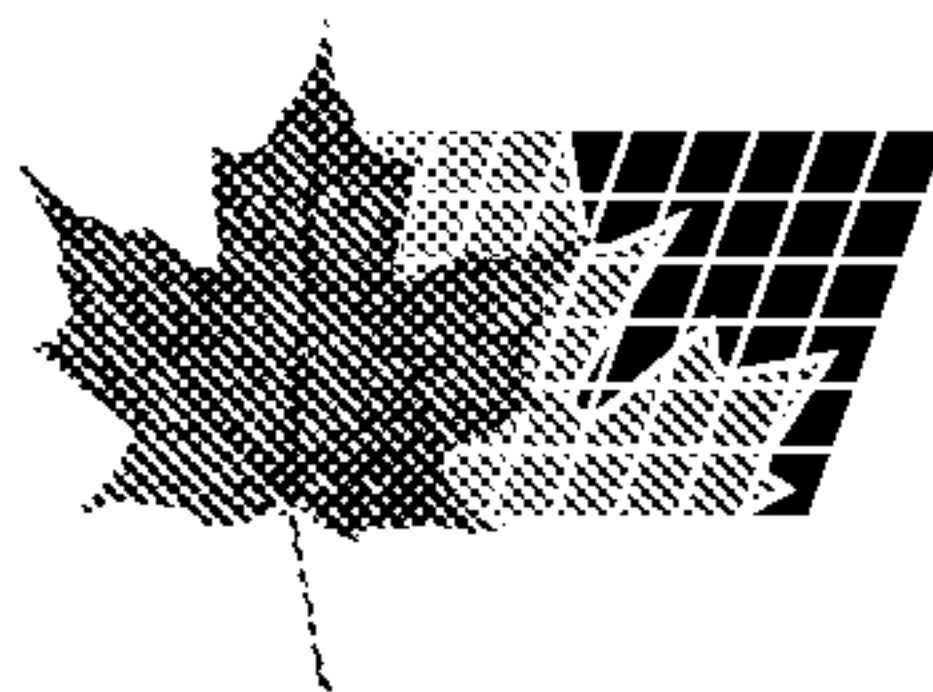




(11) (21) (C) **2,021,762**  
(22) 1990/07/23  
(43) 1991/01/26  
(45) 2000/09/12

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(51) Int.Cl.<sup>5</sup> C12N 15/51, A61K 39/29, C12N 1/21, C12N 5/10, C12P 21/02,  
C07K 14/02

(30) 1989/07/25 (385,342) US

(30) 1989/08/03 (389,184) US

(54) **ANTIGENES ET METHODES**

(54) **ANTIGENS AND METHODS THEREFOR**

(57) A composite particle comprising at least two polypeptides corresponding to all or part of a protein having the biological activity of one of the hepatitis B surface antigens, wherein the particle presents at least two antigenic determinants provided by the S-protein, preS2-protein or preS1-protein, said particle optionally further comprising host specific lipids. The invention also provides a modified hepatitis B surface antigen L protein which may be used alone or incorporated into such composite particles. A particular embodiment of the modified L protein (L\*) comprises residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L protein and a particular embodiment of a composite particle has the form (L\*, S) where S is the S-protein of HBsAg. Improved hepatitis B vaccines may be prepared following expression of the above immunogens, especially in yeasts such as S. cerevisiae and Hansenula polymorpha.



Abstract

A composite particle comprising at least two polypeptides corresponding to all or part of a protein having the biological activity of one of the hepatitis B surface antigens, wherein the particle presents at least two antigenic determinants provided by the S-protein, preS2-protein or preS1-protein, said particle optionally further comprising host specific lipids. The invention also provides a modified hepatitis B surface antigen L protein which may be used alone or incorporated into such composite particles.

A particular embodiment of the modified L protein (L\*) comprises residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L protein and a particular embodiment of a composite particle has the form (L\*, S) where S is the S-protein of HBsAg.

Improved hepatitis B vaccines may be prepared following expression of the above immunogens, especially in yeasts such as S. cerevisiae and Hansenula polymorpha.

Novel antigens and methods therefor

The invention relates to a microorganism selected from the group of methylophilic yeast genera, a DNA molecule containing an expression cassette, a vector suitable for transformation of a methylophilic yeast, a process for the preparation of a microorganism, a composite particle containing at least two polypeptides corresponding to all or part of a protein having the biological activity of hepatitis B surface antigen, a method for the preparation of composite particles and to compositions, vaccines and test kits containing same.

The invention also relates to the construction of microorganism strains synthesizing modified large surface proteins of hepatitis B virus, especially hepatitis B surface L antigens which are modified at various regions of the coding sequence to alter and enhance the biological activity thereof.

Hepatitis is a wide spread, serious infectious disease, which may be caused by several agents. One important cause was identified as hepatitis B virus (HBV), a small virion of about 43 nm diameter containing a peculiar genome, which consists of a 3,200 base pair double-stranded DNA circle having a single stranded gap of about 200 base pairs. Infection with this virion results in an acute, sometimes fatal disease. Eighty five percent of affected persons recover after about three months of illness, about 1% suffers from acute necrosis of liver tissue, resulting in death within a short period, and about 10% suffer from a recurring outbreak of the disease. Approximately 4% of infected persons develop a chronic carrier state with an increased risk of primary hepatocellular carcinoma or

01  
02  
03 liver cirrhosis.  
04

05 Infection mostly occurs via perinatal or parenteral  
06 transmission of infectious virions, for example in the  
07 course of blood transfusions or by use of contaminated  
08 injection syringes or tips, or by sexual contact.  
09 Therefore there is an urgent need for reliable  
10 diagnostic means for detection of HBV and for a vaccine  
11 protecting people at high risk of exposure, such as  
12 spouses of chronic carriers, travellers to areas of  
13 high HBV endemicity, newborns of chronic carriers,  
14 homosexuals, prostitutes and drug abusers, which should  
15 be affordable at low costs. Furthermore in third world  
16 countries there exists a need for an inexpensive  
17 vaccine for mass immunisation programmes.  
18

19 In addition to viral particles consisting of the core  
20 (HBcAg) and capsid or surface (HBsAg) antigen proteins  
21 enclosing the circular, partially double stranded DNA  
22 genome the blood of infected individuals may contain  
23 substantial quantities of HBsAg particles containing  
24 the surface antigen protein and host lipids. These  
25 particles have been purified from human serum and  
26 formulated as vaccines which have been shown to confer  
27 protection against HBV infection (Szmuness et al,  
28 1980).  
29

30 In this application certain publications are referenced  
31 by mention of the first author and the year of  
32 publication within parenthesis. Full citation for  
33 these references listed according to their alphabetical  
34 order may be found at the end of the specification.

01  
02  
03 The preparation of vaccines from serum derived HBsAg  
04 has been hampered by the limited supply of infected  
05 blood and by the need to undergo long and  
06 rigorous testing to ensure the removal and/or  
07 inactivation of potential infectious agents.  
08

09 Hepatitis B virus (HBV) infection in humans is  
10 associated with the occurrence in the serum of various  
11 structures carrying the hepatitis B surface antigen  
12 (HBsAg) [Tiollais et al, Nature, 17:489 (1985)]. In  
13 addition to infectious virions, filamentous and  
14 spherical particles of 22 nm in diameter (containing  
15 about 100 envelope proteins) are present which are  
16 formed by association of host-derived lipids with the  
17 three hepatitis surface proteins: the major (S), middle  
18 (M) and large (L) proteins.  
19

20 These proteins share the same sequence of 226 amino  
21 acids codons on the HBV genome, known as the S protein  
22 coding sequence. The entire amino acid coding sequence  
23 which immediately precedes the S protein coding  
24 sequence on the HBV genome is referred to herein as the  
25 preS coding sequence. The preS coding sequence codes  
26 for a 55 amino acid sequence which immediately precedes  
27 the S protein, called the preS2 region, and, depending  
28 on the virus subtype, either a 108 or 119 amino acid  
29 sequence which immediately precedes the preS2 region,  
30 called the preS1 region.  
31

32 The entire preS coding sequence thus codes for 163 (55  
33 preS2 + 108 preS1) amino acids in ay subtypes and for  
34 174 (55 preS2 + 119 preS1) amino acids in most ad  
35 subtypes. Sequence comparisons between the preS coding  
36 sequences in the genomes of ad and ay isolates have

shown that the first eleven codons of the preS1 region of ad isolates are not present in ay subtypes. In agreement with the generally adopted nomenclature, throughout this application the codon and amino acid numbering of ad subtypes is followed, meaning that HBV genomes of ad isolates encode a preS region of 174 amino acids numbered 1 to 174 while the 163 amino acids preS region of ay subtypes are numbered 12 to 174.

The major protein (S) is encoded by the 226 amino acid codons S gene and exists in glycosylated and non-glycosylated forms.

The middle protein (M) includes the preS2 region and the S protein (M protein: 55 plus 226 amino acids). The M protein is a glycoprotein present in two forms according to the extent of glycosylation [Stibbe et al, J. Virol., 46:626-628 (1983)]. The 55 amino acids encoded by the preS2 region are hydrophilic and contain an immunodominant epitope located at the surface of the envelope gene (residues 132-137). This epitope is disulfide bond-independent and reported to be more immunogenic than epitopes of the S protein. [Neurath et al, Science, 224:392-394 (1984); Neurath et al., Nature, 315:154-156 (1985)]. The preS2 sequence also contains a receptor for polymerized human serum albumin (pHSA) [Machida et al, Gastroent., 86:910-918 (1984)]. This receptor can also bind monomeric HSA and may mediate attachment of HBV to hepatocytes, which also have a receptor for pHSA. It has been suggested that such binding could lead to tolerance or auto-immune reactions in humans.

The large protein (L) includes the preS1 region, the preS2 region and the S coding sequence (L protein: 108

(or 119) plus 55 plus 226 amino acids). It is present in glycosylated and non-glycosylated forms. The L protein is of variable length according to the subtype, e.g., 389 and 400 amino acids long for the ay and ad subtypes, respectively. The preS1 translation product is also involved in the attachment of HBV to hepatocytes.

Because the promoter for the S and M specific transcripts is embedded within the open reading frame of the L protein, transformation of mammalian cells with DNA encoding the complete open reading frame for the L protein may result in synthesis of all three surface proteins. In mammalian cells, HBsAg particles are secreted [Tiollais et al, cited above]. In mammalian systems expression of L protein causes an inhibition of secretion of HBsAg particles. [See, e.g., Ou et al, J. Virol., 61:782 (1987)]. This phenomenon has been suggested to be related to the presence on the L protein of an N-myristoyl group, causing interference with the budding or secretion process [Persing et al, Science, 234:1388-1391 (1986); Persing et al, J. Virol., 61:1672-1677 (1987)].

For ease of reference, the following Table A sets out the DNA coding sequences and amino acid sequences for the preS1, preS2 and S regions of the HBV proteins discussed herein. Amino acid numbers are above the codons in parentheses and are numbered from the first ATG codon present in the open reading frame for the envelope proteins found in HBV adw isolate sequence which was subcloned on plasmid pRIT10616. As the first eleven codons of this ad sequence are not present in any of the expression cassettes described in the present application these sequences are not given in Table A.

Plasmid pRIT10616, harboured in E. coli K12 strain 600, is on deposit at the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number ATCC 39131.

Table APRE-S1 REGIONNcoI

(12)

ATG GGG ACG AAT CTT TCT GTT CCC AAC CCT CTG GGA TTC TTT  
Met Gly Thr Asn Leu Ser Val Pro Asn Pro Leu Gly Phe Phe

(26)

CCC GAT CAT CAG TTG GAC CCT GCA TTC GGA GCC AAC TCA AAC  
Pro Asp His Gln Leu Asp Pro Ala Phe Gly Ala Asn Ser Asn

(40)

AAT CCA GAT TGG GAC TTC AAC CCC ATC AAG GAC CAC TGG CCA  
Asn Pro Asp Trp Asp Phe Asn Pro Ile Lys Asp His Trp Pro

(54)

GCA GCC AAC CAG GTA GGA GTG GGA GCA TTC GGG CCA GGG CTC  
Ala Ala Asn Gln Val Gly Val Gly Ala Phe Gly Pro Gly Leu

(68)

ACC CCT CCA CAC GGC GGT ATT TTG GGG TGG AGC CCT CAG GCT  
Thr Pro Pro His Gly Gly Ile Leu Gly Trp Ser Pro Gln Ala

(82)

CAG GGC ATA TTG ACC ACA GTG TCA ACA ATT CCT CCT CCT GCC  
Gln Gly Ile Leu Thr Thr Val Ser Thr Ile Pro Pro Pro Ala

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(96)

TCC ACC AAT CGG CAG TCA GGA AGG CAG CCT ACT CCC ATC TCT  
Ser Thr Asn Arg Gln Ser Gly Arg Gln Pro Thr Pro Ile Ser

(110)

(119)

CCA CCT CTA AGA GAC AGT CAT CCT CAG GCC  
Pro Pro Leu Arg Asp Ser His Pro Gln Ala

PRE-S2 REGION

(120)

ATG CAG TGG AAT TCC ACT GCC TTC CAC CAA GCT CTG CAG GAT  
Met Gln Trp Asn Ser Thr Ala Phe His Gln Ala Leu Gln Asp

(134)

CCC AGA GTC AGG GGT CTG TAT TTT CCT GCT GGT GGC TCC AGT  
Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly Gly Ser Ser

(148)

TCA GGA ACA GTA AAC CCT GCT CCG AAT ATT GCC TCT CAC ATA  
Ser Gly Thr Val Asn Pro Ala Pro Asn Ile Ala Ser His Ile

(162)

(174)

TCG TCA AGC TCC GCG AGG ACT GGG GAC CCT GTG ACG AAC  
Ser Ser Ser Ser Ala Arg Thr Gly Asp Pro Val Thr Asn

S PROTEIN

(175)

ATG GAG AAC ATC ACA TCA GGA TTC CTA GGA CCC CTG CTC GTG  
Met Glu Asn Ile Thr Ser Gly Phe Leu Gly Pro Leu Leu Val

TTA CAG GCG GGG TTT TTC TTG TTG ACA AGA ATC CTC ACA ATA  
Leu Gln Ala Gly Phe Phe Leu Leu Thr Arg Ile Leu Thr Ile

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|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| CCG | CAG | AGT | CTA | GAC | TCG | TGG | TGG | ACT | TCT | CTC | AAT | TTT | CTA |
| Pro | Gln | Ser | Leu | Asp | Ser | Trp | Trp | Thr | Ser | Leu | Asn | Phe | Leu |
| GGG | GGA | TCA | CCC | GTG | TGT | CTT | GGC | CAA | AAT | TCG | CAG | TCC | CCA |
| Gly | Gly | Ser | Pro | Val | Cys | Leu | Gly | Gln | Asn | Ser | Gln | Ser | Pro |
| ACC | TCC | AAT | CAC | TCA | CCA | ACC | TCC | TGT | CCT | CCA | ATT | TGT | CCT |
| Thr | Ser | Asn | His | Ser | Pro | Thr | Ser | Cys | Pro | Pro | Ile | Cys | Pro |
| GGT | TAT | CGC | TGG | ATG | TGT | CTG | CGG | CGT | TTT | ATC | ATA | TTC | CTC |
| Gly | Tyr | Arg | Trp | Met | Cys | Leu | Arg | Arg | Phe | Ile | Ile | Phe | Leu |
| TTC | ATC | CTG | CTG | CTA | TGC | CTC | ATC | TTC | TTA | TTG | GTT | CTT | CTG |
| Phe | Ile | Leu | Leu | Leu | Cys | Leu | Ile | Phe | Leu | Leu | Val | Leu | Leu |
| GAT | TAT | CAA | GGT | ATG | TTG | CCC | GTT | TGT | CCT | CTA | ATT | CCA | GGA |
| Asp | Tyr | Gln | Gly | Met | Leu | Pro | Val | Cys | Pro | Leu | Ile | Pro | Gly |
| TCA | ACA | ACA | ACC | AAT | ACG | GGA | CCA | TGC | AAA | ACC | TGC | ACG | ACT |
| Ser | Thr | Thr | Thr | Asn | Thr | Gly | Pro | Cys | Lys | Thr | Cys | Thr | Thr |
| CCT | GCT | CAA | GGC | AAC | TCT | ATG | TTT | CCC | TCA | TGT | TGC | TGT | ACA |
| Pro | Ala | Gln | Gly | Asn | Ser | Met | Phe | Pro | Ser | Cys | Cys | Cys | Thr |
| AAA | CCT | ACG | GAT | GGA | AAT | TGC | ACC | TGT | ATT | CCC | ATC | CCA | TCG |
| Lys | Pro | Thr | Asp | Gly | Asn | Cys | Thr | Cys | Ile | Pro | Ile | Pro | Ser |
| TCC | TGG | GCT | TTC | GCA | AAA | TAC | CTA | TGG | GAG | TGG | GCC | TCA | GTC |
| Ser | Trp | Ala | Phe | Ala | Lys | Tyr | Leu | Trp | Glu | Trp | Ala | Ser | Val |
| CGT | TTC | TCT | TGG | CTC | AGT | TTA | CTA | GTG | CCA | TTT | GTT | CAG | TGG |
| Arg | Phe | Ser | Trp | Leu | Ser | Leu | Leu | Val | Pro | Phe | Val | Gln | Trp |

TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT TCA GCT ATA TGG  
 Phe Val Gly Leu Ser Pro Thr Val Trp Leu Ser Ala Ile Trp  
 ATG ATG TGG TAT TGG GGG CCA AGT CTG TAC AGC ATC GTG AGT  
 Met Met Trp Tyr Trp Gly Pro Ser Leu Tyr Ser Ile Val Ser

CCC TTT ATA CCG CTG TTA CCA ATT TTC TTT TGT CTC TGG GTA  
 Pro Phe Ile Pro Leu Leu Pro Ile Phe Phe Cys Leu Trp Val

(400)

TAC ATT TAA

Tyr Ile Stop

Viral transcription initiation signals located 5' to the translation initiation codons control the expression of each of the preS1, preS2 and S proteins in vivo. Upon translation in the mammalian cell, the proteins may be glycosylated, resulting in a set of surface antigens as demonstrated in the following table.

| <u>Protein</u> | <u>Length<br/>(amino<br/>acids)</u> | <u>Designation</u> | <u>Glycosylation<br/>of S-protein<br/>Sequences</u> | <u>Glycosylation<br/>of preS2-<br/>specific<br/>domains</u> |
|----------------|-------------------------------------|--------------------|-----------------------------------------------------|-------------------------------------------------------------|
| P24            | 226                                 | S-protein          | -                                                   | -                                                           |
| GP27           | 226                                 | S-protein          | +                                                   | -                                                           |
| GP33           | 281                                 | preS2-protein      | -                                                   | +                                                           |
| GP36           | 281                                 | preS2-protein      | +                                                   | +                                                           |
| P39*           | 389                                 | preS1-protein      | -                                                   | -                                                           |
| GP42*          | 389                                 | preS1-protein      | +                                                   | -                                                           |

\*: Subtype ayw; the preS1-proteins derived from adw-subtype are about 1.0 to 1.5 kDa larger, which is in agreement with an additional 11 amino acids at the N-terminal end (Heermann et al., 1984).

01  
02  
03 Since the DNA sequence of the surface antigen coding  
04 region became known, several attempts have been made to  
05 produce S protein by virtue of recombinant DNA  
06 technology. Burrell et al.(1979) and Murray et al.  
07 (1980) as well as several other researchers reported  
08 the expression of HBsAg in E. coli. Valenzuela et al.  
09 (1982) reported the expression of HBsAg in yeast.  
10 Following disruption of yeast cells transformed with a  
11 vector containing the S protein coding sequence under  
12 control of the yeast alcohol dehydrogenase I promoter,  
13 spherical particles containing HBsAg could be observed  
14 which are similar to those found in sera of individuals  
15 infected with HBV. In European Patent Application 0  
16 226 846 (Tschopp et al.) the production of HBV  
17 S-protein in the yeast Pichia is disclosed. Protein  
18 synthesis is under the control of a regulatory region  
19 responsive to methanol.

20  
21 However, although vaccines containing exclusively  
22 S-antigen particles are generally highly protective  
23 (Szmuness et al., 1980) some hosts respond poorly to  
24 such preparations. To overcome this unpredictable  
25 failure of the vaccination, systems for expression of  
26 preS2-protein and preS1-protein have been developed.  
27 PreS2 is known to be even more immunogenic than  
28 S-protein. For example, upon immunisation of mice with  
29 subviral particles bearing preS2-and S-specific  
30 epitopes, the antibody response to preS2 exceeds the  
31 anti-S-response (Milich et al., 1985). Furthermore it  
32 has been observed that immunisation with preS2  
33 containing particles may also induce an  
34 anti-S-response. The role of preS1- and preS2-proteins  
35 in immunisation and the specificity of the T- and  
36 B-cell recognition of HBV surface antigen proteins has  
37 recently been reviewed (Milich, 1988, with references

therein). Valenzuela et al. (1985) disclose the expression of the entire preS2- protein coding sequence in yeast transformed with a plasmid containing the corresponding viral sequences. It has to be noted, that in yeast transcription, initiation has to be directed by yeast promoters preceding the respective coding region, since the viral transcription initiation signals within the HBV-ORF are not recognised by the yeast transcription system.

Itoh et al. (1986) and others also disclose expression of the preS2-protein in yeast and assembly into particles. Dehoux et al. (1986) disclose expression of a 39 kD preS1-protein in yeast and state that this is assembled into a particle form. A report by Imamura et al. (1987) discloses expression in S. cerevisiae of both the preS2-protein and the preS1-protein. These authors state that the large protein was found as a relatively stable, non-glycosylated product of 42 kD molecular weight which was not readily assembled into particles.

Glycosylation of the surface proteins produced in the yeast S. cerevisiae differs from glycosylation observed in naturally occurring particles isolated from individuals infected with HBV. The S-protein is not glycosylated, whereas preS2-protein and preS1-protein are produced in N-linked glycosylated and non-glycosylated forms. N-linked chains of the high mannose type were identified, as well as O-linked oligosaccharide chain(s) (Itoh et al., 1986; Langley et al., 1988).

Expression of HBV surface antigen in mammalian cells has also been performed. Michel et al. (1984)

disclosed the synthesis of HBsAg in Chinese Hamster ovary (CHO) cells transfected with a plasmid carrying the preS2-protein coding sequence. Persing et al. (1985) disclosed the expression of three HBsAg related polypeptides of 24,000, 27,000 and 35,000 daltons in mouse L cells transformed with a preS2-protein coding sequence. The three polypeptides obtained may be organised into complex immuno reactive HBsAg particles of about 22 nm diameter.

McLachlan et al. (W088/06185) reported on the expression of various HBV related proteins in vertebrate cells. However, the expression of preS-antigen in mammalian cells is generally hampered by the fact that due to relative overproduction of preS1-protein relative to S-protein secretion of HBsAg particles is inhibited (Ou et al., 1987). Moreover the ratio of the different S related proteins cannot be controlled. Finally culturing of animal cells is an expensive approach which results in high costs for the product obtained.

Although vaccines presently described and in use have great efficacy, a certain percentage of persons receiving such vaccines, particularly immunocompromised persons, e.g., hemodialysis patients, are non- or slow responders [See, e.g., Hadler et al, New Engl. J. Med., 315:209-215 (1986); and Bruguera et al, Post Grad. Med. J., 63:155-158 (1987)].

There thus remains a need in the art for methods and compositions useful in preparing additional effective vaccines to HBV.

01  
02  
03 According to a first aspect of the present invention  
04 there is provided a microorganism selected from the  
05 group of methylotrophic yeast genera, carrying  
06

07 A) more than one copy of an expression cassette (ec1)  
08 coding for a first polypeptide and/or one or more than  
09 one copy of an expression cassette (ec2) coding for a  
10 second polypeptide, optionally in addition one or more  
11 than one expression cassette (ec3) coding for a third  
12 polypeptide, or  
13

14 B) one copy of an expression cassette (ec1) coding  
15 for a first polypeptide and one or more than one copy  
16 of an expression cassette (ec2) coding for a second  
17 polypeptide, optionally in addition one or more than  
18 one expression cassette (ec3) coding for a third  
19 polypeptide,  
20

21 wherein the expression cassettes comprise:  
22

23 a) a regulon R which is responsive to methanol and/or  
24 depletion of catabolite repressing carbon sources;  
25

26 b) an open reading frame coding for all or part (or  
27 parts) of a protein having the biological activity of  
28 one of the hepatitis B surface antigens;  
29

30 c) optionally a DNA sequence serving as a  
31 transcription terminator T;  
32

33 wherein R exerts control on the transcription of the  
34 open reading frame and T directs polyadenylation and/or  
35 termination of transcription of a produced mRNA.  
36

01  
02  
03 The advantage of the above aspect of the invention is  
04 that a means is provided for obtaining composite  
05 particles containing various ratios of pre S-and  
06 S-protein thereby allowing the large scale production  
07 of these composite particles at low costs.  
08

09 The present invention also provides a composite  
10 particle containing at least two polypeptides  
11 corresponding to all or part of a protein having the  
12 biological activity of a hepatitis B surface antigen,  
13 wherein the particle presents at least two antigenic  
14 determinants provided by the S-protein, preS2-protein  
15 or preS1-protein, said particle optionally further  
16 comprising host specific lipids.  
17

18 The following terms used throughout the specification  
19 are defined as follows:  
20

21 An ''expression cassette'' is defined as any discrete  
22 region of DNA which functions in a host cell as a  
23 complete gene expression unit.  
24

25 A ''complete gene expression unit'' is a structural  
26 gene and the promoter and regulatory regions required  
27 for its transcription and translation.  
28

29 A ''functional DNA coding region'' means a DNA coding  
30 sequence which, when fused in phase to the S protein  
31 coding sequence, does not interfere with the assembly  
32 of an HBsAg mixed particle.  
33

34 A ''functional derivative'' means a coding sequence  
35 having amino acid alterations which do not interfere  
36 with particle formation and which retain immunogenicity  
37 or other functionality. Such functional derivatives

can be prepared by conventional site specific mutagenesis or by other standard techniques.

A ''vector'' is defined as DNA which can carry and maintain the DNA fragment of the invention, including, for example, phages and plasmids. These terms are understood by those of skill in the art of genetic engineering.

The invention, which comprises further subjects, is now described in a more detailed manner by the following description, examples and figures.

