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(54) Title: RECOMBINANT PLAGUE VACCINE

(57) Abstract

The present invention relates to a recombinant vaccine to protect animals against plague. More particularly, the invention includes recombinant molecules containing isolated nucleic acid molecules that encode proteins from *Yersinia*, *Pasteurella*, or *Francisella* bacteria expressed in eukaryotic cells. In one embodiment, the recombinant molecule is an animal virus genome; in another embodiment the recombinant molecule is a recombinant plasmid. The present invention also includes recombinant viruses comprising a recombinant animal virus genome and recombinant cells comprising either a recombinant virus or a recombinant plasmid. The present invention further includes therapeutic compositions comprising such recombinant molecules, viruses and cells, as well as methods to protect animals from plague.

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RECOMBINANT PLAGUE VACCINE

Field of the Invention

The present invention relates to a recombinant vaccine to protect animals against plague. More particularly, the invention includes recombinant molecules containing isolated nucleic acid molecules that encode antigens from *Yersinia*, *Pasteurella*, or *Francisella* expressed in eukaryotic cells.

Background of the Invention

Plague remains a significant problem in the western United States. *Yersinia pestis* is enzootic in several wild animal reservoirs, particularly squirrels and wild mice. The disease is also frequently epizootic in prairie dogs. Plague and plague-like diseases caused by other species of *Yersinia*, *Pasteurella*, and *Francisella* are also enzootic in wild reservoirs. Although the enzootic reservoirs are not a severe direct threat to humans, infection of domestic cats from wild reservoirs is a very serious problem in that several pet owners and veterinarians have contracted infection from these animals; see, for example, Doll et al., 1994, *Am. J. Trop. Med. Hyg., 51,* 109-114. For this reason, an effective vaccine to protect cats (and thus cat owners and veterinarians) against plague is needed. Additionally, plague is one of the factors responsible for the diminishing population of black-footed ferrets (*Mustela nigripes*) in the western United States. In addition to contracting plague themselves, this nearly extinct species relies solely on prairie dogs as its food source; thus epizootic episodes of plague in prairie dog populations in the black-footed ferrets' habitat contribute to the depletion of this species; see, for example, Williams et al., 1994, *J. Wildl. Dis., 30,* 581-585. Thus, methods to control plague in the wild reservoirs are also needed. It would be particularly useful to reduce plague in these wild reservoirs through the release of baited, orally delivered vaccines.

Current plague vaccine formulations suffer from significant problems. Commercially available vaccines include a formulation comprising formalin-killed *Yersinia pestis* (Cutter USP, available from Greer Laboratories, Lenoire, NC) and a formulation comprising a modified live *Yersinia pestis* (*Y. pestis* EV76-6). Although both of these vaccine formulations protect mice from lethal plague challenge (i.e., up to 5×10^3 50% lethal doses (LD_{50}s) for the killed vaccine and up to 5×10^6 LD_{50}s for the

modified live vaccine), these formulations result in severe side effects, and thus have not come under wide-spread use in either humans or other animals; see, for example, Russell et al., 1995, *Vaccine, 13*, 1551-1556. As used herein, the term "LD_{50}" refers to the number of bacterial cells in a dose that, when administered to a group of animals, will kill 50% of the animals in that group. When a strain of *Y. pestis* is administered to a group of mice, the LD_{50} is typically about one bacterial cell.

In several animal models, protection from challenge with virulent *Yersinia pestis* has been correlated with increased antibody titer toward the Fraction 1 (F1) capsular antigen. In previous studies, F1 antigen administered as an isolated subunit vaccine proved to be protective against plague challenge in mice, but less protective than the killed whole cell vaccines or avirulent live vaccines described above; see, for example, Simpson et al., 1990, *Am. J. Trop. Med. Hyg., 43,* 389-96. Furthermore, animals were not well protected by intragastric administration of the vaccine formulation; see, for example, Thomas et al., 1992, *Am. J. Trop. Med. Hyg., 47,* 92-7. The complete nucleotide sequence encoding a *Y. pestis* F1 antigen has been disclosed in Galyov et al., 1990, *FEBS Lett., 277,* 230-232, which is incorporated herein by reference.

Prokaryotic recombinant cell-based plague vaccines have also been disclosed. For example, a live recombinant *Salmonella typhimurium* expressing the F1 antigen was protective in mice against a low-level *Y. pestis* challenge, i.e., less than 50 LD_{50}s. This vaccine formulation was hampered by instability of the construct *in vivo;* see, for example PCT Publication No. WO 95/18231, published July 6, 1995, by Titball et al. Another example discloses vaccines comprising *Salmonella minnesota*, expressing F1; see, for example Russian Publication No. RU 2046145, published October 20, 1995, by Anisimov et al. However, the safety of live attenuated *Salmonella* vaccines is questionable, and different attenuated *Salmonella* species would be required to protect different animals due to the species specificity of *Salmonella* infection (e.g., *S. typhimurium* primarily infects mice and *S. typhi* primarily infects humans).

Another antigen being evaluated for vaccine potential is the V antigen on the *lcr* plasmid of *Y. pestis*. This protein is highly antigenic and when administered with F1 antigen, the two subunits are nearly as protective as the attenuated live *Y. pestis* vaccine; see, for example, Leary et al., 1995, *Infect. Immun., 63,* 2854-2858, Williamson et al.,

1995, *FEMS Immunol. Med. Microbiol., 12,* 223-230, and PCT Publication No. WO 95/24475, published September 14, 1995, by Titball et al. The V antigen, however, has been shown to be associated with suppression of gamma interferon and TNF-alpha *in vivo.*

Raccoon poxvirus (RCN) has been shown to be a very safe and effective vaccine vector in a variety of animal species, and particularly for cats. Protection against rabies, feline panleukopenia, and feline infectious peritonitis viruses has been demonstrated in cats vaccinated with recombinant RCN expressing antigens from the respective viruses; see, for example U.S. Patent No. 5,266,313, issued November 30, 1993, by Esposito et al., PCT Publication No. WO 93/01284, published January 21, 1993, by Scott et al, and European Publication No. EP 94306917.9, published May 10, 1995, by Wasmoen et al. A particularly attractive feature of recombinant raccoon poxvirus vaccine formulations in cats is that the vaccine can be delivered orally, which is a preferred administration route both for cats and for wildlife. Advantages of oral administration include a high incidence of injection site-associated sarcomas in cats, and the propensity of an orally delivered vaccine to induce mucosal immunity. The inventors are not aware of any prior use of raccoon poxvirus for the expression of an antigen from a bacterial pathogen. In fact, only one citation was found for the expression of a protein from a bacterial pathogen in an animal virus vector; see, PCT Publication No. WO 90/15872, published December 27, 1990, by Fischetti et al.

Similarly, nucleic acid immunization is apparently a safe, inexpensive method of protecting animals from disease; see, for example, Wolff et al., 1990, *Science 247,* 1465-1468. Nucleic acid vaccines are administered to an animal in a fashion to enable expression of protective proteins in the animal. A number of delivery methods for nucleic acid vaccines are known in the art including either intramuscular or intradermal injection, intradermal scarification using skin-test applicators, and particle bombardment (e.g. "gene-gun") delivery; see, for example, Raz et al., *Proc. Natl. Acad. Sci. USA, 93,* 5141-5145, U.S. Patent No. 5,204,253, issued April 20, 1993, by Bruner et al., and PCT Publication No. WO 95/19799, published July 27, 1995, by McCabe.

Summary of the Invention

The present invention relates to a novel recombinant molecule in which one or more nucleic acid molecules encoding antigens from *Yersinia, Pasteurella*, or *Francisella* are operatively linked to one or more eukaryotic transcription control regions, such that the antigen(s) are expressed in eukaryotic cells. Two primary embodiments of the present invention are a recombinant virus and a recombinant plasmid. Other embodiments include a recombinant animal virus genome and a recombinant eukaryotic cell. Also included in the present invention are methods to produce a recombinant molecule, a recombinant plasmid, a recombinant animal virus genome, a recombinant animal virus, and a recombinant eukaryotic cell of the present invention. Although it is uncommon to express a bacterial antigen in a live viral vector or in a eukaryotic cell, the idea is attractive because eukaryotic expression provides the possibility of inducing improved humoral, mucosal and cell-mediated immune responses.

The present invention also includes therapeutic compositions, such as vaccines, that are capable of protecting an animal from contracting plague. Therapeutic compositions of the present invention include recombinant molecules that include isolated nucleic acid molecules that encode *Yersinia, Pasteurella*, or *Francisella* antigens, operatively linked to eukaryotic transcription control regions. Such therapeutic compositions include a recombinant animal virus genome, a recombinant virus and a recombinant cell expressing one or more antigens derived from *Yersinia, Pasteurella*, or *Francisella,* and a recombinant plasmid that expresses one or more antigens from *Yersinia, Pasteurella*, or *Francisella* when the plasmid is delivered into a eukaryotic cell. A preferred therapeutic composition of the present invention also includes an excipient, an adjuvant and/or a carrier. Also included in the present invention is a method to protect an animal from plague, which includes administering to the animal a therapeutic composition of the present invention.

Another embodiment of the present invention is an isolated nucleic acid molecule encoding a *Yersinia pestis* antigen fused, in frame, with a eukaryotic membrane anchor domain.

Preferred embodiments of the present invention include (a) a recombinant raccoon poxvirus genome that includes an isolated nucleic acid molecule encoding a *Yersinia pestis* antigen operatively linked to a poxvirus transcription control region, (b) a recombinant raccoon poxvirus including such a recombinant genome, (c) a recombinant cell including such a recombinant genome, (d) a recombinant plasmid that includes an isolated nucleic acid molecule encoding a *Yersinia pestis* antigen operatively linked to a eukaryotic transcription control region, and (e) a recombinant cell that includes such a recombinant plasmid. A particularly preferred eukaryotic transcription control region is the human cytomegalovirus (HCMV) immediate-early promoter.

Particularly preferred embodiments of the present invention include, but are not limited to, an isolated nucleic acid molecule nYpF1anc$_{576}$ having nucleic acid sequence SEQ ID NO:7, and encoding protein PYpF1anc$_{192}$, having amino acid sequence SEQ ID NO:8, and the complement of SEQ ID NO:7; recombinant molecules vRCN-p11-nYpF1(a)sec$_{544}$, pCMV-nYpF1(b)sec$_{544}$, pCMV-nYpF1anc$_{576}$, and pCMV-nYpF1mat$_{474}$; recombinant virus RCN:p11-nYpF1(a)sec$_{544}$; and recombinant cell BSC-1:RCN:p11-nYpF1(a)sec$_{544}$.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses recombinant molecules that comprise isolated nucleic acid molecules encoding antigens from *Yersinia, Pasteurella*, or *Francisella* that are operatively linked to eukaryotic transcription control regions and, as such, are expressed under eukaryotic transcription control in eukaryotic cells. Also included in the present invention are therapeutic compositions comprising the claimed recombinant molecules, which are useful to protect animals from plague. Further included in the present invention are methods, using the claimed therapeutic compositions, to protect animals from plague.

Expression of *Yersinia, Pasteurella*, or *Francisella* antigens from eukaryotic transcription control regions is a novel aspect of the current invention. While not being bound by theory, the inventors believe that the current invention will have significant utility as a plague vaccine, in that the embodiments of the current invention will be efficacious in controlling plague in animal populations (including in wild animal populations); the claimed vaccines will be economical to make and use, will be safer,

and will have significantly reduced side-effects relative to currently available vaccines to control plague.

Preferred embodiments of the present invention include recombinant live virus vaccines and genetic immunization (i.e. naked nucleic acid) vaccines. Live virus vaccines and genetic immunization vaccines are advantageous because they are believed to confer more vigorous and longer-lasting immunity than subunit or killed vaccines. While not being bound by theory, it is believed that such advantages are due to the ability of the genetic information carried by the virus or the recombinant molecule to enter the cells of the treated animal, and to direct the expression of a protective compound, such as a protective protein or a protective RNA, for extended periods of time. Thus, therapeutic compositions of the present invention need not be administered frequently.

One particularly preferred embodiment of the present invention is an orally delivered plague vaccine for domestic cats. Cats are susceptible to the pneumonic form of the disease which is much more easily transmitted to other animals, including humans. While not being bound by theory, the inventors believe that oral delivery of a therapeutic composition of the present invention will induce mucosal immunity, which will more effectively control pneumonic plague.

As used herein, the terms *Yersinia, Pasteurella*, and *Francisella* refer to bacterial genera, and as such, include any species belonging to any of these genera. Particularly preferred bacterial species to target using embodiments of the present invention include *Yersinia pestis* (previously referred to as *Pasteurella pestis,* the name having changed about 1971) , *Yersinia pseudotuberculosis, Yersinia enterocolitica*, *Pasteurella multocida,* and *Francisella tularensis*, with *Y. pestis* being even more preferred, as this species is believed to be the most common etiologic agent of plague.

A *Yersinia, Pasteurella*, or *Francisella* antigen refers to an antigen derived from any portion of a *Yersinia, Pasteurella*, or *Francisella* bacterium that is capable of being expressed from an isolated nucleic acid molecule, and that, when administered to an animal as an immunogen, will produce a humoral, mucosal, and/or cellular immune response against *Yersinia, Pasteurella*, or *Francisella* in that animal. The ability of a candidate antigen to effect an immune response can be measured using techniques

known to those skilled in the art, some of which are disclosed herein. It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, an isolated nucleic acid molecule refers to one or more isolated nucleic acid molecules, or at least one isolated nucleic acid molecule. As such, the terms "a" (or "an), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. Furthermore, a compound "selected from the group consisting of" refers to one or more of the compounds in that group, including mixtures (i.e., combinations) of two or more of the compounds.

An antigen of the present invention includes not only full-length antigens but also homologs of full-length *Yersinia, Pasteurella*, or *Francisella* antigens, including smaller portions of such antigens. As used herein, the term homolog refers to any closely related antigen or epitope capable of eliciting an immune response to the native antigen. Examples of homologs include *Yersinia, Pasteurella*, or *Francisella* proteins in which amino acids have been deleted (e.g. a truncated version of the protein, such as a peptide), inserted, inverted, substituted, and/or derivatized (e.g., by glycosylation, phosphorylation, acetylation, myristylation, prenylation, palmitoylation, amidation and/or addition of glycerophosphatidyl inositol) such that the homolog includes at least one epitope capable of eliciting an immune response against *Yersinia, Pasteurella*, or *Francisella*. As used herein, an epitope refers to the smallest portion of an antigen that is capable of eliciting an immune response in an animal. The minimal size of a protein epitope, as defined herein, is about five amino acids. It is to be noted, however, that such an epitope might comprise a portion of the antigen other than the amino acid sequence, e.g., a carbohydrate moiety.

Yersinia, Pasteurella, or *Francisella* antigen homologs can also be the result of natural allelic or strain variation, natural mutation or laboratory-induced mutation in the genes that encode the antigens. As used herein, a gene includes all nucleic acid sequences related to a nucleic acid molecule that encodes a protein, such as regulatory regions that control production of the protein encoded by that nucleic acid molecule (such as, but not limited to, transcription, translation or post-translation control regions) as well as the coding region itself. An allelic variant, strain variant, or variant (used

interchangeably herein) of an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella*, or *Francisella* antigen refers to a nucleic acid molecule encoding an antigen at essentially the same locus (or loci) in the genome as the nucleic acid molecule in question but which, due to natural strain variations caused by, for example, mutation or recombination, has a similar but not identical nucleic acid sequence. Such variants typically encode proteins having similar activity to that of the protein encoded by the nucleic acid molecule to which they are being compared, but they do not necessarily have identical amino acid sequences. Allelic and strain variants can also comprise alterations in the 5' or 3' untranslated regions of a gene comprising the nucleic acid molecule (e.g., in regulatory control regions). Allelic and strain variants are well known to those skilled in the art and would be expected to be found to varying extents among the surface antigen genes of a pathogenic microorganism. Also included in the definition of variants are laboratory-induced mutants, such as variants arising due to errors incorporated into a nucleic acid molecule encoding an antigen during PCR amplification. Such errors can alter the nucleic acid sequence of a gene in question, and, as such, may also alter the amino acid sequence resulting in, for example, an amino acid substitution or the introduction of a stop (termination) codon, thus truncating the resultant antigen. Such a mutant is included as a variant if the gene in question encodes a protein having similar activity to that of the protein encoded by the gene to which it is being compared, e.g., in the case of the present invention, the protein must still be capable of eliciting an immune response in an animal against a *Yersinia, Pasteurella*, or *Francisella* native antigen.

It will be appreciated by those skilled in the art that a bacterial antigen expressed in a recombinant eukaryotic cell might be altered in ways that will vary its presentation to an animal's immune system. While not being bound by theory, the inventors believe that antigens secreted from a cell, anchored to the cell's membrane, or remaining in the cytoplasm of the cell will be processed and reacted to differently by an animal's immune system. In such a way, the immune response to a particular antigen can be altered from, for example, a completely humoral response to one that includes a shift toward a cellular immune response. Methods to introduce alterations into antigens of the current invention are known to those skilled in the art, and examples are disclosed herein. For

example, a nucleic acid molecule encoding a secreted protein can usually be converted into a nucleic acid molecule encoding a cytoplasmic protein by deleting the secretory signal segment from the nucleic acid molecule. Also, a nucleic acid molecule encoding a secreted protein can be engineered to be anchored to a cell's membrane by fusing a portion of a nucleic acid molecule encoding the antigen in-frame with an appropriate heterologous nucleic acid molecule encoding a membrane anchor domain.

