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(54) Title: HYBRID PROTEINS OR POLYPEPTIDES

(57) Abstract

A hybrid protein or polypeptide which comprises the epitopes or mimotopes of a plurality of different protein antigens may be used to stimulate an immune response against the different antigens. A recombinant virus such as vaccinia virus, may include a nucleotide sequence capable of being expressed as such a hybrid protein or polypeptide.

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"HYBRID PROTEINS OR POLYPEPTIDES"

This invention relates to hybrid proteins or polypeptides which may be used as a means of simultaneously immunising against a plurality of protein antigens, and to methods for the production thereof. In particular, this invention relates to hybrid proteins or polypeptides containing multiple epitopes in the one molecule.

In recent years there has been rapid progress in the characterisation of defined protein antigens which are crucial in the development of host-protective immune responses to foreign pathogens. The vast majority of B-cell epitopes which are recognised appear to be non-linear or conformational epitopes. Only in the case of a few antigens (e.g. the foot and mouth disease virus VP1 140-162 peptide and the polio virus VP1, 1-14 peptide) has it be demonstrated that a linear array of amino acid

sequences constitute a functional portion of the protein which can be blocked with antibodies.

Vaccination with synthetic peptides in various forms of conjugation to carrier molecules and with a variety of adjuvant formulations have been used to elicit antibodies which are able to recognise the native protein molecule and in some cases to immunise against disease.

Recent experiments have suggested that non-linear conformational epitopes might be mimicked by synthetic linear sequences (termed "mimotopes") which might also be used as immunogens.

However there is an accumulating body of data which suggests that higher order structural features or additional sequence elements may be required in addition to the basic epitope to elicit the appropriate antibody and T-cell responses relevant to an effective immune response. This may mean that chemically synthesised peptides suitable for effective immunisation may need to be larger than first envisaged and peptide vaccines may therefore not turn out to be as economical to manufacture as was first envisaged. This may mean that although the linear peptide sequence is the target of host protective immune responses, it may need to be present in the context of a larger protein molecule produced in bacteria, yeast, recombinant viruses or other commercially viable expression system.

One of the striking features of studies to date on the protein antigens of Plasmodium falciparum has been the preponderance of tandem arrays of repetitive amino acid sequences. In the majority of cases these sequences

are the immunodominant epitopes of the antigen and there are early indications that some may be the targets of host protective immune responses. These epitopes appear to be recognised as linear determinants.

An effective vaccine against malaria may well require the simultaneous immunisation with a "cocktail" of defined antigen molecules (or peptides related to the critical epitopes). It is also likely that any large scale vaccination of human population in malaria endemic areas will also be carried out in parallel with vaccination with other defined antigen vaccines against other diseases prevalent in the region (e.g. Hepatitis B). Accordingly, the possibility of immunising with a hybrid protein made up of a number of peptide sequences which are known to be epitopes in natural infections with malaria has been investigated.

The prevalence of repetitive peptide sequences as dominant antigenic determinants in many of the antigens of P.falciparum has provided a unique opportunity to investigate vaccination procedures involving the presentation of a plurality of short peptide sequences in the one hybrid protein molecule.

According to the present invention, there is provided a hybrid protein or polypeptide which comprises the epitopes or mimotopes of a plurality of different protein antigens.

The term "hybrid" as used herein means a non-naturally occurring protein or polypeptide.

Preferably, the hybrid protein or polypeptide

comprises more than one "repeat" of each epitope or mimotope in order to maximise its immunogenicity in the hybrid molecule, even if the epitope is represented only once in the native antigen molecule.

In one particularly preferred aspect of the invention, in the hybrid protein or polypeptide broadly described above at least one of the epitopes or mimotopes is an epitope or mimotope of an antigen of P.falciparum.

The present invention also extends to the cloning of a DNA molecule that encodes the above described hybrid protein or polypeptide by incorporating the DNA molecule into a suitable vector which, in an appropriate host cell, is capable of expressing the hybrid molecule in vitro. Accordingly, in another aspect of the present invention there is provided a DNA sequence comprising a nucleotide sequence capable of being expressed as a hybrid protein or polypeptide, said hybrid protein or polypeptide comprising the epitopes or mimotopes of a plurality of different protein antigens.

The present invention also extends to a recombinant DNA molecule comprising a DNA sequence as outlined above, operatively linked to an expression control sequence. The invention extends further to a vector comprising a DNA sequence as outlined above, as well as to a host cell containing such a vector.

As broadly described above, the hybrid polypeptide of the present invention provides a means of simultaneously immunising against a plurality of protein antigens, and accordingly the present invention also encompasses a composition for stimulating immune responses

against a plurality of protein antigens which comprises a hybrid protein or polypeptide as broadly described above, together with a pharmaceutically acceptable carrier therefor, and optionally further comprising an adjuvant. In an alternative embodiment, the invention provides a composition for stimulating immune responses against a plurality of protein antigens which comprises a virus or microorganism in association with a pharmaceutically acceptable carrier, and optionally further comprising an adjuvant, the virus or molecule having inserted therein a DNA molecule comprising a nucleotide sequence capable of being expressed as a hybrid protein or polypeptide as broadly described above.

The present invention also extends to a method of stimulating immune responses which comprises administering a composition as described above.

In one particularly preferred embodiment, the present invention provides a recombinant virus, particularly a recombinant vaccinia virus, or a composition comprising such a virus, characterised in that it includes a nucleotide sequence capable of being expressed as a hybrid protein or polypeptide as broadly described above. Preferably also, the virus further includes a sequence capable of being expressed as a surface or membrane-associated polypeptide segment in association with said hybrid protein or polypeptide to locate the hybrid protein or polypeptide on or at the surface of virus infected cells.

The following detailed description of the simultaneous delivery of a number of potentially important epitopes of P.falciparum in one hybrid protein, is given

by way of illustration of the present invention. It will be readily appreciated by those skilled in the art that the techniques described can be readily adapted to other malarial antigens or epitopes or to non-malarial linear epitopes from other protein antigens.

In the drawings:

Figure 1 is a schematic representation of the P.falciparum malaria genes used in this study indicating the location of their tandem repeating epitopes and the construction of the hybrid S-antigen gene containing the multiple repeating elements used to express the hybrid protein using recombinant vaccinia viruses;

Figure 2 is a diagrammatic representation of the construction of the plasmid vectors used in the cloning of the hybrid genes for expression in recombinant vaccinia viruses;

Figure 3 shows the sequences of the synthetic oligonucleotides used in the construction of the hybrid genes;

Figure 4 shows the steps used in the construction of one of the hybrids containing the NANP repeat of the CS protein;

Figure 5 shows the method of construction of hybrid genes containing multiple inserts;

Figure 6 is a diagram showing the hybrid genes constructed and tested in recombinant vaccinia (V series) and expressed in bacteria using the glutathione S-transferase bacterial expression vector system (Sj

series);

Figure 7 shows the sequence of some of the clones shown in Figure 6 to check the copy number and orientation of inserts;

Figure 8 shows the expression of the hybrid proteins in bacteria transformed with the vaccinia virus transfection plasmids containing the hybrid genes under the control of vaccinia and bacterial promoter elements. Expression was detected using the colony immunoassay procedure with anti-MESA repeat antisera on lysates of IPTG induced bacterial colonies. Only clones V7 and VS5367 containing the MESA repeat sequence in the correct orientation gave a positive signal in this assay with the anti-MESA antiserum;

Figure 9 shows the expression of some of the hybrid proteins described in Figure 6 in recombinant vaccinia infected cells;

Figure 10 shows the antibody responses of one of the rabbits immunised with recombinant vaccinia virus expressing a hybrid protein comprising five repetitive epitopes from four separate malarial antigens (pS5367.8 Figures 1 and 6) measured in an ELISA assay in which the plates were coated with synthetic peptides of the various repeating epitopes.

Figure 11 shows the purified glutathione S-transferase/hybrid protein fusion polypeptides produced by bacteria containing the Sj series of constructs shown in Figure 6.

