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| <p>(21) International Application Number: PCT/US99/15568</p> <p>(22) International Filing Date: 9 July 1999 (09.07.99)</p> <p>(30) Priority Data: 60/092,416 10 July 1998 (10.07.98) US</p> <p>(71) Applicant (for all designated States except US): U.S. MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES [US/US]; Department of the Army, 504 Scott Street, Frederick, MD 21702 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): LEE, John, S. [US/US]; 1807 Elizabeth Court, Hagerstown, MD 21740 (US). PUSHKO, Peter [US/US]; 917 Seminole Road, Frederick, MD 21701 (US). PARKER, Michael, D. [US/US]; Apartment T-13, 1001 Carroll Parkway, Frederick, MD 21701 (US). SMITH, Jonathan, F. [US/US]; 6936 Eylers Valley Flint Road, Sabillasville, MD 21780 (US). WELKOS, Susan, L. [US/US]; 1594 Dockside Drive, Frederick, MD 21701 (US).</p> <p>(74) Agents: HARRIS, Charles, H.; United States Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702 (US) et al.</p> | | <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p> |
| <p>(54) Title: ANTHRAX VACCINE</p> | | |
| <p>(57) Abstract</p> <p>Using the nontoxic PA protein from <i>B. anthracis</i>, a method and composition for use in inducing an immune response which is protective against anthrax in subjects is described.</p> | | |

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TITLE OF THE INVENTION

Anthrax Vaccine

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FIELD OF THE INVENTION

This invention relates to vaccines for bacterial toxins from *Bacillus anthracis*.

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INTRODUCTION

Anthrax is a disease cause by the sporulating bacteria *Bacillus anthracis*. Humans working with animal products are at risk from contracting anthrax. Areas such as Iran, Turkey, Iraq, Pakistan, and sub-Saharan Africa are hyperendemic for anthrax, although the organism can be found in most areas of the world. Anthrax manifests disease in three different ways. Inhalation, gastrointestinal, and cutaneous anthrax result from inhaling spores, ingesting spores in contaminated meat, or contacting spores in an open wound, respectively. Untreated inhalation or gastrointestinal anthrax has a case fatality rate of essentially 100 percent while cutaneous anthrax has a case fatality rate of up to 25 percent. Previous research has shown that the protective antigen (PA) produced by *B. anthracis* can protect mice from anthrax. Even though the Anthrax vaccine is FDA licensed, reactogenicity is mild to moderate.

Therefore, there is a need for an efficacious vaccine for anthrax useful for protecting humans.

SUMMARY OF THE INVENTION

5 The present invention satisfies the need discussed above. The present invention relates to a method and composition for use in inducing an immune response which is protective against infection with anthrax.

10 In this vaccine strategy, a gene coding for a protein of interest is cloned into a VEE virus vector in place of the VEE virus structural genes; the result is a self-replicating RNA molecule, a replicon, that encodes its own replicase and transcriptase functions,
15 and in addition makes abundant quantities of mRNA encoding the foreign protein. When replicon RNA is transfected into eukaryotic cells along with two helper RNAs that express the VEE structural proteins (glycoproteins and nucleocapsid), the replicon RNA is
20 packaged into VEE virus-like particles by the VEE virus structural proteins, which are provided in trans. Since the helper RNAs lack packaging signals necessary for further propagation, the resulting VEE replicon particles (VRPs) which are produced are
25 infectious for one cycle but are defective thereafter. Upon infection of an individual cell with a VRP, an abortive infection occurs in which the infected cell produces the protein of interest in abundance, is ultimately killed by the infection, but does not
30 produce any viral progeny (Pushko *et al.*, 1997, *Virology* 239, 389-401).

 The PA gene which contains a prokaryotic secretory signal and the entire 83 kDa coding sequence (Welkos *et al.*, 1988, *Gene* 69, 287-300), was inserted

into the VEE replicon vaccine vector (Figure 1) and have demonstrated high level expression of this bacterial protein in eukaryotic cells in culture. Mice, either the C57BL/6 strain or the A/J strain, inoculated with VRP containing the PA-replicon produced high specific antibody titers and were protected from developing anthrax when challenged subcutaneously with *B. anthracis*.

Therefore, it is one object of the present invention to provide a VEE virus replicon vector comprising a VEE virus replicon and DNA fragments encoding the PA protein from *B. anthracis*.

It is another object of the present invention to provide a self replicating RNA comprising the VEE virus replicon and any of the *B. anthracis* fragments described above.

It is another object of the present invention to provide infectious VEE virus replicon particles produced from the VEE virus replicon RNA described above.

It is further an object of the invention to provide an immunological composition for the protection of mammals against *B. anthracis* infection comprising VEE virus replicon particles containing any of the *B. anthracis* fragments described above or a combination of different VEE virus replicons each having a different *B. anthracis* fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. Diagram of the protective antigen (PA) replicon constructs. The PA replicons are similar to

the full-length VEE RNA except that the open reading frame encoding the VEE structural proteins was replaced with the PA genes.

Figure 2. Western blot of BHK cell lysates showing expression of anthrax protective antigen from recombinant VEE replicons. a) uninfected cell lysate; b) recombinant expression product from *B. anthracis*; c) infected cell lysate.

Figure 3A and 3B. ELISA titers for mice (A, C57Bl/6 mice; B, A/J mice) immunized with PA-VRP. GMTs only includes those mice that survived challenge. a) Received 0.2 ml on day 0 and 28. b) Received 10^7 iu on day 0 and 28. c) Received 10^7 iu PA-VRP on day 0, 28, 56, and 84; C57Bl/6 mice that died, titer = 100; A/J mice that died, titer = 400. d) Received 10^7 iu PA-VRP on day 0, 28, and 56; C57Bl/6 mice that died, titers = 400, 100, 100; A/J mice that died, titers = 102400, 100, 1600. e) Received 10^7 iu PA-VRP on day 0, and 28; C57Bl/6 mice that died, GMT = 1313; A/J mice that died, GMT = 1766. Mice were challenged 28 days after last inoculation with 18-32 LD₅₀ Sterne strain for C57Bl/6 and 12-25 LD₅₀ Sterne strain for A/J mice.

Figure 4. Schematic representation of constructs containing PA.

DETAILED DESCRIPTION

In the description that follows, a number of terms used in recombinant DNA, virology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Replicon. A replicon is equivalent to a full length virus from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site.

Transcription of the RNA from the replicon yields an RNA capable of initiating infection of the cell identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA *in trans*. This is typically done with two helpers also called defective helper RNAs. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequences for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then be inoculated into animals similar to parent virus. The replicon

particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nonstructural proteins and the product of the
5 heterologous gene cloned in place to the structural proteins.

The VEE virus replicon is a genetically reorganized version of the VEE virus genome in which the structural proteins genes are replaced with a gene
10 from an immunogen of interest, in this invention, the *B. anthracis* proteins. The result is a self replicating RNA (replicon) that can be packaged into infectious particles using defective helper RNAs that encode the glycoprotein and capsid proteins of the VEE
15 virus.

Subject. Includes both human, animal, e.g., horse, cattle, donkey, monkey, pig, dog, guinea pig, mouse, hamster, avian e.g., chicken, pheasant or turkey, fish and other marine animals, and insects
20 such as mosquito.