Brief Description of the Figures:

Figure 1:

Construction of plasmid pME4 from pHARS1. The HARS1 fragment has been transferred to the single PvuII site of former pHARS1, the sequences between the former position of HARS and the beta-lactamase gene as well as the 5' end of the beta-lactamase gene itself have been deleted.

Figure 2:

Schematic representation of lambda-phages carrying genomic Hansenula polymorpha DNA fragments comprising

- a) the MOX gene
- b) the FMD gene.

Figure 3:

Map of plasmid pT-24. This plasmid contains a DNA fragment encompassing the terminator of the MOX gene

cloned into the SmaI-site of the pUC19 multiple purpose cloning site.

Figure 4:

Maps of plasmid pRB-S-269 and pRB-S-271 expressing the S-gene under control of the MOX- or FMD-promoter, respectively.

Figure 5:

Maps of plasmids pBC, pMS-2, pFS-9. The latter two plasmids are derived from pBC containing the Hansenula polymorpha URA3 gene by insertion of expression cassettes containing the MOX promoter (pMS-2) or the FMD promoter (pFS-9) into the URA3 gene coding sequence.

Figure 6:

Map of plasmid pHK2. This plasmid contains a kanamycin gene (derived from Tn5) under control of the ADH1-promoter of Saccharomyces cerevisiae. In addition plasmid pHK2 comprises the HARS1 sequence.

Figure 7:

Maps of plasmids pRB-S-322 and pRB-S-326. The functional unit consisting of the kanamycin resistance gene and ADH1-promoter as present on plasmid pHK was inserted into the HARS regions of plasmid pRB-S-269 and pRB-S-271.

Figure 8:

Construction of pMPS-22 from pMOX-P/T1. The preS gene of Hepatitis B virus was inserted into the EcoRI site

01  
02  
03 of plasmid pMOX-P/T1. In the resulting plasmid pMPS-22  
04 the preS gene is regulated by the MOX promoter.  
05

06 Figure 9:

07  
08 Map of plasmid pMPS-9. For the construction of this  
09 plasmid the preS expression cassette of pMPS-22 was  
10 inserted between the XhoI and StuI site of the  
11 Hansenula fragment of original plasmid pBC.  
12

13 Figure 10:

14  
15 Map of the Hansenula polymorpha URA3 gene and adjacent  
16 DNA-regions.  
17

18 Figure 11:

19  
20 A diagram illustrating the structure of plasmid pMPT121  
21 in which foreign gene sequences can be inserted under  
22 the control of the MOX promoter.  
23

24 Figure 12:

25  
26 A diagram illustrating the structure of plasmid pKL\*1  
27 containing an expression cassette for L\* protein under  
28 control of the MOX promoter.  
29

30 Figure 13:

31  
32 A schematic diagram showing the steps for constructing  
33 an E. coli plasmid harbouring a Gly13 deleted L  
34 protein-expression cassette, pRIT12998.  
35

01  
02  
03 Figure 14:  
04

05 A schematic diagram illustrating the procedure for  
06 obtaining a yeast plasmid pRIT12979 capable of  
07 expressing a Gly13 deleted L protein.  
08

09 Figure 15:  
10

11 A schematic diagram illustrating the construction of  
12 the plasmid pRIT13191 containing the coding sequence  
13 for an L protein deleted for amino acids 53-132 and  
14 146-174.  
15

16 Figure 16  
17

18 A schematic diagram illustrating the construction of  
19 yeast expression plasmids pRIT13192 and pRIT13193  
20 containing the expression cassettes for the L\* and  $\Delta$ Gly  
21 L\* proteins respectively.  
22

23 The microorganism according to the first aspect of the  
24 invention may be any member of a methylotrophic yeast  
25 genus, which has been manipulated to carry more than  
26 one copy of an expression cassette ec1 and optionally  
27 one or more than one expression cassette ec2 and/or  
28 optionally one or more than one expression cassette ec3  
29 or at least one expression cassette of two or more  
30 different types. The expression cassettes need only  
31 differ in the nature of the encoded protein to be  
32 expressed and in general comprise the following  
33 components:  
34

- 35 a) A regulon R which is responsive to methanol and/or  
36 depletion of catabolite repressing carbon  
37 sources. The term 'regulon' as used throughout

01  
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03 the application comprises any cis-acting DNA  
04 involved in the regulation of expression of a  
05 given structural gene. The term thus embraces  
06 sequences preceding a given coding sequence, for  
07 example, a promoter and sequences which are  
08 recognised by transcription factors or other  
09 proteins involved in transcription regulation.  
10 The meaning of the term 'regulon' is thus not  
11 limited to sequences corresponding to known  
12 promoters. Also included are DNA sequences  
13 exhibiting the same responsiveness to methanol as  
14 known regulons of methanol inducible genes, i.e.  
15 all functionally equivalent sequences.  
16

17 Recently several regulons exhibiting the above  
18 outlined properties became known. For example  
19 Ledeboer et al. (1985) disclose the regulatory DNA  
20 sequence preceding the methanol oxidase (MOX)  
21 structural gene of Hansenula polymorpha. In EP 85  
22 201 235.0 to Unilever NV, a system for the  
23 expression of proteins is disclosed, which  
24 involves the use of the promoters regulating the  
25 expression of methanol oxidase and DHAS in vivo.  
26 In EP 87 110 417 to Rhein Biotech GmbH, a system  
27 suitable for expression of foreign proteins under  
28 control of the formate dehydrogenase promoter  
29 (FMD) is proposed. Ellis et al. (1985) disclose  
30 the isolation of methanol regulatable genes from  
31 Pichia pastoris. These and other promoters  
32 obtained or obtainable from genes of  
33 methylotrophic yeast, which are potentially  
34 involved in methanol utilisation, all have in  
35 common that they respond to either addition of  
36 methanol and/or depletion of a carbon source with

enhanced synthesis of the respective gene regulated by them. Also included are all DNA sequences which exhibit the same responsiveness.

b) An open reading frame (ORF) coding for part(s) or all of the protein having the biological activity of one of the hepatitis B surface antigens. As already mentioned, hepatitis B surface antigens of given subtype comprise a set of about six different proteins, differing in length and glycosylation pattern. These proteins exhibit different antigenic determinants and also seem to have different functions for the viable virus. For eliciting a preS immune response it is not necessarily required to provide the respective complete preS-sequence, but it may be sufficient to include just those codons encoding the relevant epitope into the open reading frame. Furthermore a protein having the biological activity of one of the hepatitis B surface antigens also includes proteins having undergone exchanges of amino acids which do not interfere with their biological activity.

c) Optionally a DNA sequence serving as a terminator T. As the terminator any sequence may be used, which efficiently terminates transcription in the respective host organism. Terminators may be for example sequences prone to give formation of hairpin structures and/or polyadenylation sites in transcribed RNA. In a preferred embodiment the terminator is derived from a methanol responsive gene of the same organism and/or from the same gene as the regulon was obtained.

01  
02  
03 The three components are arranged in operable linkage  
04 in order to enable the regulon to regulate  
05 transcription and the terminator to terminate  
06 transcription of the open reading frame between them.  
07

08 The microorganism according to the present invention  
09 has the invaluable advantage, that because of the  
10 presence of more than one copy of an expression  
11 cassette coding for a first polypeptide and/or one or  
12 more than one copy of each of the different additional  
13 expression cassettes, transcription and translation of  
14 the encoded protein(s) may occur in highly enhanced  
15 amounts. The microorganism according to the present  
16 invention provides the possibility to obtain any  
17 desired ratio of a first to a second polypeptide within  
18 the cell. Thus, upon assembly of protein to form  
19 particles composite particles of any composition may be  
20 obtained. Furthermore owing to the production of high  
21 amounts of different proteins in close proximity these  
22 proteins are more liable to form particles comprising  
23 more than one polypeptide species than particles known  
24 in the art.  
25

26 The microorganism according to the present invention  
27 preferably expresses all or part of the S-protein from  
28 a first expression cassette (ec1) since this  
29 constitutes the structural scaffold of any particle  
30 which may be formed. In one embodiment the  
31 microorganism further contains a second expression  
32 cassette (ec2) which expresses a polypeptide  
33 representing all or part of the preS1-protein, for  
34 example the preS1 specific domains localised at the  
35 N-terminal end of this protein or all or part of the  
36 preS2-protein. In a further embodiment the

01  
02  
03 microorganism may contain three different types of  
04 expression cassette to enable simultaneous expression  
05 of three polypeptides comprising all or part of the  
06 S-protein, all or part of the preS1-protein and all or  
07 part of the preS2-protein.

08  
09 Furthermore, the use of regulons derived from  
10 methylotropic yeast enables the repression,  
11 derepression or induction of a cell culture by addition  
12 or depletion of a carbon source or by addition of  
13 methanol which is cheaply available.

14  
15 The group of methylotrophic yeasts comprises members of  
16 the following genera: Candida, Kloeckera,  
17 Saccharomyces, Rhodotorula, Torulopsis and Pichia,  
18 preferably Hansenula. In the most preferred embodiment  
19 a microorganism according to the present invention is  
20 derived from Candida boidinii, Pichia pastoris or  
21 Hansenula polymorpha. Yeasts from these genera are  
22 well known to persons skilled in the art and their  
23 general culturing and growth conditions are  
24 established.

25  
26 A useful strain of the preferred methylotrophic yeast  
27 Hansenula may be any strain of the species Hansenula  
28 polymorpha. In the most preferred embodiment the  
29 strain to be used has the identifying characteristics  
30 of Hansenula polymorpha RB 10(DSM 5215). RB 10 is a  
31 ura3 mutant of Hansenula polymorpha ATCC 34438.

32  
33 In a preferred embodiment the microorganism according  
34 to the present invention is capable of efficient  
35 utilisation of glycerol as a carbon source. Glycerol  
36 is a non-catabolite repressing carbon source which is

01  
02  
03 converted into energy with different efficiency by  
04 known methylotrophic yeasts. Yeast species of the  
05 genus Hansenula are able to metabolise glycerol with  
06 high efficiency, whereas S. cerevisiae grows poorly on  
07 this carbon source. In addition, growth of Hansenula  
08 polymorpha on glycerol results in very little ethanol  
09 by-production which otherwise causes problems during  
10 large-scale fermentation.

11  
12 The regulons used for the expression cassettes of the  
13 present invention are preferably derived from yeast of  
14 the well known genera mentioned above. Regulons which  
15 have been characterised in detail are preferred, such  
16 as the regulating region from various methanol  
17 responsive genes of Hansenula polymorpha, for example  
18 the methanol oxidase gene, the FMD gene, DAS gene or  
19 catalase gene. The regulating regions of most of these  
20 genes are already sequenced and the sequences published  
21 (e.g. Janowicz et al., 1985; Ledebøer et al., 1985, EP  
22 87 110 417). The technical procedure of isolating  
23 these regulons from appropriate microorganism is  
24 facilitated by the knowledge of the respective DNA  
25 sequences and corresponding restriction patterns.

26  
27 As a terminator any sequence may be included which  
28 efficiently serves to terminate the nascent mRNA  
29 regardless of whether it is derived from a eukaryotic  
30 or prokaryotic gene. Preferred terminators are derived  
31 from yeast genes. Use of termination sequences  
32 obtained from the methanol responsive genes cited above  
33 is most preferred.

34  
35 It is another advantage of the microorganism according  
36 to the present invention that a surprisingly high

01  
02  
03 number of expression cassettes can be introduced into  
04 the respective host cell and be stably maintained. In  
05 general, there are two possibilities to achieve stable  
06 maintenance of specific genetic information:

07  
08 The information is either encoded chromosomally or it  
09 is localised on an autonomously replicating vector,  
10 which may be a plasmid, a cosmid, a virus or the like.  
11 Normally higher gene dosages are provided by  
12 transformation of the desired host organism using high  
13 copy number autonomously replicating plasmids or  
14 lysogenic viruses. These plasmids or viruses are well  
15 known in the art and usually contain a replicon and a  
16 selective marker gene enabling replication and stable  
17 maintenance of the vector. However, viruses are often  
18 subject to conversion to lytic cycles and plasmids are  
19 prone to being lost under certain conditions and  
20 furthermore often require continuous selection  
21 pressure. Moreover even with high copy number plasmids  
22 a copy number of not more than about 50 to 75 can  
23 generally be obtained in yeast. On the other hand,  
24 introduction of desired genetic information, e.g. an  
25 expression cassette, into the host genome normally  
26 requires a certain degree of homology between at least  
27 part of the DNA sequence to be inserted, preferably the  
28 termini of said molecule, and the site of insertion.  
29 Thus the number of potential integration sites for  
30 homologous recombination of a given molecule is  
31 limited, in most cases to just one. This number is  
32 slightly more elevated for homologous recombination  
33 with genes present in multiple copies or with  
34 transposon-like structures such as Ty (Jacobs et al.,  
35 1988). In order to promote the integrative  
36 recombination event, it is preferable to use vectors

01  
02  
03 lacking a replicon but carrying a selective marker  
04 gene, which confers a detectable phenotypic trait to  
05 the transformed cell and allows identification under  
06 selective conditions.

07  
08 Random recombination is normally a rarely observed  
09 event. However according to the present invention, it  
10 is possible to introduce numerous copies of any given  
11 DNA sequence into one or more chromosomes of the  
12 genome, by insertion due to random recombination. To  
13 obtain transformants carrying as many as some hundred  
14 copies of the DNA sequence desired, it is preferable to  
15 use autonomously replicating plasmids for  
16 transformation, which by the simultaneous events of  
17 integration of some copies and replication of others  
18 can provide a large pool of integratable copies, which  
19 are inserted during subsequent cell cycles.  
20 Surprisingly it was possible to obtain methylotrophic  
21 yeast strains carrying many expression cassettes.

22  
23 Preferred yeast strains, such as yeast strains of the  
24 genus Hansenula, contain about 2 - 500, preferably 15  
25 to 200, especially 20 to 150 copies of a desired DNA  
26 sequence. Once the desired number of integrated  
27 expression cassettes is obtained further integration  
28 processes can be prevented by cycles of growth on  
29 non-selective medium to segregate out the remaining  
30 plasmid copies.

31  
32 A special feature of microorganisms according to the  
33 present invention is the phenomenon of multimeric  
34 integration. Upon transformation with a vector capable  
35 of autonomous replication Hansenula polymorpha was  
36 found to integrate several repeats of the plasmid used  
37 for transformation. Repetitions of up to about 100  
38 copies of the introduced plasmid were observed. This

allows the presence and integration of a high number of copies of foreign DNA sequences without jeopardising the cell's viability by insertion into essential genes. Surprisingly integrants containing multimeric DNA were shown to be stable.

For the purpose of designing vaccines containing composite particles a constant ratio of the different polypeptides of said particles is desirable. In order to meet this need the present invention provides microorganisms having a selected ratio of different expression cassettes. For instance, a microorganism according to the present invention may carry between 1 to 100 copies of an expression cassette (ec1) encoding all or part of a protein having the biological activity of S-protein and 1 copy or several copies of an expression cassette (ec2) encoding a protein having the biological activity of preS2- or preS1-protein.

Preferred microorganisms carrying a ratio of between 2 copies of ec1 to one copy of ec2 to 30 copies of ec1 to one copy of ec2 more especially in the ratio of 5 to 10 copies of ec1 to one copy of ec2 or 6-8 copies of ec1 to one copy of ec2. A third expression cassette can be introduced in the same ratio as ec2 or 6-8 copies of ec1 to one copy of ec3.

In addition to the possibility of preparing strains containing integrated copies of the expression cassette, it is also possible to use microorganisms containing autonomously replicating vectors comprising autonomously replicating sequences (ARS) and the desired expression cassette. Presence and maintenance of vectors containing ARS are well known in the art.

Subjects of the present invention are also DNA

01  
02  
03 molecules containing an expression cassette (ec1)  
04 coding for a first polypeptide and an expression  
05 cassette (ec2) coding for a second polypeptide and/or  
06 an expression cassette (ec3) coding for a third  
07 polypeptide, wherein the expression cassettes comprise:

08  
09 a) a regulon R which is responsive to methanol and/or  
10 depletion of catabolite-repressing carbon sources;

11  
12 b) an open reading frame coding for part (or parts)  
13 or all of a protein having the biological activity of  
14 one of the hepatitis B surface antigens;

15  
16 c) optionally a DNA sequence serving as a terminator  
17 T;

18  
19 wherein R exerts control on the transcription of the  
20 open reading frame and T directs polyadenylation and/or  
21 termination of transcription of the mRNA produced.

22  
23 For the properties of the regulon, the open reading  
24 frame and the terminator referral is made to the above  
25 provided information. Also for these molecules it is  
26 preferable to derive both regulon and terminator from a  
27 methylotrophic yeast selected from the genera Candida,  
28 Kloeckera, Saccharomyces, Rhodotorula, Torulopsis,  
29 Pichia and Hansenula. In the most preferred embodiment  
30 the regulon and the terminator are derived from a gene  
31 involved in methanol utilisation, for example the  
32 MOX-gene, the FMD gene, the DAS gene or the catalase  
33 gene. Hansenula polymorpha is a preferred  
34 microorganism as a source for these genes. For the  
35 identity of the polypeptides encoded by ec1, ec2 and  
36 ec3, referral is made to the explanation given above.

37  
38 The DNA molecule according to the present invention may

01  
02  
03 be composed of fragments obtained from the respective  
04 natural sources. The DNA coding for any of the  
05 hepatitis B surface antigens can be isolated from DNA  
06 of intact viruses, also called Dane particles.  
07 Isolation, cloning and sequencing of HBV virion DNA are  
08 disclosed in Charnay et al. (1979), Galibert et al.  
09 (1979), Sninsky et al. (1979) Valenzuela et al. (1979).  
10

11 The choice of restriction enzymes for cloning and DNA  
12 manipulation depends on the kind of subtype of  
13 hepatitis virus used. The subtypes differ not only by  
14 length, but also with respect to their individual  
15 restriction patterns which are subject to normal  
16 mutation events. It is possible for any person skilled  
17 in the art to identify the restriction fragment  
18 carrying the desired coding region, for example by  
19 cleavage of the isolated DNA using different sets of  
20 restriction endonucleases, subjecting the samples to  
21 gel electrophoresis and Southern blotting or equivalent  
22 methods and identifying the restriction fragment using  
23 a probe corresponding to a known surface gene  
24 sequence. If necessary, the DNA may be sequenced and  
25 further manipulated, for example by treatment with  
26 endonucleases, in vitro mutagenesis, primer repair or  
27 other known techniques in order to create a fragment  
28 having the desired size, carrying the desired sequence  
29 and having the appropriate termini. As is known to any  
30 one skilled in the art, the termini may be trimmed to  
31 have any desired sequence by digestion with  
32 exonucleases, filling in with the Klenow fragment of  
33 DNA-polymerase I and addition or substitution of DNA by  
34 the use of synthetic oligodeoxyribonucleotides as  
35 linkers and adapters.  
36

37 The DNA sequence coding for the respective hepatitis

01  
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03 surface antigen may then be further combined with an  
04 appropriate regulon and/or terminator, which are  
05 obtained by known techniques from the genome of a  
06 methylophilic yeast. The respective fragments are  
07 adapted accordingly, in order to be in a suitable  
08 position relative to the translation initiation codon  
09 and the translation termination codon of the coding DNA  
10 sequence. Ligation is also carried out according to  
11 known procedures. Since these methods are well known  
12 in the art, no further explanations are provided. For  
13 a detailed description of techniques used in molecular  
14 cloning, 'Molecular Cloning' by Maniatis et al.  
15 (1982) may be consulted.

16  
17 Instead of ligating DNA sequences isolated exclusively  
18 from the respective natural sources, it is also  
19 possible to synthesise part or all of the desired  
20 expression cassette by means of chemical synthesis.  
21 Chemical synthesis may be conducted according to known  
22 procedures. Chemical synthesis of long  
23 oligodeoxyribonucleotides is already offered on a  
24 commercial basis. Furthermore, any combination of  
25 fragments synthesised chemically and fragments obtained  
26 from the respective natural source may be appropriate  
27 for the purposes of the present invention.

28  
29 A further subject of the present invention is the  
30 provision of the URA3 gene obtained from a  
31 methylophilic yeast. The gene was identified by  
32 complementation of the corresponding pyr F mutation of  
33 E. coli strain MB 1000 with plasmids constructed by  
34 random insertion of Hansenula polymorpha restriction  
35 fragments into a suitable vector. A plasmid was  
36 identified which complemented the deficiency of the  
37 recipient strain. The newly identified gene comprises

approximately 1400 bp by measurement on agarose gels.  
Its restriction pattern is shown in Figure 10 below.

In order to facilitate the construction the  
amplification and introduction of the expression  
cassette into the host microorganism it is advisable to  
insert this onto a vector suitable for transformation  
of a methylotrophic yeast. This vector may either be a  
linear or circular plasmid or a virus. It is possible  
to use so called integrative vectors, i.e. vectors  
lacking any autonomously replicating sequence (ARS).  
If these vectors are not integrated they will be lost  
during subsequent cell cycles.

In one preferred embodiment the vector also comprises  
such ARS DNA sequences capable of directing autonomous  
replication. Preferably these sequences are derived  
from the respective host microorganism. In the case of  
methylotrophic yeast there are several ARS described,  
for example HARS, and PARS (Roggenkamp et al., 1986;  
Cregg et al., 1985). These sequences contain a region  
of DNA which functions to stably maintain the vector as  
an extrachromosomal entity within the host cell.

In order to facilitate the identification of  
transformed host organisms it is further preferred to  
provide selective marker-genes suitable for selection  
of the desired microorganism following transformation.  
In yeast plasmids known in the art these selective  
marker-genes often correspond the DNA sequences coding  
for enzymes involved in the amino acid or nucleic acid  
metabolism, which are defective in the recipient host  
organism to be transformed.

The same strategy may be applied for selection of  
transformed methylotropic yeasts. Genes, which are

commonly used for selection of auxotrophic Saccharomyces cerevisiae strains include the LEU2, HIS3, TRP5, ARG4 and URA3 genes. Genes obtained from Saccharomyces cerevisiae may also be used as selective markers in transformation of methylotrophic yeast; however, growth of such transformants on minimal medium could be considerably impaired. Therefore, in a preferred embodiment of the present invention, methylotrophic yeast strains deficient in the function of any one gene involved in normal metabolism are transformed with a DNA molecule carrying a wild type homologous marker gene. The present invention provides for the first time a homologous marker gene for selection for transformants in Hansenula polymorpha, namely the URA3 gene. The characteristics of this gene, i.e. its restriction fragment pattern, is discussed above.

As an alternative way of selecting transformants use can be made of antibiotic resistance genes. This is a well-known approach for bacterial transformation and has been shown to be applicable to S. cerevisiae (Jimenez and Davies, 1980).

However, for methylotrophic yeasts so far nothing is known regarding their behaviour upon contact with antibiotics such as chloramphenicol or gentamycin G418. This is believed to be the first report of selection of transformed methylotrophic yeast by subjecting the same to selective concentrations of growth inhibiting aminoglycoside antibiotics following the transformation procedure. Surprisingly, the aminoglycoside phosphotransferase derived from Tn5 (Grindley, 1980) could be shown to be active also in methylotrophic yeasts when regulated by a promoter obtained from bakers yeast. Inactivation of G418 is

01  
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03 effective also in strains where only one or two copies  
04 of the gene are present in the cell, i.e. the gene is  
05 not required in a high copy number. According to the  
06 present invention this is a very efficient  
07 transformation/selection system in methylotrophic  
08 yeasts.

09  
10 In the most preferred embodiment the vector according  
11 to the present invention is selected from the vectors  
12 pMS-2, pFS-9, pRB-S, pRB-S-269, pRB-S-271, pRB-S-322,  
13 pRB-S-326, pMPS22, pMPS21, pMPS9. The construction and  
14 use of these vectors will be described below in more  
15 detail.

16  
17 A further subject of the present invention is a process  
18 for the preparation of a microorganism as described  
19 above, where a methylotrophic yeast host organism is  
20 transformed with a vector carrying the expression  
21 cassettes ec1 and/or ec2 and/or ec3 and wherein upon  
22 replication of the vector at least some of the  
23 expression cassettes are integrated by recombination  
24 with the chromosome of the yeast host organism,  
25 preferably as a multimeric repeat. As a consequence,  
26 the transformed methylotrophic organism may contain  
27 several copies of any introduced expression cassette  
28 dispersed over the whole genome, but also with a high  
29 probability that multimeric repeats of the expression  
30 cassettes are localised in one site of the genome.  
31 This type of recombination is found to occur especially  
32 in Hansenula. However, the methylotrophic yeast host  
33 organism may also be selected from any other species of  
34 methylotrophic yeast genera, for example, Candida,  
35 Kloeckera, Saccharomyces, Rhodotorula, Torulopsis and  
36 Pichia.

37  
38 In the most preferred embodiment the host organism is

selected from the species Hansenula polymorpha, exemplified by a strain having the identifying characteristics of Hansenula polymorpha RB 10. Hansenula polymorpha RB 10 was deposited with the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' on February 17, 1989 and received the accession No. DSM5215. H. polymorpha DSM 5215 forms a further aspect of this invention.

While it is preferred to perform the transformation using any one of the above-described vectors according to the present invention, it will be appreciated that other vectors may be constructed and used.