The choice of the carrier protein

The carrier protein used to express the multiple epitopes will vary according to the intended mode of presentation of antigen and the sequences to be expressed. In many cases the best carrier protein will need to be determined empirically as the sequence of the peptides being inserted will have differing effects on the stability of the resultant fusion protein.

One of the proteins chosen as the carrier protein in this example is a modified version of one of the malarial S-antigens. The family of S-antigen molecules has to date not been implicated as potential malaria vaccine candidates primarily because the immunodominant repeat portion of these molecules is remarkably variant. These repeating structures vary enormously, not only in their amino acid composition which greatly affects their immunological properties, but in their length and copy number. These radical variations in protein structure do not affect their behaviour as secreted proteins.

The transmembrane anchor sequence from a mouse immunoglobulin gene has been added to the native S-antigen molecule of the FC27 isolate of P.falciparum and it has been shown that the hybrid protein is correctly presented as an integral membrane protein on the surface of eukaryotic cells (Langford et.al., (1986)). This hybrid protein contains 13 copies of an 11 amino acid repeating epitope. Clearly this repeating sequence does not prevent the transport of the hybrid protein to the cell surface. The present example replaces this simple repetitive sequence with other sequences in the hope that the new hybrid protein will be transported to the cell surface.

The mode of presentation of the recombinant antigen

The hybrid antigens could be delivered using a live recombinant virus presentation system. We have used recombinant vaccinia viruses as an example of this approach.

The hybrid protein could also be expressed in bacteria or eukaryotic cells and then purified for use as a vaccine. As an example of this approach we have made use of a newly developed expression vector that vastly simplifies the purification of foreign proteins produced in bacteria (Australian Patent Application PI2195/87). Proteins are produced as fusion polypeptides with the soluble Schistosma japonicum enzyme glutathione S-transferase (GST) and can then be easily purified by affinity purification on glutathione coupled to agarose beads. Pure fusion polypeptides can then be eluted from the beads using free glutathione prior to further purification for use as a vaccine.

The choice of linear sequences for insertion in the hybrid protein.

The sequences used in the construction of the hybrid proteins described herein were selected from a large number of repetitive malarial antigens with some regard to the potential of these antigens as vaccine molecules. Thus the dominant immunogenic epitope of the P.falciparum sporozoite (NANP) was an obvious choice as peptide and recombinant protein preparations of this basic sequence are presently being used in human vaccine trials (Zavala et.al. (1985)).. Similarly recent results on the protection of Aotus monkeys with fragments of the RESA molecule (Collins et.al. (1986)) have focused attention on two regions of repeating amino acids with sequences DDEHVEEPTVA and EENVEHDA. There are many other candidate vaccine antigens of P.falciparum many of which contain

immunodominant repetitive amino acid sequences. From these the GESKET and QSGASA repetitive sequences of the MESA and Pf195 major merozoite surface antigens, respectively, have been chosen as further examples.

Epitopes from other non-malarial antigens could also be included as well as mimotopes of conformation epitopes. Inserting these sequences as repetitive elements may also be desirable. The observations on the immunodominance of the repeating peptide sequences of malarial antigens suggest that simply repeating these peptide sequences may dramatically increase their immunogenicity.

As well as incorporating the relevant epitopes involved in antibody responses it may also be advantageous to incorporate T-cell recognition sequences which, as well as being of importance in the development of T-cell responses, may also provide T-cell help for amanistic antibody responses when the recipient is exposed to intact pathogen.

General methods for the construction of the hybrid proteins

Prior to the construction of the hybrid genes containing the new repeating sequences the plasmid containing the hybrid anchored S-antigen gene described previously (Langford et.al. (1986)) was modified to facilitate the cloning and immunological screening of the new hybrid proteins. These modifications included

(a) The inclusion of a bacterial promoter upstream of the S-antigen gene and the vaccinia promoter. This was achieved by first removing the ClaI site in the pBR322 region of the vector pGS62 (Figure 2a) by partial digestion with ClaI, Klenow "filling-in" and

religation. Into the now unique ClaI site a TaqI fragment from the strong hybrid LacUV5/Trp bacterial promoter was inserted generating the new vector pGS62 tac (shown in figure 2b). Using this vector the expression of genes inserted into the multiple cloning site (MCS) of the vector can be checked in bacteria as well as in virus-infected cells.

(b) The creation of a unique BamHI cloning site in the body of the S-antigen gene. This was achieved by first deleting the BamHI site from the MCS of the vector pGS62tac by BamHI digestion, Klenow "filling-in" and religation to generate the new plasmid pGS62-tac [BamΔ](figure 2c). The 1088 bp EcoRI fragment from pVA20 which also contains a BamHI site was cloned into the EcoRI site of the MCS of pGS62tac BamHIΔ to generate the plasmid pCL4 (figure 2d).

(c) The deletion of the repeating sequences of the S-antigen gene. To achieve this the isolated EcoRI fragment of pV8 (Langford et.al., (1986)) was digested with Sau3A. This enzyme cuts once in each of the S-antigen repeats. The non-repeat fragments 5' and 3' to the repeat portions were isolated and ligated into the EcoRI site of the vector pUC8 to generate the new construct pSAD AhaI0. The Sph 1 linkered mouse membrane IgG1 cDNA fragment encoding the transmembrane anchor sequence was then cloned into the unique Sph 1 site of this plasmid to generate the new plasmid pA10. The EcoRI insert from this insert was then cloned into the unique EcoRI site of the plasmid pGS62tac BamHIΔ to generate the plasmid pLK8 (shown in figure 2e). The plasmid pLK8 contains the S-antigen gene with less than one full copy of the 33bp repeating sequence and with a unique BamHI site

located within this remaining partial repeat sequence. It is into this site that appropriately engineered sequences encoding new antigenic determinants can be cloned thus neatly replacing the S-antigen repeating epitope.

Replacement of the S-antigen repetitive sequences with new repeating epitopes.

The incoming sequences in the situation described in this example need to have BamHl "sticky ends" and to be engineered in such a way that the reading frame of the whole hybrid gene is maintained. This requires that the total length of the insert DNA should be an equal multiple of 3 base pairs and that the reading frame be in phase with the GAT codon of the GGATCC BamHl site at the 5' end of the (coding strand of the) insert.

It is also believed that the epitope to be expressed should be repeated as many times as possible to maximise its immunogenicity even if this epitope is represented only once or a few times in the native antigen molecule.

To achieve this, in this example the gene sequences encoding the repeating peptide sequences have been chemically synthesised as short oligonucleotides. Chemical synthesis of the oligonucleotides also enables the codons to be "mammalianised" for optimal expression in recombinant vaccinia virus-infected mammalian cells. This may be of importance in the optimal expression of foreign proteins from species such as P.falciparum which exhibit a strong bias in their preferred codon usage away from that seen in mammalian cells. However, the insert could also be derived from naturally occurring sequences as long as

it fulfils the length and phase requirements described above.

Complementary oligonucleotide sequences (Figure 3) were synthesised on an Applied Biosystems oligonucleotide synthesiser and purified by HPLC. These were then kinased prior to annealing and ligation in the standard way. The oligonucleotides were designed so that the ends were complimentary in one orientation only, ensuring that only "head to tail" ligation of the double stranded monomers was possible. The ligated fragments were then size fractionated on a low gelling temperature agarose gel and DNA molecules in the size range from 180 to 600bp were isolated and purified from the agarose. These size fractionated molecules were then ligated with BamHl cut calf intestinal phosphatase treated, pLK8 plasmid DNA in the presence of two kinased synthetic oligonucleotides with the sequence 5'-GATCCC-3' and 5'-GATCGG-3". These adaptors were designed to allow the ligation of the 3' overhanging CC and GG ends of the repeating oligonucleotide fragment to the 5' overhanging GATC sticky ends of the BamHl cut pLK8 and pCL4 and to ensure that the insert sequence was "in frame" with the S-antigen sequence (see example shown in Figure 4).

Recombinant bacterial clones containing the inserted sequence were selected by colony hybridization using the gamma -[^{32}P]-ATP kinased oligonucleotides used in the initial cloning.

