtgtgctg gtcggggcat tgcgtgccca cagccaggca ttactatta aattgaaaaa agatgtgtct attccgaccg tggaagaact
 gctggctgcg cacaatccgt gggcgaaagt cgttcgaac gatcgggaaa tcaatgctg tgagctaacc ccagtgccg
 ttaccggcac gctgaccacg ccggtaggcc gcctgcgtaa gctgaatatg ggaccagagt tcctgtcagc ctttaccgtg
 ggcgaccagc tgctgtgggg ggccgcggag ccgtgcgtc ggatgcttcg tcaactggcg taa (SEQ ID NO: 9)

→ connect to the *Ascl*

Ascl:

ggcgcgcc → connect to *HCV-IRES*

Hepatitis C virus-IRES (bases 36-341 of GenBank accession no. AJ242651):

atcactcccc tgtgaggaa tactgtcttc acgcagaaag cgcctagcca tggcgtagt
 atgagtgtcg tgcagcctcc aggaccccc ctccggggag agccatagt gtctgcggaa
 ccggtgagta caccggaatt gccaggacga ccgggtcctt tcttgatca acccgctcaa
 tgcctggaga ttggggcgtg cccccgccag actgctagcc gagtagtgtt gggtcgcgaa
 aggccttggt gtagtgcctg atagggtgct tgcgagtgc ccgggaggtc tcttagaccg
 tgcacc (SEQ ID NO: 10) → connect to multiple cloning site

Multiple cloning site:

atg gtt aac gcg gcc gct taa tta ata aat aaa taa (SEQ ID NO: 11) → connect to *SFV 3-prime untranslated sequence*

Semliki forest virus-3-prime untranslated region (bases 1-262 of GenBank accession no. V01398):
 gttagggtta ggcaatggca ttgatatagc aagaaaattg aaaacagaaa aagttagggt aagcaatggc atataacat aactgtataa
 cttgtaacaa agcgcaacaa gacctgcgca attggccccc tggtccgcct cacggaaact cggggcaact catattgaca cattaattgg
 caataattgg aagcttacat aagcttaatt cgacgaataa ttggattttt atttattttt gcaattggtt ttaatatatt cc
 (SEQ ID NO: 12) → connect to *Phi-8 3-prime polymerase binding site*

Phi-8 segment M 3-prime Polymerase binding site (bases 4677-4741 of GenBank accession no. AF226852):

actgttgataaacaggaccggaagggttaacccgagaggggggagtgaggcttcggcctccacttc(SEQ ID NO: 13) → connect to *PstI*

PstI:

ctgcag

Example 8. Extraction and purification of rdsRNAs

To demonstrate that strain MPC51 LSMtb4 produces nucleocapsids, single clones were grown at 28°C in M9 medium supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml) and D-Glutamate (50 µg/ml). Cultures were started at OD₆₀₀ <0.1 and cells were harvested during exponential growth, between OD₆₀₀ 0.6 and 0.8. Cells were harvested by centrifugation at 8000 rpm for 5 min. To lyse cells, the bacteria were resuspended in 5 ml PBS and lysed at 20,000 psi in a French pressure cell (Thermo Electron). The lysates were subjected to centrifugation at 8,000 x g for 5 min and the supernatants were applied to 10-30% (w/v) sucrose gradients containing 10 mM potassium phosphate (pH 7.3; Sigma, St. Louis, MO, Cat. No. P5379) and 1 mM magnesium chloride (Sigma, St. Louis, MO, Cat. No. M1028). The gradients were placed in a JS24.15 rotor in an Avanti J-30i centrifuge (Beckman Coulter, Fullerton, CA, Cat. No.363118). After centrifugation at 23,000 rpm and 23°C for 90 min, the nucleocapsids formed a sharp band that was collected and stored separately at -80°C. The remaining contents of the tubes were fractionated in 1 ml aliquots and stored at -80°C. The presence of rdsRN's in these aliquots was verified by immunoblotting with capsid-specific antisera and by RT-PCR using primers designed to amplify (+) and (-) strand LSMtb4 RNA.

An improved rdsRN purification procedure has been designed as follows. A 500 ml culture of *S.flexneri* MPC51 bearing rdsRN LSMtb4 will be grown at 28°C to an OD600=0.8, and the cells pelleted. The rdsRN's will be purified using a multi-step filtration and centrifugation process. The cell pellet will first be lysed using an Invensys APV Microfluidizer (Lake Wills, WI) and clarified by centrifugation. The clarified supernatant will then be processed by tangential-flow filtration (TFF) using a Pellicon system (Millipore Inc., Billerica, MA) with a 0.45 µm pore size element (Millipore #P2HV MPC 05, or equivalent). Free nucleic acids will then be digested with Benzonase (for 30 minutes at 25°C).

A second filtration step will then be used to concentrate the rdsRN's and wash out medium components, digested nucleic acids, and the nucleases. Tangential flow filtration using a 100 kDa spiral wound ultrafiltration module (Millipore #CDUF 006 LH, or equivalent) will be used to concentrate the product and exchange the buffer into phosphate-buffered saline or other client-specified buffer of choice. Following tangential flow filtration, one-half of the partially purified rdsRN's will then be precipitated by the addition of NaCl and PEG, and then resuspended in a small volume of a phosphate buffer (Hoogstraten *et al.*, Virology, 272:218-224, 2000). Two small analytical scale gradient purifications will then be implemented using a portion (10 – 25%) of the PEG precipitated and resuspended material. Resuspended rdsRN's will be layered onto both preformed sucrose and opti-prep gradients and centrifuged overnight. The rdsRN band (identified by immunoblot and RT-PCR analysis) will be collected and the gradient material removed by dialysis against a buffer specified by the contracting laboratory. Purified material (both pre- and post-gradient) will then be processed to remove residual Endotoxin (if needed) using Q-ion exchange chromatography and/or Acticlean Etox™ (Sterogene, Carlsbad, CA). Final purified rdsRN's will be aliquoted and stored at 4°C.

Aliquots will be taken at each stage of the purification process and analyzed by immunoblot and RT-PCR.

Example 9. Immunogenicity of rdsRN's in mice

Given that this invention is based on the RNA-dependent RNA polymerase of a bacteriophage, it is pertinent to determine whether phi-8 polymerase is functional in eukaryotes. The ability of purified nucleocapsids to elicit an antibody response against the mycobacterial antigen package TBS encoded on rS and rM will be tested by vaccinating 6-8 week old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, Cat. No.000651) with purified nucleocapsids.

Five groups, each consisting of five mice, will be vaccinated as follows: 10 µg empty procapsid, 10 ng nucleocapsid, 100 ng nucleocapsid, 1 µg nucleocapsid, and 10 µg nucleocapsid. The mice will receive a priming vaccination on day 0 and receive two booster vaccinations on days 14 and 42. All vaccinations will be intramuscular by injecting nucleocapsids into the hind legs of each mouse.

To measure humoral responses to the TBS antigens, sera will be collected before and at 10-day intervals after each vaccination. About 100 µl of blood per mouse will be collected into individual tubes from each mouse-tail vein and allowed to clot by incubating for 4 hr on ice. After centrifugation in a microfuge for 5 min, the sera will be transferred to fresh tubes and stored at -20°C.

Solid phase ELISA will be utilized to quantitate IgG responses to the TBS antigens. Purified soluble *mycobacterial* antigens are suspended in PBS at a concentration of 2 µg/ml and is used to coat 96-well microtiter ELISA plates. The plates are incubated overnight at 4°C followed by four washes with 0.05% (v/v) TBS-Tween solution. The plates are then blocked at room temperature for 1 hr with blotto (5% (w/v) non-fat dried milk in TBS). Plates are then washed with TBS-Tween solution, as above. Sera are diluted in blotto and threefold serial dilutions, beginning at 1:30, are added in duplicates to the plates so that volume per well is 100 µl. Pre-immunization serum is included in each ELISA as a negative control. Plates are incubated for 2 hr at room temperature followed by four washes with TBS-Tween solution. For detection, the secondary antibody is alkaline phosphatase labeled affinity-purified goat anti-mouse IgG (heavy chain specific) (Accurate Chemical and Scientific Corporation, Westbury, NY, Cat. No. SBA103004). The secondary antibody is diluted 1:2000 in TBS, 2% (w/v) non-fat dry milk, and 5% (v/v) lamb serum, 100 µl of which is added to each well and incubated at room temperature for 1 hr. Color is developed by sequential 15 min incubations in 100 µl of substrate followed by 100 µl amplifier of Invitrogen's ELISA amplification system (Cat No. 19589-019). Absorbance is determined at 490 nm using a SpectraMax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). End-point titers are calculated by taking the inverse of the last serum dilution that produced an increase in absorbance at 490 nm that is greater than the mean of the negative control row plus three standard error values.

Cellular immunity may be measured by intracellular cytokine staining (also referred to as intracellular cytokine cytometry) or by ELISPOT (Letsch A. *et al.*, Methods 31:143-49; 2003). Both

methods allow the quantitation of antigen-specific immune responses, although ICS also adds the simultaneous capacity to phenotypically characterize antigen-specific CD4⁺ and CD8⁺ T-cells. Such assays can assess the numbers of antigen-specific T cells that secrete IL-2, IL-4, IL-5, IL-6, IL-10 and IFN- (Wu *et al.*, AIDS Res. Hum. Retrovir. 13: 1187; 1997). ELISPOT assays are conducted using commercially-available capture and detection mAbs (R&D Systems and Pharmingen), as described (Wu *et al.*, Infect. Immun. 63:4933; 1995) and used previously (Xu-Amano *et al.*, J. Exp. Med. 178:1309; 1993); (Okahashi *et al.*, Infect. Immun. 64: 1516; 1996). Each assay includes mitogen (Con A) and ovalbumin controls.