In one embodiment, the present invention relates to a recombinant DNA molecule that includes a VEE replicon and a DNA sequence encoding the non-toxic
25 protective antigen (PA) from *B. anthracis*. The sequence for PA has been determined and has been deposited in GenBank at accession no. M22589. The PA sequence encodes a prokaryotic secretory signal in addition to the entire sequence encoding the 83 Kd PA
30 (SEQ ID NO:1). Other nucleic acid sequences related to this invention include sequences wherein the secretory signal has been removed (MAT-PA) (SEQ ID NO:2), or replaced with other secretory signals known to people in the art such as the tissue plasminogen
35 activator (TPA) secretory signal resulting in a DNA

fragment encoding TPA-PA (SEQ ID NO:3). Nucleic acid sequences included in this invention are sequences encoding the active form of PA, a 63 kDa protein, termed PA63 (SEQ ID NO:4). In addition, isolated
5 nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the *B. anthracis* proteins described and specified in
10 SEQ ID NO:5 (TPA-PA), SEQ ID NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63). Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate
15 variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli*).

Nucleic acid molecules of the present invention
20 may be in the form of RNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the
25 sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For
30 example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or
35 substantially) DNA molecules in solution. Isolated

RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such
5 molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences
10 of at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is
15 position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1.

The present invention further relates to variants
20 of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the *B. anthracis* polypeptides described above. Variants may occur naturally, such as a
25 natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occurring variants may be produced by known mutagenesis techniques. Such
30 variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid
35 substitutions, deletions, or additions. Especially

preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of the *B. anthracis* polypeptides disclosed herein or portions thereof.

5 Also preferred in this regard are conservative substitutions.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The
10 vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The
15 cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with
20 ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. When the DNA sequences described above are in a
25 replicon expression system, such as the VEE replicon described above, the proteins can be expressed *in vivo*. The DNA sequence for any of the *B. anthracis* proteins described above can be cloned into the multiple cloning site of a replicon such that
30 transcription of the RNA from the replicon yields an infectious RNA containing the *B. anthracis* protein or proteins of interest. Use of helper RNA containing sequences necessary for encapsulation of the viral transcript will result in the production of viral
35 particles containing replicon RNA which are able to

infect a host and initiate a single round of replication resulting in the expression of the *B. anthracis* proteins. Such replicon constructs include those listed in Table 1.

5 Table 1
Replicon

| Plasmid Name | expresses |
|----------------------|--|
| 10 p3014-TPA-PA | TPA signal, 5 amino acids of C terminal end of prokaryotic secretory signal, and the 83kDa mature protein. |
| p3014-PA | 29 amino acid prokaryotic secretory signal, and the 83kDa mature protein secreted by <i>B. anthracis</i> |
| 15 p3014-MAT-PA | 83kDa mature protein |
| p3014-PA63 | 63kDa protein (minus 20kDa N-terminal end that is naturally cleaved off by furin proteases at host cell membrane surface) |
| 20 p3014-sub-domains | subdomain #1: from nucleotide 1891 to 2391; subdomain #2: from nucleotide 2392 to 2658; subdomain #3: from nucleotide 2659 to 3351; subdomain #4: from nucleotide 3352 to 3669; subdomain #5: from nucleotide 3670 to 4098 (Subdomains are numbered according to Welkos et al., 1988, supra, determined on subdomains present in the crystal structure, Petosa, et al., 1997, <i>Nature</i> 385, 833-838). |
| 25 | |
| 30 | |
| 35 | |

The VEE constructs containing anthrax proteins can be used as a DNA vaccine, or for the production of RNA molecules as described below.

40 In another embodiment, the present invention relates to RNA molecules resulting from the transcription of the constructs described above. The RNA molecules can be prepared by *in vitro* transcription using methods known in the art and described in the Examples below. Alternatively, the

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RNA molecules can be produced by transcription of the constructs *in vivo*, and isolating the RNA. These and other methods for obtaining RNA transcripts of the constructs are known in the art. Please see Current
5 Protocols in Molecular Biology. Frederick M. Ausubel et al. (eds.), John Wiley and Sons, Inc. The RNA molecules can be used, for example, as a nucleic acid vaccine, or to transfect cells along with RNA from helper plasmids, one of which expresses VEE
10 glycoproteins and the other VEE capsid proteins, as described above, in order to obtain replicon particles.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection,
15 electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. et al. (Eds), Wiley & Sons, Inc. All documents cited herein supra and infra are hereby
20 incorporated in their entirety by reference thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for
25 example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences
30 when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions,

and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of protein of the invention, such as glutathione S-transferase. The recombinant molecule can be suitable for transfecting eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as baby hamster kidney (BHK) cells, MRC-5 cells and vero cells, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus (CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase

expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein or polypeptide specified as amino acid sequence in SEQ ID NO:5 (TPA-PA), SEQ ID NO:6 (PA), SEQ ID NO:7 (MAT-PA), and SEQ ID NO:8 (PA63).

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

The recombinant or fusion protein can be used as a vaccine for immunity against anthrax infection or as a diagnostic tool for detection of bacillus anthracis. The transformed host cells can be used to analyze the effectiveness of drugs and agents which inhibit anthrax or *B. anthracis* proteins, such as host

proteins or chemically derived agents or other proteins which may interact with *B. anthracis* proteins of the present invention to inhibit its function. A method for testing the effectiveness of an anti-anthrax drug or agent can for example be the rat anthrax toxin assay (Ivins et al. 1984, *Infec. Immun.* 52, 454-458 and Ivins et al. 1986) or a skin test in rabbits for assaying antiserum against anthrax toxin (Belton and Henderson, 1956, *Br. J. Exp. Path.* 37, 156-160).

In another embodiment, the present invention relates to an anthrax vaccine comprising one or more replicon particles derived from one or more replicons encoding one or more *B. anthracis* proteins or polypeptides as described above. The present invention relates a method for providing immunity against anthrax said method comprising administering one or more replicon particles containing any combination of the *B. anthracis* proteins to a subject such that a protective immune reaction is generated.

Vaccine formulations of the present invention comprise an immunogenic amount of a replicon particle, resulting from one of the replicon constructs described above, or a combination of replicon particles as a multivalent vaccine, in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered. An amount of from about 10^2 to 10^7 per dose is suitable, more or less can be used depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the replicon particles disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, orally and by topical application of the virus (typically carried in the pharmaceutical formulation) to an airway surface. Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the replicon as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed. An "immunogenic amount" is an amount of the replicon particles sufficient to evoke an immune response in the subject to which the vaccine is administered.

When the replicon RNA or DNA is used as a vaccine, the replicon RNA or DNA can be administered directly using techniques such as delivery on gold beads (gene gun), delivery by liposomes, or direct injection, among other methods known to people in the art. Any one or more constructs or replicating RNA described above can be use in any combination effective to elicit an immunogenic response in a subject. Generally, the nucleic acid vaccine administered may be in an amount of about 1-5 ug of

nucleic acid per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine
5 to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in
10 which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent
15 dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses
20 expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

The following MATERIALS AND METHODS were used in the examples that follow.

Plasmids. Construction of the VEE replicon, capsid (C-) helper, and glycoprotein (GP-) helper
25 plasmids was previously described (Pushko, 1997, supra). The PA gene from nucleotide 1804 to 4098 (SEQ ID NO:1) (Welkos et al., 1988, supra) was cloned into the VEE replicon plasmid as a BamHI/BamHI fragment
30 utilizing a shuttle vector. TPA-PA (SEQ ID NO:3), MAT-PA (SEQ ID NO:2), and PA63 (SEQ ID NO:4) were PCR amplified using forward and reverse primers which also contained the ClaI recognition site. The PCR products were agarose gel purified and ligated into the T/A
35 vector pCR2.1 (Invitrogen, Inc.). The PA genes, after

digestion of pCR2.1-PA's with ClaI, were ligated into the VEE replicon plasmid. The Lassa nucleocapsid replicon (Lassa N-replicon) was constructed as previously described (Pushko, 1997, supra) and used as
5 a negative control replicon.