Plasmid DNA was isolated from the positive clones and digested with restriction enzymes to determine the clones which contained the longest inserts. A number of these were then sequenced using the double stranded DNA

sequencing procedure on alkaline denatured plasmid DNA preparations to select a clone with an insert in the correct orientation and to confirm its predicted sequence (Figure 7). These plasmid DNA were then transfected directly into WT vaccinia virus-infected cells to produce TK^- recombinant virus as described previously (Langford et.al. (1986)).

Increasing the length of the repeating epitope by further subcloning.

The new insert in the hybrid gene is flanked by Sau3A sites which allow the insert to be isolated and purified from the recombinant plasmid. However only one BamHl site at the 5' of the coding strand of the insert is regenerated in this hybrid (see Figure 4). Thus the recombinant plasmid can be linearised with BamHl, phosphatase treated and ligated with the isolated Sau3A fragment. Plasmid DNA was prepared from the transformed bacteria resulting from this cloning and digested with restriction enzymes to select clones with double (or triple) inserts. These were then sequenced to determine which contained new inserts in the correct orientation and to confirm the predicted sequence. These new hybrids again have a unique BamHl site at the 5' end of the insert, and this process can be repeated many times over thereby increasing the size of the insert to any desired length. In this example hybrid genes containing 16(V6), 32(V66) and 48(V666) copies of the 12bp repeat of the CSP gene and 10 (V3) and 20 (V33) copies of the 24bp repeat of the RESA gene have been produced (see Figure 6).

Construction of hybrid genes encoding multiple repeating epitopes.

The inserts from all the clones constructed in

pLK8 and pCL4 could be isolated as Sau3A fragments and cloned into the unique BamH1 site of any other construct. This allows a polymer of inserts in order to be built in as a new BamH1 site will be created following the insertion of each new insert. As the Sau3A fragment can ligate in either orientation into the BamH1 site the orientation needs to be checked in each case by DNA sequencing. A number of these hybrid genes including one construct (V55367) containing inserts encoding the S-antigen, CS, RESA 3', RESA 5' and MESA repeats were cloned into recombinant vaccinia viruses. These constructs are shown in Figure 6.

Expression and purification of hybrid protein vaccines in bacteria

BamH1/EcoR1 fragments of the genes described in the V-series of constructs shown in Figure 6 were subcloned into the BamH1/EcoR1 cloning sites at the 3' end of the S. japonicum glutathione S-transferase (GST) gene in the expression vector pGEX-1 (or an earlier version of the vector; pSj10 BamΔ10) as described in Australian Patent Application No.PI 2195/87. In this vector system the GST gene is under the control of the strong, IPTG inducible trp/lac (tac) promoter (Amann et al., 1983).

This expression system has been used to purify a wide range of parasite and non-parasite polypeptides, the majority of which are produced as abundant, soluble and stable fusion proteins that can be purified on glutathione-agarose.

RESULTS

Checking the clones by sequencing.

All clones shown in Figure 6 have been sequenced at various stages in their construction and again immediately prior to transformation into virus infected

cells to produce the viral recombinants. Instability of the repetitive elements of Plasmodial antigen genes has been a frequent problem in their manipulation in recombinant bacteria. However, in this example, there was no evidence that the copy-number of the repeating sequences of any of the hybrids was altered during the cloning procedures. Plasmid DNAs were sequenced directly following alkaline denaturation using the double stranded sequencing procedures adapted from those described by Chen and Seeburg (1985). Synthetic oligonucleotide primers complimentary to S-antigen sequences located 3'(5'-AATGGATTAATAGAAGG-3') and 5'(5'-GCTTTCCATGTCCTTCAGC-3') to the BamHl cloning site in pLK8 allowed the sequence and orientation of the insert to be checked from each side. As a rapid check for orientation of the insert normally only one dideoxy sequencing reaction was necessary. For example in the sequencing gel shown in Figure 7B only "A" reactions were performed. In this example, clones with inserts with the sequence 5'-AACGCCAACCCC-3' in the correct orientation would be expected to produce a sequence pattern made up of uniformly spaced doublets interrupted by gaps of 4 nucleotides in length. This pattern is interrupted every 192bp in the clones containing multiple inserts of the CS repeating sequence because of the 5'-GATCCC-3' adaptor sequences at the end of each single insert sequence.

Checking for the expression of the inserted sequences in bacteria.

The strong hybrid bacterial promoter located upstream of the S-antigen gene in these hybrid constructs enables the expression of the hybrid protein to be checked in bacteria transformed with the plasmid DNA. In the example shown in Figure 8, a number of bacterial clones

containing hybrid genes with RESA 24bp repeat inserts were picked into arrays on nitrocellulose, replicated and grown in the presence of IPTG. Colonies were then lysed with SDS and SDS/chloroform. Filters were then blocked and then probed with antibodies raised in rabbits to chemically synthesised polypeptides encoding the repeat epitopes conjugated to KLH. This was followed by I^{125} protein A binding, washing and autoradiography. The expression of the specific insert sequences could be demonstrated in bacteria for most hybrids prior to their insertion into vaccinia virus.

Checking for the expression of the hybrid proteins in recombinant vaccinia infected cells.

Once recombinant vaccinia clones had been identified, monolayers of BSC-1 cells were infected with the viruses and incubated for 18hr. Cells were then harvested and precipitated by centrifugation. The pellets were then suspended in SDS sample buffer, boiled and centrifuged to remove insoluble material. This sample was then subjected to SDS/PAGE and immunoblotting using the rabbit anti-peptide antisera. Figure 9 shows the expression of a number of these hybrid proteins. In most cases a stable antigenic protein is produced.

Immunisation of animals.

Rabbits were immunised with 10^8 PFU of each recombinant virus by intradermal injection on the middle of the back. Sera were collected at weekly intervals and assayed in an ELISA in which synthetic peptides of the repeating epitopes, conjugated to BSA or β-galactosidase fusion polypeptides of malarial antigens purified from bacteria, were used to coat the plates. Antibody responses specific to the sequences encoded by the inserts

could be detected in all cases.

In Figure 10, the antibody responses of one of many rabbits immunised with the virus expressing the V55367 hybrid protein are shown. Antibodies recognising the CSP, MESA, RESA 8-amino acid, RESA 11-amino acid and S-antigen repeating epitopes could be detected in all animals following immunisation with this single hybrid protein.

Hybrid proteins produced in bacteria

Constructs shown in Figure 6 (Sj series) expressed abundant, stable fusion proteins once the hydrophobic mouse IgG transmembrane region sequence was removed (by cleaving the hybrid genes with SphI followed by religation thus releasing the transmembrane gene region). The fusion proteins could be purified on glutathione agarose beads and eluted using free glutathione, yielding up to 10mg of purified protein per liter of bacterial culture. Commassie stained polyacrylamide gel analysis of these purified proteins are shown in Figure 11.

Protein purified from cloned SjS53 was used to immunize rabbits and mice producing antibodies which were capable of recognizing each of the repeating epitopes contained in the hybrid molecule (data not shown). These purified hybrid proteins also proved to be excellent substitutes for synthetic peptides as diagnostic reagents in ELISAs and cell proliferation assays.

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3. Collins et.al. (1986) Nature, 323, p.259.

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CLAIMS

1. A hybrid protein or polypeptide which comprises the epitopes or mimotopes of a plurality of different protein antigens.

2. A hybrid protein or polypeptide according to claim 1, which comprises more than one repeated sequence of each of said epitopes or mimotopes.

3. A hybrid protein or polypeptide according to claim 1, wherein at least one of said epitopes or mimotopes is an epitope or mimotope of an antigen of P. falciparum.

4. A hybrid protein or polypeptide according to claim 3, wherein at least one of said epitopes or mimotopes is selected from the group of epitopes of P. falciparum antigens consisting of NANP, DDEHVEEPTVA, EENVEHDA, GESET and QSGASA.

5. A hybrid protein or polypeptide according to claim 1, further comprising a surface or membrane-associated polypeptide segment.