In order to obtain microorganisms containing more than one copy of an expression cassette (ec1) and optionally one or more than one copy of a second expression cassette (ec2), the following process comprising several steps is the most convenient way. According to the process, the methylotrophic host organism a) is transformed with a first vector and initially grown on selection medium, followed by incubation under non-selective conditions for several generations, b) mitotically stable transformants containing more than one expression cassette (ec1) coding for a first polypeptide are selected, c) said transformants are optionally cured of residual autonomously replicating plasmids, d) said transformants are optionally transformed with a second vector and again initially grown on selective medium, followed by incubation under non-selective conditions for several generations, e) mitotically stable transformants containing more than one expression cassette (ec1) coding for a first polypeptide and optionally one or more than one expression cassette (ec2) coding for a second polypeptide are selected, f) said transformants are

01  
02  
03 optionally cured of residual autonomously replicating  
04 plasmids, steps d) to f) are optionally repeated using  
05 a third vector.  
06

07 According to this process, it is possible to increase  
08 the number of expression cassettes of one type, for  
09 instance, ecl, by subjecting the cells to several  
10 subsequent cycles of transformation, growth under  
11 selective/non-selective conditions, selection of  
12 transformants and optionally curing of free plasmids.  
13 Alternatively, two or more types of expression  
14 cassettes can be introduced by either using a single  
15 type of vector containing two or more different  
16 expression cassettes or subjecting the cells to the  
17 above described cycles, wherein transformation is  
18 performed using different vectors, carrying different  
19 expression cassettes. The process according to the  
20 present invention thus provides a variety of  
21 possibilities in order to obtain a desired  
22 microorganism.  
23

24 A subject of the present invention is also a composite  
25 particle, comprising at least two polypeptides  
26 corresponding to part or all of a protein having the  
27 biological activity of one of the hepatitis B surface  
28 antigens, wherein the particles present at least two  
29 antigenic determinants provided by the S-protein,  
30 preS2-protein or preS1-protein, said particle  
31 optionally containing host specific lipids. In  
32 general, the particles are made of a majority of a  
33 protein having the antigenic characteristics of the  
34 S-protein, which also constitutes about 80 to 90% of  
35 naturally occurring 20 nm particles. The composite  
36 particles further may comprise either any protein  
37 having the antigenicity of preS1-protein specific  
38 domains, i.e. peptides corresponding to at least part

01  
02  
03 of the N-terminal 108 (or 119) amino acids of  
04 preS1-protein, or/and any protein having the  
05 antigenicity of preS2 specific domains, i.e. peptides  
06 corresponding to at least part of the 55 amino acid  
07 preS2-specific domain.

08  
09 The presence of two or more different proteins having  
10 the biological activity of hepatitis B surface antigens  
11 in one particle is a new approach for the design of  
12 yeast-derived anti-hepatitis B vaccine. Yeast-derived  
13 particles or aggregates obtained so far contain  
14 exclusively either the S protein, or the preS2-protein  
15 or the preS1-protein, i.e. only one of the three  
16 possible proteins. While it has been shown by  
17 Valenzuela et al. (1985) and Itoh et al. (1986) that  
18 preS2-protein is assembled into particles,  
19 preS1-protein is reported to form either a mixture of  
20 small particles (2 or 3 nm in diameter) or a  
21 polydisperse population of large aggregates some 15 to  
22 50 nm in diameter (Kniskern et al., 1988; Imamura et  
23 al., 1987).

24  
25 Rutgers et al. (1988) report that on preS2-protein and  
26 preS1-protein particles or aggregates, the S-protein  
27 encoded epitopes are masked as these particles are less  
28 reactive in the AUSRIA assay. This may have a  
29 deleterious effect on the immune response with respect  
30 to S-protein when particles composed of homogenous preS  
31 protein species are used as immunogens for vaccine  
32 purposes.

33  
34 Thus, the present invention provides for the first time  
35 composite particles comprising more than one species of  
36 hepatitis B surface antigen. By analogy with particles  
37 comprising only one protein produced in S. cerevisiae,  
38 the particles according to the present invention will  
39 normally comprise host-specific lipids.  
40

01  
02  
03 In a preferred embodiment the composite particles  
04 according to the present invention comprise more than  
05 1% preS1-protein, the rest of the protein being  
06 S-protein and optionally some preS2-protein. Preferred  
07 particles, however, contain 1-50%, especially 1-25%  
08 preS1-protein, the rest being the same as above. In  
09 especially preferred embodiments, the ratio of  
10 preS1-protein to S-protein is between 1:1 and 1:100,  
11 preferably 1:2, 1:8 or 1:15.

12  
13 The particles according to the present invention are at  
14 least partially glycosylated. A proportion of the pre  
15 S1-proteins in methylotrophic yeasts are glycosylated,  
16 as indicated by the detection of bands with molecular  
17 weights of 45 kD, 38 kD and 39 kD. It will be  
18 appreciated by one skilled in the art that the number  
19 and size of preS1 protein species observed by  
20 immunoblotting may present some apparent variability.  
21 Detection of the 45, 38 and 39 kD bands will depend,  
22 inter alia, on their resolution during polyacrylamide  
23 gel electrophoresis, interference from other yeast  
24 proteins and variations in the efficacy of the  
25 procedures used in immunoblotting. Further, the method  
26 chosen for the preparation of crude cell extracts and  
27 the specific composition of the buffer used for  
28 extraction may also influence the detection of a  
29 particular band. The relative proportions of the three  
30 species may also vary with growth conditions.  
31 Furthermore, estimates of molecular weight are also  
32 subject to experimental variation and depend on the  
33 standards used for calibration. Glycosylation of  
34 S-protein has not been observed.

35  
36 The composite particles described above are  
37 conveniently prepared by cultivating a methylotrophic  
38 yeast strain according to the present invention in a

01  
02  
03 suitable culture medium. Using a regulated system of  
04 expression, the synthesis of particle components can be  
05 controlled to avoid their expression affecting cell  
06 growth. Thus at any desired cell density, methanol  
07 regulon-controlled protein synthesis may be derepressed  
08 to permit expression of the heterologous protein. Upon  
09 coordinated expression of different types of hepatitis  
10 B surface antigens in close proximity, particle  
11 formation occurs. These particles can be isolated from  
12 the culture by means of well-known techniques, for  
13 example isopycnic centrifugation, chromatography and  
14 other methods known in the art.

15  
16 The same method may also be applied in order to obtain  
17 particles or aggregates comprising only one species of  
18 surface antigen, when appropriate strains are used.

19  
20 In a preferred embodiment, protein synthesis directed  
21 by the expression cassette starts upon depletion of  
22 repressing carbon sources. The cells, which were  
23 previously subject to catabolite repression, begin  
24 expression upon dilution of the repressing agent. The  
25 effect of derepression can be further enhanced by  
26 addition of methanol, which results in induction of a  
27 methanol responsive regulon. Addition of methanol  
28 results in up to 10 fold enhancement of heterologous  
29 protein synthesis.

30  
31 In a preferred embodiment, the culture medium contains  
32 glycerol as a carbon source. Glycerol is a carbon  
33 source having a minimal catabolite repressing effect.  
34 Glycerol concentrations of up to 0.3% do not effect  
35 repression. Therefore, when using a culture medium  
36 containing glycerol, this carbon source does not have  
37 to be removed completely before the optional addition  
38 of methanol.  
39

01  
02  
03 Fermentation of the methylotrophic yeast strains  
04 according to the present invention is carried out  
05 between 30 to 40°C using a medium maintained at pH 3 to  
06 7, preferably pH 4 to 6. Examples of media are given  
07 below.

08  
09 In a further preferred embodiment of this invention the  
10 composite particle may comprise any of the modified L  
11 proteins disclosed both generically and specifically  
12 hereinbelow.

13  
14 In a particularly preferred embodiment the modified L  
15 protein corresponds to a 280 amino acid polypeptide  
16 containing residues 12-52, 133-145 and 175-400 relative  
17 to the ORF on a HB virus of ad serotype.

18  
19 In yet a further preferred embodiment of this invention  
20 the composite particle may contain hepatitis B surface  
21 antigen polypeptides of differing subtypes to broaden  
22 the range of protection. That is the S-protein may be  
23 of ad subtype specificity and the preS1-protein and/or  
24 the modified L protein and/or the preS2-protein of ay  
25 subtype or vice versa.

26  
27 The subject of the present invention is further a  
28 composition of matter comprising the new composite  
29 particle or hepatitis B surface antigen protein  
30 produced according to the present invention. Further  
31 components of the inventive composition may be  
32 stabilizers, buffering substances, diluents, salts such  
33 as NaCl, or sugars.

34  
35 As outlined above, the initial purpose of the present  
36 invention is to provide a vaccine against hepatitis B

01 virus. Therefore, a vaccine comprising a composite  
02 particle as described above or hepatitis B surface  
03 antigen produced according to the present invention is  
04 a further subject of the present application. The  
05 vaccine may comprise further substances, for example  
06 salts, stabilizers, adjuvants, and/or other  
07 physiologically acceptable additives. The vaccine  
08 comprising composite particles elicits formation of  
09 antibodies directed against at least two of the  
10 possible antigenic determinants of hepatitis B surface  
11 antigens. Due to the presence of preS1 specific and  
12 preS2 specific domains, even individuals who did not  
13 respond to presently available vaccines, may be  
14 protected against hepatitis B infection. In general  
15 the vaccine according to the present invention will  
16 evoke a broader immune response than present vaccines  
17 and furthermore can be produced at lower costs due to  
18 the high amount of expression per cell and the high  
19 cell density.  
20  
21

22  
23 The present invention has also proved to be useful for  
24 the development of a test kit. By providing a  
25 composition comprising the composite particles  
26 according to the present invention, it is possible to  
27 monitor the formation of immune complexes formed  
28 between antibodies of an infected individual with such  
29 particles. The test kit requires further provision of  
30 a detection agent, for example, a goat/anti-human IgG  
31 antibody labelled by any of the known techniques. The  
32 antibody, which can of course also be derived from any  
33 other mammal, may for example be coupled to an enzyme  
34 or may be radioactively labelled to effect detection.  
35 The advantage of a test kit responsive to antibodies  
36 against preS1 and preS2 specific domains is the  
37 possibility of early detection of HBV infection, since

01  
02  
03 anti-preS1 and anti-preS2 antibodies appear prior to  
04 anti-S antibodies during HBV infection in man (Petit et  
05 al., 1986). Thus the test kit according to the  
06 invention provides a convenient means for early  
07 diagnosis of this highly infective disease.

08  
09 In a further aspect the present invention provides a  
10 modified large surface protein (hereinafter modified L  
11 protein) of hepatitis B virus (HBV). One such modified  
12 L protein has an L protein sequence characterized by  
13 the absence of sites preferentially attacked by  
14 proteases. Elimination of these sites provides a less  
15 protease sensitive molecule, which upon expression  
16 produces a protein readily purified in particle form.

17  
18 Another aspect of the present invention is a modified L  
19 protein characterized by the absence of an amino acid  
20 necessary for myristylation of the protein.

21  
22 In yet another aspect the present invention includes a  
23 modified L protein which is characterised by the  
24 absence of several potential glycosylation sites.  
25 Elimination of the glycosylation of the L protein  
26 improves the presentation of epitopes which are  
27 shielded, distorted or modified by glycosylation of the  
28 protein which occurs during expression in yeast hosts.  
29 The modified, deglycosylated L protein presents the  
30 protective B epitopes and significant T<sub>H</sub> epitopes of  
31 the preS1, preS2 and S region in their correct  
32 conformation, well exposed on the surface of the  
33 resulting particle.

34  
35 A further aspect of the present invention includes a  
36 modified L protein characterised by a deletion of a  
37 part of the preS2 region, which includes the human

serum albumin (pHSA) binding site. This modification of the L protein thereby eliminates the potential tolerance or auto-immune problems linked to the presence of the pHSA receptor without losing the major B epitopes of the preS2 region.

In still a further aspect of this invention, a modified L protein is provided which is characterized as non-glycosylated, and having diminished protease sensitivity and diminished pHSA binding capacity. This protein contains the preS1 region sequences corresponding to amino acid residues 12 to 52 fused to preS2 region sequences corresponding to amino acid residues 133-145, followed by the entire coding sequence for the S protein in a continuous reading frame.

The modified L proteins of this invention may be expressed not only in methylotrophic yeasts as thus far discussed but also in other yeast expression systems which produce the proteins as particles retaining the major antigenic sites encoded by the three domains preS1, preS2 and S. The modified L proteins may also be co-expressed with the S protein in yeast to form particles of mixed subunit composition. Other expression systems, e.g., bacterial, insect, mammalian may also produce the modified L proteins in various forms.

As another aspect this invention also provides novel DNA sequences coding for the modified L proteins of the present invention. In another aspect of this invention, such DNA sequences may be incorporated in vectors under the control of appropriate regulatory sequences.

01  
02  
03 As still another aspect there is provided a method for  
04 producing a modified L protein of HBV as described  
05 above involving culturing a yeast cell transformed with  
06 a yeast expression plasmid expressing a selected  
07 modified L protein gene sequence under control of  
08 suitable regulatory sequences and employing suitable  
09 culture conditions. The modified L protein may also be  
10 co-expressed in a yeast host with S protein. The  
11 resulting L protein may be isolated from the culture or  
12 cell lysate by conventional techniques. The modified L  
13 protein may be isolated in the form of particles of  
14 either mixed or homogeneous subunit composition  
15 depending on its co-expression with S protein.  
16

17 As still a further aspect, the invention provides a  
18 method for producing modified L proteins in other  
19 expression systems, including bacterial expression  
20 systems; insect cell expression systems, e.g.,  
21 baculovirus systems; mammalian cell expression  
22 systems; vaccinia virus systems and plant expression  
23 systems.  
24

25 Still a further aspect of this invention are vaccines  
26 comprising one or more modified L proteins of this  
27 invention, alone or in combination with other vaccinal  
28 agents and pharmaceutically acceptable vaccinal  
29 carriers and adjuvants. The vaccines of this invention  
30 comprising the modified L proteins described herein may  
31 also include the modified L protein in the form of a  
32 particle of mixed subunit composition with other  
33 peptides i.e. S protein.  
34

35 From the foregoing it will be appreciated that the  
36 novel modified large (L) hepatitis B surface protein  
37 molecules may be used alone or with other HBsAg or HBV

components to provide vaccinal agents for use in immunizing persons against HBV infection. The invention also involves expressing a modified L protein in a suitable host cell under suitable regulatory control.

The modified L protein of the present invention is characterized by deletion, insertion or modification of a variety of amino acid sequences in the preS region. It is to be understood that the L molecule to be modified and expressed here has already been deleted for its first eleven amino acids and contains 163 amino acids in the preS1 region (see co-pending U.S. patent application SN 009,325. The resulting reduction in size of the preS region facilitates particle formation in the host cell and enhances various biophysical properties of the molecule, which may make the modified L particle particularly desirable for vaccinal manufacture and use. For example, the shortened preS regions of the present invention may enable better presentation of the S and preS epitopes on the particle for interaction with the immune system, thereby enhancing immunogenicity.

In one embodiment, the present invention provides a modified L protein deleted for the Gly 13 amino acid residue in the preS1 region. The L protein expressed in yeast carries a myristyl group covalently attached in an amide linkage, as disclosed in copending U.S. patent application SN 07/009,325. An N-terminal Gly residue is an essential requirement for the myristylation of a protein [Towler and Gordon, Ann. Rev. Biochem., 57:69-99 (1988)]. Therefore, one

01  
02  
03 suitable modified L protein of this invention is  
04 encoded by DNA having a deletion of the Gly13 codon  
05 (GGG) from the nucleotide sequence encoding the L  
06 protein. This deletion results in the synthesis of a  
07 non-myristylated L protein.

08  
09 As used herein the symbol  $\Delta$  denotes deletion:  
10 Thus, for example,  $\Delta$ Gly13 modified L means modified L  
11 protein lacking amino acid residue 13 (glycine).  
12

13 Another embodiment of the present invention is a  
14 modified L protein deleted for one or more of its  
15 potential glycosylation sites. The L protein expressed  
16 in yeast carries an N-linked oligosaccharide chain at  
17 Asn123 and several O-linked mannose chains linked to  
18 Ser and Thr residues in both the preS1 and preS2  
19 regions of the molecule as described in European patent No.  
20 EP 0 278 940 B1, [see, also,  
21 Rutgers et al, ''Viral Hepatitis and Liver Disease''  
22 ed. A. J. Zuckerman, A. R. Liss, New York, pp. 304-308  
23 (1988)]. To remove the N-glycosylation site which is  
24 characterized by the sequence Asn-X-(Ser or Thr), where  
25 X may be any amino acid, the sequence at amino acid  
26 positions 123-125 is altered. The N-linked  
27 glycosylation site may be eliminated by deleting or  
28 replacing the amino acids at positions 123 and 125,  
29 deleting amino acid 124 or deleting the entire 123-125  
30 sequence. To destroy the O-linked glycosylation  
31 sites, all or some of the Ser and Thr residues in the L  
32 sequence are deleted from, or replaced with, other  
33 amino acids in the protein. The L protein expressed  
34 from illustrative plasmid pRIT13192 is characterized as  
35 wholly or partially non-glycosylated.  
36

Another modified L protein of the invention contains a deletion in the preS2 sequence at amino acid residues 120-132 of the preS2 region responsible for binding of polymerized human serum albumin (pHSA) [see Machida et al, cited above]. pHSA binding is greatly diminished on the proteins lacking this sequence.

Another modified L protein according to the invention is characterized by deletion or alteration of the protease sensitive sites. Protease sensitive peptide bonds exist in the preS sequence behind the Arg residues around position 100 in the preS1 region [Heermann et al., Intervirology 28, 14-25 (1987)] and behind amino acid residues Arg137, Gly149 and Arg167. Deletions or other modifications introduced in the coding region of the L protein to remove some or all of the codons for these residues produce an L protein less sensitive to protease action.

The deletions introduced in the coding region of the L protein to form modified L proteins preferably leave untouched the codons for amino acids 21-47 and 133-145. These sequences are believed to provide the modified protein with the capacity to induce preS-specific antibodies correlated with immune protection [see, e.g., Itoh et al, *supra*].

Construction of an exemplary yeast expression plasmid pRIT12979 encoding the Gly13 L protein is described in Example F.1 below. Analysis of the modified L protein expression from plasmid pRIT12979 for expression level, post-translational modifications and macromolecular assembly is described in Example F.2.

Another modified L protein, devoid of all three of the above mentioned properties, i.e. to function as a

substrate to yeast glycosylation enzymes, pHSA binding capacity and protease sensitivity, and modified by removal of the codons for amino acid residues 53-132 and 146-174 of the preS region is illustrated in plasmid pRIT13192, described in Example F.3 below. Plasmid pRIT13192 contains the codons for residues 12-52 and 133-145 of the preS region. Analysis of L protein expression from plasmid pRIT13192 is also described herein (Example F.4). The L protein expressed from pRIT13192 thus shows a greatly reduced pHSA binding capacity, an improved protease resistance and less glycosylation.

Yet another exemplary modified L protein of the present invention combines the deletions introduced in the L protein coding sequences of pRIT12979 and pRIT13192. The L protein expressed by this sequence codes for a non-myristylated, less glycosylated, less protease sensitive L protein with diminished pHSA binding capacity. Construction of the yeast expression plasmid pRIT13193 bearing this modified L sequence insert is also described in detail in Example F.3 below.

Modified L protein coding sequences, including the entire S coding sequence, for use in the present invention can be prepared from these S and preS protein coding sequences of Table A by conventional site specific mutagenesis [see, e.g., Botstein et al, Science, 229:1193 (1985)] or recombinant DNA techniques, [see, e.g., T. Maniatis et al, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory (1982)]. The amino acid position numbers referred to below will correspond with the position numbers in Table A for clarity. However, other published versions of the S, and preS sequences can be

01  
02  
03 used in preparing the modified L proteins of this  
04 invention by one of skill in the art.

05  
06 For example, the S protein can be isolated from DNA  
07 extracted from Dane particles in infected human serum  
08 by filling in the single strand region with a DNA  
09 polymerase, preferably the endogenous polymerase,  
10 followed by digestion with a restriction endonuclease.  
11 The choice of endonuclease will depend, in part, on the  
12 particular Dane particles. For example, the HBsAg  
13 coding sequence of HBV DNA of certain Dane particles of  
14 the adw serotype can be isolated on a single BamHI  
15 fragment; the HBsAg coding sequence of HBV DNA of  
16 certain Dane particles of the ayw serotype can be  
17 isolated on a HhaI fragment. HBV DNA of Dane particles  
18 of the same serotype may also exhibit different  
19 patterns of restriction sites.

20  
21 Construction of the modified L protein sequences of  
22 this invention can be accomplished by use of  
23 intermediate vectors. Techniques for cloning DNA  
24 fragments in phages are disclosed, for example, by  
25 Charnay et al, Nature, 286:893-895 (1980) and Tiollais,  
26 United Kingdom Patent Application 2,034,323.

27  
28 The modified L protein of this invention may be  
29 constructed in a recombinant DNA molecule or vector. A  
30 recombinant vector of this invention comprises the  
31 modified L protein coding sequence of this invention  
32 operatively linked to a regulatory region. Such vector  
33 may also additionally contain a replicon depending upon  
34 preference and/or the host to be employed. By the term  
35 ''replicon'' is meant that minimum region of DNA which  
36 functions to maintain stably, and extrachromosomally, a  
37 recombinant vector in a eukaryotic or prokaryotic host  
38 organism transformed with such vector. Such replicons

01  
02  
03 are well-known in the art. By the term 'regulatory  
04 region' is meant any DNA sequence which contains a  
05 promoter region and other sequences necessary for  
06 transcription of a coding sequence which regulates  
07 transcription of a structural gene's coding sequence.  
08 Such regulatory regions are well-known in the art.

09  
10 Such a vector can be prepared by conventional  
11 techniques such as by inserting the modified protein  
12 coding sequence of this invention into a vector already  
13 containing a replicon and regulatory region in such a  
14 way that the DNA molecule is operatively linked to such  
15 regulatory region. This DNA molecule, or an expression  
16 cassette containing the modified L protein coding  
17 sequence, can be constructed by conventional  
18 techniques, such as by ligating the L coding sequence  
19 to the regulatory sequence.

20  
21 Restriction of DNA to prepare DNA fragments used in the  
22 invention, ligation of such fragments to prepare  
23 recombinant DNA molecules used in the invention and  
24 insertion into microorganisms are carried out by known  
25 techniques, such as techniques disclosed in the  
26 previously and subsequently cited references.  
27 Conditions are selected to avoid denaturation of the  
28 DNA and enzymes. Restriction enzymes and ligases used  
29 in carrying out this invention are commercially  
30 available and should be used in accordance with  
31 instructions included therewith. T4 DNA ligase is the  
32 preferred ligase.

33  
34 The various fragments and final constructions used for  
35 the expression of the modified L proteins, and the  
36 components of the vectors carrying these coding  
37 sequences for expression in host cells in the method of  
38 the present invention may be joined together using with

01  
02  
03 conventional methods known to those of skill in the  
04 art. In many cases, genes have been isolated and  
05 restriction mapped, as well as sequenced. To that  
06 extent, one can select the sequence of interest, such  
07 as the HBV envelope protein coding sequence, by  
08 restriction of the gene, employing further manipulation  
09 as necessary such as resection with Bal31, in vitro  
10 mutagenesis, primer repair, or the like, to provide a  
11 fragment of a desired size, including the desired  
12 sequence, and having the appropriate termini. Linkers  
13 and adapters can be used for joining sequences, as well  
14 as replacing lost sequences, where a restriction site  
15 employed was internal to the region of interest. The  
16 various fragments which are isolated, may be purified  
17 by electrophoresis, electroeluted, ligated to other  
18 sequences, cloned, reisolated and further manipulated.

19  
20 The use of regulatory regions for controlling  
21 transcription of the modified L gene of interest may  
22 allow for growing the host cells to high density with  
23 no or low levels of expression of the structural gene,  
24 and then inducing expression by changing the  
25 environmental conditions, such as nutrient,  
26 temperature, and the like.

27  
28 The vector carrying the L protein coding sequence is  
29 transformed into, and expressed in a selected host cell  
30 by conventional techniques. The cell is then cultured  
31 in appropriate culture media, i.e., media which enable  
32 the host to survive and express the modified L protein  
33 in recoverable quantity. For example, where a yeast  
34 host cell is selected, an exemplary medium for such use  
35 is Yeast Nitrogen Base minimal medium. The appropriate  
36 culture media may be selected by one of skill in the  
37 art depending upon the particular host employed in the  
38 method.  
39

The isolation of the modified L protein molecule of the invention may be from a cell lysate or extract of the host cell culture medium depending upon the cell's ability to secrete the modified protein. Recovery of the protein of this invention is performed by conventional protein isolation techniques.

For producing modified L proteins of this invention, a suitable host cell or cell line may be selected. Suitable cells or cell lines may be yeast cells. Many strains of yeast cells known to those skilled in the art are available as host cells for expression of the modified L proteins of the present invention. In the practice of this invention, any species of yeast may be employed. In one aspect, the examples below employ Saccharomyces, specifically S. cerevisiae. Numerous strains of S. cerevisiae are available from public depositories and laboratories, e.g., American Type Culture Collection, Rockville, MD.

In another aspect Hansenula polymorpha may be used.

Other yeast species may also be employed including, without limitation, Hansenula, Pichia, Candida, Kluyveromyces and Schizosaccharomyces.

Additionally, other species of eukaryotic host cells, e.g., mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells, may be employed as hosts cells for expression of the L proteins described herein. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U. S. Patent 4,419,446. Other suitable mammalian

cell lines, are the monkey COS-1 cell line, and the CV-1 cell line.

It will be realised that some of the deletions introduced in the L protein coding sequence described above have altered the internal sequences engaged in the transcription and translation of M and S specific mRNA in mammalian cells. Such a modified L protein coding sequence allows, therefore, independent expression of S and modified L protein in a mammalian cell harbouring their individual expression cassettes.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of E. coli (e.g., HB101, MC1061 and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Some viruses may also be employed to express the L proteins of this invention, such as baculoviruses or vaccinia viruses. Selection of appropriate host cells is within the ability of one of skill in the art, as is the selection of appropriate culture conditions, media and vector components appropriate to expression in a selected host cell.

In the expression systems described above and below in the Examples, any known promoter capable of controlling

-52-

1 the expression of the protein coding sequences in the  
2 selected host cell may be selected. Where the host cell  
3 of choice of a yeast cell, the promoters useful for  
4 expressing the modified L protein cassettes may include  
5 the strong promoter TDH3 or other constitutive or  
6 regulated promoters which allow modulation of the level  
7 of the L protein depending on the composition of the  
8 growth medium. Additionally, for example, PGK, ARG3, and  
9 APH1 are illustrative promoters useful in the vectors of  
10 the present invention.

11  
12 The present invention also provides for expression of  
13 the modified L proteins of this invention as part of an  
14 HBsAg particle of mixed polypeptide composition. The  
15 method involves coexpressing in a yeast cell a first  
16 protein S and a second protein X-S, where S is  
17 substantially the major surface protein of HBV and where  
18 X, for purposes of the present invention, is a modified  
19 preS sequence encoded by a functional DNA coding region  
20 fused in phase to the S coding sequence.

21  
22 According to this method the S protein coding sequence  
23 is coexpressed with the modified L protein amino acid  
24 sequence. The coding regions of the L protein, as well  
25 as the sequence for the S protein may be constructed as  
26 described above using conventional chemical synthetic or  
27 recombinant DNA technology. The modified L proteins are  
28 incorporated together with S proteins into particles of  
29 mixed subunit composition.