Production of VRP. Plasmid templates for the PA-replicons, C-helper, GP-helper, and the Lassa N-replicon were linearized by digestion with NotI at a unique site downstream from the coding sequences, and
10 capped run-off transcripts were prepared *in vitro* using T7 RNA polymerase. Packaging of the replicons into VEE replicon particles (VRPs) was accomplished by electroporating the replicon RNA and the two helper RNAs into BHK cells. VRPs were harvested between 20
15 and 27 hours after transfection and purified from cell culture supernatants by ultracentrifugation through a discontinuous sucrose gradient (20%). After reconstituting the pelleted VRP in 1/50 volume phosphate buffered saline, the VRPs were stored at -
20 70°C.

Analysis of expression products and titration of VRP. Subconfluent monolayers were infected with PA-VRP's (either TPA-PA, PA, MAT-PA, or PA63) or Lassa N-VRP (m.o.i. = 2) or cell suspensions
25 were electroporated with replicon RNA. Cells were harvested at approximately 20-24 hours and expressed proteins were separated by SDS-PAGE. Visualization of PA proteins was accomplished using a chemiluminescence western blot assay and antibodies specific for each
30 protein. Titration of VRPs was accomplished by infecting subconfluent monolayers with increasing dilutions of purified VRP. Antigen positive cells were visualized in an indirect immunofluorescence assay using a monoclonal antibody specific for each protein,

or in a direct immunofluorescence assay using an FITC-conjugated monkey anti-Lassa serum.

Immunization of mice. Mice were inoculated 1 to 4 times at 28 day intervals with 10^5 to 10^7 5 infectious units (iu) of either PA-VRP or Lassa N-VRP (negative control). Positive control mice for the anthrax study were inoculated subcutaneously with 0.2 ml of anthrax vaccine at 28 day intervals. Serum for ELISA was obtained 2 days before each inoculation and 10 3 days before challenge.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with protective antigen (gift from Joe Farchaus, Bacteriology Division) (1 ug/ml) in PBS and allowed to absorb overnight at 4°C. 15 Four fold serum dilutions in blocking buffer were applied to the plates and incubated at 37°C for 1 hour. After washing, an anti-mouse secondary antibody (HRP conjugated) was added to the plate and incubated for an additional hour at 37°C. After washing, bound 20 antibody was detected colormetrically using ABTS as a substrate.

Challenge of mice.

Anthrax challenge: C57BL/6 or A/J mice were challenged subcutaneously with 12-32 LD₅₀ *B. anthracis* 25 (Sterne) 31 days after the last inoculation.

Example 1

Packaging and expression of PA-replicon

The PA-replicon was efficiently packaged into VRPs using the double helper system. Stock solutions 30 contained about 10^8 infectious units of purified PA-VRP per milliliter. No replication competent VEE virus was detected in any of the preparations. A western blot of cell lysates, generated from BHK cells infected with PA-VRP, demonstrates a product of

approximately 85 kDa (Figure 2). Because the PA-VRP expression product contains a 29 amino acid prokaryotic secretory signal, which is cleaved from the standard, the PA product from the BHK cells
5 appears larger than the PA standard.

Example 2

Protection against challenge with *B. anthracis*

The results of the animal studies demonstrated that PA from *B. anthracis* could be used to immunize
10 and protect mice from a lethal challenge of *B. anthracis* (Sterne strain). Although the Sterne strain is attenuated in some animals, it is virulent in mice. Controls in the experiment included mice vaccinated with the current human vaccine (anthrax vaccine
15 absorbed, AVA) which protected all mice, and mice immunized with the Lassa N-replicon which expressed an antigen irrelevant to anthrax protection which failed to protect the mice. Figure 3A shows ELISA titers and survival for C57Bl/6 mice inoculated with two to four
20 doses of PA-VRP. Seventy percent of the C57Bl/6 mice which received three inoculations of the PA-VRP were protected whereas 90% of the C57Bl/6 mice which received four inoculations were protected from an otherwise lethal challenge of *B. anthracis* (Sterne
25 strain). The geometric mean of the serum ELISA titers to PA of C57Bl/6 mice given four doses of the PA-replicon was 150500, as compared to 264 for mice that were inoculated with the negative control replicon. Figure 3B shows ELISA titers and survival for A/J mice
30 inoculated with two to four doses of PA-VRP. Three inoculations of the PA-VRP protected 70% of the A/J mice, whereas four inoculations protected 90% of the A/J mice from an otherwise lethal challenge of the *B. anthracis* (Sterne strain). The geometric mean of the
35 serum ELISA titers against PA in A/J mice given four

doses of the PA-replicon was 223336 as compared to 100 for mice which were inoculated with the negative control replicon. As expected, none of the mice (from either strain) which were inoculated with the negative control replicon survived challenge with *B. anthracis* (Sterne strain).

Discussion/Conclusion

Since VEE virus replicates in the cytoplasm of eukaryotic cells, the VEE replicon vaccine vector is a useful tool for the expression of prokaryotic genes in eukaryotic cells. Cytoplasmic expression of genes alleviates the difficulties imposed by splicing and nuclear transport of mRNA. We used the VEE replicon as a way to express the prokaryotic PA gene in eukaryotic cells and to develop a new vaccine candidate against anthrax.

The human AVA contains mostly PA protein and is presumed to protect humans by eliciting an antibody response that can neutralize the PA portion of anthrax toxin produced by invading *B. anthracis*. After neutralization of the toxin, the immune system destroys the invading bacteria. In order to model the human disease, we chose to use mice and the Sterne strain of *B. anthracis*. The Sterne strain produces anthrax toxin (encoded on the pX01 plasmid) but lacks a capsule (encoded on the pX02 plasmid). Mice exposed to large quantities of Sterne (ranging from 10^3 to 10^8 spores for the different mouse strains) are not able to overcome the effects of the toxin produced by the bacteria so they succumb to infection. We hypothesized that by immunizing the mice with PA, thereby inducing neutralizing antibodies to the PA

portion of the toxin produced by a challenge inoculum, that the mice would be protected.

A VEE replicon was constructed which expressed the PA gene from *B. anthracis* and was evaluated for *in vitro* expression, *in vivo* immunogenicity, and protective efficacy. C57Bl/6 and A/J mice demonstrated increasing antibody responses to PA when inoculated with increasing doses of PA-VRP. The two mouse strains inoculated with up to four doses of PA-VRP displayed increased protection against a lethal challenge with *B. anthracis* (Sterne strain). The use of mice and the Sterne strain of *B. anthracis* useful as a model system for studying the immunogenicity and efficacy of replicon based anthrax vaccines.

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What is claimed is:

1. A recombinant DNA construct comprising:
 - (i) a vector, and
 - (ii) at least one nucleic acid5 fragment comprising any combination of *B. anthracis* proteins selected from the group consisting of PA, MAT-PA, TPA-PA, and PA63.
- 10 2. A recombinant DNA construct according to claim 1 wherein said vector is an expression vector.
3. A recombinant DNA construct according to claim 1 wherein said vector is a prokaryotic vector.
- 15 4. A recombinant DNA construct according to claim 1 wherein said vector is a eukaryotic vector.
5. The recombinant DNA construct of claim 1 wherein said vector is a VEE virus replicon vector.
- 20 6. The recombinant DNA construct according to claim 5 wherein said construct is p3014-PA.
7. The recombinant DNA construct according to claim 5 wherein said construct is p3014-TPA-PA.
- 25 8. The recombinant DNA construct according to claim 5 wherein said construct is p3014-MAT-PA.
- 30 9. The recombinant DNA construct according to claim 5 wherein said construct is p3014-PA63.
- 35 10. Self replicating RNA produced from a construct chosen from the group consisting of: p3014-PA, p3014-TPA-PA, p3014-MAT-PA, and p3014-P63

11. Infectious alphavirus particles produced from packaging the self replicating RNA of claim 10.

5 12. A pharmaceutical composition comprising infectious alphavirus particles according to claim 11 in an effective immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

10 13. A host cell transformed with a recombinant DNA construct according to claim 5.