Yersinia, Pasteurella, or *Francisella* antigens of the current invention can include any antigen, including homologs thereof, derived from these bacterial genera, that, when administered to an animal as an immunogen, using techniques known to those skilled in the art, will produce a humoral, mucosal, and/or cellular immune response against *Yersinia, Pasteurella,* or *Francisella* in that animal. Preferred *Yersinia, Pasteurella,* and *Francisella* antigens include *Yersinia pestis, Yersinia pseudotuberculosis, Yersinia enterocolitica, Pasteurella multocida,* and *Francisella tularensis* antigens. Preferred *Yersinia pestis* antigens of the present invention include F1 antigens, V antigens, pesticin antigens, W antigens, pH 6 antigens, superoxide dismutase antigens, *Yersinia* outer protein (YOP) antigens, high molecular weight iron-regulated membrane protein antigens, murine toxin antigens, and/or hemin storage protein antigens. More preferred *Yersinia pestis* antigens of the present invention include F1 antigens and V antigens. An even more preferred *Yersinia pestis* antigen is an F1 antigen. The most preferred antigens of the present invention include $PYpF1sec_{170}$, $PYpF1anc_{192}$, $PYpF1anc_{171}$, $PYpF1mat_{150,}$ and $PYpF1mat_{149,}$ the amino acid sequences of which are presented herein as SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:21, respectively, as well as homologs and smaller portions, as small as a single epitope, of such antigens. It is to be understood that other *Yersinia, Pasteurella,* or *Francisella* species, share at least some of the above preferred antigens with *Y. pestis* (see, for example, Carter et al., 1980, *Infect. Immun., 28,* 638-40), but also comprise other antigens useful in the present invention.

As used herein, the term "isolated nucleic acid molecule" refers to a nucleic acid molecule derived, at least partially, from *Yersinia, Pasteurella,* or *Francisella.* An isolated nucleic acid molecule is a nucleic acid molecule that has been removed from its natural milieu. As such, the term "isolated" does not necessarily reflect the extent to

which the nucleic acid molecule has been purified. An isolated nucleic acid molecule can include DNA, RNA, or derivatives of either DNA or RNA. An isolated nucleic acid molecule can be single-stranded or double-stranded. Certain isolated nucleic acid molecules for which the nucleic acid sequence of the coding strand is disclosed in a SEQ ID NO are also recognized to include a complementary strand, the nucleic acid sequence of which can be easily determined by one skilled in the art; such complementary sequences are included as part of the present invention. An isolated nucleic acid molecule can be obtained from its natural source, or can be produced using, for example, recombinant nucleic acid technology or chemical synthesis.

According to the present invention, an isolated nucleic acid molecule encodes at least one *Yersinia, Pasteurella,* or *Francisella* antigen, examples of such antigens being disclosed herein. In one embodiment, the antigen is expressed by (i.e. under the direction of) a recombinant molecule of which the isolated nucleic acid molecule is a part, via operative linkage of that nucleic acid molecule to a eukaryotic transcription control region. Although the phrase "nucleic acid molecule" primarily refers to the physical nucleic acid molecule and the phrase "nucleic acid sequence" primarily refers to the sequence of nucleotides on the nucleic acid molecule, the two phrases can be used interchangeably, especially with respect to a nucleic acid molecule, or a nucleic acid sequence, being capable of encoding a *Yersinia, Pasteurella,* or *Francisella* antigen.

An isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen can be produced using a number of methods known to those skilled in the art; see, for example, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, and Ausubel et al.,1993, *Current Protocols in Molecular Biology*, Greene/Wiley Interscience. Sambrook et al, ibid., and Ausubel et al., ibid., are incorporated by reference herein in their entireties. For example, nucleic acid molecules can be produced and/or modified using a variety of techniques including, but not limited to, by classic mutagenesis and recombinant DNA techniques (e.g., site-directed mutagenesis, chemical treatment, restriction enzyme cleavage, ligation of nucleic acid fragments and/or PCR amplification), or synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic acid molecules and combinations thereof.

Isolated nucleic acid molecules of the present invention may also (a) contain secretory signal segments (i.e., nucleic acid sequences encoding a secretory signal peptide) to enable an expressed *Yersinia*, *Pasteurella* or *Francisella* antigen of the present invention to be secreted from the cell that produces the antigen, (b) contain nucleic acid sequences encoding membrane anchor domains that lead to the expression of nucleic acid molecules of the present invention as antigens anchored to the cell membrane of the host cell such that the antigenic domain is outside of the cell, and/or (c) contain other fusion sequences which lead to the expression of nucleic acid molecules of the present invention as various fusion proteins. Nucleic acid sequences encoding signal peptides, membrane anchor domains or other protein domains are fused in-frame with isolated nucleic acid molecules encoding *Yersinia*, *Pasteurella* or *Francisella* antigens of the present invention by methods known to those skilled in the art. As used herein, the term "fused in-frame" indicates that two or more heterologous nucleic acid molecules are combined such that a single contiguous amino acid sequence is encoded. Examples of suitable signal segments and membrane anchor segments are disclosed herein. Isolated nucleic acid molecules of the present invention may also include intervening and/or untranslated sequences surrounding and/or within the isolated *Yersinia, Pasteurella,* or *Francisella* nucleic acid sequences.

Suitable signal segments include any signal segment encoding a signal peptide capable of directing the secretion of an antigen of the present invention. As used herein, the term "signal segment" refers to a nucleic acid molecule encoding a secretory signal peptide, and the term "signal peptide" refers to the peptide domain capable of directing secretion of an antigen of the present invention. Typically, signal segments encode peptides of about 15 to 50 amino acids in length, and are located at the 5' end of a nucleic acid molecule encoding a secreted protein, but they can be located at other (i.e., internal) positions within a nucleic acid molecule. Preferred signal segments include, but are not limited to, endogenous signal segments of the isolated *Yersinia, Pasteurella,* or *Francisella* nucleic acid molecules of the present invention, as well as tissue plasminogen activator (t-PA) , interferon, interleukin, growth hormone, histocompatibility, and viral envelope glycoprotein signal segments.

Suitable membrane anchor segments include any membrane anchor segment that encodes a peptide domain capable of anchoring an antigen of the present invention into a eukaryotic cell membrane. As used herein, the term "membrane anchor segment" refers to the nucleic acid molecule encoding a peptide domain capable of anchoring an antigen of the present invention to a eukaryotic cell membrane. Typically, a membrane anchor segment encodes a protein domain comprising hydrophobic amino acids. Membrane anchor segments can be located either at the 5' end or the 3' end of a nucleic acid molecule encoding an anchored protein. A membrane anchor segment located at the 5' end (e.g., as in a class II transmembrane glycoprotein gene) usually also functions as a secretory signal segment. Preferred membrane anchor segments include, but are not limited to, vesicular stomatitis virus (VSV) glycoprotein, respiratory syncytial virus G protein, herpesvirus glycoprotein, immunoglobulin, and glycosyl-phosphotidylinositol membrane anchor segments. Particularly preferred membrane anchor segments include the canine herpesvirus glycoprotein G, glycoprotein E and glycoprotein I membrane anchor segments.

Suitable intervening and/or untranslated sequences include, but are not limited to, any sequences that enhance or regulate expression of a *Yersinia, Pasteurella,* or *Francisella* antigen of the present invention. Preferred untranslated sequences include the human cytomegalovirus (HCMV) intron-A sequence and the encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES).

Isolated nucleic acid molecules of the present invention can include any nucleic acid molecule capable of encoding a *Yersinia, Pasteurella,* or *Francisella* antigen of the current invention. Preferred isolated nucleic acid molecules encode at least one *Yersinia pestis* antigen of the present invention, including, but not limited to, an F1 antigen, a V antigen, a pesticin antigen, a W antigen, a pH 6 antigen, a superoxide dismutase antigen, a YOP antigen, a high molecular weight iron-regulated membrane protein antigen, a murine toxin antigen, and a hemin storage protein antigen. More preferred isolated nucleic acid molecules encode a *Yersinia pestis* F1 antigen and /or a V antigen. An even more preferred isolated nucleic acid molecule encodes a *Yersinia pestis* F1 antigen. Particulary preferred isolated nucleic acid molecules of the present invention include nYpF1(a)sec_{544}, nYpF1(b)sec_{544}, nYpF1sec_{510}, nYpF1anc_{576}, nYpF1anc_{513}, nYpF1mat_{474},

nYpF1mat$_{450}$, and nYpF1mat$_{447}$, the coding strand nucleotide sequences of which are represented herein as SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:22, respectively, as well as variants of these nucleic acid molecules and/or smaller portions of these nucleic acid molecules, capable of encoding at least one epitope (i.e., at least about 15 nucleotides). It is to be understood that isolated nucleic acid molecules encoding antigens from other *Yersinia, Pasteurella,* or *Francisella* species, as disclosed above, are also useful in the present invention.

Isolated nucleic acid molecules nYpF1(a)sec$_{544}$ and nYpF1(b)sec$_{544}$, the nucleic acid sequence of the coding strands of which are denoted herein as SEQ ID NO:1 and SEQ ID NO:4, respectively, each encode a full-length, unprocessed F1 antigen of 170 amino acids, denoted herein as PYpF1sec$_{170}$, having an amino acid sequence denoted herein as SEQ ID NO:2, assuming an initiation codon extending from about nucleotide 17 through about nucleotide 19 of SEQ ID NO:1 or SEQ ID NO:4, respectively, and a stop codon extending from about nucleotide 527 through about nucleotide 529 of SEQ ID NO:1 or SEQ ID NO:4, respectively. As disclosed in Galyov, et al., *ibid.*, PYpF1sec$_{170}$ includes an N-terminal signal peptide sequence of about 21 amino acids extending from about amino acid 1 to about amino acid 21 of SEQ ID NO:2. The mature (i.e., processed) form of PYpF1sec$_{170}$ is represented by PYpF1mat$_{149}$, having the amino acid sequence SEQ ID NO:21. PYpF1mat$_{149}$ is encoded by nucleic acid molecule nYpF1mat$_{447}$, having the coding strand nucleotide sequence represented by SEQ ID NO:22, assuming a first codon extending from about nucleotide 1 through about nucleotide 3 of SEQ ID NO:22. The coding region encoding PYpF1sec$_{170}$, not including the stop codon, is represented by nucleic acid molecule nYpF1sec$_{510}$, having the coding strand nucleic acid sequence represented by SEQ ID NO:3.

Isolated nucleic acid molecule nYpF1mat$_{474}$, denoted herein as SEQ ID NO:11, encodes PYpF1mat$_{150}$, a predicted mature F1 antigen of about 150 amino acids, the sequence of which is presented herein as SEQ ID NO:12, assuming an initiation codon extending from about nucleotide 7 through about nucleotide 9 of SEQ ID NO:11 and a stop codon extending from about nucleotide 457 through about nucleotide 459 of SEQ ID NO:11. The coding region encoding PYpF1mat$_{150}$, not including the stop codon, is

represented by nucleic acid molecule nYpF1mat$_{450}$, having the coding strand nucleic acid sequence represented by SEQ ID NO:13. While not being bound by theory, an expressed antigen encoded by nYpF1mat$_{474}$ would be expected to stay in the cytoplasm of the cell in which it is expressed, since it lacks a secretory signal segment.

Isolated nucleic acid molecule nYpF1anc$_{576}$, the coding strand of which is denoted herein as SEQ ID NO:7, comprises a coding region, not including the stop codon, encoding PYpF1anc$_{192}$, a novel fusion protein of about 192 amino acids comprising an F1 antigen of about 134 amino acids linked to the membrane anchor domain of the canine herpesvirus (CHV) glycoprotein G (i.e., about amino acid 358 through about amino acid 415 of CHV gG, disclosed as SEQ ID NO:10 in pending U.S. Patent Application Serial No. 08/602,010, by Haanes, et al., filed Feb. 15, 1996; this application is incorporated herein by reference in its entirety). Methods to construct nYpF1anc$_{576}$ are disclosed herein. While not being bound by theory, a protein encoded by nYpF1anc$_{576}$ would be expected to be secreted from the cytoplasm of a eukaryotic cell such that its C-terminal region is lodged in the cell's plasma membrane, and its N-terminal region is extending outside the cell. The amino acid sequence of PYpF1anc$_{192}$ is presented herein as SEQ ID NO:8, assuming an initiation codon extending from about nucleotide 1 through about nucleotide 3 of SEQ ID NO:7. PYpF1anc$_{192}$ includes an N-terminal signal peptide sequence of about 21 amino acids extending from about amino acid 1 to about amino acid 21 of SEQ ID NO:8. The mature form of PYpF1anc$_{192}$ is represented by PYpF1anc$_{171}$, having the amino acid sequence SEQ ID NO:10. PYpF1anc$_{171}$ is encoded by nucleic acid molecule nYpF1anc$_{513}$, having the coding strand nucleotide sequence represented by SEQ ID NO:9.

Another embodiment of the present invention is an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen that also includes at least one additional isolated nucleic acid molecule fused in-frame such that a multivalent antigen is encoded. Such a multivalent antigen can be produced by joining two or more isolated nucleic acid molecules together in such a manner that the resulting nucleic acid molecule is expressed as a multivalent antigen containing epitopes from at least two heterologous antigens, or portions thereof. Such a multivalent antigen can comprise two or more isolated nucleic acid molecules encoding *Yersinia, Pasteurella* or *Francisella*

antigens, or can comprise one or more isolated nucleic acid molecules in addition to those encoding *Yersinia, Pasteurella* or *Francisella* antigens, such that the multivalent antigen is capable of protecting an animal from diseases caused by other infectious agents in addition to *Yersinia, Pasteurella* or *Francisella.*

Examples of multivalent antigens include, but are not limited to, *Yersinia, Pasteurella* or *Francisella* antigen of the present invention attached to one or more antigens protective against one or more other infectious agents, such as, but not limited to: viruses (e.g., caliciviruses, distemper viruses, hepatitis viruses, herpesviruses, immunodeficiency viruses, infectious peritonitis viruses, leukemia viruses, panleukopenia viruses, parvoviruses, picornaviruses, rabies viruses, other cancer-causing or cancer-related viruses); bacteria (e.g., *Leptospira, Rochalimaea*); fungi and fungal-related microorganisms (e.g., *Candida, Cryptococcus, Histoplasma*); and other parasites (e.g., *Babesia, Cryptosporidium, Eimeria, Encephalitozoon, Hepatozoon, Isospora, Microsporidia, Neospora, Nosema, Plasmodium, Pneumocystis, Toxoplasma*, as well as helminth parasites).

A recombinant molecule of the present invention includes at least one isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen, operatively linked to a eukaryotic transcription control region. Such a molecule contains heterologous nucleic acid sequences, that is, nucleic acid sequences that are not naturally found adjacent to the isolated nucleic acid molecules of the present invention and that are derived from species other than *Yersinia, Pasteurella,* or *Francisella.* A recombinant molecule can be either RNA or DNA, can have components from prokaryotic as well as eukaryotic sources, and must have the ability, by methods described herein, to enter eukaryotic cells and direct expression of isolated nucleic acid molecules of the present invention in those eukaryotic cells. In the case of the present invention, the recombinant molecule is typically a recombinant animal virus genome or a recombinant plasmid.

According to the present invention, an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella* or *Francisella* antigen is operatively linked to a eukaryotic transcription control region. The phrase "operatively linked" refers to the combining of an isolated nucleic acid molecule of the present invention with a eukaryotic

transcription control region in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, a eukaryotic transcription control region is a nucleic acid sequence which controls the initiation, elongation and termination of transcription in a eukaryotic cell. Particularly important transcription control regions are those which control transcription initiation, such as promoter and enhancer sequences. Suitable transcription control regions include any transcription control region that can function in at least one recombinant eukaryotic cell of the present invention. A variety of such transcription control regions are known to those skilled in the art. Preferred transcription control regions include those which function in mammalian cells, such as, but not limited to, promoter and enhancer sequences from alphaviruses (such as Sindbis virus), vaccinia virus, raccoon poxvirus, other poxviruses, adenovirus, adeno-associated virus, cytomegaloviruses (preferably the intermediate early promoter, preferably in conjunction with intron-A), other herpesviruses, simian virus 40 (preferably the early promoter), retroviruses (such as Rous sarcoma virus), and picornaviruses (particularly an internal ribosome entry site, or IRES, enhancer region). Other preferred transcription control regions include those derived from mammalian genes such as actin, heat shock protein, bovine growth hormone transcription control regions, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins).

One type of recombinant molecule of the present invention comprises an animal virus genome. Such a recombinant molecule contains an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen of the present invention, operatively linked to at least one eukaryotic transcription control region capable of effectively regulating expression of the nucleic acid molecule(s) in the cell to be transformed. Eukaryotic transcription control regions for recombinant virus genomes of the present invention include any that would function in the virus of choice. As used herein, a recombinant animal virus genome can comprise a heterologous eukaryotic transcription control region, i.e., a transcription control region that is non-native to the particular animal virus genome, being, for example, derived from another animal virus

genome or from any other suitable eukaryotic gene. Suitable heterologous transcription control regions are disclosed herein. A recombinant animal virus genome of the present invention can also comprise an endogenous eukaryotic transcription control region, i.e., a transcription control region that is normally found in that virus. Such endogenous transcription control regions can be situated at their normal (i.e., natural) position in the viral genome, or at a non-natural position, i.e. inserted into another viral gene or into an intergenic region of the viral genome. Suitable recombinant virus genomes of the present invention include a poxvirus genome, a herpesvirus genome, an alphavirus genome (for example, from a Sindbis virus), a picornavirus genome (for example, from a poliovirus or a mengovirus), a retrovirus genome, an adenovirus genome, or an adeno-associated virus genome. A preferred recombinant virus genome of the present invention comprises a poxvirus genome, for example, an orthopoxvirus, a parapoxvirus, an entomopoxvirus, or an avipoxvirus (i.e., fowlpox) genome. A more preferred recombinant virus genome comprises an orthopoxvirus genome, particularly a vaccinia virus or a raccoon poxvirus genome.

Preferred transcription control regions for a recombinant virus genome of the present invention include those that function in poxviruses. It will be appreciated by those skilled in the art that poxviruses undergo all aspects of viral replication, including transcription, in the cytoplasm of the infected host cell, and as such, have specialized, viral-encoded transcription-related proteins and recognition sequences. In particular, transcription of genes encoded on a poxvirus genome requires a poxvirus promoter. Examples of poxvirus promoters include early/late promoters (i.e., working at early and late times in the infectious cycle of the virus) and late promoters. Particularly preferred poxvirus promoters include the vaccinia virus p7.5 (early/late) promoter (see, for example, Cochran et al., 1985, *J. Virol. 54*, 30-37), the vaccinia virus p11 (late) promoter (see, for example, Bertholet et al., 1986, *EMBO J. 5*, 1951-1957), and the synthetic pSYN (late) promoter (see, for example, Davison, et al., 1990, *Nucl. Acids Res. 18*, 4825-4826).