Example 10: Segment-L expression

As described in Examples 3 and 4 above, segment-wtL is introduced into the bacterial packaging strain as an extrachromosomally replicating plasmid. Clearly, a more stable method of expressing wtL is by integration into the bacterial chromosome. A truncated copy of wtL that lacks the *pac* sequence may be integrated into the chromosome to create a procapsid producing strain, in which case, full-length wild-type or recombinant segment-L RNA must subsequently be electroporated into integrates along with rM and rS RNA to complete packaging of all three segments. Alternatively, full-length wtL is integrated into the chromosome, thereby eliminating the need to subsequently introduce wtL RNA by electroporation.

Chromosomal integration may be achieved by homologous recombination using a temperature sensitive plasmid (herein referred to as "TS"), plasmids that can replicate only at a permissive temperature (30°C), but not at non-permissive temperatures (42°C and above) (Kretschmer *et al.*, J. Bacteriol. 124:225; 1975); (Hashimoto and Sekiguchi, J. Bacteriol. 127:1561; 1976). Examples of TS plasmids include pMAK705, pTSA29, pTSC29, pTSK29, all of which are pSC101 derivatives (Hamilton *et al.*, J. Bacteriol. 171:4617; 1989); (Phillips, Plasmid 41:78; 1999).

The use of TS plasmids in allelic exchange has been described in detail elsewhere (Hashimoto and Sekiguchi, J. Bacteriol. 127:1561; 1976); (Hamilton *et al.*, J. Bacteriol. 171:4617; 1989); (Phillips, Plasmid 41:78; 1999). Briefly, the wtL sequence is cloned into a TS plasmid such that it is flanked by sequences of the gene to be deleted. The plasmid carrying the cloned gene is then electroporated into the target bacteria and the cells are grown at 42°C to allow cointegrate formation, that is, the initial recombination event between homologous sequences of the chromosome and the plasmid. Cointegrates are selected by growing the cells on media that is supplemented with the antibiotic marker that is carried on the plasmid. Given that the plasmid does

not replicate at 42°C, only cointegrates will be antibiotic resistant. Cointegrates carry the plasmid origin of replication in the chromosome, replication from which is deleterious to the cell when cointegrates are subsequently grown at 30°C in the presence of antibiotic. Thus, at 30°C a second recombination event (resolution) occurs resulting in plasmid regeneration. Single colonies are then tested for antibiotic resistance at 42°C, so that antibiotic sensitive colonies no longer have plasmid integrated into the chromosome. To cure the cells of the plasmid generated by the second recombination, the cells are grown at 42°C without antibiotic.

In yet another approach, a TS plasmid may be used to express wtL, much like in Example 3, however, the bacteria are initially grown at permissive temperature only. The wtL is cloned under the control of a bacterial promoter, such as T7, from which it is expressed at 30°C. Following the introduction of rM and rS RNA by electroporation, the procapsids encoded by wtL package and replicate both RNAs. The cells may then be cured of the plasmid by growing at 42°C and no antibiotic. The resulting plasmid-free cells will continue to replicate RNA and may be used as bacterial vaccine vectors that do not carry regulatory concerns associated with plasmids, such as introduction of antibiotic resistance.

Example 11. Construction of additional packaging and delivery strains

As discussed above, the double-stranded RNA bacteriophage (dsRP) $\Phi 8$, a member of the *Cystoviridae*, possesses a three-segmented genome (designated S, M, and L) and was originally isolated from *Pseudomonas syringae*. The genes encoding the proteins necessary for formation of a functional nucleocapsid (NC) are as follows: the RNA-dependent RNA polymerase P2, the packaging enzyme P4, and the structural proteins P1 and P7. All of these are encoded on the L segment of the genome.

Taking advantage of this, we have designed recombinant segments -S and -M which include segment-specific packaging sequences at the 5' end, followed by an *asd* allele (in recombinant Segment-M) to complement a corresponding mutation in a bacterial carrier strain, gene 8 of bacteriophage $\Phi 8$ (in recombinant Segment-S), the Hepatitis C virus (HCV) IRES, a multiple cloning site (MCS) for insertion of antigens of interest, an SFV polymerase binding sequence, and a 3' $\Phi 8$ polymerase binding site. In each recombinant segment construct, the *asd* allele or gene 8 is under the translational control of a prokaryotic ribosomal binding site and the antigens/reporters of interest are under the translational control of the eukaryotic HCV IRES. Recombinant segment-S and -M are cloned into the *PstI* site of pGEM-3Z. Each recombinant segment is encoded

downstream of the T7 promoter of the parent vector for in vitro transcription of plus strand recombinant segment RNA.

To provide a mechanism for assembly, propagation and delivery of the rdsNC, an attenuated invasive *Shigella flexneri* 2a was created. This strain, MPC51, is a derivative of the *asd*- *S. flexneri* strain 15D into which a *murI* deletion mutation was introduced. The *asd* defect is complemented by the rdsNC encoded *asd* allele and the *murI* mutation results in the inability of the strain to synthesize D-glutamate; hence, this strain is incapable of synthesizing a proper cell wall, which promotes lysis of the bacterial cell after invasion of a eukaryotic cell. As measured by gentamicin protection assay, the HeLa cell invasive behavior of the Δasd , $\Delta murI$ double mutant MPC51 was similar to 15D and MPC51pYA3342 (plasmid encoding *asd*). In addition, this strain was further modified by removal of the kanamycin resistance gene previously inserted in the chromosomal *asd* locus by means well known to those skilled in the art. The resultant strain, *Shigella flexneri* NCD1, is thus free of introduced antibiotic resistance markers, still retains chromosomal deletions of the *asd* and *murI* genes, and is acceptable for pharmacologic use in humans under current regulatory requirements. NCD1 has also been shown to be invasive in HeLa and Caco-2 cells in a manner similar to the parent strain.

Using the techniques described herein a series of rdsRN's in *S. flexneri* MPC51 and NCD1 have been produced. Both strains were stably transformed with a plasmid encoding segment L (pLM2653). Assembly of empty procapsids in strains carrying this plasmid was confirmed by electron microscopy and immunoblotting of native and denatured whole cell lysates. A novel antigen expression cassette encoding a fusion of *Mycobacterium tuberculosis* antigens 85A, 85B, and 10.4 (TBS) was cloned into the MCS of recombinant segments -S and -M under translational control of the HCV IRES. The plasmids encoding these constructs were linearized and fluorinated RNA transcripts of the segments were synthesized *in vitro*. *S. flexneri* MPC51pLM2653 was electroporated with these fluorinated RNA transcripts. This resulted in *Shigella* strain MPC51 bearing rdsRN, designated MSTBS3. The presence of double-stranded RNA of each segment was confirmed by RT-PCR and the nucleocapsids of the novel rdsRN in the *Shigella flexneri* MPC51 propagating vector was visualized by electron microscopy.

Recombinant segment-S and segment-M constructs were assembled as described in Examples 2 and 4 with the following modifications. The β -galactosidase gene was cloned downstream of the Encephalocarditis virus (EMCV) IRES in each segment. The *asd* allele on

recombinant segment-M was replaced with an aminoglycoside phospho-transferase (*aphA*) gene under the translational control of the gene 10 ribosomal binding site. *In vitro* synthesized ssRNA from each recombinant segment was electroporated into NCD1pLM2653. Kanamycin resistant colonies were selected and grown in liquid M9 minimal media. The presence of double-stranded RNA's from segment-S and segment-M were verified by RT-PCR and the presence of capsid protein in the plasmid-cured rdsRN carrying strain was verified by Western blotting of whole cell lysates with antisera specific to the P4 protein of $\Phi 8$. This represents an additional selection system for the assembly and propagation of rdsRN's in a bacterial packaging strain.

Studies were carried out to show that *S flexneri* MPC51, in addition to packaging and propagating the rdsRN, is able to invade mammalian cells and is properly attenuated such that it does not propagate but rather dies after invasion of mammalian cells and thus lyses enabling the intracellular delivery of the rdsRN. An rdsRN designated MSTBS3 was created by electroporating MPC51pLM2653 with recombinant segments-S and -M encoding a fusion of Mtb antigens 85A, 85B, and TB10.4. The presence of rdsRN's encoding these immunogens was confirmed by RT-PCR and Western blot analysis of the resultant shigella strain MPC51 bearing rdsRN MSTBS3. To demonstrate the capacity of MPC51 + MSTBS3 to invade and subsequently lyse to deliver rdsRN MSTBS3, semi-confluent monolayers of HeLa and Caco-2 cells were infected for a period of one hour with MSTBS3 at an MOI of 100 in a series of multi-well dishes. Gentamicin sulfate was then added to each well at a concentration of 150 $\mu\text{g/ml}$ for one hour to kill all extracellular bacteria. Cells were then washed twice with DMEM and the media was replaced with DMEM plus 10% FBS and allowed to incubate. HeLa and Caco-2 cells in individual wells were lysed with Triton X-100 at 3-4 hour intervals beginning with the completion of the gentamicin treatment and surviving (thus intracellular) bacteria were plated in dilution for enumeration.

As shown in Figure 16A, viable bacterial vector counts were reduced by 95% in HeLa cells at 24 hours and 99% at 36 hours, indicating that bacterial cells were surviving long enough to escape the endosome and thus deliver the rdsRN's but were also dying off at a sufficient rate to allow timely delivery of rdsRN's and avoid cytotoxicity resulting from survival or multiplication of the bacterial vector. Similarly, as shown in Figure 16B viable bacterial vector counts from Caco-2 cells were reduced to <5% at 24 hours and <1% at 40 hrs, with no detectable surviving organisms at 64 hours. This clearly demonstrates that this packaging and vector strain is capable of invading

mammalian cells, then dies and lyses due to introduced *asd* and *murI* mutations allowing release of the rdsRN particles into the cytoplasm of the mammalian cell.