 14. A host cell according to claim 13 wherein said host cell is prokaryotic.

15 15. A host cell according to claim 13 wherein said host cell is eukaryotic.

 16. A method for producing *B. anthracis* protein
20 comprising culturing the cells according to claim 14 under conditions such that said DNA fragment is expressed and said protein is produced.

 17. A method for producing *B. anthracis* protein
25 comprising culturing the cells according to claim 15 under conditions such that said DNA fragment is expressed and said protein is produced.

 18. A vaccine for anthrax comprising viral
30 particles containing one or more replicon RNA encoding any combination of *B. anthracis* proteins chosen from the group consisting of PA, PA-MAT, TPA-PA, and PA63.

 19. A pharmaceutical composition comprising the
35 self replication RNA of claim 14 in an effective

immunogenic amount in a pharmaceutically acceptable carrier and/or adjuvant.

20. A pharmaceutical composition comprising one
5 or more recombinant DNA constructs chosen from the
group consisting of p3014-PA, p3014-TPA-PA, p3014-MAT-
PA, and p3014-P63 in a pharmaceutically acceptable
amount, in a pharmaceutically acceptable carrier/and
or adjuvant.

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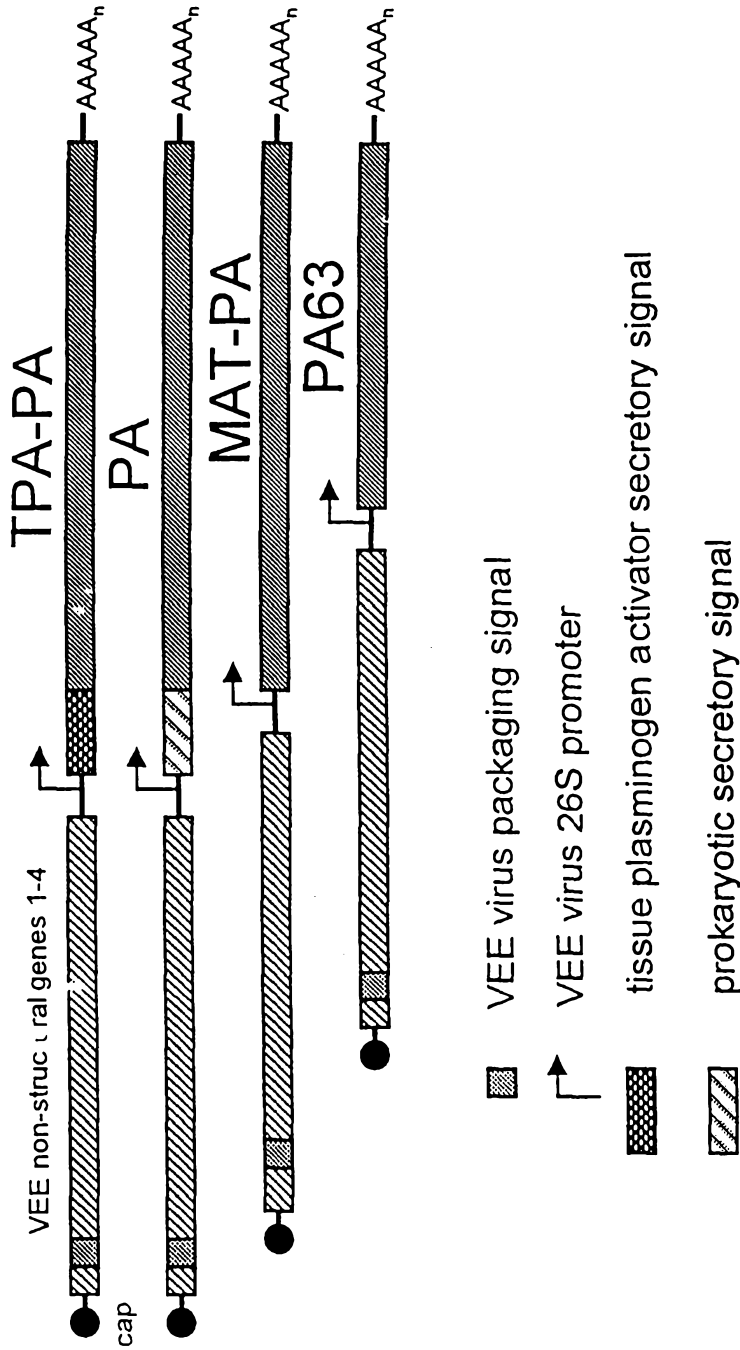


Figure 1. Diagram of the protective antigen (PA) replicon constructs. The PA replicons are similar to the full-length VEE RNA except that the open reading frame encoding the VEE structural proteins was replaced with the PA genes.

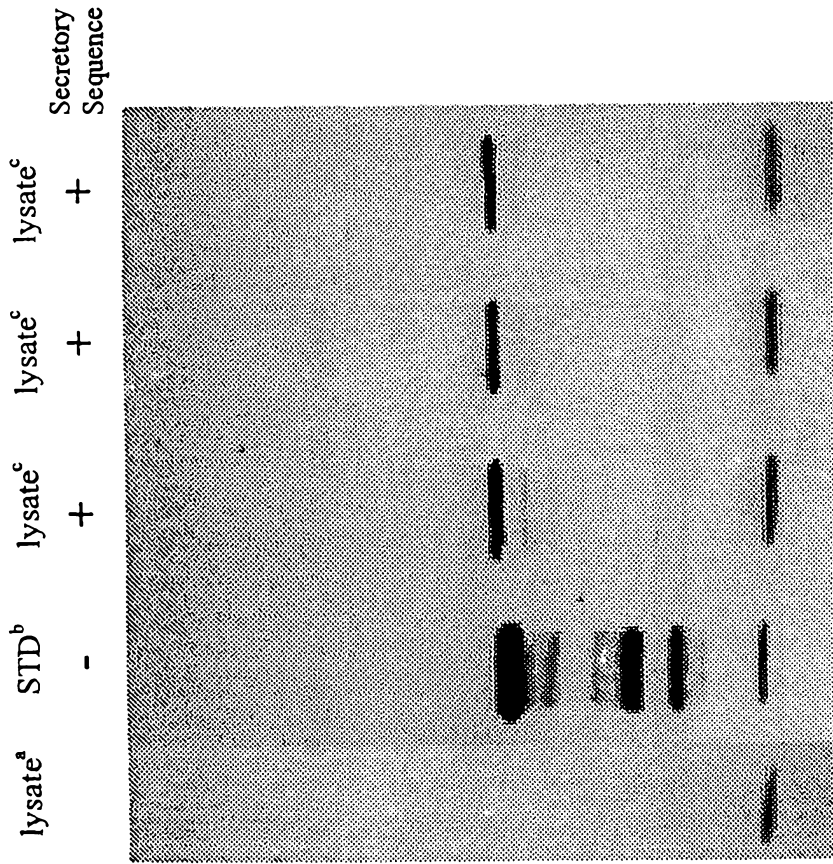


Figure 2. Western blot of BHK cell lysates showing expression of anthrax protective antigen from recombinant VEE replicons. a) uninfected cell lysate; b) recombinant expression product from *B. anthracis*; c) infected cell lysate.