Many recombinant virus genomes of the present invention, particularly poxvirus genomes, are very large. One skilled in the art will know that a portion of genes in such viruses are dispensable for growth of the virus (often referred to as non-essential genes).

As such, one or more isolated nucleic acid molecules encoding a *Yersinia, Pasteurella,* or *Francisella* antigens, operatively linked to eukaryotic transcription control regions suitable for a particular virus, can be located (preferably by insertion) in one or more non-essential genes. Alternatively, an isolated nucleic acid molecule can be located between genes in the viral genome, i.e., in an intergenic region. In some recombinant virus genomes, an isolated nucleic acid molecule of the present invention can be located in an essential gene. If the resulting recombinant virus is to undergo replication, this latter group of insertions requires that the essential gene be complemented in the infected host cell, either by that cell being stably transformed with the essential gene, transiently transformed with the essential gene, or having that gene supplied on a helper virus.

In a recombinant vaccinia virus or raccoon poxvirus genome of the present invention, an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen can be located in any suitable non-essential gene or intergenic region. Preferred non-essential genes include a thymidine kinase gene, a hemagglutination gene, an anti-inflammatory gene, and an A-type inclusion gene. Preferred anti-inflammatory genes include a soluble cytokine receptor gene, a serpin gene, a complement receptor gene and an immunoglobulin receptor gene. Particularly preferred non-essential genes are the thymidine kinase genes of raccoon poxvirus and vaccinia virus.

Another recombinant molecule of the present invention comprises a recombinant plasmid which includes an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen operatively linked to a eukaryotic transcription control region. A recombinant plasmid of the current invention has utility as a genetic immunization vaccine to protect an animal against plague. A preferred recombinant plasmid contains an origin of replication for propagation in a bacterial host (e.g., *Escherichia coli*), a mode of selection, such as an antibiotic resistance gene, and a suitable cloning site for isolated nucleic acid molecules of the present invention. Any suitable eukaryotic transcription control region can be used, such as, but not limited to, those disclosed herein. Particularly preferred transcription control regions include, but are not limited to, a HCMV immediate-early promoter (preferably in conjunction with

intron-A), a Rous sarcoma virus long terminal repeat, and an SV40 early promoter. The incorporation of polyadenylation sequences, for example, bovine growth hormone or SV40 polyadenylation sequences, is also preferred. A preferred recombinant plasmid can also include an enhancer region, for example, a HCMV intron-A sequence or an EMCV-IRES sequence.

A recombinant molecule of the present invention is a molecule that can include at least one of any isolated nucleic acid molecule that encodes an antigen from *Yersinia, Pasteurella,* or *Francisella*, operatively linked to at least one eukaryotic transcription control region capable of effectively regulating expression of the nucleic acid molecule(s) in the cell to be transformed, examples of which are disclosed herein. Preferred recombinant molecules include at least one of the following nucleic acid molecules: nYpF1(a)sec_{544}, nYpF1(b)sec_{544}, nYpF1sec_{510}, nYpF1anc_{576}, nYpF1anc_{513}, nYpF1mat_{474}, nYpF1mat_{450}, and nYpF1mat_{447}. Particularly preferred recombinant molecules of the present invention include vRCN-p11-nYpF1(a)sec_{544}, vRCN-p11-nYpF1anc_{576}, vRCN-p11-nYpF1mat_{474}, pCMV-nYpF1(b)sec_{544}, pCMV-nYpF1anc_{576}, and pCMV-nYpF1mat_{474}. The term "vRCN" refers to a recombinant raccoon poxvirus genome and the term "pCMV" refers to a recombinant plasmid comprising the HCMV immediate-early transcription control region. Details regarding the production of recombinant molecules of the present invention are disclosed herein.

Another embodiment of the present invention is a recombinant virus. A recombinant virus of the present invention includes any animal virus comprising a suitable recombinant molecule, i.e. a recombinant virus genome, of the present invention. Suitable recombinant viruses of the present invention include poxviruses, herpesviruses, alphaviruses (for example Sindbis virus), picornaviruses (for example, poliovirus or mengovirus), retroviruses, adenoviruses, and adeno-associated viruses. A preferred recombinant virus of the present invention comprise a poxvirus, for example, an orthopoxvirus, a parapoxvirus, an entomopoxvirus, or an avipoxvirus (i.e., fowlpox). A more preferred recombinant virus comprises a recombinant orthopoxvirus, particularly a vaccinia virus or a raccoon poxvirus. An example of a more preferred embodiment of the present invention is a recombinant raccoon poxvirus (RCN) comprising a nucleic acid molecule encoding an F1 antigen of *Yersinia pestis* operatively linked to a vaccinia

virus p11 promoter, as disclosed in Examples 1 and 2 below. Particularly preferred recombinant viruses of the present invention comprise recombinant raccoon poxviruses RCN:p11-nYpF1(a)sec_{544}, RCN:p11-nYpF1anc_{576}, and RCN:p11-nYpF1mat_{474}, with RCN:p11-nYpF1(a)sec_{544} being the more preferred.

One embodiment of the present invention is an attenuated recombinant virus. As used herein, an attenuated virus is a virus that results in less pathogenicity than its wild-type counterpart when used to infect an animal. A preferred attenuated virus of the present invention causes little or no pathogenicity when used to infect an animal. An attenuated recombinant virus can be produced by inactivating a viral gene that, due to that gene's inactivation, results in an attenuated virus. Methods to inactivate a gene are disclosed herein. An attenuated recombinant virus can be identified by exposing animals to the virus and measuring clinical signs, such as fever, lesions, or viremia, in those animals compared to similar animals exposed to the wild-type virus. Clinical signs to measure vary with each individual virus, and are known to one skilled in the art. Suitable viral genes to inactivate in order to produce an attenuated recombinant virus include any gene that when inactivated leads to an attenuated virus. A preferred attenuated recombinant virus of the present invention is a virus having a recombinant genome in which a heterologous nucleic acid molecule, i.e., one encoding a *Yersinia, Pasteurella,* or *Francisella* antigen, is inserted into a viral gene, the insertion resulting in an attenuated virus. A particularly preferred attenuated recombinant virus of the present invention is an attenuated recombinant vaccinia virus or raccoon poxvirus. A particularly preferred method of attenuation of a recombinant vaccinia virus or raccoon poxvirus is by insertion of a heterologous nucleic acid molecule into the thymidine kinase (tk) locus.

An attenuated recombinant virus of the present invention, particulary an attenuated recombinant raccoon poxvirus, has utility, for example, as a therapeutic composition to protect an animal from plague. While not being bound by theory, the inventors believe that a recombinant raccoon poxvirus need not be further attenuated for use as a live viral vaccine in most animals due to the low pathogenicity of wild-type RCN. See, for example, Esposito, et al., 1989, *Vaccines 89*, Cold Spring Harbor Labs Press, 403-408.

Another embodiment of the present invention is a recombinant cell comprising a eukaryotic host cell transformed with at least one of any recombinant molecule of the present invention. Suitable and preferred recombinant molecules with which to transform cells are disclosed herein. The terms "transform" or "transformed", as used in the present invention, refer to any way in which a recombinant molecule can be inserted into a cell. Transformation techniques include, but are not limited to, transfection, viral infection with a recombinant virus, viral transduction with a recombinant virus, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism.

Suitable host cells to transform include any cell that can be transformed with a recombinant molecule of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule (e.g., nucleic acid molecules encoding one or more proteins of the present invention and/or other proteins useful in the production of a multivalent vaccines). Host cells of the present invention can be any cell capable of producing at least one antigen of the present invention. Preferred host cells primarily include mammalian cells. Most preferred host cells include BHK (baby hamster kidney) cells, MDCK cells (normal dog kidney cell line), CRFK cells (normal cat kidney cell line), BSC-1 cells (African monkey kidney cell line used, for example, to culture raccoon poxvirus), COS (e.g., COS-7) cells, and Vero cells. Particularly preferred host cells are BHK cells, BSC-1 cells, MDCK cells, CRFK cells, CV-1 cells, COS cells, Rat-2 cells, Vero cells, and non-tumorigenic mouse myoblast G8 cells (e.g., ATCC CRL 1246). Additional appropriate cell hosts include other kidney cell lines, other fibroblast cell lines (e.g., human, murine or chicken embryo fibroblast cell lines), myeloma cell lines, Chinese hamster ovary cells, mouse NIH/3T3 cells, $LMTK^{31}$ cells and/or HeLa cells.

A recombinant cell of the present invention can include any eukaryotic host cell transformed with at least one of any recombinant molecule of the present invention. A preferred recombinant cell includes at least one isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen operatively linked to at least one eukaryotic transcription control region capable of effectively regulating expression of the

nucleic acid molecule(s) in that cell; particularly preferred nucleic acid molecules to include are nYpF1(a)sec_{544}, nYpF1(b)sec_{544}, nYpF1sec_{510}, nYpF1anc_{576}, nYpF1anc_{513}, nYpF1mat_{474}, nYpF1mat_{450}, and/or nYpF1mat_{447}. A more preferred recombinant cell includes one or more of the following recombinant molecules: vRCN-p11-nYpF1(a)sec_{544}, vRCN-p11-nYpF1anc_{576}, vRCN-p11-nYpF1mat_{474}, pCMV-nYpF1(b)sec_{544}, pCMV-nYpF1anc_{576}, and pCMV-nYpF1mat_{474}. Particularly preferred recombinant cells of the present invention include BSC-1:RCN:p11-nYpF1(a)sec_{544}, BSC-1:RCN-p11-nYpF1anc_{576}, BSC-1:RCN-p11-nYpF1mat_{474}, as well as any animal cells comprising pCMV-nYpF1(a)sec_{544}, pCMV-nYpF1anc_{576}, and pCMV-nYpF1mat_{474}.

One embodiment of the present invention is a therapeutic composition that, when administered to an animal in an effective manner, is capable of protecting that animal from plague. Therapeutic compositions of the present invention include a recombinant molecule comprising an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen operatively linked to a eukaryotic transcription control region. Suitable therapeutic compositions include recombinant animal virus genomes, recombinant viruses, recombinant plasmids and recombinant cells as disclosed herein. In order to protect an animal from plague, a therapeutic composition of the present invention is administered to the animal in an effective manner prior to infection in order to prevent disease, reduce disease symptoms and/or prevent transmission of the disease from asymptomatic carriers (i.e., as a preventative vaccine). Therapeutic compositions of the present invention can be administered to any animal susceptible to such therapy, preferably to mammals, and more preferably to cats, primates, rodents, ungulates, bears, dogs, camels, and pigs. Preferred animals to protect against plague include domestic cats, humans, bobcats, cougars, domestic dogs, coyotes, foxes, rock squirrels, ground squirrels, prairie dogs, black footed ferrets, domestic ferrets, pronghorn antelope, badgers, bears, wild boars, domestic pigs, camels, chipmunks, red deer, mule deer, fishers, foxes, gerbils, martens, urban mice, wild mice, polecats, rabbits, urban rats, wild rats, tree squirrels, and voles. Even more preferred animals to protect against plague include domestic cats, domestic dogs, humans, rock squirrels, California ground squirrels, prairie dogs, domestic ferrets, and black-footed ferrets.

As used herein, the term plague refers to the group of diseases most normally caused by the bacterium *Yersinia pestis*, but also, in some cases, similar diseases caused by other species within the genera *Yersinia, Pasteurella,* or *Francisella*. As such, plague includes, but is not limited to, diseases such as bubonic plague, septicemic plague, pneumonic plague, urban plague, rat plague, wild rodent plague, sylvatic plague, campestral plague, high plains plague, disseminated intravascular coagulopathy, la peste bubonique, The Pest, The Black Plague, and The Black Death.

Another embodiment of the present invention is a therapeutic composition to protect an animal from plague that also includes at least one additional isolated nucleic acid molecule encoding an antigen from a pathogen other than *Yersinia, Pasteurella* or *Francisella*, operatively linked to one or more eukaryotic transcription control regions, such that a multivalent therapeutic composition is produced. Such a multivalent therapeutic composition can be produced by combining one or more additional isolated nucleic acid molecules into a recombinant molecule of the present invention, or by combining one or more recombinant molecules with a recombinant molecule of the present invention. When administered to an animal, such a multivalent therapeutic composition is able to direct the expression of one or more antigens in the cells of that animal such that the animal is protected from diseases caused by other infectious agents in addition to *Yersinia, Pasteurella* or *Francisella*.

Examples of multivalent therapeutic compositions include, but are not limited to, a *Yersinia, Pasteurella* or *Francisella* antigen of the present invention plus one or more antigens protective against one or more other infectious agents, such as, but not limited to: viruses (e.g., caliciviruses, distemper viruses, hepatitis viruses, herpesviruses, immunodeficiency viruses, infectious peritonitis viruses, leukemia viruses, panleukopenia viruses, parvoviruses, picornaviruses, rabies viruses, other cancer-causing or cancer-related viruses); bacteria (e.g., *Leptospira*, *Rochalimaea*); fungi and fungal-related microorganisms (e.g., *Candida*, *Cryptococcus*, *Histoplasma*); and other parasites (e.g., *Babesia*, *Cryptosporidium*, *Eimeria*, *Encephalitozoon*, *Hepatozoon*, *Isospora*, *Microsporidia*, *Neospora*, *Nosema*, *Plasmodium*, *Pneumocystis*, *Toxoplasma*, as well as helminth parasites).

Therapeutic compositions of the present invention can be formulated in an excipient that the animal to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosol, m- or o-cresol, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration.

In one embodiment of the present invention, a therapeutic composition can include an adjuvant. Adjuvants are agents that are capable of enhancing the immune response of an animal to a specific antigen. Suitable adjuvants include, but are not limited to, cytokines, chemokines, and compounds that induce the production of cytokines and chemokines (e.g., granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), erythropoietin (EPO), interleukin 2 (IL-2), interleukin-3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12), interferon gamma, interferon gamma inducing factor I (IGIF), transforming growth factor beta, RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage inflammatory proteins (e.g., MIP-1 alpha and MIP-1 beta), and *Leishmania* elongation initiating factor (LEIF)); bacterial components (e.g., endotoxins, in particular superantigens, exotoxins and cell wall components); aluminum-based salts; calcium-based salts; silica; polynucleotides; toxoids; serum proteins, viral coat proteins; block copolymer adjuvants (e.g., Hunter's Titermax™ adjuvant (Vaxcel™, Inc. Norcross, GA), Ribi adjuvants (Ribi ImmunoChem

Research, Inc., Hamilton, MT); and saponins and their derivatives (e.g., Quil A (Superfos Biosector A/S, Denmark). Protein adjuvants of the present invention can be delivered in the form of the protein themselves or of nucleic acid molecules encoding such proteins using the methods described herein.

In one embodiment of the present invention, a therapeutic composition can include a carrier. Carriers include compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release vehicles, biodegradable implants, liposomes, bacteria, viruses, other cells, oils, esters, and glycols. While not being bound by theory, an advantage of a therapeutic composition comprising a recombinant virus is that a carrier is usually not required.

One embodiment of the present invention is a controlled release formulation that is capable of slowly releasing a composition of the present invention into an animal. As used herein, a controlled release formulation comprises a composition of the present invention in a controlled release vehicle. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. Other controlled release formulations of the present invention include liquids that, upon administration to an animal, form a solid or a gel *in situ*. Preferred controlled release formulations are biodegradable (i.e., bioerodible).

Some preferred therapeutic compositions of the present invention include at least a portion of a recombinant virus genome, comprising a recombinant virus vaccine. Preferred recombinant virus genomes include those based on alphaviruses, poxviruses, adenoviruses, adeno-associated viruses, picornaviruses, herpesviruses, and retroviruses, with those based on poxviruses being particularly preferred.

A therapeutic composition comprising a recombinant virus of the present invention includes a recombinant molecule of the present invention that is packaged in a viral coat and that can be expressed in an animal after administration. Preferably, the recombinant molecule produces attenuated virus. A number of recombinant viruses can be used, including, but not limited to, those based on alphaviruses, poxviruses,

adenoviruses, adeno-associated viruses, picornaviruses, herpesviruses, and retroviruses. Preferred recombinant virus vaccines are those based on poxviruses.

A therapeutic composition comprising a genetic immunization (i.e., naked nucleic acid) vaccine of the present invention includes an isolated nucleic acid molecule of the present invention operatively linked to a eukaryotic transcription control region in a recombinant molecule of the present invention. A genetic immunization vaccine of the present invention can comprise one or more nucleic acid molecules of the present invention in the form of, for example, a dicistronic recombinant molecule. Any suitable eukaryotic transcription control region can be used. Particularly preferred transcription control regions include the HCMV intermediate early promoter (preferably in conjunction with intron-A), Rous sarcoma virus long terminal repeat, and tissue-specific transcription control regions, as well as transcription control regions endogenous to viral vectors if viral vectors are used. The incorporation of polyadenylation sequences and enhancers are also preferred.

A recombinant cell vaccine of the present invention includes recombinant eukaryotic cells of the present invention that express one or more *Yersinia, Pasteurella,* or *Francisella* antigens of the present invention. Preferred recombinant cells for this embodiment include BHK, CV-1, myoblast G8, COS (e.g., COS-7), Vero, MDCK and CRFK recombinant cells. Recombinant cell vaccines of the present invention can be administered in a variety of ways but have the advantage that they can be administered orally, preferably at doses ranging from about 10^8 to about 10^{12} cells per kilogram body weight. Recombinant cell vaccines can comprise whole cells, cells stripped of cell walls or cell lysates.

The present invention also includes methods to protect an animal from plague using a therapeutic composition of the current invention. According to this embodiment, a recombinant molecule of the present invention can be administered to an animal in a fashion to enable the recombinant molecule to enter one or more cells of the animal, such that an antigen encoded by an isolated nucleic acid molecule contained therein is expressed into a protective antigen in the animal. Recombinant molecules of the present invention can be delivered to an animal by a variety of methods including, but not limited to, (a) administering a genetic immunization vaccine, e.g., naked DNA or RNA

molecules, such as is taught, for example, in Wolff et al., *ibid.*, or (b) administering a nucleic acid molecule packaged as a recombinant virus vaccine or as a recombinant cell vaccine (i.e., the nucleic acid molecule is delivered by a viral or cellular vehicle).