Example 12. Development and demonstration of methods for the purification of rdsRN from packaging strains

Methods for the purification of rdsRN's from bacterial packaging strains were developed methods and their utility was. In a first experiment, a 15 liter culture of MPC51 carrying rdsRN MSTBS3 was grown in M9 media in a BioFlo IV fermentor. The culture was grown at 28°C to an OD₆₀₀ of 1.0. The cell pellet was harvested by centrifugation and stored at 4°C pending nucleocapsid purification. A small sample was fixed and processed for EM analysis to verify NC production. Pellets were resuspended in 250ml of cold PBS (with protease inhibitors). Material was passed thru an APV Microfluidizer using 7,000 psi. Debris was pelleted at 3,000rpm for 10 minutes at 4°C. Supernatant was collected in a sterile bottle and pellets were resuspended in 250ml of the same buffer. This same process was repeated 3 more times with the following psi: 7000, 9000, 13000. After each pass thru the microfluidizer the debris was pelleted, supernatant collected and the pellet was resuspended in PBS. The remaining pellet was stored at 4°C. The pooled supernatants were passed thru a Pellicon 0.45µm tangential flow filtration device. The flow-thru was then processed with a 100kD cut-off Spiral Filter (Millipore). The spiral filter retentate was aseptically passed thru a sterile 0.45 ZapCap filter to decrease likelihood of contamination and then stored at 4°C.

Western blot analysis of cell lysates, pellets, and filter fractions with antisera to capsid protein P4 showed that the P4 protein was present in the 15L fermentation, the first 3 clarified lysates and very little remained in the residual pellet. P4 was also present in the spiral filter retentate as expected, but none was seen in the pellicon retentate indicating that the material was smaller than 0.45 µm in diameter but greater than 100 kDa in mass as one would expect for a fully formed rdsRN.

In order to further purify, as well as potentially concentrate samples, a 40-60% Sucrose and OptiPrep gradients were prepared as follows: 5.5mls of the 60% solution was added to the bottom of the ultraclear centrifuge tube and 5.5mls of the 40% solution was added on top. 1ml of material was then layered on top of the gradients and the tubes were centrifuged overnight at 25,000rpm at 4°C in an SW41 rotor. Since no visible bands were seen in the gradients which would have

allowed for extraction of material in a more concentrated volume, 1ml samples were collected from the top of the gradients so that the entire gradient could be analyzed for NC content.

75 μ l of each fraction (11 total) were mixed with 4X loading dye and boiled for 10 minutes. Samples were resolved on NuPAGE 4 – 12 % Bis-Tris Gels then transferred to PVDF. After transfer the blots were blocked 30 minutes in TBST + 0.5% BSA, shaking at room temperature. Primary antibody (α -P4 antibody) was added in TBST at a 1:2500 dilution overnight, shaking at 4°C. Blot was washed in TBST and then incubated with secondary antibody (α -rabbit IgG HRP linked whole antibody) at 1:5000 in TBST for 1 hour, shaking at room temperature. The blots were washed in TBST then developed using ECL reagents and exposed to film. As shown in Figure 17, P4 nucleocapsid protein was present in the first 6 fractions, but present in the highest concentration in fraction 3, representing partially purified nucleocapsid MSTBS3.

In an additional experiment, MPC51 carrying MSTBS3 were grown in 400mL of M9 medium in 1L shaker flasks at 28°C to an OD₆₀₀ of 1.2. Cells were pelleted at 6000 x g for 10 minutes at 4°C and resuspended in 20mL of ice-cold "Buffer A" (PBS pH 7.4 containing 200mM NaCl and 2mM MgSO₄.) Cells were lysed using three passes through a French Press. The sample was centrifuged for 10 minutes at 8000 x g at 4°C. The supernatant was concentrated using Centricon YM-100 concentrators. The retentate was combined and pelleted on a 10-20% sucrose gradient for 2h at 22,000rpm in a SW28 rotor at 20 °C. The pellet was resuspended in a minimal volume of "Buffer A" and used for further testing. Preliminary Western blot data indicate the presence of P4 in the final pellet.

This example shows the successful development and implementation of protocols for the purification of rdsRN's from bacterial packaging strains.

Example 13. Mouse immunogenicity study

In order to investigate the ability of the rdsRN propagated and delivered by bacterial packaging strains to elicit an immune response in mice, groups of 5 Balb/c mice were vaccinated intranasally with MPC51+MSTBS3 as outlined in the Table 1. Control groups 1 and 2 received saline intramuscularly or MPC51 carrying empty capsids intranasally, respectively. Positive control group 6 received 5x10⁹ pfu of an adenovirus vector vaccine encoding the same TB antigens as MSTBS3. This positive control vaccine has been extensively characterized in mice, guinea pigs, non-human primates and humans. Spleens were harvested from vaccinated animals 8 weeks post-immunization and analyzed using an immunogen specific ICS/FACS assay.

Table 1. Design of murine antigenicity study demonstrating the immune response to RNA encoded TB antigens of rdsRN MSTBS3.

VACCINE	# OF MICE	DOSE	ROUTE
1. Saline	5	100 μ l	im
2. MPC51	5	5×10^6 cfu	in
3. MSTBS3	5	5×10^6 cfu	in
4. MSTBS3	5	5×10^5 cfu	in
5. MSTBS3	5	5×10^4 cfu	in
6. Ad35.TBS	5	5×10^9 pfu	im

Mice vaccinated with MPC51 carrying rdsRN MSTBS3 (Groups 3-5) generated antigen-specific IFN- γ producing CD8+ T cells at a frequency greater than or equal to Ad35.TBS (Figure 18). The MPC51 *Shigella* vector carrying empty capsids and saline did not elicit any measurable antigen-specific immune responses. Similarly, mice vaccinated with MSTBS3 generated greater percentages of antigen-specific TNF- α producing CD8+ T cells than the Ad35.TBS vaccinated mice. This clearly shows that the *Shigella flexneri* MPC51 rdsRN vaccine MSTBS3 was capable of delivering antigen encoding RNA nucleocapsids into a live animal, that the nucleocapsid RNA encoding *Mycobacterium tuberculosis* antigens was translated in the live animal, and that the translated antigens elicited immune responses equal to or greater than those elicited by one of the worlds leading TB vaccine candidates (Ad35.TBS).

Example 14. Studies in primates

The ability of *Shigella* MPC51 vectored rdsRN MSTBS3 to elicit antigen-specific immune responses in rhesus macaques was also studied. A total of 22 monkeys were weight and sex distributed into five vaccine groups of 4 animals each plus a saline control group of 2 as shown in table 2. Under sedation, monkeys in groups 1 and 2 received 50 ml saline or *S. flexneri* MPC51 carrying empty capsids in 50 ml saline respectively via orogastric feeding tube. Group 3 received a BCG vaccination intradermally. Groups 4-6 received 10^9 , 10^{10} , or 10^{11} cfu of MPC51 carrying MSTBS3 in PBS via orogastric feeding tube. Each group was boosted with the same vaccine or control at four weeks, with the exception of group3 which was boosted with 10^{10} cfu MSTBS3.

Table 2. Design of a primate immunogenicity study to evaluate immune responses elicited by orogastric immunization with rdsRN MSTBS3.

Group	n	Prime (week 0)	Boost (week 4)
1	2	Saline ig	Saline ig
2	4	MPC51 1x10 ¹¹ CFU ig	MPC51 1x10 ¹¹ CFU ig
3	4	rBCG AFR-01 5x10 ⁵ CFU id	MSTBS3 1x10 ¹⁰ CFU ig
4	4	MSTBS3 1x10 ⁹ CFU ig	MSTBS3 1x10 ⁹ CFU ig
5	4	MSTBS3 1x10 ¹⁰ CFU ig	MSTBS3 1x10 ¹⁰ CFU ig
6	4	MSTBS3 1x10 ¹¹ CFU ig	MSTBS3 1x10 ¹¹ CFU ig

Blood was drawn for analysis of cellular immune responses at two week intervals. FACSIA analysis of antigen-specific immune responses in whole blood revealed CD4+ and CD8+ T cell responses to Ag85A, Ag85B, and TB10.4 encoded by the RNA in the MSTBS3 nucleocapsid (Figure 19A-D). This example shows that *Shigella flexneri* MPC51 can carry nucleocapsids, deliver them to mammalian tissues, and that the nucleocapsid produced RNA can be translated in the mammalian tissue to elicit a desirable immune response in an animal model very close to humans.

Example 15. Additional examples of packaging strain constructs: inclusion of Hepatitis C virus X region

The 3' Nontranslated Region (NTR) of Hepatitis C virus includes a sequence known as the X region (Ito *et al*, J Virol 72(11):8789-8796; 1998, Song *et al*, J Virol, 80(23): 11579–11588; 2006). This RNA sequence (GenBank Acc NC_004102) has been shown to bind the polypyrimidine tract binding protein (PTB) of mammalian cells. As the HCV and other IRES sequences also bind PTB, it has been shown that the inclusion of this sequence is necessary to stabilize the interaction between the IRES and the 40s ribosomal subunit of eukaryotic cells to produce active translation of sequences downstream of the HCV IRES and that this sequence is necessary to cause an active HCV infection. This stabilization of a closed loop eukaryotic translation complex with a 3'

nontranslated sequence is not unique to HCV, and has been shown to enhance translation from a variety of IRES-including sequences, as well as non-IRES dependent sequences.