6. A hybrid protein or polypeptide according to claim 1, further comprising a T-cell recognition sequence.

7. A DNA molecule comprising a nucleotide sequence capable of being expressed as a hybrid protein or polypeptide according to any one of claims 1 to 6.

8. A recombinant DNA molecule comprising a nucleotide sequence according to claim 7, operatively linked to an expression control sequence.

9. A cloning vector comprising a nucleotide sequence according to claim 7.

10. A host cell comprising a cloning vector according to claim 9.

11. A composition for stimulating an immune response against a plurality of different protein antigens which comprises a hybrid protein or polypeptide according to any one of claims 1 to 6, together with a pharmaceutically acceptable carrier or diluent.

12. A composition according to claim 11, further comprising an adjuvant.

13. A method of stimulating an immune response which comprises administering a composition according to claim 11 or claim 12.

14. A recombinant virus comprising a nucleotide sequence capable of being expressed as a hybrid protein or polypeptide according to any one of claims 1 to 6.

15. A recombinant virus according to claim 14, wherein said virus is vaccinia virus.

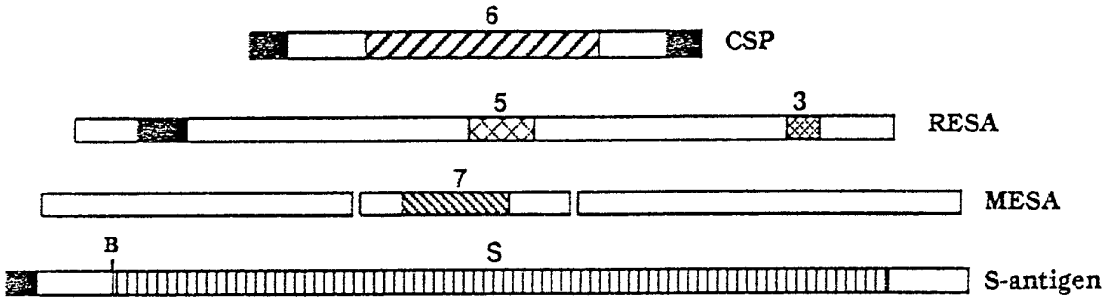
16. A composition for stimulating an immune response against a plurality of different protein antigens, which

comprises a recombinant virus according to claim 14 or claim 15, together with a pharmaceutically acceptable carrier or diluent.

17. A composition according to claim 16, further comprising an adjuvant.

18. A method of stimulating an immune response which comprises administering a composition according to claim 16 or claim 17.

P. falciparum genes with tandem repeating sequences



Hybrid S-antigen gene constructs

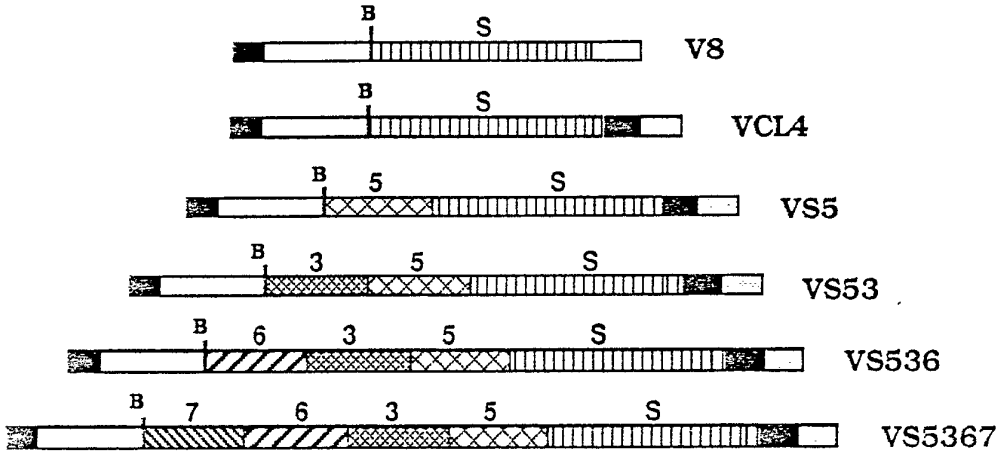
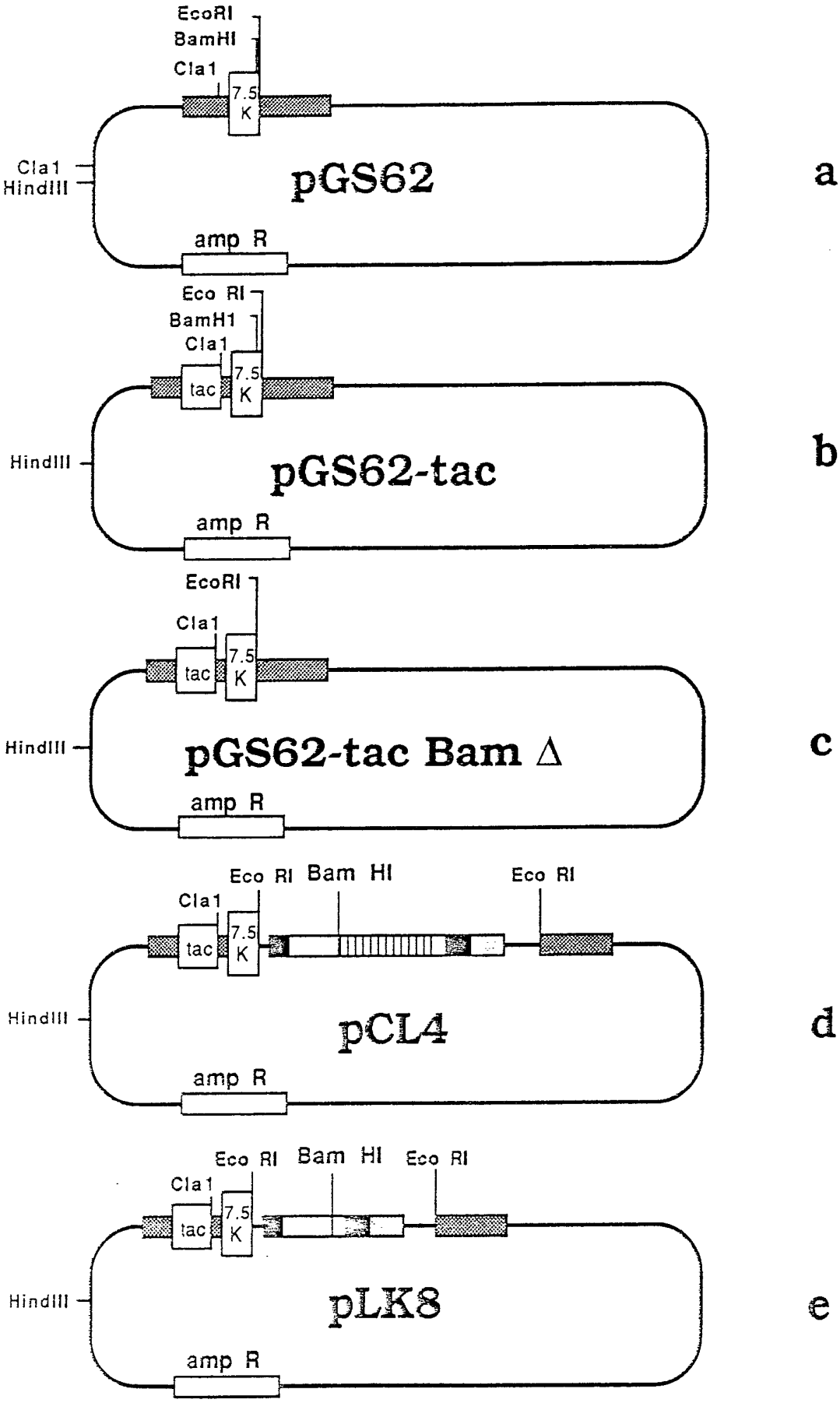


