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VaccinesTHIS SHEET HAS BEEN
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5 The present invention relates to a novel hybrid protein, its use in medicine, particularly in the prevention of malaria infections and vaccines containing it.

10 Malaria, is one of the world's major health problems with 2 to 4 million people dying from the disease each year. One of the most acute forms of the disease is caused by the protozoan parasite, Plasmodium falciparum which is responsible for most of the mortality attributable to Malaria.

15 The life cycle of P. falciparum is complex, requiring two hosts, man and mosquito for completion. The infection of man is initiated by the inoculation of sporozoites in the saliva of an infected mosquito. The sporozoites migrate to the liver and there infect hepatocytes where they differentiate, via the exoerythrocytic intracellular stage, into the merozoite stage which infects red blood cells (RBC) to initiate cyclical replication in the asexual blood stage. The cycle is completed by the differentiation of a number of merozoites in the RBC into sexual stage gametocytes which are ingested by the mosquito, where they develop through a series of stages in the midgut to produce sporozoites which migrate to the salivary gland.

25 The sporozoite stage of P. falciparum has been identified as a potential target of a malaria vaccine. The major surface protein of the sporozoite is known as circumsporozoite protein (CS Protein). This protein from strain 7G8 has been cloned, expressed and sequenced (Dame et al Science 225 (1984) p593). The protein from strain 7G8 is characterised by having a central immunodominant repeat region comprising a tetrapeptide Asn-Ala-Asn-Pro repeated 37 times but interspersed with four minor repeats Asn-Val-Asp-Pro. In other strains the number of major and minor repeats vary as well as their relative position. This central portion is flanked by an N and C terminal portion composed of non-repetitive amino acid sequences designated as the repeatless portion of the CS protein.

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It has been shown that irradiated sporozoites can provide significant protection against experimental human malaria (Am. J. Trop. Med. Hyg. 24: 297-402, 1975). However, production difficulties makes the use of



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irradiated sporozoite impractical from the point of view of producing a vaccine.

- Several groups have proposed subunit vaccines based on the
- 5 circumsporozoite protein. Two of these vaccines have undergone clinical testing; one is a synthetic peptide, the other is a recombinant protein (Ballou et al Lancet: i 1277 (1987) and Herrington et al Nature 328:257 (1987).
- 10 These vaccines were successful in stimulating an anti-sporozoite response. Nonetheless, the magnitude of the response was disappointing, with some vaccinees not making a response at all. Furthermore, the absence of "boosting" of antibody levels on subsequent injections and results of in
- 15 vitro lymphocyte proliferation assays suggested that T-cells of most of these volunteers did not recognise the immuno-dominant repeat. Nonetheless, one vaccinee in each study did not develop parasitemia.

- The present invention provides a new, improved antigen for use in malaria vaccines which not only produces a humoral response, but also a
- 20 cellular immune response. Preferably the antigen induces the production of neutralising antibodies against the immunodominant repeat. Most preferably, the antigen should also elicit effector T cell mediated immune responses of the CD4⁺ and CD8⁺ cytotoxic T lymphocyte (CTL) type and of the delayed type hypersensitivity type and also, preferably be able to
- 25 induce T helper (TH) memory cells.

- Accordingly, the present invention provides a hybrid protein comprising substantially all the C-terminal portion of the CS protein, four or more
- 30 tandem repeats of the immunodominant region, and the Surface antigen from Hepatitis B virus (HBsAg). Preferably the hybrid protein comprises a sequence which contains at least 160 amino acids which is substantially homologous to the C-terminal portion of the CS protein. The CS protein may be devoid of the last 12 amino-acids from the C terminal.

- 35 In particular there is provided a protein which comprises a portion of the CS protein of *P. falciparum* substantially as corresponding to amino acids 210-398 of *P. falciparum* 7G8 fused in frame via a linear linker to the N-terminal of HBsAg. The linker may comprise a portion of preS2 from

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HBsAg.

The present invention also provides DNA sequences encoding the proteins of the present invention.

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A particularly preferred embodiment is the hybrid protein designated RTS. The amino acid sequence of RTS is shown in figure 5. This hybrid consists of:

- 10 ° A methionine-residue, encoded by nucleotides 1059 to 1061, derived from the Sacchromyes cerevisiae TDH3 gene sequence. (Musti A.M. et al Gene 1983 25 133-143.
- 15 ° Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.
- 20 ° A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 210 to 398 of the circumsporozoite protein (CSP) of Plasmodium falciparum strain 7G8 (Dame et al supra).
- 25 ° An amino acid (Arg) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.
- 30 ° Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to 1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (9).
- 35 ° A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype).

In an alternative embodiment there is provided a hybrid protein designated RTS* which was generated using the CSP gene sequence from P. falciparum NF54 (Mol. Biochem Parisitol. 35 : 185-190, 1989) and
35 comprises substantially all of the region 207 to 395 of the CS protein from P falciparum NF54.

In particular RTS* comprises:



- A Methionine, encoded by nucleotides 1059 to 1061, derived from the TDH3 gene sequence.
- 5 • Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.
- 10 • A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 207 to 395 of the circumsporozoite protein (CSP) of *Plasmodium falciparum* strain NF54 (Mol.Biochem.Parasitol, 35:185-190, 1989).
- 15 • An amino acid (Gly) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.
- Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to 1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (Nature 280:815-819, 1979).
- 20 • A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype) (Nature 280:815-819, 1979).

25

The amino acid sequence of RTS* is depicted in figure 9.

An expression cassette containing RTS* was constructed and comprises the following features:

30

- A promoter sequence, extending from nucleotide 1 through 1058, derived from the S.cerevisiae TDH3 gene.
- 35 • An open reading frame starting at nucleotide 1059 and extending to nucleotide 2330. This open reading frame is immediately followed by a translational stop codon, TAA (nucleotides 2331 to 2333). The open reading frame encodes the amino acids specifying the hybrid RTS* protein.



- A transcription termination sequence contained within the sequence extending from base pair 2334 to 3504, derived from the S. cerevisiae ARG3 gene.

5

The nucleotide sequence is depicted in figure 10.

The DNA sequences encoding the proteins of the present invention are, in a preferred embodiment flanked by transcriptional control elements, preferably derived from yeast genes and incorporated into an expression vector.

10

Such vectors are a further aspect of the invention. A preferred promoter is the promoter from the S. cerevisiae TDH3 gene Musti et al supra).

15

The invention also relates to a host cell transformed with a vector according to the invention. Host cells can be prokaryotic or eukaryotic but preferably, are yeast, such as S. cerevisiae. In such a host, the hybrid protein, for example RTS will be expressed as lipoprotein particle. The chosen recipient yeast strain preferably already carries in its genome several integrated copies of an hepatitis B S expression cassette. The resulting strain synthesizes two polypeptides, S and RTS (or other hybrid protein of the invention), that spontaneously co-assemble into mixed (for example RTS, S or RTS*, S) lipoprotein particles. These particles, advantageously present the CSP sequences of the hybrid at their surface. These mixed particles also form part of the present invention. Advantageously the ratio of RTS: S or RTS* : S in these mixed particles is 1:4.

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The present invention also relates to vaccines comprising an immunoprotective amount of a protein or particle according to the invention in admixture with a suitable diluent or carrier.

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In the vaccine of the invention, an aqueous solution of the hybrid may be used directly. Alternatively, the protein with or without prior lyophilisation can be mixed or absorbed with any of the known adjuvants which include but are not limited to alum, muramyl dipeptide, saponins such as Quil A.

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An immunostimulant may alternatively or in addition be included. In a preferred embodiment this immunostimulant will be 3 Deacylated monophosphoryl lipid A (3D-MPL).

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3 Deacylated monophosphoryl lipid A is known from US patent No. 4,912,094 and UK patent application No. 2,220,211 (Ribi) and is available from Ribi Immunochem, Montana, USA.

- 10 The protein of the present invention may also be encapsulated into microparticles such as liposomes.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, 15 Baltimore, Maryland, U.S.A., 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877.

Conventional adjuvants may be used, but a preferred immunostimulant is 3-deacylated monophosphoryl lipid A (3D-MPL).

20

Typically when 3D-MPL is used the antigen and 3D-MPL are delivered with alum or presented in an oil in water emulsion or multiple oil in water emulsions. The incorporation of 3D-MPL is advantageous since it is a stimulator of effector T-cells responses.

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Accordingly a preferred embodiment of the present invention provides a vaccine comprising a hybrid protein as herein described, preferably RTS, or RTS* in combination with 3D-MPL and a carrier. Typically the carrier will be an oil in water emulsion or alum.

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In a most preferred embodiment the hybrid protein is presented as a particle or mixed particle as herein described.

The amount of the protein of the present invention present in each vaccine 35 dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected



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that each does will comprise 1-1000 μ g of protein, preferably 1-200 μ g most preferably 10-100 μ g. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects
5 will preferably receive a boost in about 4 weeks, followed by repeated boosts every six months for as long as a risk of infection exists. The immune response to the protein of this invention is enhanced by the use of adjuvant and or an immuno stimulant.

10 The proteins of the present invention are preferably expressed in yeast, and especially those belonging to the genus Saccharomyces.

A further aspect of the present invention is to provide a process for the preparation of hybrid protein of the invention, which process comprises
15 expressing DNA sequence encoding the protein, in a suitable host, preferably a yeast, and recovering the product.

It is particularly preferred to express the protein of the invention in a Sacchromyces strain. When RTS, for example is expressed in such strains
20 it spontaneously assembles into multimeric lipoprotein particles.

These particles are highly immunogenic and induce a strong humoral response, as well as immunological memory and also are capable of inducing effector T cells of the CTL and DTH types.

25 A further aspect of the invention lies in a method of treating a patient susceptible to plasmodium infections by administering an effective amount of a vaccine as hereinbefore described.

Example 11. CONSTRUCTION OF THE RTS.S strain RIT4383

5 The S. cerevisiae strain, RIT4383 used for production of particles containing both the S and RTS polypeptides, carries separate expression cassettes for each protein. The S gene expression cassette has been integrated in 5 to 6 copies at least two sites in genome using a linear
10 RTS gene expression cassette has been integrated in 2 to 3 copies at one or 2 sites in the genome, using a linear integration vector similar to the one employed for the integration of the S gene cassette. Expression from both types of cassette is driven by a promoter derived from the yeast TDH3 gene.

15

1.1 CONSTRUCTION OF THE S EXPRESSION CASSETTE AND INTEGRATIVE VECTOR (pRIT13034)

20 The S gene expression cassette (Figure 1A) identical to that found in strain RIT4376 (1) and consists of a 1058 bp TDH3 promoter fragment, 681 bp of S gene sequence, a 6 bp spacer containing an EcoR1 linker and a 1165 bp fragment carrying the Arg3 transcription terminator. The S coding sequence was derived by subcloning from a complete genomic clone (pRIT10616) of a virus of (adw serotype).

25

The structure of the Ty vector, pRIT13034, used for integration of the S expression cassette into the yeast genome, is shown in Figure 2. The construction and use of this type of vector for integration of expression cassettes in the yeast genome is described in Jacobs et al. (2). pRIT13034
30 contains the S gene cassette, the URA3 gene and the CUP1 gene inserted within a copy of a Ty element cloned on pUC9. The URA3 gene and the CUP1 gene both provide selectable markers that allow to distinguish transformed yeast colonies from untransformed colonies. pUC9 is described by Vieira & Messing (3), the URA3 fragment is from pFL44 (F. Lacroute, CNRS, Strasbourg), the CUP1 fragment is from pCUP5 (T. Butt, SKF Labs, Philadelphia), the Ty element is from Juniaux et al. (4) and the S gene cassette from pRIT12353. Digestion of pRIT13034 with XhoI
35 endonuclease liberates the 8500bp linear fragment shown in Figure 3



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which can integrate into the genome by homologous recombination of the free ends with resident Ty elements.

Example 2

5

2. CONSTRUCTION OF STRAIN Y1295

The recipient strain, EJ cup1D-3d (trp1, leu2, ura3, cup1 , gal1D, MAT α) was used for initial introduction by transformation of the linear vector
10 fragment from pRIT13034. This strain contains a single disrupted cup1 locus, cup1 .

After transformation with the linear XhoI fragment, Ura⁺ colonies were isolated and screened for copper resistance. The more resistant
15 transformants had integrated two to five copies of the vector as determined by Southern blotting analysis. Two transformant colonies were retained, MS9 with an estimated 3 to 4 copies and MS23 with 4 to 5 copies of the integrated linear vector. Strain MS23 was then crossed with strain EJ cup1D-7b (trp1, ura3, cup1 , gal1D, MAT α) and a
20 haploid ascospore recovered to give strain MS23-2a.

This strain was then backcrossed to MS9 and a Leu⁻, Trp⁻ haploid segregant obtained (MS54-2c) containing 5 to 6 copies of the integrated expression cassette. Southern blotting showed that MS54-2C contained 4
25 to 5 tandem copies of the integration vector at one locus and a further single copy integrated at a different locus. All 5 to 6 copies of the expression cassette were intact as digestion of total yeast cell DNA with HindIII endonuclease to liberate the cassette fragment and Southern blotting with an S gene specific probe gave a single 3 kb band as expected.
30 A spontaneous Trp⁺ revertant of this strain was obtained, MS54-2c-T, and given the laboratory accesssion number Y1295.

Example 3 CONSTRUCTION OF THE RTS-EXPRESSION CASSETTE

35 The expression cassette for the RTS hybrid protein was constructed by a multistep cloning procedure and was cloned in the E.coli yeast shuttle vector Yep13(6) yielding a plasmid Yep13RTS (Fig.4). The structure of the cassette is shown in figure 1B. Its entire nucleotide sequence was



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determined either by direct sequencing (as for the coding sequence and parts of the flanking control sequences) or by consultation of the relevant literature (as for parts of the promoter and terminator sequences). The DNA sequence is illustrated in figure 5. It contains the following

5 elements:

- A promotor sequence, extending from nucleotide 1 through 1058, derived from the S. cerevisiae TDH3 gene.
- 10 An open reading frame starting at nucleotide 1059 and extending to nucleotide 2330. This open reading frame is immediately followed by a translational stop codon, TAA (nucleotides 2331 to 2333). The open reading frame encodes the amino acids specifying the hydrid RTS protein.
- 15 A transcription termination sequence contained within the sequence extending from base pair 2334 to 3504, derived from the S. cerevisiae ARG3 gene (Crabeel et al EMBO J. 1983 2: 205-212).
- 20 The amino acid sequence of the hydrid RTS protein, encoded by nucleotides 1059 to 2330 is indicated in figure 5 and contains the following elements:
- A methionine-residue, encoded by nucleotides 1059 to 1061, derived
- 25 from the TDH3 gene sequence.
- Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.
- 30 A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 210 to 398 of the circumsporozoite protein (CSP) of Plasmodium falciparum strain 7G8 (8).
- 35 An amino acid (Arg) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.
- Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to



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1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (9).

5 A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype).

Example 4 CONSTRUCTION OF THE RTS CASSETTE INTEGRATIVE VECTOR pRIT13539

10 The Construction of the RTS Cassette integrative vector pRIT13539 is shown on figure 6.

The RTS expression cassette was inserted on the Ty based integrative vector pRIT13144. This vector is a derivative of pRIT12927 (2) in which
15 the LEU2 gene was inserted in the Sall site of the Ty element as a Sall-XhoI fragment isolated from the vector pCV9 (7). Thus, after insertion of the RTS expression cassette into pRIT13144, the resulting plasmid, pRIT13539, contains, in addition to the expression cassette, the yeast
20 LEU2 gene as selective marker (figure 5). Digestion of pRIT13539 with BglII endonuclease liberates a 6.800 bp linear fragment shown in figure 7 which can integrate into the genome by homologous recombination of the free ends with resident Ty elements.

25 Example 5 TRANSFORMATION OF STRAIN Y1295 AND GENERATION OF STRAIN RIT4383 (Y1530)

To obtain a strain expressing both S and RTS proteins, Y1295 was transformed with the 6800 bp linear BglII fragment (figure 7) with selection for Leu⁺ colonies. Several integrants containing sets of both
30 expression cassettes present in the genome at various ratio were obtained. One selected transformant, expressing the RTS and S protein in a ratio of approximately 1:4 was given the official designation RIT4383 (the laboratory accession number is Y1530).

35 Example 5b Transformation of Strain Y1295 and Generation of Strain Y1631.

A similar (to RTS) construct was generated using the CSP gene sequence



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derived from P.falciparum strain NF54 (Mol.Biochem.Parasitol.35:185-190, 1989). The fusion protein obtained will be designated RTS* to distinguish it from the construct obtained with the CSP derived from P.falciparum strain 7G8.

5

The sequence of the expression cassette is shown in figure 9. It contains the following elements:

- 10 • A promoter sequence, extending from nucleotide 1 through 1058, derived from the S.cerevisiae TDH3 gene.
- 15 • An open reading frame starting at nucleotide 1059 and extending to nucleotide 2330. This open reading frame is immediately followed by a translational stop codon, TAA (nucleotides 2331 to 2333). The open reading frame encodes the amino acids specifying the hybrid RTS* protein.
- 20 • A transcription termination sequence contained within the sequence extending from base pair 2334 to 3504, derived from the S. cerevisiae ARG3 gene.

This expression cassette encoding this RTS* fusion protein was transformed and integrated into the genome of yeast strain Y1295, using the same approach as described previously for the RTS construct.

- 25 Transformed clones expressing both the S and RTS* proteins were obtained. One clone was selected expressing the two proteins in a ratio of approximately 4S:1RTS*. The clone was given the laboratory accession number Y1631.

30 Example 6

PRELIMINARY CHARACTERIZATION OF THE STRAIN RIT4383

35 6.1 Analysis by immunoblotting

Cell free extracts prepared from RIT 4383 were analysed by immunoblot using various antibodies:



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- a monoclonal antibody directed toward the S protein (Mab HBS1)
- a monoclonal antibody directed toward the repeat part of the RTS protein (Mab 167)
- 5 • a rabbit serum directed toward the repeat-less sequences of the RTS protein (rabbit serum no. 20).

10 In yeast strain RIT4383, two expressed products were recognized by monoclonal antibody HBS1: a 24KD protein corresponding to the S protein and a 46KD RTS hybrid protein. The RTS hybrid protein is also detected by antibodies directed toward repeat and non-repeat epitopes of the CSP. These results indicate clearly that strain RIT4383 simultaneously expresses the two S and RTS antigens at a ratio RTS/S of approximately 1:4.

15

6.2 CsCl density gradient centrifugation

20 The formation of particles in strain RIT4383 was analyzed by CsCl density gradient centrifugation. Crude extract ($\pm$ 15 mg of total protein) was analyzed on a 10 ml 1.5 M CsCl gradient (68 hours at 40.000 rpm, + 8°C in a Beckman 50 Ti rotor). Fractions (0.5ml) were collected and analyzed by a radioimmunoassay specific for HBsAg (AUSRIA), by an RTS specific ELISA and by immunoblot using an anti-HBsAg monoclonal antibody.

25

As shown in Figure 8, ELISA, RIA and Western blot peaks appear at the same fraction (no. 13) of the gradient corresponding to a buoyant density of $\rho = 1.21$ suggesting that mixed particles, containing both S and RTS monomers, are formed in this strain.

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Example 7

PREPARATION OF THE SEED LOTS

35

Production procedure for master seed lot

Strain Y1530 (RIT4383) is first grown for 48 hours at 30°C in Petri dishes containing 20 ml sterile YNB (Difco) supplemented with



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5 dextrose (0.1%) and 1.8% (w/v) agar (Difco). The surface growth is suspended into sterile YNB broth (Difco) supplemented with dextrose (1%) and glycerol (10%). This suspension is distributed under aseptic conditions into 2 ml sterile polypropylene stoppered tubes (1 ml per tube) and stored at -70°C.

Production procedure for working seed lot

10 One master seed tube is rapidly thawed, and its contents are streaked with a platinum loop on Petri dishes prepared as described above. After incubation at 30°C for 63 hours, part of the surface growth is transferred to a 2 L conical flask containing 400 ml of sterile YNB broth (Difco) supplemented with dextrose (2%). The culture is incubated at 30°C for 24 hours before being centrifuged
15 (15 min at 6300 x g) under aseptic conditions. The pellet is resuspended into sterile YNB broth (Difco) supplemented with dextrose (1%) and glycerol (10%). This is distributed under aseptic conditions into 2 ml sterile glass stoppered tubes (0.5 ml per tube) and stored at -70°C.

20

Example 8: FERMENTATION

Preparation of the inoculum

25 (a) Growth on solid medium

One vial of the working seed lot is rapidly thawed and spread onto Petri dishes containing sterile YNB broth (Difco) supplemented with dextrose (1%) and 1.8% (w/v) agar (Difco). The Petri dishes
30 are incubated for 48 hours at 30°C.

(b) Growth of inoculum

35 The surface growth of one Petri dish is suspended in sterile YNB broth (Difco) supplemented with dextrose (2%) and distributed equally into two conical flasks (2 L, 400 ml liquid per flask). The flasks are incubated for 24 hours at 30°C on a rotary shaker.

Fermentation

5 The fermentor vessel (20 L total volume) containing 5 L of dieionized water supplemented with $(\text{NH}_4)_2\text{SO}_4$ (40 g) is sterilized in-situ at 121°C for 30 minutes using clean, pre-filtered steam at 2 bar g pressure. After cooling to room temperature, the liquid volume in the fermentor is adjusted to 4L, and 1 L of filter-sterilized HB4 solution is added. The fermentation is begun by adding the inoculum (800 ml) from the two conical flasks.

10

The fermentation is run using the fed-batch technique whereby the culture is continuously fed with a solution of the following composition.

15

- 5 L HB4 solution;
- 4 L dextrose 80% (sterilized at 121°C).

20

The culture density increases by aerobic growth at 30°C and pH 5 (maintained by addition of NH_4OH). Dissolved oxygen is maintained about 50% saturation by adjustment of airflow and agitation speed. The rate of addition of the feed is predetermined to maximize growth rate and minimize formation of by-product ethanol.

25

The fermentation is stopped after 40-90 hours. At the end of the fermentation, the total culture volume is 10-18 L, and the dry cell weight is between 30 and 100 g/L. The culture is rapidly cooled down to $15\text{-}25^\circ\text{C}$, and the yeast cells are recovered from the broth by centrifugation. The concentrated yeast cells are washed once

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with phosphate buffer (50 mM) before being re-centrifuged and subsequently stored at -70°C in polyethylene bags.

**HB4 Medium Composition**

Component	Quantity
KH ₂ PO ₄	41.00 g
MgSO ₄ .7H ₂ O	23.50 g
CaCl ₂ .2H ₂ O	4.70 g
NaCl	0.30 g
FeCl ₃ .6H ₂ O	50.00 mg
H ₃ BO ₃	28.00 mg
MnSO ₄ .H ₂ O	22.40 mg
ZnSO ₄ .7H ₂ O	22.40 mg
Na ₂ MoO ₄ .2H ₂ O	11.20 mg
KI	5.60 mg
CaCl ₂ .6H ₂ O	5.10 mg
CuSO ₄ .5H ₂ O	2.24 mg
Biotin	2.70 mg
Folic acid	2.70 mg
Inositol	2.70 mg
Ca Pantothenate	0.54 mg
Pyridoxin.HCl	0.54 mg
Thiamin.HCl	0.54 mg
Niacin	1.20 mg
P-amino benzoic acid	0.60 mg
Riboflavin	0.60 mg
HCl (37°C)	5.00 ml
Deionized water (up to)	1.00 litre

Example 9: Extraction and Purification of RTS/SEXTRACTION PROCEDURE

5

9.1 Preparation of cell suspension

Frozen concentrated yeast cells (at -70°C) are thawed down to -30°C overnight and subsequently thawed out to $5-15^{\circ}\text{C}$ by placing the polyethylene bags containing the cells in water ($10-20^{\circ}\text{C}$). A yeast cell suspension is made with a phosphate buffer solution (pH 8.0) containing the following ingredients : Ethylenediamine tetraacetic acid 9EDTA), pMethylsulfonyl Fluoride (PMSF), isopropanol and tween 20.

15

9.2 Cell disruption

Cells are disrupted in a bead mill containing lead-free glass beads (0.49-0.70 mm diameter). The grinding chamber is cooled by circulation of refrigerating fluid at -20°C , in such a way that the temperature of the homogenate at the outlet of the grinding chamber does not exceed 15°C . The liquid flowrate is 6 L/hour and the agitation speed is 3000 rpm. This process is performed twice. The pH of the resulting homogenate is 6.7 to 7.5.

25

9.3 Polyethyleneglycol clarification

Polyethyleneglycol 400 (PEG 400) is added to the disrupted cell suspension to give a final concentration of 10% (30 minutes below 10°C , pH 6.1) and a partially-clarified supernatant is recovered by centrifugation (J21B Beckman centrifuge, JA10 rotor at 17,000 g for 1 hour).

30

9.4 Methanol clarification

35

Methanol is added at pH 7 to the PEG-clarified antigen to give the proportion of 1 volume of methanol for 5 volumes of PEG-clarified antigen. The clarified antigen is obtained at 17,000 g for 20



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minutes by centrifugation (J21B Beckman centrifuge, JA10 rotor).

9.5 Adsorption/Desorption on colloidal silica

5 The crude antigen is adsorbed overnight onto 1.5% (w/v) colloidal silica (Aerosil 380, Degussa) at 4°C.

10 After washing (3 times) the pellet by successive centrifugation and resuspension in NaCl 0.9% (w/v), the antigen is desorbed using a 10mM pyrophosphate buffer, pH 9.5, containing 1% TWEEN 20.

15 The desorbing buffer volume corresponds to 1/8 of the methanol clarified antigen solution. The desorbed antigen solution is recovered by ultracentrifugation in a L 8.70 Beckman ultracentrifuge rotor R19 at 50,000 g for 60 minutes.

9.6 Diafiltration

20 Before the purification steps, the desorbed antigen is washed by ultrafiltration with 5 volumes of urea 6M, NaCl 500 mM, TRIS-HCl 20 mM at pH 8.5 in order to eliminate much of the proteic and lipidic contaminants.

25 Then the buffer is exchanged in the same system (Ultrasette, FILTRON fitted with polysulfone membranes with a 300 kD nominal cut-off) with 5 volumes of TRIS-HCl 10 mM (pH 8.1).

9.7 Ion exchange chromatography on DEAE-TSK 650 (M)

30 The clarified solution is applied to an anion-exchange column (DEAE-TSK 650 (M)) equilibrated in a 10 mM TRIS-buffer, pH 8.1. After washing successively with 2 volumes of 10 mM TRIS-HCl buffer pH 8.1 and 3 volumes of 10 mM TRIS-HCl buffer pH 8.1 supplemented with 40 mM NaCl, the antigen is desorbed with less
35 than one volume of 10 mM TRIS-HCl buffer pH 8.1 containing 150 mM NaCl. The antigen-containing fractions are pooled.

9.8 Hydrophobic interaction chromatography on Butyl-TSK 650 (M)



5 After NaCl addition up to a final concentration of 650 mM NaCl, the antigen solution is loaded on a Butyl-TSK 650 (M) column equilibrated with a 20 mM TRIS-HCl buffer, 600 mM NaCl (pH 8.1). Most of the antigen passes through while most of the impurities bind to the gel.

9.9 Concentration by ultrafiltration

10 The HIC pool is concentrated by ultrafiltration in an Ultrasette system (FILTRON) fitted with polysulfone membranes with a 300 kDa nominal cut-off.