Genetic immunization vaccines of the present invention can be administered by a variety of methods. Suitable delivery methods include, for example, intramuscular injection, subcutaneous injection, intradermal injection, intradermal scarification, particle bombardment, oral application, and nasal application, with intramuscular injection, intradermal injection, intradermal scarification and particle bombardment being preferred. A preferred single dose of a genetic immunization vaccine ranges from about 1 nanogram (ng) to about 1 milligram (mg), depending on the route of administration and/or method of delivery, as can be determined by those skilled in the art. Examples of administration methods are disclosed, for example, in U.S. Patent No. 5,204,253, by Bruner, et al., issued April 20, 1993, PCT Publication No. W0 95/19799, published July 27, 1995, by McCabe, and PCT Publication No. WO 95/05853, published March 2, 1995, by Carson, et al. Genetic immunization vaccines of the present invention can be contained in an aqueous excipient (e.g., phosphate buffered saline) alone or with a carrier (e.g., lipid-based vehicles), or it can be bound to microparticles (e.g., gold particles).

When administered to an animal, a recombinant virus vaccine of the present invention infects cells within the immunized animal and directs the production of a *Yersinia, Pasteurella,* or *Francisella* antigen that is capable of protecting the animal from plague. For example, a recombinant virus vaccine comprising an isolated nucleic acid molecule encoding a *Yersinia, Pasteurella,* or *Francisella* antigen of the present invention is administered according to a protocol that results in the animal producing a sufficient immune response to be protected from a plague challenge. A preferred single dose of a recombinant virus vaccine of the present invention is from about 1×10^4 to about 1×10^8 virus plaque forming units (pfu) per animal. Administration protocols are well-known to those skilled in the art, with subcutaneous, intramuscular, intradermal, intranasal, and oral administration routes being preferred. A particularly preferred method of administration, especially in cats, for a recombinant virus vaccine of the present invention is by oral delivery. Since RCN, for example, has been shown to be

effective in cats when administered orally, the induction of a strong mucosal response is a possibility. As such, a preferred therapeutic composition to administer to an animal is a recombinant RCN comprising an isolated nucleic acid molecule encoding the F1 antigen of *Yersinia pestis*. A particularly preferred therapeutic composition comprises RCN:p11-nYpF1sec$_{544}$.

The efficacy of a therapeutic composition of the present invention to protect an animal from plague can be tested in a variety of ways including, but not limited to, detection of protective antibodies, detection of cellular immunity within the treated animal, or challenge of the treated animal with *Yersinia, Pasteurella,* or *Francisella* (preferably *Yersinia pestis*) to determine whether the treated animal is resistant to disease. In one embodiment, therapeutic compositions can be tested in a target animal and then serum from that vaccinated animal can be transferred to animal models such as mice, to test for protection by passive immunity. Such techniques are known to those skilled in the art.

Preferred embodiments of the present invention include (a) a recombinant raccoon poxvirus genome that includes an isolated nucleic acid molecule encoding a *Yersinia pestis* antigen operatively linked to a poxvirus transcription control region, (b) a recombinant raccoon poxvirus including such a recombinant genome, (c) a recombinant cell including such a recombinant genome, (d) a recombinant plasmid that includes an isolated nucleic acid molecule encoding a *Yersinia pestis* antigen operatively linked to a eukaryotic transcription control region, and (e) a recombinant cell that includes such a recombinant plasmid. A particularly preferred eukaryotic transcription control region is the human cytomegalovirus (HCMV) immediate-early promoter. Other preferred embodiments include therapeutic compositions comprising a recombinant raccoon poxvirus genome, a recombinant raccoon poxvirus, a recombinant cell, and/or a recombinant plasmid as described above.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

Examples

It is to be noted that the Examples include a number of molecular biology, microbiology, immunology and biochemistry techniques considered to be known to

those skilled in the art. Disclosure of such techniques can be found, for example, in Sambrook, et al., *ibid.*, Ausubel, et al., *ibid.*, and related references.

Example 1:

This example discloses the production of a recombinant raccoon poxvirus capable of expressing a secreted *Y. pestis* F1 antigen.

A. Recombinant molecule pKB3poly-nYpF1(a)sec_{544}, containing a nucleic acid molecule encoding the F1 antigen of *Yersinia pestis* operatively linked to a vaccinia virus p11 late promoter transcription control region was produced in the following manner. The pKB3poly poxvirus shuttle vector was created by modifying a region of plasmid pKB3 (P_{11}-type) plasmid (described in U.S. Patent No. 5,348,741, by Esposito et al., issued September 20, 1994) such that the initiation codon linked to the p11 promoter was mutated and additional unique polylinker restriction sites were added. The resulting poxvirus vector, referred to as pKB3poly, requires the insert DNA to provide the ATG initiation codon when inserted downstream of the p11 promoter. The pKB3poly vector was designed such that foreign DNA cloned into the polylinker region of pKB3poly vector will recombine into the thymidine kinase (tk) gene of a wild-type orthopoxvirus.

Plasmid YPR1 (Simpson, et al., *ibid.*, obtained from the National Institutes of Health Rocky Mountain Laboratories, Hamilton, MT) was used as a template for PCR amplification of the F1 nucleic acid molecule using sense primer EJH031 5' ACG CGC**GTCGAC**G AGGTAATATA TGAAAAAAAT CAG 3'; denoted herein as SEQ ID NO:14 (*Sal*I site in bold) and antisense primer EJH032 5' CGC**GGATCC**C TATATGGATT ATTGGTTAGA TACGG 3'; denoted herein as SEQ ID NO:15 (*Bam*HI site in bold). These primers were synthesized based on a published *Y. pestis* F1 nucleotide sequence, available in Galyov, et al., *ibid.* The PCR amplified product was digested with restriction endonucleases *Sal*I and *Bam*HI and gel purified, resulting in a double stranded nucleic acid molecule of about 544 base-pairs denoted herein as nYpF1(a)sec_{544}, the sequence of which is denoted herein as SEQ ID NO:1. It is to be noted that the PCR fragment amplified from plasmid YPR1 was not derived from the same strain of *Yersinia pestis* as the published sequence and, as such, may comprise an allelic or strain variant of the published sequence. SEQ ID NO:1 contains an open

reading frame of about 510 nucleotides, assuming a start codon extending from about nucleotide 17 through about nucleotide 19 of SEQ ID NO:1 and a termination codon extending from about nucleotide 527 through about 529 of SEQ ID NO:1. The coding region is denoted herein as nYpF1sec$_{510}$, the coding strand of which is presented herein as SEQ ID NO:3. SEQ ID NO:3 encode a full-length F1 protein of about 170 amino acids, denoted herein as PYpF1sec$_{170}$, the sequence of which is presented herein as SEQ ID NO:2. As disclosed in Galyov, et al., *ibid.*, PYpF1sec$_{170}$ includes an N-terminal signal peptide sequence of about 21 amino acids extending from about amino acid 1 to about amino acid 21 of SEQ ID NO:2. In its native milieu, this signal peptide directs the secretion of F1 across the inner and outer *Y. pestis* membranes where it is assembled into a capsule around the bacterial cell. The mature form of PYpF1sec$_{170}$ is represented by PYpF1mat$_{149}$, having the amino acid sequence SEQ ID NO:21. PYpF1mat$_{149}$ is encoded by nucleic acid molecule nYpF1mat$_{447}$, having the coding strand nucleotide sequence represented by SEQ ID NO:22, assuming a first codon extending from about nucleotide 1 through about nucleotide 3 of SEQ ID NO:22.

The PCR-amplified fragment comprising nYpF1(a)sec$_{544}$ was ligated into *Bam*HI and *Sal*I-digested and gel-purified pKB3poly transfer vector, resulting in recombinant molecule pKB3poly-nYpF1(a)sec$_{544}$. Plasmid DNA comprising pKB3poly-nYpF1(a)sec$_{544}$ was purified using Qiagen columns (available from Qiagen, Chatsworth, CA).

B. A recombinant raccoon poxvirus capable of expressing *Y. pestis* F1 antigen was produced as follows. BSC-1 African green monkey kidney cells (obtained from American Type Culture Collection (ATCC), Rockville, MD) were infected at a multiplicity of infection (MOI) of 0.05 with wild type raccoon poxvirus RCN CDC/V71-I-85A (obtained from Dr. Joseph Esposito; Esposito et al., 1985, *Virology 143*, 230-251) and were then transfected with pKB3poly-nYpF1(a)sec$_{544}$ plasmid DNA by calcium phosphate precipitation to form recombinant cell BSC1:RCN:p11-nYpF1(a)sec$_{544}$ by recombination of pKB3poly-nYpF1(a)sec$_{544}$ with the wild-type RCN DNA at the tk locus. The resulting recombinant virus, denoted RCN:p11-nYpF1(a)sec$_{544}$, was plaque purified twice in RAT-2 rat embryo, thymidine kinase mutant cells (available from ATCC) in the presence of bromodeoxyuridine (BUDR, available from Sigma Chemical

Company, St. Louis, MO) to select for tk⁻ recombinants. A tk⁻ recombinant virus was plaque purified a third time on BSC-1 cells without BUDR.

Example 2:

This example demonstrates expression of cellular and secreted forms of *Y. pestis* F1 antigen in RCN:p11-nYpF1(a)sec_{544}-infected cells.

Expression of *Y. pestis* F1 antigen in RCN:p11-nYpF1(a)sec_{544}-infected cells and its secretion from the cells was monitored by the following method. BSC-1 cells were plated into 6 well polystyrene dishes in about 2 ml of MEM medium (available from Life Technologies, Inc., Gaithersburg, MD) containing 5 % fetal bovine serum (FBS) per well. The cells were allowed to grow overnight at 37°C with 5% CO_2. The medium was removed from the cells and replaced with about 2ml of MEM containing 1.0 % FBS. The cells were then infected with RCN:p11-nYpF1(a)sec_{544} at an MOI of approximately 0.025 pfu/cell and were further incubated for about 2 days at 37°C, 5% CO_2 until 100% cytopathic effect (CPE) was observed. The culture was harvested by scraping the infected cells into the medium. The culture was centrifuged at 6000 RPM in a table-top centrifuge for 6 min. at room temperature.

The supernatant and cells were prepared for western blot analysis as follows. The cell pellet was washed in PBS and resuspended in 50 µl of 1x loading buffer (125 mM Tris, pH 6.8, 4% SDS, 0.05% Bromophenol blue, 20% glycerol, and 10% β-mercaptoethanol.). The cell lysate was heated to 95°C for 5 min., and then filtered through a 0.45 µm filter unit, for example, an Ultrafree-MC™ 0.45 µm filter unit, available from Millipore Corp., Bedford, MA. An about 1.9 cm^2 equivalent of the filtered sample (about 10 µl) was analyzed by western blot as described below. The supernatant from the infected cells (about 2 ml) was centrifuged at 14,000 rpm for 5 min. at room temperature in a microcentrifuge and was then concentrated to 100 µl in an ultrafiltration device with a 10-kD molecular weight cutoff, for example, a Microcon-10™ unit, available from Amicon, Inc., Beverly, MA, according to the manufacturer's instructions. The supernatant was combined with an equal volume of 2X loading buffer and heated to 95°C for 5 min. An about 1.9 cm^2 equivalent of the prepared concentrated supernatant (about 20 µl) was analyzed by western blot as described below.

The cell lysate and concentrated supernatant fractions of the RCN:p11-nYpF1(a)sec_{544}-infected BSC-1 cells, prepared as described above, F1 antigen purified from *Y. pestis* (obtained from the Centers for Disease Control (CDC), Fort Collins, CO), and appropriate wild-type virus infected-cell controls were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis using monospecific polyclonal rabbit anti-F1 antigen antiserum (obtained from the CDC). Both the RCN:p11-nYpF1(a)sec_{544} infected-cell lysate and supernatant fractions revealed a eukaryotic version of F1 antigen that migrated as a doublet with apparent molecular weights ranging from about 17 kD to about 22 kD. Upon comparing the cm^2-adjusted equivalent amounts of the cell and supernatant fractions analyzed on the western blot, it appeared that the supernatant fraction contained about 4 times more F1 antigen than the infected cell lysate fraction. While not being bound by theory, this result suggests that F1 antigen is secreted from the RCN:p11-nYpF1(a)sec_{544}-infected BSC-1 cells, which implies that the bacterial signal segment contained on nYpF1(a)sec_{544} is functional in eukaryotic cells (resulting in an antigen equivalent to PYpF1mat_{149}). The 17-kD band migrated at a position very similar, if not identical, to that of F1 antigen purified from *Yersinia pestis*. As deduced from the nucleotide sequence, the predicted native size of *Y. pestis* F1, with the signal peptide removed, is 15.5 kD (Galyov, et al., *ibid*). Native-expressed F1 antigen is known to be glycosylated, which accounts for its larger observed size of 17 kD; see, for example Bennett, et al., 1974, *J. Bacteriol., 117*, 48-55. Since the RCN-expressed F1 antigen (the lower and more abundant band of the doublet) ran at an apparent molecular weight similar, if not identical, to native F1, it was probably also post-translationally modified. While not being bound by theory, the protein migrating with a 22-kD apparent molecular weight could represent another form of post-translational modification. A third, larger immunoreactive protein band, migrating at a molecular weight significantly greater than 90 kD but less than 250 kD was observed in the infected-cell lysate fraction (but not in the supernatant fraction). While not being bound by theory, this band could represent a multimeric form of F1 (common in *Y. pestis*, see, for example, Bennett, et al., *ibid.*) or, alternatively, could represent F1 protein bound tightly to a cellular or viral protein.

Example 3:

This example discloses the immunization of mice with recombinant virus RCN:p11-nYpF1(a)sec_{544}, and the generation of antibodies in the immunized animals.

A. Virus stocks for immunization of mice were prepared as follows. BSC-1 cells were seeded into 40-225 cm^2 flasks in MEM containing 5 % FBS and incubated at 37°C for 2-3 days until a confluent monolayer was formed. The cells were infected at an MOI of 0.01 pfu/cell with RCN:p11-nYpF1(a)sec_{544}. The virus stock was treated with 0.125mg/ml trypsin for 15 min. at 37°C just prior to infecting the cells. The infected cells were incubated for 36-48 hrs. at 37°C until 100% CPE was obtained. The infected cells were detached from the flasks with sterile glass beads and the culture was centrifuged at 5000 rpm for 15 min in a table-top centrifuge at room temperature. The infected cells were resuspended in 30 ml of cold 10 mM Tris buffer, pH 9.0 and homogenized with 40 strokes in a dounce homogenizer on ice. The homogenized sample was centrifuged at 300 x g for 5 min at 5-10°C in a tabletop centrifuge. The supernatant was saved on ice. The pellet was resuspended in 10 ml of cold 10 mM Tris, pH 9.0, and homogenized with 20 strokes in a dounce homogenizer on ice. The sample was centrifuged as before and the supernatant was removed and combined with the first supernatant. The combined supernatant was sonicated on ice for 3 pulses at 6 watts of 15 seconds each with a hand held sonicator, for example, a VirSonic60™ sonicator (available from The VirTis Co., Inc., Gardiner, NY). The supernatant was then layered onto three 13-ml cushions of 36% sucrose (in 10 mM Tris, pH 9.0) and centrifuged for 80 min. at 32,900 x g, at 4°C using, for example, a Model J2-21M ultracentrifuge fitted with a JA-20 rotor, available from Beckman Instruments, Inc., Fullerton CA, to pellet the virus particles. The pelleted virus was resuspended in 4 ml of cold 1 mM Tris, pH 9.0 and sonicated on ice with 2 pulses of 15 seconds each. The virus was aliquotted and stored at -70°C. Prior to vaccinating animals, an aliquot was thawed and titered by plaque assay, using techniques familiar to those skilled in the art.

B. To evaluate the immunogenicity in mice of recombinant virus RCN:p11-nYpF1(a)sec_{544} expressing the *Y. pestis* F1 capsular antigen, four groups of AJ mice, all about three weeks old, were immunized by injection into the footpad, as follows. Group 1, consisting of 60 mice, received about 1 X 10^8 plaque forming units (pfu) of RCN:p11-

nYpF1(a)sec_{544} in about 30 µl of diluent (1 mM Tris, pH 9.0). Group 2, consisting of 36 mice, received about 1 µg of F1 protein purified from *Y. pestis* (obtained from the CDC) also in about 30 µl of diluent. Group 3, consisting of 36 mice, received about 1 X 10^8 pfu of a control RCN virus, RCN-lacZ, in about 30 µl of diluent. RCN-lacZ comprises the gene encoding *E. coli* beta-galactosidase driven by the vaccinia p7.5 promoter, which was inserted into the tk locus of RCN by a method similar to that described in Example 1B above. Group 4, consisting of 36 mice, received about 30 µl of diluent.

C. The response to F1 antigen by the immunized mice was measured by enzyme-linked immunosorbent assay (ELISA) assay as follows. Blood was collected from all immunized mice 5 days prior to infection and at days 10, 20, 30, 37 and 58 post-infection. Serum samples were prepared by methods well known to those skilled in the art. The serum samples were tested for anti-F1 antibodies using an ELISA for total IgM/IgG, performed as follows. Individual wells of 96-well ELISA plates were coated with purified F1 antigen (about 1.0 µg in about 50 µl carbonate buffer, pH 9.6 per well), and were incubated overnight at 4°C or at 37°C for 2 hours. Plates were washed with Tris-buffered saline with 0.1% Tween-20 (TBST), and then blocked with 200 µl of blocking buffer (0.03% bovine serum albumin in TBST), by incubating at 37°C for 1 hour or overnight at 4°C. Following the blocking step and further washing, the mouse serum samples (1:40 and 1:640 dilutions in 50 µl total volume, diluted in TBST) were added to duplicate wells on the plates. Known negative and positive-control mouse serum samples, diluted as the test serum samples, were also added on each plate. The plates were incubated at 37°C for 1 hour. After another wash, horseradish peroxidase labeled goat anti-mouse conjugate (50 µl of a 1:2000 dilution, available from Jackson ImmunoResearch Lab., Inc., West Grove, PA) was added to each well, and incubated at 37°C for 1 hour. The plates were then washed and 50 µl of peroxidase substrate was added to each well (using, for example, the ABTS™ peroxidase substrate available from Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), and incubated for 15 minutes at room temperature. Finally, the reaction was stopped by the addition of 50 µl of stop solution (1% SDS). The ELISA plates were read on an ELISA plate reader set at 405 nm. The average and standard deviations (SD) of the negative controls at 1:40 and 1:640 dilutions were calculated, and any specimen that was the average +3SD was

considered positive. The mean O.D. and standard deviations of the ELISA for serum samples taken on days 20, 30, and 37 are summarized in Table 1.