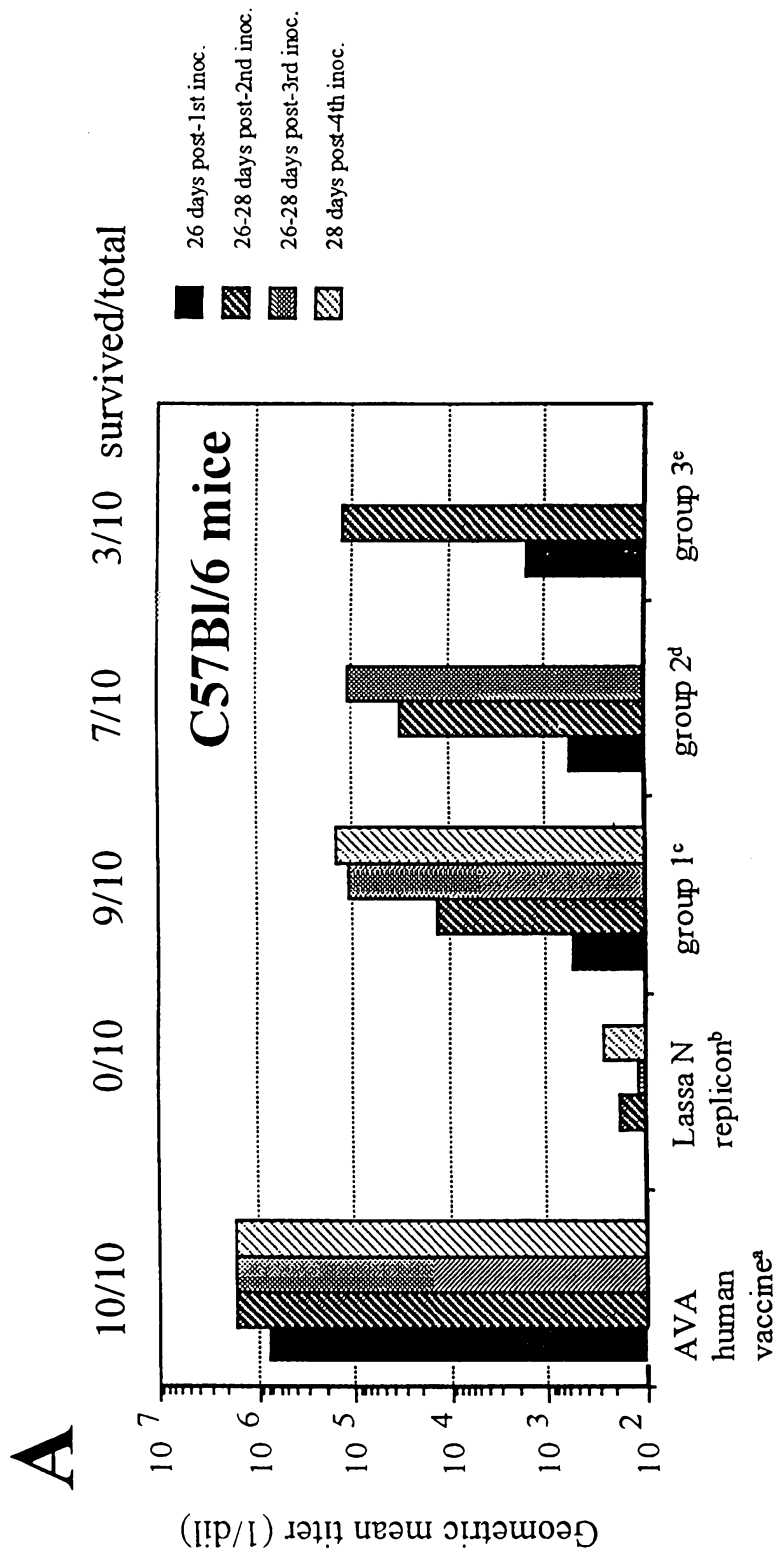


FIGURE 3

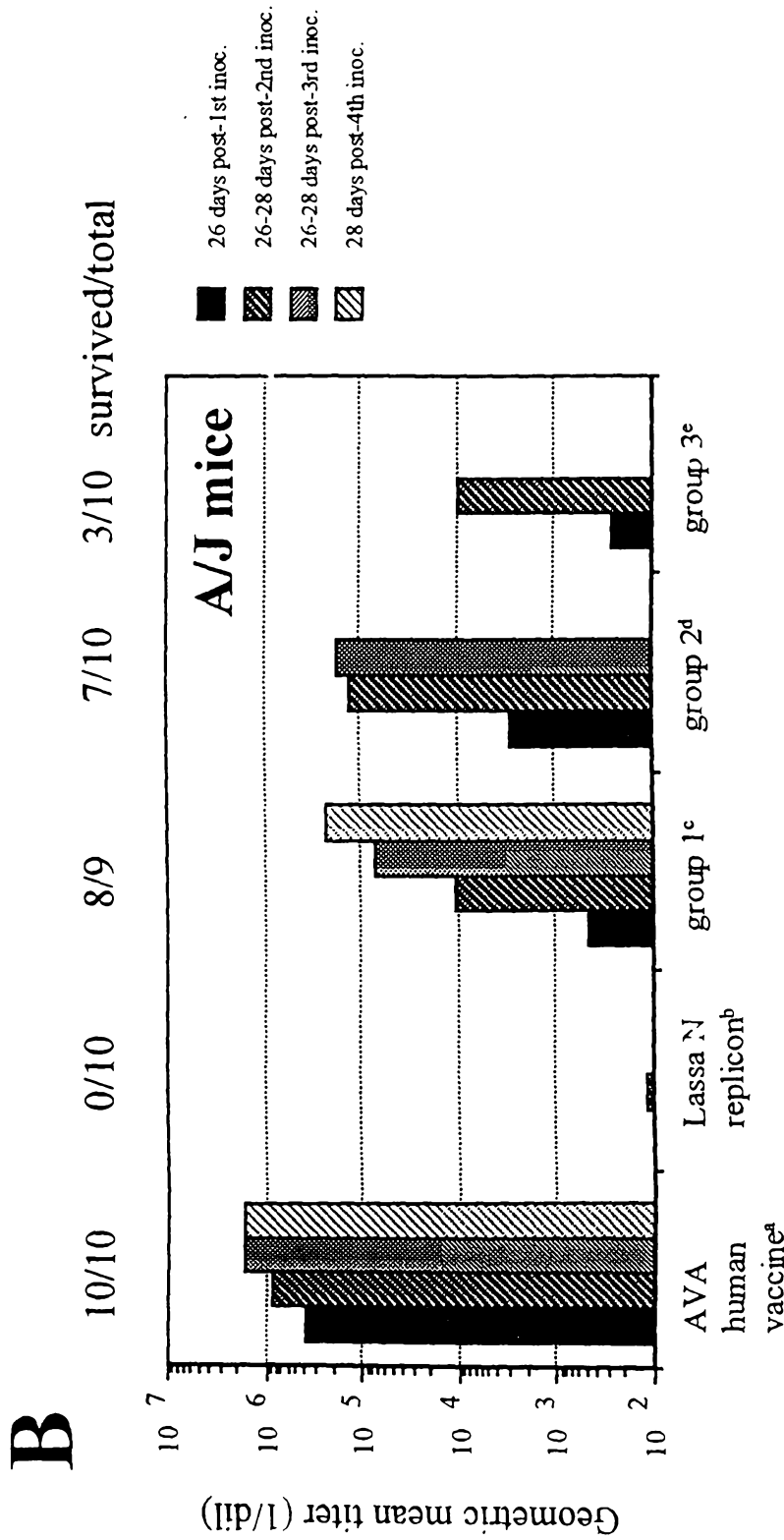
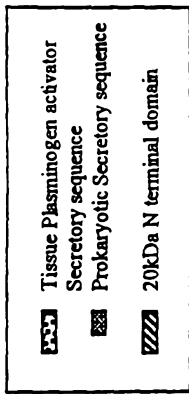
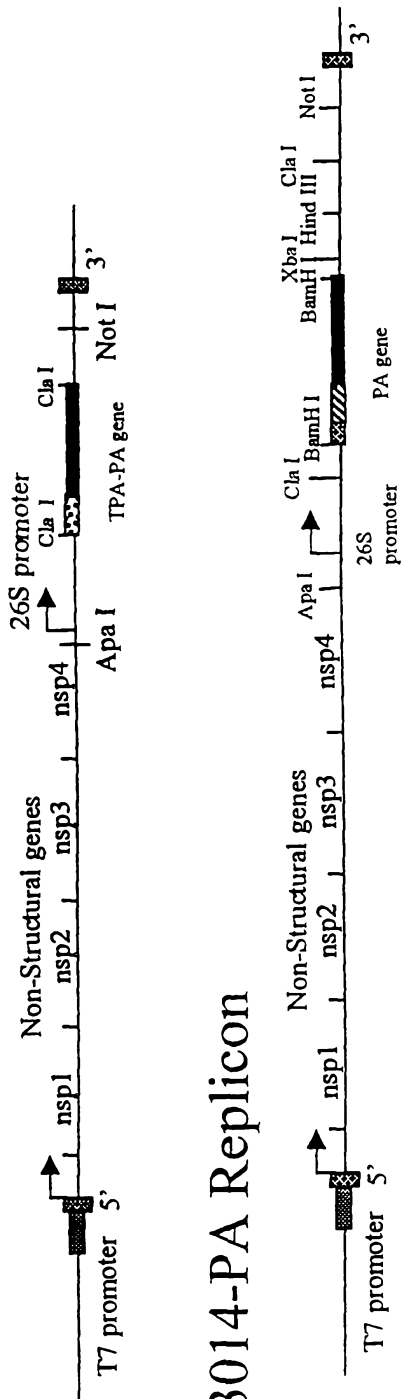