Figure 1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Cla1
7.5 K
Cla1
HindIII
pGS62
amp R
a
Eco RI
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HindIII
pGS62-tac
amp R
b
EcoRI
Cla1
tac
7.5 K
HindIII
pGS62-tac Bam Δ
amp R
c
Eco RI
Bam HI
Eco RI
Cla1
tac
7.5 K
HindIII
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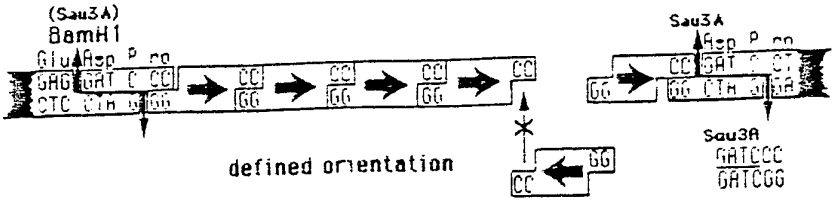
amp R
e

Figure 2



RESA 5'repeat (5)

```
     D   D   E   H   V   E   E   P   T   V   A
   Asp Asp Glu His Val Glu Glu Pro Thr Val Ala
   GAC GAC GAG CAC GTG GAG GAG CCC ACC GTG GCC
GG CTG CTG CTC GTG CAC CTC CTC GGG TGG CAC C

                                  GACGACGAGCACGTGGAGGAGCCCACCGTGGCC
                                  CCACGGTGGGCTCCTCCACGTGCTCGTCGTCGG
```

RESA 3'repeat (3)

```
     E   E   N   V   E   H   D   A
   Glu Glu Asn Val Glu His Asp Ala
   GAG GAG AAC GTG GAG CAC GAC GCC      GAGGAGAACGTGGAGCACGACGCC
GG CTC CTC TTG CAC CTC GTG CTG C        CGTCGTGCTCCACGTTCTCCTCGG
```

CS repeat (6)

```
     N   A   N   P
   Asn Ala Asn Pro
   AAC GCC AAC CCC                    AACGCCAACCCC
GG TTG CGG TTG G                      GGTTGGCGTTGG
```

MESA repeat (7)

```
     G   E   S   K   E   T
   Gly Glu Ser Lys Glu Thr
   GGC GAG AGC AAG GAG ACC          GGCGAGAGCAAGGAGACC
GG CCG CTC TCG TTC CTC T            TCTCCTTGCTCTCGCCGG
```

195K repeat (8)

```
     Q   S   G   A   S   A
   Gln Ser Gly Ala Ser Ala
   CAG AGC GGC GCC AGC GCC
GG GTC TCG CCG CGG TCG C

                                       NarI
                                  CAGAGCGGCGCCAGCGCC
                                  GGCTGGCGCCGCTCTGGG
```

Figure 3

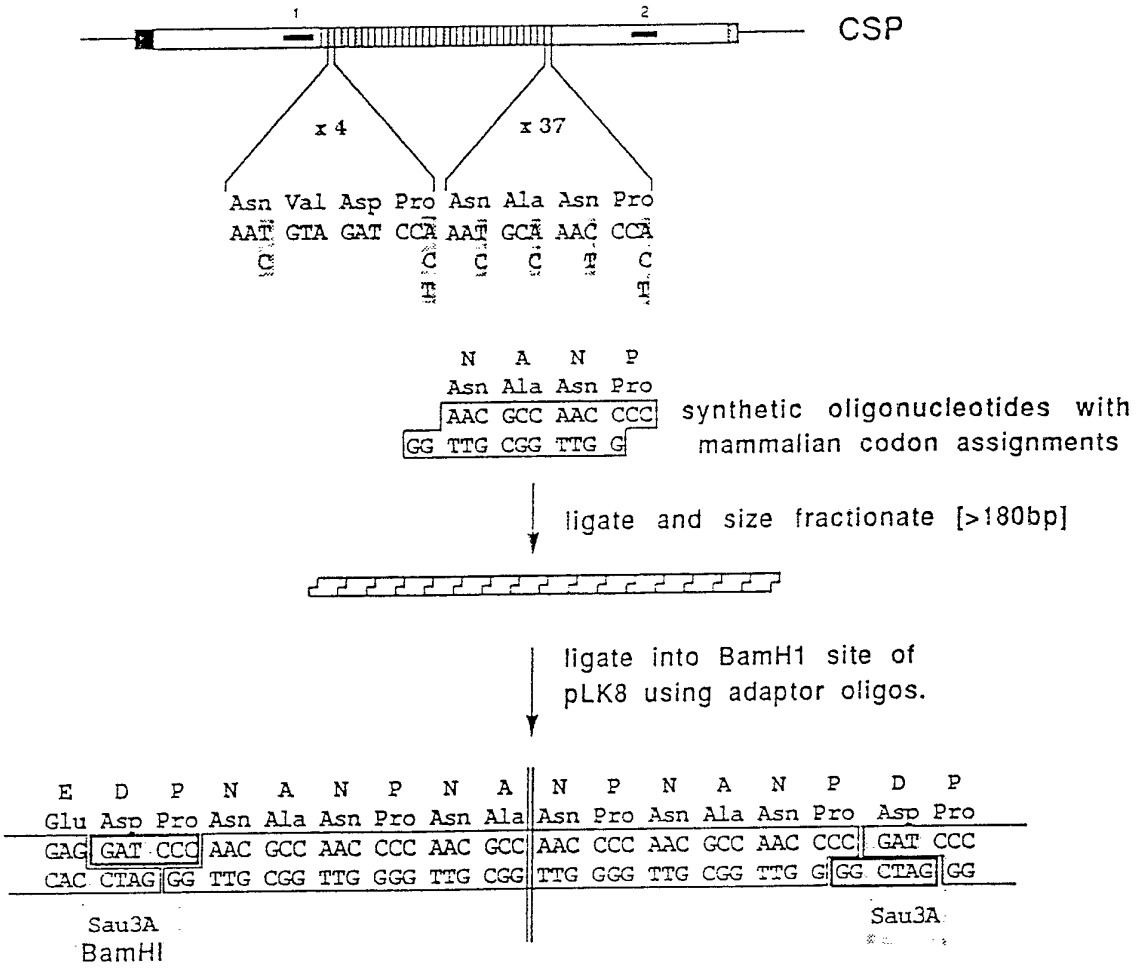

1
2
CSP
x 4
x 37
Asn Val Asp Pro Asn Ala Asn Pro
AAT GTA GAT CCA AAT GCA AAC CCA
C C C C T C
T T
N A N P
Asn Ala Asn Pro
AAC GCC AAC CCC
GG TTG CGG TTG G
synthetic oligonucleotides with mammalian codon assignments
ligate and size fractionate [>180bp]
ligate into BamH1 site of pLK8 using adaptor oligos.
E D P N A N P N A N P N A N P D P
Glu Asp Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asp Pro
GAG GAT CCC AAC GCC AAC CCC AAC GCC AAC CCC AAC GCC AAC CCC GAT CCC
CAC CTAG GG TTG CGG TTG GGG TTG CGG TTG GGG TTG CGG TTG G GG CTAG GG
Sau3A
BamHI
Sau3A