9.10 Ultracentrifugation in a CsCl gradient

15 CsCl is added to the Butyl-TSK pool to give a final concentration of 1.5 M.

20 After 65 hours in a 50.2 Ti Beckman rotor at 270,000 g, the antigen-containing fractions are collected.

9.11 Size exclusion chromatography on SEPHACRYL S300 (HR Type)

25 In order to exchange the buffer and to eliminate low molecular weight contaminants, the antigen solution is applied to a SEPHACRYL S300 HR column. The elution buffer is 10 mM phosphate, containing 150 mM NaCl (pH 7.4).

STERILE FILTRATION

30 After dilution to between 150 and 400 µg Lowry/ml and pH adjustment to 6.8, the purified antigen is sterilized by filtration through a 0.22 µm sterile filter. The resulting solution contains purified RTS/S particles.

35

Example 10: IMMUNOLOGICAL CHARACTERIZATION OF RTS/S

10.1 Antigenicity



In order to test the antigenicity of the RTS/S particles, a number of ELISA's were performed, combining monoclonal antibodies directed against the different epitopes.

5

The monoclonal antibodies (MoAbs) used are :

- MoAb R10 :
 - specific for the repeat sequence (NANP) of the region.
 - IgM isotype.
- MoAb RL117:
 - specific for the non-repeat sequence of the CS region.
- MoAb RF1 :
 - specific for the S sequence of the HBsAg.
 - IgG1 isotype.

10 MoAbs R10 and RL117 were prepared in house by fusion of Sp2/OAg 14 myeloma cells with splenocytes of Balb/C mice immunized with partially pure RTS-like particles containing both repeat and non-repeat regions of the CSP sequence.

15 Three batches of the candidate RTS/S vaccine were analyzed : batches Nos. 24M31, 24M32 and 24M34.

10.2 Reaction with monoclonal antibody R10 (anti-repeat)

20 The anti-repeat MoAb R10 was used in a "sandwich" ELISA. Samples to be tested were incubated in microtiter plates previously coated with MoAb R10. The same MoAb coupled to peroxidase was then added. After one hour incubation at 37°C and washing, color was developed by addition of Orthophenylene-diamine-H₂O₂. Absorbance was measured at 490 nm and plotted versus the antigen concentration.

25

Results

30 The three batches : 24M31, 24M32 and 24M34 reacted positively and consistently with MoAb R10, thus confirming the presence and accessibility of the repeat epitopes on the RTS/S particles. The



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amount of antigen necessary to reach 50% of maximum binding was 51.2, 38.2 and 60.6 ng for batches 24M31, 24M31 and 24M34 respectively.

5 10.3 Reaction with monoclonal antibody RL117 (anti-repeatless)

10 The reactivity of MoAb RL117 with RTS,S particles was analyzed in a "sandwich" ELISA test. MoAb RL117 was coupled to peroxidase and used for the detection of the RTS/S particles, while MoAb R10, specific for the repeat region, was used for the capture of particles.

15 Briefly, samples to be tested were incubated in microtiter plates previously coated with MoAb R10. RL117 coupled to peroxidase was then added, and, after one hour incubation at 37°C and washing, the coloration was developed by addition of Orthophenylene-diamine-H₂O₂. Absorbance was measured at 490 nm and plotted versus the antigen concentration.

20 Results

25 The three batches : 24M31, 24M32 and 24M34 reacted positively and consistently with MoAbs RL117 and R10, thus confirming the presence and accessibility of repeat and ono-repeat epitopes on the same RTS/S particles. The amount of antigen necessary to reach 50% of maximum binding was 169.2, 117.6 and 181.1 ng for batches 24M31, 24M32 and 24M34 respectively

10.4 Reaction with monoclonal antibody RF1 (anti-S)

30 The presence of S-epitopes in RTS/S particles was shown by a "sandwich" ELISA test, using MoAb RF1 coupled to peroxidase for detection. The R10 MoAb was used directly onto the microtiter plates in order to capture the RTS,S particles.

35 In summary, samples to be tested were incubated in microtiter plates previously coated with MoAb R10. MoAb RF1 coupled to peroxidase was then added. After incubation at 37°C for 1 hour and washing, color development was allowed by addition of



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Orthophenylene-diamine- H_2O_2 . Absorbance was measured at 490 nm and plotted versus the antigen concentration.

Results

5

The three batches : 24M31, 24M32 and 24M34 reacted positively and consistently with MoAbs RF1 and R10, thus confirming the presence and accessibility of the repeat and Septopes on the same RTS/S particles. The amount of antigen necessary to reach 50% of maximum binding was 52.3, 55.2 and 106.2 ng for batches 24M31, 24M32 and 24M34 respectively.

10

Example 11: IMMUNOGENICITY IN VIVO OF RTS/S PARTICLES

15 11.1 Immunogenicity studies

Studies on the immunogenicity of (RTS,S) particles were performed in mice and in Cercopithecus aethiops monkeys.

20

In mice, anti-CSP antibodies were analyzed by ELISA test using the R32tet32 antigen for the detection of the anti-repeat antibodies. R32tet32 consists of 32 copies of the NANP (major) repeat fused to a 32 amino acid portion of the tetracyclin resistance gene of plasmid pBR322. The recombinant antigen is produced in Escherichia coli.

25

Antibodies (Abs) directed against the non repeated sequence of the CS protein were measured by ELISA test using the RLF antigen.

The RLF antigen consists of the complete CS protein flanking region devoid of repeat fused to the first 81 amino acids of the NS1 protein of influenza virus. The RLF antigen is produced in E. coli.

30

Mice sera were serially diluted, starting at 1:100, and titers are expressed as the reciprocal of the dilution corresponding to an optical density of 1.0 in the ELISA test (1). Measurements of anti-CSP Abs were performed on individual sera and the geometric mean titer (GMT) was calculated.

35

In order to analyze the anti-carrier response, anti-HBs antibody titers were also measured (pooled sera only).



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In mice experiments, Balb/C mice (H-2d haplotype) usually used for the immunogenicity of HBsAg were also used to evaluate the immunogenicity of RTS,S particles. The intraperitoneal (i.p.) and the subcutaneous (s.c.) routes of immunization were compared and the effect of the immunostimulant 3-deacylated monophosphoryl lipid A (3D-MPL) on the immune response was also tested.

The immunogenicity of the (RTS,S) vaccine was also tested in a similar way in *Cercopithecus aethiops* monkeys.

Selected individual or pooled sera were also tested for their capacity to inhibit the in vitro invasion of a human hepatoma cell line (HepG2) by *P. falciparum* sporozoites (ISI assay (2)).

11.2 Immunogenicity in mice

EXPERIMENT 1 : Immunogenicity of clinical (RTS,S) batches adsorbed on A1(OH)₃

Method

Immunization :

Groups of 10 Balb/C mice were injected twice intraperitoneally at one month interval with 1 µg of each of three (RTS,S) batches previously adsorbed on A1(OH)₃ (batches 24M/31, 24M/32 and 24M/34). Control mice were injected with HBsAg (Engerix-B, batch ENG611B4). On days 30 and 45, the mice were bled and the antibody titers were measured on individual sera.

Serological methods :

The anti-R32tet32 and the anti-RLF titers were measured by ELISA using respectively R32tet32 and RLF as coating antigens. The microplates were incubated with the serum sample (12 two-fold serial dilutions starting at 1:100) to be tested. Mouse Abs were reacted with biotinylated anti-mouse Ig followed by streptavidin : biotinylated horseradish peroxidase complex and orthophenylene-



-24-

diamine/H₂O₂. The optical density was measured at 490 nm. The titers were expressed as the reciprocal of the dilution corresponding to an optical density of 1.0 (50 % maximal binding). For each group of mice, the geometric mean titer (GMT) was calculated.

5 The anti-HBs antibody titer was calculated according to the Hollinger formula (3) and expressed in mIU/ml.

Results

10 Anti-CSP response :

Strong anti-R32tet32 and anti-RLF responses were observed for each (RTS,S) batch tested. No significant difference between the batches was noted. A remarkable booster effect was observed after
15 the second dose. Mice immunized with HBsAg ("Engerix-B") were used as negative control in this experiment.

Anti-HBs response :

20 The (RTS,S) batches induce antibodies directed against the HBsAg carrier protein. The assay was performed on pooled sera only.

EXPERIMENT 2 : Effect of the immunostimulant 3D-MPL on
the immunogenicity of (RTS,S) particles in
25 Balb/C mice.

We analyzed the effect of the 3D-MPL on the immunogenicity of the (RTS,S) vaccine in mice. Both the intraperitoneal (i.p.) and the subcutaneous (s.c.) routes of immunization were tested.

30 A. Immunization by the ip route

Method

Immunization:

35

Groups of 10 Balb/C mice were injected twice intraperitoneally at one month interval with 1 µg of (RTS,S) (batch 24M/34) adsorbed on Al(OH)₃ alone or on Al(OH)₃ + 3D-MPL (50 µg/dose). Control mice



-25-

were injected with NaCl 150 mM. One days 30 and 45, the mice were blend and the antibody titers measured on individual sera.

Serological method:

5

The serological methods were the same as in the first experiment described above.

Results

10

Anti-CSP response:

15

The (RTS,S) vaccine batch induces strong anti-R32tet32 and anti-RLF responses in both formualtions. In all cases, a significant booster effect is observed following the second immunization. Titers obtained with the formulation containing 3D-MPL are in all cases higher than the aluminium alone formulation, and a statistically significant increase was observed in the primary anti-R32tet32 response ($p = 0.02$). A group of mice injected with NaCl 150 mM was used as negative control in this experiment.

20

Anti-HBs response:

25

Both formualtions of the (RTS,S) vaccine, injected by the i.p. route, induced a strong anti-HBs response. A significant booster effect was observed following second immunization with either formulation.

B. Immunization by the s.c. route

30

Method

Immunization:

35

Groups of 10 Ba1b/C mice were injected twice subcutaneously at one month interval with 1 μ g of (RTS,S), (batch 24M/34) adsorbed on Al(OH)₃ alone or on Al(OH)₃ + 3D-MPL (50 μ g/dose). Control mice were injected with NaCl 150 mM. One days 30 and 45, the



-26-

mice were blend and the antibody titers measured on individual sera.

5 The serological methods were the same as in the first experiment described above.

Results

10 Anti-CSP response:

The (RTS,S) vaccine batch induced positive response against R32tet32 and RLF in both formulations. In all cases, a significant booster effect was observed following the second immunization. Statistically significant higher titers were observed with the 15 3D-MPL formulation on day 45, both for the anti-R32tet32 and anti-RLF responses ($p = 0.18$ and $p = 2.9$ respectively). In general, however, titers were lower than those obtained with the i.p. route. A group of mice injected with NaCl 150 mM was used as negative control in this experiment.

20 Anti-HBs response:

Both formulations of the (RTS,S) vaccine, injected by the s.c. route induced a good anti-HBs response. A significant booster effect was 25 observed following second immunization with either formulation. As observed for the anti-CSP responses, the anti-HBs response was lower by this route of immunization as compared to the i.p. route.

11.3 Immunogenicity in Cercopithecus aethiops

30 The immunogenicity was evaluated in Cercopithecus aethiops monkeys with clinical batch 24M/32 adsorbed on Al(OH)₃.

35 Methods

Immunization:

Five monkeys wer injected intramuscularly on days 0, 28 and 84



-27-

with 20 μ g of (RTS,S) particles adsorbed on Al(OH)₃ (0.5 mg Al+++). The animals were bled on days 0, 14, 28, 42, 56, 66 and 98. Antibodies directed against R32tet32, RLF and HBs antigens were measured.

5

Serological methods:

A anti-R32tet32 and the anti-RLF antibody titers were measured by ELISA using respectively the R32tet32 and the RLF antigens coated on microplates. The plates were then incubated with the serum sample to be tested (12 two-fold serial dilutions starting at 1:10). Monkey antibodies were detected by biotinylated anti-human Ig followed by streptavidin biotinylated horseradish peroxidase complex and orthophenylenediamine/H₂O₂. The optical density was measured at 490 nm. The titers were expressed as the reciprocal of the dilution corresponding to an optical density of 1.0 (50% maximal binding). For each group, the geometric mean titer (GMT) was calculated. The anti-HBs antibody titers were calculated according to the Hollinger formula (Hollinger et al., 1982) and expressed in mIU/ml.

10

15

20

Results

Anti-CSP response:

25

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The (RTS,S) vaccine induced a positive response against both R32tet32 and RLF antigens in all 5 monkeys. A significant booster effect was observed 14 days following second immunization (day 42). A slow decrease of antibody titers was then observed up to the third immunization. The titers again increase 14 days following the third immunization (day 98), except in the case of the anti-RLF response of monkey Jo. 352.

35

The anti-R32tet32 titers reached after the third immunization (day 98) are not however higher than those observed after the second immunization (day 42).

In the case of the anti-RLF response (with the exception of monkey



-28-

JO 353), an increase of titers is observed following the third immunization (day 98) relative to post second immunization levels (day 42).

5 Anti-HBs response:

All monkeys raised an anti-HBs response with significant booster effects following second (day 42) as well as third (day 98) immunization.

10

Biological activity of antibodies raised against the (RTS,S) particle

15

As a measure of the biological function of the antibodies induced by the (RTS,S) vaccine, pooled mice and individual monkeys sera were tested by the Inhibition of Sporozoite Invasion (ISI) assay (Hollingdale et al., 1984). This assay measured the capacity of the anti-CSP antibodies to inhibit the in vitro invasion of a human hepatoma cell line (HepG2) by P. falciparum sporozoites.

20

Results

25

The results of this experiment are presented in Tables 1 and 2. The ISI data are expressed as % inhibition relative to the activity of a pre-immune control serum taken as 0% inhibition. For reference, the anti-R32tet32 and RLF antibody titers of the tested sera are included. Table 1 shows that all mice sera tested have very high ISI activity. Table 2 shows that all 5 monkeys have also a very ISI activity on day 98 compared with the corresponding immune serum.

30

Conclusion

35

The (RTS,S) particles induced, both in mice and monkeys, a high antibody response directed against the repeat and non-repeat epitopes of the CSP and against the S protein of the HBsAg carrier.

The primary antibody response in mice was enhanced by the presence of 3D-MPL.



Antibody titers obtained after intraperitoneal injection were higher than those obtained after immunization by the subcutaneous route.

- 5 The antibodies elicited in the two animal species effectively prevented invasion of cultured human hepatoma cells by P. falciparum sporozoites.



-30-

Table 1: ISI activity of sera from Balb/C mice immunized with TS.S

Pool of Mice sera	A-R32tet32 Titer	A-RLF Titer	ISI (%)
Pool i.p A1+3D-MPL Day 45	606544	242408	100%
Pool i.p Alum only Day 45	87005	160284	98%
Pool i.p A1+3D-MPL Day 45	15333	99002	94%
Pool s.c A1+3D-MPL Day 45	5102	20453	86%
Pool of Negative Controls	205	205	0%



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Table 2: ISI activity of individual monkey sera immunized with RTS.S

Monkey #	Serum	A-R32tet32 titer	A-RLF titer	ISI (%)
J0 352	DAY 0	<50	<50	0%
	DAY 98	1762	1250	98%
J0 353	DAY 0	<50	<50	0%
	DAY 98	1574	32218	90%
J0 354	DAY 0	<50	<50	0%
	DAY 98	3751	31034	98%
J0 356	DAY 0	<50	<50	0%
	DAY 98	1495	18544	95%
J0 357	DAY 0	<50	<50	0%
	DAY 98	1420	30727	98%

Reference

- (1) Harford N, Cabezon T, Colau B, et al., "Construction and
Characterization of a *Saccharomyces Cerevisiae* Strain (RIT4376)
5 Expressing Hepatitis B Surface Antigen", Postgrad Med J 63, Supp.
2: 65-70, 1987.
- (2) Jacobs E, Rutgers T, Voet P, et al., "Simultaneous Synthesis and
Assembly of Various Hepatitis B Surface Proteins in *Saccharomyces*
10 *cerevisiae*", Gene 80: 279-291, 1989.
- (3) Vieira J and Messing J, "The pUC plasmids, an M13mp7-Derived
System for Insertion Mutagenesis and Sequencing with Synthetic
Universal Primers", *Gene 19: 259-268, 1982.
15
- (4) Juniaux Embo J 1: 1125-1131, 1982.
- (5) Hinnen A, Hicks JB, and Fink GR, "Transformation of Yeast", Proc
Natl Acad Sci USA 75: 1929-1933, 1980.
20
- (6) Broach JR, Strathern JN, and Hicks JB, "Transformation in Yeast
Development of a Hybrid Cloning Vector and Isolation of the CAN 1
Gene", Gene 8: 121-133, 1979.
- 25 (7) Zhang H, et al., "Double Stranded SDNA Sequencing as a Choice
for DNA Sequencing", Nucleic Acids Research 16: 1220, 1988.
- (8) Dame JB, Williams JL, Mc Cutchan TF, et al., "Structure of the
Gene Encoding the Immunodominant Surface Antigen on the
30 Sporozoites of the Human Malaria Parasite *Plasmodium*
falciparum", Science 225: 593-599, 1984.
- (9) Valenzuela P, Gray P, Quiroga M, et al., "Nucleotide Sequences of
the Gene Coding for the Major Protein of Hepatitis B Virus Surface
35 Antigen", Nature 280: 815-819, 1979.

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-33-
Claims

1. A hybrid protein comprising substantially all the C-terminal portion of the CS protein of Plasmodium, four or more tandem repeats of the CS protein immunodominant region, and the surface antigen from Hepatitis B Virus (HBsAg).
2. A hybrid protein comprising a sequence of CS protein of P.falciparum substantially as corresponding to amino acids 210-398 of P.falciparum 7G8 CS protein or 207-395 of P. falciparum NF54 CS protein fused in frame via a linear linker to the N-terminal of HBsAg.
3. A hybrid protein as claimed in claim 1 or 2 wherein the CS protein is fused to HBsAg.
4. A hybrid protein comprising the following amino acid sequences:
 - a) an N-terminal methionine residue
 - b) Met Ala Pro
 - c) a stretch of 189 amino acids corresponding to amino acids 210 to 398 of CS protein P.falciparum 7G8 or 207-395 of CS protein P. falciparum NF54
 - d) Arg
 - e) Pro Val Thr Asn from hepatitis B Pre S₂ protein.
 - f) a stretch of 226 amino acids specifying the S protein of hepatitis B virus
6. The hybrid protein designated RTS.
7. The hybrid protein designated RTS*.
8. A DNA sequence encoding a hybrid protein as claimed in claims 1 to 7.
9. A vector containing a DNA sequence as claimed in claim 8 said sequence being linked to transcriptional control elements.
10. A host transformed with a vector of claim 9.



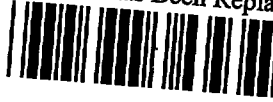
11. A host as claimed in claim 10 wherein the host is S. cerevisiae.
12. A host as claimed in claim 11 additionally transformed with a
5 gene encoding the Hepatitis B surface antigen.
13. A multimeric lipoprotein particle comprising a hybrid protein of
claims 1 to 7.
- 10 14. A mixed multimeric lipoprotein particle comprising a hybrid
protein of claims 1 to 7 and Hepatitis B surface antigen.
- 15 15. A mixed multimeric lipoprotein of claim 14 comprising RTS, or
RTS* and the surface antigen of Hepatitis B virus.
- 16 16. A particle as claimed in claim 14 or 15 wherein the ratio of hybrid
protein to surface antigen is 1:4.
- 20 17. A vaccine comprising an immunoprotective amount of a particle or
protein according to any of claims 1 to 7 or claims 13 to 16 in admixture
with a suitable diluent or carrier.
- 25 18. A vaccine as claimed in claim 17 additionally comprising 3-
Deacylated monophosphoryl lipid A.
- 30 19. A vaccine as claimed in claim 18 additionally comprising alum.
20. A vaccine as claimed in claim 18 presented in an oil in water
emulsion.
- 35 21. A method of treating a patient susceptible to plasmodium
infections comprising administering an effective amount of vaccine
according to any of claims 17 to 20.
21. A process for the production of a hybrid protein according to any of
claims 1 to 7 which process comprises expressing a DNA sequence
encoding the protein in a suitable host and recovering the product.



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22. A process for the production of particles as claimed in claims 12 to 15 comprising expressing a DNA sequence encoding a protein of claims 1 to 7 and hepatitis B surface antigen in a *Sacchromyces* strain.

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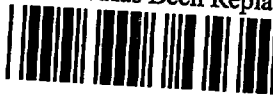
- 36 -
Abstract

Vaccines

- 5 A novel hybrid protein is provided which comprises a portion of the CS protein of *P. falciparum* and the surface antigen of Hepatitis B virus.

The use of this protein for vaccination purposes is disclosed.

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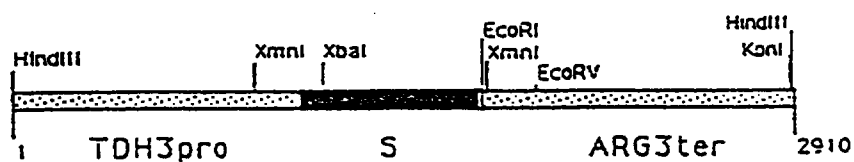


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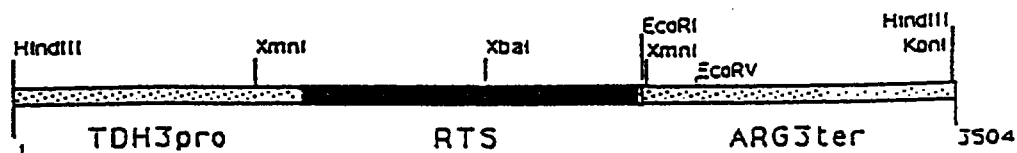


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Figure 1: Restriction endonuclease map of (A) the S expression cassette and (B) the RTS expression cassette.
The extent of each coding sequence is indicated by the black bar.



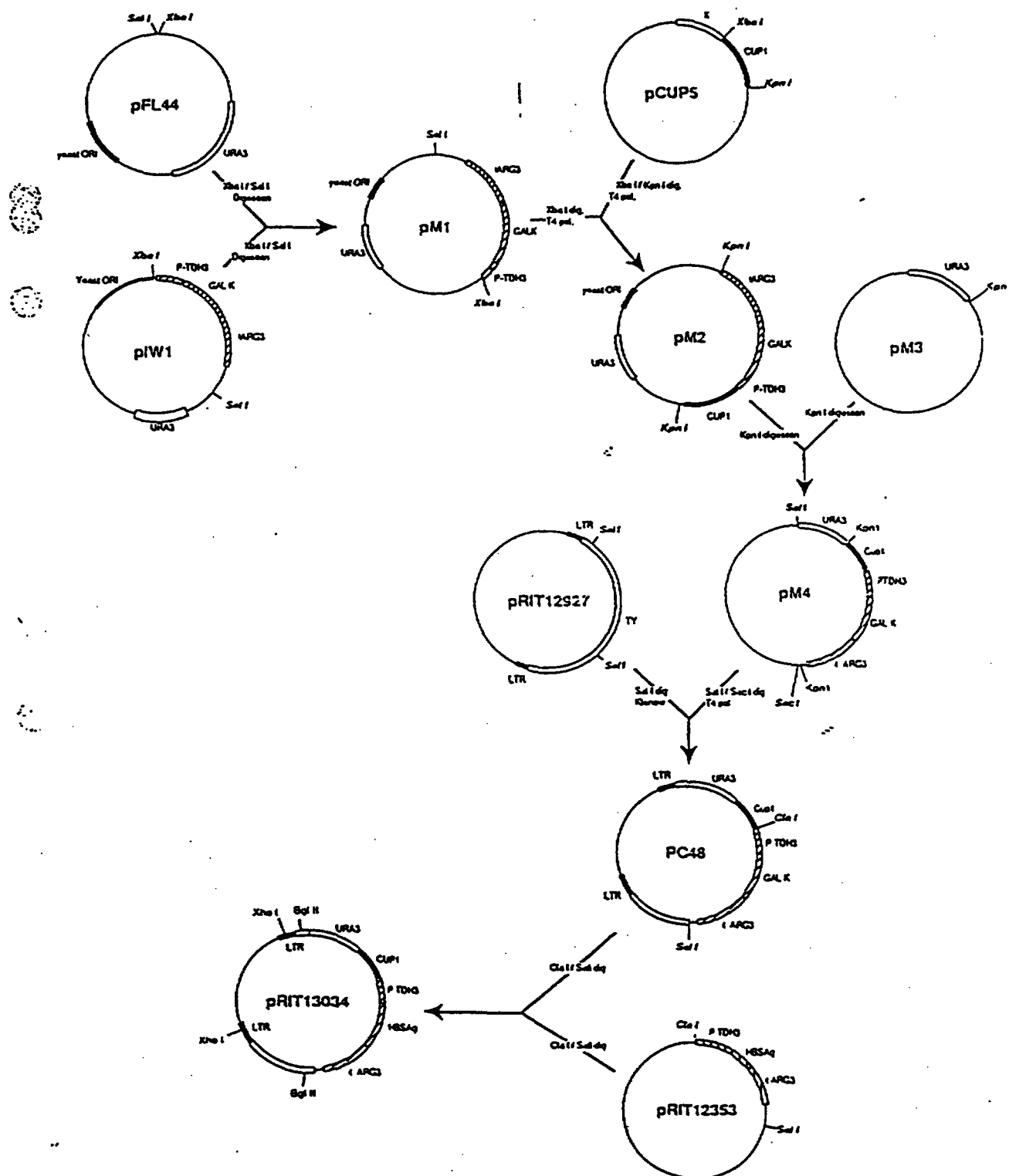
A



B

Figure 2: Construction of plasmid pRIT13034 -

Digestion with *Xho*I endonuclease liberates a 8.5 kb linear DNA fragment carrying the S expression cassette for integration into the yeast chromosome by homology of the free ends with resident Ty elements.



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Figure 3: Restriction map of the 8.5 kb linear XhoI fragment from pRIT13034
The linear fragment contains the URA3 and CUP1 genes for selection of transformed yeast cells together with the S expression cassette.

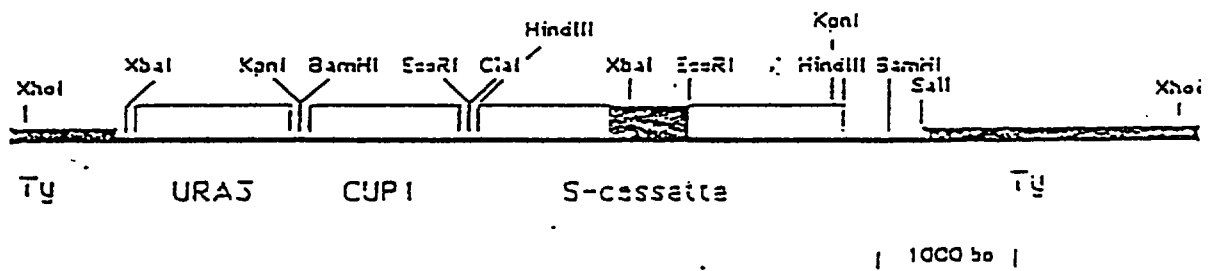




Figure 4: Construction of the RTS expression cassette and its cloning in plasmid Yep13.