30  
31

01  
02  
03 In the construction of the X-S proteins comprising  
04 modified L proteins of the present invention, S is  
05 preferably the entire mature surface protein (about 226  
06 amino acids) of HBV (see Table A). Alternatively, the  
07 S protein may be encoded by an S protein coding  
08 sequence of the HBV genome, wherein S is substantially  
09 the same as an authentic HBsAg S protein. Truncates of  
10 S can also be employed in the construction of the  
11 modified L protein containing mixed particles, as long  
12 as particle assembly is not adversely affected.  
13

14 The S protein coding sequence is placed into a first  
15 expression cassette employing conventional techniques  
16 to allow expression of the S protein in the yeast  
17 host. The S protein coding sequence or a substantial  
18 portion thereof is employed in a fusion with portions  
19 of the preS coding sequence and placed into a second  
20 expression cassette to allow production of a modified L  
21 protein of this invention.  
22

23 A vector, constructed by conventional techniques as  
24 described above, carrying the S protein expression  
25 cassette and one or more vectors carrying the modified  
26 L protein expression cassette are transformed into into  
27 a yeast host cell by conventional techniques.  
28

29 Alternatively, S protein expression cassettes and  
30 expression cassettes for one or more modified L  
31 proteins are placed together on the same vector which  
32 is transformed into a yeast host cell.  
33

34 Vectors that can be used are Ty integrative vectors as  
35 described in detail in co-pending U.S. patent  
36 application SN 386,401. Another alternative method  
37 employs compatible autonomously replicating vectors

that carry the desired expression cassettes and transform the yeast host cell.

Examples of vector systems are described in detail in Mellor et al., Yeast, 2:145 (1986); Boeke et al., Cell, 40,491 (1985), Boeke et al., Cell, 42:507 (1985) and copending U.S. patent applications SN 101,463; 233,631; 368,401 and 009,325 (European Patent Application Publication No. 0 278 940).

Additionally other species of host cells may be employed for the expression of the composite HBsAg particles of mixed polypeptide composition described herein. Suitable eukaryotic hosts are the same as those listed hereinabove for expression of the modified L proteins of the invention, for example mammalian cells, such as CHO cells. Insect cells and viruses such as baculoviruses and vaccinia viruses mentioned hereinabove may also be used.

A preferred embodiment of the method according to the present invention employs Ty integrative vectors carrying the S protein expression cassette and autonomously replicating plasmid vectors carrying the modified L protein expression cassette. The use of Ty vectors carrying the S protein expression cassette allows these sequences to be stably integrated into the chromosomes of the yeast host cell. Such integrations are stable due to their low frequency of excision. The vectors are thus homogeneously distributed in the cell population, allowing a similar rate of protein synthesis in each cell.

Transformation into these cells of autonomously replicating plasmids carrying the expression cassette for the modified L protein ensures that for a definite amount of S protein synthesis in each cell, the level of modified L protein synthesis varies according to the copy number of the plasmid present in the cell.

In a further preferred embodiment both the S protein expression cassette and the modified L protein expression cassette may be inserted on Ty integrative vectors and separately integrated into yeast host cells of opposite mating types. Strains carrying the desired numbers of S cassette and modified L cassette

1 integration events may then be crossed to obtain  
2 diploid cells expressing both the S and modified L  
3 polypeptides. If desired haploid segregants carrying  
4 both types of expression cassette may be obtained by  
5 sporulating such diploids.

6  
7 The cell may be cultured in appropriate culture media,  
8 i.e., media which enable the host to express the S  
9 protein and modified L protein in particles of mixed  
0 subunit composition in recoverable quantity.

1 Resulting modified L proteins will be produced  
2 simultaneously in yeast cells together with the S  
3 protein and assembled as mixed composite particles.  
4 Such particles are herein defined as a multimeric  
5 assembly of two or more polypeptides into a particulate  
6 structure, which is therefore characterised by a  
7 composite nature being of mixed polypeptide  
8 composition. The different peptides are disposed in a  
9 spatial association at the surface of the mixed  
0 particles. The presence of the novel modified L  
1 protein on the HBsAg particle will elicit an immune  
2 response similar to that associated with recognition of  
3 the native determinant. The present invention thereby  
4 enables the production of HBsAg particles in yeast  
5 which mimic natural particles insofar as they contain  
6 the essential antigenic sites from the S, M and L  
7 proteins of HBV.

8 The isolation of the mixed particle of the invention  
9 from a cell lysate or extract of the culture medium is  
0 performed by conventional protein isolation  
1 techniques.

01  
02  
03 In the practice of this method of this invention, any  
04 species of yeast host for which transformation, cloning  
05 and expression systems are available may be employed in  
06 developing 'mixed particles' of HBsAg.

07  
08 In other yeast genera no Ty sequences are present and  
09 therefore vector systems must be designed to provide  
10 coexpression of the S and modified L protein coding  
11 sequences in these genera to create the mixed particles  
12 described. Therefore, expression cassettes may be  
13 designed for integration into the yeast chromosomes  
14 employing other repetitive DNA sequences or plasmids  
15 such as ARS based plasmids in yeast such as Pichia,  
16 allowing integration into the chromosomes. An example  
17 of such applicable ARS plasmids is described in Cregg  
18 et al, Mol. Cell. Biol., 5(12):3376 (1985).

19  
20 In the expression systems described above, e.g. both  
21 the Ty expression systems and others, the regulatory  
22 element comprises a promoter which effects RNA  
23 polymerase binding and transcription. Regulatable,  
24 i.e., inducible or derepressible, promoters, can also  
25 be used. A variety of useful promoters are available  
26 for expression of heterologous polypeptides in yeast.  
27 These include the copper inducible metallothionein gene  
28 (CUP1) promoter and the constitutive promoter of the  
29 glycolytic genes, glyceraldehyde-3-phosphate  
30 dehydrogenase (TDH3) and alcohol dehydrogenase (ADH).  
31 Regions for transcription termination in yeast are  
32 derived from the 3' end of any of several yeast genes,  
33 for example, the gene for iso-1-cytochrome C (CYC1).  
34 Expression systems for use in Kluyveromyces are  
35 disclosed, e.g., in PCT W083/04050, Hollenberg et al;  
36 in Schizosaccharomyces, e.g., in EP-A-107170, Yamamoto;  
37 in Pichia, e.g., in Cregg et al, Bio/Technology, 5:479

(1987); and in Hansenula, e.g., in EP-A-299108, Hollenberg et al.

Specifically provided by the examples of this invention are S. cerevisiae strains capable of synthesizing S protein as a result of the genomic integration of S protein expression cassettes directed by Ty based vectors. One such vector is pRIT13133-L.

Another Ty1 vector, pRIT13034-L, contains the same expression cassette and URA3 marker but contains in addition the CUP1 gene which can be used as an additional marker in CUP1<sup>S</sup> or cup1 $\Delta$  recipient yeast strains.

Preferred yeast strains are devoid of functional chromosomal CUP1 genes, which are sensitive to copper toxicity, in order to readily select transformants having integrated several copies of vectors. Two preferred cup1 $\Delta$  yeast strains are EJ cup1  $\Delta$  3d and EJ cup1  $\Delta$  7b.

Yeast strains containing several integrated copies of either pRIT13133-L or prRIT1034-L are described in the Examples below.

Transformation into these yeast strains of autonomously replicating yeast expression plasmids carrying an expression cassette for a modified L protein is also described in the Examples below.

01  
02  
03 Diploid yeast strains carrying several integrated  
04 copies of both S and modified L expression cassettes  
05 are described in Example F.10 below.  
06

07 The method of the present invention for producing such  
08 modified L proteins alone or in particles of mixed  
09 subunit composition has advantageous utility in the  
10 production of vaccines to pathogenic microorganisms.  
11 For example, the modified L proteins described in the  
12 various vaccine formulations comprising hepatitis B  
13 surface antigen and may provide benefits not obtained  
14 with known HBV vaccines. The HBsAg particle of mixed  
15 subunit composition containing a modified L protein  
16 resembles particles or filaments or virions present in  
17 the blood of HBV infected persons that contain preS2  
18 and preS1 epitopes exposed at their surface in addition  
19 to epitopes from the major proteins and may also prove  
20 useful in novel vaccine formulations. The modified L  
21 proteins or mixed particles of mixed subunit  
22 composition containing them may have immunogenic  
23 properties that are not obtained with individual  
24 mixtures of S, M and L proteins.  
25

26 In further embodiment of this invention the particles  
27 of mixed subunit composition may contain modified L  
28 proteins and S proteins with different subtype  
29 determinants so as to broaden the range of protection.  
30 That is the modified L protein may be of any subtype  
31 specificity and the S protein of any subtype or vice  
32 versa.  
33

34 Thus this invention encompasses vaccines containing the  
35 modified L proteins of the invention or a particle  
36 containing the modified L protein either in mixed (S,  
37 modified L) or homogeneous (modified L protein) subunit

01  
02  
03 form. Such vaccine will contain an immunoprotective  
04 quantity of the particles or modified L proteins, i.e.,  
05 enough of the proteins or particles are administered to  
06 elicit sufficient protective antibody response against  
07 HBV without serious side effects. Such vaccines may be  
08 prepared by conventional techniques. For example, a  
09 vaccine for stimulating protection against HBV  
10 infection in humans may contain the particle and/or the  
11 modified L proteins described above and a suitable  
12 conventional carrier. The particle or the L protein  
13 may be in an aqueous solution buffered at physiological  
14 pH for direct use. Alternatively, the protein or  
15 particle can be admixed or adsorbed with a conventional  
16 adjuvant, such as aluminum hydroxide or aluminum  
17 phosphate. Such a particle or modified L protein may  
18 also be combined with other immunogens to prepare  
19 combination vaccines. See, e.g., New Trends and  
20 Developments in Vaccines, eds. Voller et al, University  
21 Park Press, Baltimore, MD (1978).

22  
23 These modified L proteins, alone or in particles of  
24 mixed or homogeneous subunit composition, as described,  
25 thus broaden the spectrum of antibodies against HBV,  
26 and may provide an earlier response to viral challenge  
27 by the person vaccinated with the vaccines containing  
28 these proteins. Additionally the vaccines containing  
29 these modified L proteins may enable persons who do not  
30 respond to S proteins to mount a preS response, or may  
31 augment or enhance the S response.

32  
33 Another use for the particles of this invention and/or  
34 the modified L proteins may be as probes or reagents  
35 for detection of HBV or HBV derived proteins or  
36 anti-HBV antibodies in biological samples by various  
37 conventional immunoassays and the like.  
38

01  
02  
03 It will be appreciated that all vaccines described  
04 herein can be administered by an appropriate route,  
05 e.g., by the subcutaneous, intravenous or intramuscular  
06 routes. The amount of the immunogen in each vaccine  
07 dose is selected by the attending physician with regard  
08 to consideration of the patient's age, weight, sex,  
09 general physical condition and the like. The amount to  
10 induce an immunoprotective response in the patient  
11 without significant adverse side effects may vary  
12 depending upon the immunogen employed and the optional  
13 presence of an adjuvant. Generally, it is expected  
14 that each dose will comprise 1-1000 micrograms of  
15 protein, preferably 1-200 micrograms. Initial doses  
16 may be optionally followed by repeated boosts, where  
17 desirable.

18  
19 Accordingly the present invention further provides a  
20 method of treating or preventing hepatitis B infection  
21 in humans which comprises giving to a patient in need  
22 thereof a protective amount of a vaccine according to  
23 the invention.

24  
25 Especially preferred vaccines according to the  
26 invention are those which comprise composite particles  
27 of structure (L\*, S) where L\* is as defined below and S  
28 denotes the S-protein of hepatitis B surface antigen,  
29 most preferably expressed in S. cerevisiae or H.  
30 polymorpha as described herein.

31  
32 As used herein the symbol L\* denotes a modified L  
33 protein comprising residues 12-52, 133-145 and 175-400  
34 relative to the large open reading frame on HB virus of  
35 ad serotype shown in Table A hereinabove. The symbol  
36  $\Delta$ gly L\* similarly denotes a modified L protein  
37 comprising residue 12 followed by residues 14-52,  
38 followed by residues 133-145 and 175-400.

39  
40 The following Examples illustrate the invention.  
41

01  
02  
03 Materials  
04

05 1. Plasmids  
06

07 Plasmids mentioned in the specification are  
08 described in Part A below.  
09

10 2. Yeast strains  
11

12 Hansenula polymorpha RB 10 (ura 3) is a mutant of  
13 Hansenula polymorpha ATCC 34438, obtained by  
14 mutagenesis using ethylmethansulfonate (EMS)  
15 essentially as described by Roggenkamp et al.  
16 (1986).  
17

18 3. Enzymes  
19

20 Restriction endonucleases, T4 DNA ligase and other  
21 enzymes used, were obtained either from  
22 Boehringer, Mannheim, FRG or Bethesda Research  
23 Laboratories, Eggenstein, FRG. The enzymes were  
24 used according to the instructions of the  
25 respective manufacturer.  
26

27 4. Immunological Reagents  
28

29 a) S1.1: mouse monoclonal antibody specific for  
30 the S1 domain of preS1-protein (SmithKline  
31 Biologicals, Rixensart, Belgium)  
32

33 b) S2.1, S2.2 and S2.5; mouse monoclonal  
34 antibodies specific for the S2-domain of hepatitis  
35 B surface antigen (SmithKline Biologicals,  
36 Rixensart, Belgium).  
37

38 Antibodies specific for the S1-domain and  
39 S2-domain of hepatitis B surface antigen are

referred to as 'preS-antibodies'.

c) RF6: Mouse monoclonal antibody specific for the S domain of hepatitis B surface antigen (H. Thomas, Royal Free Hospital, London).

d) RF1: Mouse monoclonal antibody specific for a conformational dependant epitope located in the S-region (H. Thomas, Royal Free Hospital, London).

e) HBS1: Mouse monoclonal antibody directed against a denaturation and reduction resistant epitope of plasma-derived HBsAg, located in the S-region (SmithKline Biologicals Rixensart, Belgium). This monoclonal competes in binding experiments with Mab RF6.

Antibodies a), b) and e) were prepared by the applicant according to standard procedures by fusion of mouse spleen cells with myeloma cells. For the method reference is made to Köhler and Milstein, 1975. Methods are disclosed in Goding, Monoclonal Antibodies, Principles and Practice, Academic Press, London 1983.

f) AUSRIA<sup>TM</sup> (Abbott Laboratories, Wiesbaden-Delkenheim, FRG and Louvain-La-Neuve, Belgium): commercial kit containing polyclonal antibodies directed against native conformational epitopes.

g) AUSZYME<sup>TM</sup> (Abbott Laboratories, Wiesbaden-Delkenheim, FRG and Louvain-La-Neuve, Belgium): commercial kit containing monoclonal antibodies directed against a native conformational epitope.

The antibodies mentioned under f), g) are commercially available. They do not react with

monomeric hepatitis B surface antigen.

h) AUSAB<sup>TM</sup> (Abbott Laboratoires, Louvain-La-Neuve, Belgium): Commercial kit for detection of anti HBsAg-antibodies.

## 5. Media

Pepton 190, yeast extract, Casamino acids and yeast nitrogen base used for preparation of media were purchased from Gibco Ltd., Paisley, U.K.

In order to prepare agar plates, 18g agar (Gibco Ltd.) per litre were added prior to autoclaving the respective medium.

### a) SMR: Semi-rich non-selective medium

|                                                                         |         |
|-------------------------------------------------------------------------|---------|
| Pepton 190                                                              | 2 g/l   |
| Yeast extract                                                           | 1 g/l   |
| Casamino acids (Pepton 5)                                               | 3 g/l   |
| Yeast nitrogen base W/O (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 1.4 g/l |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>                         | 5 g/l   |
| Carbon sources: glucose                                                 | 20 g/l  |
| or glycerol                                                             | 20 g/l  |
| or methanol                                                             | 10 g/l  |

SMR containing methanol is also called Met-SMR.

### b) YNB: Selective medium

|                                                                             |         |
|-----------------------------------------------------------------------------|---------|
| Yeast nitrogen base without (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 1.4 g/l |
| (Gibco or Difco)                                                            |         |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>                             | 5 g/l   |
| Carbon sources: glucose                                                     | 20 g/l  |
| or glycerol                                                                 | 20 g/l  |
| or methanol                                                                 | 10 g/l  |

c) YEPD: Non-selective rich medium.

|               |        |
|---------------|--------|
| Yeast extract | 10 g/l |
| Pepton 190    | 20 g/l |
| Glucose       | 20 g/l |

If necessary, 1 mg gentamycin G418 was added per 1 ml of medium.

d) S-medium

| <u>COMPONENT</u>                                    | <u>AMOUNT PER LITRE</u> |
|-----------------------------------------------------|-------------------------|
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>     | 5 g                     |
| KH <sub>2</sub> PO <sub>4</sub>                     | 4 g                     |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O                | 2 g                     |
| NaCl                                                | 0.1 g                   |
| CaCl <sub>2</sub> ·2H <sub>2</sub> O                | 0.4 g                   |
| L-Histidine monohydrochloride                       | 10 mg                   |
| LD-Methionine                                       | 9 mg                    |
| LD-Tryptophan                                       | 9 mg                    |
| H <sub>3</sub> BO <sub>3</sub>                      | 2 mg                    |
| CuSO <sub>4</sub> ·5H <sub>2</sub> O                | 0.16 mg                 |
| KI                                                  | 0.4 mg                  |
| FeCl <sub>3</sub> ·6H <sub>2</sub> O                | 0.8 mg                  |
| MnSO <sub>4</sub> ·H <sub>2</sub> O                 | 1.6 mg                  |
| Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O | 0.8 mg                  |
| ZnSO <sub>4</sub> ·7H <sub>2</sub> O                | 1.6 mg                  |
| Biotin                                              | 0.16 mg                 |
| Ca-pantothenate                                     | 32 mg                   |
| Folic acid                                          | 0.16 mg                 |
| Inositol                                            | 160 mg                  |
| Niacin                                              | 0.4 mg                  |
| p-Amino-benzoic acid                                | 0.2 mg                  |
| Pyridoxine hydrochloride                            | 32 mg                   |
| Riboflavin                                          | 0.2 mg                  |
| Thiamine hydrochloride                              | 32 mg                   |

01  
02  
03 II Methods  
0405 1. Handling of DNA  
06

07 Cleavage with restriction endonucleases, ligation  
08 using T4 DNA ligase, phosphorylation of DNA,  
09 isolation of DNA from E. coli, separation of DNA  
10 by gel electrophoresis, and all other techniques  
11 belonging to the field of gene technology used in  
12 these applications were essentially performed as  
13 described by Maniatis et al., 1982.  
14

15 2. Transformation of yeast  
16

17 Methylophilic yeast is transformed by the  
18 methods described previously (Roggenkamp et al.,  
19 1986).  
20

21 The method using intact frozen cells is preferred  
22 (Klebe et al., 1983).  
23

- 24 a) Transformed cells are identified by  
25 complementation of *ura3* deficiency or by  
26 resistance to gentamycin.  
27

28 Resistance against gentamycin G418 was checked by  
29 plating the cells on YEPD plates containing 25 ml  
30 solidified medium. After 6 hours of growth on the  
31 plates 1 ml aqueous solution containing 25 mg  
32 gentamycin 418 was applied onto each plate and the  
33 incubation continued for 2 to 3 days. Gentamycin  
34 G418 (Trade Mark Geneticin) was obtained from  
35 Gibco Ltd.  
36

01  
02  
03 b) Segregation of mitotically stable transformants  
04 was performed by selecting transformants from the  
05 selective transformation plate and allowing them  
06 to grow in a liquid selective medium (YNB or YEPD  
07 with G418 at 1 mg/ml) for about 20 generations  
08 (two passages). After this growth period cells  
09 were inoculated into a non-selective medium (SMR  
10 or YEPD) and allowed to grow for another 40-60  
11 generations (5-6 passages). The cells were then  
12 plated on a selective plate (YNB or YEPD with G418  
13 at 1 mg/ml) and clones still expressing the  
14 selective marker were retained.  
15

16 3. Isolation of yeast DNA  
17

18 Total DNA from yeast was prepared as described in  
19 Struhl et al. (1979).  
20

21 4. Analysis of yeast DNA  
22

23 The structure of the DNA in yeast transformants  
24 was analysed by digestion with appropriate  
25 restriction endonucleases followed by agarose gel  
26 electrophoresis. DNA fragments were then  
27 transferred to nitrocellulose and hybridized with  
28 the appropriate radioactively labelled DNA probe  
29 (Southern 1975).  
30

31 5. Immuno assays  
32

33 Western Blot Analysis  
34

35 a) Amersham procedure  
36

37 Proteins obtained from crude extracts or from

01  
02  
03 gradient fractions were separated by  
04 SDS-polyacrylamide gel electrophoresis (Laemmli,  
05 1970) using 12.5% separating, 5% stacking gels.  
06 The protein bands were transferred onto  
07 nitrocellulose essentially as described by Towbin  
08 et al. (1979).

09  
10 The antigenic material was detected by reacting  
11 the blot with specific antibodies. The  
12 antigen-antibody complex was visualised by  
13 reacting the filter with biotinylated antibody RPN  
14 1001 (Amersham Buchler GmbH and Co. Ltd.,  
15 Braunschweig, FRG) in a 1:300 dilution followed by  
16 an incubation with peroxidase-streptavidin-complex  
17 RPN 1051 (Amersham). All steps of the experiment  
18 were performed according to the instructions of  
19 the manufacturer.

- 20  
21 b) Alternatively, the filter was preincubated with  
22 0.05% Tween<sup>TM</sup>(w/v) in PBS. Following incubation  
23 with the given monoclonal antibody, antibody  
24 binding was detected by successive incubation with  
25 biotinylated sheep anti-mouse IgG (RPN 1021,  
26 Amersham) in a 1:250 dilution containing 0.05%  
27 Tween 20<sup>TM</sup>, streptavidin-biotinylated horse radish  
28 peroxidase complex (RPN 1051, Amersham, dilution  
29 1:400 in PBS containing 1% gelatin) and finally 30  
30 µl H<sub>2</sub>O<sub>2</sub> and 10 ml HRP Colour Development reagent  
31 containing 4-chloro-1-naphthol (3 mg/ml methanol)  
32 in 50 ml PBS. Between incubations the filter was  
33 washed with PBS containing 0.05% Tween 20<sup>TM</sup>

34  
35 Quantification of produced monomeric antigens (S  
36 and preS) was achieved by comparison of samples of

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- 68 -

J2051

0.01-0.3  $\mu$ g of yeast-derived purified HBsAg  
(SmithKline Biologicals) to 3-5 different  
dilutions of crude protein extracts of Hansenula  
containing 2 to 20  $\mu$ g total cell protein.

The protein concentration was determined using a  
BioRad protein assay kit (BioRad Munich, FRG).

01  
02  
03 EXAMPLES: PART A  
04

05 Plasmids  
06

07 Example A.1  
08

09 Construction of plasmids enabling expression of protein  
10 in Hansenula polymorpha  
11

12 a) Construction of a basic plasmid containing an  
13 Hansenula polymorpha autonomously replicating sequence  
14 (pME4).  
15

16 The parental plasmid used to construct plasmids  
17 containing the S-gene under control of Hansenula  
18 promoters is pME4, which is already disclosed in the  
19 Ph.D thesis of M. Eckart, 1988. pME4 is a derivative of  
20 YIp5 (Struhl et al., 1979; Stinchcomb et al., 1980)  
21 obtained by the following modifications:  
22

23 The Hansenula polymorpha autonomously replicating  
24 sequence HARS1 is cloned into the SalI site of YIp5 as  
25 disclosed in Roggenkamp et al. (1986) to result in  
26 pHARS. Plasmid pHARS was then reconstructed by  
27 deleting and reinserting the 440 base pair HARS1-SalI  
28 fragment into the PvuII site of the same vector. The  
29 SalI sticky ends of the fragment were made blunt ended  
30 prior to ligation by incubation with exonuclease VII  
31 followed by reaction with Klenow polymerase. This  
32 reconstruction resulted in a single SalI site in the  
33 tetracycline resistance gene of the pBR322 part of the  
34 plasmid. The intermediate plasmid thus obtained was  
35 again reconstructed in order to obtain a beta-lactamase  
36 gene lacking the original signal sequence wherein the  
37 signal sequence is replaced by the linker shown below;  
38 for details, see Eckart (1988):  
39

|    | <u>SalI</u> |     | <u>EcoRI</u> |     | <u>BstEII</u> |     |     |     |             |
|----|-------------|-----|--------------|-----|---------------|-----|-----|-----|-------------|
| 04 | GTC         | GAC | GAA          | TTC | AAA           | ATG | TCC | GGT | CAC         |
| 05 | CAG         | CTG | CTT          | AAG | TTT           | TAC | AGG | CCA | GTG         |
| 06 |             |     |              |     |               | Met | Ser | Gly | His         |
| 07 |             |     |              |     |               |     |     |     |             |
| 08 |             |     |              |     |               |     |     |     | B-lactamase |

09 This step yielded plasmid pME4 as shown in figure 1.

10  
11 b) MOX and FMD promoter fragments.

12  
13 The isolation of the MOX promoter from H. polymorpha is  
14 disclosed in Eckart, 1988. For the purposes of the  
15 present invention a genomic EcoRI-SalI fragment of  
16 1525 bp containing the upstream control region of the  
17 MOX gene and the codons for the five first amino acids  
18 of the MOX protein was cloned into a pBR322  
19 derivative. A schematic representation of the MOX-gene  
20 is given in Fig. 2. This intermediate plasmid was  
21 cleaved using EcoRI and treated with nuclease Bal31 and  
22 subsequently ligated with EcoRI linkers. Thus a series  
23 of promoter fragments having EcoRI 3' ends and SalI 5'  
24 ends were obtained by subsequent cleavage with  
25 restriction endonuclease SalI. The positions of the  
26 various deletion endpoints were established by DNA  
27 sequencing according to the method of Maxam and  
28 Gilbert, 1977. For the purpose of further experiments  
29 described in this application a plasmid designated  
30 pBR322-MP containing a deletion up to position minus 3  
31 (when the first base of the MOX translation initiation  
32 codon is base plus 1) was used. The plasmid pBR322-MP  
33 is described in Eckart, 1988.