We have designed and constructed recombinant segments-S and -M including this sequence between the RNA encoded sequence of interest and the 3' polymerase binding site to enhance translation of RNA encoded proteins of interest in the eukaryotic cell. This sequence further deletes the requirement for polyadenylation of rdsRN produced RNA to promote high efficiency translation. While the specific enhancement of translation from HCV and EMCV IRES sequences has been demonstrated using this NTR, it should be obvious to those knowledgeable in the art that a variety of viral or eukaryotic NTR's could also be used in such a manner to enhance eukaryotic translation of RNA encoded in rdsRN's.

Example 16. Enhancement of amount of RNA produced by rdsRN's

We have further designed a unique means by which to utilize a mutant alphavirus replicon to enhance the amount of RNA of interest produced by rdsRN's. The non structural proteins (NSP's) 1-4 of alphaviruses (including, but not limited to, Sindbis Virus and Venezuelan Equine Encephalitis (VEE) virus) are required and sufficient for rapid, high volume production of minus strand and then plus strand RNA from these plus strand RNA viruses (Rice et al, J. Virol 72(8), 6546-6553: 1998). The replicase of VEE is one of the highest velocity RNA-dependent RNA polymerases known. The first protein of VEE and other alphaviruses is the NSP1234 polyprotein. This product is autocleaved first to produce NSP123 and NSP4. In this context the viral replicase is specific for production of full length minus strand transcript using the viral plus strand as template. Proteolytic processing of NSP123 into NSP1, NSP2, and NSP3 via a catalytic site in NSP2 then produces a replicase complex of NSP1-4 as independent subunits that produce plus strand transcripts (viral mRNA) encoding viral proteins.

We have designed and constructed a recombinant segment-L that encodes a VEE replicase that is permanently specific for minus strand synthesis. Starting from GenBank sequence LO465, 3' to the segment-L packaging sequence we have placed an EMCV IRES followed by RNA sequence encoding the VEE isolate P676 replicase with the following modifications. The codon encoding Cys 1012 in NSP2 of the poly protein has been changed from TGT to GGC thus encoding a Gly at aa 1012. This eliminates the proteolytic activity of NSP2 and thus leaves NSP123 permanently fused. An opal codon at bp 5682 of the reference sequence (within the NSP3 coding sequence) has been changed from TGA to AGA to encode an Arg residue and allow full length translation of NSP123,

i.e. NSPs1, 2, and 3 are translated together as a single polypeptide chain. This sequence is followed by a second IRES which directs the independent translation of NSP4 with an added initiation and termination codon. This is followed by the stabilizing HCV X region and the segment-L polymerase binding site. When translated in a eukaryotic cell, this recombinant segment-L produces a stable, fused NSP123 polyprotein and NSP4 that assembles as a protein complex that is specific for the plus strand RNA-dependent synthesis of minus strand RNA.

We have thus also designed recombinant segments –S and –M similar to those previously described with the following modifications to the eukaryotic expression cassettes. Upstream of the expression cassette in each segment we have placed the 45 bp conserved 5' NTR of VEE P676. This sequence is followed by the RNA of interest anti-coded (*ie* encoded as if this were a minus strand of the nucleocapsid reading 5' to 3'). This is followed by a similarly anti-coded EMCV IRES and the 188 base VEE P676 3' NTR including the repeated sequence elements (RSE) (Pfeffer *et al*, Virology, 240, 100-108: 1998), the conserved 19 nucleotide sequence and the HCV X region.

rdsRN's produced using this system thus produce and secrete a recombinant segment-L encoding a minus strand synthesis-specific VEE replicase complex and recombinant segments -S and -M that are recognized by the replicase as plus strand VEE sequence due to the presence of the VEE 5' and 3' NTR's. This high velocity replicase complex then rapidly transcribes minus strand copies of the recombinant segments, thus producing a vastly enlarged pool of RNA's of interest down stream of a eukaryotic IRES due to their being anti-coded on the plus strand of the rdsRN.

This represents not only a novel means of amplifying the amount of rdsRN RNA of interest in a eukaryotic cell, it is a completely novel design and use of a modified alphavirus amplicon not previously reported.

Example 17. Response of Human Cells to Shigella Vectored rdsRN's

The innate immune system serves as a first line of defense system against invading pathogens, including bacteria or viruses. Eukaryotic cells possess the inherent capability to recognize components of viruses and microbes via a number of cell surface and intracellular germline-encoded pattern-recognition receptors (PRRs) such as the Toll-like receptors (TLRs), the Nod-like (nucleotide-binding oligomerization domain) receptors, and the RNA helicases RIG-I (retinoic acid-inducible gene-I) and MDA5 (melanoma differentiation associated gene 5). Binding of viral or bacterial components by these receptors mediates up-regulation and production of antibacterial and antiviral effectors.

Jawed vertebrates which evolved an adaptive immune system also developed the interferon cytokine family that is dedicated to autocrine and paracrine signaling of the presence of infection and facilitates communication among cells that provide protection against infectious agents, including viruses and intracellular bacteria. Similarly, they may activate mechanisms within an infected cell intended to limit the infection by interruption of cellular processes or degradation of foreign material. Interferon (IFN)- α and - β comprise the type I IFN family and were first identified as humoral factors that confer an antiviral state on cells. Among the autocrine IFN-induced effector and modulator proteins essential for the antiviral actions of type I IFNs are the RNA-dependent protein kinase (PKR), the 2',5'-oligoadenylate synthetase (OAS), RNase L, and the Mx protein GTPases. Double-stranded or highly structured RNA plays a central role in modulating protein phosphorylation and RNA degradation catalyzed by the IFN-inducible PKR kinase which halts RNA translation and the OAS-dependent RNase L which degrades RNA, respectively, and also in RNA editing by the IFN-inducible RNA-specific adenosine deaminase (ADAR1). The expression of IFN- α/β is effectively controlled by transcription factors of the IFN regulatory factor (IRF) family. For example, double-stranded RNA and lipopolysaccharide, when recognized by TLR3 and TLR4 respectively, lead to IRF-3 and IRF-7 activation; TLR7 and TLR9 detect single-stranded RNA and CpG DNA and stimulate IRF-5 and IRF-7 via a MyD88-dependent pathway also involving IRAK1/4 and TRAF6.

Most successful viral pathogens of mammals have evolved mechanisms of blocking these autocrine and paracrine responses, enabling them to establish infection. Moreover, certain viruses or double-stranded RNA activate TLR-independent PRR responses, which signal via the cytosolic RNA helicases RIG-I and/or MDA5 through the adapter molecule IPS-1 (interferon-promoter stimulator 1) thereby stimulating IRF-3 and IRF-7 dependent transcription of specific response genes (1, 4, 10–13). Examples of viral proteins evolved to overcome this response include, but are not limited to, the NSP1 protein of rotavirus which binds IRF-3 and prevents nuclear translocation, the C12R protein of ectromelia virus which binds IFN- α/β , NS1 of influenza which prevents nuclear translocation of IRF-3 and interferes with RIG-1 dependent signaling, and the NS3/4A protease of HCV which specifically cleaves the MAV and TRIF proteins involved in signaling the transcription of IFN- α/β .

We have found that invasion of human cell lines with *Shigella*-vectored rdsRN's activates the upregulation of IFN- β production. RT-PCR analysis of HeLa cell lysates with primers specific

for IFN- β mRNA clearly demonstrated the presence of IFN- β message in cells infected with *Shigella* alone or with *Shigella* carrying nucleocapsids (Figure 20A and B). Briefly, semi-confluent monolayers of HeLa cells were invaded for 1 hour with *S. flexneri* 15D, MPC51, and rdsRN's 5TBC and S4-GFP at an MOI of 100 in a 6 well plate at 37°C. 5TBC and S4-GFP are *Shigella* strains carrying rdsRN encoding TB antigens and green fluorescent protein, respectively. Cells were washed twice with DMEM and medium containing 150 μ g/ml gentamicin was added to the cells for 1 hr to kill extracellular bacteria. Subsequently, cells were then washed twice, and DMEM with 10% FBS was added and allowed to incubate for 20h. Cells were washed twice with PBS and total RNA was isolated using an RNeasy mini kit (Qiagen). One μ g of total RNA was reverse transcribed from each well using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). The generated cDNA was amplified by semiquantitative RT-PCR using specific primers for IFN- β and housekeeping gene GAPDH. The resultant products were analyzed by agarose gel electrophoresis.

Microarray analysis of the expression of known human genes involved in type I IFN responses to microbial or viral pathogens was performed using total RNA isolated from HeLa cells invaded with *Shigella flexneri* 15D and *Shigella flexneri* rdsRN LGP3. These studies revealed a 2-38-fold induction of the 10 isoforms of IFN- α , a 22-fold induction of IFN- β , a 9-fold induction of RNase-L, and a 13 fold-induction of MX1 in response to *S. flexneri* invasion. As IRF-3 dependent upregulation of OAS-dependent RNaseL causes degradation of RNA, presumably including rdsRN produced RNA, and IFN- β mediated phosphorylation of PKR results in a halting of RNA translation within the infected cell, the autocrine type I IFN response to *Shigella*-rdsRN invasion clearly reduces the capacity of the rdsRN encoded RNA to be translated or stably expressed in a mammalian cell.

We have identified key viral genes which we have included in both the *Shigella* packaging strain and in the encoded RNA of the rdsRN's. The NSP1 gene of rotavirus encodes a protein which binds IRF-3 and prevents its translocation into the nucleus – a key step in the activation of the type I IFN response. Expression of this protein from the packaging strain and rdsRN will effectively block subsequent activation of the type I IFN upon its elaboration or release into the eukaryotic cytoplasm. Expression of soluble forms of the C12R IFN- α/β receptor of ectromelia virus from the packaging strain and rdsRN will effectively bind and render inactive any IFN- α/β expressed from cells invaded by *Shigella* or other invasive packaging strains and allow much higher expression of rdsRN encoded immunogens and stabilize any other rdsRN RNA of interest in the eukaryotic cell.