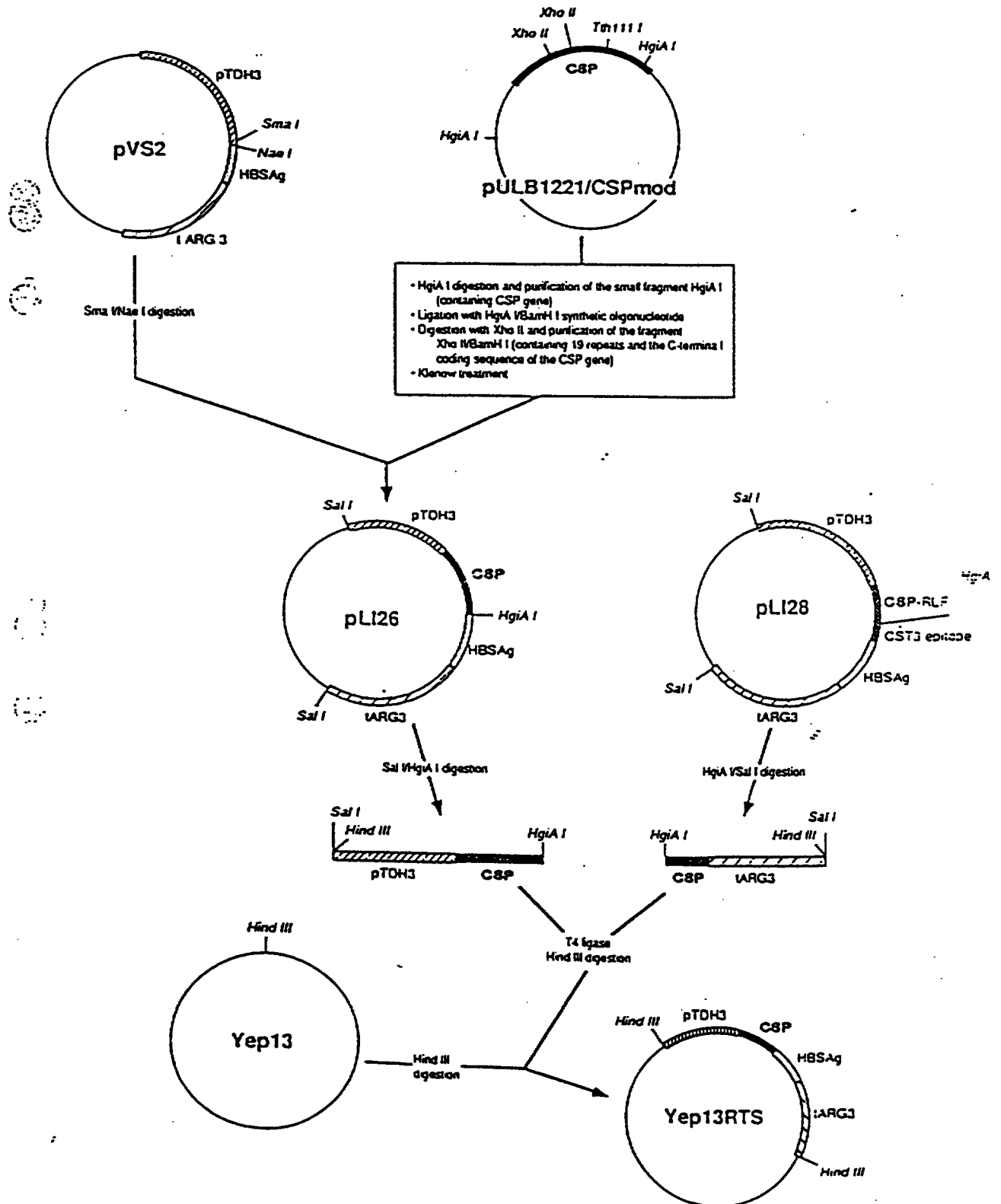




Figure 5: Nucleotide sequence of the RTS expression cassette and predicted translation product of the RTS-HBsAg hybrid protein.
The translation product initiated from the TDH3 ATG codon is shown below the DNA sequence.

10	30	50
AAGCTTACCAGTTCTCACACGGAACACCACTAATGGACACAAATTCGAAATACTTTGACC		
70	90	110
CTATTTTCGAGGACCTTGTCACCTTGAGCCCAAGAGAGCCAAGATTTAAATTTTCCTATG		
130	150	170
ACTTGATGCAAAATCCCAAAGCTAATAACATGCAAGACACGTACGGTCAAGAAGACATAT		
190	210	230
TTGACCTCTTAACTGGTTCAGACGCGACTGCCTCATCAGTAAGACCCGTTGAAAAGAACT		
250	270	290
TACCTGAAAAAACGAATATATACTAGCGTTGAATGTTAGCGTCAACAACAAGAAGTTTA		
310	330	350
ATGACGCGGAGGCCAAGGCAAAAAGATTCCCTTGATTACGTAAGGGAGTTAGAATCATTTT		
370	390	410
GAATAAAAAACACGCTTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGA		
430	450	470
ATACGTAAATAATTAATAGTAGTGATTTTCCTAACTTTATTTAGTCAAAAATTAGCCTTT		
490	510	530
TAATTCTGCTGTAAACCCGTACATGCCCAAAATAGGGGGCGGGTTACACAGAATATATAAC		
550	570	590
ATCGTAGGTGTCTGGGTGAACAGTTTATCCCTGGCATCCACTAAATATAATGGAGCTCGC		
610	630	650
TTTTAAGCTGGCATCCAGAAAAAAAAGAATCCCAGCACCAAAATATTGTTTCTTCACC		
670	690	710
AACCATCAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCACAAACAG		
730	750	770
GCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGATGACAC		
790	810	830
AAGGCAATTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTACCTTCTGC		



850	870	890
TCTCTCTGATTGGAAGCTGAAAAAAGGTTGAAACCAGTTCCTGAAATTATTC		
910	930	950
CCTACTTGACTAATAAGTATATAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTAT		
970	990	1010
TTCTTAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAACACCA		
1030	1050	1070
AGAACTTAGTTTCGAATAAACACACATAAACAAACAAAATGATGGCTCCCGATCCTAATG MetMetAlaProAspProAsnA		
1090	1110	1130
CAAATCCAAATGCAAACCCAAATGCAAACCCAAACGCAAACCCCAATGCAAATCCTAATG laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA		
1150	1170	1190
CAAACCCCAATGCAAATCCTAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATG laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA		
1210	1230	1250
CAAACCCAAACGCAAACCCCAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATG laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA		
1270	1290	1310
CAAACCCAAATGCAAACCCCAATGCAAACCCCAATGCAAATCCTAATAAAACAATCAAG laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnLysAsnAsnGlnG		
1330	1350	1370
GTAATGGACAAGGTCACAATATGCCAATGACCCAAACCGAAATGTAGATGAAATGCTA lyAsnGlyGlnGlyHisAsnMetProAsnAspProAsnArgAsnValAspGluAsnAlaA		
1390	1410	1430
ATGCCAACAATGCTGTAAAAATAATAATAACGAAGAACCAAGTGATAAGCACATAGAAC snAlaAsnAsnAlaValLysAsnAsnAsnAsnGluGluProSerAspLysHisFleGluG		
1450	1470	1490
AATATTTAAAGAAAATAAAAAATTCTATTTCAACTGAATGGTCCCATGTAGTGTAACCTT lnTyrLeuLysLysIleLysAsnSerIleSerThrGluTrpSerProCysSerValThrC		
1510	1530	1550
GTGGAAATGGTATTCAAGTTAGAATAAAGCCTGGCTCTGCTAATAAACCTAAAGACGAAT ysGlyAsnGlyIleGlnValArgIleLysProGlySerAlaAsnLysProLysAspGluL		
1570	1590	1610
TAGATTATGAAATGATATTGAAAAAAATTGTAAATGGAAAGTGCTCGAGTGTGT euAspTyrGluAsnAspIleGluLysLysIleCysLysMetGluLysCysSerSerValP		
1630	1650	1670



TTAATGTCGTAAGTAGTCGACCTGTGACGAACATGGAGAACATCACATCAGGATTCCTAG
heAsnValValAsnSerArgProValThrAsnMetGluAsnIleThrSerGlyPheLeuG

1690

1710

1730

GACCCCTGCTCGTGTTACAGGCGGGGTTTTCTTGTTGACAAGAATCCTCACAATACCGC
lyProLeuLeuValLeuGlnAlaGlyPhePheLeuLeuThrArgIleLeuThrIleProG

1750

1770

1790

AGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTCTAGGGGGATCACCCGTGTGTCTTG
lnSerLeuAspSerTrpTrpThrSerLeuAsnPheLeuGlyGlySerProValCysLeuG

1810

1830

1850

GCCAAAATTGCGAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTC
lyGlnAsnSerGlnSerProThrSerAsnHisSerProThrSerCysProProIleCysP

1870

1890

1910

CTGGTTATCGCTGGATGTGTCTGCGGCGTTTATCATATTCCTCTTCATCCTGCTGCTAT
roGlyTyrArgTrpMetCysLeuArgArgPheIleIlePheLeuPheIleLeuLeuLeuC

1930

1950

1970

GCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCTCTAA
ysLeuIlePheLeuLeuValLeuLeuAspTyrGlnGlyMetLeuProValCysProLeuI

1990

2010

2030

TTCCAGGATCAACAACAACCAATACGGGACCATGCAAAACCTGCACGACTCCTGCTCAAG
leProGlySerThrThrThrAsnThrGlyProCysLysThrCysThrThrProAlaGlnG

2050

2070

2090

GCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCACCTGTA
lyAsnSerMetPheProSerCysCysCysThrLysProThrAspGlyAsnCysThrCysI

2110

2130

2150

TTCCCATCCCATCGTCCTGGGCTTTTCGCAAAATACCTATGGGAGTGGGCCCTCAGTCCGTT
leProIleProSerSerTrpAlaPheAlaLysTyrLeuTrpGluTrpAlaSerValArgP

2170

2190

2210

TCTCTGGCTCAGTTTACTAGTGCCATTTGTTTTCAGTGGTTCGTAGGGCTTTCCCCCACTG
heSerTrpLeuSerLeuLeuValProPheValGlnTrpPheValGlyLeuSerProThrV

2230

2250

2270

TTTGGCTTTTACGCTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCGTGA
alTrpLeuSerAlaIleTrpMetMetTrpTyrTrpGlyProSerLeuTyrSerIleValS

2290

2310

2330

GTCCCTTTATACCGCTGTTACCAATTTTCTTTTGTCTCTGGGTATACATTTAACGAATTC
erProPheIleProLeuLeuProIlePhePheCysLeuTrpValTyrIleEnd

2350

2370

2390

CAAGCTGAAACAATTCAAAGGTTTTCAAATCAATCAAGAAGTGTCTCTGTGGCTGATCC



2410 2430 2450
AAACTACAAATTTATGCATTGTCTGCCAAGACATCAAGAAGAAGTTAGTGATGATGTCTT

2470 2490 2510
TTATGGAGAGCATTCCATAGTCTTTGAAGAAGCAGAAAACAGATTATATGCAGCTATGTC

2530 2550 2570
TGCCATTGATATCTTTGTTAATAATAAAGGTAATTTCAAGGACTTGAAATAATCCTTCTT

2590 2610 2630
TCGTGTTCTTAATAACTAATATATAAATACAGATATAGATGCATGAATAATGATATACAT

2650 2670 2690
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2710 2730 2750
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2770 2790 2810
AAAAATTTAAATATACACTGCCAACAAATTCCTTCGAGTTGTCCAATTTACCACTTTTA

2830 2850 2870
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2890 2910 2930
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2950 2970 2990
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3010 3030 3050
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3070 3090 3110
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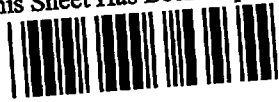
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3190 3210 3230
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3250 3270 3290
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3370

3390

3410

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3470

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3490

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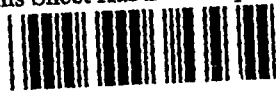
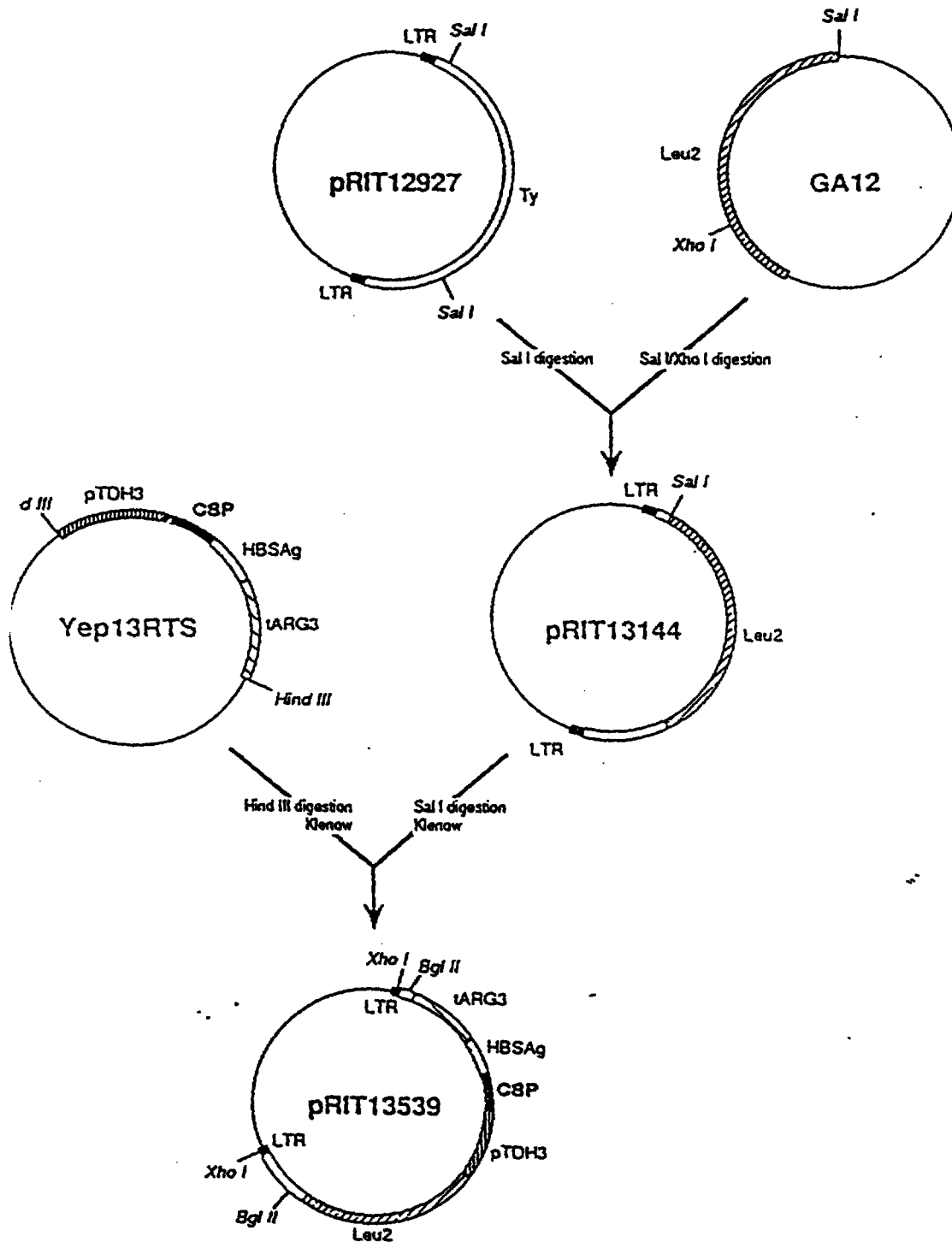


Figure 6: Construction of plasmid pRIT13539

Digestion with BglII endonuclease liberates a 6.8 Kb linear DNA fragment carrying the RTS expression cassette for integration into the yeast chromosome by homology of the free ends with resident Ty elements.

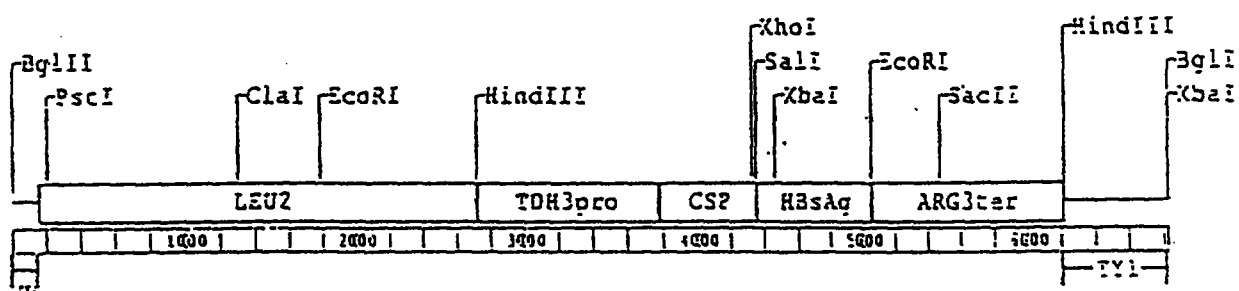


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Figure 7: Restriction map of the 6.8 Kb linear BglII fragment from pRIT13539
The linear fragment contains the LEU2 gene for selection of transformed yeast cells together with the RTS expression cassette.



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Figure 3

CsCl density analysis of a cell-free extract prepared from strain RIT4383

Fractions were analyzed by a radioimmunoassay specific for HBsAg (AUSRIA), and by an RTS-specific ELISA. The top panel shows the immunoblot analysis of fractions using an anti-S Mab. The buoyant density (ρ) of the peak fraction was calculated from a measure of its refractive index.

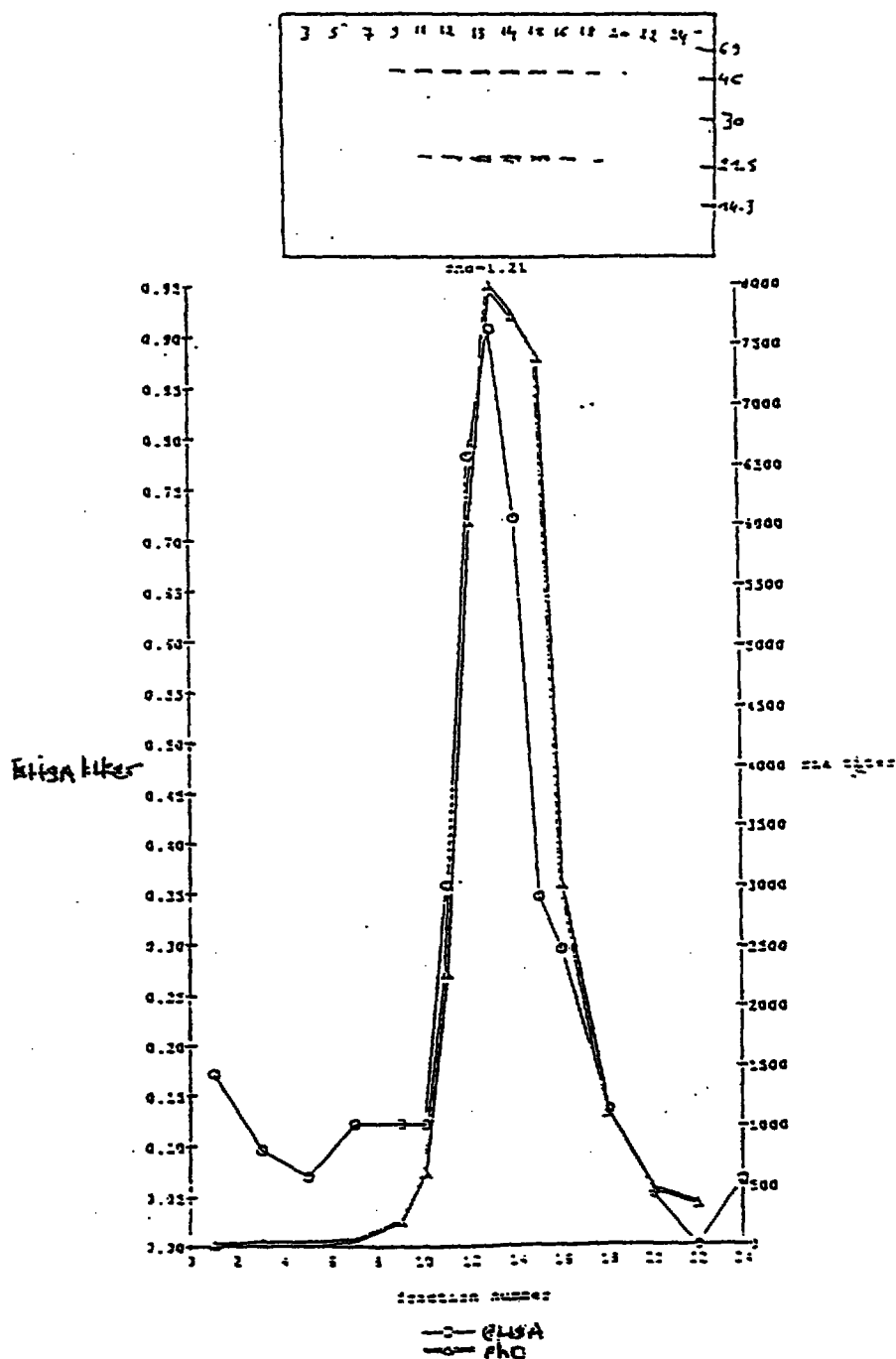


Figure 9: Nucleotide sequence of the RTS* expression cassette and predicted translation product of the hybrid protein.

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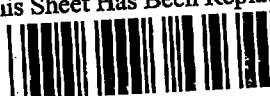
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70 90 110
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130 150 170
ACTTGATGCAAATTCCTAAAGCTAATAACATGCAAGACACGTACGGTCAAGAAGACATAT
190 210 230
TTGACCTCTTAAGTGGTTCAGACGCGACTGCCTCATCAGTAAGACCCGTTGAAAAGAACT
250 270 290
TACCTGAAAAAACGAATATATACTAGCGTTGAATGTTAGCGTCAACAACAAGAAGTTTA
310 330 350
ATGACGCGGAGGCCAAGGCCAAAAGATTCTTGATTACGTAAGGGAGTTAGAATCATTTT
370 390 410
GAATAAAAAACACGCTTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGA
430 450 470
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490 510 530
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550 570 590
ATCGTAGGTGTCTGGGTGAACAGTTTATCCCTGGCATCCACTAAATATAATGGAGCTCGC
610 630 650
TTTTAAGCTGGCATCCAGAAAAAAAAGAATCCCAGCACCAAAATATTGTTTTCTTCACC
670 690 710
AACCATCAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCACAAACAG
730 750 770
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790 810 830
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850 870 890
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1030	1050	1070
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1090	1110	1130
CAAATCCAAATGCAAACCCAAACGCAAACCCCAATGCAAATCCTAATGCAAACCCCAATG laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA		
1150	1170	1190
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1210	1230	1250
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1270	1290	1310
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1330	1350	1370
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1390	1410	1430
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1450	1470	1490
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1510	1530	1550
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1570	1590	1610
ATGATATTGAAAAAAAATTTGTAAATGGAAAAATGTTCCAGTGTGTTAATGTCGTAA snAspIleGluLysLysIleCysLysMetGluLysCysSerSerValPheAsnValValA		
1630	1650	1670
ATAGTTCAATAGGATTAGGGCCTGTGACGAACATGGAGAACATCACATCAGGATTCCTAG snSerSerIleGlyLeuGlyProValThrAsnMetGluAsnIleThrSerGlyPheLeuG		

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lyProLeuLeuValLeuGlnAlaGlyPhePheLeuLeuThrArgIleLeuThrIleProG

1750 1770 1790
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1810 1830 1850
GCCAAAATTGCGAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTC
lyGlnAsnSerGlnSerProThrSerAsnHisSerProThrSerCysProProIleCysP

1870 1890 1910
CTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTAT
roGlyTyrArgTrpMetCysLeuArgArgPheIleIlePheLeuPheIleLeuLeuLeuC

1930 1950 1970
GCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCTCTAA
ysLeuIlePheLeuLeuValLeuLeuAspTyrGlnGlyMetLeuProValCysProLeuI

1990 2010 2030
TTCCAGGATCAACAACAACCAATACGGGACCATGCAAAACCTGCACGACTCCTGCTCAAG
leProGlySerThrThrThrAsnThrGlyProCysLysThrCysThrThrProAlaGlnG

2050 2070 2090
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lyAsnSerMetPheProSerCysCysCysThrLysProThrAspGlyAsnCysThrCysI

2110 2130 2150
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2170 2190 2210
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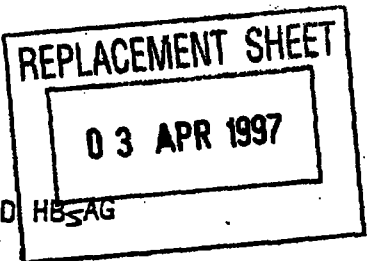
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2530 2550 2570
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2650 2670 2690
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2770 2790 2810
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2950 2970 2990
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3070 3090 3110
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3130 3150 3170
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3190 3210 3230
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3250 3270 3290
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3370 3390 3410
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3430 3450 3470
TAGGTTTGTAACTAAGCTCCTTCTGAACGCTGTCTCAATTTTCATCATCTTTCGGGATCT
3490
CTGGTACCAAAATTGGATAAGCTT

WVYI*

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HYBRID PROTEIN BETWEEN CS FROM PLASMODIUM AND HB SAG

The present invention relates to a novel hybrid protein, its use in medicine, particularly in the prevention of malaria infections and vaccines containing it.

Malaria, is one of the world's major health problems with 2 to 4 million people dying from the disease each year. One of the most acute forms of the disease is caused by the protozoan parasite, Plasmodium falciparum which is responsible for most of the mortality attributable to Malaria.

The life cycle of P. falciparum is complex, requiring two hosts, man and mosquito for completion. The infection of man is initiated by the inoculation of sporozoites in the saliva of an infected mosquito. The sporozoites migrate to the liver and there infect hepatocytes where they differentiate, via the exoerythrocytic intracellular stage, into the merozoite stage which infects red blood cells (RBC) to initiate cyclical replication in the asexual blood stage. The cycle is completed by the differentiation of a number of merozoites in the RBC into sexual stage gametocytes which are ingested by the mosquito, where they develop through a series of stages in the midgut to produce sporozoites which migrate to the salivary gland.

The sporozoite stage of P. falciparum has been identified as a potential target of a malaria vaccine. The major surface protein of the sporozoite is known as circumsporozoite protein (CS Protein). This protein from strain 7G8 has been cloned, expressed and sequenced (Dame et al Science 225 (1984) p593). The protein from strain 7G8 is characterised by having a central immunodominant repeat region comprising a tetrapeptide Asn-Ala-Asn-Pro repeated 37 times but interspersed with four minor repeats Asn-Val-Asp-Pro. In other strains the number of major and minor repeats vary as well as their relative position. This central portion is flanked by an N and C terminal portion composed of non-repetitive amino acid sequences designated as the repeatless portion of the CS protein.

It has been shown that irradiated sporozoites can provide significant protection against experimental human malaria (Am. J. Trop. Med. Hyg. 24: 297-402, 1975). However, production difficulties makes the use of

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irradiated sporozoite impractical from the point of view of producing a vaccine.

Several groups have proposed subunit vaccines based on the circumsporozoite protein. Two of these vaccines have undergone clinical testing; one is a synthetic peptide, the other is a recombinant protein (Ballou *et al* Lancet: i 1277 (1987) and Herrington *et al* Nature 328:257 (1987).

These vaccines were successful in stimulating an anti-sporozoite response. Nonetheless, the magnitude of the response was disappointing, with some vaccinees not making a response at all. Furthermore, the absence of "boosting" of antibody levels on subsequent injections and results of in vitro lymphocyte proliferation assays suggested that T-cells of most of these volunteers did not recognise the immuno-dominant repeat. Nonetheless, one vaccinee in each study did not develop parasitemia.