34  
35 The isolation and characterisation of the FMD promoter  
36 is disclosed in European Patent Application 87 110  
37 417.0. For the purposes of the present invention a 1.4

01 kb BamHI fragment encompassing about 1000 bp of the  
 02 upstream promoter region was cloned into the BamHI site  
 03 of pUC19. A clone was selected, in which the insert  
 04 was orientated in a way to have the single EcoRI-site  
 05 of pUC19 at the 3' end of the inserted fragment. A  
 06 schematic representation of the genomic clone  
 07 containing the FMD gene including its promoter is shown  
 08 in Figure 2B. A series of nuclease Bal31 deletions  
 09 starting from the EcoRI site within then pUC19-linker  
 10 was prepared in order to obtain plasmids containing the  
 11 promoter without sequences of the structural gene.  
 12 Following Bal31 treatment the DNA was ligated to EcoRI  
 13 linkers, the DNA was cleaved with BamHI and the  
 14 BamHI-EcoRI fragments were cloned into pBR322. A  
 15 variety of deletions were obtained. Deletions to  
 16 positions minus 5 and minus 9 from the first ATG were  
 17 most efficient in later experiments. For the further  
 18 experiments described in this application a plasmid  
 19 carrying a promoter fragment containing the deletion to  
 20 position -9 from the first ATG was used (plasmid  
 21 pBR322-FMD-P-9). A schematic representation of the  
 22 MOX- and FMD promoter fragments used for construction  
 23 of further plasmids is shown below.

24 i) MOX promoter fragment (-3)

|    |  |            |                  |          |
|----|--|------------|------------------|----------|
| 25 |  | SalI       |                  | EcoRI    |
| 26 |  |            |                  |          |
| 27 |  |            | -15              | -3       |
| 28 |  | ( 1500 bp) | ----AAACTACAAAAC | GGAATTCC |
| 29 |  |            |                  | Linker   |

ii) FMD promoter fragment (-9)

BamHI

EcoRI

-14 -9

( 1000 bp) ----TTCATC

GGAATTCC

Linker

C) Terminator

The isolation of a transcription terminator from the MOX gene is disclosed in Eckart, 1988. A schematic representation of the 3' region of the MOX gene is shown below:

Arg Phe Stop

-----

AGA TTC TAA

SalI-----Asp718-----EcoRV-----NruI

< 1477 bp >< 327bp >

< 284 bp >< 368bp >

The SalI-NruI fragment shown in the scheme above was subcloned into pBR322 between the SalI and NruI sites and this plasmid was then cleaved using restriction endonuclease Asp718 resulting in a linearisation of the plasmid due to a single Asp718 site within the MOX open reading frame, and subjected to Bal31 deletions.

By DNA sequence analysis of the resulting fragments a terminator fragment of about 320 bp was identified (Eckart, 1988) which still functions as terminator. This blunt ended terminator fragment (GACATACC--315bp-EcoRV) was ligated into the SmaI site of pUC19. Orientation of the fragment was established

01  
02  
03 by sequence analysis (Maxam and Gilbert, 1977). The  
04 resulting clone pT-24 is shown in Figure 3. This clone  
05 was used to construct expression vectors as shown  
06 below.

07  
08 d) Selective marker genes

09  
10 pME4 (described above, see Fig. 1) was cleaved with  
11 EcoRI and the sticky ends were filled in with Klenow  
12 polymerase. Plasmid pT-24 was digested with EcoRI and  
13 HindIII and the fragment comprising the pUC19 linker  
14 and the MOX terminator fragment isolated. The sticky  
15 ends of this fragment were made blunt ended by  
16 treatment with Klenow polymerase and the blunt ended  
17 fragment ligated with pME4. The resulting plasmid was  
18 called pP/T-24. The cloning sites derived from pUC19  
19 were later on used for further cloning steps.  
20

21 Plasmid pP/T-24 contains the S. cerevisiae URA3 gene as  
22 a selective marker. As a further selective marker a  
23 gene conferring resistance against chloramphenicol was  
24 introduced. This gene was isolated from vector pBR325  
25 as a 1.7 kb AatII-ClaI fragment, subjected to a short  
26 Bal31 digestion for removal of about four base pairs on  
27 each side, and made blunt-ended using Klenow fill-in  
28 reaction. Subsequently the fragment was ligated with  
29 pP/T-24, which had been cleaved with NruI. The  
30 resulting plasmid showing chloramphenicol resistance  
31 was called pP/T-24-C.  
32

33 Example A.2

34  
35 Construction of a plasmid containing the Hepatitis B  
36 surface antigen gene in functional combination with the  
37 Hansenula polymorpha terminator.  
38

01  
02  
03 In order to obtain a functional unit comprising the  
04 Hepatitis B S-gene and a terminator efficient in  
05 Hansenula polymorpha the S-gene was introduced as an  
06 NcoI-EcoRI fragment of 683 bp into the BamHI site of  
07 the plasmid pP/T-24C provided by the pUC19 linker  
08 portion of said plasmid. The 683 bp fragment was  
09 obtained from pRIT12331 which is a conventional pUC9  
10 based E. coli vector containing the 683 bp NcoI-EcoRI  
11 DNA fragment encoding the S-protein from NcoI at the  
12 ATG codon (CCATGG) to EcoRI beyond the TAA stop codon  
13 (TAACGAATTC). The surface antigen coding sequence was  
14 derived by conventional recombinant DNA techniques from  
15 pRIT10616 disclosed in EP-A-0278940 and in Harford et  
16 al., 1987. pRIT10616 contains the genome of a HBV virus  
17 of adw serotype cloned on pACYC184 and was deposited  
18 with the American Type Culture Collection under the  
19 terms of the Budapest Treaty on June 2, 1982 under  
20 Accession Number ATCC39131.

21  
22 The sticky ends of the S-gene fragment and of the BamHI  
23 cleaved pP/T-24C were made blunt ended by filling in  
24 prior to ligation. Ligation of the fragment in the  
25 proper orientation regenerates adjacent BamHI and NcoI  
26 sites on the construction immediately 5' to the S-gene  
27 coding region. The resulting plasmid is called pRB-S.

### 28 29 Example A.3

30  
31 Construction of plasmids containing a functional  
32 expression cassette comprising Hansenula polymorpha  
33 derived promoters, Hepatitis B surface antigen gene and  
34 Hansenula polymorpha terminator.

35  
36 The pBR322 derivatives pBR322-MP and pBR322-FMD-P-9  
37 containing the MOX or FMD promoters, respectively,

were digested with EcoRI and the sticky ends were filled in using Klenow polymerase. The plasmids were then cleaved with SalI. The resulting fragment containing the MOX promoter comprises exclusively Hansenula polymorpha genomic DNA, whereas the fragment comprising the FMD promoter contains in addition 275 bp of pBR322 sequences (position bp 375 to bp 650 of pRB322 map).

Plasmid pRB-S was cut at the regenerated BamHI site, the site was made blunt ended by filling-in with Klenow polymerase and subsequently the plasmid was digested with SalI. The promoter fragments having a SalI sticky end and a blunt end were ligated with the large SalI-blunt end fragment of pRB-S containing the S-gene coding region and the Hansenula polymorpha terminator. The resulting plasmids are called pRB-S-269 (MOX promoter) and pRB-S-271 (FMD promoter, minus 9 deletion) and contain the S-gene under control of the Hansenula polymorpha promoter and terminator as shown in Figure 4. The sequence surrounding the fusion between promoters and S-gene is shown in the schematic representation below.

a) MOX - promoter fusion (pRB-S-269)

|              |                 |
|--------------|-----------------|
| -3           | +1              |
| <---AAAAC    | GGAATTGATCC ATG |
|              | ----->          |
| MOX promoter | S-gene          |

b) FMD - promoter (-9) fusion

|              |                 |
|--------------|-----------------|
| -9           | +1              |
| <---TTCCATC  | GGAATTGATCC ATG |
|              | ----->          |
| FMD promoter | S-gene          |

Plasmids constructed identically to pRB-S-271 and pRB-S-269, but lacking the insertion of the 440 bp restriction fragment carrying the HARS1 sequences were called pRB-S-269I and pRB-S-271I. These plasmids were used to obtain integrants containing 1 to 3 copies of the expression cassette.

#### Example A.4

Construction of integrative vectors containing the S-gene.

a) Construction of plasmid pBC containing Hansenula polymorpha autonomously replicating sequences and H. polymorpha URA3 gene.

Plasmid pBC\* is derived from plasmid YRp7 (Tschumper and Carbon, 1980) containing the Saccharomyces cerevisiae ARS1 sequence. This autonomously replicating sequence was inactivated by insertion of a 5400 bp BglII-SphI fragment encompassing the Hansenula polymorpha URA3 gene. Furthermore the Hansenula autonomously replicating sequence 1 (HARS1) was cloned into the SalI site of the tetracycline resistance gene (Fig. 5).

b) Insertion of expression cassettes into plasmid pBC.

Plasmid pRB-S-269 was digested with BspMII and SalI.

The resulting fragment bearing the expression cassette comprising the MOX promoter, the Hepatitis B surface antigen encoding region and terminator was made blunt ended and ligated to plasmid pBC following cleavage with XhoI and StuI and filling in of the XhoI sticky ends. The resulting plasmid pMS-2 contains the S-gene

01  
02  
03 expression cassette surrounded by flanking sequences of  
04 the Hansenula polymorpha URA3 gene fragment.  
05

06 For integration of the S-gene expression cassette into  
07 the Hansenula polymorpha genome plasmid pMS2 was  
08 cleaved with NheI and the resulting fragment of 6.1 kb  
09 was used to transform the cells.  
10

11 Vector pFS-9 was constructed in an analogous way except  
12 that the expression cassette was isolated from  
13 pRB-S-271 containing the FMD promoter.  
14

15 A detailed description of the resulting plasmids is  
16 provided by way of Figure 5.  
17

18 Example A.5  
19

20 Construction of plasmids comprising the S-gene  
21 expression cassette and an additional selective marker  
22 gene.  
23

24 a) Construction of the basic plasmid pHK2  
25

26 For construction of plasmid pHK2 (Fig. 6; deposited  
27 under the terms of the Budapest Treaty with the DSM  
28 (DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND  
29 ZELLKULTUREN GmbH) under Accession number 5328 on April  
30 27, 1989) the kanamycin resistance gene was isolated  
31 from transposon Tn5 (Grindley and Joyce, 1980).  
32

33 Unnecessary sequences were deleted by treatment of the  
34 structural gene with nuclease Bal31. The resulting  
35 fragment comprising the kanamycin structural gene  
36 including the kanamycin terminator was ligated with the  
37 Saccharomyces cerevisiae ADH1 promoter (Hitzeman et al.  
38 1981). Furthermore the HARS1 sequence was introduced  
39 as a 440 bp SalI fragment into the plasmid.

b) Construction of kanamycin resistant strains derived from pRB-S-269 and pRB-S-271.

The HindIII fragment from pHK encompassing the kanamycin resistance coding region (3.5 kb) was cloned into the HindIII site present in the HARS1-sequence of plasmids pRB-S-269 and pRB-S-271. The resulting plasmids are called pRB-S-322 and pRB-S-326 respectively and are shown in Figure 7.

#### Example A.6

Construction of a parental plasmid for expression of pre-S proteins.

Plasmid pP/T-24 (Example A.2) was reconstructed in order to obtain a plasmid pMOX - P/T1 (Fig. 8) carrying a universal expression cassette comprising the MOX promoter, a multiple cloning site and the MOX terminator.

In order to insert a linker containing restriction sites for restriction endonucleases SalI, EcoRI, BglII and BamHI the pUC19 linker of plasmid pP/T-24 was partly replaced by synthetic DNA containing restriction sites for the above mentioned enzymes.

Plasmid pP/T-24 was cleaved with restriction endonucleases BamHI and SalI. These sites originate from the pUC19 linker (BamHI) and pME4 (SalI). The linker molecule containing restriction sites for SalI, EcoRI, BglII and BamHI was inserted. The resulting plasmid pT1 was digested with EcoRI and SalI and the MOX promoter was inserted as a 1.5 kb EcoRI-SalI fragment yielding plasmid pT2. The relevant areas of plasmid pT2 are shown below:

SaliI

EcoRI

BglII

BamHI

-1500

-3

-----//---AAAAAC GGAATTC AGATCT GGATCC AGCTT-----

&lt;-----

MOX promoter

-----&gt;

Terminator

Furthermore plasmid pT2 contains the Hansenula polymorpha ARS and Saccharomyces cerevisiae URA3 gene as in plasmid pRB-269 (Fig. 3).

The next construction step was the replacement of the Saccharomyces cerevisiae URA3 gene by the URA3 gene derived from Hansenula polymorpha. A clone containing this gene was isolated as described below in example A.9. The URA3 gene was isolated as a 1.2 kb BamHI-BglII fragment. Whereas the BglII site is an original genomic site, the BamHI site was the result of ligating a Sau3A site with a BamHI site used to construct the genomic library. The sticky ends of this BamHI-BglII fragment were filled in by Klenow polymerase and inserted into the AvaI site of pBR322, which also had been filled in with Klenow polymerase. The AvaI site was regenerated by the ligation and the resulting plasmid was designated pURA3-P1.

Plasmid URA3-P1 was digested with NruI and BspMII. These sites originate from pBR322, positions bp 974 and bp 1664 of the conventional pBR322 map. Plasmid pT2 was subjected to a complete digestion with NruI (bp 974 of pBR322) and partial digestion with BspMII.

01  
02  
03 There are 2 BspMII sites in pT2: The first in pBR322  
04 (bp 1664), the second is located in the MOX  
05 terminator. The NruI-BspMII fragment of pT2 (1800 bp)  
06 contains the URA3 gene of Saccharomyces cerevisiae.  
07 The NruI-BspMII fragment from URA3-P1 carrying the  
08 Hansenula polymorpha URA3 gene was cloned into pT2  
09 replacing the S. cerevisiae URA3 gene located on the  
10 corresponding NruI-BspMII fragment. The resulting  
11 plasmid pT3 contains the MOX terminator, a linker, the  
12 MOX promoter and the Hansenula polymorpha URA3 gene  
13 following one another. Plasmid pT3 lacks however a  
14 functional  $\beta$ -lactamase gene as in parental plasmid  
15 pME4. To replace this defective  $\beta$ -lactamase gene by a  
16 functional gene pBR322 was digested with EcoRI, the  
17 sticky ends filled-in with Klenow polymerase and  
18 ligated to a 8 bp Asp718 linker, followed by digestion  
19 with PvuI (position 3738 of the pBR322 map). The 633  
20 bp Asp718-PvuI fragment thus obtained bears the  
21 original promoter and translation initiation codon of  
22 the  $\beta$ -lactamase gene.

23  
24 Plasmid pT3 was subjected to cleavage by Asp718 (the  
25 Asp718 is located at the end of the MOX terminator) and  
26 to a partial digestion with PvuI. The Asp718-PvuI  
27 fragment obtained as described above was then inserted  
28 into the pretreated pT3 yielding the plasmid pMOX-P/T1.  
29

30 Plasmid pMOX-P/T1 is shown in Figure 8. This plasmid  
31 contains a functional beta-lactamase gene.  
32

Example A.7

Construction of plasmids expressing pre-S proteins.

The pre-S gene encoding the preS1-S2-S-protein (large protein) of Hepatitis B virus was obtained as a NcoI-HindIII fragment from pRIT12816. pRIT12816 is a conventional pBR322 based E. coli vector containing a 1185 bp NcoI-HindIII fragment encoding the preS1-S2-S-protein from NcoI at the ATG codon (CCATGG) to HindIII at 15 bp beyond the TAA stop codon (....AAGCTT). The preS1-S2-S-coding sequence fragment was derived by conventional cloning techniques from pRIT10616 disclosed in EP-A-0278940 and in Harford et al., 1987. A 861 bp fragment encoding the preS2-S sequence can also be recovered from this by conventional means. The NcoI-HindIII fragment was made blunt ended by filling-in with Klenow polymerase and cloned into the also made blunt ended EcoRI site of pMOX-P/T1. As a consequence of correct ligation the EcoRI site in front of the preS1 structural gene is regenerated by NcoI/EcoRI blunt ended ligation. The construction expressing the pre S-gene under control of the MOX promoter is called pMPS-22. This is shown in Figure 8.

The analogous construction containing the FMD promoter is called pMPS-21.

Example A.8

Construction of a vector containing a preS expression cassette.

The SalI-Asp718 expression cassette from plasmid pMPS22 was made blunt ended by filling in and inserted between

the filled in XhoI site and StuI sites of plasmid pBC described in Example A4a above. The resulting plasmid is called pMPS-9 and shown in Figure 9.

#### Example A.9

Cloning of the Hansenula polymorpha URA3 gene.

The URA3 gene of Hansenula polymorpha was cloned by complementation of the corresponding pyrF mutation of E. coli strain MB 1000.

Genomic DNA fragments ranging in size from 6 to 9 kbp were isolated via partial digestion with Sau3A, separation on a low melting agarose gel and isolation of the appropriate DNA fraction.

The purified fragments were ligated into the unique BamHI site of vector YRp7, containing an autonomous replicating sequence of Hansenula polymorpha inserted into the SalI site. The resulting ligation mixture was used to transform E. coli strain MB 1000 and transformants were selected on minimal plates without uracil. Transformants obtained were analysed and the plasmids were isolated. A sub-fragment as shown in Fig. 10 was isolated, which mediated the restoration of the URA<sup>+</sup> phenotype in E. coli, Saccharomyces cerevisiae and Hansenula polymorpha orotidine 5'-monophosphate decarboxylase mutants. The structure of the cloned DNA was shown to be identical to that of the genomic URA3 region by Southern blotting. A restriction map of the Hansenula polymorpha URA3 gene fragment is shown in Figure 10.

Part BExpression of Hepatitis B surface Antigen (HBsAg)  
in Hansenula polymorphaExample B.1Construction of Hansenula polymorpha strains containing  
the S-gene

Hansenula polymorpha RB 10 was transformed with the above described plasmids pRB-S-269, pRB-S-269-I, pRB-S-271, pRB-S-271-I. Several clones bearing autonomously replicating plasmids (transformants) or integrated foreign DNA (integrants) were obtained.

These transformants/integrants were then tested for the expression of S-gene using immuno assays. Two test systems were used: Western blotting and AUSRIA or AUSZYME tests (Abbott). The production of HBsAg monomer was analysed by Western blotting using monoclonal RF6 antibodies. Abbott's AUSRIA or AUSZYME Kits were used according to the instructions of the manufacturer to estimate the amount of HBsAg particles produced in Hansenula. Transformants showing a stable and high expression level were selected and their DNA analysed by Southern blotting following electrophoretic separation.

## a) Transformants

Several colonies bearing the freely replicating plasmid were obtained from Hansenula polymorpha transformed with plasmids pRB-S-269 and pRB-S-271. Southern analysis of the total DNA obtained from these transformants digested with different restriction

01  
02  
03 endonucleases showed the expected restriction pattern.  
04

05 b) Integrants  
06

07 Hansenula polymorpha can be transformed with the  
08 integrative vectors pRB-S-269-I and pRB-S-271-I at a  
09 high frequency. Integrants grown on complete medium  
10 (YEPD) are mitotically stable. Alternatively,  
11 mitotically stable maintenance of a foreign DNA in  
12 Hansenula can also be obtained by the procedure  
13 described above, wherein an autonomously replicating  
14 vector, preferably a high copy number plasmid such as  
15 pRB-S-269 or pRB-S-271 integrates spontaneously into  
16 the genome. The resulting integrants also show high  
17 mitotic stability of the foreign DNA.  
18

19 Both methods mentioned above were used to obtain  
20 strains efficiently expressing the S-gene in  
21 non-selective complete medium. 3 strains were  
22 retained; the strain having the identification number  
23 352, which was transformed with pRB-S-271, wherein the  
24 S-gene is regulated by the FMD-promoter, and strains  
25 No. 415 and 416, which were transformed with pRB-S-269  
26 containing a MOX regulated S-gene, were used for  
27 further experiments.  
28

29 Example B.2  
30

31 Mitotic stability of foreign DNA in integrants  
32

33 The stability of the recombinant strains 352, 415 and  
34 416 was tested by the following procedure: Strains were  
35 grown in non-selective SMR medium for 40 generations.  
36 Twelve independent colonies were isolated from the  
37 cultures before and after the 40-generation period.

01  
02  
03 The structure of the foreign DNA and the copy number of  
04 the expression cassette were examined in the isolated  
05 clones by Southern blot analysis in combination with  
06 agarose gel electrophoresis of restriction fragments  
07 and Pulsed Field Gradient Gel Electrophoresis of total  
08 chromosomes. The latter technique allows the  
09 separation and analysis of yeast chromosomes (Schwartz  
10 and Cantor, 1984).  
11

### 12 Pulsed Field Gel Electrophoresis

13

14 Cells from strains 415, 416 and 352 were subjected to  
15 gentle lysis in the sample wells of an 0.7% w/v agarose  
16 gel. The in situ liberated chromosomes were separated  
17 using an LKB-Pharmacia Pulsaphor Plus apparatus  
18 according to the instructions of LKB-Pharmacia.  
19 Following PFGE the DNA was transferred to  
20 nitrocellulose according to the procedure of Southern  
21 (1975). The blot was submitted to hybridisation using  
22 <sup>32</sup>P-labelled probes specific either for the MOX and/or  
23 FMD gene or the URA3 gene of Hansenula polymorpha, the  
24 latter being known to be present as a single copy in  
25 the genome.  
26

27 Comparison of the signals derived from possible  
28 multimeric integrants with those of single copy genes  
29 (intrinsic markers) allowed an estimation of the copy  
30 number of the relevant expression cassettes present  
31 inside the cell. The analysis of chromosomes by PFGE  
32 demonstrates that clone 415 has about 30 to 50 copies of  
33 the expression cassette, whereas clone 416 contains  
34 about 5 - 6 expression cassettes and clone 352 about 10  
35 expression cassettes. The analysis showed that the  
36 foreign DNA is integrated into the Hansenula genome.  
37

Southern Analysis

Strains 352, 415 and 416 were grown for 40 generations in SMR medium. Twelve independent colonies were isolated from each batch before and after the above mentioned growth period. DNA preparations from these sub-clones were then subjected to Southern analysis after cleavage with various restriction enzymes and using  $^{32}\text{P}$ -labelled DNA-probes. Analysis of the restriction patterns showed that the expression cassettes were intact and that the introduced DNA was genetically stable. The analysis further revealed that the genome of strain 415 contains long repeats with several copies of the plasmid. The plasmid is deduced to be arranged in this repeat in a head to tail manner.

Example B.3Expression studies

Recombinant strains were routinely analysed for the presence of HBsAg by Western Blotting and AUSRIA/AUSZYME tests.

## a) Growth of Cultures and Western blotting

Strains 352, 415 and 416 were grown in 11 flasks in 200 ml SMR-glycerol medium at  $37^{\circ}\text{C}$  with vigorous aeration. At the beginning of the stationary phase, which is characterised by the depletion of glycerol, 50 ml/l of a five fold concentrated SMR medium lacking glycerol was added together with methanol to a final concentration of 10 g/l. Samples of 10 ml volume were taken from the culture prior to addition of methanol and at 24 hours following addition of methanol. For

01  
02  
03 the purpose of comparison the same strains were grown  
04 also in SMR medium containing 30 g/l glucose  
05 (derepression of promoters). The cells were harvested  
06 in the late log-phase.  
07

08 From the harvested cells crude protein extracts were  
09 prepared by suspending a cell pellet corresponding to  
10 about 0.1 g dry weight in 5 ml of PE buffer (0.5 M  
11 NaCl, 0.1% Triton X-100, 10 mM phosphate buffer pH 7.5,  
12 2 mM PMSF). Approximately an equal volume of glass  
13 beads of 0.45 mm diameter was added until the mixture  
14 became almost solid. The mixture was subjected to  
15 vigorous shaking for 4 minutes at 4°C using a Braun MSK  
16 homogenisation (B. Braun and Diessel Biotech GmbH,  
17 Melsungen, FRG). Subsequently, 4 ml of PE buffer was  
18 added and the homogenate was mixed and centrifuged 5  
19 minutes, at 4°C, 40,000xg. Aliquots of 5 to 25 µg of  
20 the supernatant were subjected to SDS-polyacrylamide  
21 gel electrophoresis, using purified HBsAg (0.05 to 0.5  
22 µg) as a control. The presence of antigenic material  
23 was demonstrated by transferring the protein onto  
24 nitrocellulose according to the procedure of Towbin  
25 (1979) and detection with the monoclonal antibody RF6  
26 as described in Method 5a above. The Western blots  
27 allow an estimation of the amount of antigen produced  
28 in clones 352, 415 and 416, by reference to the known  
29 amounts of pure yeast-derived HBsAg.  
30

31 The results are summarized in Table 1. In methanol  
32 induced flask-cultures (SMR medium) with a cell density  
33 of about 5 g dry weight/l the S-protein constitutes  
34 about 2 to 5% of the total extracted protein of  
35 transformed strains.  
36

01  
02  
03 The comparison of expression in cells grown under  
04 conditions of induction (methanol), derepression  
05 (glycerol) and repression (glucose) demonstrates a very  
06 stringent control of the MOX and FMD promoters.  
07 Expression is completely blocked in glucose grown  
08 cells. In glycerol, synthesis of HBsAg is about 30% of  
09 the level obtained in induced cells.  
10

11 b) Antigenicity of HBsAg expressed in Hansenula  
12 polymorpha.  
13

14 The antigenicity of the material contained in crude  
15 extracts was measured using an Abbott AUSZYME test  
16 based on monoclonal antibodies. The results were  
17 expressed as arbitrary units (absorbance at 492 nm per  
18 0.1 µg protein; dilution of crude extracts and the  
19 reaction were performed in 150 mM NaCl, 25 mM phosphate  
20 buffer pH 7.4, 0.01% BSA, 0.01% Tween 20). The  
21 reactivity with the AUSZYME test indicates the presence  
22 of conformational epitopes characteristic for sub-viral  
23 particles.  
24

TABLE 1 HBsAg production in strains  
expression S-gene

| Strain | Promoter | Carbon Source | HBsAg<br>estimated<br>by Western<br>blotting<br>mg/100mg<br>protein | HBsAg<br>estimated<br>by AUSZYME<br>assay<br>arbitrary<br><u>A<sub>492</sub> units</u><br>0.1µg protein |
|--------|----------|---------------|---------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|
| 352    | FMD      | Glucose       | No signal                                                           | 0.3                                                                                                     |
|        |          | Glycerol      | 0.8-1.2                                                             | 10.0                                                                                                    |
|        |          | Methanol      | 2.5-3.0                                                             | 25.0                                                                                                    |
| 415    | MOX      | Glucose       | No signal                                                           | 0.1                                                                                                     |
|        |          | Glycerol      | 1.0-1.5                                                             | 12.0                                                                                                    |
|        |          | Methanol      | 4.0-5.0                                                             | 45.0                                                                                                    |
| 416    | MOX      | Glucose       | No signal                                                           | No signal                                                                                               |
|        |          | Glycerol      | 0.5-0.8                                                             | 5.0                                                                                                     |
|        |          | Methanol      | 2.0-3.0                                                             | 22.0                                                                                                    |

c) Density gradient centrifugation of crude extracts  
of H. polymorpha

The crude extracts obtained according to the procedure described above were subjected to density gradient centrifugation either using CsCl or sucrose gradients. Sucrose equilibrium sedimentation gradient centrifugation was performed by applying 100 to 200 µg (200 µl) of the crude protein extract on top of a 20 to

01  
02  
03 50% sucrose gradient in 50 mM NaCl, 2 mM EDTA, 25 mM  
04 NaPO<sub>4</sub>, pH 7.2. Tubes having a volume of 5 ml were  
05 centrifuged for 18 hours at 40,000 rpm in a Beckman SW  
06 50.1 rotor.