Table 1

	Day 20	Day 30	Day 37
Group 1	.611 ± .19	.569 ± .185	.781 ± .336
Group 2	1.064 ± .116	.954 ± .105	.920 ± .25
Group 3	.113 ± .016	.083 ± .004	.141± .04
Group 4	.087± .01	-	-

The ELISA results demonstrate that the RCN:p11-nYpF1(a)sec_{544}-immunized mice (Group 1) produced antibodies against *Y. pestis* F1 antigen that, at least by day 37, approached the antibody levels produced by mice immunized with purified F1 antigen (Group 2).

Example 4:

This Example discloses the production of recombinant plasmids encoding various forms of the *Y. pestis* F1 antigen.

A. Eukaryotic expression vector pPVXC was produced as follows. Vector pRc/RSV (available from Invitrogen Corp., San Diego, CA) was cleaved by restriction enzyme *Pvu*II, and the 2963-base pair *Pvu*II fragment was gel purified. That fragment was self-ligated to form vector pRc/RSV(*Pvu*), which contains a Rous Sarcoma Virus (RSV) long terminal repeat, a multiple cloning site, a bovine growth hormone polyadenylation sequence, a bacterial origin of replication, and an ampicillin resistance gene. Expression vector pPVXC was produced by introducing a *Hin*dIII/*Ssp*I fragment containing the HCMV intermediate early promoter and first intron (i.e., intron-A) into pRc/RSV(Pvu) that had been cleaved by *Hin*dIII and *Nru*I. This manipulation removed sequences encoding the RSV long terminal repeat from pRc/RSV(*Pvu*).

B. A recombinant plasmid, denoted herein as pCMV-nYpF1(b)sec_{544}, in which a nucleic acid molecule encoding a full-length F1 protein is operatively linked to the HCMV immediate-early transcription control region, was produced as follows. Nucleic acid molecule nYpF1(b)sec_{544}, which encodes PYpF1sec_{170} (i.e., SEQ ID NO:2), was produced by PCR amplification of that molecule from plasmid YPR1(described in

Example 1) using forward primer JO-1, having nucleic acid sequence 5'GGC**AAGCTT**G AGGTAATATA TGAAAAAAAT CAG 3', represented herein as SEQ ID NO:16 (*Hind*III site in bold); and reverse primer JO-2 having nucleic acid sequence 5' GGC**GAATTC**C TATATGGATTA TTGGTTAGAT ACGG 3', represented herein as SEQ ID NO:17 (*Eco*RI site in bold). These primers were synthesized based on a published *Y. pestis* F1 nucleotide sequence (Galyov, et al., *ibid.*) as described in Example 1. The only differences between YpF1(a)sec_{544} and YpF1(b)sec_{544} are the 5' and 3' restriction enzyme sites. The PCR amplified product was digested with restriction endonucleases *Hind*III and *Eco*RI and gel purified, resulting in a double stranded nucleic acid molecule of about 544 base-pairs denoted herein as nYpF1(b)sec_{544}, the sequence of the coding strand of which is denoted herein as SEQ ID NO:4. The coding sequence, open reading frame and the mature processed protein encoded by SEQ ID NO:4 are all identical to those described in Example 1 for SEQ ID NO:1. It is to be noted that the PCR fragment amplified from plasmid YPR1 was not derived from the same strain of *Yersinia pestis* as the published sequence and, as such, may comprise an allelic or strain variant of the published sequence. Recombinant molecule pCMV-nYpF1(b)sec_{544} was produced by ligating nucleic acid molecule nYpF1(b)sec_{544} into pPVXC that had been cleaved by *Hind*III and *Eco*RI and gel purified.

C. A recombinant plasmid, identified herein as pCMV-nYpF1anc_{576}, capable of expressing an F1 antigen fused to a eukaryotic membrane anchor domain, was produced as follows. Nucleic acid molecule nCgGanc$_{192}$, which encodes the membrane anchoring domain of the canine herpesvirus (CHV) glycoprotein gG gene was produced by PCR amplification from CHV viral DNA using forward primer JO-3, having a sequence 5' GGGAT**GACGT CGTC**GGTTAT AATAATTGTA ATACCC 3', represented herein as SEQ ID NO:18 (*Tth*111I site in bold), and reverse primer JO-4 having nucleic acid sequence 5'GGC**GAATTC**T TAAATATCAT AAAAATTTAA TTTCTGGGG 3', represented herein as SEQ ID NO:19 (*Eco*RI site in bold). The PCR amplified product was digested with restriction endonucleases *Tth*111I and *Eco*RI and gel purified, resulting in a double stranded nucleic acid molecule of about 192 base-pairs denoted herein as nCgGanc$_{192}$, the coding strand sequence of which is denoted herein as SEQ ID NO:5. SEQ ID NO:5 comprises nucleotides about 1072-1248 of SEQ ID NO:9 in

pending U.S. Patent Application Serial No. 08/602,010, *ibid.* Translation of SEQ ID NO:5 yields a protein of about 61 amino acids, denoted herein as $PCgGanc_{61}$, the amino acid sequence of which is presented in SEQ ID NO:6. Recombinant molecule pCMV-$nYpF1anc_{576}$ was produced by digesting pCMV-$nYpF1(b)sec_{544}$ (produced as described in Example 4B) with *Tth*111I and *Eco*RI, gel purifying the larger restriction fragment from this digest, and ligating this fragment with $nCgGanc_{192}$. This manipulation resulted in the first 418 nucleotides of $nYpF1(b)sec_{544}$ being fused in-frame with $nCgGanc_{192}$. The fusion produced coding region $nYpF1anc_{576}$, the coding strand sequence of which is denoted herein as SEQ ID NO:7. Translation of SEQ ID NO:7 yields a protein of about 192 amino acids, denoted $PYpF1anc_{192}$, the amino acid sequence of which is presented in SEQ ID NO:8, assuming an initiation codon extending from about nucleotide 1 through about nucleotide 3 of SEQ ID NO:7. $PYpF1anc_{192}$ includes an N-terminal signal peptide sequence of about 21 amino acids extending from about amino acid 1 to about amino acid 21 of SEQ ID NO:8. The mature form of $PYpF1anc_{192}$ is represented by $PYpF1anc_{171}$, having the amino acid sequence SEQ ID NO:10. $PYpF1anc_{171}$ is encoded by nucleic acid molecule $nYpF1anc_{513}$, having the coding strand sequence represented by SEQ ID NO:9.

D. A recombinant plasmid, denoted herein as pCMV-$nYpF1mat_{474}$, capable of expressing a non-secreted form of F1 antigen, was produced as follows. Nucleic acid molecule $nYpF1mat_{474}$, which encodes a non-secreted form of the *Y. pestis* F1 antigen, was produced by PCR amplification of that molecule from plasmid YPR1 (described in Example 1) using forward primer JO-5, having nucleic acid sequence 5' CCC**AAGCTT**A TGGACGATTT AACTGCAAGC ACC 3', represented herein as SEQ ID NO:20 (*Hind*III site in bold); and reverse primer JO-2 having nucleic acid sequence 5' GGC**GAATTC**C TATATGGATT ATTGGTTAGA TACGG 3', represented herein as SEQ ID NO:17 (*Eco*RI site in bold). The PCR amplified product was digested with restriction endonucleases *Hind*III and *Eco*RI and gel purified, resulting in a double stranded nucleic acid molecule of about 474 base-pairs, denoted herein as $nYpF1mat_{474}$, the coding strand nucleotide sequence of which is denoted herein as SEQ ID NO:11. Translation of SEQ ID NO:11 yields a protein of about 150 amino acids, denoted $PYpF1mat_{150}$, the amino acid sequence of which is presented in SEQ ID NO:12,

assuming a start codon spanning from about nucleotide 7 to about nucleotide 9 of SEQ ID NO:11 and a stop codon spanning from about nucleotide 457 to about nucleotide 459 of SEQ ID NO:11. The coding region of PYpF1mat$_{150}$ is referred to herein as nYpF1mat$_{450}$, the coding strand sequence of which is represented in SEQ ID NO:13. Recombinant molecule pCMV-nYpF1mat$_{474}$ was produced by ligating nucleic acid molecule nYpF1mat$_{474}$ into pPVXC that has been cleaved by *Hind*III and *Eco*RI and gel purified.

Example 5:

This example demonstrates the production of a recombinant protein from a recombinant plasmid of the present invention.

Transient expression of PYpF1sec$_{170}$ from recombinant plasmid pCMV-nYpF1(b)sec$_{544}$ in baby hamster kidney cells (BHK, available from ATCC) was performed as follows. Briefly, six-well polystyrene tissue culture plates were seeded with about 3 x 10^5 BHK cells/well in 2 ml of MEM NEAA Earle's salts (available from Irvine Scientific, Santa Ana, CA), supplemented with 100 mM L-glutamine and 5% FBS (complete growth media). Cells were grown to about 80% confluence (about 48 hr). The recombinant plasmid to be transfected, produced as described in Example 4B, was purified using Qiagen columns (available from Qiagen) per manufacturer's instructions. Using polystyrene plates, about 0.5 µg of recombinant plasmid pCMV-nYpF1(b)sec$_{544}$ was mixed with about 100 µl OptiMEM medium (available from LTI). About 10 µl Lipofectamine (available from LTI) was mixed with about 500 µl OptiMEM. The recombinant plasmid mixture was then added to the Lipofectamine mixture and incubated at room temperature for about 30 min. After incubation, about 500 µl OptiMEM was added and the entire mixture was overlaid onto the BHK cells that had been rinsed with OptiMEM. Cells were incubated for 4 hours at 37°C, 5% CO_2, 90% relative humidity. The transfection mixture was then removed and replaced with about 1 ml of OptiMEM.

Transfected cells were incubated at 37°C, 5% CO_2, 90% relative humidity for about 24 hr or about 48 hr, at which times the cell supernatants and cells were harvested separately. The media was removed, the cells were washed twice with about 2 ml PBS and were then scraped off the plate in about 1.5 ml PBS. The cells were then pelleted by

centrifugation, the PBS was removed and the cells were frozen at -70°C. The cell-supernatants were frozen without any further manipulations.

Cell and supernatant samples were subjected to SDS PAGE and immunoblot analyses by methods similar to those described in Example 2 above, except that 10 µl samples of supernatant were assayed without being concentrated. Rabbit anti-F1 antigen antiserum, as described in Example 2, was immunoreactive with antigens expressed by the cells and supernatants harvested about 48 hours after transfection with plasmid pCMV-nYpF1(b)sec_{541}.

Example 6:

This example discloses the production of a recombinant plasmid encoding a secreted form of the *Y. pestis* F1 antigen.

A. Eukaryotic expression vector pPVXC-tPA was produced as follows. A double-stranded cassette comprising the tissue plasminogen activator (t-PA) signal peptide sequence (see, for example, Wang, RF and Mullins, JI, 1995, Gene 153 (2), 197-202) was constructed by annealing two complementary synthetic oligonucleotides: JO-6 having nucleic acid sequence 5'AGCTTCAATC **ATG**GATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC AGTCTTCGTT TCGGCCGGCC CGGGAT3' (partial *Hind*III and *Eco*RV sites underlined, t-PA initiation codon in bold, *Nae*I site in double underline); and JO-7 having nucleic acid sequence 5'ATCCCGGGCC GGCCGAAACG AAGACTGCTC CACACAGCAG CAGCACACAG CAGAGCCCTC TCTTCATTGC ATCCATGATT GA3' (partial *Eco*RV and *Hind*III sites underlined, *Nae*I site in double underline). These oligonucleotides were annealed by methods known by those skilled in the art to produce an about 82-base-pair cassette with a 4-nucleotide overhang on the 5' end. This cassette comprises the coding strand encoding the t-PA signal peptide sequence, $ntPA_{69}$, extending from nucleotide 11 to nucleotide 79 of JO-6. Translation of $ntPA_{69}$ yields a protein of about 23 amino acids, denoted herein as $PtPA_{23}$. The resulting double-stranded cassette was cloned into the pPVXC plasmid (described in Example 4A) which had been previously digested with *Hind*III and *Eco*RV and gel purified. The resulting expression vector, pPVXC-tPA, contains the t-PA signal peptide sequence followed by an *Nae*I restriction site into which a protein coding sequence may be inserted in-frame.

B. A recombinant plasmid, denoted herein as pCMV-ntPA/YpF1sec$_{534}$, in which a nucleic acid molecule encoding the mature F1 protein fused in-frame with the t-PA signal peptide sequence is operatively linked to the HCMV immediate-early transcription control region, was produced as follows. The nucleic acid molecule nYpF1mat$_{481}$ which encodes the mature F1 protein was produced by PCR amplification from recombinant plasmid pCMV-nYpF1(b)sec$_{544}$ (described in Example 4B) using forward primer JO-8, having nucleic acid sequence 5'GGC**GCCGGC**G CAGATTTAAC TGCAAGCACC3' (*Nae*I site in bold), and reverse JO-9 having nucleic acid sequence 5' GGC**CTCGAG**C GGAATTCTTA GGATCCTTGG TTAGATACTG TTACGG 3' (*Xho*I site in bold, stop codon underlined). The resulting PCR product was digested with restriction endonucleases *Nae*I and *Xho*I and gel purified, resulting in a double-stranded nucleic acid molecule of about 481 base pairs denoted herein as nYpF1mat$_{481}$. This sequence comprises a region of SEQ ID NO:1 extending from nucleotide 63 to nucleotide 512, which encodes a portion of SEQ ID NO:2 extending from amino acid 14 through amino acid 163. Recombinant molecule pCMV-ntPA/YpF1mat$_{534}$ was produced by ligating nucleic acid molecule nYpF1mat$_{481}$ into pPVXC-tPA that had been digested with *Nae*I and *Xho*I and gel purified. This manipulation results in nYpF1mat$_{481}$ being fused in-frame with ntPA$_{69}$. The fusion produces coding region ntPA/YpF1sec$_{534}$. Translation of ntPA/YpF1sec$_{534}$ yields a protein of about 178 amino acids, denoted herein as PtPA/YpF1sec$_{178}$.

Example 7:

This Example demonstrates the production of recombinant protein from recombinant plasmid pCMV-ntPA/YpF1sec$_{534}$.

Transient expression of PtPA/YpF1sec$_{178}$ from recombinant plasmid pCMV-ntPA/YpF1sec$_{534}$, produced as described in Example 6, by transfection into CHO cells, was performed as described in Example 5. Cell and supernatant samples were subjected to SDS PAGE followed by western blot analysis by methods similar to those described in Example 2, but with some slight modifications. The cell pellets were quantified by spectrophotometric absorbance at 600nm. The cells were then centrifuged and resuspended in a volume of 2X loading buffer so that 20 µl were equivalent to 0.1 O.D. units. Gels were loaded with 5 µl of cell sample per lane (0.025 OD units).

Supernatants were concentrated approximately 67-fold with Microcon-10™ unit, and gels were loaded with 15 µl concentrated supernatant sample per lane. Rabbit anti-F1 antigen antiserum, as described in Example 2, was immunoreactive with antigens of about 18 kD expressed by both the cells and supernatants harvested about 48 hours after transfection with plasmid pCMV-ntPA/YpF1sec$_{534}$.

The expression of PtPA/YpF1sec$_{178}$ was also detected by immunofluorescence following transient transfection of BHK cells. Briefly, six-well polystyrene tissue culture plates were seeded with about 3x10^5 BHK cells per well in 2 ml of MEM NEAA Earle's salts (available from Irvine Scientific, Santa Ana, CA), supplemented with 100 nM L-glutamine and 5% FBS (complete growth media). Recombinant molecule pCMV-ntPA/YpF1sec$_{534}$ was purified using Qiagen® Maxiprep columns, according to the manufacturer's instructions. The purified recombinant plasmid (1.0 µg) was mixed with 100 µl of OptiMEM® media and incubated for 10 min at room temperature. A mixture of 10 µl of Lipofectamine® and 100 µl of OptiMEM® media (reagents available from Life Technologies Inc. (LTI) was incubated for 10 min at room temperature. The recombinant plasmid mixture was then added to the Lipofectamine mixture and incubated at room temperature for 30 min. After incubation, 800 µl of OptiMEM was added, and the entire mixture was overlaid onto BHK cells that had been rinsed with OptiMEM. Cells were incubated for 5 hours at 37°C, 5% CO_2, and 90% relative humidity. The transfection mixture was then removed and replaced with 2 ml of DMEM containing 10% FBS and incubated for about 24 to 48 hours. For immunofluorescence assays, transfected cells were rinsed three times with 1X PBS and then fixed in a Methanol/Acetone (50/50) solution for 5 min on ice. Fixed cells were rinsed three times with PBS. Rabbit anti-F1 antigen antiserum (1:50 dilution in PBS) was added, and the cells were incubated for 1 hr at 37°C. Following three rinses with PBS, a secondary a FITC-conjugated anti-rabbit antibody (available from Kirkegaard & Perry, Gaithersburg, MD), diluted 1:25 in 0.25% Evans Blue/PBS was added and incubated for 1 hour at 37°C. Cells were then rinsed three times, overlaid with 50% glycerol and examined with a fluorescence microscope. Rabbit anti-F1 antigen antiserum was immunoreactive with antigens expressed by cells transfected with plasmid pCMV-ntPA/YpF1sec$_{534}$.

Example 8:

This example describes the immunization of mice with recombinant molecules of the present invention and the generation of antibodies in the immunized animals.

A. Plasmid DNA for immunizations was produced as follows. Plasmid DNAs described below were purified using Qiagen® megacolumns, per manufacturer's instructions. The endotoxin level of each preparation was measured by QCL-1000 kit (available from Biowhittaker, Walkersville, MD) prior to animal immunization, and was found to be 0.043 Endotoxin Units/ug of DNA, an acceptable level for animal immunizations.