The present invention provides a new, improved antigen for use in malaria vaccines which not only produces a humoral response, but also a cellular immune response. Preferably the antigen induces the production of neutralising antibodies against the immunodominant repeat. Most preferably, the antigen should also elicit effector T cell mediated immune responses of the CD4⁺ and CD8⁺ cytotoxic T lymphocyte (CTL) type and of the delayed type hypersensitivity type and also, preferably be able to induce T helper (TH) memory cells.

Accordingly, the present invention provides a hybrid protein comprising substantially all the C-terminal portion of the CS protein, four or more tandem repeats of the immunodominant region, and the Surface antigen from Hepatitis B virus (HBsAg). Preferably the hybrid protein comprises a sequence which contains at least 160 amino acids which is substantially homologous to the C-terminal portion of the CS protein. The CS protein may be devoid of the last 12 amino-acids from the C terminal.

In particular there is provided a protein which comprises a portion of the CS protein of *P. falciparum* substantially as corresponding to amino acids 210-398 of *P. falciparum* 7G8 fused in frame via a linear linker to the N-terminal of HBsAg. The linker may comprise a portion of preS2 from



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HBsAg.

The present invention also provides DNA sequences encoding the proteins of the present invention.

A particularly preferred embodiment is the hybrid protein designated RTS. The amino acid sequence of RTS is shown in figure 5. This hybrid consists of:

- ° A methionine-residue, encoded by nucleotides 1059 to 1061, derived from the Sacchromyes cerevisiae TDH3 gene sequence. (Musti A.M. et al Gene 1983 25 133-143.
- ° Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.
- ° A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 210 to 398 of the circumsporozoite protein (CSP) of Plasmodium falciparum strain 7G8 (Dame et al supra).
- ° An amino acid (Arg) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.
- ° Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to 1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (9).
- ° A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype).

In an alternative embodiment there is provided a hybrid protein designated RTS* which was generated using the CSP gene sequence from P. falciparum NF54 (Mol. Biochem Parisitol. 35 : 185-190, 1989) and comprises substantially all of the region 207 to 395 of the CS protein from P falciparum NF54.

In particular RTS* comprises:

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- A Methionine, encoded by nucleotides 1059 to 1061, derived from the TDH3 gene sequence.
- Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.
- A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 207 to 395 of the circumsporozoite protein (CSP) of *Plasmodium falciparum* strain NF54 (Mol.Biochem.Parasitol, 35:185-190, 1989).
- An amino acid (Gly) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.
- Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to 1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (Nature 280:815-819, 1979).
- A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype) (Nature 280:815-819, 1979).

The amino acid sequence of RTS* is depicted in figure 9.

An expression cassette containing RTS* was constructed and comprises the following features:

- A promoter sequence, extending from nucleotide 1 through 1058, derived from the S.cerevisiae TDH3 gene.
- An open reading frame starting at nucleotide 1059 and extending to nucleotide 2330. This open reading frame is immediately followed by a translational stop codon, TAA (nucleotides 2331 to 2333). The open reading frame encodes the amino acids specifying the hybrid RTS* protein.

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- A transcription termination sequence contained within the sequence extending from base pair 2334 to 3504, derived from the S. cerevisiae ARG3 gene.

The nucleotide sequence is depicted in figure 10.

The DNA sequences encoding the proteins of the present invention are, in a preferred embodiment flanked by transcriptional control elements, preferably derived from yeast genes and incorporated into an expression vector.

Such vectors are a further aspect of the invention. A preferred promoter is the promoter from the S. cerevisiae TDH3 gene Musti *et al supra*).

The invention also relates to a host cell transformed with a vector according to the invention. Host cells can be prokaryotic or eukaryotic but preferably, are yeast, such as S. cerevisiae. In such a host, the hybrid protein, for example RTS will be expressed as lipoprotein particle. The chosen recipient yeast strain preferably already carries in its genome several integrated copies of an hepatitis B S expression cassette. The resulting strain synthesizes two polypeptides, S and RTS (or other hybrid protein of the invention), that spontaneously co-assemble into mixed (for example RTS, S or RTS*, S) lipoprotein particles. These particles, advantageously present the CSP sequences of the hybrid at their surface. These mixed particles also form part of the present invention. Advantageously the ratio of RTS: S or RTS* : S in these mixed particles is 1:4.

The present invention also relates to vaccines comprising an immunoprotective amount of a protein or particle according to the invention in admixture with a suitable diluent or carrier.

In the vaccine of the invention, an aqueous solution of the hybrid may be used directly. Alternatively, the protein with or without prior lyophilisation can be mixed or absorbed with any of the known adjuvants which include but are not limited to alum, muramyl dipeptide, saponins such as Quil A.

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An immunostimulant may alternatively or in addition be included. In a preferred embodiment this immunostimulant will be 3 Deacylated monophosphoryl lipid A (3D-MPL).

3 Deacylated monophosphoryl lipid A is known from US patent No. 4,912,094 and UK patent application No. 2,220,211 (Ribi) and is available from Ribi Immunochem, Montana, USA.

The protein of the present invention may also be encapsulated into microparticles such as liposomes.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A., 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877.

Conventional adjuvants may be used, but a preferred immunostimulant is 3-deacylated monophosphoryl lipid A (3D-MPL).

Typically when 3D-MPL is used the antigen and 3D-MPL are delivered with alum or presented in an oil in water emulsion or multiple oil in water emulsions. The incorporation of 3D-MPL is advantageous since it is a stimulator of effector T-cells responses.

Accordingly a preferred embodiment of the present invention provides a vaccine comprising a hybrid protein as herein described, preferably RTS, or RTS* in combination with 3D-MPL and a carrier. Typically the carrier will be an oil in water emulsion or alum.

In a most preferred embodiment the hybrid protein is presented as a particle or mixed particle as herein described.

The amount of the protein of the present invention present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected

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that each dose will comprise 1-1000 μ g of protein, preferably 1-200 μ g most preferably 10-100 μ g. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects will preferably receive a boost in about 4 weeks, followed by repeated boosts every six months for as long as a risk of infection exists. The immune response to the protein of this invention is enhanced by the use of adjuvant and or an immuno stimulant.

The proteins of the present invention are preferably expressed in yeast, and especially those belonging to the genus Saccharomyces.

A further aspect of the present invention is to provide a process for the preparation of hybrid protein of the invention, which process comprises expressing DNA sequence encoding the protein, in a suitable host, preferably a yeast, and recovering the product.

It is particularly preferred to express the protein of the invention in a Saccharomyces strain. When RTS, for example is expressed in such strains it spontaneously assembles into multimeric lipoprotein particles.

These particles are highly immunogenic and induce a strong humoral response, as well as immunological memory and also are capable of inducing effector T cells of the CTL and DTH types.

A further aspect of the invention lies in a method of treating a patient susceptible to plasmodium infections by administering an effective amount of a vaccine as hereinbefore described.

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Example 11. CONSTRUCTION OF THE RTS.S strain RIT4383

The S. cerevisiae strain, RIT4383 used for production of particles containing both the S and RTS polypeptides, carries separate expression cassettes for each protein. The S gene expression cassette has been integrated in 5 to 6 copies at least two sites in genome using a linear integration vector with homology to resident Ty retrotransposons. The RTS gene expression cassette has been integrated in 2 to 3 copies at one or 2 sites in the genome, using a linear integration vector similar to the one employed for the integration of the S gene cassette. Expression from both types of cassette is driven by a promoter derived from the yeast TDH3 gene.

1.1 CONSTRUCTION OF THE S EXPRESSION CASSETTE AND INTEGRATIVE VECTOR (pRIT13034)

The S gene expression cassette (Figure 1A) identical to that found in strain RIT4376 (1) and consists of a 1058 bp TDH3 promoter fragment, 681 bp of S gene sequence, a 6 bp spacer containing an EcoR1 linker and a 1165 bp fragment carrying the Arg3 transcription terminator. The S coding sequence was derived by subcloning from a complete genomic clone (pRIT10616) of a virus of (adw serotype).

The structure of the Ty vector, pRIT13034, used for integration of the S expression cassette into the yeast genome, is shown in Figure 2. The construction and use of this type of vector for integration of expression cassettes in the yeast genome is described in Jacobs et al. (2). pRIT13034 contains the S gene cassette, the URA3 gene and the CUP1 gene inserted within a copy of a Ty element cloned on pUC9. The URA3 gene and the CUP1 gene both provide selectable markers that allow to distinguish transformed yeast colonies from untransformed colonies. pUC9 is described by Vieira & Messing (3), the URA3 fragment is from pFL44 (F. Lacroute, CNRS, Strasbourg), the CUP1 fragment is from pCUP5 (T. Butt, SKF Labs, Philadelphia), the Ty element is from Juniaux et al. (4) and the S gene cassette from pRIT12353. Digestion of pRIT13034 with XhoI endonuclease liberates the 8500bp linear fragment shown in Figure 3

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which can integrate into the genome by homologous recombination of the free ends with resident Ty elements.

Example 2

2. CONSTRUCTION OF STRAIN Y1295

The recipient strain, EJ cup1D-3d (trp1, leu2, ura3, cup1 , gal1D, MAT α) was used for initial introduction by transformation of the linear vector fragment from pRIT13034. This strain contains a single disrupted cup1 locus, cup1 .

After transformation with the linear XhoI fragment, Ura⁺ colonies were isolated and screened for copper resistance. The more resistant transformants had integrated two to five copies of the vector as determined by Southern blotting analysis. Two transformant colonies were retained, MS9 with an estimated 3 to 4 copies and MS23 with 4 to 5 copies of the integrated linear vector. Strain MS23 was then crossed with strain EJ cup1D-7b (trp1, ura3, cup1 , gal1D, MAT α) and a haploid ascospore recovered to give strain MS23-2a.

This strain was then backcrossed to MS9 and a Leu⁻, Trp⁻ haploid segregant obtained (MS54-2c) containing 5 to 6 copies of the integrated expression cassette. Southern blotting showed that MS54-2C contained 4 to 5 tandem copies of the integration vector at one locus and a further single copy integrated at a different locus. All 5 to 6 copies of the expression cassette were intact as digestion of total yeast cell DNA with HindIII endonuclease to liberate the cassette fragment and Southern blotting with an S gene specific probe gave a single 3 kb band as expected. A spontaneous Trp⁺ revertant of this strain was obtained, MS54-2c-T, and given the laboratory accesssion number Y1295.

Example 3 CONSTRUCTION OF THE RTS-EXPRESSION CASSETTE

The expression cassette for the RTS hybrid protein was constructed by a multistep cloning procedure and was cloned in the E.coli yeast shuttle vector Yep13(6) yielding a plasmid Yep13RTS (Fig.4). The structure of the cassette is shown in figure 1B. Its entire nucleotide sequence was

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determined either by direct sequencing (as for the coding sequence and parts of the flanking control sequences) or by consultation of the relevant literature (as for parts of the promotor and terminator sequences). The DNA sequence is illustrated in figure 5. It contains the following elements:

A promotor sequence, extending from nucleotide 1 through 1058, derived from the S. cerevisiae TDH3 gene.

An open reading frame starting at nucleotide 1059 and extending to nucleotide 2330. This open reading frame is immediately followed by a translational stop codon, TAA (nucleotides 2331 to 2333). The open reading frame encodes the amino acids specifying the hybrid RTS protein.

A transcription termination sequence contained within the sequence extending from base pair 2334 to 3504, derived from the S. cerevisiae ARG3 gene (Crabeel et al EMBO J. 1983 2: 205-212).

The amino acid sequence of the hybrid RTS protein, encoded by nucleotides 1059 to 2330 is indicated in figure 5 and contains the following elements:

A methionine-residue, encoded by nucleotides 1059 to 1061, derived from the TDH3 gene sequence.

Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.

A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 210 to 398 of the circumsporozoite protein (CSP) of Plasmodium falciparum strain 7G8 (8).

An amino acid (Arg) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.

Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to

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1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (9).

A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype).

Example 4 CONSTRUCTION OF THE RTS CASSETTE INTEGRATIVE VECTOR pRIT13539

The Construction of the RTS Cassette integrative vector pRIT13539 is shown on figure 6.

The RTS expression cassette was inserted on the Ty based integrative vector pRIT13144. This vector is a derivative of pRIT12927 (2) in which the LEU2 gene was inserted in the SalI site of the Ty element as a SalI-XhoI fragment isolated from the vector pCV9 (7). Thus, after insertion of the RTS expression cassette into pRIT13144, the resulting plasmid, pRIT13539, contains, in addition to the expression cassette, the yeast LEU2 gene as selective marker (figure 5). Digestion of pRIT13539 with BglII endonuclease liberates a 6.800 bp linear fragment shown in figure 7 which can integrate into the genome by homologous recombination of the free ends with resident Ty elements.

Example 5 TRANSFORMATION OF STRAIN Y1295 AND GENERATION OF STRAIN RIT4383 (Y1530)

To obtain a strain expressing both S and RTS proteins, Y1295 was transformed with the 6800 bp linear BglII fragment (figure 7) with selection for Leu⁺ colonies. Several integrants containing sets of both expression cassettes present in the genome at various ratio were obtained. One selected transformant, expressing the RTS and S protein in a ratio of approximately 1:4 was given the official designation RIT4383 (the laboratory accession number is Y1530).

Example 5b Transformation of Strain Y1295 and Generation of Strain Y1631.

A similar (to RTS) construct was generated using the CSP gene sequence

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derived from P.falciparum strain NF54 (Mol.Biochem.Parasitol.35:185-190, 1989). The fusion protein obtained will be designated RTS* to distinguish it from the construct obtained with the CSP derived from P.falciparum strain 7G8.

The sequence of the expression cassette is shown in figure 9. It contains the following elements:

- A promoter sequence, extending from nucleotide 1 through 1058, derived from the S.cerevisiae TDH3 gene.
- An open reading frame starting at nucleotide 1059 and extending to nucleotide 2330. This open reading frame is immediately followed by a translational stop codon, TAA (nucleotides 2331 to 2333). The open reading frame encodes the amino acids specifying the hybrid RTS* protein.
- A transcription termination sequence contained within the sequence extending from base pair 2334 to 3504, derived from the S. cerevisiae ARG3 gene.

This expression cassette encoding this RTS* fusion protein was transformed and integrated into the genome of yeast strain Y1295, using the same approach as described previously for the RTS construct. Transformed clones expressing both the S and RTS* proteins were obtained. One clone was selected expressing the two proteins in a ratio of approximately 4S:1RTS*. The clone was given the laboratory accession number Y1631.

Example 6

PRELIMINARY CHARACTERIZATION OF THE STRAIN RIT4383

6.1 Analysis by immunoblotting

Cell free extracts prepared from RIT 4383 were analysed by immunoblot using various antibodies:

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- a monoclonal antibody directed toward the S protein (Mab HBS1)
- a monoclonal antibody directed toward the repeat part of the RTS protein (Mab 167)
- a rabbit serum directed toward the repeat-less sequences of the RTS protein (rabbit serum no. 20).

In yeast strain RIT4383, two expressed products were recognized by monoclonal antibody HBS1: a 24KD protein corresponding to the S protein and a 46KD RTS hybrid protein. The RTS hybrid protein is also detected by antibodies directed toward repeat and non-repeat epitopes of the CSP. These results indicate clearly that strain RIT4383 simultaneously expresses the two S and RTS antigens at a ratio RTS/S of approximatively 1:4.

6.2 CsCl density gradient centrifugation

The formation of particles in strain RIT4383 was analyzed by CsCl density gradient centrifugation. Crude extract ($\pm$ 15 mg of total protein) was analyzed on a 10 ml 1.5 M CsCl gradient (68 hours at 40.000 rpm, + 8°C in a Beckman 50 Ti rotor). Fractions (0.5ml) were collected and analyzed by a radioimmunoassay specific for HBsAg (AUSRIA), by an RTS specific ELISA and by immunoblot using an anti-HBsAg monoclonal antibody.

As shown in Figure 8, ELISA, RIA and Western blot peaks appear at the same fraction (no. 13) of the gradient corresponding to a buoyant density of $\rho = 1.21$ suggesting that mixed particles, containing both S and RTS monomers, are formed in this strain.

Example 7

PREPARATION OF THE SEED LOTS

Production procedure for master seed lot

Strain Y1530 (RIT4383) is first grown for 48 hours at 30°C in Petri dishes containing 20 ml sterile YNB (Difco) supplemented with

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dextrose (0.1%) and 1.8% (w/v) agar (Difco). The surface growth is suspended into sterile YNB broth (Difco) supplemented with dextrose (1%) and glycerol (10%). This suspension is distributed under aseptic conditions into 2 ml sterile polypropylene stoppered tubes (1 ml per tube) and stored at -70°C.

Production procedure for working seed lot

One master seed tube is rapidly thawed, and its contents are streaked with a platinum loop on Petri dishes prepared as described above. After incubation at 30°C for 63 hours, part of the surface growth is transferred to a 2 L conical flask containing 400 ml of sterile YNB broth (Difco) supplemented with dextrose (2%). The culture is incubated at 30°C for 24 hours before being centrifuged (15 min at 6300 x g) under aseptic conditions. The pellet is resuspended into sterile YNB broth (Difco) supplemented with dextrose (1%) and glycerol (10%). This is distributed under aseptic conditions into 2 ml sterile glass stoppered tubes (0.5 ml per tube) and stored at -70°C.

Example 8: FERMENTATION

Preparation of the inoculum

(a) Growth on solid medium

One vial of the working seed lot is rapidly thawed and spread onto Petri dishes containing sterile YNB broth (Difco) supplemented with dextrose (1%) and 1.8% (w/v) agar (Difco). The Petri dishes are incubated for 48 hours at 30°C.

(b) Growth of inoculum

The surface growth of one Petri dish is suspended in sterile YNB broth (Difco) supplemented with dextrose (2%) and distributed equally into two conical flasks (2 L, 400 ml liquid per flask). The flasks are incubated for 24 hours at 30°C on a rotary shaker.

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Fermentation

The fermentor vessel (20 L total volume) containing 5 L of dieionized water supplemented with $(\text{NH}_4)_2\text{SO}_4$ (40 g) is sterilized in-situ at 121°C for 30 minutes using clean, pre-filtered steam at 2 bar g pressure. After cooling to room temperature, the liquid volume in the fermentor is adjusted to 4L, and 1 L of filter-sterilized HB4 solution is added. The fermentation is begun by adding the inoculum (800 ml) from the two conical flasks.

The fermentation is run using the fed-batch technique whereby the culture is continuously fed with a solution of the following composition.

- 5 L HB4 solution;
- 4 L dextrose 80% (sterilized at 121°C).

The culture density increases by aerobic growth at 30°C and pH 5 (maintained by addition of NH_4OH). Dissolved oxygen is maintained about 50% saturation by adjustment of airflow and agitation speed. The rate of addition of the feed is predetermined to maximize growth rate and minimize formation of by-product ethanol.

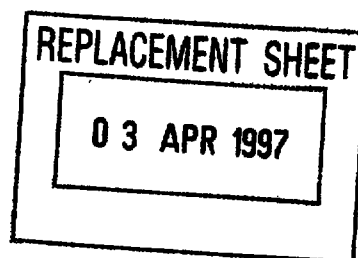
The fermentation is stopped after 40-90 hours. At the end of the fermentation, the total culture volume is 10-18 L, and the dry cell weight is between 30 and 100 g/L. The culture is rapidly cooled down to 15-25°C, and the yeast cells are recovered from the broth by centrifugation. The concentrated yeast cells are washed once with phosphate buffer (50 mM) before being re-centrifuged and subsequently stored at -70°C in polyethylene bags.

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HB4 Medium Composition

Component	Quantity
KH ₂ PO ₄	41.00 g
MgSO ₄ .7H ₂ O	23.50 g
CaCl ₂ .2H ₂ O	4.70 g
NaCl	0.30 g
FeCl ₃ .6H ₂ O	50.00 mg
H ₃ BO ₃	28.00 mg
MnSO ₄ .H ₂ O	22.40 mg
ZnSO ₄ .7H ₂ O	22.40 mg
Na ₂ MoO ₄ .2H ₂ O	11.20 mg
KI	5.60 mg
CaCl ₂ .6H ₂ O	5.10 mg
CuSO ₄ .5H ₂ O	2.24 mg
Biotin	2.70 mg
Folic acid	2.70 mg
Inositol	2.70 mg
Ca Pantothenate	0.54 mg
Pyridoxin.HCl	0.54 mg
Thiamin.HCl	0.54 mg
Niacin	1.20 mg
P-amino benzoic acid	0.60 mg
Riboflavin	0.60 mg
HCl (37°C)	5.00 ml
Deionized water (up to)	1.00 litre



Example 9: Extraction and Purification of RTS/S

EXTRACTION PROCEDURE

9.1 Preparation of cell suspension

Frozen concentrated yeast cells (at -70°C) are thawed down to -30°C overnight and subsequently thawed out to $5-15^{\circ}\text{C}$ by placing the polyethylene bags containing the cells in water ($10-20^{\circ}\text{C}$). A yeast cell suspension is made with a phosphate buffer solution (pH 8.0) containing the following ingredients : Ethylenediamine tetraacetic acid 9EDTA), pMethylsulfonyl Fluoride (PMSF), isopropanol and tween 20.

9.2 Cell disruption

Cells are disrupted in a bead mill containing lead-free glass beads (0.49-0.70 mm diameter). The grinding chamber is cooled by circulation of refrigerating fluid at -20°C , in such a way that the temperature of the homogenate at the outlet of the grinding chamber does not exceed 15°C . The liquid flowrate is 6 L/hour and the agitation speed is 3000 rpm. This process is performed twice. The pH of the resulting homogenate is 6.7 to 7.5.

9.3 Polyethyleneglycol clarification

Polyethyleneglycol 400 (PEG 400) is added to the disrupted cell suspension to give a final concentration of 10% (30 minutes below 10°C , pH 6.1) and a partially-clarified supernatant is recovered by centrifugation (J21B Beckman centrifuge, JA10 rotor at 17,000 g for 1 hour).

9.4 Methanol clarification

Methanol is added at pH 7 to the PEG-clarified antigen to give the proportion of 1 volume of methanol for 5 volumes of PEG-clarified antigen. The clarified antigen is obtained at 17,000 g for 20

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minutes by centrifugation (J21B Beckman centrifuge, JA10 rotor).

9.5 Adsorption/Desorption on colloidal silica

The crude antigen is adsorbed overnight onto 1.5% (w/v) colloidal silica (Aerosil 380, Degussa) at 4°C.

After washing (3 times) the pellet by successive centrifugation and resuspension in NaCl 0.9% (w/v), the antigen is desorbed using a 10mM pyrophosphate buffer, pH 9.5, containing 1% TWEEN 20.

The desorbing buffer volume corresponds to 1/8 of the methanol clarified antigen solution. The desorbed antigen solution is recovered by ultracentrifugation in a L 8.70 Beckman ultracentrifuge rotor R19 at 50,000 g for 60 minutes.

9.6 Diafiltration

Before the purification steps, the desorbed antigen is washed by ultrafiltration with 5 volumes of urea 6M, NaCl 500 mM, TRIS-HCl 20 mM at pH 8.5 in order to eliminate much of the proteic and lipidic contaminants.

Then the buffer is exchanged in the same system (Ultrasette, FILTRON fitted with polysulfone membranes with a 300 kD nominal cut-off) with 5 volumes of TRIS-HCl 10 mM (pH 8.1).

9.7 Ion exchange chromatography on DEAE-TSK 650 (M)

The clarified solution is applied to an anion-exchange column (DEAE-TSK 650 (M)) equilibrated in a 10 mM TRIS-buffer, pH 8.1. After washing successively with 2 volumes of 10 mM TRIS-HCl buffer pH 8.1 and 3 volumes of 10 mM TRIS-HCl buffer pH 8.1 supplemented with 40 mM NaCl, the antigen is desorbed with less than one volume of 10 mM TRIS-HCl buffer pH 8.1 containing 150 mM NaCl. The antigen-containing fractions are pooled.

9.8 Hydrophobic interaction chromatography on Butyl-TSK 650 (M)

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After NaCl addition up to a final concentration of 650 mM NaCl, the antigen solution is loaded on a Butyl-TSK 650 ~~mM~~ column equilibrated with a 20 mM TRIS-HCl buffer, 600 mM NaCl (pH 8.1). Most of the antigen passes through while ~~most~~ of the impurities bind to the gel.

9.9 Concentration by ultrafiltration

The HIC pool is concentrated by ultrafiltration in an Ultrasette system (FILTRON) fitted with polysulfone membranes with a 300 kDa nominal cut-off.

9.10 Ultracentrifugation in a CsCl gradient

CsCl is added to the Butyl-TSK pool to give a final concentration of 1.5 M.

After 65 hours in a 50.2 Ti Beckman rotor at 270,000 g, the antigen-containing fractions are collected.

9.11 Size exclusion chromatography on SEPHACRYL S300 (HR Type)

In order to exchange the buffer and to eliminate low molecular weight contaminants, the antigen solution is applied to a SEPHACRYL S300 HR column. The elution buffer is 10 mM phosphate, containing 150 mM NaCl (pH 7.4).

STERILE FILTRATION

After dilution to between 150 and 400 μ g Lowry/ml and pH adjustment to 6.8, the purified antigen is sterilized by filtration through a 0.22 μ m sterile filter. The resulting solution contains purified RTS/S particles.

Example 10: IMMUNOLOGICAL CHARACTERIZATION OF RTS/S

10.1 Antigenicity

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In order to test the antigenicity of the RTS/S particles, a number of ELISA's were performed, combining monoclonal antibodies directed against the different epitopes.

The monoclonal antibodies (MoAbs) used are :

- MoAb R10 :
 - specific for the repeat sequence (NANP) of the CSP region.
 - IgM isotype.
- MoAb RL117:
 - specific for the non-repeat sequence of the CSP region.
- MoAb RF1 :
 - specific for the S sequence of the HBsAg.
 - IgG1 isotype.

MoAbs R10 and RL117 were prepared in house by fusion of Sp2/OAg 14 myeloma cells with splenocytes of Balb/C mice immunized with partially pure RTS-like particles containing both repeat and non-repeat regions of the CSP sequence.

Three batches of the candidate RTS/S vaccine were analyzed : batches Nos. 24M31, 24M32 and 24M34.

10.2 Reaction with monoclonal antibody R10 (anti-repeat)

The anti-repeat MoAb R10 was used in a "sandwich" ELISA. Samples to be tested were incubated in microtiter plates previously coated with MoAb R10. The same MoAb coupled to peroxidase was then added. After one hour incubation at 37°C and washing, color was developed by addition of Orthophenylene-diamine-H₂O₂. Absorbance was measured at 490 nm and plotted versus the antigen concentration.

Results

The three batches : 24M31, 24M32 and 24M34 reacted positively and consistently with MoAb R10, thus confirming the presence and accessibility of the repeat epitopes on the RTS/S particles. The

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amount of antigen necessary to reach 50% of maximum binding was 51.2, 38.2 and 60.6 ng for batches 24M31, 24M31 and 24M31 respectively.

10.3 Reaction with monoclonal antibody RL117 (anti-repeatless)

The reactivity of MoAb RL117 with RTS,S particles was analyzed in a "sandwich" ELISA test. MoAb RL117 was coupled to peroxidase and used for the detection of the RTS/S particles, while MoAb R10, specific for the repeat region, was used for the capture of particles.