Figure 4

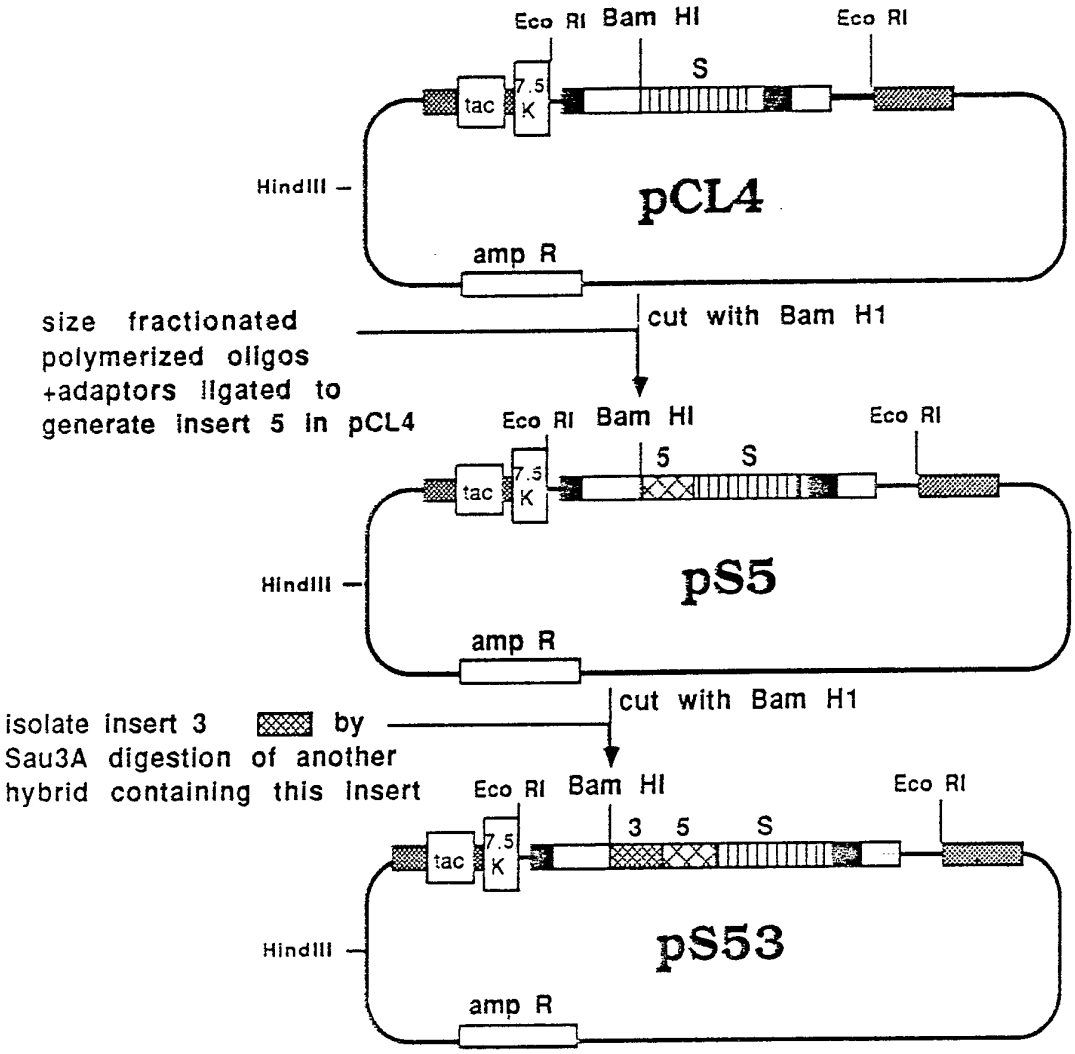

Eco RI
Bam HI
Eco RI
S
tac
7.5 K
HindIII
pCL4
amp R
size fractionated polymerized oligos +adaptors ligated to generate insert 5 in pCL4
cut with Bam H1
Eco RI
Bam HI
Eco RI
5
S
tac
7.5 K
HindIII
pS5
amp R
isolate insert 3 by Sau3A digestion of another hybrid containing this insert
cut with Bam H1
Eco RI
Bam HI
Eco RI
3
5
S
tac
7.5 K
HindIII
pS53
amp R

Figure 5

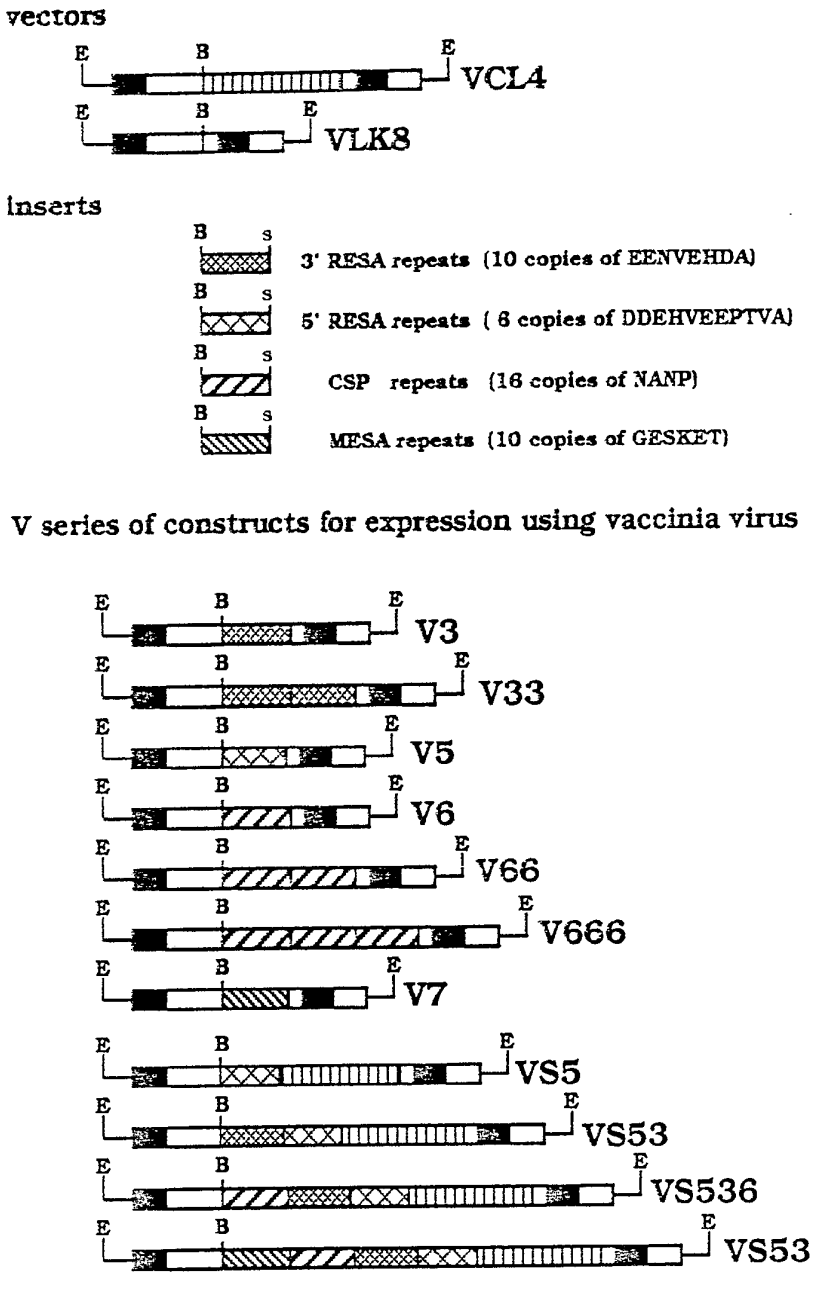

vectors
E
B
E
VCL4
E
B
E
VLK8
inserts
B
S
3' RESA repeats (10 copies of EENVEHDA)
B
S
5' RESA repeats (6 copies of DDEHVEEPTVA)
B
S
CSP repeats (16 copies of NANP)
B
S
MESA repeats (10 copies of GESKET)
V series of constructs for expression using vaccinia virus
E
B
E
V3
E
B
E
V33
E
B
E
V5
E
B
E
V6
E
B
E
V66
E
B
E
V666
E
B
E
V7
E
B
E
VS5
E
B
E
VS53
E
B
E
VS536
E
B
E
VS5367
Sj series of constructs for expression and purification in bacteria
BE
pGEX-1 (S. japonicum -glutathione S-transferase)
B
E
Sj3
B
E
Sj5
B
E
Sj7
B
E
Sj35
B
E
SjS
B
E
SjS5
B
E
SjS53