These findings regarding the type I IFN response to bacterial rdsRN packaging strains and to rdsRN's themselves and the novel method of expression of viral antagonists of this response from the packaging strain and the rdsRN are key to the elaboration of stable RNA from rdsRN's in the eukaryotic cell and any subsequent translation of this RNA of interest into proteins designed to elicit a biological response.

While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

CLAIMS

We claim:

1. A bacterial strain for packaging, producing and/or delivering genes or RNA, comprising
 - a) genomic DNA comprising at least one selectable phenotypic mutation;
 - b) nucleic acid sequences encoding genes necessary for nucleocapsid production;
 - c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity;
 - d) dsRNA sequences contained within said one or more nucleocapsids, said dsRNA sequences encoding at least:
 - i) a gene product that complements said at least one selectable phenotypic mutation, and
 - ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence;and
 - e) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.
2. The bacterial strain of claim 1, wherein said nucleic acid sequences that stabilize a closed loop eukaryotic translation complex comprise nucleic acid sequences that bind a mammalian polypyrimidine tract binding protein.
3. The bacterial strain of claim 2, wherein said nucleic acid sequences that bind a mammalian polypyrimidine tract binding protein comprise a 3' non-translated region.
4. The bacterial strain of claim 3, wherein said 3' non-translated region is region X of hepatitis C virus.
5. The bacterial strain of claim 1, further comprising nucleic acid sequences encoding alphavirus non-structural proteins 1, 2, 3, and 4.
6. The bacterial strain of claim 5, wherein alphavirus non-structural protein 2 is a mutant non-structural protein 2 that is devoid of proteolytic activity.

7. The bacterial strain of claim 5, wherein alphavirus non-structural proteins 1, 2, and 3 are translated together as a single polypeptide.
8. The bacterial strain of claim 5, wherein alphavirus non-structural protein 4 is translated separately from alphavirus non-structural proteins 1, 2, and 3.
9. The bacterial strain of claim 5, wherein a protein complex formed from said alphavirus non-structural proteins 1, 2, 3, and 4 is specific for plus strand RNA-dependent synthesis of minus strand RNA.
10. A recombinant double-strand RNA nucleocapsid (rdsRN), comprising
 - a) proteins with RNA packaging and RNA polymerase activity;
 - b) dsRNA sequences encoding at least:
 - i) a gene product, and
 - ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence;
 - and
 - c) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.
11. The rdsRN of claim 10, wherein said nucleic acid sequences that stabilize a closed loop eukaryotic translation complex comprise nucleic acid sequences that bind a mammalian polypyrimidine tract binding protein.
12. The rdsRN of claim 11, wherein said nucleic acid sequences that bind a mammalian polypyrimidine tract binding protein comprise a 3' non-translated region.
13. The rdsRN of claim 12, wherein said 3' non-translated region is region X of hepatitis C virus.
14. The rdsRN of claim 10, further comprising nucleic acid sequences encoding alphavirus non-structural proteins 1, 2, 3, and 4.

15. The rdsRN of claim 14, wherein alphavirus non-structural protein 2 is a mutant non-structural protein 2 that is devoid of proteolytic activity.
16. The rdsRN of claim 14, wherein alphavirus non-structural proteins 1, 2, and 3 are translated together as a single polypeptide.
17. The rdsRN of claim 14, wherein alphavirus non-structural protein 4 is translated separately from alphavirus non-structural proteins 1, 2, and 3.
18. The rdsRN of claim 14, wherein a protein complex formed from said alphavirus non-structural proteins 1, 2, 3, and 4 is specific for plus strand RNA-dependent synthesis of minus strand RNA.
19. A vaccine preparation, comprising,
bacterial cells, comprising
- a) genomic DNA comprising at least one selectable phenotypic mutation;
 - b) nucleic acid sequences encoding genes necessary for nucleocapsid production
 - c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity;
 - d) dsRNA sequences contained within said nucleocapsid, said RNA sequences encoding at least:
 - i) a gene product, and
 - ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence;
- and
- e) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.
20. A vaccine preparation, comprising,
recombinant double-strand RNA nucleocapsids (rdsRNs), comprising
- a) proteins with RNA packaging and RNA polymerase activity;
 - b) dsRNA sequences encoding at least:

- i) a gene product that complements at least one selectable phenotypic mutation, and
- ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence; and
- iii) nucleic acid sequences encoding genes necessary for phage or virus nucleocapsid production;

and

- c) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.

21. A method of creating a recombinant bacterium for use as a bacterial packaging strain, comprising the steps of

introducing at least one selectable phenotypic mutation into genomic DNA of a bacterium;
genetically engineering said bacterium to contain DNA encoding functional double-stranded RNA phage nucleocapsid proteins; and

inserting into said bacterium mRNA segments encoding

- i. at least one gene encoding a functional product that complements said at least one selectable phenotypic mutation;
- ii. functional double-stranded RNA phage nucleocapsid proteins;

and

- iii) nucleic acid sequences that stabilize a closed loop eukaryotic translation complex.

22. A bacterial strain for packaging, producing and/or delivering genes or RNA, comprising

- a) genomic DNA comprising at least one selectable phenotypic mutation;
- b) nucleic acid sequences encoding genes necessary for nucleocapsid production;
- c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity;
- d) dsRNA sequences contained within said one or more nucleocapsids, said dsRNA sequences encoding at least:

- i) a gene product that complements said at least one selectable phenotypic mutation, and
- ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence;

and

e) nucleic acid sequences encoding one or more proteins that interfere with a host cell type I interferon (IFN) response.

23. The bacterial strain of claim 22, wherein said one or more proteins binds to type I IRF-3 and blocks its activation.

24. The bacterial strain of claim 23, wherein said one or more proteins is NSP1 of rotavirus.

25. The bacterial strain of claim 22, wherein said one or more proteins binds and renders inactive IFN- α or IFN- β or both.

26. The bacterial strain of claim 25, wherein said one or more proteins is a C12R IFN- α/β receptor from ectromelia virus.

27. A recombinant double-strand RNA nucleocapsid (rdsRN), comprising

a) proteins with RNA packaging and RNA polymerase activity;

b) dsRNA sequences encoding at least:

i) a gene product, and

ii) an RNA of interest operably linked to a eukaryotic translation initiation sequence;

and

c) nucleic acid sequences encoding one or more proteins that interfere with a host cell type I interferon (IFN) response.

28. The rdsRN of claim 27, wherein said one or more proteins binds to IRF-3 and blocks its activation.

29. The rdsRN of claim 28, wherein said one or more proteins is NSP1 of rotavirus.

30. The rdsRN of claim 27, wherein said one or more proteins binds and renders inactive IFN- α or IFN- β or both.

31. The rdsRN of claim 30, wherein said one or more proteins is a C12R IFN- α/β receptor from ectromelia virus.

32. A vaccine preparation, comprising,
bacterial cells, comprising

- a) genomic DNA comprising at least one selectable phenotypic mutation;
- b) nucleic acid sequences encoding genes necessary for nucleocapsid production
- c) one or more nucleocapsids comprising proteins with RNA packaging and RNA polymerase activity;
- d) dsRNA sequences contained within said nucleocapsid, said RNA sequences encoding at least:
 - i) a gene product, and
 - ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence;

and

- e) nucleic acid sequences encoding one or more proteins that interfere with a host cell interferon (IFN) response.

33. A vaccine preparation, comprising,

recombinant double-strand RNA nucleocapsids (rdsRNAs), comprising

- a) proteins with RNA packaging and RNA polymerase activity;
- b) dsRNA sequences encoding at least:
 - i) a gene product that complements at least one selectable phenotypic mutation, and
 - ii) an RNA encoding an immunogen operably linked to a eukaryotic translation initiation sequence; and
 - iii) nucleic acid sequences encoding genes necessary for phage or virus nucleocapsid production;

and

c) nucleic acid sequences encoding one or more proteins that interfere with a host cell interferon (IFN) response.

34. A method of creating a recombinant bacterium for use as a bacterial packaging strain, comprising the steps of

introducing at least one selectable phenotypic mutation into genomic DNA of a bacterium; genetically engineering said bacterium to contain DNA encoding functional double-stranded

RNA phage nucleocapsid proteins; and

inserting into said bacterium mRNA segments encoding

i. at least one gene encoding a functional product that complements said at least one selectable phenotypic mutation;

ii. functional double-stranded RNA phage nucleocapsid proteins;

and

iii) nucleic acid sequences encoding one or more proteins that interfere with a host cell interferon (IFN) response.

35. A recombinant alphavirus replicon, comprising

nucleic acid sequences encoding alphavirus non-structural proteins 1, 2, 3, and 4, wherein alphavirus non-structural protein 2 is a mutant non-structural protein 2 that is devoid of proteolytic activity, and wherein alphavirus non-structural proteins 1, 2, and 3 are translated together, and non-structural protein 4 is translated separately.

36. The recombinant alphavirus replicon of claim 35, wherein said nucleic acid sequences are from an alphavirus selected from the group consisting of Sindbis virus and Venezuelan equine encephalitis.

37. The alphavirus replicon of claim 36, wherein said nucleic acid sequences are from Venezuelan equine encephalitis.

38. The alphavirus replicon of claim 37, wherein a codon encoding cysteine at position 1012 in non-structural protein 2 is changed to encode an amino acid that is not cysteine.

39. The alphavirus replicon of claim 37, wherein said amino acid that is not cysteine is glycine.
40. The alphavirus replicon of claim 35, wherein a protein complex formed from said alphavirus non-structural proteins 1, 2, 3, and 4 is specific for plus strand RNA-dependent synthesis of minus strand RNA.
41. The alphavirus replicon of claim 35, further comprising an internal ribosome entry site (IRES) which directs independent translation of NSP4.