Briefly, samples to be tested were incubated in microtiter plates previously coated with MoAb R10. RL117 coupled to peroxidase was then added, and, after one hour incubation at 37°C and washing, the coloration was developed by addition of Orthophenylene-diamine-H₂O₂. Absorbance was measured at 490 nm and plotted versus the antigen concentration.

Results

The three batches : 24M31, 24M32 and 24M34 reacted positively and consistently with MoAbs RL117 and R10, thus confirming the presence and accessibility of repeat and non-repeat epitopes on the same RTS/S particles. The amount of antigen necessary to reach 50% of maximum binding was 169.2, 117.6 and 181.1 ng for batches 24M31, 24M32 and 24M34 respectively

10.4 Reaction with monoclonal antibody RF1 (anti-S)

The presence of S-epitopes in RTS/S particles was shown by a "sandwich" ELISA test, using MoAb RF1 coupled to peroxidase for detection. The R10 MoAb was used directly onto the microtiter plates in order to capture the RTS,S particles.

In summary, samples to be tested were incubated in microtiter plates previously coated with MoAb R10. MoAb RF1 coupled to peroxidase was then added. After incubation at 37°C for 1 hour and washing, color development was allowed by addition of

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Orthophenylene-diamine- H_2O_2 . Absorbance was measured at 490 nm and plotted versus the antigen concentration.

Results

The three batches : 24M31, 24M32 and 24M34 reacted positively and consistently with MoAbs RF1 and R10, thus confirming the presence and accessibility of the repeat and Sepitopes on the same RTS/S particles. The amount of antigen necessary to reach 50% of maximum binding was 52.3, 55.2 and 106.2 ng for batches 24M31, 24M32 and 24M34 respectively.

Example 11: IMMUNOGENICITY IN VIVO OF RTS/S PARTICLES

11.1 Immunogenicity studies

Studies on the immunogenicity of (RTS,S) particles were performed in mice and in *Cercopithecus aethiops* monkeys.

In mice, anti-CSP antibodies were analyzed by ELISA test using the R32tet32 antigen for the detection of the anti-repeat antibodies. R32tet32 consists of 32 copies of the NANP (major) repeat fused to a 32 amino acid portion of the tetracyclin resistance gene of plasmid pBR322. The recombinant antigen is produced in *Escherichia coli*. Antibodies (Abs) directed against the non repeated sequence of the CS protein were measured by ELISA test using the RLF antigen. The RLF antigen consists of the complete CS protein flanking region devoid of repeat fused to the first 81 amino acids of the NS1 protein of influenza virus. The RLF antigen is produced in *E. coli*. Mice sera were serially diluted, starting at 1:100, and titers are expressed as the reciprocal of the dilution corresponding to an optical density of 1.0 in the ELISA test (1). Measurements of anti-CSP Abs were performed on individual sera and the geometric mean titer (GMT) was calculated.

In order to analyze the anti-carrier response, anti-HBs antibody titers were also measured (pooled sera only).

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In mice experiments, Balb/C mice (H-2d haplotype) usually used for the immunogenicity of HBsAg were also used to evaluate the immunogenicity of RTS,S particles. The intraperitoneal (i.p.) and the subcutaneous (s.c.) routes of immunization were compared and the effect of the immunostimulant 3-deacylated monophosphoryl lipid A (3D-MPL) on the immune response was also tested.

The immunogenicity of the (RTS,S) vaccine was also tested in a similar way in *Cercopithecus aethiops* monkeys.

Selected individual or pooled sera were also tested for their capacity to inhibit the in vitro invasion of a human hepatoma cell line (HepG2) by *P. falciparum* sporozoites (ISI assay (2)).

11.2 Immunogenicity in mice

EXPERIMENT 1 : Immunogenicity of clinical (RTS,S) batches adsorbed on Al(OH)₃

Method

Immunization :

Groups of 10 Balb/C mice were injected twice intraperitoneally at one month interval with 1 µg of each of three (RTS,S) batches previously adsorbed on Al(OH)₃ (batches 24M/31, 24M/32 and 24M/34). Control mice were injected with HBsAg (Engerix-B, batch ENG611B4). On days 30 and 45, the mice were bled and the antibody titers were measured on individual sera.

Serological methods :

The anti-R32tet32 and the anti-RLF titers were measured by ELISA using respectively R32tet32 and RLF as coating antigens. The microplates were incubated with the serum sample (12 two-fold serial dilutions starting at 1:100) to be tested. Mouse Abs were reacted with biotinylated anti-mouse Ig followed by streptavidin : biotinylated horseradish peroxidase complex and orthophenylene-

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diamine/H₂O₂. The optical density was measured at 490 nm. The titers were expressed as the reciprocal of the dilution corresponding to an optical density of 1.0 (50 % maximal binding). For each group of mice, the geometric mean titer (GMT) was calculated. The anti-HBs antibody titer was calculated according to the Hollinger formula (3) and expressed in mIU/ml.

Results

Anti-CSP response :

Strong anti-R32tet32 and anti-RLF responses were observed for each (RTS,S) batch tested. No significant difference between the batches was noted. A remarkable booster effect was observed after the second dose. Mice immunized with HBsAg ("Engerix-B") were used as negative control in this experiment.

Anti-HBs response :

The (RTS,S) batches induce antibodies directed against the HBsAg carrier protein. The assay was performed on pooled sera only.

EXPERIMENT 2: Effect of the immunostimulant 3D-MPL on the immunogenicity of (RTS,S) particles in Balb/C mice.

We analyzed the effect of the 3D-MPL on the immunogenicity of the (RTS,S) vaccine in mice. Both the intraperitoneal (i.p.) and the subcutaneous (s.c.) routes of immunization were tested.

A. Immunization by the ip route

Method

Immunization:

Groups of 10 Balb/C mice were injected twice intraperitoneally at one month interval with 1 µg of (RTS,S) (batch 24M/34) adsorbed on Al(OH)₃ alone or on Al(OH)₃ + 3D-MPL (50 µg/dose). Control mice

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were injected with NaCl 150 mM. One days 30 and 45, the mice were bled and the antibody titers measured on individual sera.

Serological method:

The serological methods were the same as in the first experiment described above.

Results

Anti-CSP response:

The (RTS,S) vaccine batch induces strong anti-R32tet32 and anti-RLF responses in both formulations. In all cases, a significant booster effect is observed following the second immunization. Titers obtained with the formulation containing 3D-MPL are in all cases higher than the aluminium alone formulation, and a statistically significant increase was observed in the primary anti-R32tet32 response ($p = 0.02$). A group of mice injected with NaCl 150 mM was used as negative control in this experiment.

Anti-HBs response:

Both formulations of the (RTS,S) vaccine, injected by the i.p. route, induced a strong anti-HBs response. A significant booster effect was observed following second immunization with either formulation.

B. Immunization by the s.c. route

Method

Immunization:

Groups of 10 Balb/C mice were injected twice subcutaneously at one month interval with 1 μ g of (RTS,S), (batch 24M/34) adsorbed on Al(OH)₃ alone or on Al(OH)₃ + 3D-MPL (50 μ g/dose). Control mice were injected with NaCl 150 mM. One days 30 and 45, the

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mice were bled and the antibody titers measured on individual sera.

The serological methods were the same as in the first experiment described above.

Results

Anti-CSP response:

The (RTS,S) vaccine batch induced positive response against R32tet32 and RLF in both formulations. In all cases, a significant booster effect was observed following the second immunization. Statistically significant higher titers were observed with the 3D-MPL formulation on day 45, both for the anti-R32tet32 and anti-RLF responses ($p = 0.18$ and $p = 2.9$ respectively). In general, however, titers were lower than those obtained with the i.p. route. A group of mice injected with NaCl 150 mM was used as negative control in this experiment.

Anti-HBs response:

Both formulations of the (RTS,S) vaccine, injected by the s.c. route induced a good anti-HBs response. A significant booster effect was observed following second immunization with either formulation. As observed for the anti-CSP responses, the anti-HBs response was lower by this route of immunization as compared to the i.p. route.

11.3 Immunogenicity in Cercopithecus aethiops

The immunogenicity was evaluated in *Cercopithecus aethiops* monkeys with clinical batch 24M/32 adsorbed on Al(OH)₃.

Methods

Immunization:

Five monkeys were injected intramuscularly on days 0, 28 and 84

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with 20 µg of (RTS,S) particles adsorbed on Al(OH)₃ (0.5 mg Al+++). The animals were bled on days 0, 14, 28, 42, 56, 66 and 98. Antibodies directed against R32tet32, RLF and HBs antigens were measured.

Serological methods:

A anti-R32tet32 and the anti-RLF antibody titers were measured by ELISA using respectively the R32tet32 and the RLF antigens coated on microplates. The plates were then incubated with the serum sample to be tested (12 two-fold serial dilutions starting at 1:10). Monkey antibodies were detected by biotinylated anti-human Ig followed by streptavidin biotinylated horseradish peroxidase complex and orthophenylenediamine/H₂O₂. The optical density was measured at 490 nm. The titers were expressed as the reciprocal of the dilution corresponding to an optical density of 1.0 (50% maximal binding). For each group, the geometric mean titer (GMT) was calculated. The anti-HBs antibody titers were calculated according to the Hollinger formula (Hollinger et al., 1982) and expressed in mIU/ml.

Results

Anti-CSP response:

The (RTS,S) vaccine induced a positive response against both R32tet32 and RLF antigens in all 5 monkeys. A significant booster effect was observed 14 days following second immunization (day 42). A slow decrease of antibody titers was then observed up to the third immunization. The titers again increase 14 days following the third immunization (day 98), except in the case of the anti-RLF response of monkey Jo 352.

The anti-R32tet32 titers reached after the third immunization (day 98) are not however higher than those observed after the second immunization (day 42).

In the case of the anti-RLF response (with the exception of monkey

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JO 353), an increase of titers is observed following the third immunization (day 98) relative to post second immunization levels (day 42).

Anti-HBs response:

All monkeys raised an anti-HBs response with significant booster effects following second (day 42) as well as third (day 98) immunization.

Biological activity of antibodies raised against the (RTS,S) particle

As a measure of the biological function of the antibodies induced by the (RTS,S) vaccine, pooled mice and individual monkeys sera were tested by the Inhibition of Sporozoite Invasion (ISI) assay (Hollingdale et al., 1984). This assay measured the capacity of the anti-CSP antibodies to inhibit the in vitro invasion of a human hepatoma cell line (HepG2) by P. falciparum sporozoites.

Results

The results of this experiment are presented in Tables 1 and 2. The ISI data are expressed as % inhibition relative to the activity of a pre-immune control serum taken as 0% inhibition. For reference, the anti-R32tet32 and RLF antibody titers of the tested sera are included. Table 1 shows that all mice sera tested have very high ISI activity. Table 2 shows that all 5 monkeys have also a very ISI activity on day 98 compared with the corresponding immune serum.

Conclusion

The (RTS,S) particles induced, both in mice and monkeys, a high antibody response directed against the repeat and non-repeat epitopes of the CSP and against the S protein of the HBsAg carrier.

The primary antibody response in mice was enhanced by the presence of 3D-MPL.

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Antibody titers obtained after intraperitoneal injection ~~were~~ higher than those obtained after immunization by the subcutaneous route.

The antibodies elicited in the two animal species effectively prevented invasion of cultured human hepatoma cells by P. falciparum sporozoites.

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Table 1: ISI activity of sera from Balb/C mice immunized with TS.S

Pool of Mice sera	A-R32tet32 Titer	A-RLF Titer	ISI (%)
Pool i.p A1+3D-MPL Day 45	606544	242408	100%
Pool i.p Alum only Day 45	87005	160284	98%
Pool i.p A1+3D-MPL Day 45	15333	99002	94%
Pool s.c A1+3D-MPL Day 45	5102	20453	86%
Pool of Negative Controls	205	205	0%

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Table 2: ISI activity of individual monkey sera immunized with RTS,S

Monkey #	Serum	A-R32 ^{ten} 32 titer	A-RLF titer	ISI (%)
J0 352	DAY 0	<50	<50	0%
	DAY 98	1762	1250	98%
J0 353	DAY 0	<50	<50	0%
	DAY 98	1574	32218	90%
J0 354	DAY 0	<50	<50	0%
	DAY 98	3751	31034	98%
J0 356	DAY 0	<50	<50	0%
	DAY 98	1495	18544	95%
J0 357	DAY 0	<50	<50	0%
	DAY 98	1420	30727	98%

Reference

- (1) Harford N, Cabezon T, Colau B, et al., "Construction and Characterization of a *Saccharomyces Cerevisiae* Strain (RIT4376) Expressing Hepatitis B Surface Antigen", Postgrad Med J 63, Supp. 2: 65-70, 1987.
- (2) Jacobs E, Rutgers T, Voet P, et al., "Simultaneous Synthesis and Assembly of Various Hepatitis B Surface Proteins in *Saccharomyces cerevisiae*", Gene 80: 279-291, 1989.
- (3) Vieira J and Messing J, "The pUC plasmids, an M13mp7-Derived System for Insertion Mutagenesis and Sequencing with Synthetic Universal Primers", *_Gene 19: 259-268, 1982.
- (4) Juniaux Embo J 1: 1125-1131, 1982.
- (5) Hinnen A, Hicks JB, and Fink GR, "Transformation of Yeast", Proc Natl Acad Sci USA 75: 1929-1933, 1980.
- (6) Broach JR, Strathern JN, and Hicks JB, "Transformation in Yeast Development of a Hybrid Cloning Vector and Isolation of the CAN 1 Gene", Gene 8: 121-133, 1979.
- (7) Zhang H, et al., "Double Stranded SDNA Sequencing as a Choice for DNA Sequencing", Nucleic Acids Research 16: 1220, 1988.
- (8) Dame JB, Williams JL. Mc Cutchan TF, et al., "Structure of the Gene Encoding the Immunodominant Surface Antigen on the Sporozoites of the Human Malaria Parasite *Plasmodium falciparum*", Science 225: 593-599, 1984.
- (9) Valenzuela P, Gray P, Quiroga M, et al., "Nucleotide Sequences of the Gene Coding for the Major Protein of Hepatitis B Virus Surface Antigen", Nature 280: 815-819, 1979.



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Claims

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1. A hybrid protein comprising substantially all the C-terminal portion of the CS protein of Plasmodium, four or more tandem repeats of the CS protein immunodominant region, and the surface antigen from Hepatitis B Virus (HBsAg).
2. A hybrid protein comprising a sequence of CS protein of P.falciparum substantially as corresponding to amino acids 210-398 of P.falciparum 7G8 CS protein or 207-395 of P. falciparum NF54 CS protein fused in frame via a linear linker to the N-terminal of HBsAg.
3. A hybrid protein as claimed in claim 1 or 2 wherein the CS protein is fused to HBsAg.
4. A hybrid protein comprising the following amino acid sequences:
 - a) an N-terminal methionine residue
 - b) Met Ala Pro
 - c) a stretch of 189 amino acids corresponding to amino acids 210 to 398 of CS protein P.falciparum 7G8 or 207-395 of CS protein P. falciparum NF54
 - d) Arg
 - e) Pro Val Thr Asn from hepatitis B Pre S₂ protein.
 - f) a stretch of 226 amino acids specifying the S protein of hepatitis B virus
6. The hybrid protein designated RTS.
7. The hybrid protein designated RTS*.
8. A DNA sequence encoding a hybrid protein as claimed in claims 1 to 7.
9. A vector containing a DNA sequence as claimed in claim 8 said sequence being linked to transcriptional control elements.
10. A host transformed with a vector of claim 9.

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11. A host as claimed in claim 10 wherein the host is S. cerevisiae
12. A host as claimed in claim 11 additionally transformed with a gene encoding the Hepatitis B surface antigen.
13. A multimeric lipoprotein particle comprising a hybrid protein of claims 1 to 7.
14. A mixed multimeric lipoprotein particle comprising a hybrid protein of claims 1 to 7 and Hepatitis B surface antigen.
15. A mixed multimeric lipoprotein of claim 14 comprising RTS, or RTS* and the surface antigen of Hepatitis B virus.
16. A particle as claimed in claim 14 or 15 wherein the ratio of hybrid protein to surface antigen is 1:4.
17. A vaccine comprising an immunoprotective amount of a particle or protein according to any of claims 1 to 7 or claims 13 to 16 in admixture with a suitable diluent or carrier.
18. A vaccine as claimed in claim 17 additionally comprising 3-Deacylated monophosphoryl lipid A.
19. A vaccine as claimed in claim 18 additionally comprising alum.
20. A vaccine as claimed in claim 18 presented in an oil in water emulsion.
21. A method of treating a patient susceptible to plasmodium infections comprising administering an effective amount of vaccine according to any of claims 17 to 20.
21. A process for the production of a hybrid protein according to any of claims 1 to 7 which process comprises expressing a DNA sequence encoding the protein in a suitable host and recovering the product.

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22. A process for the production of particles as claimed in ~~claims 12 to~~
15 comprising expressing a DNA sequence encoding a protein of claims 1
to 7 and hepatitis B surface antigen in a *Sacchromyces* strain.

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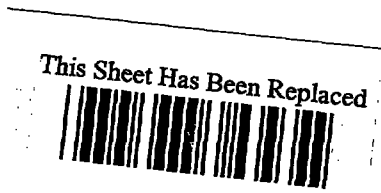
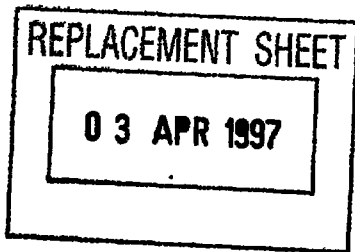
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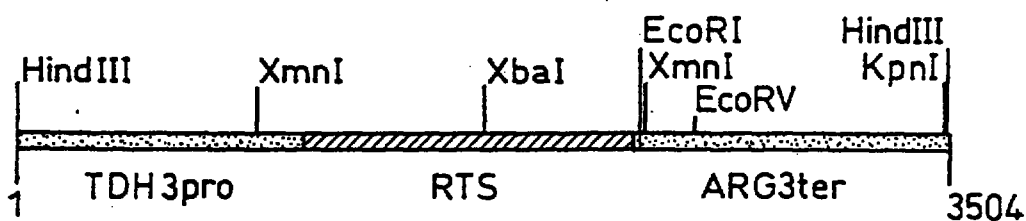
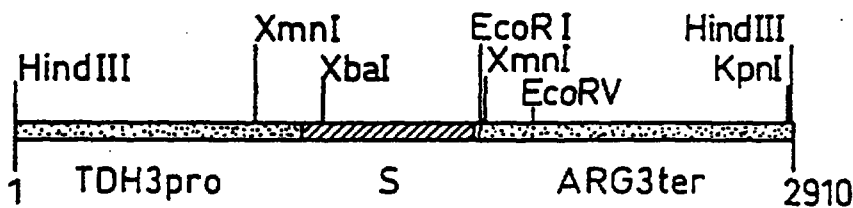
HYBRID PROTEIN BETWEEN CS FROM PLASMODIUM AND HB_sAGABSTRACT

A novel hybrid protein is provided which comprises a portion of the CS protein of *P. falciparum* and the surface antigen of Hepatitis B virus. The use of this protein for vaccination purposes is disclosed.

FIGURE 7



Restriction endonuclease map of (A) the S expression cassette and (B) the RTS expression cassette.
The extend of each coding sequence is indicated by the black bar.

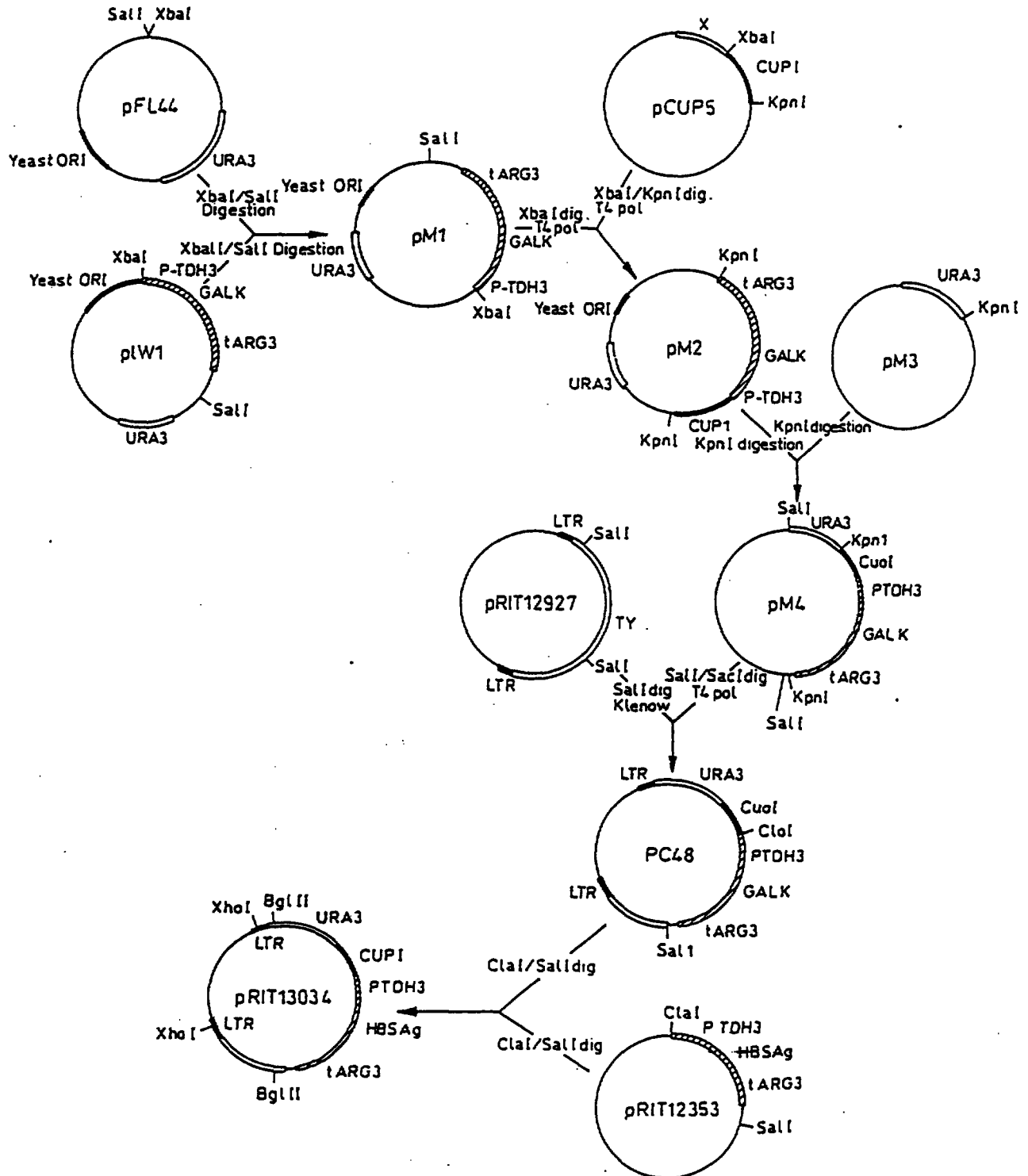


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REPLACED**Construction of plasmid pRIT13034**

Digestion with XhoI endonuclease liberates a 8.5 Kb linear DNA fragment carrying the S expression cassette for integration into the yeast chromosome by homology of the free ends with resident Ty elements.

**Fig.2**

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Restriction map of the 8.5 Kb linear XhoI fragment from pRIT13034
The linear fragment contains the URA3 and CUP1 genes for selection of transformed yeast cells together with the S expression cassette.

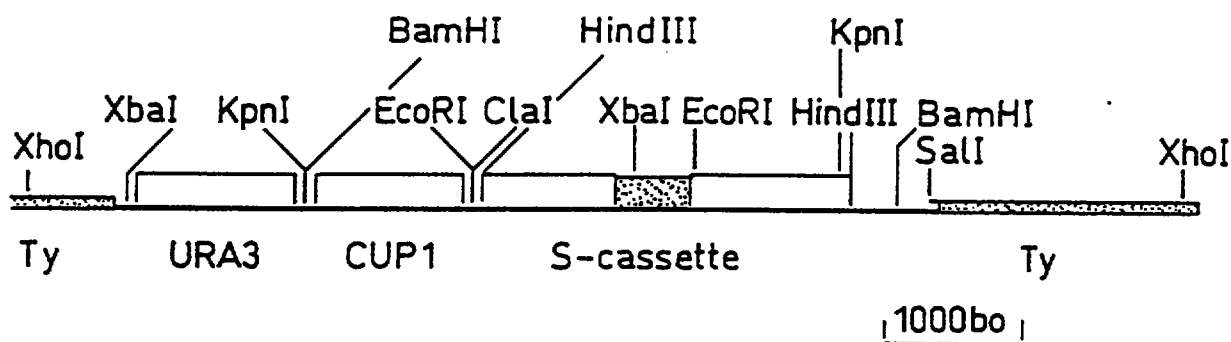
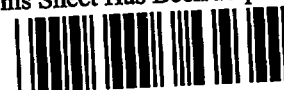


Fig.3

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Construction of the RTS expression cassette and its cloning in plasmid Yep13.