Figure 6

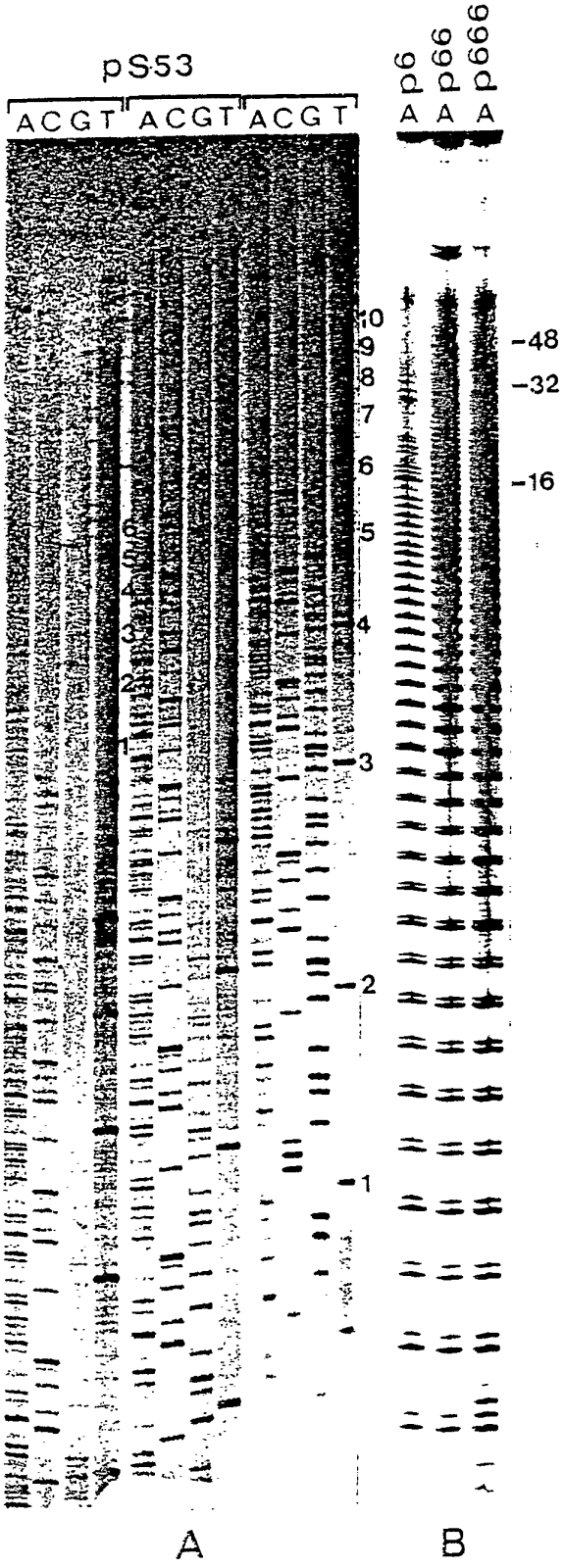


Figure 7

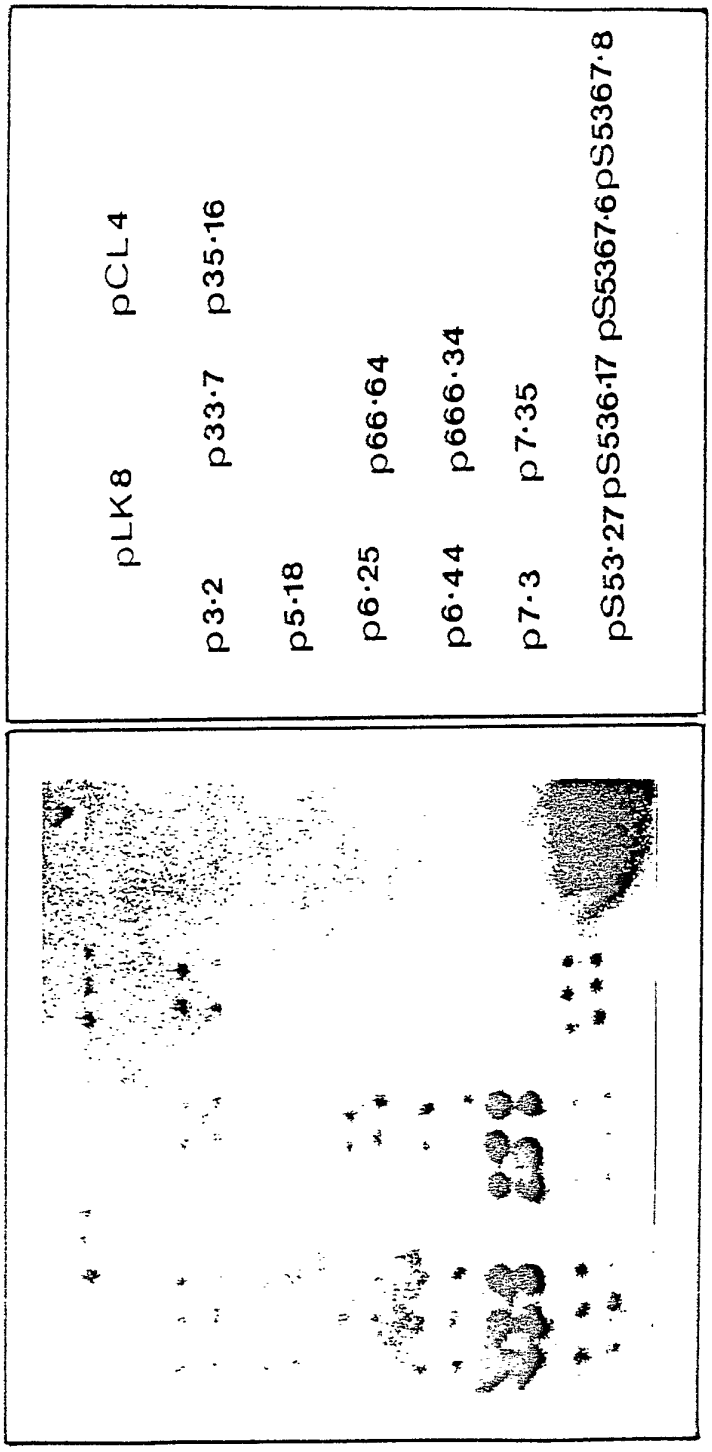

pLK8
pCL4
p3·2
p33·7
p35·16
p5·18
p6·25
p66·64
p6·44
p666·34
p7·3
p7·35
pS53·27
pS53617
pS5367·6
pS5367·8

Figure 8


parasite infected RBCs
RBCs
VS5367
V5
Marker
$M_r X 10^{-3}$
200
100
96
67
43
anti RESA 11mer