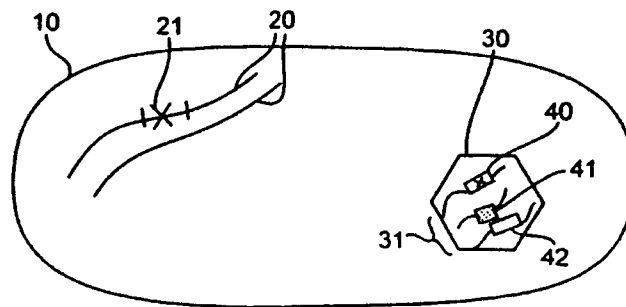


Figure 1

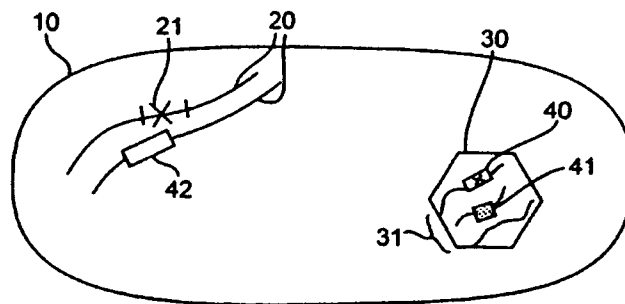


Figure 2

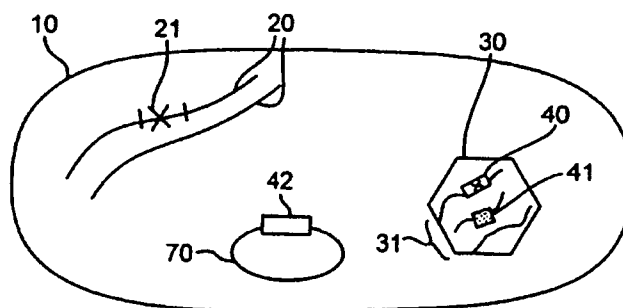


Figure 3

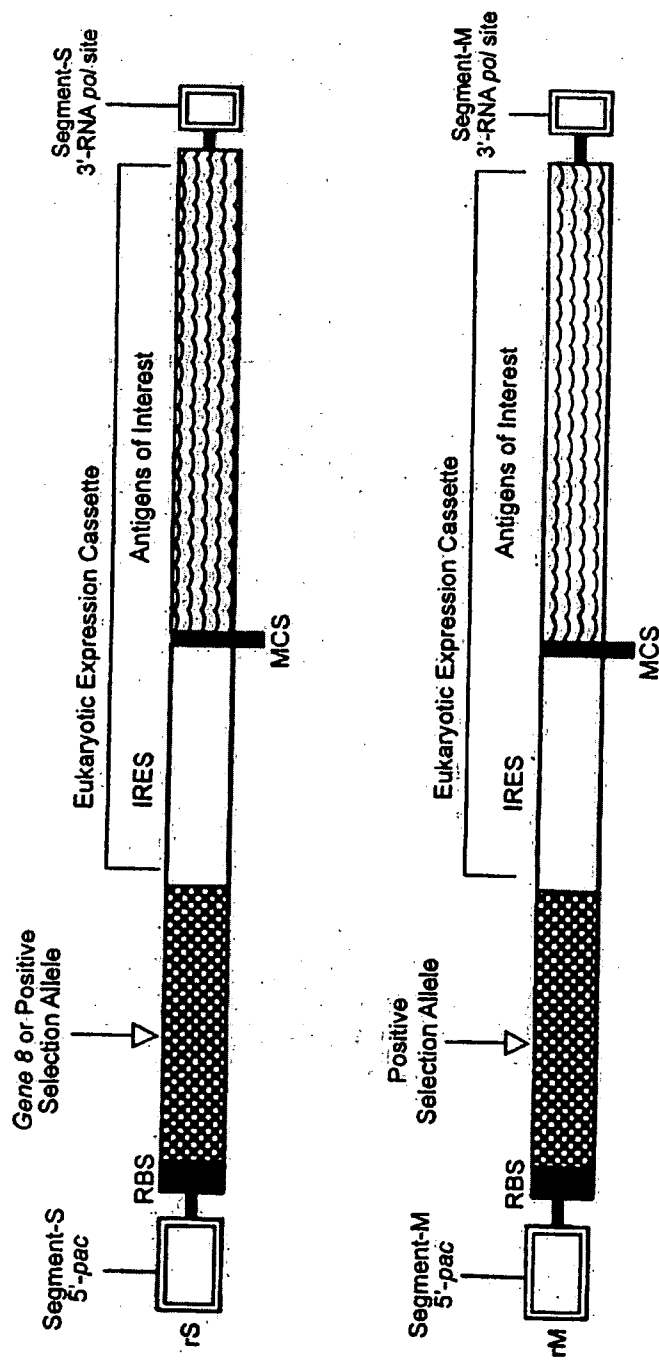


Figure 4

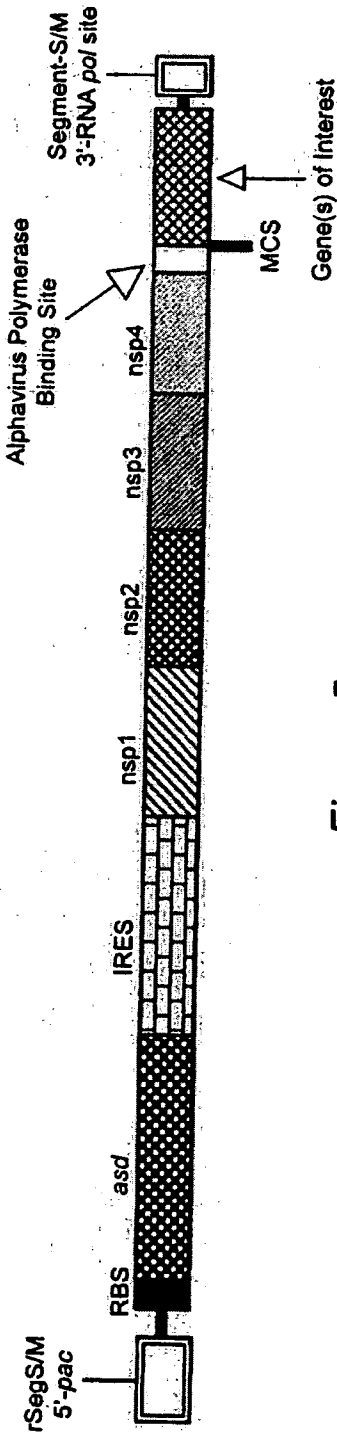


Figure 5

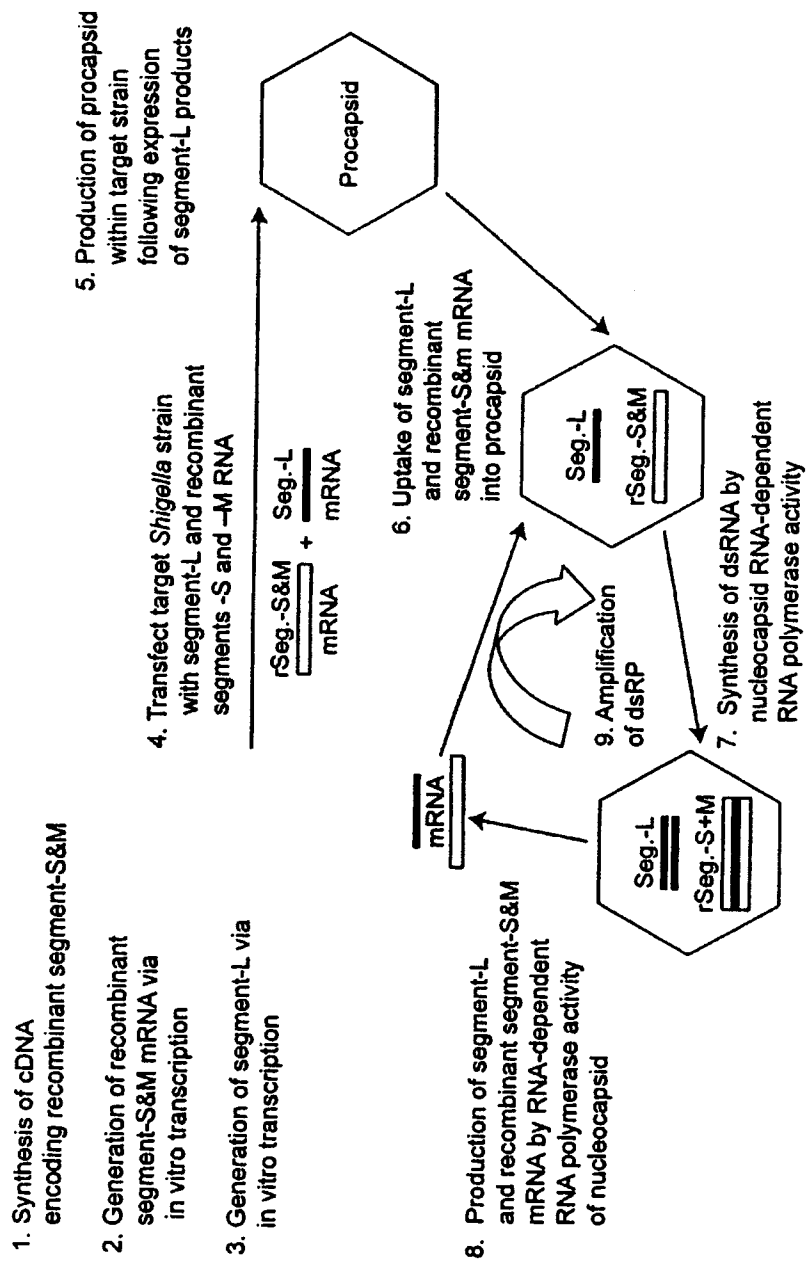


Figure 6

As measured by gentamicin protection assay,
1.0 x 10⁶ – 1.2 x 10⁶ HeLa per well, MOI = 100