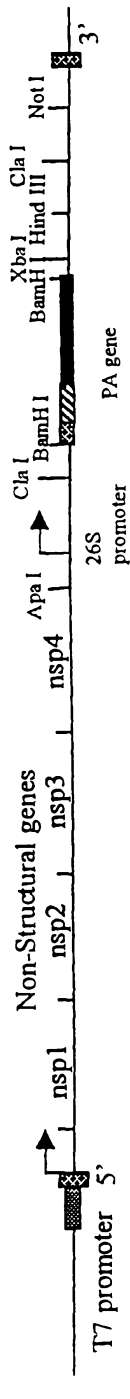
Figure 3. ELISA titers for mice immunized with PA-VRP. GMTs only includes those mice that survived challenge. a) Received 0.2 ml on day 0 and 28. b) Received 10⁷ iu on day 0 and 28. c) Received 10⁷ iu PA-VRP on day 0, 28, 56, and 84; C57Bl/6 mice that died, titer = 100; A/J mice that died, titer = 400. d) Received 10⁷ iu PA-VRP on day 0, 28, and 56; C57Bl/6 mice that died, titers = 400, 100, 100; A/J mice that died, titers = 102400, 100, 1600. e) Received 10⁷ iu PA-VRP on day 0, and 28; C57Bl/6 mice that died, GMT = 1313; A/J mice that died, GMT = 1766. Mice were challenged 28 days after last inoculation with 18-32 LD₅₀ Sterne strain for C57Bl/6 and 12-25 LD₅₀ Sterne strain for A/J mice.



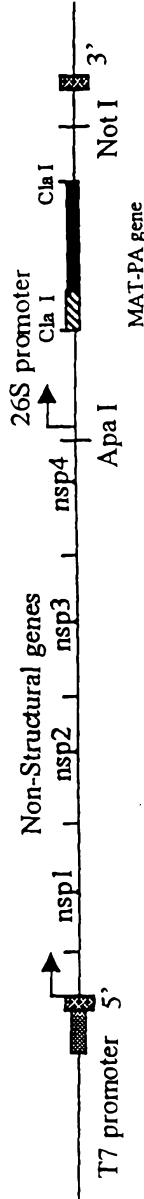
p3014-TPA-PA Replicon



p3014-PA Replicon



p3014-MAT-PA Replicon



p3014-PA63 Replicon

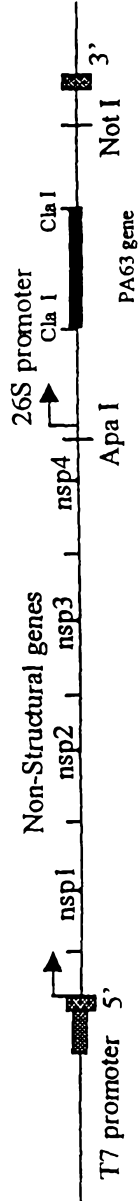


FIGURE 4

TPA-PA amino acid sequence SEQ ID NO: 15

1 MDAMKRGLLCCVLLLCGAVFVSASEVIQAEVKQENRLLNESESS
 44 SQGLLGYYFSDLNFPQAPMVVTSSTTGDLSPSSELENIPSENQYFQSAIWSGFIVKVK
 102 SDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD
 160 FKLYWTDSDQNKKEVISSDNLQLPELKQKSSNSRKKRSTASGPTVPRDNDGIPDSLEV
 218 EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV
 276 SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG
 334 NAEVHASFDFDIGGSVSAGFSNSNSSTVAIDHLSLSLAGERTWAETMGLNTADTARLNAN
 392 IRYVNTGTAPIYINVLPPTS LVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
 450 NAQDDFSSTPITMNYNLFLELEKTKQLRLDQVYGNIAIYNFENGRVVRVDTGSNWSE
 508 VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMLKEALKI AFGENEPN
 566 GNLYQYQKIDITEFDNFQDQTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKR
 624 FHYDRNNAIVGADES VVKEAHREVINSSTEGLLL NIDKDIRKILSGYIVEIEDTEGLK
 682 EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPYISNPNYKVNVAVTKENTIINPS
 706 ENGDTSTNGIKKILIFSKKGYEIG