07  
08 Cesium chloride gradient centrifugation was performed  
09 by diluting 1 volume of the crude protein extract with  
10 1 volume of 3 M CsCl in 25 mM phosphate buffer pH 7.4  
11 Centrifugation was performed at 4°C, 40,000 rpm in a  
12 Beckman 50 Ti rotor for 40 hours.

13  
14 The gradients were subjected to fractionation and the  
15 various fractions tested using RF6 antibodies in  
16 Western blotting or using AUSRIA and/or AUSZYME  
17 assays. It could be shown that a peak of HBsAg  
18 material with a density of about 1.16 to 1.19 g/ml is  
19 formed in a CsCl density gradient. The material  
20 present in this peak reacts very well not only with RF6  
21 antibodies in Western blots but also with the AUSRIA  
22 and AUSZYME assays. This density as well as reactivity  
23 in AUSRIA/AUSZYME tests indicate the presence of  
24 conformational epitopes characteristic for particles.  
25 Moreover, in a further experiment the density of this  
26 material was compared to sub-viral particles isolated  
27 from human serum. It could be shown that both  
28 materials are found at a comparable density by  
29 isopycnic centrifugation in CsCl solutions.

30  
31 Sucrose gradient centrifugation of crude extracts gave  
32 peak fractions, which reacted with AUSRIA and AUSZYME  
33 and which constituted at least 80% of the HBsAg as  
34 shown by analyses of gradient fractions in parallel  
35 with AUSRIA/AUSZYME and Western blotting. The residual  
36 20% of the material is found mainly in the top and

01  
02  
03 bottom fractions of the gradient and react only in  
04 Western blot analysis, indicating that this material  
05 constitutes the monomeric form of the protein.  
06 Analysis of peak fractions obtained from CsCl gradients  
07 also revealed that at least 80% of the material forms  
08 particles.

09  
10 The Hansenula derived particles are resistant to  
11 proteolysis. Incubation of crude protein extracts  
12 containing particles for several hours at various  
13 temperatures reveals only a very minor degradation as  
14 shown by testing the AUSZYME values and analysing the  
15 intactness of the polypeptide by denaturing SDS-PAGE  
16 followed by Western blot. The material was further  
17 analysed by electron microscopy. The electron  
18 microscopy pictures clearly show particles with a  
19 diameter of about 22 nm.

20  
21 The above described results were obtained for all 3  
22 strains (352, 415 and 416).  
23

## Part C

Expression of preS1-S2-S protein in Hansenula polymorpha

Example C.1

Contruction of Hansenula polymorpha strains containing the preS1-S2-S gene

Hansenula polymorpha RB 10 was transformed with the 5570 bp PvuI-Asp718 fragment derived from pMPS-22, encompassing the URA3 gene and preS1 gene under control of the MOX promoter (see Figure 8). Furthermore, the corresponding fragment carrying the preS1 gene under control of the FMD promoter ( -9 deletion) was obtained from plasmid pMPS-21. The integrants resulting from these transformations were shown to contain 1 to 2 expression cassettes by Southern analysis. Integrants containing the preS1 gene under control of MOX promoter are referred to as preS-AM; integrants containing the preS gene under control of FMD promoter are referred to as preS-AF.

Integrants containing several expression cassettes were obtained by transforming Hansenula polymorpha with autonomously replicating plasmids pMPS22 and pMPS21 bearing the HARS sequence. Integrants were formed by spontaneous integration of these plasmids as described in ''Materials and Methods'', item 2b. These types of transformants were given the general designation preS-BM (MOX promoter) and preS-BF (FMD-promoter).

Strains preS-AM-405, preS-BM-402, preS-BM-403 and preS-BM-454 wherein expression of the preS1-gene is regulated by the MOX promoter were retained for further analysis.

01  
02  
03 2. Mitotic stability  
04

05 Mitotic stability of the foreign DNA present in the  
06 four strains identified in Example C.1 above was tested  
07 by Southern analysis of 12 independent subclones  
08 isolated before and after a 40 generations growth  
09 period as described in PART B. The analysis confirmed  
10 the genetic stability of the integrated vectors (see  
11 also an example in PART D.3).  
12

13 3. Expression of preS protein in Hansenula polymorpha  
14

15 Strains preS-BM-402, preS-BM-403, preS-AM-405 and  
16 preS-BM-454 were grown on SMR medium containing  
17 glycerol followed by induction of the cells with  
18 methanol (10 g/l) exactly as described in Part B.  
19 Cells were harvested 25 hours following addition of  
20 methanol and crude extracts were prepared as described  
21 in Part B.  
22

23 a) 12 µg amounts of crude extracts of strains  
24 preS-BM-402, preS-AM-405, and preS-BM-454 were analysed  
25 by polyacrylamide SDS-gel electrophoresis followed by  
26 Western blotting using a mixture of monoclonal  
27 antibodies (S1.1, S2.2 and S2.5) as described in  
28 ''Methods 5 (a)'' . Both types of strains showed an  
29 antigenic double band at 38/39 kD and a further band at  
30 45 kD.  
31

32 Strain preS-AM-405 showed a lower content of the 45 kD  
33 species relative to the 39 kD materials than did strain  
34 preS-BM-402; strain preS-BM-454 showed the highest  
35 relative content of the 45 kD species.  
36

37 The electrophoretic pattern of preS-protein from the

above strains was compared with antigen derived from human serum particles. Both human and yeast preparations show a band at about 38/39 kD when compared on Western blots.

b) A variation in the expression level for different strains was also seen. These differences were presumably due to variations in copy number of the expression cassette between the strains. The preS-antigen was estimated by Western blotting to constitute 0.1 to 1.5% of the total cell protein in different preS-A and preS-B strains as given in Table 2.

Crude extracts were also tested with the AUSZYME assay for the presence of particles. The results as shown in Table 2 demonstrate a very low reactivity expressed as A<sub>492</sub> units per 0.1 µg protein.

TABLE 2 - Expression of preS in H. polymorpha

| Clone       | Estimated PreS<br>protein by<br>Western<br>blotting<br>mg preS1/100mg | AUSZYME<br>A <sub>492</sub> units<br>per 0.1 µg<br>protein |
|-------------|-----------------------------------------------------------------------|------------------------------------------------------------|
| preS-AM-405 | 0.1-0.2                                                               | 0.01                                                       |
| preS-BM-402 | 0.3-0.5                                                               | 0.02                                                       |
| preS-BM-403 | 0.4-0.6                                                               | 0.02                                                       |
| preS-BM-454 | 1.2-1.5                                                               | 0.1-0.2                                                    |

01  
02  
03 c) Glycosylation: Strain preS-BM-454 characterised  
04 by high production of preS protein (about 1.5% of total  
05 cell protein) and a pronounced 45 kD band was chosen  
06 for further studies on glycosylation. PreS protein  
07 from preS-BM-454 was analysed by incubating the crude  
08 protein extracts with EndoH endoglycosidase. The  
09 following data clearly show that the 45 kD species is a  
10 glycosylated form of preS protein.  
11

12 Upon incubation of 45 µg crude protein for 0.5, 1.0 and  
13 24 hours with 0.5 mU EndoH endoglycosidase according to  
14 the instructions of the manufacturer (Boehringer  
15 Mannheim, FRG), the 45 kD band shifted to the apparent  
16 molecular weight of 42 kD. This shift indicates that  
17 the 45 kD species is a N-glycosylated form of preS  
18 protein, bearing one 'core' group of about 3000 D.  
19

20 Tunicamycin, known to be a potent inhibitor of  
21 N-glycosylation, was also used to study the  
22 glycosylation of preS protein in Hansenula polymorpha.  
23 Strain preS-BM-454 was grown to a density of  $A_{600} =$   
24 10.0 in YNB medium containing glucose (10 g/l). 10 ml  
25 of this culture was inoculated into 100 ml of YNB  
26 containing 10 g/l methanol and 50 µg/ml tunicamycin.  
27 The pH value of the medium was kept constant at 7.2 to  
28 7.5. A control experiment was conducted without use of  
29 tunicamycin. The cells were harvested 10, 15 and 30  
30 hours following inoculation of the medium containing  
31 tunicamycin.  
32

33 Crude protein extracts were analysed by Western  
34 blotting. The studies demonstrated that in the cells  
35 grown in the presence of tunicamycin a single band at  
36 42 kD and a double band at 38/39 kD are visible. In

01  
02  
03 the control experiment the 42 kD band was replaced by a  
04 45 kD band. Similar results were obtained with  
05 extracts from strains preS-BM-402, preS-BM-403 and  
06 preS-AM-405, which however produce much lower amounts  
07 of the 45 kD protein species (not more than 5 to 10% of  
08 total pre-S-protein synthesised). The results suggest  
09 that the 42 kD band represents an O-glycosylated form  
10 of preS-protein.  
11

12 d) Density gradient centrifugation  
13

14 Crude extracts from strain preS-AM-405 and strains  
15 preS-BM-402, preS-BM-403, preS-BM-454 were subjected to  
16 analysis by sucrose and CsCl gradient centrifugations.  
17 The conditions were described in Part B 3(c). The  
18 gradient fractions were tested by Western blotting and  
19 by AUSZYME tests.  
20

21 The analysis revealed that in contrast to strains  
22 expressing S antigen (PART B) or both preS1 and S  
23 antigens (PART D), the preS-strains do not efficiently  
24 form sub-viral particles. Neither isopycnic CsCl nor  
25 sucrose gradient centrifugation revealed formation of a  
26 band of the required density. This was also confirmed  
27 by the fact that the HBsAg-related material from  
28 gradient fractions and crude extracts reacted poorly  
29 with the AUSRIA or AUSZYME tests.  
30

PART DFormation of composite particles containing both preS  
and S antigensExample D.1Construction of strains

Several strains were constructed which are characterised by different ratios of preS1 to S expression and different total expression levels.

These differences were achieved by variation of the copy number of the expression cassettes per genome. Additional variability was obtained by placing the genes under the control of different Hansenula promoters.

Three basic types of recombinant strains were obtained as follows:

- a) Strains containing one copy of the preS1 gene and one copy of the S gene (A - strains)

The 6.6 kb NheI DNA fragment containing the preS1 cassette and the URA3 gene was isolated from plasmid pMPS9 (Figure 9). A NheI DNA fragment containing the S gene was isolated from plasmid pMPS2 (Figure 5). The fragments were ligated using T4 DNA ligase and joined fragments consisting of one copy of each of the genes were isolated from agarose gels. These fragments were used to transform Hansenula polymorpha strain RB10 to uracil independence.

01  
02  
03 The resulting transformants were analysed by Southern  
04 blotting and clones containing one integrated copy of  
05 each cassette were selected. Strain preS/S-A-293 was  
06 retained for further analysis.

07  
08 b) Strains containing one preS expression cassette  
09 and several S expression cassettes (B - strains)

10  
11 Hansenula polymorpha was transformed with the 5570 bp  
12 Asp718-PvuI fragment from pMPS-22 containing the preS1  
13 expression cassette. Transformants bearing one  
14 integrated copy of the cassette were selected. The  
15 strain retained was preS-AM-405 described in part C.1  
16 above.

17  
18 Strain preS-AM-405 was then transformed with  
19 autonomously replicating plasmids pRB-S-326  
20 (FMD-promoter) or pRB-S-322 (MOX-promoter) (Figure 7)  
21 containing the S gene under control of the respective  
22 promoters. Both plasmids in addition bear a gene  
23 encoding resistance against gentamycin G418 as a  
24 selective marker. The Kan gene is under the control of  
25 the Saccharomyces cerevisiae ADH1 promoter (Figure 7)  
26 as described above. Transformation yielded several  
27 strains containing always one integrated copy of the  
28 preS1 cassette and multiple integrated copies (30 to  
29 100) of the S gene containing cassette as shown by  
30 Southern blot and PFGE analyses.

31  
32 Strains preS/S-B-431, preS/S-B-432 and preS/S-B-433  
33 were retained for further analysis. Strain  
34 preS/S-B-431 carries a MOX - promoter controlled  
35 S-gene, whereas preS/S-B-432 and preS/S-B-433 contain  
36 expression cassettes comprising the FMD promoter.  
37

01  
02  
03 c) Strains containing several expression cassettes of  
04 both the S and preS genes (C-strains)  
05

06 Hansenula polymorpha was transformed with the  
07 autonomously replicating plasmid pMPS-22 containing the  
08 pre-S1 gene (Figure 8). Transformation yielded several  
09 strains containing variable numbers of expression  
10 cassettes integrated into the genome. Isolates  
11 containing 2 to 20 preS1 expression cassettes were  
12 identified by Southern blotting. Those strains were  
13 described in Part C as preS-BM-402, preS-BM-403 and  
14 preS-BM-454 and express different amounts of preS  
15 antigen. These preS strains were then again  
16 transformed with autonomously replicating plasmids  
17 pRB-S-322 (MOX - promoter) and pRB-S-326 (FMD -  
18 promoter), which bear the S-gene (Figure 7).  
19 Resistance against G418 was used as a transformation  
20 marker. Selection for stable integrants yielded  
21 several clones for each transformation some of which  
22 were further analysed. Strains retained for further  
23 analysis were preS/S-C-452, preS/S-C-465 derived from  
24 strain preS-BM-402, strain preS/S-C-466 derived from  
25 strain preS-BM-403, strain preS/S-C-448-C4 derived from  
26 strain preS-BM-454 wherein the S-gene expression  
27 cassettes comprise the FMD promoter. Table 3 provides  
28 detailed information about the origin and designation  
29 of the above described strains.  
30

TABLE 3

Characteristics of strains expressing both pres and S-protein

| Strain          | Estimated number of pres cassettes | Estimated number of S-cassettes | Total expression of pres/S (Western) mg/100mg | Reactivity with AUSZYME arbitrary A492 units * | Pres to S ratio estimated from Immunoblotting |
|-----------------|------------------------------------|---------------------------------|-----------------------------------------------|------------------------------------------------|-----------------------------------------------|
| pres/S-A-293    | 1                                  | 1                               | ca. 0.3-0.4                                   | 5.0                                            | ca: 1:1                                       |
| pres/S-B-431    | 1                                  | ca.10                           | 1.5-2.0                                       | 10-15                                          | ca: 1:15                                      |
| pres/S-B-432    | 1                                  | ca.10-15                        | 2.5-3.0                                       | 10-20                                          | ca: 1:15                                      |
| pres/S-B-433    | 1                                  | ca.20                           | 2.0-2.5                                       | 10-15                                          | ca: 1:20                                      |
| pres/S-C-452    | several                            | >30-50                          | 3.0-4.0                                       | 20-30                                          | ca: 1:8                                       |
| pres/S-C-453    | several                            | >30-60                          | 3.5-4.5                                       | 15-25                                          | ca: 1:10                                      |
| pres/S-C-465    | several                            | >30-50                          | 3.5-4.5                                       | 25-35                                          | ca: 1:5                                       |
| pres/S-C-466    | several                            | >50-80                          | 3.5-4.5                                       | 25-35                                          | ca: 1:8                                       |
| pres/S-C-448-C4 | >20-30                             | >10-20                          | 3.0-3.5                                       | 10-20                                          | ca: 5:3                                       |

\* Numbers represent the lowest and highest values determined for a given strain.

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Example D.2Expression of preS and S-proteins in H. polymorpha

Expression of preS and S-proteins in different transformant strains of H. polymorpha was tested by immunoblotting as described above in Materials and Methods 5a and by the AUSZYME assay. A summary of the results and the properties of the various strains tested is given in Table 3.

Strains expressing both the preS and S-proteins were grown and induced as described in part B item 3 except that 30 ml culture volumes were used. Crude cell extracts were prepared as described above and subjected to SDS-polyacrylamide gel electrophoreses followed by Western blotting using RF6 or a mixing of S1.1 and S2.1 monoclonal antibodies for detection. 12 to 15 µg amounts of crude extracts from methanol induced cells of strains preS/S-A-293, preS/S-B-431, preS/S-B-432, preS/S-B-433, preS/S-C-453, preS/S-C-465, preS/S-C-466 and preS/S-C-448-C4 were immunoblotted using monoclonal RF6 for detection. This analysis demonstrates that a spectrum of strains can be obtained which express different ratios of preS to S-protein. From these and other immunoblots the ratio of preS to S-protein in strain preS/S-B-431 was estimated to be about 1:15, in strain preS/S-C-452 to be about 1:8 and in strain preS/S-448-C4 to be about 5:3. The strains were found to differ also in their level of productivity both by immunoblotting and by AUSZYME assay as shown in Table 3. Cell extracts from these strains were also immunoblotted using monoclonal S1.1 for detection. In most strains the preS protein is detected as the doublet protein band at 38-39 kD with lesser amounts of the 45 kD band (which is estimated to comprise only

about 5% of the total preS-protein detected). In contrast strain preS/S-C-448-C4 expresses approximately equivalent amounts of the 38-39 kD and 45 kD species.

### Example D.3

#### Stability of the foreign DNA

The stability of the foreign DNA in the above described preS/S transformants and integrants was analysed as described in part B. The studies indicate a very good mitotic stability of the recombinant clones. Southern analysis of 12 independent colonies isolated before and following a 40-generation fermentation of strains preS/S-453 and preS/S-431 demonstrated that in all cases the same restriction fragment pattern was visible. The preS/S strains listed in Table 3 were also analysed by Pulsed Field Gradient Electrophoresis. The separated chromosomes were blotted onto nitrocellulose membranes and then hybridised with different radioactive DNA probes. The procedure allows estimation of the number of copies of the S-expression cassette and proved conclusively that these preS/S strains are integrants. The results of these experiments are summarised in Table 3 above.

### Example D.4

#### Isolation and characterisation of composite particles

##### a) Partial purification of composite particles from *Hansenula polymorpha*.

A fermenter culture of *Hansenula polymorpha* strain preS/S-B-431 was grown in S-medium described above in Materials, 5d and the cells recovered by centrifugation

01  
02  
03 after 78 hours induction with methanol. The cell  
04 pellet was resuspended at a concentration of about  
05 120 g dry cell weight per litre in buffer containing  
06 0.5% (w/v) Tween 20, 2 mM EDTA, 4 mM PMSF, 5% (v/v)  
07 isopropanol and 50 mM sodium phosphate buffer pH 9.0.  
08 Cells were broken in a bead mill (Dynomill™ Type KDL,  
09 Bachofen AG, Basel, Switzerland) containing 0.45-0.7 mm  
10 diameter glass beads using 4 passages at a flow rate of  
11 6 liters per hour and a residence time of 7 minutes in  
12 the chamber.

13  
14 The cell extract was clarified by centrifugation for 45  
15 minutes at 16000 g at 4°C and the supernatant  
16 recovered.

17  
18 1% (w/v) colloidal silica (Aerosil 380™, Degussa,  
19 Frankfurt, FRG) was then added to the supernatant with  
20 stirring and the mixture was stirred overnight at 4°C  
21 to permit adsorption of HBsAg. The silica was  
22 recovered by centrifugation for 30 minutes at 4000 g at  
23 4°C and washed twice with 0.9% (w/v) NaCl by  
24 resuspension of the pellet and recentrifugation. HBsAg  
25 adsorbed to the silica was desorbed by resuspension of  
26 the washed pellet in 10 mM sodium pyrophosphate pH 9.5  
27 and incubation for 3 hours at 37°C with constant  
28 stirring. The volume of sodium pyrophosphate buffer  
29 added was about one eighth to one tenth of the initial  
30 clarified cell extract volume. The solution was then  
31 centrifuged at 17,000 g for 60 minutes at 4°C and the  
32 supernatant recovered.

33  
34 The supernatant was made 1.5 M with CsCl and the  
35 mixture centrifuged to isopycnic equilibrium for 70  
36 hours at 45000 rpm in a 50.2 Ti rotor (Spinco Division,  
37 Beckman Instruments, Palo Alto, USA). The resulting

CsCl gradients was fractionated and fractions tested for the presence of HBsAg using an Elisa kit (Enzygnost, Behring-Werke AG, Marburg, FRG) as described by the manufacturer. Peak fractions were pooled and dialysed against 10 mM sodium/potassium phosphate, 0.15 M NaCl pH 6.8. Assays made on this material and the crude cell extract showed that 48% of total AUSTRIA reactive material, less than 3% of total proteins, less than 0.2% of total sugars and less than 2% of total lipids were recovered in this procedure.

A cell pellet of Hansenula polymorpha strain preS/S-C-452 was also extracted by exactly the same procedure with similar results.

Samples of the partially purified HBsAg preparations from the preS/S-B-431 and preS/S-C-452 strains were examined by gel electrophoresis and immunoblotting using monoclonals HBS1 and S1.1 for detection of HBsAg related proteins as described in Part D.4 below.

Immunoblotting with monoclonal HBS1 showed the presence of a reactive band at 24 kD and a further band at about 38/39 kD in both preparation. Immunoblotting with monoclonal S1.1 detected only the preS1-protein related band at 38/39 kD in both preparations.

This shows that both the preS1-protein and S-protein species copurify through colloidal silica adsorption and isopycnic CsCl density gradient centrifugation.

b) CsCl density gradient centrifugation of composite particles.

A fermenter culture of H. polymorpha strain preS/S-B-431 was grown in S-medium (Materials, 5d) and

01  
02  
03 harvested after 73 hours induction with methanol. The  
04 cell pellet was recovered by centrifugation and  
05 resuspended and a cell extract prepared by passage  
06 through a French Press as described below. Cellular  
07 debris was removed from the crude lysate by  
08 centrifugation for 30 minutes at 17000 g. Crude cell  
09 extracts were then centrifuged to equilibrium at  
10 245,000 g in 1.5 M CsCl, phosphate buffered saline pH  
11 7.5. Gradients were fractionated and each fraction  
12 analysed for antigenicity using ELISA tests specific  
13 for HBsAg (Enzygnost™: Behringwerke AG, Marburg, FRG)  
14 and for preS1-protein. The Enzygnost tests measures  
15 only assembled HBsAg particles, not protein monomers.  
16 The preS1-protein specific Elisa test employs murine  
17 monoclonal antibody S1.1 for antigen capture and  
18 detection and is described below in Part D.5.  
19

20 A peak of HBsAg activity was observed at a density of  
21 1.18-1.19 g/cm<sup>3</sup> in the CsCl gradient. A major peak of  
22 preS1-protein activity was observed at this same  
23 density together with a minor peak at a density of 1.26  
24 g/cm<sup>3</sup>.  
25

26 The gradient fractions were also analysed for the  
27 presence of preS1-protein S-protein by immunoblotting.  
28 After sodium dodecylsulfate polyacrylamide gel  
29 electrophoresis according to Laemmli (1970) and  
30 blotting to nitrocellulose according to Towbin et al.  
31 (1979), the nitrocellulose sheet was incubated with a  
32 rabbit polyclonal serum raised against serum derived  
33 HBsAg. Detection of antibody binding was by the  
34 alkaline phosphatase method.  
35

36 A major protein band at 24 kD corresponding to the S  
37 protein and a double band at 38/39 kD were observed.  
38 The maximum activity in the immunoblotting for all

three protein species was in CsCl gradient fractions with a density of 1.18 - 1.19 g/cm<sup>3</sup> which corresponds to the peak of HBsAg activity measured in the Enzygnost test. This indicates that the majority of HBV surface antigen proteins in the crude extract are present in a lipoproteic structure with a density characteristic of HBsAg.

A further nitrocellulose sheet was incubated with murine monoclonal antibody S1.1. This antibody detected only the protein bands at 38/39 kD in fractions corresponding to a CsCl density of 1.18-1.19 g/cm<sup>3</sup>. The crude cell extracts were also subjected to rate-zonal centrifugation on a sucrose gradient. Crude cell extracts were loaded on 5-20% (w/v) sucrose gradients made up in 50 mM Tris-HCl buffer pH 8.1 and centrifuged at 288,000 g for 200 minutes. Gradients were fractionated and the fractions analysed for the presence of HBsAg by the Enzygnost Elisa test and for the presence of preS1-protein containing HBsAg by capture of antigen with the HBsAg specific antibodies from the Enzygnost test and detection with preS1-protein specific Mab S1.1. The Enzynost assay showed a peak of HBsAg activity sedimenting through the gradient.

The Mab.1.1 Elisa showed the same peak as the Enzygnost assay but with a shoulder of activity extending into higher sucrose densities. This shoulder of material with a higher sedimentation coefficient is eliminated when crude extracts from Hansenula polymorpha strain preS/S-B-431 were partially purified by adsorption/desorption on colloidal silica and centrifugation in a CsCl gradient before analysis by sucrose density gradient centrifugation. These results

show that both HBsAg and preS1-protein cosediment in CsCl density gradients with a density characteristic of lipoproteic particles.

c) Antigenicity of composite HBsAg particles expressed in H. polymorpha

HBV surface antigen particles were partially purified from a crude extract of H. polymorpha strain preS/S-B-431 as described above in item 3a. This preparation was tested for reaction with murine monoclonal antibodies (Mabs) directed against epitopes located on the preS1, preS2 and S regions of the surface antigen protein and for the presence of the receptor for polymers of human serum albumin (pHSA-receptor) on the preS2 region.