15D	AVG= 1.34 x 10 ⁵ cfu
MPC51	AVG= 2.23 x 10 ⁴ cfu
MPC51pYA3342	AVG= 7.40 x 10 ⁴ cfu

Figure 7

immunoblot of whole cell
lysate passed through 100 kDa
filter under native and
denaturing conditions, probed
with anti-P4

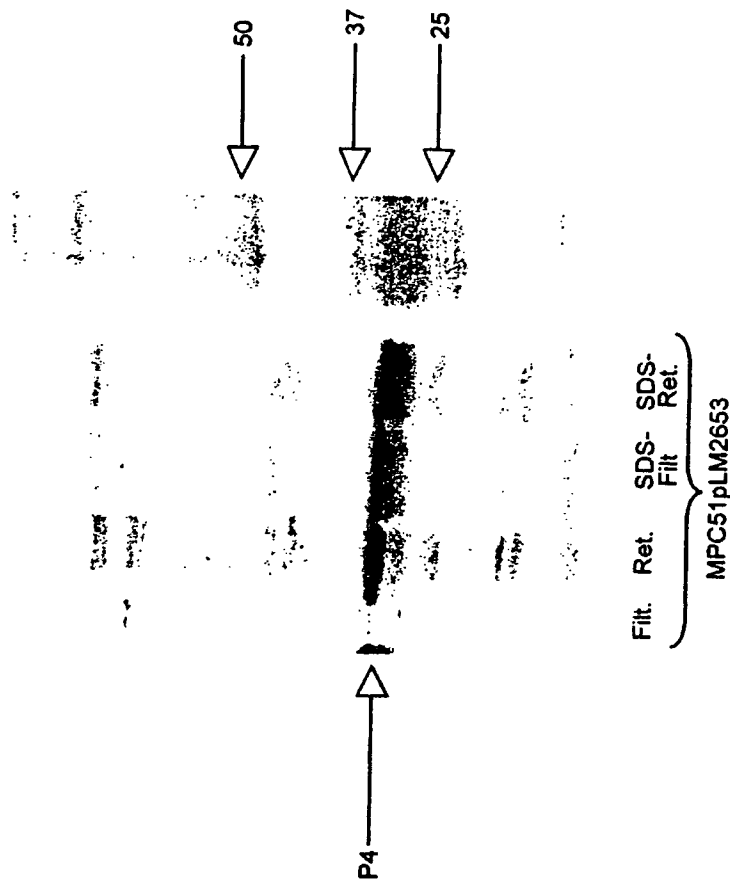


Figure 8



Figure 9



MPC51pLM2653 carrying rdsRP LSMtb4,
reverse transcription step performed with only
+ or - strand specific primer, respectively