TPA-PA gene sequence SEQ ID NO: 3

1 atgg atgcaatgaa gagaggctc tgctgtgtgc tgctgctgtg tggagcagtc
 55 ttcgtttcgg ctacgagagt gattcaggca gaagttaaac aggagaaccg gttattaat
 115 gaatcagaat caagttccca ggggttacta ggatactatt ttagtgattt gaattttcaa
 175 gcacccatgg tggttacctc ttctactaca ggggattat ctattcctag ttctgagtta
 235 gaaaatattc catcgaaaa ccaatatttt caatctgeta ttgggtcagg atttatcaaa
 295 gttagaaga gtgatgaata tacatttgct acttccgetg ataatcatgt aacaatgtgg
 355 gtagatgacc aagaagtgat taataaagct tctaattcta acaaaatcag attagaaaa
 415 ggaagattat atcaataaaa aattcaatat caacgagaaa atcctactga aaaaggattg
 475 gatttcaagt tgtactggac cgatttctca aataaaaaag aagtgatttc tagtgataac
 535 ttacaattgc cagaattaaa acaaaaatct tcgaactcaa gaaaaagcg aagtacaagt
 595 gctggacctc cggttccaga ccgtgacaat gatggaatcc ctgattcatt agaggtagaa
 655 ggatatacgg ttgatgtcaa aaataaaaaga acttttctt caccatggat ttctaatt
 715 catgaaaaga aaggattaac caaatataaa tcatctctctg aaaaatggag cacggcttct
 775 gatccgtaca gtgatttcca aaaggttaca ggacggattg ataagaatgt atcaccagag
 835 gcaagacacc cccttggtgc agcttatecg attgtacatg tagatatgga gaatattatt
 895 ctctcaaaaa atgaggatca atccacacag aatactgata gtgaaacgag aacaataagt
 955 aaaaatactt ctacaagtag gacacatact agtgaagtac atggaatgc agaagtgc
 1015 gcgtcgttct ttgatattgg tgggagtgtc tctgcaggat ttagtaattc gaattcaagt
 1075 acggtcgcga ttgatcattc actatctcta gcaggggaaa gaacttgggc tgaacaatg
 1135 ggtttaaata ccgctgatac agcaagatta aatgccaata ttagatatgt aaacttggg
 1195 acggtcccaa tctacaacgt gttaccaacg acttcgtagg tgttaggaaa aaatcaaca
 1255 ctccgcgaca ttaaagctaa ggaaaaccaa ttaagtcaaa tacttgcacc taataattat
 1315 tatccttcta aaaaacttggc gccaatcgca ttaaatgcac aagacgattt cagtttact
 1375 ccaattacaa tgaattacaa tcaatttctt gagttagaaa aaacgaaaca attaagatta
 1435 gatcggatc aagtatatgg gaatatagca acatacaatt ttgaaaatgg aagagtggg
 1495 gtggatacag gctcgaactg gagtgaagtg ttaccgcaaa ttcaagaac aactgcacgt
 1555 atcattttta atggaaaaga tttaaatctg gtgaaaaggc ggatagcggc ggttaatcct
 1615 agtgatecat tagaaacgac taaaccggat atgacattaa aagaagccct taaaatagca
 1675 tttggattta acgaaccgaa tggaaactta caatatcaag ggaaagacat aaccgaattt
 1735 gattttaatt tcgatcaaca aacatctcaa aatatcaaga atcagttagc ggaattaac
 1795 gcaactaaca tatatactgt attagataaa atcaaattaa atgcaaaaat gaatatttta
 1855 ataagagata aacgttttca ttatgataga aataacatag cagttggggc ggatgagtca
 1915 gtagttaagg aggctcatag agaagtaatt aattcgtcaa cagagggatt attgttaaat
 1975 attgataagg atataagaaa aatattatca ggttatattg tagaaattga agatactgaa
 2035 gggcttaaag aagttataaa tgacagatat gatatgttga atatttctag ttacggcaa
 2095 gatggaaaaa catttataga ttttaaaaaa tataatgata aattaccgtt atatataagt
 2155 aatcccaatt ataaggtaaa tgtatatgct gttactaaag aaaacactat tattaatcct
 2215 agtgagaatg gggatactag taccaacggg atcaagaaaa ttttaattct ttctaaaaa
 2275 gcctatgaga taggataa

PA amino acid sequence SEQ ID NO: 6

1 MKKRKVLIPLMALSTILVSSTGNLEVIQAEVVKQENRLLNESESS
 45 SQGLLGYFSDLNFOAPMVVTSSTTGDLSIPSELENI PSENQYFQSATWSGFIVKVK
 103 SDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKGLD
 161 FKLYWTDSONKKEVISSDNLQLPELKQKSSNSRKRSTASAGPTVPDRDNDGIPDSLEV
 219 EGYTVDVKNKRTFLSPWISNIHEKGLTKYKSSPEKWTASDPYDFEKVTEGRIDKNV
 277 SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG
 335 NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAE TMGLNTADTARLNAN
 393 IRVNTGTAPIYNVLP TTSVLVKGKQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
 451 NAQDDFSSPTITMNYNQFLELEKTKQLRLD TDQVYGN IATYNFENGRVVRVDTGSNWSE
 509 VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAPGFNEPN
 567 GNLQYQGKDI TEFDNF DQOTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILIRDKR
 625 FHYDRNNIAVGADES VVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLK
 683 EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPYISNPYKVN VYAVTKENT IINPS
 707 ENGDTSTNGIKKILIFSKKGYEIG

PA gene sequence - SEQ ID NO: 4

1 atgaaa aacgaaaagt gttaatacca ttaatggcat tgtctacgat attagtttca
 58 agcacaggta attagaggt gattcaggca gaagttaaac aggagaaccg gttattaat
 118 gaatcagaat caagttccca ggggttacta ggatactatt ttagtgattt gaattttcaa
 178 gcacccatgg tggttacctc ttctactaca ggggatttat ctattcctag ttctgagtta
 238 gaaaatattc catcggaaaa ccaatatttt caatctgcta tttggtcagg atttatcaaa
 298 gttaagaaga gtgatgaata tacatttgc tcttcgctg ataatcatgt aacaatgtgg
 358 gtatgtgacc aagaagtgat taataaagct tctaattcta acaaaatcag attagaaaaa
 418 ggaagattat atcaataaaa aattcaatat caacgagaaa atcctactga aaaaggattg
 478 gattcaagt tgtactggac cgattctcaa aataaaaaag aagtgatttc tagtgataac
 538 ttacaattgc cagaattaa acaaaaatct tcgaactcaa gaaaaagcg aagtacaagt
 598 gctggaccta cggttccaga ccgtgacaat gatggaatcc ctgattcatt agaggtagaa
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 718 catgaaaaga aaggattaac caaatataaa tcatctcctg aaaaatggag caeggcttct
 778 gatccgtaca gtgatttcga aaagggtaca ggacggattg ataagaatgt atcaccagag
 838 gcaagacacc cccttgtggtc agettatccg attgtacatg tagatatgga gaattattt
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 958 aaaaataactt ctacaagttag gacacatact agtgaagtac atggaaatgc agaagtgc
 1018 gcgtcgttct ttgatattgg tgggagtgtc totgcaggat ttagtaattc gaattcaagt
 1078 acggtcgcga ttgatcattc actatctcta gcaggggaaa gaacttgggc tgaacaatg
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 1258 ctgcgcgcaa ttaaagctaa ggaaaaccaa ttaagtcaaa tacttgacc taataattt
 1318 tacccttcta aaaacttggc gccaatcgca ttaaatgcac aagacgattt cagttctact
 1378 ccaattacaa tgaattacaa tcaatttctt gagttagaaa aaacgaaaca attagatta
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 1558 atcattttta atggaaaaga tttaaatctg gttagaaaggc ggatagcggc ggttaatcct
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 1678 tttggattta acgaaccgaa tggaaactta caatatcaag ggaaagacat aaccgaattt
 1738 gattttaatt tcgatcaaca aacatctcaa aatatcaaga atcagttagc ggaattaaac
 1798 gcaactaaca tatatactgt attagataaa atcaaatcaa atgcaaaaat gaattattta
 1858 ataagagata aacgttttca ttatgataga aataacatag cagttggggc ggatgagtca
 1918 gtagttaagg aggctcatag agaagtaatt aattcgtcaa cagagggatt attgtaaat
 1978 attgataagg atataagaaa aatattatca ggttatattg tagaaattga agatactgaa
 2038 gggcttaagg aagttataaa tgacagatca gatatgttga atatttctag ttacggcaa
 2098 gatggaaaaa ctttatagaa ttttaaaaaa tataatgata aattaccgtt atataaagt
 2158 aatcccaatt ataaggtaaa tgtatatgct gttactaaag aaaacactat tattaatcct
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 2278 ggctatgaga taggataa