C1. Reaction with MabS1.1

A murine monoclonal antibody (Mab) S1.1 directed against a preS1 epitope of the HBV surface antigen protein was used to detect the presence of this site on the composite particle by an ELISA assay. MabS1.1 was coated on the wells of a microtitre plate (Immunoplate I; Nunc, Gibco Europe, Ghent, Belgium) and incubated with the partially purified crude extract. After washing to remove non-bound material, capture of reactive particles was revealed by incubation with MabS1.1 coupled to horse radish peroxidase by the method of Wilson and Nakame, 1978.

Colour development to reveal second antibody binding was with orthophenylenediamine as chromogen. Absorbance was read at 490 nm (with 620 nm for the reference filter) in an Intermed Immunoreader<sup>TM</sup>, NJ 2000 (Analisis, Ghent, Belgium).

The partially purified extract from H. polymorpha strain preS/S-B-431 gave a positive reaction in the test indicating capture by and binding MabS1.1 which is specific for the preS1 region. Surface antigen particles containing only S-protein purified from S. cerevisiae RIT4376 (Harford et al., 1987) gave no reaction in this test.

C2. Reaction with MabS2.5

Murine monoclonal antibody S2.5 is directed against the preS2 region of the HBV surface antigen. This Mab was used in an ELISA test to determine the presence of the preS2 epitope on the composite particles. MabS2.5 was coated on wells of a microtitre plate and incubated with the partially purified cell extract from H. polymorpha preS/S-B-431. Antigen binding was revealed by incubation with MabS2.5 coupled with horseradish peroxidase and colour development as described above. The partially purified cell extract from H. polymorpha preS/S-B-431 gave a positive reaction in the test indicating the presence of the preS2 region epitope on the surface antigen particles. Surface antigen particles containing only S-protein purified from S. cerevisiae RIT4376 gave no reaction in the test.

C3. Reaction with Mabs RF1 and S1.1 (HRP)

Murine monoclonal antibody RF1 (H. Thomas, Royal Free Hospital, London, U.K.) is directed against a conformational dependant epitope located in the S region and was used in an ELISA test. This Mab was used to coat wells of microtitre plates and incubated with the partially purified extract of H. polymorpha strain preS/S-B-431. Particle binding was revealed by incubation with the horseradish peroxidase coupled S1.1

Mab described in section C.1 above. The partially purified extract from H. polymorpha gave a positive result in the test whereas surface antigen particles purified from S. cerevisiae RIT4376 gave no reaction.

C4. Assay for the pHSA receptor

An assay for the detection of the pHSA receptor present on the preS2 region was made according to the procedure of Pontisso et al. (1983).

Polymerised human serum albumin was coated on the wells of a microtitre plate and incubated with the partially purified cell extract of H. polymorpha strain preS/S-B-431. After washing to remove non-bound material, particles captured on the plate were detected by incubation with polyclonal anti-HBsAg rabbit antibodies labelled with I<sup>125</sup> (AUSAB kit). The partially purified extract from H. polymorpha gave a positive reaction in the test whereas HBsAg purified from S. cerevisiae RIT4376 gave no reaction in the test.

The above results show that the HBsAg reactive material present in the partially purified extract from H. polymorpha contains antigenic sites specifically bound by Mabs directed against epitopes in the preS1, preS2 and S regions of HBV surface antigen and that this material also contains a site reacting with pHSA.

d) Immune precipitation of composite particles.

To determine whether the AUSRIA reactive material present in a partially purified extract of H. polymorpha strain PreS/S-B-431 contained composite particles an immune precipitation with a monoclonal

antibody specific for the preS1 protein was performed.

Immune complexes were captured by formalin-fixed Staphylococcus aureus (Staph A) cells (Immunoprecipitin, BRL, Gaithersburg, MD, USA) using rabbit anti-mouse serum as sandwich antibody between the monoclonal antibody and the Staph A cells. Staph A cells were pre-treated as recommended by the supplier and finally washed and resuspended at 10% (w/v) in PBS. The Staph A cells (120  $\mu$ l of 10% w/v suspension) were then incubated with 20  $\mu$ l of rabbit anti-mouse serum (RAM) for 2 hours at room temperature. The Staph A-RAM complex was collected by centrifugation, washed twice with PBS containing 0.1% (v/v) Tween 20, and finally resuspended at about 10% (w/v) in PBS containing 0.1% (v/v) Tween 20.

To a partially purified preparation of particles of H. polymorpha strain preS/S-B-431 obtained as described above and to a sample of yeast-derived S-protein HBsAg particles (lot HB193, Smith Kline Biologicals, Rixensart, Belgium) both containing 2  $\mu$ g AUSRIA reactive material in 500  $\mu$ l was added 1  $\mu$ l of MabS1.1 (638  $\mu$ g protein per ml) and the mixtures were incubated for 2 hours at room temperature. 20  $\mu$ l of the Staph A-RAM complex was then added and incubation continued overnight at 4°C. Immune complexes formed were collected by centrifugation, washed 5 times with PBS containing 0.1% (v/v) Tween 20 and once with PBS before resuspending the final pellets in sample buffer for SDS polyacrylamide gel electrophoresis. The immune precipitates were analysed by immunoblotting as described below using mouse monoclonal antibody RF6 (H. Thomas, Royal Free Hospital, London) for detection of HBsAg protein species. Monoclonal antibody RF6 is directed against a denaturation and reduction resistant

epitope of plasma derived HBsAg and competes in binding experiments with monoclonal HBS1.

The immune blot showed that the immune precipitate obtained from the H. polymorpha preS/S-B-431 particles contained not only the preS1-protein but also the S-protein while the immune precipitate from the Saccharomyces derived HBsAg contained no S-protein.

This confirms that composite particles of mixed polypeptide composition are present in the material partially purified from H. polymorpha strain preS/S-B-431 cell extracts.

#### e) Electron microscopy

Partially purified HBsAg particles from H. polymorpha strain preS/S-B-431 were examined by electron microscopy. Material was diluted in 20 mM Tris-HCl buffer pH 8.2 with 0.1% (w/v) bovine serum albumin and sedimented onto copper/rhodium grids covered with a film of celloidin before staining with uranyl acetate. Examination in the electron microscope showed the presence of spheroidal to spherical particles with a mean diameter of 27 nm. This size is within the range of mean particle diameters measured by the same technique for a series of HBsAg preparations purified from S. cerevisiae strain RIT4376 (Petre, J. et al, Postgrad. Medical Suppl. 2 73-81 (1987)).

#### Example D.5

#### 5. Comparison of composite particles expressed in H. polymorpha and S. cerevisiae

Cultures of strain preS/S-C-453 of H. polymorpha and

01  
02  
03 S. cerevisiae strain Y1108 were grown in the S-medium  
04 as described in Materials, 5d. Strain Y1108 is a  
05 diploid strain of S. cerevisiae expressing both the  
06 preS1 and S-proteins from expression cassettes  
07 integrated into the genome by Ty vectors. The strain  
08 contains 5-7 copies of a preS1 expression cassette and  
09 3-4 copies of a S- expression cassette. The strain  
10 requires tryptophan for growth. Construction and  
11 chromosomal integration of such expression cassettes  
12 using Ty vectors is described in copending USSN 07/292  
13 202 filed December 30, 1988 assigned to SmithKline  
14 Beckman Coporation which is hereby incorporated as  
15 reference. Glycerol at 1.5% (w/v) was used as carbon  
16 source for growth of H. polymorpha. A series of 2  
17 litre erlenmeyer flasks containing 400 ml of medium  
18 were inoculated with H. polymorpha strain preS/S-C-453  
19 and incubated on an orbital shaker for about 33 hours  
20 at 30°C until exhaustion of glycerol. The cells from  
21 three flasks were harvested by centrifugation and the  
22 cell pellet stored at -70°C until further processing.  
23 100 ml of culture medium was removed from each of six  
24 further flasks and replaced with 100 ml of fresh medium  
25 containing 4% (w/v) methanol. Incubation was then  
26 continued.

27  
28 Three cultures were harvested by centrifugation after a  
29 further 14 hours incubation and a further three  
30 cultures harvested after 38 hours incubation in the  
31 presence of methanol. The cells were recovered by  
32 centrifugation and the cell pellet stored at -70°C  
33 until further processing.

34  
35 In a second experiment cultures of S. cerevisiae Y1108  
36 were grown as described above and harvested after 19.5  
37 hours incubation. Cultures of H. polymorpha  
38 preS/S-C-453 were also grown as described above except

01  
02  
03 that cells were harvested after 26 hours growth in  
04 glycerol medium and after 45 hours in methanol  
05 containing medium.  
06

07 Cell pellets of Hansenula polymorpha were resuspended  
08 at a concentration of about 25% wet cell pellet weight  
09 per volume in buffer containing 0.5% (w/v) Tween 20, 2  
10 mM EDTA, 4 mM PMSF (phenylmethylsulphonylfluoride), 5%  
11 (w/v) isopropanol and 50 mM sodium phosphate pH 9.0 10  
12 ml of this mixture was passaged 6 times in a French  
13 Press at 20,000 psi to break the cells. The crude cell  
14 extract was then clarified by centrifugation at 17,000  
15 g for 45 minutes at 4°C and the supernatant recovered.  
16 Cell pellets of S. cerevisiae were treated identically  
17 except that cells were resuspended at a concentration  
18 of about 50% wet cell pellet weight per volume of the  
19 buffer described above.  
20

21 The protein content of each supernatant was determined  
22 by the method of Lowry et al. (1951). The clarified  
23 cell extracts were assayed for HBSAg by AUSRIA and for  
24 the presence of a preS1 protein epitope by an Elisa  
25 assay. The monoclonal S1.1 Elisa assay was performed  
26 as follows. Wells of plastic trays (Nunc Immunoplate  
27 1, Gibco Europe, Ghent, Belgium) were coated with S1.1  
28 mouse monoclonal antibody in 50 mM sodium bicarbonate  
29 buffer pH 9.6 for 2 hours at 37°C, and the antibody  
30 solution removed. The wells were then blocked by  
31 incubation with a PBS solution containing 1% (w/v)  
32 bovine serum albumin for 1 hour at 37°C. Serial two  
33 fold dilutions of cell extracts made in PBS, 0.2% (w/v)  
34 bovine serum albumin were then added to wells and  
35 incubated 1 hour at 37°C.  
36

37 The wells were then washed 3 times with 0.9% (w/v)  
38 NaCl, 0.05% (w/v) Tween 20. Antigen capture was

01 revealed by incubation with monoclonal antibody S1.1  
02 coupled to horseradish peroxidase by the method of  
03 Wilson and Nakane (1978).  
04  
05  
06

07 Conjugated antibody in PBS, 0.2% (w/v) bovine serum  
08 albumin was added to each well and incubated for 1 hour  
09 at 37°C. Second antibody binding was revealed by  
10 addition of 100 µl of a solution containing 4 mg  
11 o-phenylenediamine (Sigma), 15 µl hydrogen peroxide  
12 dissolved in 10 ml 0.1 M KH<sub>2</sub>PO<sub>4</sub> pH 6.0 and incubation  
13 for 15 minutes at room temperature. The reaction was  
14 blocked by addition of 25 µl 1 N H<sub>2</sub>SO<sub>4</sub>, and the optical  
15 density read at 490 nm (with 620 nm for the reference  
16 filter) in an Intermed Immunoreader, NJ200 (Analisis,  
17 Ghent, Belgium). Results were calculated from the  
18 linear portion of the optical density curves obtained  
19 and expressed as Optical Density Units per mg protein.  
20

21 The amount of AUSRIA reactive HBsAg and preS1-protein  
22 determined in the cell extracts of S. cerevisiae and  
23 H. polymorpha from the two experiments are shown in  
24 Tables 4 and 5. The amount of AUSRIA reactive  
25 materials found in crude extracts of S. cerevisiae  
26 strain Y1108 is about one hundred-fold less than in  
27 extracts of H. polymorpha preS/S-C-453 induced for 38  
28 to 45 hours in methanol. The amount of preS-protein  
29 assayed by Elisa is about 3 to 8-fold less indicating  
30 that S. cerevisiae strain Y1108 makes a greater  
31 proportion of preS1-protein to S-protein than does  
32 H. polymorpha strain preS/S-C-453.  
33

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TABLE 4

AUSRIA assay of crude cell extracts

| Cell extract        | Flask  | Protein | HBsAg | (AUSRIA) % | preS1-<br>protein<br>Elisa;<br>OD<br>units<br>per mg<br>protein |
|---------------------|--------|---------|-------|------------|-----------------------------------------------------------------|
|                     | Number | (mg/ml) | total | protein    |                                                                 |
| <u>S.cerevisiae</u> | a      | 16.2    | 0.011 |            | 2.9                                                             |
| Y1108               | b      | 18.0    | 0.008 |            | 3.0                                                             |
|                     | c      | 12.0    | 0.010 |            | 4.4                                                             |
| <u>H.polymorpha</u> | a      | 6.8     | 0.077 |            | 2.4                                                             |
| preS/S-C-453        | b      | 7.4     | 0.123 |            | 2.3                                                             |
| glycerol            | c      | 9.2     | 0.143 |            | 1.7                                                             |
| culture             |        |         |       |            |                                                                 |
| Methanol            | a      | 5.5     | 1.15  |            | 9.2                                                             |
| induced             | d      | 7.0     | 1.40  |            | 11.3                                                            |
| 38 hours            | c      | 7.4     | 1.46  |            | 8.8                                                             |

TABLE 5      AUSRIA assay of crude cell extracts

| Cell extract<br>protein | Flask<br>Number | Protein<br>(mg/ml) | HBsAg<br>total | <u>(AUSRIA)</u><br>%<br>protein | preS1-<br>protein<br>Elisa;<br>OD<br>units<br>per mg<br>protein |
|-------------------------|-----------------|--------------------|----------------|---------------------------------|-----------------------------------------------------------------|
| <u>S.cerevisiae</u>     | a               | 13.3               |                | 0.009                           | 8.3                                                             |
| Y1108                   | b               | 15.0               |                | 0.015                           | 9.7                                                             |
|                         | c               | 17.1               |                | 0.013                           | 8.9                                                             |
| <u>H.polymorpha</u>     | a               | 8.4                |                | 0.070                           | 1.3                                                             |
| preS/S-C-453            | b               | 8.2                |                | 0.061                           | 0.3                                                             |
| glycerol<br>culture     | c               | 7.9                |                | 0.082                           | 0.3                                                             |
| Methanol                | a               | 6.3                |                | 1.106                           | 4.1                                                             |
| induced                 | b               | 6.5                |                | 1.314                           | 6.8                                                             |
| 45 hours                | c               | 7.7                |                | 1.688                           | 5.1                                                             |

The results were confirmed by immunoblotting of the extracts from the first experiment together with extracts of methanol induced cultures of strains 454 and 448-C4 as controls.

Samples containing 15 µg of protein were electrophoresed through 12.5% separating, 5% stacking gels according to the method of Laemmli (1971) and subjected to immunoblotting as described above in Materials and Methods 5a using monoclonal HBS1 for detection.

Extracts from strain preS/S-C-453 show a strongly reactive band at 24 kD related to the S-protein and a band at 38-39 kD related to preS-protein. A 45 kD band is barely detectable. In contrast extracts from S. cerevisiae strain Y1108 show weak bands at 24 kD and 45 kD. The 38-39 kD doublet band of preS-protein was barely detectable. The immunoblotting result confirms that S. cerevisiae expresses less S and preS-proteins than methanol induced H. polymorpha and that the preS protein expressed by S. cerevisiae is predominantly the glycosylated 45 kD form.

#### Example D.6

Myristoylation of preS protein in H. polymorpha.

Cultures of strain LR9, strain 452 and 453 were grown in shake flasks on minimal medium with 2.5% v/v glycerol for 24 hours and methanol (1%, v/v) was then added. 6 hours after methanol addition, tritium labelled myristic acid was added and the cultures incubated for a further 16 hours. Cells were collected, washed and disrupted with glass beads in the presence of 1% SDS and β-mercaptoethanol. The

01  
02  
03 extracted proteins were separated by SDS-PAGE gel  
04 electrophoresis and visualised by immunoblotting with  
05 monoclonal HBS1 and by fluorography. The results  
06 showed that a band corresponding to the 38/39 Kd preS1  
07 protein was labelled in the extracts of stains 452 and  
08 453 but not in the extract from LR9. This is  
09 consistent with post translational removal of the  
10 N-terminal methionine and myristoylation of the glycine  
11 residue at the second position of the polypeptide  
12 (Towler and Gordon, Ann. Rev. Biochem., 57:69-99,  
13 1988).

14  
15 Example D.7

16  
17 Immunogenicity of preS antigens from *H. polymorpha*

18  
19 Crude protein extracts were prepared from methanol  
20 grown cultures of strains 452 and 454 and adsorbed onto  
21 aluminium hydroxide at 1 mg/ml final concentration.  
22 The preS1 epitope content of each extract was measured  
23 in an ELISA assay using monoclonal antibody S1.1 and  
24 preS1 protein partially purified from *S. cerevisiae* as  
25 standard. Immune complexes for injection were also  
26 formed by incubating 600 µl of S1.1 monoclonal  
27 antibody with 1 ml of Sepharose Protein G 4 Fast Flow  
28 (obtained from Pharmacia LKB, Brussels, Belgium) for 2  
29 hours at 4°C. The mixture was then centrifuged, washed  
30 with PBS and the pellet resuspended in 1 ml PBS. 250  
31 µl of this suspension was added to 3.4 ml of each  
32 crude cell extract and the mixture incubated for 1 hour  
33 at 20°C.

34  
35 Following incubation the Sepharose Protein G/S1.1/crude  
36 protein mixture was centrifuged and the pellet  
37 resuspended in 10 mM sodium phosphate buffer. Half of  
38

01  
02  
03 this material was adsorbed onto aluminium hydroxide at  
04 1 mg/ml final concentration.

05  
06 Groups of 5 Balb/c mice were injected intraperitoneally  
07 with the four preparations. One month later the mice  
08 received an intraperitoneal booster injection of 1 mcg  
09 of aluminium hydroxide adsorbed, partially purified  
10 particles from a methanol grown culture of strain 453.  
11 The mice were bled 15 days later.  
12 The titres of anti-S, anti-preS2 and anti-preS1  
13 antibodies in the sera were determined in the same way  
14 as described hereinbelow.

15  
16 Table 6 shows the respective antibody titres induced in  
17 Balb/c mice expressed as Geometric Mean Titer.

18  
19 The results show that anti-preS antibodies are induced  
20 in mice when injected with preS1-S2-S containing  
21 extract (strain 454) or with extract containing  
22 composite particles (strain 452) and boosted with  
23 composite particles (strain 453).  
24

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Table 6

| Preparation | mcg of   | $\alpha$ -HBS | $\alpha$ -120-145 | $\alpha$ 12-32 | $\alpha$ 32-47 |
|-------------|----------|---------------|-------------------|----------------|----------------|
|             | antigen  | GMT in        | GMT (c)           | GMT (c)        | GMT (c)        |
|             | injected | mIU/m         |                   |                |                |
|             | (a)(b)   |               |                   |                |                |
| Strain 454  |          |               |                   |                |                |
| crude       |          |               |                   |                |                |
| extract     | 222      | 17755         | 688               | 240            | 612            |
| Immune ppt  | 155      | 616           | 518               | 59             | 1400           |
| Strain 452  |          |               |                   |                |                |
| crude       |          |               |                   |                |                |
| extract     | 48       | 124803        | 426               | 59             | <100           |
| Immune ppt  | 48       | 119903        | 487               | 64             | 784            |

Notes

(a) Relative to preS1-S2-S antigen from S. cerevisiae

(b) All mice received a booster injection at day 30 of particles partially purified from strain 453.

(c) Titres are expressed as the inverted dilution giving an OD of 1.0 in the assay.

Part EExample E.1

Expression of composite particles containing both modified preS1-S2-S (L\*) and S antigens of single or mixed subtype.

(1) Construction of a L\* (ad) coding sequence

A modified preS1-S2-S (L\*) coding sequence suitable for direct insertion on the Hansenula expression vector pMPT-121 (Figure 11) was constructed as follows. Plasmid DNA of pRIT13192 described hereinbelow was digested with HindIII endonuclease to obtain linear fragments. About 1 ng of this DNA was mixed with oligonucleotides BC74 and BC75 and subjected to polymerase chain reaction (PCR) amplification.

PCR technology is well known and is disclosed in 'PCR Protocols, A guide to methods and applications'; Innes M.A. et al. (Eds.) Academic Press Inc., San Diego CA. 1989.

Oligonucleotides BC74 and BC75 have the sequences shown below and were synthesised by conventional phosphoramidite chemistry.

BC74 5' CCC AGA TCT AAG CTT ATT AAA TGT ATA CCC A 3'

BC75 5' CCC GAA TTC AAA ATG GGG ACG AAT CTT TCT 3'

Oligonucleotide BC74 has homology to the 3' end of the L\* coding sequence on pRIT13192 together with a HindIII and BglII restriction site extension. Oligonucleotide BC75 has homology to the 5' end of the L\* coding sequence on pRIT13192 together with an EcoRI site and a

01  
02  
03 short leader sequence before the ATG codon as  
04 extension. About 1 ng of template pRIT13192 DNA was  
05 mixed with 50 mM KCl, 10 mM Tris-HCl pH8.3, 1.5mM.  
06 MgCl<sub>2</sub>, 0.01% (w/v) gelatin, 200 µM each of dATP, dCTP,  
07 dGTP and dTTP, 1µM of each oligonucleotide primer and  
08 2.5 units of Taq polymerase in a final volume of 100 µl  
09 using commercially available DNA amplification reagents  
10 (Gene Amp<sup>TM</sup> DNA Amplification Reagent kit from Perkin  
11 Elmer Cetus; obtainable from Vander Heyden, Brussels).  
12 The mixture was subjected to 25 cycles of denaturation  
13 (2 min, 92°), annealing (2 min, 48°) and extension (2  
14 min, 72°) using a DNA Thermal Cyclor (Perkin Elmer  
15 Cetus; obtained from Vander Heyden, Brussels). One ng  
16 of the modified, amplified L\* coding sequence fragment  
17 so obtained was then reamplified in a second round with  
18 the same oligonucleotides in the same conditions and  
19 purified from the reaction mixture by ethanol  
20 precipitation.

21  
22 An aliquot of about 250 ng of the 870 bp adapted L\*  
23 fragment DNA was digested with EcoRI and BglII  
24 endonucleases and cloned between the EcoRI and BglII  
25 sites of a pBR327 derivative plasmid, pRIT12555 to give  
26 pRIT13488. pRIT12555 consists of the pBR327 replicon  
27 with a BglII site introduced by linker insertion  
28 between the EcoRI and ClaI sites of the original.  
29 plasmid.

30  
31 A further aliquot of the adapted L\* fragment was  
32 digested with EcoRI and BglII endonucleases and  
33 inserted between the EcoRI and BglII sites of the  
34 plasmid pMPT121 (Figure 11). This places the L\* coding  
35 sequence between the MOX promoter and the MOX  
36 terminator on an expression plasmid suitable for  
37 introduction into H. polymorpha by transformation. DNA

sequencing of the resulting plasmid, pL\*-11, showed that the L\* coding region sequence was correct.

(2) Construction of an S-(ay) coding sequence

pRIT13490 is a pBR322 derivative plasmid containing a complete preS2-S coding sequence obtained by conventional recombinant DNA manipulations from pRIT10601 which contains the complete genome of a hepatitis B virus of ay subtype cloned on pBR322. pRIT10601 was deposited with the American Type Culture Collection, Rockville MD on June 2, 1982 under Accession Number ATCC 39132.

It will be appreciated by one of skill in the art that in the following manipulations described below pRIT13490 could be substituted by pRIT10601 or any other cloned HBV fragment containing a complete S-(ay) coding sequence. DNA of pRIT13490 was digested with HindIII endonuclease to linearize the plasmid and mixed with the oligonucleotide BC74 described above and with oligonucleotide BC73 which has homology to the 5' end of the S coding sequence on pRIT13490 together with an EcoRI site and a short leader sequence before the ATG codon as extension.

BC73 5' CCC GAA TTC AAA ATG GAG AAC ATC ACA TCA 3'

The mixture of HindIII treated pRIT13490 and oligonucleotides was amplified by 2 rounds of PCR as described above to generate a 700 bp S-(ay) coding sequence fragment flanked by EcoRI and BglII restriction sites. About 250 ng of this material was digested with EcoRI and BglII endonucleases and cloned between the EcoRI and BglII sites of pRIT12555 to give

pRIT13438. The correctness of the S-(ay) coding sequence on pRIT13438 was verified by DNA sequencing.

(3) Construction of a L\* (ad/ay) coding sequence

About 1 ng of the PCR amplified fragment used to construct pRIT13488 was further amplified by 2 rounds of PCR amplification in the presence of oligonucleotides BC75 (described above) and BC30 having the following sequence.

BC30 5' ACG AGT CTA GAC TCT GCG GTA 3'

BC30 is homologous to the region around the XbaI site of the S coding sequence derived from the pRIT10616 HB virus clone of ad subtype. A 280 bp fragment was recovered from the amplification and about 250 ng of this was digested with EcoRI and XbaI endonucleases.

About 1 ng of the PCR amplified fragment used to construct pRIT13438 was further amplified by 2 rounds of PCR amplification in the presence of oligonucleotides BC74 (described above) and BC12 having the following sequence.

BC12 5' ATG GAG AAC ATC ACA TCA 3'

BC12 is homologous to the first 6 codons of the S coding sequences of the HB virions cloned on pRIT10601 and pRIT10616. Following PCR amplification about 250 ng of the 700 bp fragment was digested with XbaI and BglII endonucleases.

The two endonuclease digested, PCR amplified fragments were then ligated with EcoRI, BglII digested DNA of pRIT12555.

01  
02  
03 A plasmid, pRIT13491, was recovered consisting of  
04 pRIT12555 together with the EcoRI-XbaI fragment from  
05 the PCR amplification of the fragment used to construct  
06 pRIT13488 and the XbaI-BglII fragment from the PCR  
07 amplification of the fragment used to construct  
08 pRIT13438. DNA sequencing of the L\* coding insert on  
09 pRIT13491 showed that an error had been introduced at  
10 the 5th amino acid of the S coding region. To correct  
11 this, the PCR amplified fragment used to construct  
12 pRIT13488 above was digested with EcoRI and XbaI  
13 endonucleases and used to replace the corresponding  
14 EcoRI-XbaI fragment on pRIT13491 which contained the  
15 error. The resulting plasmid pRIT13489 specifies a  
16 ''hybrid'' L\* coding sequence in which the part from  
17 the ATG initiation codon to the XbaI site is derived  
18 from ad subtype virus sequence and the part from the  
19 XbaI site to the termination codon is derived from ay  
20 subtype virus sequence. The corresponding polypeptide  
21 will have the ay subtype.