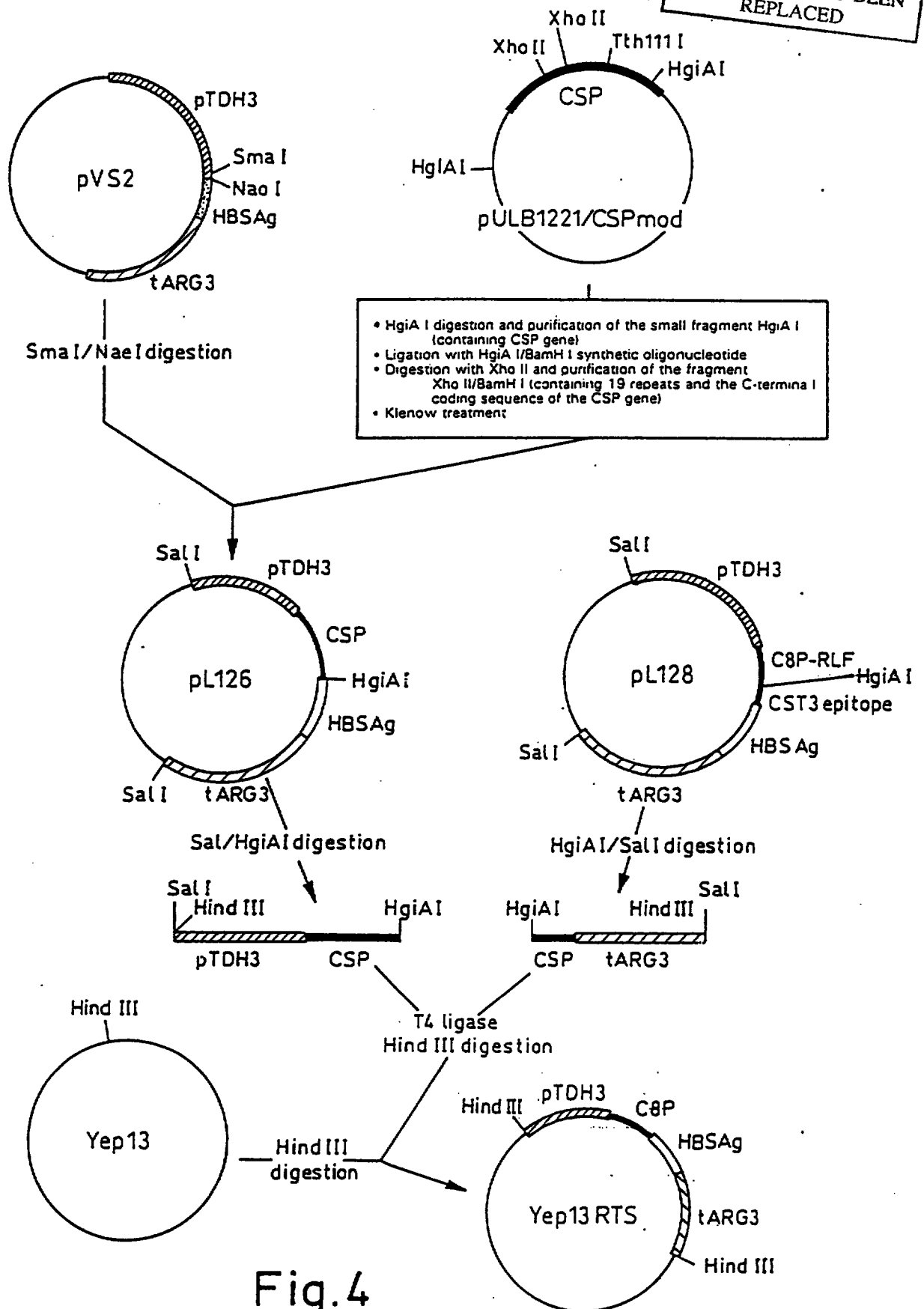
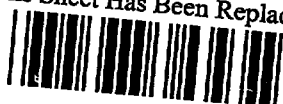