Figure 9

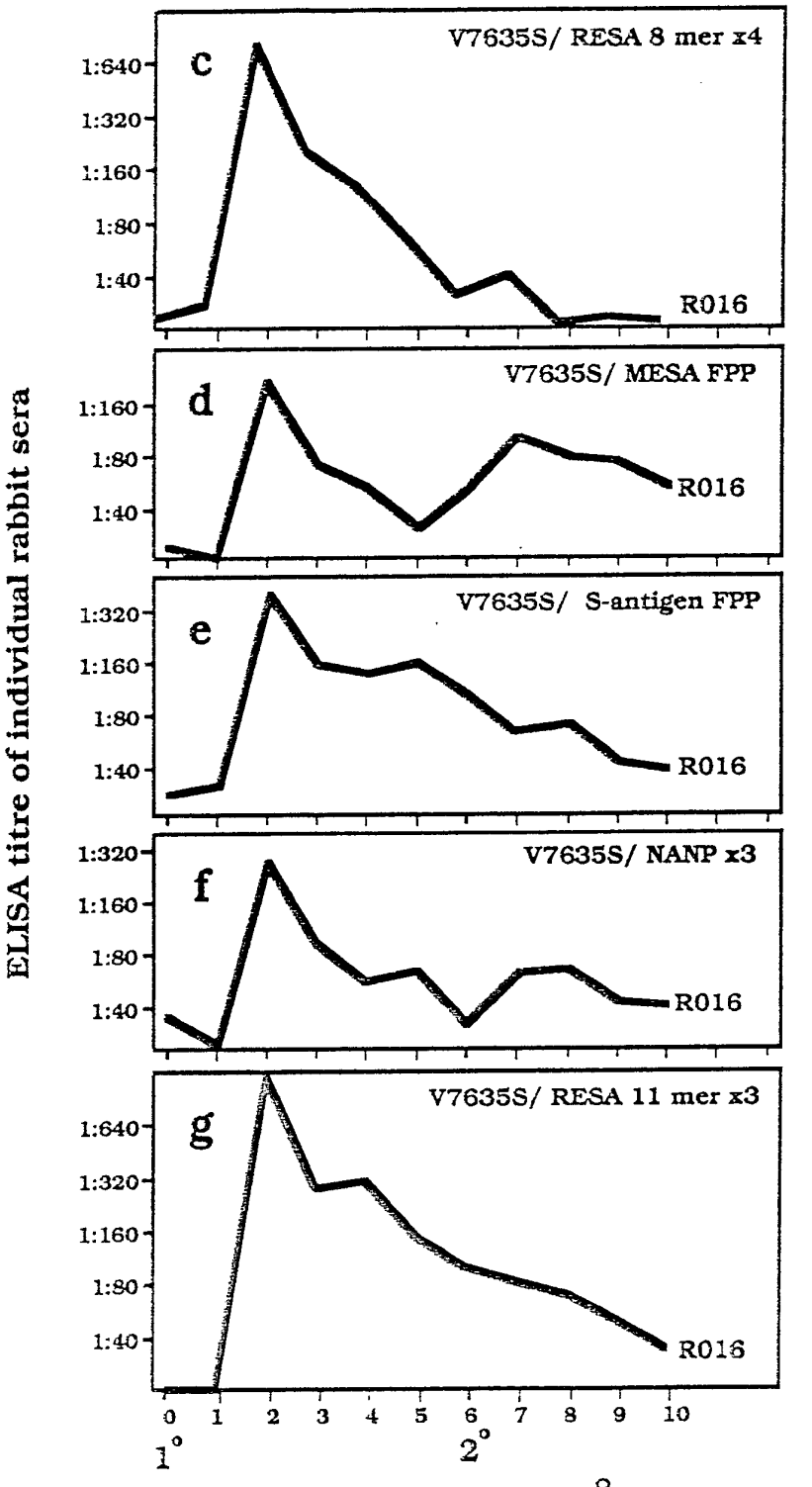

V7635S/ RESA 8 mer x4
c
1:640
1:320
1:160
1:80
1:40
R016
V7635S/ MESA FPP
d
1:160
1:80
1:40
R016
V7635S/ S-antigen FPP
e
1:320
1:160
1:80
1:40
R016
V7635S/ NANP x3
f
1:320
1:160
1:80
1:40
R016
V7635S/ RESA 11 mer x3
g
1:640
1:320
1:160
1:80
1:40
R016
ELISA titre of individual rabbit sera
0 1 2 3 4 5 6 7 8 9 10
1°
2°
weeks after immunization with 10^8 PFU of virus

Figure 10

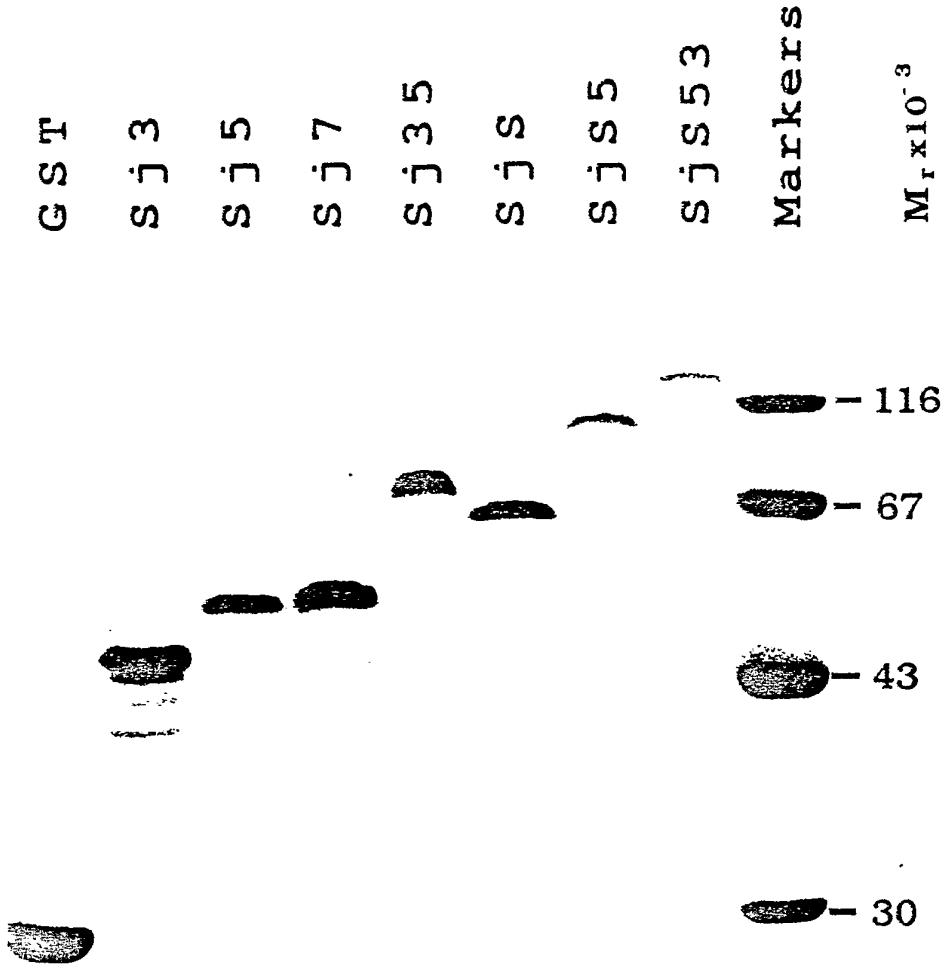

GST
sj3
sj5
sj7
sj35
sjs
sjs5
sjs53
Markers
$M_r \times 10^{-3}$
116
67
43
30

Figure 11

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00352

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07K 15/12, C07H 21/04, C07G 17/00, C12N 1/20, 7/00, 15/00, A61K 39/44

II. FIELDS SEARCHED

Minimum Documentation Searched †

Classification System	Classification Symbols
IPC	WPI, WPIL Keyword: Plasmodium falciparum

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ‡

AU : C07K 15/12, C07G 7/00, C12N 15/00
Chemical Abstracts Keyword: Plasmodium falciparum
GenBank, EMBL, NBRF, Kyoto Databases

III. DOCUMENTS CONSIDERED TO BE RELEVANT §

Category *	Citation of Document, ‖ with indication, where appropriate, of the relevant passages ‖	Relevant to Claim No. ‖
X,Y,E	AU,A, 74536/87 (THE WELLCOME FOUNDATION LTD) 7 January 1988 (07.01.88)	(1,3,4,7-18)
Y,P	Patarroyo, M.E. et al, Nature, Volume 328, issued 13 August 1987 (London), "Induction of protective immunity against experimental infection with malaria using synthetic peptides", see pages 629-632.	(11-13).
A	AU,A, 56037/86 (THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH) 16 October 1986 (16.10.86)	
A	Aslund, L. et al, Proc. Natl. Acad. Sci. USA, Volume 84, issued March 1987 (USA), "Synthetic gene construct expressing a repeated and highly immunogenic epitope of the Plasmodium falciparum antigen Pf155", see pages 1399-1403.	
A	AU,A, 52934/86 (SMITHKLINE BECKMAN CORPORATION AND THE UNITED STATES OF AMERICA) 14 August 1986 (14.08.86)	

(continued)

* Special categories of cited documents: ‖
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 January 1988 (20.01.88)	(05.02.88) 5 FEBRUARY 1988
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	S.D. BARKER

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A Good, M.F. et al, Science, Volume 235, issued 27 February 1987 (Washington DC USA), "Construction of Synthetic Immunogen : Use of New T-Helper Epitope on Malaria Circumsporozoite Protein", see pages 1059-1062.

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers .... because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00352

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members					
AU 52934/86	DK	623/86	EP	192626	HU	41844
	IL	77787	JP	62000037	PT	81974
	ZA	8600874				
AU 56037/86	DK	5966/86	EP	217877	IL	78476
	WO	8606075				

END OF ANNEX