B. Mice were immunized with plasmid DNA using the following method. Four groups of BALB/c mice of about three weeks of age were immunized by intramuscular injection as follows. Group 1, consisting of 5 mice, received about 100 μg of pPVXC plasmid DNA (produced as described in Example 4) in about 30 μl of diluent (TE). Group 2, consisting of 5 mice, received about 100 μg of pCMV-nYpF1(b)sec_{544} plasmid DNA (produced as described in Example 4) in about 30 μl of diluent (TE). Group 3, consisting of 5 mice, received about 100 μg of pCMV-ntPA/YpF1sec_{534} plasmid DNA (produced as described in Example 6) in about 30 μl of diluent (TE). Group 4, consisting of 5 mice, received about 1 μg of F1 protein purified from *Y. pestis* (as described in Example 2) also in about 30 μl of diluent.

C. The immune response to F1 antigen in the immunized mice was measured by enzyme-linked immunosorbent assay (ELISA) as follows. Blood was collected from all immunized mice 5 days prior to infection and at days 10, 20, 30, 40 post-vaccination. Serum samples were prepared by methods well known to those skilled in the art. The serum samples were tested for anti-F1 antibodies using an ELISA for total IgM/IgG, performed as described in Example 3C. The antibody titers, geometric means, and standard deviations of the ELISA for serum samples taken on days -5 (i.e., 5 days prior to infection), 10, 20, 30, and 40 are summarized in Table 1.

Table 1

Mouse #	Group	Day -5	Day 10	Day 20	Day 30
Group 1: 1	CMV	<40	<40	<40	<40
2	CMV	<40	40	<40	40
3	CMV	<40	<40	<40	40
4	CMV	<40	<40	<40	40
5	CMV	<40	<40	<40	40
Group 2: 1	CMV/F1	<40	<40	<40	<40
2	CMV/F1	<40	<40	<40	<40
3	CMV/F1	<40	<40	<40	<40
4	CMV/F1	<40	<40	<40	<40
5	CMV/F1	<40	<40	<40	<40
Group 3: 1	CMV/tPA-F1	<40	<40	640	2,560
2	CMV/tPA-F1	<40	40	160	2,560
3	CMV/tPA-F1	<40	160	640	10,240
4	CMV/tPA-F1	<40	2,560	10,240	10,240
5	CMV/tPA-F1	<40	<40	640	2,560
Geom Mean			1.98	2.87	3.63
St. Deviat			0.78	0.65	0.32
Group 4:1	F1	<40	10,240	10,240	40,960
2	F1	<40	10,240	2,560	2,560
3	F1	<40	640	640	10,240
4	F1	<40	640	640	2,560
5	F1	<40	10,240	640	2,56
Geom Mean			3.47	3.13	3.74
St. Dev			0.65	0.53	0.53

The results show that the pCMV-ntPA/YpF1sec$_{534}$ plasmid was highly immunogenic in mice inducing high levels of antibodies in all immunized mice. At day 30 post-vaccination, anti-F1 antibody titers in mice vaccinated with the pCMV-ntPA/YpF1sec$_{534}$ plasmid were equivalent to those detected in animals vaccinated with the F1 protein.

Example 9:

This example discloses the production of a recombinant raccoon poxvirus containing the EMCV IRES and a fused *Y. pestis* tPA-F1 antigen.

A. Eukaryotic expression vector pCITE-tPA was constructed as follows. A double-stranded cassette comprising the tissue plasminogen activator (t-PA) signal peptide sequence (Wang et al, *ibid.*) was constructed by annealing two complementary synthetic oligonucleotides: JO-10, having nucleotide sequence 5'ATGCAATGAA GAGAGGGCTC TGCTGTGTGC TGCTGCTGTG TGGAGCAGTC TTCGTTTCTG

CCGGCCCGGG TATACG3'; and JO-11 having nucleotide sequence 5' GATCCGTATA CCCGGGCCGG CAGAAACGAA GACTGCTCCA CACAGCAGCA GCACACAGCA GAGCCCTCTC TTCATTGCAT3'. The annealed sequence contains three blunt cutting restriction sites: *Nae*I (double underline); *Sma*I (bold); and *Bst*1107I (single underline); located at the 3' end of the annealed signal sequence. The annealed cassette lacks the t-PA initiation codon at the 5' end, and is designed to blunt ligate to the *Msc*I site in pCITE-4a (available from Novagen), thus utilizing the EMCV IRES preferential ATG. This changes the first amino acid from a lysine to a glutamic acid, which is a conservative change and the protein retains its original hydrophobicity. The annealed cassette was ligated into the pCITE 4a+ plasmid that had been previously digested with *Msc*I and *Bam*HI and gel purified. The resulting nucleic acid molecule was designated pCITE-tPA.

B. Recombinant molecule pCMV-IRES-tPA containing the tPA signal peptide sequence operatively linked to a CMV promoter and EMCV IRES was constructed as follows. A DNA fragment containing IRES-tPA, denoted herein as nIRES-tPA, was PCR amplified from pCITE-tPA using forward primer JO-12 having nucleotide sequence 5'A**GGCGCGCC***G TCGAC*GTTAT TTTCCACCAT ATTGCCG3'(*Asc*I site in bold, *Sal*I site in italics), and reverse primer JO-13 having nucleotide sequence 5'C**GAATTC***GGA TCC*GTATACC3' (*Eco*RI site in bold, *Bam*HI site in italics, and *Bst*11071 site underlined). Recombinant molecule pCMV-IRES-tPA was produced by ligating nucleic acid molecule nIRES-tPA that had been digested with *Asc*I and *Eco*RI into a modified pCMV-intA vector. This modified pCMV-intA vector was created by annealing, using techniques known to those skilled in the art, the following two complementary synthetic oligonucleotides: JO-14 having nucleotide sequence 5'*AGCTT***GGCGC GCC**G3' (*Hin*dIII site in italics, *Asc*I site in bold, first base of *Bam*HI site underlined), and JO-15 having nucleotide sequence 5'GATCC**GGCGC GCC***A*3' (*Bam*HI site underlined, *Asc*I site in bold, first base of *Hin*dIII site underlined). The annealed oligonucleotides were ligated into the pCMV-intA plasmid that had been digested with *Hin*dIII and *Bam*HI and gel purified.

C. Recombinant molecule pCMV-IRES-ntPA/YpF1sec$_{525}$, containing a nucleic acid molecule encoding the mature F1 antigen of *Y. pestis* fused in-frame with the t-PA

signal peptide sequence and operatively linked to a CMV promoter and EMCV IRES, was produced as follows. Nucleic acid molecule nYpF1mat$_{468}$ was PCR amplified from pCMV-nYpF1(b)sec$_{544}$ (described in Example 4B) using forward primer JO-8 and reverse primer JO-16, having nucleic acid sequence 5'CG**GAATTC**TT A*GGATCC*TTG GTTAGATACG GTTACGG3' (*Eco*RI site in bold, stop codon underlined, *Bam*HI site in italics). The resulting PCR product was digested with restriction endonucleases *Ngo*MI and *Eco*RI and gel purified, resulting in a double-stranded molecule of 468 base pairs denoted herein as nYpF1mat$_{468}$. Recombinant molecule pCMV-IRES-ntPA/YpF1sec$_{525}$ was produced by ligating nucleic acid molecule nYpF1mat$_{468}$ into the pCMV-IRES-tPA vector that had been digested with *Ngo*MI and *Eco*RI and gel purified.

D. A recombinant raccoon poxvirus (RCNV) capable of expressing *Y. pestis* F1 antigen was produced as follows. Recombinant cell Vero:RCN:IRES-ntPA/YpF1sec$_{525}$ containing nucleic acid molecule ntPA/YpF1sec$_{525}$ operatively linked to a vaccinia virus p11 late promoter transcription control region and EMCV IRES was produced in the following manner. Recombinant molecule pCMV-IRES-ntPA/YpF1sec$_{525}$ was digested with *Sal*I and *Eco*RI to generate a fragment containing nucleic acid molecule ntPA/YpF1sec$_{525}$ operatively linked to a CMV promoter and EMCV IRES. This fragment was then cloned into a RCN transfer vector that had been digested with *Sal*I and *Eco*RI and gel purified. The resulting plasmid was recombined into a raccoon pox virus in Vero cells as described in Example 1B to form recombinant cell Vero:RCN:IRES-ntPA/YpF1sec$_{525}$. The resulting recombinant virus, denoted RCN:IRES-ntPA/YpF1sec$_{525}$, was plaque purified as described in Example 1B.

Example 10:

This example demonstrates enhanced expression of *Y. pestis* F1 antigen in cells infected with a recombinant raccoon pox virus of the present invention.

Expression of *Y. pestis* F1 antigen in RCN:IRES-ntPA/YpF1sec$_{525}$-infected cells was monitored by the following method. Vero cells were plated at 7 x10^5 cells/well in six well dishes with MEM+5%FBS one day prior to infection. Cells were infected in duplicate at an MOI of approximately 0.5 with viruses that had pre-treated with trypsin (1 mg/ml) for 15 min at 37°C. Upon infection, the media was changed to MEM without FBS, and the infected cells were incubated for about 24 to 48 hr. The viruses used

included: (a) wild type raccoon poxvirus RCN CDC/V71-I-85A (described in Example 1B); (b)RCN:p11-nYpF1(a)sec_{544} (produced as described in Example 1B); and (c) RCN:IRES-ntPA/YpF1sec_{525}. The infected cells were harvested by washing cells into media, and recovering the cells by centrifugation at 10,000 rpm for 5 min at room temperature. The supernatants and cells were prepared for western blot analysis as described in Example 2. About 10 µl of each cell fraction and 30 µl of each concentrated media sample were loaded on a 4-20% SDS-PAGE gel and run for 1 hr at 200 V. The separated proteins were transferred to nitrocellulose using a Bio-Rad transfer apparatus at 100 V for 1 hr. The filter was subjected to western blot analysis using polyclonal rabbit anti-F1 antigen antiserum (described in Example 2). Filters were scanned and analyzed for density with a NIH image program. Analysis of the results, in comparison with a known quantity of F1 antigen, indicated that the presence of the EMCV IRES motif led to an about two-fold increase in F1 protein production; that is, cells infected with RCN:IRES-ntPA/YpF1sec_{525} produced about twice as much protein as did cells infected with RCN:p11-nYpF1(a)sec_{544}.

Example 11:

This example discloses the production of recombinant mengoviruses containing several forms of the *Y. pestis* F1 antigen.

A. Recombinant molecule pMV-nYpF1mat_{450}, encoding the mature F1 antigen in frame with the polyprotein coding region of mengovirus, was prepared as follows. The mature F1 coding region was amplified from recombinant molecule pCMV-IRES-ntPA/YpF1sec_{525} (produced as described in Example 9C) using forward primer JO-17, having the nucleotide sequence 5'GGG**GCTAGC**A GATTTAACTG CAAGCACCAC and reverse primer JO-18 having the nucleotide sequence 5'GGG**GCTAGC**T GGTTAGATAC GGTTACGGTT ACAGCAGC (*Nhe*I sites shown in bold). The amplified fragment was purified using QIAquick™ PCR purification kit (available from Qiagen Inc., Valencia, CA) as recommended by manufacturer. The PCR-amplified fragment was re-circularized by ligation, digested with restriction endonuclease *Nhe*I, and gel purified, resulting in a double-stranded nucleic acid molecule of about 450 base pairs denoted herein as nYpF1mat_{450}. Recombinant molecule pMV-nYpF1mat_{450} was produced by ligating nucleic acid molecule nYpF1mat_{450} into menogvirus plasmid

pCoCe (available from the University of Wisconsin, Madison, WI; see also U.S. Patent No. 5,229,111, by Duke et al, issued July 20, 1993) that had been digested with *Nhe*I and gel purified. This manipulation resulted in nYpF1mat$_{450}$ being fused in-frame with the sequence encoding the mengovirus polyprotein.

B. Recombinant molecule pMV-ntPA/YpF1sec$_{519}$, encoding the mature F1 antigen fused with the t-PA signal peptide sequence, and in frame with the polyprotein coding region of mengovirus, was prepared as follows. The mature F1 coding region fused to the t-PA signal peptide sequence was amplified from recombinant molecule pCMV-IRES-ntPA/YpF1sec$_{525}$ (produced as described in Example 9C) using forward primer JO-14, having the nucleotide sequence 5'GGG**GCTAGC**C GATGCAATGA AGAGAGGGCT CT 3' and reverse primer JO-13 (*Nhe*I site shown in bold). The amplified fragment was purified and digested with restriction endonuclease *Nhe*I as described in Example 11A, resulting in a double-stranded nucleic acid molecule of about 519 base pairs denoted herein as ntPA/YpF1sec$_{519}$. Recombinant molecule pMV-ntPA/YpF1sec$_{519}$ was produced by ligating nucleic acid molecule ntPA-YpF1sec$_{519}$ into the mengovirus pCoCe plasmid described in Example 11A that had been digested with *Nhe*I and gel purified. This manipulation resulted in ntPA/YpF1sec$_{519}$ being fused in-frame with the sequence encoding the mengovirus polyprotein.

C. Recombinant molecule pMV-ntPA/YpF1anc$_{705}$, encoding the mature F1 antigen fused with the t-PA signal peptide sequence and the CHV gG membrane anchor sequence (described in Example 4C), and in frame with the polyprotein coding region of mengovirus, was prepared as follows. The mature F1 coding region fused to the t-PA signal peptide sequence was amplified from recombinant molecule pCMV-IRES-ntPA/YpF1sec$_{525}$ as in Example 11B using forward primer JO-14, and reverse primer JO-15 having nucleotide sequence 5'CGGAATTCTT A**GGATCC**TTG GTTAGATACG GTTACGG-3' (*Bam*HI site shown in bold). An about 196 bp fragment was then amplified from CHV genomic DNA (described in Example 4C) using forward primer JO-16 having nucleotide sequence 5'CG**GGATCC**AA TGGTTATAAT AATTGTAATA CCC -3' (*Bam*HI site shown in bold), and reverse primer JO-17 having nucleotide sequence 5'AAC**GCTAGC**A GAATATCATA AAATAATAAT TTCTG-3' (*Nhe*I site shown in bold). These two resulting PCR-

amplified fragments were purified and digested with *Bam*HI. The fragments were then ligated in the presence of polynucleotide kinase, digested with *Nhe*I, and gel purified, producing nucleic acid molecule ntPA/YpF1anc$_{705}$. Recombinant molecule pMV-ntPA/YpF1anc$_{705}$ was produced by ligating nucleic acid molecule ntPA/YpF1anc$_{705}$ into the mengovirus pCoCe plasmid described in Example 11A that had been digested with *Nhe*I and gel purified. This manipulation resulted in ntPA/YpF1anc$_{705}$ being fused in-frame with the sequence encoding the mengovirus polyprotein.

Example 12:

This example describes the production of recombinant mengoviruses from the recombinant molecules described in Example 11.

A. Recombinant mengovirus RNA was produced from recombinant molecules pMV-nYpF1mat$_{450}$, pMV-ntPA/YpF1sec$_{519}$, and pMV-ntPA/YpF1anc$_{705}$ using *in vitro* transcription, as follows. Two μg of each recombinant molecule was linearized with *Bam*HI (pMV-nYpF1mat$_{450}$ and pMV-ntPA/YpF1sec$_{519}$) or *Hin*dIII (pMV-ntPA/YpF1anc$_{705}$) in a 50 μl reaction mix. Each DNA was extracted once with phenol/$CHCl_3$ and precipitated. RNA from each recombinant molecule was then synthesized by *in vitro* transcription using MEGAscript™ kit (available from Ambion Inc., USA) in 20 μl of reaction volume containing 1 μg of each template DNA essentially as described by manufacturer, producing mengovirus RNA molecules rMV-nYpF1mat$_{450}$, rMV-ntPA/YpF1sec$_{519}$, and rMV-ntPA/YpF1anc$_{705}$. The yield of each mengovirus RNA was evaluated by running an aliquot of RNA on 1% non-denaturing agarose gel.

B. Recombinant mengoviruses were produced by RNA electroporation into HeLa cells, as follows. HeLa cells were grown in a T225 cm^2 tissue culture flask to ~80% confluency in D-MEM-10%, which is D-MEM media supplemented with 10% FBS, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 1X MEM vitamins mixture (all reagents available from LTI). The cells were trypsinized using a standard protocol and resuspended in D-MEM-10%. The cells were washed three times in ice-cold OPTI-MEM I media (available from LTI) and resuspended in 500 μl aliquots of $5x10^6$ cells for each RNA sample. Approximately 2 μg of each mengovirus RNA, produced as described in Example 12A, was added to an aliquot of cells and the

mixtures were immediately subjected to two pulses of electrical discharge in BTX-500 electroporation device (Electro cell manipulator 600, BTX Inc., Santa Clara, CA) with the following settings: 400 V, 800 μF, 13 ohms. After incubation for 5-10 min at room temperature, the transfected recombinant cells, denoted herein as HeLa:MV-nYpF1mat$_{450}$, HeLa:MV-ntPA/YpF1sec$_{519}$, and HeLa:MV-ntPA/YpF1anc$_{705}$ were resuspended in 10 ml of OPTI-MEM supplemented with 1% of FBS and were transferred into tissue culture flasks. Infectious viruses, denoted herein as MV-nYpF1mat$_{450}$, MV-ntPA/YpF1sec$_{519}$, and MV-ntPA/YpF1anc$_{705}$ were collected after complete CPE was observed, at approximately 2 days post electroporation. The infected cells were lysed by two freeze-thaw cycles followed by clarification of cell lysates by centrifugation for 15 min at 3,000 rpm in GP8R refrigerated centrifuge (Forma Scientific Inc.).