Fig.4

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Figure 5: Nucleotide sequence of the RTS expression cassette and predicted translation product of the RTS-HBsAg hybrid protein. The translation product initiated from the TDH3 ATG codon is shown below the DNA sequence.

```

      10              30              50
AAGCTTACCAGTTCTCACACGGAACACCACTAATGGACACAAATTCGAAATACTTTGACC

      70              90              110
CTATTTTCGAGGACCTTGTCACCTTGAGCCCAAGAGAGCCAAGATTTAAATTTTCCTATG

     130              150              170
ACTTGATGCAATTCCTCAAAGCTAATAACATGCAAGACACGTACGGTCAAGAAGACATAT

     190              210              230
TTGACCTCTTAACTGGTTCAGACGCGACTGCCTCATCAGTAAGACCCGTTGAAAAGAACT

     250              270              290
TACCTGAAAAAACGAATATATACTAGCGTTGAATGTTAGCGTCAACAACAAGAAGTTTA

     310              330              350
ATGACGCGGAGGCCAAGGCAAAAAGATTCCCTTGATTACGTAAGGGAGTTAGAATCATTTT

     370              390              410
GAATAAAAAACACGCTTTTTCAGTTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGA

     430              450              470
ATACGTAAATAATTAATAGTAGTGATTTTCCTAACTTTATTTAGTCAAAAATTAGCCTTT

     490              510              530
TAATTCTGCTGTAACCCGTACATGCCCAAATAGGGGGCGGGTTACACAGAATATATAAC

     550              570              590
ATCGTAGGTGTCTGGGTGAACAGTTTATCCCTGGCATCCACTAAATATAATGGAGCTCGC

     610              630              650
TTTTAAGCTGGCATCCAGAAAAAAAAGAATCCCAGCACCAAATATTGTTTTCTTCACC

     670              690              710
AACCATCAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCACAAACAG

     730              750              770
GCAAAAAACGGGCACAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGATGACAC

     790              810              830
AAGGCAATTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTACCTTCTGC

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890
TCTCTCTGATTGGAAGCTGAAAAAAGGTTGAAACCAGTTCCTGAAATTATCC

910 930 950
CCTACTTGACTAATAAGTATATAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTAT

970 990 1010
TTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAACACCA

1030 1050 1070
AGAACTTAGTTTCGAATAAACACACATAAACAAACAAATGATGGCTCCCGATCCTAATG
MetMetAlaProAspProAsnA

1090 1110 1130
CAAATCCAAATGCAAACCCAAATGCAAACCCAAACGCAAACCCCAATGCAAATCCTAATG
laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA

1150 1170 1190
CAAACCCCAATGCAAATCCTAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATG
laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA

1210 1230 1250
CAAACCCAAACGCAAACCCCAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATG
laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA

1270 1290 1310
CAAACCCCAATGCAAACCCCAATGCAAACCCCAATGCAAATCCTAATAAAACAATCAAG
laAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnLysAsnGlnG

1330 1350 1370
GTAATGGACAAGGTCACAATATGCCAAATGACCCAAACCGAAATGTAGATGAAAATGCTA
lyAsnGlyGlnGlyHisAsnMetProAsnAspProAsnArgAsnValAspGluAsnAlaA

1390 1410 1430
ATGCCAACAAATGCTGTAAAAAATAATAATAACGAAGAACCAAGTGATAAGCACATAGAAC
snAlaAsnAsnAlaValLysAsnAsnAsnAsnGluGluProSerAspLysHisIleGluG

1450 1470 1490
AATATTTAAAGAAAATAAAAAATTCTATTTCAACTGAATGGTCCCATGTAGTGTAATT
lnTyrLeuLysLysIleLysAsnSerIleSerThrGluTrpSerProCysSerValThrC

1510 1530 1550
GTGGAAATGGTATTCAAGTTAGAATAAAGCCTGGCTCTGCTAATAAACCTAAAGACGAAT
ysGlyAsnGlyIleGlnValArgIleLysProGlySerAlaAsnLysProLysAspGluL

1570 1590 1610
TAGATTATGAAATGATATTGAAAAAAATTTGTAAATGGAAAAGTGCTCGAGTGTGT
euAspTyrGluAsnAspIleGluLysLysIleCysLysMetGluLysCysSerSerValP

1630 1650 1670

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TTAATGTCGTAAATAGTCGACCTGTGACGAACATGGAGAACATCACATCAGGATTCCTAG
heAsnValValAsnSerArgProValThrAsnMetGluAsnIleThrSerGlyPheLeuG

1690

1710

1730

GACCCCTGCTCGTGTTACAGGCGGGGTTTTCTTGTTGACAAGAATCCTCACAATACCGC
lyProLeuLeuValLeuGlnAlaGlyPhePheLeuLeuThrArgIleLeuThrIleProG

1750

1770

1790

AGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTCTAGGGGGATCACCCGTGTGTCTTG
lnSerLeuAspSerTrpTrpThrSerLeuAsnPheLeuGlyGlySerProValCysLeuG

1810

1830

1850

GCCAAAATTTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCTCCAATTTGTG
lyGlnAsnSerGlnSerProThrSerAsnHisSerProThrSerCysProProIleCysP

1870

1890

1910

CTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCTCTGCTGCTAT
roGlyTyrArgTrpMetCysLeuArgArgPheIleIlePheLeuPheIleLeuLeuLeuC

1930

1950

1970

GCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCTCTAA
ysLeuIlePheLeuLeuValLeuLeuAspTyrGlnGlyMetLeuProValCysProLeuI

1990

2010

2030

TTCCAGGATCAACAACAACCAATACGGGACCATGCAAAACCTGCACGACTCCTGCTCAAG
leProGlySerThrThrThrAsnThrGlyProCysLysThrCysThrThrProAlaGlnG

2050

2070

2090

GCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCACCTGTA
lyAsnSerMetPheProSerCysCysCysThrLysProThrAspGlyAsnCysThrCysI

2110

2130

2150

TTCCCATCCCATCGTCCTGGGCTTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTT
leProIleProSerSerTrpAlaPheAlaLysTyrLeuTrpGluTrpAlaSerValArgP

2170

2190

2210

TCTCTTGGCTCAGTTTACTAGTGCCATTTGTTTCAGTGGTTCGTAGGGCTTTCCCCACTG
heSerTrpLeuSerLeuLeuValProPheValGlnTrpPheValGlyLeuSerProThrV

2230

2250

2270

TTTGGCTTTTTCAGCTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCGTGA
alTrpLeuSerAlaIleTrpMetMetTrpTyrTrpGlyProSerLeuTyrSerIleValS

2290

2310

2330

GTCCCTTTTATACCGCTGTTACCAATTTTCTTTTGTCTCTGGGTATACATTTAACGAATTC
erProPheIleProLeuLeuProIlePhePheCysLeuTrpValTyrIleEnd

2350

2370

2390

CAAGCTGAAACAATTCAAAGGTTTTCAAATCAATCAAGAACTTGTCTCTGTGGCTGATCC

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2410 2430 2450
AAACTACAAATTTATGCATTGTCTGCCAAGACATCAAGAAGAAGTTAGTGATGATGTCTT

2470 2490 2510
TTATGGAGAGCATTCCATAGTCTTTGAAGAAGCAGAAAACAGATTATATGCAGCTATGTC

2530 2550 2570
TGCCATTGATATCTTTGTTAATAATAAAGGTAATTTCAAGGACTTGAAATAATCCTTCTT

2590 2610 2630
TCGTGTTCTTAATAACTAATATATAAATACAGATATAGATGCATGAATAATGATATACAT

2650 2670 2690
TGATTATTTTGCAATGTCAATTAAAAAATGTTAGTAAACTATGTTACATTCCA

2710 2730 2750
AGCAAATAAAGCACTTGGTTAAACGAAATTAACGTTTTTAAGACAGCCAGACCGGGTCT

2770 2790 2810
AAAAATTTAAATATACACTGCCAACAAATTCCTTCGAGTTGTCCAATTTACCACCTTTTA

2830 2850 2870
TATTTTCATCAACTTCAGCAGATTCAACCTTCTCACATAGAACATTGGAATAAACAGCCT

2890 2910 2930
TAACACCACCTTTCAAGTTTGACAGCGTAATATGAGGAATTTGTTTTGACAACACAACC

2950 2970 2990
CTTTAATTTTCTCATTGTTTTTCATCAATTATGCATCCATCTTTATCTTTAGACAGTTCCA

3010 3030 3050
CTACAATAGCAATAGTTTTTTCATCCCAACATAGTTTTTCGAGCCTAAAATTCAGTTTGT

3070 3090 3110
CGGTCGTTTTTACCTGCGTATTTTGGTTATTACCAGAGCCTTGTGCATTTTCTATGCGGT

3130 3150 3170
TGTTATTGTACTCCGTTATCTGGTCAGTGTATCTGTTACAATATGATTCCACAACCTTTTT

3190 3210 3230
TGCTCTTTTTTCACGGGACGACATGACATGACCTAATGTTATATGAAGTTCCTTCTGAAC

3250 3270 3290
TTTTCCACTAGCTAGTAAATGCTTGAATTTCTCAGTCAGCTCTGCATCGCTAGCAATACA

3310 3330 3350
CCTCTTGACCAATTCAATAATTTTCATCGTAGTTTTCTATTTAGCTGAGATATATGTAGGT

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3370

3390

3410

TTAATTAACTTAGCGTTTTTTGTTGATTATTGTTGCCTTTACCAACTATTTTCTCACAG

3430

3450

3470

TAGGTTTGTAATCTAAGCTCCTTCTGAACGCTGTCTCAATTTTCATCATCTTTCGGGATCT

3490

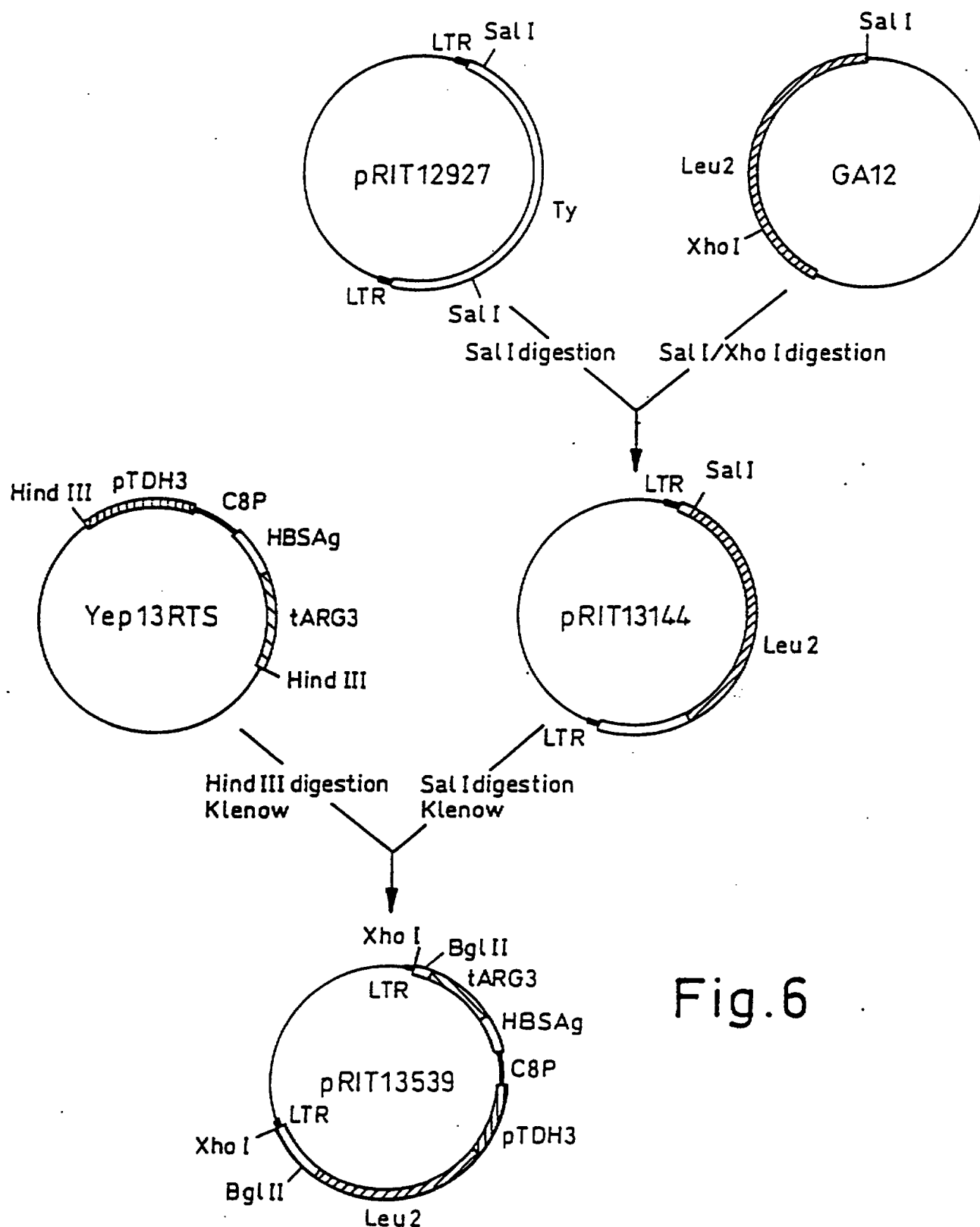
CTGGTACCAAATTGGATAAGCTT



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Construction of plasmid pRIT13539

Digestion with BglII endonuclease liberates a 6.8 Kb linear DNA fragment carrying the RTS expression cassette for integration into the yeast chromosome by homology of the free ends with resident Ty elements.

**Fig.6**

Restriction map of the 6.8 Kb linear BglII fragment from pRIT13539
 The linear fragment contains the LEU2 gene for selection of transformed yeast
 cells together with the RTS expression cassette.

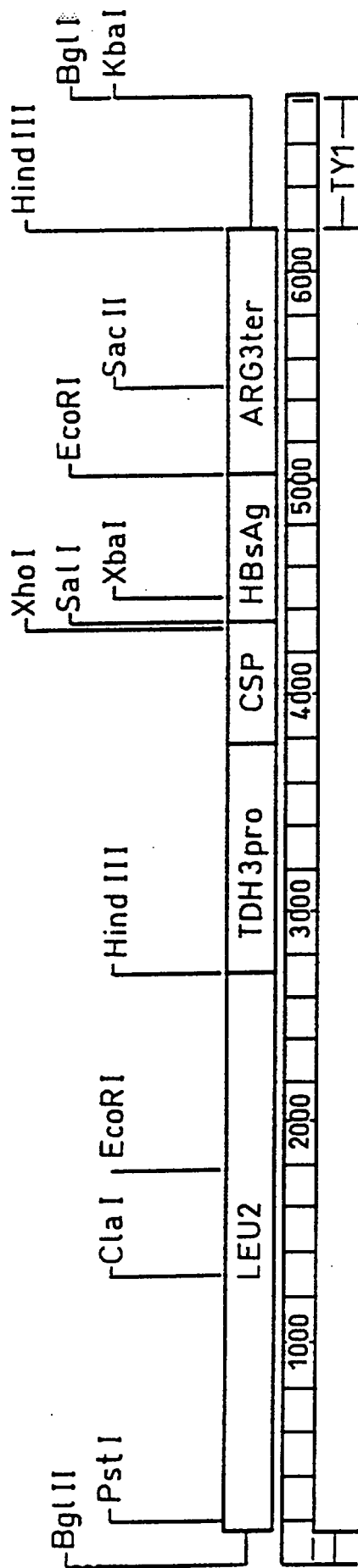


Fig. 7

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Cscl density analysis of a cell-free extract prepared from strain RIT4383. Fractions were analyzed by a radioimmunoassay specific for HBsAg (AUSRIA) and by an RTS-specific ELISA. The top panel shows the immunoblot analysis of fractions using an anti-S Mab. The buoyant density (ρ) of the peak fraction was calculated from a measure of its refractive index.

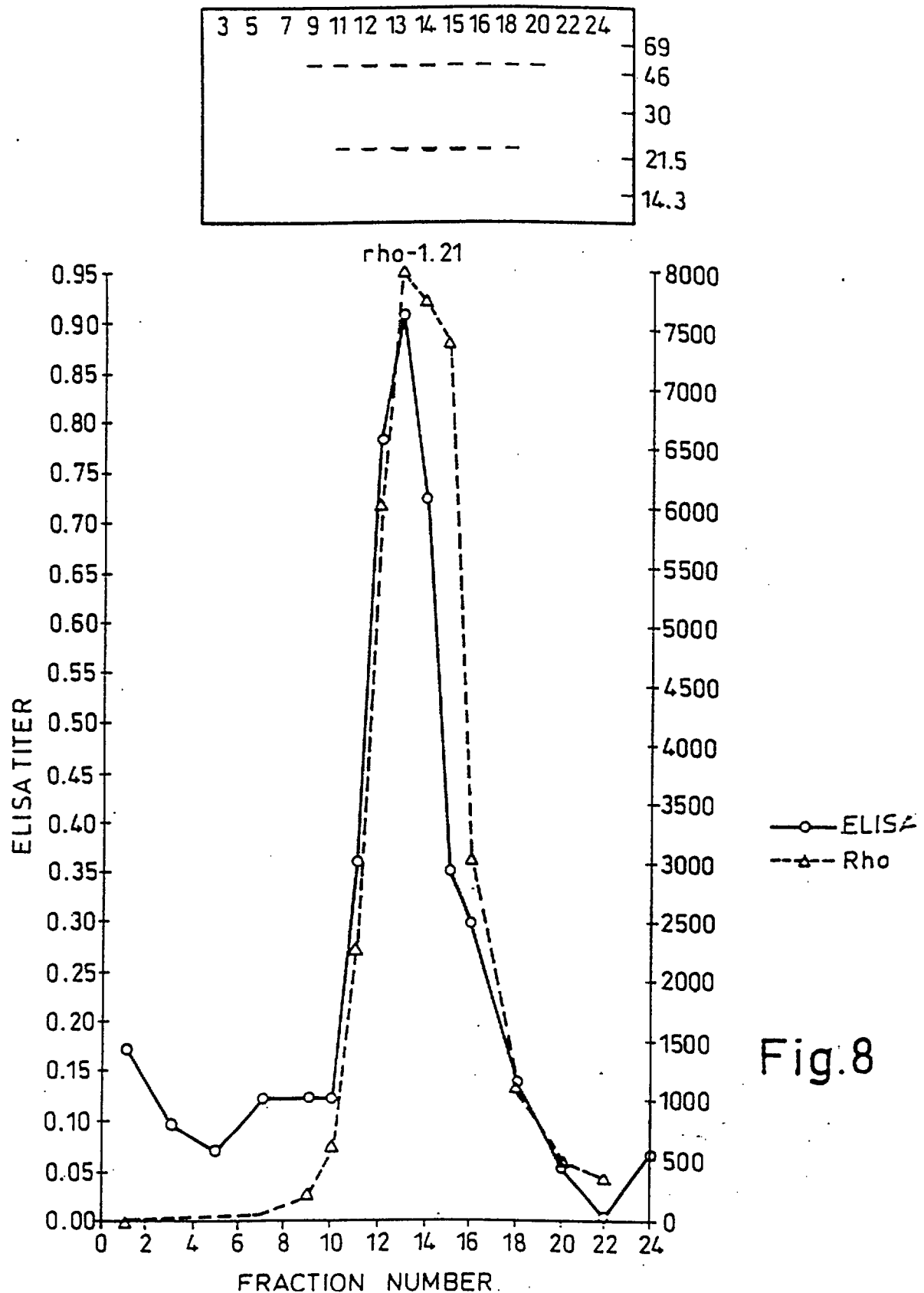


Fig.8

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Figure 9: Nucleotide Sequence of the RTS* expression cassette and predicted translation product of the hybrid protein.

Met Met Ala Pro Asp Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala
1 5 10 15

Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala
20 25 30

Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala
35 40 45

Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala
50 55 60

Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Lys
65 70 75 80

Asn Asn Gln Gly Asn Gly Gln Gly His Asn Met Pro Asn Asp Pro Asn
85 90 95

Arg Asn Val Asp Glu Asn Ala Asn Ala Asn Ser Ala Val Lys Asn Asn
100 105 110

Asn Asn Glu Glu Pro Ser Asp Lys His Ile Lys Glu Tyr Leu Asn Lys
115 120 125

Ile Gln Asn Ser Leu Ser Thr Glu Trp Ser Pro Cys Ser Val Thr Cys
130 135 140

Gly Asn Gly Ile Gln Val Arg Ile Lys Pro Gly Ser Ala Asn Lys Pro
145 150 155 160

Lys Asp Glu Leu Asp Tyr Ala Asn Asp Ile Glu Lys Lys Ile Cys Lys
165 170 175

Met Glu Lys Cys Ser Ser Val Phe Asn Val Val Asn Ser Ser Ile Gly
180 185 190

Leu Gly Pro Val Thr Asn Met Glu Asn Ile Thr Ser Gly Phe Leu Gly

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195

200

205

Pro Leu Leu Val Leu Gln Ala Gly Phe Phe Leu Leu Thr Arg Ile Leu
210 215 220

Thr Ile Pro Gln Ser Leu Asp Ser Trp Trp Thr Ser Leu Asn Phe Leu
225 230 235 240

Gly Gly Ser Pro Val Cys Leu Gly Gln Asn Ser Gln Ser Pro Thr Ser
245 250 255

Asn His Ser Pro Thr Ser Cys Pro Pro Ile Cys Pro Gly Tyr Arg Trp
260 265 270

Met Cys Leu Arg Arg Phe Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys
275 280 285

Leu Ile Phe Leu Leu Val Leu Leu Asp Tyr Gln Gly Met Leu Pro Val
290 295 300

Cys Pro Leu Ile Pro Gly Ser Thr Thr Thr Asn Thr Gly Pro Cys Lys
305 310 315 320

Thr Cys Thr Thr Pro Ala Gln Gly Asn Ser Met Phe Pro Ser Cys Cys
325 330 335

Cys Thr Lys Pro Thr Asp Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser
340 345 350

Ser Trp Ala Phe Ala Lys Tyr Leu Trp Glu Trp Ala Ser Val Arg Phe
355 360 365

Ser Trp Leu Ser Leu Leu Val Pro Phe Val Gln Trp Phe Val Gly Leu
370 375 380

Ser Pro Thr Val Trp Leu Ser Ala Ile Trp Met Met Trp Tyr Trp Gly
385 390 395 400

Pro Ser Leu Tyr Ser Ile Val Ser Pro Phe Ile Pro Leu Leu Pro Ile
405 410 415

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Phe Phe Cys Leu Trp Val Tyr Ile

420

AAGCTTACCA GTTCTCACAC GGAACACCAC TAATGGACAC AAATTCGAAA TACTTTGACC

60

CTATTTTCGA GGACCTTGTC ACCTTGAGCC CAAGAGAGCC AAGATTAA TTTTCCTATG

120

ACTTGATGCA AATTCCCAAA GCTAATAACA TGCAAGACAC GTACGGTCAA GAAGACATAT

180

TTGACCTCTT AACTGGTTCA GACGCGACTG CCTCATCAGT AAGACCCGTT GAAAAGAAGT

240

TACCTGAAAA AAACGAATAT ATACTAGCGT TGAATGTTAG CGTCAACAAC AAGAAGTTTA

300

ATGACGCGGA GGCCAAGGCA AAAAGATTCC TTGATTACGT AAGGGAGTTA GAATCATTTT

360

GAATAAAAAA CACGCTTTTT CAGTTCGAGT TTATCATTAT CAATACTGCC ATTTCAAAGA

420

ATACGTAAAT AATTAATAGT AGTGATTTTC CTAACCTTAT TTAGTCAAAA ATTAGCCTTT

480

TAATTCTGCT GTAACCCGTA CATGCCCAAA ATAGGGGGCG GGTTACACAG AATATATAAC

540

ATCGTAGGTG TCTGGGTGAA CAGTTTATCC CTGGCATCCA CTAAATATAA TGGAGCTCGC

600

TTTTAAGCTG GCATCCAGAA AAAAAAAGAA TCCCAGCACC AAAATATTGT TTTCTCACC

660

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- 16 -

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AACCATCAGT TCATAGGTCC ATTCTCTTAG CGCAACTACA GAGAACAGGG GCACAAACAG
720

GCAAAAAACG GGCACAACTT CARTGGAGTG ATGCAACCTG CCTGGAGTAA ATGATGACAC
780

AAGGCAATTG ACCCAGCAT GTATCTATCT CATTTCTTA CACCTTCTAT TACCTTCTGC
840

TCTCTCTGAT TTGGAAAAAG CTGAAAAAAA AGGTTGAAAC CAGTCCCTG AAATTATTCC
900

CCTACTTGAC TAATAAGTAT ATAAAGACGG TAGGTATTGA TTGTAATTCT GTAAATCTAT
960

TTCTTAACT TCTTAAATC TACTTTTATA GTTAGTCTTT TTTTAGTTT TAAACACCA
1020

AGAACTTAGT TTCGAATAAA CACACATAAA CAAACAAAT GATGGCTCCC GATCCTAATG
1080

CAATCCAA TGCAAAACCA AACGCAACC CCAATGCAAA TCCTAATGCA AACCCCAATG
1140

CAATCCTAA TGCAATCCT AATGCCAATC CAAATGCAAA TCCAAATGCA AACCCAAACG
1200

CAAACCCAA TGCAATCCT AATGCCAATC CAAATGCAAA TCCAAATGCA AACCCAAATG
1260

CAAACCCAA TGCAAAACCC AATGCAATC CTAATAAAA CAATCAAGGT AATGGACAAG
1320

GTCACAATAT GCCAATGAC CCAAACCGAA ATGTAGATGA AAATGCTAAT GCCAACAGTG
1380

CTGTAAAAA TAATAATAAC GAAGAACCA GTGATAAGCA CATAAAGAA TATTAAACA
1440

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03 APR 1997

AAATACAAAA TTCTCTTTCA ACTGAATGGT CCCCATGTAG TGTAACCTGT GGAAATGGTA
1500

TTCAAGTTAG AATAAAGCCT GGCTCTGCTA ATAAACCTAA AGACGAATTA GATTATGCAA
1560

ATGATATTGA AAAAAAATT TGTAATGG AAAAATGTT CAGTGTGTTT AATGTCGTAA
1620

ATAGTTCAAT AGGATTAGGG CCTGTGACGA ACATGGAGAA CATCACATCA GGATTCCTAG
1680

GACCCCTGCT CGTGTTACAG GCGGGGTTTT TCTTGTTGAC AAGAATCCTC ACAATACCGC
1740

AGAGTCTAGA CTCGTGGTGG ACTTCTCTCA ATTTTCTAGG GGGATCACCC GTGTGTCTTG
1800

GCCAAAATTC GCAGTCCCCA ACCTCCAATC ACTCACCAAC CTCCTGTCCT CCAATTTGTC
1860

CTGGTTATCG CTGGATGTGT CTGCGGCGTT TTATCATATT CCTCTTCATC CTGCTGCTAT
1920

GCCTCATCTT CTTATTGGTT CTTCTGGATT ATCAAGGTAT GTTGCCCGTT TGTCTCTAA
1980

TTCCAGGATC AACAACAACC AATACGGGAC CATGCAAAAC CTGCACGACT CCTGCTCAAG
2040

GCAACTCTAT GTTCCCTCA TGTTGCTGTA CAAAACCTAC GGATGGAAAT TGCACCTGTA
2100

TTCCCATCCC ATCGTCCTGG GCTTTCGCAA AATACCTATG GGAGTGGGCC TCAGTCCGTT
2160

TCTCTTGGCT CAGTTTACTA GTGCCATTTG TTCAGTGGTT CGTAGGGCTT TCCCCACTG
2220

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03 APR 1997

TTTGGCTTTC AGCTATATGG ATGATGTGGT ATTGGGGGCC AAGTCTGTAC AGCATCGTGA
2280

GTCCCTTTAT ACCGCTGTTA CCAATTTTCT TTTGTCTCTG GGTATACATT TAACGAATTC
2340

CAAGCTGAAA CAATTCAAAG GTTTTCAAAT CAATCAAGAA CTTGTCTCTG TGGCTGATCC
2400

AAACTACAAA TTTATGCATT GTCTGCCAAG ACATCAAGAA GAAGTTAGTG ATGATGTCTT
2460

TTATGGAGAG CATTCCATAG TCTTTGAAGA AGCAGAAAAC AGATTATATG CAGCTATGTC
2520

TGCCATTGAT ATCTTTGTTA ATAATAAAGG TAATTTCAAG GACTTGAAAT AATCCTTCTT
2580

TCGTGTTCTT AATAACTAAT ATATAAATAC AGATATAGAT GCATGAATAA TGATATACAT
2640

TGATTATTTT GCAATGTCAA TTAAAAAAA AAAATGTTAG TAAACTATG TTACATTCCA
2700

AGCAAATAAA GCACTTGGTT AAACGAAATT AACGTTTTTA AGACAGCCAG ACCGCGGTCT
2760

AAAAATTAA ATATACACTG CCAACAAATT CTTTCGAGTT GTCCAATTTC ACCACTTTTA
2820

TATTTTCATC AACTTCAGCA GATTCAACCT TCTCACATAG AACATTGGAA TAAACAGCCT
2880

TAACACCACT TTCAAGTTTG CACAGCGTAA TATGAGGAAT TTTGTTTTGA CAACACAACC
2940

CTTTAATTTT CTCATTGTTT TCATCAATTA TGCATCCAIC TTTATCTTTA GACAGTCCA
3000

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REPLACEMENT SHEET

03 APR 1997

CTACAATAGC AATAGTTTTT TCATCCCAAC ATAGTTTTTC GAGCCTAAAA TTCAGTTTGT
3060

CGGTCGTTTT TACCTGCGTA TTTTGGTTAT TACCAGAGCC TTGTGCATTT TCTATGCGGT
3120

TGTTATTGTA CTCGTTATC TGGTCAGTGT ATCTGTTACA ATATGATTCC ACAACTTTTT
3180

TGCCTCTTTT TCACGGGACG ACATGACATG ACCTAATGTT ATATGAAGTT CCTTCTGAAC
3240

TTTTCCACTA GCTAGTAAAT GCTTGAATTT CTCAGTCAGC TCTGCATCGC TAGCAATACA
3300

CCTCTTGACC AATTCAATAA TTTCATCGTA GTTTTCTATT TAGCTGAGAT ATATGTAGGT
3360

TTAATTAACT TAGCGTTTTT TGTGATTAT TGTGCGCTT ACCAACTATT TTTCTCACAG
3420

TAGGTTTGTA ATCTAAGCTC CTTCTGAACG CTGTCTCAAT TTCATCAICT TTCGGGATCT
3480

CTGGTACCAA AATTGGATAA GCTT
3504

-2-
irradiated sporozoite impractical from the point of view of producing a vaccine.

Several groups have proposed subunit vaccines based on the circumsporozoite protein. Two of these vaccines have undergone clinical testing; one is a synthetic peptide, the other is a recombinant protein (Ballou *et al* Lancet: i 1277 (1987) and Herrington *et al* Nature 328:257 (1987).

These vaccines were successful in stimulating an anti-sporozoite response. Nonetheless, the magnitude of the response was disappointing, with some vaccinees not making a response at all. Furthermore, the absence of "boosting" of antibody levels on subsequent injections and results of in vitro lymphocyte proliferation assays suggested that T-cells of most of these volunteers did not recognise the immuno-dominant repeat. Nonetheless, one vaccinee in each study did not develop parasitemia.

The present invention provides a new, improved antigen for use in malaria vaccines which not only produces a humoral response, but also a cellular immune response. Preferably the antigen induces the production of neutralising antibodies against the immunodominant repeat. Most preferably, the antigen should also elicit effector T cell mediated immune responses of the CD4⁺ and CD8⁺ cytotoxic T lymphocyte (CTL) type and of the delayed type hypersensitivity type and also, preferably be able to induce T helper (TH) memory cells.

Accordingly, the present invention provides a hybrid protein consisting of substantially all the C-terminal portion of the CS protein, four or more tandem repeats of the immunodominant region, and the Surface antigen from Hepatitis B virus (HBsAg). Preferably the hybrid protein comprises a sequence which contains at least 160 amino acids which is substantially homologous to the C-terminal portion of the CS protein. The CS protein may be devoid of the last 12 amino-acids from the C terminal.

In particular there is provided a protein which comprises a portion of the CS protein of *P. falciparum* substantially as corresponding to amino acids 210-398 of *P. falciparum* 7G8 fused in frame via a linear linker to the N-terminal of HBsAg. The linker may comprise a portion of preS2 from

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HBsAg.

The present invention also provides DNA sequences encoding the proteins of the present invention.

A particularly preferred embodiment is the hybrid protein designated RTS. The amino acid sequence of RTS is shown in figure 5. This hybrid consists of:

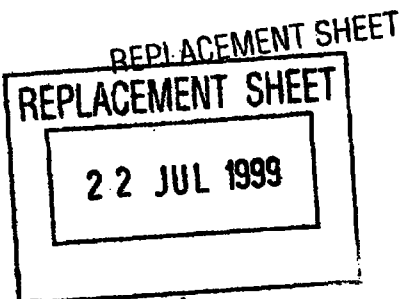
- ° A methionine-residue, encoded by nucleotides 1059 to 1061, derived from the Sacchromyes cerevisiae TDH3 gene sequence. (Musti A.M. et al Gene 1983 25 133-143.
- ° Three amino acids, Met Ala Pro, derived from a nucleotide sequence (1062 to 1070) created by the cloning procedure used to construct the hybrid gene.
- ° A stretch of 189 amino acids, encoded by nucleotides 1071 to 1637 representing amino acids 210 to 398 of the circumsporozoite protein (CSP) of Plasmodium falciparum strain 7G8 (Dame et al supra).
- ° An amino acid (Arg) encoded by nucleotides 1638 to 1640, created by the cloning procedure used to construct the hybrid gene.
- ° Four amino acids, Pro Val Thr Asn, encoded by nucleotides 1641 to 1652, and representing the four carboxy terminal residues of the hepatitis B virus (adw serotype) preS2 protein (9).
- ° A stretch of 226 amino acids, encoded by nucleotides 1653 to 2330, and specifying the S protein of hepatitis B virus (adw serotype).

In an alternative embodiment there is provided a hybrid protein designated RTS* which was generated using the CSP gene sequence from P. falciparum NF54 (Mol. Biochem Parasitol. 35 : 185-190, 1989) and comprises substantially all of the region 207 to 395 of the CS protein from P falciparum NF54.

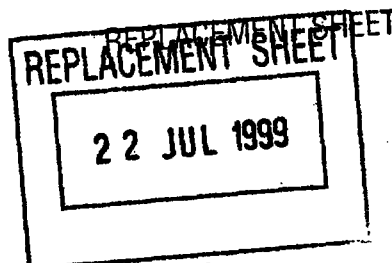
In particular RTS* consists of:



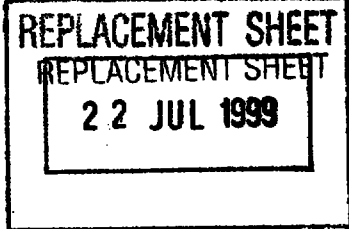
-33-
Claims



1. A hybrid protein comprising substantially all the C-terminal portion of the CS protein of Plasmodium, four or more tandem repeats of the CS protein immunodominant region, and the surface antigen from Hepatitis B Virus (HBsAg).
2. A hybrid protein comprising a sequence of CS protein of P.falciparum substantially as corresponding to amino acids 210-398 of P.falciparum 7G8 CS protein or 207-395 of P. falciparum NF54 CS protein fused in frame via a linear linker to the N-terminal of HBsAg.
3. A hybrid protein as claimed in claim 1 wherein the CS protein is fused to N-terminal of HBsAg.
4. A hybrid protein comprising the following amino acid sequences:
 - a) an N-terminal methionine residue
 - b) Met Ala Pro
 - c) a stretch of 189 amino acids corresponding to amino acids 210 to 398 of CS protein P.falciparum 7G8
 - d) Arg
 - e) Pro Val Thr Asn from hepatitis B Pre S₂ protein.
 - f) a stretch of 226 amino acids specifying the S protein of hepatitis B virus
5. A hybrid protein consisting of the following amino-acid sequences:
 - a) an N-terminal methionine
 - b) Met Ala Pro
 - c) a stretch of 189 amino acids corresponding to amino acids 207 to 395 of CS protein P.falciparum NF54
 - d) Gly



- e) Pro Val Thr Asn from hepatitis B Pres₂ protein
f) a stretch of 226 amino acids specifying the S protein of hepatitis B virus
6. A DNA sequence encoding a hybrid protein as claimed in any one of claims 1 to 5.
7. A vector containing a DNA sequence as claimed in claim 6 said sequence being linked to transcriptional control elements.
8. A host transformed with a vector of claim 7.
9. A host as claimed in claim 8 wherein the host is S. cerevisiae.
10. A host as claimed in claim 9 additionally transformed with a gene encoding the Hepatitis B surface antigen.
11. A multimeric lipoprotein particle comprising a hybrid protein of any one of claims 1 to 5.
12. A mixed multimeric lipoprotein particle comprising a hybrid protein of any one of claims 1 to 5 and Hepatitis B surface antigen.
13. A particle as claimed in claim 12 wherein the ratio of hybrid protein to surface antigen is approximately 1:4.
14. A vaccine comprising an immunoprotective amount of a particle or protein according to any of claims 1 to 5 or claims 11 to 12. in admixture with a suitable diluent or carrier.
15. A vaccine as claimed in claim 14 additionally comprising a saponin adjuvant.
16. A vaccine as claimed in claim 14 additionally comprising 3-Deacylated monophosphoryl lipid A.



17. A vaccine as claimed in claim 16 additionally comprising alum.

18. A vaccine as claimed in claim 16 presented in an oil in water emulsion.

19. A vaccine according to any one of claims 14 to 18, for use in the treatment of a patient susceptible to plasmodium infections.

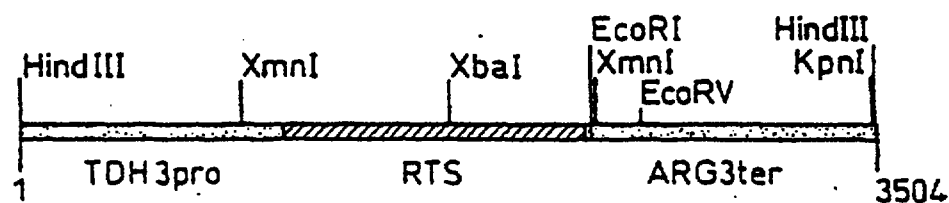
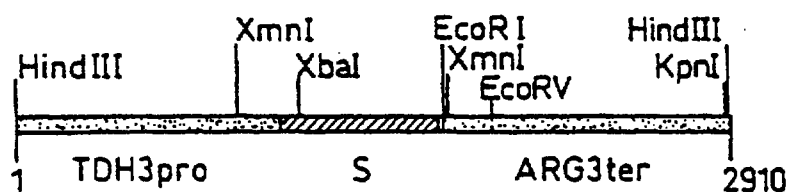
20. Use of a vaccine according to any one of claims 14 to 18 in the manufacture of a medicament for use in the treatment of a patient susceptible to plasmodium infections.

21. A process for the production of a hybrid protein according to any of claims 1 to 5 which process comprises expressing a DNA sequence encoding the protein in a suitable host and recovering the product.

22. A process for the production of particles as claimed in any one of claims 11 to 13 comprising expressing a DNA sequence encoding a protein of claims 1 to 4 and hepatitis B surface antigen in a Sacchomyces strain.

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Restriction endonuclease map of (A) the S expression cassette and (B) the RTS expression cassette.
 The extend of each coding sequence is indicated by the black bar.



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Construction of plasmid pRIT13034

Digestion with XhoI endonuclease liberates a 8.5 Kb linear DNA fragment carrying the S expression cassette for integration into the yeast chromosome by homology of the free ends with resident Ty elements.

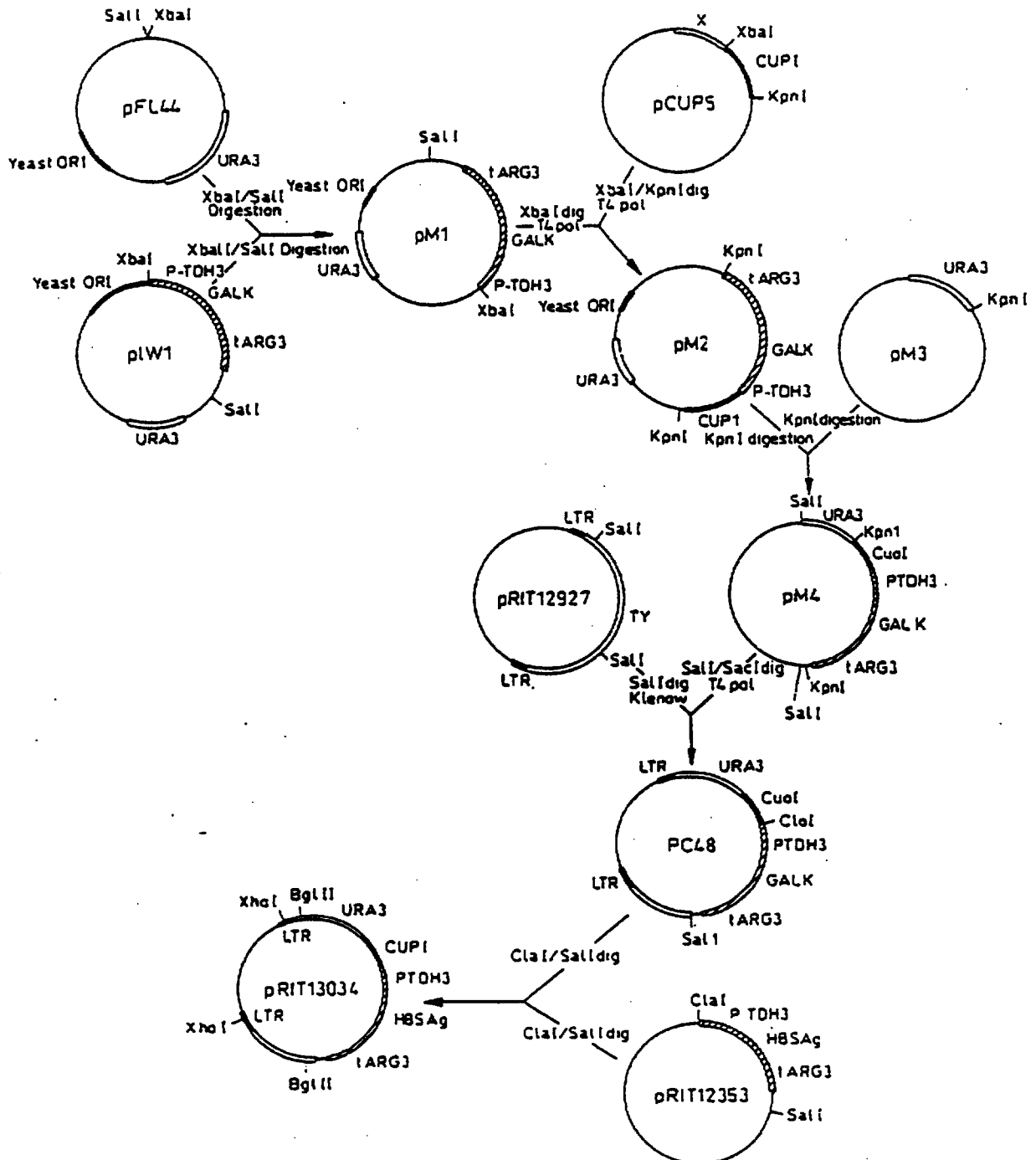
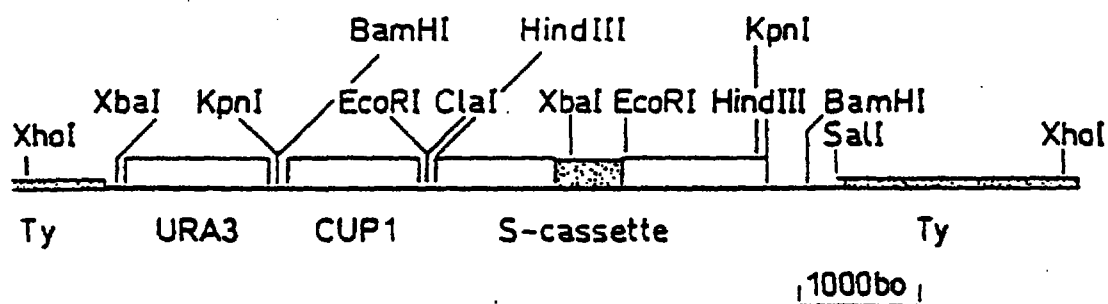


Fig.2

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Restriction map of the 8.5 Kb linear XhoI fragment from pRIT13034
The linear fragment contains the URA3 and CUP1 genes for selection of transformed yeast cells together with the S expression cassette.

**Fig.3**

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Construction of the RTS expression cassette and its cloning in plasmid Yep13.

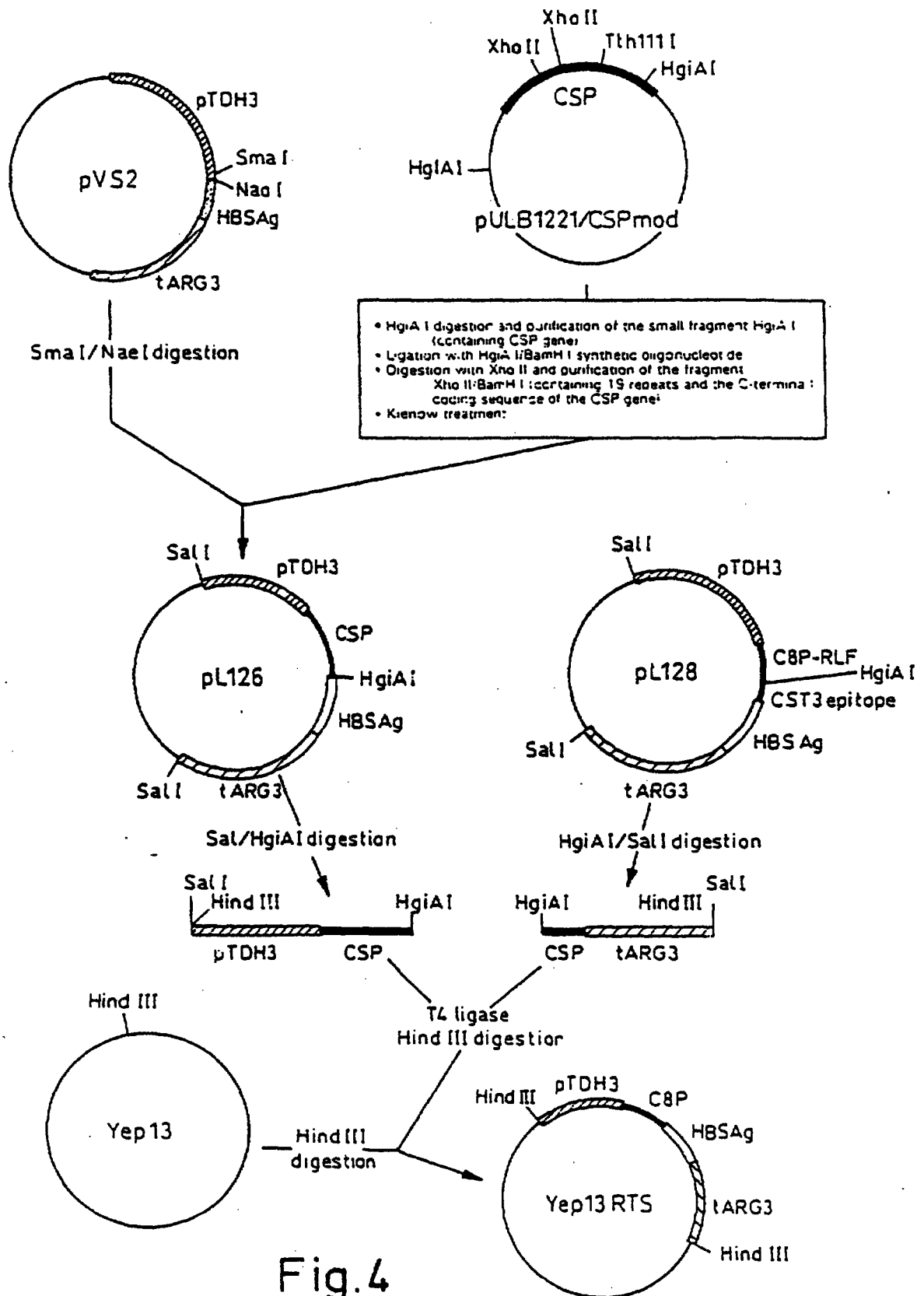


Fig.4

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Figure 5: Nucleotide sequence of the RTS expression cassette and predicted translation product of the RTS-HBsAg hybrid protein. The translation product initiated from the TDH3 ATG codon is shown below the DNA sequence.

10	30	50
AAGCTTACCAGTTCTCACACGGAACACCACTAATGGACACAAATTCGAAATACTTTGACC		
70	90	110
CTATTTTCGAGGACCTTGTCACCTTGAGCCCAAGAGAGCCAAAGATTTAAATTTTCCTATG		
130	150	170
ACTTGATGCAATTCCTCAAAGCTAATAACATGCAAGACACGTACGGTCAAGAAGACATAT		
190	210	230
TTGACCTCTTAACTGGTTCAGACGGGACTGCCTCATCAGTAAGACCCCTTGAAAAGAAGCT		
250	270	290
TACCTGAAAAAACGAATATATACTAGCGTTGAATGTTAGCCTCAACAACAAGAAGTTTA		
310	330	350
ATGACGCGGAGGCCAAGGCCAAAAGATTCCCTTGATTACGTAAGGGAGTTAGAATCATTTT		
370	390	410
GAATAAAAAACACGCTTTTTTCAGTTCGAGTTTATCATTATCAATACTGCCATTTCAAAGA		
430	450	470
ATACGTAAATAATTAATAGTAGTGATTTTCCTAACTTTATTTAGTCAAAAATTAGCCTTT		
490	510	530
TAATTCTGCTGTAACCCGTACATGCCCAAATAGGGGGCGGTTACACAGAATATATAAC		
550	570	590
ATCGTAGGTGTCTGGGTGAACAGTTTATCCCTGGCATCCACTAAATATAATGGAGCTCGC		
610	630	650
TTTTAAGCTGGCATCCAGAAAAAAAAGAATCCCAGCACCAAAATATTGTTTTCTTCACC		
670	690	710
AACCATCAGTTCATAGGTCCATTCTCTTAGCGCAACTACAGAGAACAGGGGCACAAACAG		
730	750	770
GCAAAAAACGGGCAACCTCAATGGAGTGATGCAACCTGCCTGGAGTAAATGATGACAC		
790	810	830
AAGGCAATTGACCCACGCATGTATCTATCTCATTTTCTTACACCTTCTATTACCTTCTGC		

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850 870 890
TCTCTCTGATT TTGAAAAAGCTGAAAAAAGGTTGAAACCAGTTCCTGAAATTATTCC

910 930 950
CCTACTTGACTAATAAGTATATAAGACGGTAGGTATTGATTGTAATTCTGTAAATCTAT

970 990 1010
TTCTTAAACTTCTTAAATTCTACTTTTATAGTTAGTCTTTTTTTTAGTTTFAAACACCA

1030 1050 1070
AGAACTTAGTTTCGAATAAACACACATAAACAAACAAAATGATGGCTCCCGATCCTAATG
MetMetAlaProAspProAsnA

1090 1110 1130
CAAATCCAAATGCAAACCCAAATGCAAACCCAAACGCAAACCCCAATGCAAATCCTAATG
LaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA

1150 1170 1190
CAAACCCCAATGCAAATCCTAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATG
LaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA

1210 1230 1250
CAAACCCCAAACGCAAACCCCAATGCAAATCCTAATGCCAATCCAAATGCAAATCCAAATG
LaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnA

1270 1290 1310
CAAACCCCAAATGCAAACCCCAATGCAAACCCCAATGCAAATCCTAATAAAAACAATCAAG
LaAsnProAsnAlaAsnProAsnAlaAsnProAsnAlaAsnProAsnLysAsnAsnGlnG

1330 1350 1370
GTAATGGACAAGGTCACAATATGCCAATGACCCAAACCGAAATGTAGATGAAAATGCTA
LyAsnGlyGlnGlyHisAsnMetProAsnAspProAsnArgAsnValAspGluAsnAlaA

1390 1410 1430
ATGCCAACAATGCTGTAAAAATAATAAACGAAGAACCAAGTGATAAGCACATAGAAC
snAlaAsnAsnAlaValLysAsnAsnAsnAsnGluGluProSerAspLysHisIleGluG

1450 1470 1490
AATATTTAAAGAAAATAAAAAATTCTATTTCAACTGAATGGTCCCATGTAGTGTAACTT
lnTyLeuLysLysIleLysAsnSerIleSerThrGluTrpSerProCysSerValThrC

1510 1530 1550
GTGGAAATGGTATTCAAGTTAGAATAAAGCCTGGCTCTGCTAATAAACCTAAAGACGAAT
ysGlyAsnGlyIleGlnValArgIleLysProGlySerAlaAsnLysProLysAspGluL

1570 1590 1610
TAGATTATGAAAATGATATTGAAAAAATTTGTAAATGGAAAAGTGCTCGAGTGTGT
euAspTyrGluAsnAspIleGluLysLysIleCysLysMetGluLysCysSerSerValP

1630 1650 1670

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TTAATGTCGTAAATAGTCGACCTGTGACGAACATGGAGAACATCACATCAGGATTCCTAG
heAsnValValAsnSerArgProValThrAsnMetGluAsnIleThrSerGlyPheLeuG

1690

1710

1730

GACCCCTGCTCGTGTTACAGGCGGGTTTTCTTGTTGACAAGAATCCTCACAATACCGC
lyProLeuLeuValLeuGlnAlaGlyPhePheLeuLeuThrArgIleLeuThrIleProG

1750

1770

1790

AGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTCTAGGGGGATCACCCTGTGTCTTG
lnSerLeuAspSerTrpTrpThrSerLeuAsnPheLeuGlyGlySerProValCysLeuG

1810

1830

1850

GCCAAAATTGCGAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCTCTCAATTTGTC
lyGlnAsnSerGlnSerProThrSerAsnHisSerProThrSerCysProProIleCysP

1870

1890

1910

CTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTAT
roGlyTyrArgTrpMetCysLeuArgArgPheIleIlePheLeuPheIleLeuLeuLeuC

1930

1950

1970

GCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTTGCTCTAA
ysLeuIlePheLeuLeuValLeuLeuAspTyrGlnGlyMetLeuProValCysProLeuI

1990

2010

2030

TTCCAGGATCAACAACAACCAATACGGGACCATGCAAAACCTGCACGACTCCTGCTCAAG
leProGlySerThrThrThrAsnThrGlyProCysLysThrCysThrThrProAlaGlnG

2050

2070

2090

GCAACTCTATGTTTCCCTCATGTTGCTGTACAAACCTACGGATGGAAATTGCACCTGTA
lyAsnSerMetPheProSerCysCysCysThrLysProThrAspGlyAsnCysThrCysI

2110

2130

2150

TTCCCATOCCATCGTCCTGGGCTTTCGCAAAATACCTATGGGAGTGGGCTCAGTCCGTT
leProIleProSerSerTrpAlaPheAlaLysTyrLeuTrpGluTrpAlaSerValArgP

2170

2190

2210

TCTCTTGGCTCAGTTTACTAGTGCCATTTGTTTCAGTGGTTCGTAGGGCTTTCCTCCACTG
heSerTrpLeuSerLeuLeuValProPheValGlnTrpPheValGlyLeuSerProThrV

2230

2250

2270

TTTGGCTTTTCAGCTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCGTGA
alTrpLeuSerAlaIleTrpMetMetTrpTyrTrpGlyProSerLeuTyrSerIleValS

2290

2310

2330

GTCCCTTTTATACCGCTGTTACCAATTTTCTTTTGTCTCTGGGTATACATTTAACGAATTC
erProPheIleProLeuLeuProIlePhePheCysLeuTrpValTyrIleEnd

2350

2370

2390

CAAGCTGAAACAATTCAAAGGTTTTCAAATCAATCAAGAACTTGTCTCTGTGGCTGATCC

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2410	2430	2450
AAACTACAAATTTATGCATTGTCTGCCAAGACATCAAGAAGAAGTTAGTGATGATGTCTT		
2470	2490	2510
TTATGGAGAGCATTCCATAGTCTTTGAAGAAGCAGAAAACAGATTATATGCAGCTATGTC		
2530	2550	2570
TGCCATTGATATCTTTGTTAATAATAAAGGTAATTTCAAGGACTTGAAATAATCCTTCTT		
2590	2610	2630
TCGTGTTCTTAATAACTAATATATAAATACAGATATAGATGCATGAATAATGATATACAT		
2650	2670	2690
TGATTATTTTGCAATGTCAATTAAAAAATAAATGTTAGTAAACTATGTTACATTCCA		
2710	2730	2750
AGCAAATAAAGCACTTGGTTAAACGAAATTAACGTTTTTAAGACAGCCAGACCGCGGTCT		
2770	2790	2810
AAAAATTTAAATATACACTGCCAACAATTCCTTCGAGTTGTCCAATTTCAACCACTTTTA		
2830	2850	2870
TATTTTCATCAACTTCAGCAGATTCAACCTTCTCACATAGAACATTGGAATAAACAGCCT		
2890	2910	2930
TAACACCACCTTTCAAGTTTGCACAGCGTAATATGAGGAATTTGTTTTGACAACACAACC		
2950	2970	2990
CTTTAATTTTCTCATTGTTTTCATCAATTATGCATCCATCTTTATCTTTAGACAGTTCCA		
3010	3030	3050
CTACAATAGCAATAGTTTTTTCATCCCAACATAGTTTTTCGAGCCTAAAATTCAGTTTGT		
3070	3090	3110
CGGTGCTTTTACCTGCGTATTTTGGTTATTACCAGAGCCTTGTGCATTTTCTATGCGGT		
3130	3150	3170
TGTTATTGTACTCCGTTATCTGGTCAGTGTATCTGTTACAATATGATTCCACAACCTTTT		
3190	3210	3230
TGCCCTCTTTTTCACGGGACGACATGACATGACCTAATGTTATATGAAGTTCCTTCTGAAC		
3250	3270	3290
TTTTCCACTAGCTAGTAAATGCTTGAATTTCTCAGTCAGCTCTGCATGCTAGCAATACA		
3310	3330	3350
CCTCTTGACCAATTCAATAATTTTCATCGTAGTTTTCTATTAGCTGAGATATATGTAGGT		

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- 9 -

3370 3390 3410
TTAATTAACCTTAGCGTTTTTGTGATTATTGTTGCCCTTACCAACTATTTTTCTCACAG
3430 3450 3470
TAGGTTTGTAATCTAAGCTCCTTCTGAACGCTGTCTCAATTCATCATCTTTCGGGATCT
3490
CTGGTACCAAAATTGGATAAGCTT

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Construction of plasmid pRIT13539

Digestion with BglII endonuclease liberates a 6.8 Kb linear DNA fragment carrying the RTS expression cassette for integration into the yeast chromosome by homology of the free ends with resident Ty elements.

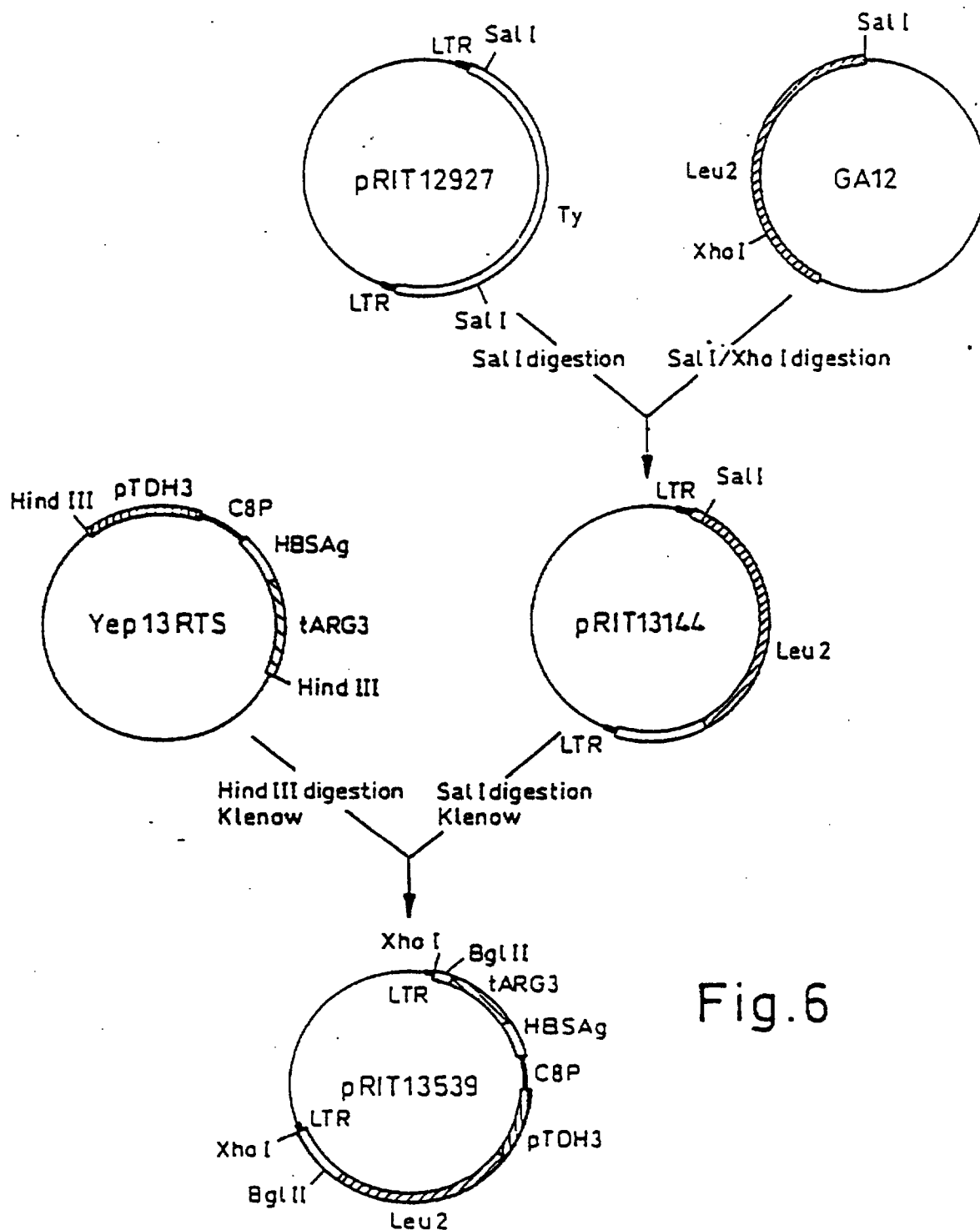


Fig.6

Restriction map of the 6.8 Kb linear Bgl II fragment from pRIT13539
 The linear fragment contains the LEU2 gene for selection of transformed yeast
 cells together with the RTS expression cassette.

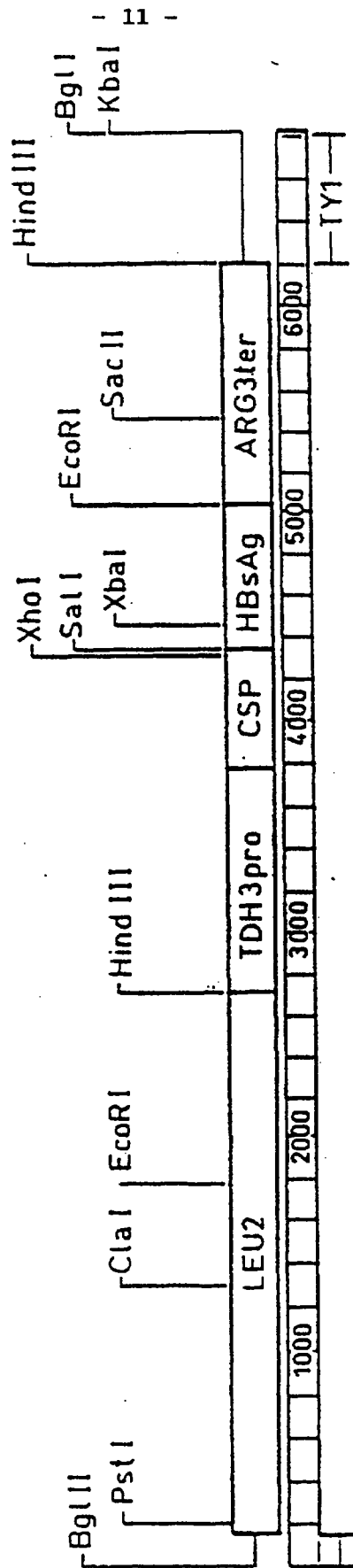


Fig. 7

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CsCl density analysis of a cell-free extract prepared from strain RIT4383. Fractions were analyzed by a radioimmunoassay specific for HBsAg (AUSRIA), and by an RTS-specific ELISA. The top panel shows the immunoblot analysis of fractions using an anti-S Mab. The buoyant density (ρ) of the peak fraction was calculated from a measure of its refractive index.

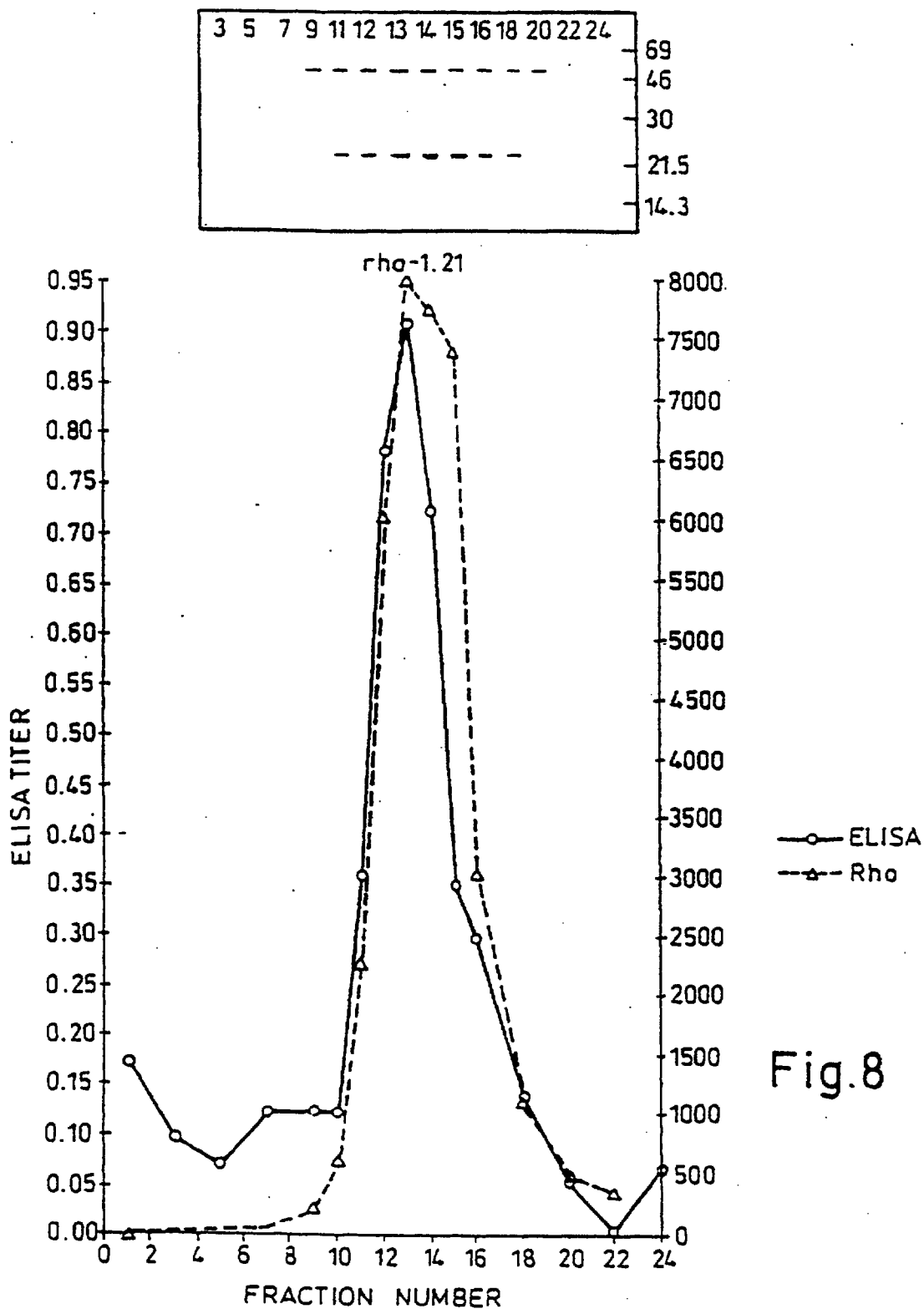
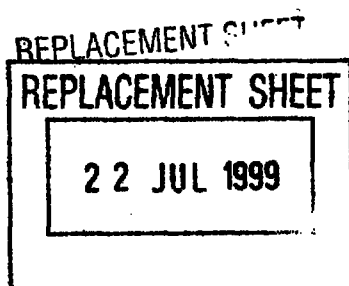


Fig.8

Figure 9: Nucleotide Sequence of the RTS* expression cassette and predicted translation product of the hybrid protein.

Met Met Ala Pro Asp Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala			
1	5	10	15
Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala			
20	25	30	
Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala			
35	40	45	
Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala			
50	55	60	
Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn Lys			
65	70	75	80
Asn Asn Gln Gly Asn Gly Gln Gly His Asn Met Pro Asn Asp Pro Asn			
85	90	95	
Arg Asn Val Asp Glu Asn Ala Asn Ala Asn Ser Ala Val Lys Asn Asn			
100	105	110	
Asn Asn Glu Glu Pro Ser Asp Lys His Ile Lys Glu Tyr Leu Asn Lys			
115	120	125	
Ile Gln Asn Ser Leu Ser Thr Glu Trp Ser Pro Cys Ser Val Thr Cys			
130	135	140	
Gly Asn Gly Ile Gln Val Arg Ile Lys Pro Gly Ser Ala Asn Lys Pro			
145	150	155	160
Lys Asp Glu Leu Asp Tyr Ala Asn Asp Ile Glu Lys Lys Ile Cys Lys			
165	170	175	
Met Glu Lys Cys Ser Ser Val Phe Asn Val Val Asn Ser Ser Ile Gly			
180	185	190	
Leu Gly Pro Val Thr Asn Met Glu Asn Ile Thr Ser Gly Phe Leu Gly			



- 14 -

195

200

205

Pro Leu Leu Val Leu Gln Ala Gly Phe Phe Leu Leu Thr Arg Ile Leu
210 215 220

Thr Ile Pro Gln Ser Leu Asp Ser Trp Trp Thr Ser Leu Asn Phe Leu
225 230 235 240

Gly Gly Ser Pro Val Cys Leu Gly Gln Asn Ser Gln Ser Pro Thr Ser
245 250 255

Asn His Ser Pro Thr Ser Cys Pro Pro Ile Cys Pro Gly Tyr Arg Trp
260 265 270

Met Cys Leu Arg Arg Phe Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys
275 280 285

Leu Ile Phe Leu Leu Val Leu Leu Asp Tyr Gln Gly Met Leu Pro Val
290 295 300

Cys Pro Leu Ile Pro Gly Ser Thr Thr Thr Asn Thr Gly Pro Cys Lys
305 310 315 320

Thr Cys Thr Thr Pro Ala Gln Gly Asn Ser Met Phe Pro Ser Cys Cys
325 330 335

Cys Thr Lys Pro Thr Asp Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser
340 345 350

Ser Trp Ala Phe Ala Lys Tyr Leu Trp Glu Trp Ala Ser Val Arg Phe
355 360 365

Ser Trp Leu Ser Leu Leu Val Pro Phe Val Gln Trp Phe Val Gly Leu
370 375 380

Ser Pro Thr Val Trp Leu Ser Ala Ile Trp Met Met Trp Tyr Trp Gly
385 390 395 400

Pro Ser Leu Tyr Ser Ile Val Ser Pro Phe Ile Pro Leu Leu Pro Ile
405 410 415

- 15 -

Phe Phe Cys Leu Trp Val Tyr Ile
420

REPLACEMENT SHEET

22 JUL 1999

AAGCTTACCA GTTCTCACAC GGAACACCAC TAATGGACAC AAATTCGAAA TACTTTGACC
50

CTATTTTCGA GGACCTTGTC ACCTTGAGCC CAAGAGAGCC AAGATTTAAA TTTTCCTATG
120

ACTTGATGCA AATTCCCAAA GCTAATAACA TGCAAGACAC GTACGGTCAA GAAGACATAT
180

TTGACCTCIT AACTGGTTCA GACGCGACTG CCTCATCAGT AAGACCCGTT GAAAAGAACT
240

TACCTGAAAA AAACGAATAT ATACTAGCGT TGAATGTTAG CGTCAACAAC AAGAAGTTTA
300

ATGACGCGGA GGCCAAGGCA AAAAGATTCC TTGATTACGT AAGGGAGTTA GAATCATTTT
360

GRATAAAAAA CAGCCTTTTT CAGTTCGAGT TTATCATTAT CAATACTGCC ATTTCAAAGA
420

ATACGTAAAT AATTAATACT AGTGATTTTC CTAACTTTAT TTAGTCAAAA ATTAGCCTTT
480

TAATTCTGCT GTAACCCGTA CATGCCCAAA ATAGGGGGCG GGTTACACAG AATATATAAC
540

ATCGTAGGTG TCTGGGTGAA CAGTTTATCC CTGGCATCCA CTAAATATAA TGGAGCTCGC
600

TTTTAAGCTG GCATCCAGAA AAAAAAAGAA TCCCAGCACC AAAATATTGT TTTCTTCACC
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REPLACEMENT SHEET

22 JUL 1999

AACCATCAGT TCATAGGTCC ATTCTCTTAG CGCAACTACA GAGAACAGGG GCACAAACAG
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GCAAAAAACG GGCACAACCT CAATGGAGTG ATGCAACCTG CCTGGAGTAA ATGATGACAC
780

AAGGCAATTG ACCCAGCAT GTATCTATCT CATTTTCTTA CACCTTCTAT TACCTTCTGC
840

TCTCTCTGAT TTGGAAAAAG CTGAAAAAAA AGGTTGAAAC CAGTTCCTG AAATTATTCC
900

CCTACTTGAC TAATAAGTAT ATAAAGACGG TAGGTATTGA TTGTAATTCT GTAAATCTAT
960

TTCTTAACT TCTTAAATC TACTTTTATA GTTAGTCTTT TTTTAGTTT TAAAACACCA
1020

AGAACTTAGT TTCGAATAAA CACACATAAA CAAACAAAAT GATGGCTCCC GATCCTAATG
1080

CAAATCCAAA TGCAAACCCA AACGCAAACC CCAATGCAAA TCCTAATGCA AACCCCAATG
1140

CAAATCCTAA TGCAAATCCT AATGCCAATC CAAATGCAAA TCCAAATGCA AACCCAAACG
1200

CAAACCCCAA TGCAAATCCT AATGCCAATC CAAATGCAAA TCCAAATGCA AACCCAAATG
1260

CAAACCCAAA TGCAAACCCC AATGCAAATC CTAATAAAAA CAATCAAGGT AATGGACAAG
1320

GTCACAATAT GCCAAATGAC CCAAACCGAA ATGTAGATGA AAATGCTAAT GCCAACAGTG
1380

CTGTAAAAAA TAATAATAAC GAAGAACCAA GTGATAAGCA CATAAAAGAA TATTTAAACA
1440

REPLACEMENT SHEET

22 JUL 1999

- 17 -

AAATACAAA TTCTCTTTCA ACTGAATGGT CCCCATGTAG TGTAAC TTGT GAAATGGTA
1590

TTCAAGTTAG AATAAAGCCT GGCTCTGCTA ATAAACCTAA AGACGAATTA GATTATGCAA
1560

ATGATATTGA AAAAAAATT TGTAATGG AAAAAATGTC CAGTGTGTTT AATGTCGTAA
1620

ATAGTTCAAT AGGATTAGGG CCTGTGACGA ACATGGAGAA CATCACATCA GGATTCCTAG
1680

GACCCCTGCT CGTGTTACAG GCGGGGTTTT TCTGTGTGAC AAGAATCCTC ACAATACCGC
1740

AGAGTCTAGA CTCGTGGTGG ACTTCTCTCA ATTTCTAGG GGGATCACCC GTGTGTCTTG
1800

GCCAAAATTC GCAGTCCCCA ACCTCCAATC ACTCACCAAC CTCCTGTCCT CCAATTTGTC
1860

CTGGTTATCG CTGGATGTGT CTGCGGCGTT TTATCATATT CCTCTTCATC CTGCTGCTAT
1920

GCCTCATCTT CTTATTGGTT CTTCTGGATT ATCAAGGTAT GTTGCCCGTT TGTCTCTAA
1980

TTCCAGGATC AACAAACAACC AATACGGGAC CATGCAAAAC CTGCACGACT CCTGCTCAAG
2040

GCAACTCTAT GTTTCCTCA TGTGCTGTA CAAAACCTAC GGATGGAAAT TGCACCTGTA
2100

TTCCCATCCC ATCGTCTGG GCTTTCGCAA AATACCTATG GGAGTGGGCC TCAGTCCGTT
2160

TCTCTGGCT CAGTTTACTA GTGCCATTG TTCAGTGGTT CGTAGGGCTT TCCCCACTG
2220

REPLACEMENT SHEET

22 JUL 1999

- 18 -

TTTGGCTTTC AGCTATATGG ATGATGTGGT ATTGGGGGCC AAGTCTGTAC AGCATCGTGA
2280

GTCCCTTTAT ACCGCTGTTA CCAATTTTCT TTTGTCTCTG GGTATACATT TAACGAATTC
2340

CAAGCTGAAA CAATTCAAAG GTTTTCAAAT CAATCAAGAA CTTGTCTCTG TGGCTGATCC
2400

AAACTACAAA TTTATGCATT GTCTGCCAAG ACATCAAGAA GAAGTTAGTG ATGATGTCTT
2460

TTATGGAGAG CATTCCATAG TCTTTGAAGA AGCAGAAAAC AGATTATATG CAGCTATGTC
2520

TGCCATTGAT ATCTTTGTTA ATAATAAAGG TAATTTCAAG GACTTGAAAT AATCCTTCTT
2580

TCGTGTTCTT AATAACTAAT ATATAAATAC AGATATAGAT GCATGAATAA TGATATACAT
2640

TGATTATTTT GCAATGTCAA TTAAAAAAA AAAATGTTAG TAAACTATG TTACATTCCA
2700

AGCAAATAAA GCACTTGGTT AAACGAAATT AACGTTTTTA AGACAGCCAG ACCGCGTCT
2760

AAAAATTTAA ATATACACTG CCAACAAATT CCTTCGAGTT GTCCAATTC ACCACTTTTA
2820

TATTTTCATC AACTTCAGCA GATTCAACCT TCTCACATAG AACATTGGAA TAAACAGCCT
2880

TAACACCACT TTCAAGTTTG CACAGCGTAA TATGAGGAAT TTTGTTTTGA CAACACAACC
2940

CTTTAATTTT CTCATTGTTT TCATCAATTA TGCATCCATC TTTATCTTTA GACAGTTCCA
3000

22 JUL 1999

- 19 -

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3060

CGGTCGTTTT TACCTGCGTA TTTTGGTTAT TACCAGAGCC TTGTGCATTT TCTATGCGGT
3120

TGTTATTGTA CTCCGTTATC TGGTCAGTGT ATCTGTTACA ATATGATTCC ACAACTTTTT
3180

TGCCCTTTTT TCACGGGACG ACATGACATG ACCTAATGTT ATATGAAGTT CCTTCTGAAC
3240

TTTTCCACTA GCTAGTAAAT GCTTGAATTT CTCAGTCAGC TCTGCATCGC TAGCAATACA
3300

CCTCTTGACC AATTCAATAA TTTTCATCGTA GTTTTCTATT TAGCTGAGAT ATATGTAGGT
3360

TTAATTAACT TAGCGTTTTT TGTGATTAT TGTTGCCTTT ACCAACTATT TTTCTCACAG
3420

TAGGTTTGTA ATCTAAGCTC CTTCTGAACG CTGTCTCAAT TTCATCATCT TTCGGGATCT
3480

CTGGTACCAA AATTGGATAA GCTT
3504