Figure 10

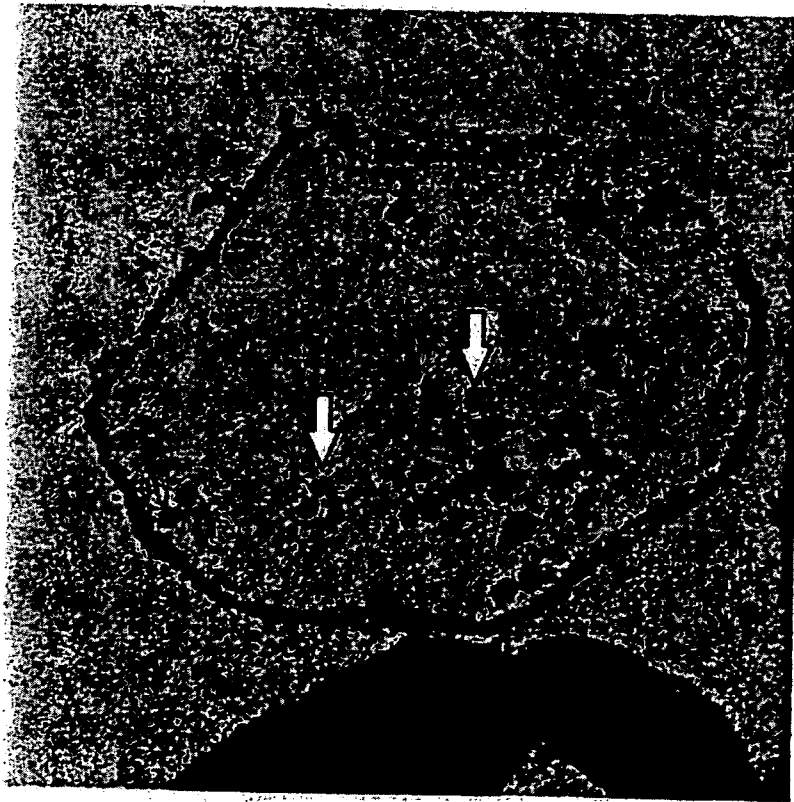


Figure 11



Figure 12



Figure 13

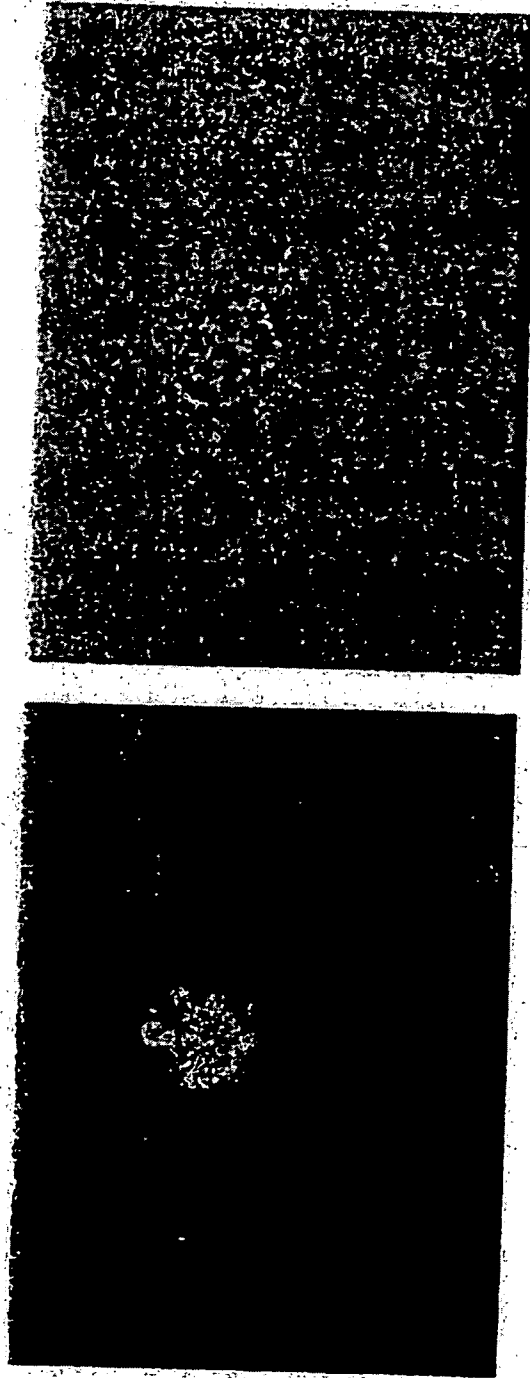


Figure 14

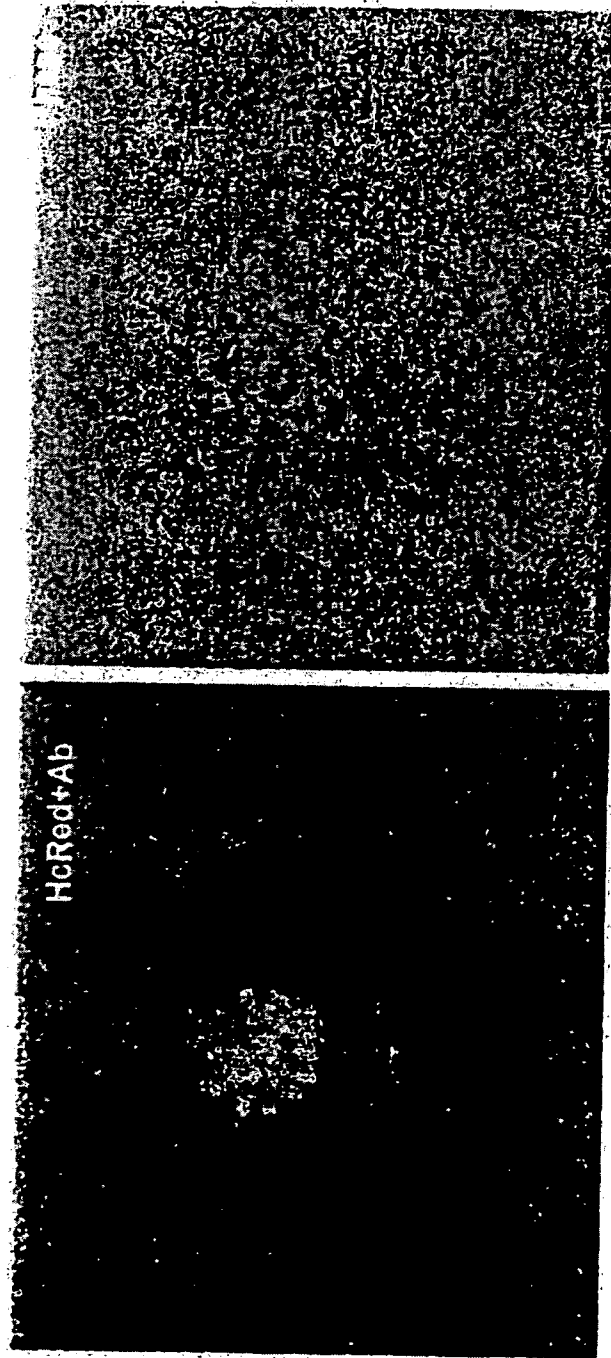


Figure 15

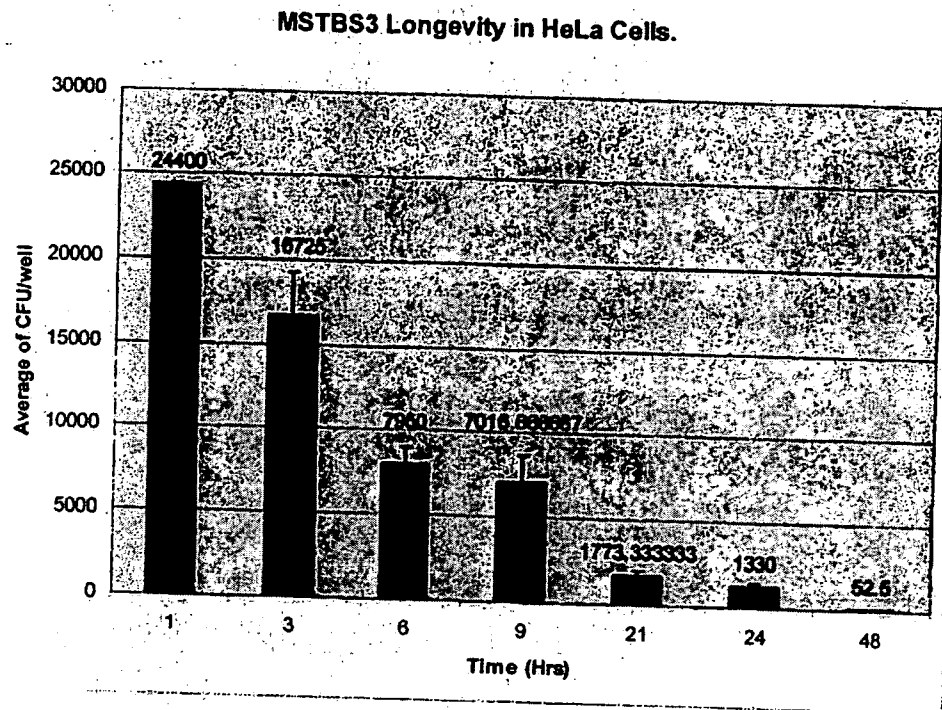
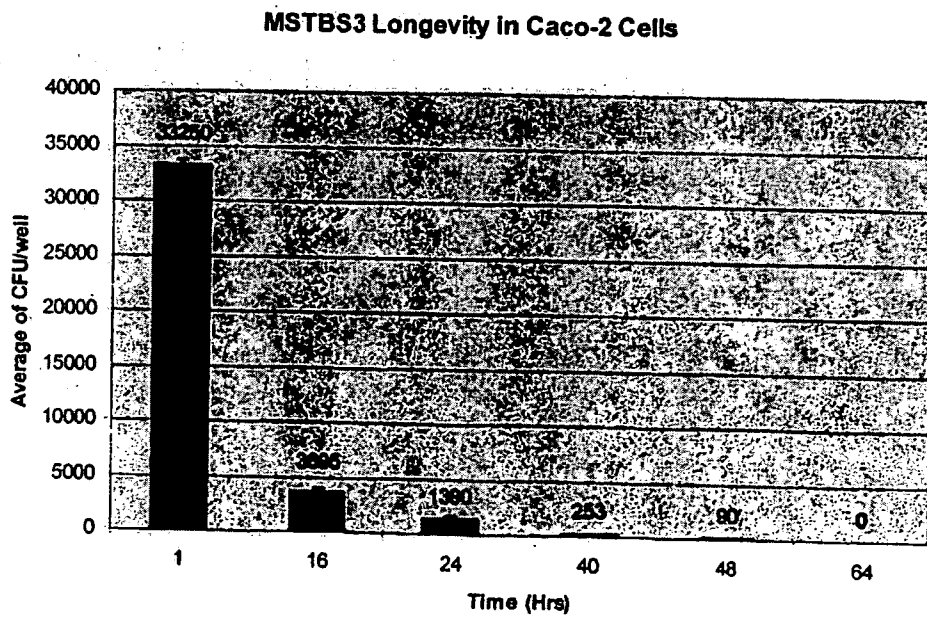


Figure 16B



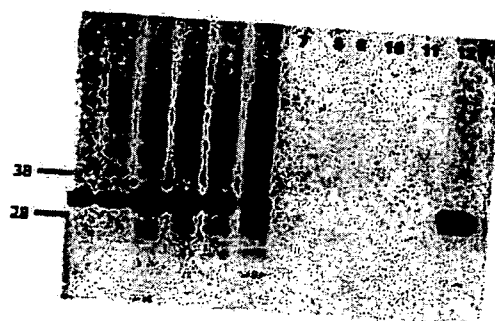


Figure 17

Figure 18A

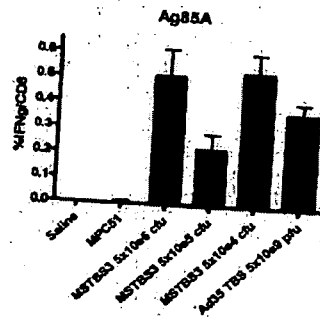


Figure 18B

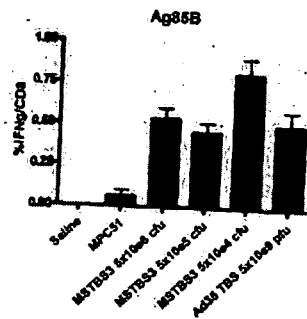
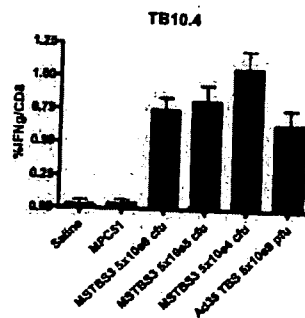


Figure 18C



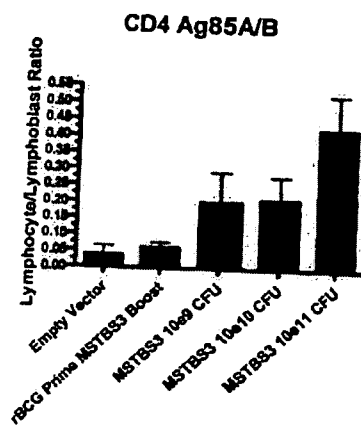


Figure 19A

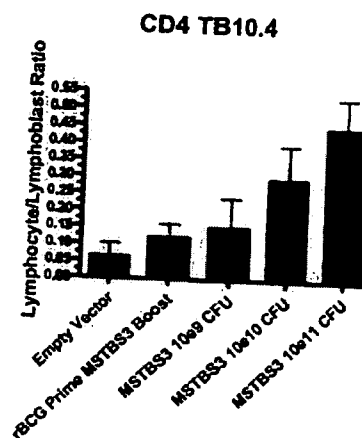


Figure 19B

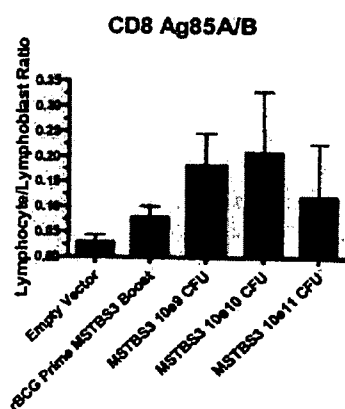


Figure 19C

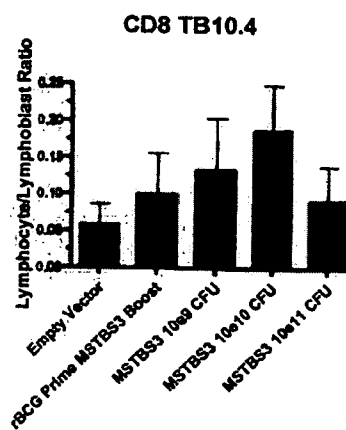
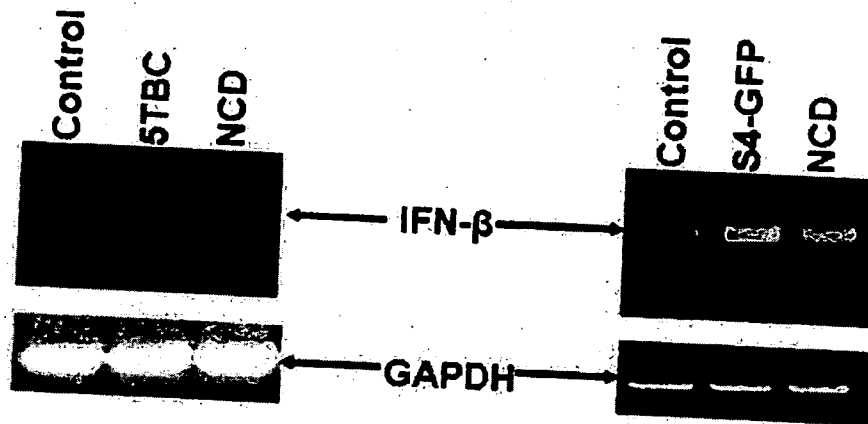


Figure 19D

*Figure 20A**Figure 20B*