Sequence Listing

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Heska Corporation
(B) STREET: 1825 Sharp Point Drive
(C) CITY: Fort Collins
(D) STATE: CO
(E) COUNTRY: US
(F) POSTAL CODE (ZIP): 80525
(G) TELEPHONE: (970) 493-7272
(H) TELEFAX: (970) 484-9505

(ii) TITLE OF INVENTION: RECOMBINANT PLAGUE VACCINE

(iii) NUMBER OF SEQUENCES: 22

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: LAHIVE & COCKFIELD, LLP
(B) STREET: 28 STATE STREET
(C) CITY: BOSTON
(D) STATE: MA
(E) COUNTRY: US
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: ASCII DOS TEXT

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/767,115
(B) FILING DATE: 04-DEC-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Rothenberger, Scott D.
(B) REGISTRATION NUMBER: 41,277
(C) REFERENCE/DOCKET NUMBER: PL-1-C1-PCT (HKV-015PC)

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 227-7400
(B) TELEFAX: (617) 742-4214

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 544 nucleotides
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) LOCATION: 17..529

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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                  Met Lys Lys Ile Ser Ser Val Ile Ala Ile Ala
                    1               5                  10

TTA TTT GGA ACT ATT GCA ACT GCT AAT GCG GCA GAT TTA ACT GCA AGC        97
Leu Phe Gly Thr Ile Ala Thr Ala Asn Ala Ala Asp Leu Thr Ala Ser
             15                  20                  25

ACC ACT GCA ACG GCA ACT CTT GTT GAA CCA GCC CGC ATC ACT CTT ACA       145
Thr Thr Ala Thr Ala Thr Leu Val Glu Pro Ala Arg Ile Thr Leu Thr
         30                  35                  40

TAT AAG GAA GGC GCT CCA ATT ACA ATT ATG GAC AAT GGA AAC ATC GAT       193
Tyr Lys Glu Gly Ala Pro Ile Thr Ile Met Asp Asn Gly Asn Ile Asp
     45                  50                  55

ACA GAA TTA CTT GTT GGT ACG CTT ACT CTT GGC GGC TAT AAA ACA GGA       241
Thr Glu Leu Leu Val Gly Thr Leu Thr Leu Gly Gly Tyr Lys Thr Gly
 60                  65                  70                  75

ACC ACT AGC ACA TCT GTT AAC TTT ACA GAT GCC GCG GGT GAT CCC ATG       289
Thr Thr Ser Thr Ser Val Asn Phe Thr Asp Ala Ala Gly Asp Pro Met
                 80                  85                  90

TAC TTA ACA TTT ACT TCT CAG GAT GGA AAT AAC CAC CAA TTC ACT ACA       337
Tyr Leu Thr Phe Thr Ser Gln Asp Gly Asn Asn His Gln Phe Thr Thr
             95                  100                 105

AAA GTG ATT GGC AAG GAT TCT AGA GAT TTT GAT ATC TCT CCT AAG GTA       385
Lys Val Ile Gly Lys Asp Ser Arg Asp Phe Asp Ile Ser Pro Lys Val
         110                 115                 120

AAC GGT GAG AAC CTT GTG GGG GAT GAC GTC GTC TTG GCT ACG GGC AGC       433
Asn Gly Glu Asn Leu Val Gly Asp Asp Val Val Leu Ala Thr Gly Ser
     125                 130                 135

CAG GAT TTC TTT GTT CGC TCA ATT GGT TCC AAA GGC GGT AAA CTT GCA       481
Gln Asp Phe Phe Val Arg Ser Ile Gly Ser Lys Gly Gly Lys Leu Ala
140                 145                 150                 155

GCA GGT AAA TAC ACT GAT GCT GTA ACC GTA ACC GTA TCT AAC CAA TAA       529
Ala Gly Lys Tyr Thr Asp Ala Val Thr Val Thr Val Ser Asn Gln
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TCCATATAGG GATCC 544

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 170 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Lys Ile Ser Ser Val Ile Ala Ile Ala Leu Phe Gly Thr Ile
1 5 10 15

Ala Thr Ala Asn Ala Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala
20 25 30

Thr Leu Val Glu Pro Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala
35 40 45

Pro Ile Thr Ile Met Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val
50 55 60

Gly Thr Leu Thr Leu Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser
65 70 75 80

Val Asn Phe Thr Asp Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr
85 90 95

Ser Gln Asp Gly Asn Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys
100 105 110

Asp Ser Arg Asp Phe Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu
115 120 125

Val Gly Asp Asp Val Val Leu Ala Thr Gly Ser Gln Asp Phe Phe Val
130 135 140

Arg Ser Ile Gly Ser Lys Gly Gly Lys Leu Ala Ala Gly Lys Tyr Thr
145 150 155 160

Asp Ala Val Thr Val Thr Val Ser Asn Gln
165 170

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 510 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATGAAAAAAA TCAGTTCCGT TATCGCCATT GCATTATTTG GAACTATTGC AACTGCTAAT    60
GCGGCAGATT TAACTGCAAG CACCACTGCA ACGGCAACTC TTGTTGAACC AGCCCGCATC   120
ACTCTTACAT ATAAGGAAGG CGCTCCAATT ACAATTATGG ACAATGGAAA CATCGATACA   180
GAATTACTTG TTGGTACGCT TACTCTTGGC GGCTATAAAA CAGGAACCAC TAGCACATCT   240
GTTAACTTTA CAGATGCCGC GGGTGATCCC ATGTACTTAA CATTTACTTC TCAGGATGGA   300
AATAACCACC AATTCACTAC AAAAGTGATT GGCAAGGATT CTAGAGATTT TGATATCTCT   360
CCTAAGGTAA ACGGTGAGAA CCTTGTGGGG GATGACGTCG TCTTGGCTAC GGGCAGCCAG   420
GATTTCTTTG TTCGCTCAAT TGGTTCCAAA GGCGGTAAAC TTGCAGCAGG TAAATACACT   480
GATGCTGTAA CCGTAACCGT ATCTAACCAA                                     510
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 544 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) LOCATION: 17..529

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
AAGCTTGAGG TAATAT ATG AAA AAA ATC AGT TCC GTT ATC GCC ATT GCA        49
                  Met Lys Lys Ile Ser Ser Val Ile Ala Ile Ala
                    1               5                  10

TTA TTT GGA ACT ATT GCA ACT GCT AAT GCG GCA GAT TTA ACT GCA AGC      97
Leu Phe Gly Thr Ile Ala Thr Ala Asn Ala Ala Asp Leu Thr Ala Ser
            15                  20                  25

ACC ACT GCA ACG GCA ACT CTT GTT GAA CCA GCC CGC ATC ACT CTT ACA     145
Thr Thr Ala Thr Ala Thr Leu Val Glu Pro Ala Arg Ile Thr Leu Thr
        30                  35                  40

TAT AAG GAA GGC GCT CCA ATT ACA ATT ATG GAC AAT GGA AAC ATC GAT     193
Tyr Lys Glu Gly Ala Pro Ile Thr Ile Met Asp Asn Gly Asn Ile Asp
    45                  50                  55

ACA GAA TTA CTT GTT GGT ACG CTT ACT CTT GGC GGC TAT AAA ACA GGA     241
Thr Glu Leu Leu Val Gly Thr Leu Thr Leu Gly Gly Tyr Lys Thr Gly
60                  65                  70                  75

ACC ACT AGC ACA TCT GTT AAC TTT ACA GAT GCC GCG GGT GAT CCC ATG     289
Thr Thr Ser Thr Ser Val Asn Phe Thr Asp Ala Ala Gly Asp Pro Met
                80                  85                  90

TAC TTA ACA TTT ACT TCT CAG GAT GGA AAT AAC CAC CAA TTC ACT ACA     337
Tyr Leu Thr Phe Thr Ser Gln Asp Gly Asn Asn His Gln Phe Thr Thr
            95                  100                 105
```

```
AAA GTG ATT GGC AAG GAT TCT AGA GAT TTT GAT ATC TCT CCT AAG GTA      385
Lys Val Ile Gly Lys Asp Ser Arg Asp Phe Asp Ile Ser Pro Lys Val
        110                 115                 120

AAC GGT GAG AAC CTT GTG GGG GAT GAC GTC GTC TTG GCT ACG GGC AGC      433
Asn Gly Glu Asn Leu Val Gly Asp Asp Val Val Leu Ala Thr Gly Ser
    125                 130                 135

CAG GAT TTC TTT GTT CGC TCA ATT GGT TCC AAA GGC GGT AAA CTT GCA      481
Gln Asp Phe Phe Val Arg Ser Ile Gly Ser Lys Gly Gly Lys Leu Ala
140                 145                 150                 155

GCA GGT AAA TAC ACT GAT GCT GTA ACC GTA ACC GTA TCT AAC CAA TAA      529
Ala Gly Lys Tyr Thr Asp Ala Val Thr Val Thr Val Ser Asn Gln
                160                 165                 170

TCCATATAGG AATTC                                                    544
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 192 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) LOCATION: 1..186

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GAC GTC GTC GGT TAT AAT AAT TGT AAT ACC CAT ATA AAG GTA ATT GGA       48
Asp Val Val Gly Tyr Asn Asn Cys Asn Thr His Ile Lys Val Ile Gly
  1               5                  10                  15

TTT GGA ACA ATT ATC TTT ATT ATT TTA TTT TTT GTT GCT GTG TTT TTT       96
Phe Gly Thr Ile Ile Phe Ile Ile Leu Phe Phe Val Ala Val Phe Phe
            20                  25                  30

TGT GGA TAT ACT TGT GTA TTA AAC TCT CGT ATT AAA ATG ATT AAC CAT      144
Cys Gly Tyr Thr Cys Val Leu Asn Ser Arg Ile Lys Met Ile Asn His
        35                  40                  45

GCT TAT ATA CAA CCC CAG AAA TTA AAT TTT TAT GAT ATT TAA GAATTC      192
Ala Tyr Ile Gln Pro Gln Lys Leu Asn Phe Tyr Asp Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 61 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Asp Val Val Gly Tyr Asn Asn Cys Asn Thr His Ile Lys Val Ile Gly
 1               5                  10                  15

Phe Gly Thr Ile Ile Phe Ile Ile Leu Phe Phe Val Ala Val Phe Phe
            20                  25                  30

Cys Gly Tyr Thr Cys Val Leu Asn Ser Arg Ile Lys Met Ile Asn His
        35                  40                  45

Ala Tyr Ile Gln Pro Gln Lys Leu Asn Phe Tyr Asp Ile
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 576 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) LOCATION: 1..576

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATG AAA AAA ATC AGT TCC GTT ATC GCC ATT GCA TTA TTT GGA ACT ATT      48
Met Lys Lys Ile Ser Ser Val Ile Ala Ile Ala Leu Phe Gly Thr Ile
 1               5                  10                  15

GCA ACT GCT AAT GCG GCA GAT TTA ACT GCA AGC ACC ACT GCA ACG GCA      96
Ala Thr Ala Asn Ala Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala
            20                  25                  30

ACT CTT GTT GAA CCA GCC CGC ATC ACT CTT ACA TAT AAG GAA GGC GCT     144
Thr Leu Val Glu Pro Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala
        35                  40                  45

CCA ATT ACA ATT ATG GAC AAT GGA AAC ATC GAT ACA GAA TTA CTT GTT     192
Pro Ile Thr Ile Met Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val
    50                  55                  60

GGT ACG CTT ACT CTT GGC GGC TAT AAA ACA GGA ACC ACT AGC ACA TCT     240
Gly Thr Leu Thr Leu Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser
 65                  70                  75                  80
```

```
GTT AAC TTT ACA GAT GCC GCG GGT GAT CCC ATG TAC TTA ACA TTT ACT     288
Val Asn Phe Thr Asp Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr
                  85                  90                  95

TCT CAG GAT GGA AAT AAC CAC CAA TTC ACT ACA AAA GTG ATT GGC AAG     336
Ser Gln Asp Gly Asn Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys
             100                 105                 110

GAT TCT AGA GAT TTT GAT ATC TCT CCT AAG GTA AAC GGT GAG AAC CTT     384
Asp Ser Arg Asp Phe Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu
         115                 120                 125

GTG GGG GAT GAC GTC GTC GGT TAT AAT AAT TGT AAT ACC CAT ATA AAG     432
Val Gly Asp Asp Val Val Gly Tyr Asn Asn Cys Asn Thr His Ile Lys
     130                 135                 140

GTA ATT GGA TTT GGA ACA ATT ATC TTT ATT ATT TTA TTT TTT GTT GCT     480
Val Ile Gly Phe Gly Thr Ile Ile Phe Ile Ile Leu Phe Phe Val Ala
145                 150                 155                 160

GTG TTT TTT TGT GGA TAT ACT TGT GTA TTA AAC TCT CGT ATT AAA ATG     528
Val Phe Phe Cys Gly Tyr Thr Cys Val Leu Asn Ser Arg Ile Lys Met
                 165                 170                 175

ATT AAC CAT GCT TAT ATA CAA CCC CAG AAA TTA AAT TTT TAT GAT ATT     576
Ile Asn His Ala Tyr Ile Gln Pro Gln Lys Leu Asn Phe Tyr Asp Ile
             180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 192 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Lys Lys Ile Ser Ser Val Ile Ala Ile Ala Leu Phe Gly Thr Ile
 1               5                  10                  15

Ala Thr Ala Asn Ala Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala
            20                  25                  30

Thr Leu Val Glu Pro Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala
        35                  40                  45

Pro Ile Thr Ile Met Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val
    50                  55                  60

Gly Thr Leu Thr Leu Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser
65                  70                  75                  80
```

```
Val Asn Phe Thr Asp Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr
                  85                  90                  95

Ser Gln Asp Gly Asn Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys
             100                 105                 110

Asp Ser Arg Asp Phe Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu
         115                 120                 125

Val Gly Asp Asp Val Val Gly Tyr Asn Asn Cys Asn Thr His Ile Lys
     130                 135                 140

Val Ile Gly Phe Gly Thr Ile Ile Phe Ile Ile Leu Phe Phe Val Ala
145                 150                 155                 160

Val Phe Phe Cys Gly Tyr Thr Cys Val Leu Asn Ser Arg Ile Lys Met
                 165                 170                 175

Ile Asn His Ala Tyr Ile Gln Pro Gln Lys Leu Asn Phe Tyr Asp Ile
             180                 185                 190
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 513 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) LOCATION: 1..513

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
GCA GAT TTA ACT GCA AGC ACC ACT GCA ACG GCA ACT CTT GTT GAA CCA      48
Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala Thr Leu Val Glu Pro
  1               5                  10                  15

GCC CGC ATC ACT CTT ACA TAT AAG GAA GGC GCT CCA ATT ACA ATT ATG      96
Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala Pro Ile Thr Ile Met
             20                  25                  30

GAC AAT GGA AAC ATC GAT ACA GAA TTA CTT GTT GGT ACG CTT ACT CTT     144
Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val Gly Thr Leu Thr Leu
         35                  40                  45

GGC GGC TAT AAA ACA GGA ACC ACT AGC ACA TCT GTT AAC TTT ACA GAT     192
Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser Val Asn Phe Thr Asp
     50                  55                  60

GCC GCG GGT GAT CCC ATG TAC TTA ACA TTT ACT TCT CAG GAT GGA AAT     240
Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr Ser Gln Asp Gly Asn
 65                  70                  75                  80
```

```
AAC CAC CAA TTC ACT ACA AAA GTG ATT GGC AAG GAT TCT AGA GAT TTT     288
Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys Asp Ser Arg Asp Phe
                    85                  90                  95

GAT ATC TCT CCT AAG GTA AAC GGT GAG AAC CTT GTG GGG GAT GAC GTC     336
Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu Val Gly Asp Asp Val
            100                 105                 110

GTC GGT TAT AAT AAT TGT AAT ACC CAT ATA AAG GTA ATT GGA TTT GGA     384
Val Gly Tyr Asn Asn Cys Asn Thr His Ile Lys Val Ile Gly Phe Gly
        115                 120                 125

ACA ATT ATC TTT ATT ATT TTA TTT TTT GTT GCT GTG TTT TTT TGT GGA     432
Thr Ile Ile Phe Ile Ile Leu Phe Phe Val Ala Val Phe Phe Cys Gly
    130                 135                 140

TAT ACT TGT GTA TTA AAC TCT CGT ATT AAA ATG ATT AAC CAT GCT TAT     480
Tyr Thr Cys Val Leu Asn Ser Arg Ile Lys Met Ile Asn His Ala Tyr
145                 150                 155                 160

ATA CAA CCC CAG AAA TTA AAT TTT TAT GAT ATT                        513
Ile Gln Pro Gln Lys Leu Asn Phe Tyr Asp Ile
                165                 170
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 171 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala Thr Leu Val Glu Pro
 1               5                  10                  15

Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala Pro Ile Thr Ile Met
            20                  25                  30

Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val Gly Thr Leu Thr Leu
        35                  40                  45

Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser Val Asn Phe Thr Asp
    50                  55                  60

Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr Ser Gln Asp Gly Asn
65                  70                  75                  80

Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys Asp Ser Arg Asp Phe
                85                  90                  95

Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu Val Gly Asp Asp Val
            100                 105                 110
```

```
Val Gly Tyr Asn Asn Cys Asn Thr His Ile Lys Val Ile Gly Phe Gly
        115                 120                 125

Thr Ile Ile Phe Ile Ile Leu Phe Phe Val Ala Val Phe Phe Cys Gly
    130                 135                 140

Tyr Thr Cys Val Leu Asn Ser Arg Ile Lys Met Ile Asn His Ala Tyr
145                 150                 155                 160

Ile Gln Pro Gln Lys Leu Asn Phe Tyr Asp Ile
                165                 170
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 474 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURES:
(A) NAME/KEY: CDS
(B) LOCATION: 7..459

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
AAGCTT ATG GCA GAT TTA ACT GCA AGC ACC ACT GCA ACG GCA ACT CTT      48
       Met Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala Thr Leu
         1               5                  10

GTT GAA CCA GCC CGC ATC ACT CTT ACA TAT AAG GAA GGC GCT CCA ATT      96
Val Glu Pro Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala Pro Ile
 15                  20                  25                  30

ACA ATT ATG GAC AAT GGA AAC ATC GAT ACA GAA TTA CTT GTT GGT ACG     144
Thr Ile Met Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val Gly Thr
                 35                  40                  45

CTT ACT CTT GGC GGC TAT AAA ACA GGA ACC ACT AGC ACA TCT GTT AAC     192
Leu Thr Leu Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser Val Asn
             50                  55                  60

TTT ACA GAT GCC GCG GGT GAT CCC ATG TAC TTA ACA TTT ACT TCT CAG     240
Phe Thr Asp Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr Ser Gln
         65                  70                  75

GAT GGA AAT AAC CAC CAA TTC ACT ACA AAA GTG ATT GGC AAG GAT TCT     288
Asp Gly Asn Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys Asp Ser
     80                  85                  90

AGA GAT TTT GAT ATC TCT CCT AAG GTA AAC GGT GAG AAC CTT GTG GGG     336
Arg Asp Phe Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu Val Gly
 95                  100                 105                 110
```