MAT-PA amino acid sequence

SEQ ID NO: 7

1 MEVKQENRLLNESESS
 17 SQGLLGYYFSDLNFOAPMVVTSSTTGDLSPSSSELENI PSENQYFQSAIWSGFIVKVKK
 75 SDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTKEGLD
 133 FKLYWTDSONKKEVITSSDNLQLPELKQKSSNSRKKRSTASAGPTVPDRDNDGIPDSLEV
 191 EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTRIDKNV
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 365 IRYVNTGTAPIYVNLPTTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
 423 NAQDDFSSPTITMNYNQFLELEKTKQLRLDQVYGNIAATYFENGRVRVDTGSNWSE
 481 VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAGGFNEPN
 539 GNLYQYQKDI TEFDNFDDQTSQNIKNQLABELNATNIYTVLDKIKLNAMNILIRDKR
 597 FHYDRNNI AVGADES VVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLK
 655 EVINDRYDMLNIISSLRQDGKTFIDFKKYNDKLPYISNPYKVVVYAVTKENTIINPS
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MAT-PA gene sequence

SEQ ID NO: 2

1 atg gaagttaaac aggagaaccg gttattaat
 34 gaatcagaat caagttccca ggggttacta ggatactatt ttagtgattt gaattttcaa
 94 gcacccatgc tggttacctc ttctactaca ggggatttat ctattcctag ttctgagtta
 154 gaaaatattc catcggaaaa ccaatatttt caatctgcta ttgggtcagg atttatcaaa
 214 gttaagaaga gtgatgaata tacatttgct acttccgctg ataatcatgt aacaatgtgg
 274 gtatgatgacc aagaagtgat taataaagct tctaattcta acaaaatcag attagaaaaa
 334 ggaagattat atcaaataaa aattcaatat caacgagaaa atcctactga aaaaggattg
 394 gatttcaagt tgtactggac cgattctcaa aataaaaaag aagtgatttc tagtgataac
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 414 gctggacctc cggttccaga cctgacaat gatggaatcc ctgattcatt agaggtagaa
 474 ggatatacgg ttgatgtcaa aaataaaaaga acttttcttt caccatggat ttctaataat
 534 catgaaaaaga aaggattaac caaatataaa tcatctcctg aaaaatggag cacggcttct
 594 gatccgtaca gtgatttcca aaaggttaca ggacggattg ataagaatgt atcaccagag
 654 gcaagacacc cccttggtgc agcttaccg attgtacatg tagatatgga gaattattt
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 774 aaaaatactt ctacaagtag gacacatact agtgaagtac atggaaatgc agaagtgcac
 834 gcgtcgttct ttgatattgg tgggagtgta tctgcaggat ttagtaatte gaattcaagt
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 1074 ctgcgcgcaa ttaaagctaa ggaaaaccaa ttaagtcaaa tacttgcaac taataattat
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 1374 atcattttta atggaaaaga tttaaatctg gtagaaaggc ggatagcggc ggttaatcct
 1434 agtgatccat tagaaacgac taaaccggat atgacattaa aagaagccct taaaatagca
 1494 tttggattta acgaaccgaa tggaaactta caatatcaag ggaaagacat aaccgaattt
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 1914 gatggaaaaa catttataga ttttaaaaaa tataatgata aattaccgtt atatataagt
 1974 aatcccaatt ataaggtaaa tgtatatgct gttactaaag aaaacactat tattaatcct
 2034 agtgagaatg gggatactag taccaaccgg atcaagaaaa ttttaatcct tctcaaaaaa
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PA63 amino acid sequence

SEQ ID NO: 8

1 MSTSAGPTVPDRDNDGIPDSLEV
 24 EGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNV
 82 SPEARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSETRTISKNTSTSRHTTSEVHG
 140 NAEVHASFFDIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNAN
 198 IRYVNTGTAPIYNVLPFTTSLVLGKNQTLATIKAKENQLSQILAPNNYPSKNLAPIAL
 256 NAQDDFSSFTITMNYNQFLELEKTKQLRLDQVYGNIAFYNFENGRVVRVDTGSNWSE
 314 VLPQIQETTARIIFNGKDLNLVERRIAAVNPSDFLETTKPDMTLKEALKIAFGFNEFPN
 372 GNLQYQKGDITEFDNFDDQTSQNIKNQLAELNATNIYTVLDDKIKLNAKMNILIRDKR
 430 FHYDRNNIavgadesvVKEAHREVINSSTEGLLLNIIDKDIRKILSGYIVEIEDTEGLK
 488 EVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPlyISNFNYKVNvyAVTKENTIINPS
 546 ENGDSTNGIKKILIFSKKGYEIG

PA63 gene sequence

SEQ ID NO: 4

1 at gagtacaagt
 13 gctggacctt cggttccaga ccgtgacaat gatggaatcc ctgattcatt agaggtagaa
 73 ggatatacgg ttgatgtcaa aaataaaaga acttttcttt caccatggat ttctaattt
 133 catgaaaaga aaggattaac caaatataaa tcatctcctg aaaaatggag cacggcttct
 193 gatccgtaca gtgatttcca aaaggttaca ggacggattg ataagaatgt atcaccagag
 253 gcaagacacc cccttggtgc agcttatccg attgtacatg tagatatgga gaattattt
 313 ctctcaaaaa atgaggatca atccacacag aatactgata gtgaaacgag aacaataagt
 373 aaaaataactt ctacaagtag gacacatact agtgaagtac atggaaatgc agaagtgc
 433 gcgtcgcttct ttgatattgg tgggagtgtt tctgcaggat ttagtaattc gaattcaagt
 493 acggtcgcaa ttgatcattc actatctcta gcaggggaaa gaacttgggc tgaacaatg
 553 ggtttaataa ccgctgatac agcaagatta aatgccaaat ttagatatgt aaatactggg
 613 acggtcccaa tctacaacgt gttaccaacg acttcgtagg tgtaggaaa aaatcaaaca
 673 ctccgacaaa ttaaagctaa ggaaaaccaa ttaagtcaaa tacttgcacc taataattat
 733 tatccttcta aaaacttggc gccaatcgca ttaaattgcac aagacgattt cagttctact
 793 ccaattacaa tgaattacaa tcaatttctt gagttagaaa aaacgaaaca attagatta
 853 gatacggatc aagtatatgg gaatatagca acatacaatt ttgaaatgg aagagtggg
 913 gtggatacag gctcgaactg gagtgaagtg ttaccgcaa ttcaagaaac aactgcacgt
 973 atcattttta atgaaaaga tttaaatctg gtagaaaggc ggatagcggc ggtaaatcct
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 1153 gattttaatt tcgatcaaca aacatctcaa aatatcaaga atcagttagc ggaattaac
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 1273 ataagagata aacgttttca ttatgataga aataacatag cagttggggc ggatgagtca
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 1393 attgataagg atataagaaa atattatca gggtatattg tagaaattga agatactgaa
 1453 gggcttaaa aagttataaa tgacagatat gatattgtga atatttctag ttacggcaa
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 1573 aatcccaatt ataaggtaaa tgtatatgct gttactaaaag aaaacactat tattaatcct
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 1693 ggctatgaga taggataa