```
GAT GAC GTC GTC TTG GCT ACG GGC AGC CAG GAT TTC TTT GTT CGC TCA     384
Asp Asp Val Val Leu Ala Thr Gly Ser Gln Asp Phe Phe Val Arg Ser
                115                 120                 125

ATT GGT TCC AAA GGC GGT AAA CTT GCA GCA GGT AAA TAC ACT GAT GCT     432
Ile Gly Ser Lys Gly Gly Lys Leu Ala Ala Gly Lys Tyr Thr Asp Ala
            130                 135                 140

GTA ACC GTA ACC GTA TCT AAC CAA TAA TCCATATAGG AATTC               474
Val Thr Val Thr Val Ser Asn Gln
        145                 150
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 150 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala Thr Leu
  1               5                  10

Val Glu Pro Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala Pro Ile
 15                  20                  25                  30

Thr Ile Met Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val Gly Thr
                35                  40                  45

Leu Thr Leu Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser Val Asn
            50                  55                  60

Phe Thr Asp Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr Ser Gln
        65                  70                  75

Asp Gly Asn Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys Asp Ser
    80                  85                  90

Arg Asp Phe Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu Val Gly
 95                 100                 105                 110

Asp Asp Val Val Leu Ala Thr Gly Ser Gln Asp Phe Phe Val Arg Ser
                115                 120                 125

Ile Gly Ser Lys Gly Gly Lys Leu Ala Ala Gly Lys Tyr Thr Asp Ala
            130                 135                 140

Val Thr Val Thr Val Ser Asn Gln
        145                 150
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 450 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATGGCAGATT TAACTGCAAG CACCACTGCA ACGGCAACTC TTGTTGAACC AGCCCGCATC 60
ACTCTTACAT ATAAGGAAGG CGCTCCAATT ACAATTATGG ACAATGGAAA CATCGATACA 120
GAATTACTTG TTGGTACGCT TACTCTTGGC GGCTATAAAA CAGGAACCAC TAGCACATCT 180
GTTAACTTTA CAGATGCCGC GGGTGATCCC ATGTACTTAA CATTTACTTC TCAGGATGGA 240
AATAACCACC AATTCACTAC AAAAGTGATT GGCAAGGATT CTAGAGATTT TGATATCTCT 300
CCTAAGGTAA ACGGTGAGAA CCTTGTGGGG GATGACGTCG TCTTGGCTAC GGGCAGCCAG 360
GATTTCTTTG TTCGCTCAAT TGGTTCCAAA GGCGGTAAAC TTGCAGCAGG TAAATACACT 420
GATGCTGTAA CCGTAACCGT ATCTAACCAA 450

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACGCGCGTCG ACGAGGTAAT ATATGAAAAA AATCAG 36

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGCGGATCCC TATATGGATT ATTGGTTAGA TACGG 35

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGCAAGCTTG AGGTAATATA TGAAAAAAAT CAG 33

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGCGAATTCC TATATGGATT ATTGGTTAGA TACGG 35

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGATGACGT CGTCGGTTAT AATAATTGTA ATACCC 36

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGCGAATTCT TAAATATCAT AAAAATTTAA TTTCTGGGG 39

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCCAAGCTTA TGGCAGATTT AACTGCAAGC ACC 33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 149 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ala Asp Leu Thr Ala Ser Thr Thr Ala Thr Ala Thr Leu Val Glu Pro
1 5 10 15

Ala Arg Ile Thr Leu Thr Tyr Lys Glu Gly Ala Pro Ile Thr Ile Met
20 25 30

Asp Asn Gly Asn Ile Asp Thr Glu Leu Leu Val Gly Thr Leu Thr Leu
35 40 45

Gly Gly Tyr Lys Thr Gly Thr Thr Ser Thr Ser Val Asn Phe Thr Asp
50 55 60

Ala Ala Gly Asp Pro Met Tyr Leu Thr Phe Thr Ser Gln Asp Gly Asn
65 70 75 80

Asn His Gln Phe Thr Thr Lys Val Ile Gly Lys Asp Ser Arg Asp Phe
85 90 95

Asp Ile Ser Pro Lys Val Asn Gly Glu Asn Leu Val Gly Asp Asp Val
100 105 110

Val Leu Ala Thr Gly Ser Gln Asp Phe Phe Val Arg Ser Ile Gly Ser
115 120 125

Lys Gly Gly Lys Leu Ala Ala Gly Lys Tyr Thr Asp Ala Val Thr Val
130 135 140

Thr Val Ser Asn Gln
145

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 447 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
GCAGATTTAA CTGCAAGCAC CACTGCAACG GCAACTCTTG TTGAACCAGC CCGCATCACT    60
CTTACATATA AGGAAGGCGC TCCAATTACA ATTATGGACA ATGGAAACAT CGATACAGAA   120
TTACTTGTTG GTACGCTTAC TCTTGGCGGC TATAAAACAG GAACCACTAG CACATCTGTT   180
AACTTTACAG ATGCCGCGGG TGATCCCATG TACTTAACAT TTACTTCTCA GGATGGAAAT   240
AACCACCAAT TCACTACAAA AGTGATTGGC AAGGATTCTA GAGATTTTGA TATCTCTCCT   300
AAGGTAAACG GTGAGAACCT TGTGGGGGAT GACGTCGTCT TGGCTACGGG CAGCCAGGAT   360
TTCTTTGTTC GCTCAATTGG TTCCAAAGGC GGTAAACTTG CAGCAGGTAA ATACACTGAT   420
GCTGTAACCG TAACCGTATC TAACCAA                                       447
```

While the various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications are adaptations are within the scope of the present invention, as set forth in the following claims.

What is claimed is:

1. A recombinant molecule comprising an isolated nucleic acid molecule that encodes an antigen selected from the group consisting of a *Yersinia* antigen, a *Pasteurella* antigen, and a *Francisella* antigen, said nucleic acid molecule being operatively linked to a eukaryotic transcription control region.

2. The recombinant molecule of Claim 1, wherein said antigen is selected from the group consisting of *Yersinia pestis, Yersinia pseudotuberculosis, Yersinia enterocolitica, Pasteurella multocida,* and *Francisella tularensis* antigens.

3. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule encodes a *Yersinia pestis* antigen.

4. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule encodes a *Yersinia pestis* antigen selected from the group consisting of an F1 antigen, a V antigen, a pesticin antigen, a W antigen, a pH6 antigen, a superoxide dismutase antigen, a *Yersinia* outer protein antigen, high-molecular weight iron-regulated membrane protein antigen, a murine toxin antigen, and a hemin storage protein antigen.

5. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule encodes a *Yersinia pestis* antigen selected from the group consisting of an F1 antigen and a V antigen.

6. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule encodes a *Yersinia pestis* F1 antigen.

7. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule is selected from the group consisting of: a nucleic acid molecule comprising a nucleic acid molecule selected from the group consisting of nYpF1(a)sec_{544}, nYpF1(b)sec_{544}, nYpF1sec_{510}, nYpF1anc_{576}, nYpF1anc_{513}, nYpF1mat_{474}, nYpF1mat_{450}, and nYpF1mat_{447}; and a nucleic acid molecule comprising a variant of a nucleic acid molecule selected from the group consisting of nYpF1(a)sec_{544}, nYpF1(b)sec_{544}, nYpF1sec_{510}, nYpF1anc_{576}, nYpF1anc_{513}, nYpF1mat_{474}, nYpF1mat_{450}, and nYpF1mat_{447}.

8. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule is selected from the group consisting of: a nucleic acid molecule

comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:22; and a nucleic acid molecule comprising a variant of a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:22.

9. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule encodes an antigen selected from the group consisting of: an antigen selected from the group consisting of $PYpF1sec_{170}$, $PYpF1anc_{192}$, $PYpF1anc_{171}$, $PYpF1mat_{150,}$ $PYpF1mat_{149}$; and an antigen encoded by a variant of a nucleic acid molecule encoding an antigen selected from the group consisting of $PYpF1sec_{170}$, $PYpF1anc_{192}$, $PYpF1anc_{171}$, $PYpF1mat_{150,}$ $PYpF1mat_{149}$.

10. The recombinant molecule of Claim 1, wherein said isolated nucleic acid molecule encodes an antigen selected from the group consisting of: an antigen having an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:21; and an antigen encoded by a variant of a nucleic acid molecule encoding an antigen having an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:21.

11. The recombinant molecule of Claim 1, wherein said recombinant molecule comprises an animal virus genome.

12. The recombinant molecule of Claim 11, wherein said eukaryotic transcription control region is heterologous to said genome.

13. The recombinant molecule of Claim 11, wherein said eukaryotic transcription control region is endogenous to said genome and is located at a position selected from the group consisting of a natural position within said genome and a non-natural position within said genome.

14. The recombinant molecule of Claim 11, wherein said animal virus genome is selected from the group consisting of a poxvirus genome, a herpesvirus genome, an alphavirus genome, a picornavirus genome, a retrovirus genome, an adenovirus genome, and an adeno-associated virus genome.

15. The recombinant molecule of Claim 11, wherein said animal virus genome is selected from the group consisting of an orthopoxvirus genome, a parapoxvirus genome, an entomopoxvirus genome, and an avipoxvirus genome.

16. The recombinant molecule of Claim 11, wherein said eukaryotic transcription control region is selected from the group consisting of an early/late poxvirus promoter, a late poxvirus promoter, a CMV immediate-early promoter, and an SV40 promoter.

17. The recombinant molecule of Claim 11, wherein said eukaryotic transcription control region is selected from the group consisting of an early/late poxvirus promoter and a late poxvirus promoter.

18. The recombinant molecule of Claim 11, wherein said genome is an orthopoxvirus genome selected from the group consisting of a raccoon poxvirus genome and a vaccinia virus genome.

19. The recombinant molecule of Claim 11, wherein said eukaryotic transcription control region is selected from the group consisting of a p7.5 promoter, a p11 promoter and a pSYN promoter.

20. The recombinant molecule of of Claim 11, wherein said isolated nucleic acid molecule is located in a region of said genome selected from the group consisting of a non-essential gene and an intergenic region.

21. The recombinant molecule of of Claim 11, wherein said isolated nucleic acid molecule is located in a non-essential gene of an orthopoxvirus genome, said non-essential gene being selected from the group consisting of a thymidine kinase gene, a hemagglutination gene, an anti-inflammatory gene, and an A-type inclusion gene.

22. The recombinant molecule of Claim 21, wherein said anti-inflammatory gene is selected from the group consisting of a soluble cytokine receptor gene, a serpin gene, a complement receptor gene, and an immunoglobulin receptor gene.

23. The recombinant molecule of Claim 1, wherein said recombinant molecule comprises vRCN-p11-nYpF1(a)sec_{544}.

24. A recombinant virus comprising a recombinant molecule as set forth in Claim 11.

25. The recombinant virus of Claim 24, wherein said virus is attenuated.

26. A recombinant virus of Claim 24, wherein said recombinant virus comprises RCN:p11-nYpF1(a)sec_{544}.

27. A recombinant cell comprising a recombinant molecule, said recombinant molecule comprising an animal virus genome as set forth in Claim 11.

28. A recombinant cell comprising a recombinant virus as set forth in Claim 24.

29. The recombinant cell of Claim 28, wherein said cell comprises BSC-1:RCN:p11nYpF1(a)sec_{544}.

30. The recombinant molecule of Claim 1, wherein said molecule comprises a recombinant plasmid.

31. The recombinant molecule of Claim 30, wherein said eukaryotic transcription control region comprises a promoter selected from the group consisting of a human cytomegalovirus immediate-early promoter, a simian virus 40 early promoter, and a Rous sarcoma virus long terminal repeat promoter.

32. The recombinant molecule of Claim 30, wherein said eukaryotic transcription control region comprises a polyadenylation region selected from the group consisting of a bovine growth hormone polyadenylation region and an SV40 polyadenylation region.

33. The recombinant molecule of Claim 30, wherein said eukaryotic transcription control region comprises a transcription enhancer region.

34. The recombinant molecule as set forth in Claim 30, wherein said recombinant molecule is selected from the group consisting of pCMV-nYpF1(b)sec_{544}, pCMV-nYpF1anc_{576}, and pCMV-nYpF1mat_{474}.

35. A recombinant cell comprising a recombinant molecule of Claim 30.

36. The recombinant cell of Claim 35, wherein said recombinant molecule is selected from the group consisting of pCMV-nYpF1(b)sec_{544}, pCMV-nYpF1anc_{576}, and pCMV-nYpF1mat_{474}.

37. A recombinant cell of Claim 35, wherein said recombinant molecule comprises a nucleic acid molecule that expresses an antigen selected from the group consisting of: an antigen selected from the group consisting of PYpF1sec_{170}, PYpF1anc_{192}, PYpF1anc_{171}, PypF1mat_{150}, and PYpF1mat_{149}; and an antigen

encoded by a variant of a nucleic acid molecule encoding an antigen selected from the group consisting of PYpF1sec$_{170}$, PYpF1anc$_{192}$, PYpF1anc$_{171}$, PYpF1mat$_{150}$, and PYpF1mat$_{149}$.

38. An isolated nucleic acid molecule encoding a *Yersinia pestis* F1 antigen fused, in-frame, with a eukaryotic membrane anchor domain.

39. The isolated nucleic acid molecule of Claim 38, wherein said eukaryotic membrane anchor domain is selected from the group consisting of a vesicular stomatitis virus glycoprotein membrane anchor domain, a respiratory syncytial virus G protein membrane anchor domain, a herpesvirus glycoprotein membrane anchor domain, an immunoglobulin membrane anchor domain, and a glycosyl phosphotidylinositol membrane anchor domain.

40. The isolated nucleic acid molecule of Claim 38, wherein said membrane anchor domain is a canine herpesvirus glycoprotein membrane anchor domain selected from the group consisting of a glycoprotein G membrane anchor domain, a glycoprotein E membrane anchor domain and a glycoprotein I membrane anchor domain.

41. The isolated nucleic acid molecule of Claim 38, wherein said nucleic acid molecule comprises nYpF1anc$_{576}$.

42. An isolated nucleic acid molecule comprising nYpF1anc$_{576}$.

43. A recombinant raccoon poxvirus genome comprising an isolated nucleic acid molecule encoding a *Yersinia pestis* F1 antigen operatively linked to a poxvirus transcription control region.

44. The recombinant genome of Claim 43, wherein said recombinant genome comprises vRCN-p11-nYpF1sec$_{544}$.

45. A recombinant raccoon poxvirus comprising a recombinant raccoon poxvirus genome, said genome comprising an isolated nucleic acid molecule encoding a *Yersinia pestis* F1 antigen operatively linked to a poxvirus transcription control region.

46. The recombinant raccoon poxvirus of Claim 45, wherein said virus comprises RCN:p11-nYpF1(a)sec$_{544}$.

47. A recombinant cell comprising a recombinant raccoon poxvirus genome, said genome comprising an isolated nucleic acid molecule encoding a *Yersinia pestis* F1 antigen operatively linked to a poxvirus transcription control region.

48. The recombinant cell of Claim 47, wherein said recombinant cell comprises vRCN-p11-nYpF1(a)sec_{544}.

49. The recombinant cell of Claim 47, wherein said recombinant cell comprising BSC-1:RCN:p11-nYpF1(a)sec_{544}.

50. A recombinant plasmid comprising an isolated nucleic acid molecule encoding a *Yersinia pestis* F1 antigen operatively linked to a eukaryotic transcription control region.

51. The recombinant plasmid of Claim 50, wherein said recombinant plasmid is selected from the group consisting of pCMV-nYpF1(b)sec_{544}, pCMV-nYpF1anc_{576}, and pCMV-nYpF1mat_{474}.

52. A recombinant cell comprising a recombinant plasmid, said plasmid comprising an isolated nucleic acid molecule encoding a *Yersinia pestis* F1 antigen operatively linked to a eukaryotic transcription control region.

53. A therapeutic composition to protect an animal from plague comprising a recombinant molecule, said recombinant molecule comprising an isolated nucleic acid molecule that encodes an antigen selected from the group consisting of a *Yersinia* antigen, a *Pasteurella* antigen, and a *Francisella* antigen, said nucleic acid molecule being operatively linked to a eukaryotic transcription control region.

54. The therapeutic composition of Claim 53, wherein said recombinant molecule is selected from the group consisting of a recombinant animal virus genome and a recombinant plasmid.

55. The therapeutic composition of Claim 53, wherein said recombinant molecule enters the cells of said animal, such that said antigen is expressed in said cells.

56. The therapeutic composition of Claim 53, wherein said therapeutic composition comprises a recombinant virus.

57. The therapeutic composition of Claim 53, wherein said therapeutic composition comprises a recombinant plasmid.

58. The therapeutic composition of Claim 53, wherein said composition further comprises a component selected from the group consisting of an excipient, an adjuvant, and a carrier.

59. The therapeutic composition of Claim 53, wherein plague comprises a disease selected from the group consisting of bubonic plague, septicemic plague, pneumonic plague, urban plague, rat plague, wild rodent plague, sylvatic plague, campestral plague, high plains plague, la peste bubonique, The Pest, The Black Plague, and The Black Death.

60. A method to protect an animal from plague comprising administering to said animal a therapeutic composition comprising a recombinant molecule, said molecule comprising an isolated nucleic acid molecule that encodes an antigen selected from the group consisting of a *Yersinia* antigen, a *Pasteurella* antigen, and a *Francisella* antigen, said nucleic acid molecule being operatively linked to a eukaryotic transcription control region.

61. The method of Claim 60, wherein said recombinant molecule is selected from the group consisting of a recombinant animal virus genome and a recombinant plasmid.

62. The method of Claim 60 comprising administering to said animal a recombinant virus.

63. The method of Claim 60 comprising administering to said animal a recombinant plasmid.

64. The method of Claim 62 wherein said therapeutic composition is administered by a method selected from the group consisting of oral, subcutaneous injection, intradermal injection, and intramuscular injection.

65. The method of Claim 62, wherein said therapeutic composition is administered orally.

66. The method of Claim 63, wherein said therapeutic composition is administered by a method selected from the group consisting of injection, oral application, nasal application, particle bombardment, or intradermal scarification.

67. The method of Claim 66, wherein said injection is administered by a method selected from the group consisting of intradermal injection and intramuscular injection.