22  
23 The EcoRI-BglII fragment from pRIT13489 containing the  
24 hybrid L\* (ad-ay) coding sequence was then inserted  
25 between the EcoRI and BglII sites on plasmid pMPT-121  
26 to give the H. polymorpha expression vector pL\* 13.

27  
28 (4) Expression in H. polymorpha of both modified  
29 preS1-S2-S (L\*) and S antigens to form composite  
30 particles.

31  
32 Several strains were constructed which were  
33 characterised by different ratios of L\* and S  
34 expression as well as different total expression  
35 levels. These differences were achieved by variation  
36 of the copy number of the expression cassette per  
37 genome.  
38

01  
02  
03 H. polymorpha strain RB10 was transformed as described  
04 above with the plasmid pL\* 11 bearing the L\*  
05 (ad)-expression cassette. Transformation yielded  
06 several strains containing variable numbers of  
07 expression cassettes integrated into the genome. Two  
08 of these strains, designated L13/1 and L19/1, were  
09 analysed in more detail. Southern blot analysis  
10 revealed that these strains contain several copies of  
11 the L\* (ad)-expression cassette. The L\*  
12 (ad)-expression level of these strains was assayed by  
13 Western blot analysis using monoclonal antibody HBS1.  
14 A band of 33 kD corresponding to L\* antigen was  
15 detected. Expression levels were estimated to be about  
16 0.5-2% of total cell protein when cells were grown in  
17 glycerol medium followed by addition of methanol.  
18

19 The L\* (ad)-expressing strains were transformed again  
20 with plasmid pRB-S-322 bearing the S(ad)-gene under  
21 control of the MOX-promoter. pRB-S-322 is described  
22 above (see Figure 7). Resistance against gentamycin  
23 was used as a transformation marker. Stable integrants  
24 were obtained employing the methods described above.  
25 Two of the strains obtained were studied in more  
26 detail: strain LMS9/1 and strain LMS26/2, both derived  
27 from recipient strain L13/1. Southern blot analysis  
28 revealed that these strains carry several copies of the  
29 L\*(ad) expression cassette as well as several copies of  
30 the S(ad) expression cassette.  
31

32 The expression of L\* and S antigens in the  
33 H. polymorpha transformants was tested by Western  
34 blotting using monoclonal antibodies HBS1 and S1.1 as  
35 described above. A spectrum of strains was obtained  
36 which express different ratios of L\* to S antigen.  
37 HBS1 antibodies detect a 24 kD protein corresponding to

01  
02  
03 S antigen, and 30 and 33 kD bands corresponding to L\*  
04 antigen. S1.1 antibodies specific for the preS1 region  
05 reveal a 33 kD band as well as the 30 kD band. The  
06 L\*/S ratios were estimated to be about 1:1 for strain  
07 LMS9/1 and about 1:10 for strain LMS26/2. Expression  
08 levels were estimated to be about 5-6% of the total  
09 cell protein when cells were grown under induced  
10 conditions with methanol.

11  
12 Glycosylation studies showed that the 33 kD protein  
13 represents a glycosylated form of the L\* antigen. When  
14 crude protein extracts from strains LMS9/1 and LMS26/2  
15 were incubated with endoglycosidase H, the 33 kD band  
16 shifted to the apparent molecular weight of 30 kD. 30  
17 kD is the expected molecular weight of L\* antigen.  
18 Thus part of the L\* antigen produced in H. polymorpha  
19 is ''core''-glycosylated while the remainder  
20 constitutes the unglycosylated form of L\*.

21  
22 In order to test for particle formation crude cell  
23 extract from strain LMS9/1 was subjected to CsCl  
24 density gradient centrifugation. Gradient fractions  
25 were analysed by immunoblotting using antibody HBS1. A  
26 peak of HBsAg-reactive material including the 24 kD S  
27 antigen and the 30 and 33 kD L\* antigen forms was  
28 observed at a density of 1.18 g/cm<sup>3</sup>. This result  
29 indicates that composite particles were formed in  
30 H. polymorpha strains coexpressing the L\* and S  
31 polypeptides.

32  
33 In a further variation of the invention a H. polymorpha  
34 strain coexpressing the L\* and S proteins was obtained  
35 by further transforming strain 415 described above.  
36 Strain 415 carries between 50-100 S(ad) expression  
37 cassettes under the control of the MOX promoter and is  
38 characterised by a high S antigen expression level.  
39

01  
02  
03 A L\* (ad) expression vector containing a  
04 kanamycin-resistance gene was constructed. A 3.5 kb  
05 NruI-fragment encoding the kanamycin-resistance gene  
06 under control of the ADHI promoter and the HARS  
07 sequence was isolated from plasmid pHK2 (Figure 6).  
08 Plasmid pL\*11 was digested with NruI and SalI  
09 endonucleases and made blunt ended by fill-in with  
10 Klenow DNA polymerase. The NruI/SalI-digest eliminated  
11 the HARS sequence from the pL\*11-vector. The  
12 NruI-fragment from pHK2 was then ligated to the blunt  
13 ended large NruI-SalI fragment of the pL\*11-vector thus  
14 introducing the kanamycin-resistance gene and  
15 re-introducing the HARS sequence which had been  
16 eliminated. The resulting plasmid is designated pKL\*1  
17 or pKL\*10 depending on the orientation of the kanamycin  
18 resistance gene cassette. The structure of pKL\*1 is  
19 shown in Figure 12. These plasmids can be introduced  
20 into H. polymorpha by transformation and selection for  
21 gentamycin resistance as described above in Methods.  
22

23 Strain 415 was transformed with pKL\*1 and pKL\*10  
24 carrying the L\*(ad) expression cassette and stable  
25 transformants were obtained after selection for  
26 resistance to gentamycin. Several strains were further  
27 analysed. Western blot analysis using antibody HBS1  
28 showed a 24 kD protein corresponding to S antigen as  
29 well as the 30 and 33 kD bands corresponding to the  
30 unglycosylated and glycosylated forms of L\* antigen  
31 respectively. In these 415 derived strains the L\*/S  
32 ratio varied from about 1:1 in strain LMS05-14-1 to  
33 about 1:20 in strain LMS05-12-1. In strains grown  
34 under fully induced conditions with methanol, L\*/S  
35 antigen expression levels amounted to an estimated 5-6%  
36 of the total cell protein. No differences between  
37 strains transformed with pKL\*1 and strains transformed  
38 with pKL\*10 were observed.  
39

01  
02  
03 (5) Co-expression of L\* and S antigens leading to  
04 composite particles with mixed subtype.

05  
06 To obtain H. polymorpha strains which produce  
07 L\*/S-particles with a mixed (ad/ay) subtype, plasmid  
08 pL\*13 bearing a L\* (ad/ay) expression cassette was  
09 transformed into H. polymorpha RB10.

10  
11 Stable clones expressing the L\* antigen can be obtained  
12 and retransformed with plasmid pRB-S-322 carrying the  
13 S(ad) expression cassette with selection for gentamycin  
14 resistance. Stable strains from the second  
15 transformation are analysed by Western blotting and  
16 AUSRIA assays to determine the respective expression  
17 levels of the L\* (ad/ay) and S(ad) antigens and the  
18 total level of surface antigen expression.

19  
20 Strain 415 expressing S(ad) protein may also be  
21 retransformed with a derivative of pL\*13 which carries  
22 a functional gentamycin resistance marker to generate  
23 stable transformants expressing both the L\* (ad/ay) and  
24 S(ad) polypeptides in the form of composite particles  
25 with both ad and ay subtype specificities.  
26

01  
02  
03 Example F.1  
04

05 Construction of plasmid pRIT12979 coding for a  
06 non-myristylated HBV large protein  
07

08 The outline of the construction of plasmid pRIT12979 is  
09 shown in Figs. 13 and 14. Fig. 13 shows the steps for  
10 constructing an E. coli plasmid harbouring a  $\Delta$ Gly13  
11 L protein-expression cassette, pRIT12998. Fig. 14  
12 illustrates the procedure for obtaining a yeast plasmid  
13 pRIT12979 capable of expressing a  $\Delta$ Gly13 L protein.  
14

15 It is understood that the Gly 13 residue is encoded by  
16 the second codon of the L protein coding sequence  
17 represented in Table A.  
18

19 The starting material for this construction is plasmid  
20 pRIT12863, a derivative of pBR327 which contains the  
21 TDH3 promoter region and the ARG3 transcription  
22 termination region [Cabezon et al., Proc. Natl. Acad.  
23 Sci. USA, 81:6594-6598 (1984)] separated by a BamHI,  
24 SmaI, EcoRI linker. A linker containing a BglII site  
25 is placed upstream of the TDH3 promoter sequences.  
26 pBR327 is described in Soberon et al, Gene, 9:287-305  
27 (1980) and was obtained from F. Bolivar (Department of  
28 Molecular Biology, National University of Mexico).  
29 This plasmid may be recovered as described in  
30 European Patent, EP 0 278 940 B1.  
31

32 The expression cassette for the non-myristylated L  
33 protein is constructed within the linker region of  
34 pRIT12863.  
35

The plasmid pRIT12816, which is an E. coli plasmid derived from pAS1 [Rosenberg et al, Methods Enzymol., 101:123-164 (1983)], harbours the coding region for the large HBV (adw serotype) protein flanked by an NcoI site (overlapping the ATG at codon position 12 in the L protein coding sequence) and by an EcoRI site beyond the stop codon. This plasmid is constructed by conventional recombinant DNA techniques [see, T. Maniatis et al, cited above].

Plasmid pRIT12816 is digested with BstXI and EcoRI and the 304bp BstXI-EcoRI fragment encoding the N-terminal portion of the preS1 region is recovered and ligated to the following synthetic BamHI-BstXI adaptor:

|                                           |             |       |
|-------------------------------------------|-------------|-------|
| BamHI                                     | <u>XbaI</u> | BstXI |
| GATCCCTCTAG ACG AAT CTT TCT GTT CCC AAC C |             |       |
| GGAGATC TGG TTA TAA AGA CAA GGG TT.       |             |       |

This fragment is inserted into pRIT12863, previously digested with BamHI and EcoRI enzymes, resulting in plasmid pRIT12996. The BamHI-BstXI synthetic adaptor harbours the N-terminal preS1 region, but does not provide the correct reading frame with the BamHI site placed at the ATG residue of the TDH3 promoter. The correct reading frame is provided by digesting pRIT12996 with BamHI and XbaI, followed by treatment with mung bean enzyme to eliminate the protruding extensions and ligation. This procedure produced plasmid pRIT12997.

Finally, the 839bp EcoRI fragment from pRIT12816 was inserted into the EcoRI site of pRIT12997 in the correct orientation to recover the complete coding

sequence for the  $\Delta$ Gly L protein. The resulting plasmid pRIT12998 contains the expression cassette coding for an L protein which is deleted for Gly13 and cannot serve as substrate for the myristoyl transferase.

To construct a yeast plasmid capable of expressing the  $\Delta$ Gly L protein, plasmid pRIT12998 was digested with BglII and SalI. The 5822 bp BglII-SalI fragment that contains the expression cassette was purified and inserted into a conventional yeast vector pRIT12741 which is a pBR327 derivative that supplies an AmpR gene, the 2 $\mu$  sequences, the E. coli replicon and the LEU2 yeast marker for selection.

The resulting plasmid pRIT12979 was used to transform the LEU- S. cerevisiae strain 10S<sub>44</sub>cir<sup>0</sup> (pep4-3, leu2-3, leu2-112), otherwise called TCY1 or Y482, which is deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number ATCC 20818. After transformation, the resulting LEU+ yeast strain is called Y720.

#### Example F.2

#### Comparative Characterisation of $\Delta$ Gly L Protein with L Protein

Yeast strain Y720 of Example F.1 was examined for  $\Delta$ Gly L protein expression in comparison with yeast strain Y587 (TCY1 transformed by pRIT12845) which expresses the L protein. pRIT12845 contains a 3370 bp expression cassette consisting of a HBV DNA derived coding sequence for the 389 amino acids of the L protein flanked 5' by, and under the control of, a 1050 bp TDH3

01  
02  
03 promoter fragment and 3' by a 1150 bp fragment carrying  
04 the ARG3 transcription terminator. pRIT12845 is  
05 described in detail in Example 16A of co-pending U.S.  
06 Patent Application SN 009,325. Yeast strain Y1017,  
07 which is TCY1 transformed with pRIT12741, served as a  
08 negative control.

09  
10 A. Myristylation

11  
12 Cells from Y720, Y587 and Y1017 were labelled with  
13 <sup>3</sup>H-myristic acid as described in co-pending U.S. Patent  
14 Application SN 009,325 . SDS-extracts of these cells  
15 were analysed by SDS polyacrylamide gel electrophoresis  
16 (SDS-PAGE), followed by both immunoblot and  
17 fluorography [See, e.g., Towler and Glaser, Proc.  
18 Natl. Acad. Sci., U.S.A., 83:2812-2816 (1986)].  
19

20 The immunoblot as described in co-pending U.S. Patent  
21 Application SN 009,325 involved detection with an S-  
22 specific monoclonal antibody (Mab), either Mab RF6 [H.  
23 Thomas, Royal Free Hospital, London] or Mab HBs1  
24 [SmithKline Biologicals]. These antibodies recognize  
25 overlapping denaturation and reduction resistant  
26 epitopes in the S protein. The immunoblot showed the  
27 expression of two forms of L protein of 38kd and 45kd  
28 in both Y720 and Y587.

29  
30 The fluorograph showed the presence of <sup>3</sup>H label in the  
31 38 kd L protein derived from Y587 cells as described in  
32 co-pending U.S. application SN 009,325. Minor amounts  
33 of label were observed in the 45kd L protein derived  
34 therefrom. In contrast, neither the 38kd nor the 45 kd  
35  $\Delta$ Gly L protein derived from Y720 cells had incorporated  
36 the label. This demonstrates that the elimination of

the glycine codon in pRIT12979 abolishes the myristylation of the  $\Delta$ Gly L protein in S. cerevisiae.

#### B. Expression level

Cells from Y720, Y587 and Y1017 were grown under identical conditions in YNB without amino acids [Difco Labs] 0.675% containing 2% glucose in Erlenmeyer flasks. Both total disrupted cells and crude extracts were analyzed by quantitative immunoblot as described in co-pending U.S. application SN 009,325.

Two-fold serial dilutions of the samples were analyzed and the immunodetection was based on the S-specific Mabs RF6 or HBS1, or a preS1-specific monoclonal antibody, S1.1 [SmithKline Biologicals]. The immunoblots displayed a 2 to 5-fold higher expression level for the  $\Delta$ Gly L protein (Y720) than the myristylated L protein in Y587. Extraction efficiency appeared to be about equal for the non-myristylated and myristylated proteins. The proportion of L protein that was found in the 45 kd glycosylated form was not changed by the elimination of the myristylation.

#### C. Lipoprotein structures

Crude extracts from Y720 and Y587 cells were subjected to CsCl equilibrium centrifugation. Gradient fractions were analyzed by immunoblot, AUSRIA assay [Abbott Labs], an ELISA assay specific for a preS1 epitope using MAb S1.1, in which MAb S1.1 was coated on the wells of a microtitre plate [Immunoplate I; Gibco Europe] and incubated with the test samples. After washing to remove non-bound material, capture of

01  
02  
03 reactive particles was revealed by incubation with Mab  
04 S1.1 coupled to horseradish peroxidase by the method of  
05 Wilson and Nakane (1978) in 'Immunofluorescence and  
06 Related Techniques', W. Knapp et al., eds., Elsevier,  
07 Amsterdam (1978). Colour development to reveal second  
08 antibody binding was with orthophenylenediamine as a  
09 chromogen. Absorbance was read at 490 nm with 620 nm  
10 for the reference filter, in a Intermed Immunoreader,  
11 NJ2000 [Analisis, Ghent, Belgium].  
12

13 The antigenic and immunoblot profile were identical.  
14 Both the  $\Delta$ Gly L and the wild type L protein banded at a  
15 density of about 1.25 g/cm<sup>3</sup>, demonstrating that both  
16 the myristylated and non-myristylated proteins are  
17 present as lipoprotein structures in crude cell  
18 extracts. Sucrose gradient velocity centrifugation of  
19 CsCl equilibrium gradient purified material showed  
20 identical sedimentation behaviour for the myristylated  
21 and non-myristylated L protein containing lipoprotein  
22 structures, indicating that these lipoprotein  
23 structures have comparable physical parameters.  
24

Example F.3Construction of plasmids coding for a modified HBV L protein devoid of potential O- and N- glycosylation sites, potential pHSA binding sites, and potential protease sensitive sites

An outline of the construction of two yeast vectors: pRIT13192, coding for the expression of a myristylated modified L protein (L\*) and pRIT13193, coding for the expression of a non-myristylated modified L protein ( $\Delta$ Gly L\*) is presented in Figs. 15 and 16.

pRIT10616, deposited as ATCC 39131, is digested with BglII, and the largest fragment ligated to the pACYC 177 replicon [Chang and Cohen, J. Bacteriol., 134:1141 (1978)], previously digested with BamHI. The intermediate plasmid obtained was digested by EcoRI and religated to obtain pRIT10633, which harbours an incomplete HBV genome from which the pACYC 184 vector has been excised.

Plasmid pRIT12331 is a pUC9 [Amersham, U.K. and Pharmacia, Sweden] derivative containing the 1500 bp HindIII-NcoI ARG3 promoter fragment from pRIT10779 [Cabezon et al, Proc. Natl. Acad. Sci. U.S.A., 81:6594-6598 (1984)] fused in the correct orientation to a 681 bp NcoI-EcoRI fragment which contains the S coding sequences with the NcoI site overlapping the ATG codon and the EcoRI site beyond and adjacent to the stop codon, inserted between the HindIII and EcoRI restriction site of pUC9.

pRIT10633 is digested with BstEII and XbaI enzymes. The 648 bp BstEII-XbaI fragment, in which the BstEII

extension was filled in by Klenow polymerase, containing the entire PreS1-PreS2 region and the N-terminal sequences of the S region was purified and inserted between the SmaI and XbaI sites of pUC12 [Amersham]. The resulting plasmid is pRIT13188.

Plasmid pRIT13188 was treated with BalI and BamHI, which deleted the preS1 region coding for the amino acids extending from residues 52 to 133 [the codons for amino acid 53 and 132 were included in deletion]. The large fragment was treated with Klenow polymerase and ligated, resulting in plasmid pRIT13189 in which the BamHI site is reformed.

The DNA corresponding to the preS amino acid region extending from residue 145 to 175 [the codons for amino acids 146-174] were deleted by digestion of pRIT13189 with BamHI and XbaI. This vector was ligated to the following synthetic adaptor:

|                                         |                |
|-----------------------------------------|----------------|
| BamHI extension                         | NcoI extension |
| GATCCCAGAGTCAGGGGTCTGTATTTTCCTGCTGGTGG  |                |
| GGTCTCAGTCCCCAGACATAAAAGGACGACCACCGTAC  |                |
| AspProArgValArgGlyLeuTyrPheProAlaGlyGly |                |
| 135                                     | 145            |

and to the NcoI-XbaI fragment from pRIT12331 containing the N-terminal portion of the S gene. The resulting plasmid is pRIT13190.

The C-terminal region of the S gene, the termination of transcription ARG3 region and the LEU2 yeast gene were added by insertion of the 4145 bp XbaI-SalI fragment

01  
02  
03 from pRIT12660 between the XbaI and SalI sites of  
04 pRIT13190. [pRIT12660 contains a 3050 bp expression  
05 cassette consisting of a HBV DNA derived coding  
06 sequence for the 281 amino acids of the M protein  
07 flanked 5' by, and under the control of, a 1050 bp TDH  
08 promoter fragment and 3' by a 1150 bp fragment carrying  
09 the ARG3 transcription terminator. pRIT 12660 is  
10 described in detail in European patent  
11 application EP 0 278 940 B1.] The resulting plasmid  
12 pRIT13191 was obtained.  
13

14 To reconstitute the expression cassettes for the L\*  
15 and  $\Delta$ Gly L\* protein the TDH3 promoter associated either  
16 to the N-terminal coding region for a L protein or to  
17 the N-terminal coding region for a  $\Delta$ Gly L protein was  
18 inserted into pRIT13191.  
19

20 The 1234 base pair EcoRI-BalI fragment was purified  
21 from pRIT12845, containing the L protein coding  
22 sequence described above and in U.S. SN 009,325, and  
23 the 1227 bp BglII-BalI fragment was purified from pRIT  
24 12979 containing the  $\Delta$ Gly L protein coding sequence as  
25 described above.  
26

27 Each fragment was mixed with the large SacI-BamHI  
28 fragment from pRIT13191 followed by treatment with T4  
29 polymerase and ligation. The resulting plasmids were  
30 designated pRIT13220 and pRIT13222. The coding  
31 sequence contained in the expression cassette present  
32 in pRIT13220 contains the amino acid residues of 12 to  
33 52 from the preS1 sequence, 133 to 145 from the preS2  
34 sequence and the entire S sequence as indicated in  
35 Table A. pRIT13222 is identical to pRIT13220 with the  
36 exception of the deletion of the Gly13 codon (GGG).  
37

The last step was the preparation from pRIT13220 and pRIT13222 of the ClaI-ClaI fragment containing each expression cassette for the L\* and  $\Delta$ Gly L\* proteins respectively and ligation of them to the ClaI-ClaI fragment from plasmid pRIT12845, giving rise to plasmids pRIT13192 and pRIT13193, respectively.

These latter plasmids were used to transform the S. cerevisiae strain TCY1, to give LEU<sup>+</sup> strains Y1139 and Y1140, respectively.

#### Example F.4

##### Expression of L\* and $\Delta$ Gly L\* in yeast

Crude yeast extracts from Y587 (L protein), Y1139 and Y1140 (L\* and  $\Delta$ GlyL\* respectively) of Example F.3 and Y724 (control S. cerevisiae strain, no expression cassette inserted) were subjected to SDS-PAGE and immunoblot detection with either the S-specific Mab HBS1 or Mab S1.1. The Y1139 and Y1140 extracts showed two bands of estimated molecular weight of about 33 and 30 kd which were specifically recognized by both the preS1 and S specific antibodies.

Treatment of the extracts with endoglycosidase H [New England Nuclear or Boehringer] or glycopepsidase F [Boehringer] resulted in the disappearance of the 33 kd band and an increase of the 30 kd band. This indicates that the 33kd band represents a glycosylated form of the L\* and  $\Delta$ Gly L\* protein of Example F.3 carrying one N- linked high mannose type glycan.

Labelling of cell cultures with <sup>3</sup>H myristic acid

01  
02  
03 followed by analysis of cell extracts by SDS-PAGE,  
04 protein blotting and either fluorography or immuno  
05 detection, showed a strong presence of  $^3\text{H}$ -label in the  
06 30 kd band and a weak incorporation of  $^3\text{H}$ -label in 33  
07 kd derived from the Y1139 extract. The  $^3\text{H}$  present in  
08 these bands was resistant to hydroxylamine treatment.  
09 No incorporation of  $^3\text{H}$ -label was observed in the  
10 corresponding protein bands from the Y1140 extract.  
11 This shows that the L\* protein as expressed in Y1139  
12 cells serves as a substrate for the yeast N-myristyl  
13 transferase and that deletion of the Gly13 residue in  
14 the  $\Delta\text{Gly}$  L\* protein of Y1140 prevents the  
15 myristoylation of the protein.  
16

17 Analysis of extracts from Y1189 and Y1140 by CsCl  
18 equilibrium and sucrose velocity gradient  
19 centrifugation indicated the presence of lipoprotein  
20 particles in the extracts.  
21

## 22 Example F.5

### 23 24 Yeast Strains Y1088 and Y1295 Containing Integrated 25 Copies of an S Protein Expression Cassette 26

27 Yeast strains harbouring several copies of an S  
28 expression cassette integrated in the genome were  
29 constructed.  
30

#### 31 A. Yeast Strain Y1088

32  
33 The Ty based linear vector pRIT13133-L described in  
34 co-pending U.S. patent application SN 368,401  
35 containing an expression cassette for the S protein  
36 (TDH3 promoter - S protein coding sequence - ARG3

01  
02  
03 termination region) was used to transform both mating  
04 types of S. cerevisiae strain 10S69d (ura3, leu2, trp1,  
05 gal1, cir<sup>0</sup>) described in co-pending U.S. patent  
06 application SN 368,401. Different haploid  
07 transformants carrying several vector copies integrated  
08 in their genomes were crossed with the haploid strains  
09 of opposite mating type. Southern blot analyses of  
10 genomic DNA isolated from segregants using an S DNA  
11 fragment probe, show 2/2 segregations of vector  
12 fragments in tetrad analyses. One haploid segregant  
13 obtained by this means, which contains 5-7 copies of  
14 vector pRIT13133-L, was named Y957. A TRP<sup>+</sup> revertant  
15 of Y957 was obtained and named Y1088.

16  
17 B. Yeast Strain Y1295  
18

19 The Ty based linear vector pRIT13034-L described in  
20 co-pending U.S. patent application SN 368,401 carries  
21 an expression cassette for the S protein identical to  
22 that in pRIT13133-L. pRIT13034-L carries the CUP1 gene  
23 as an additional marker that can be used in CUP1<sup>S</sup> or  
24 cup1Δ recipient strains.

25  
26 Two cup1Δ S. cerevisiae haploid strains (CUP1 gene  
27 disrupted in a strain containing a single copy of that  
28 gene) are preferred as recipient yeast strains for  
29 this Ty linear vector. These strains have the  
30 respective genomes: EJ cup1Δ 3d (ura 3, leu 2, trp 1,  
31 gal 1Δ, cup 1Δ, a) and EJ cup 1Δ 7b (ura 3, trp 1, gal  
32 1Δ, cup 1Δ, a).

33  
34  
35 The Ty based linear vector pRIT13034-L containing an  
36 expression cassette for the S protein was used to

transform S. cerevisiae strains EJ cup1 $\Delta$ 3d as well as EJ cup1 $\Delta$ 7b. URA3 transformants were isolated, and screened for resistance to copper toxicity. The more resistant transformants appeared to contain 2 to 5 copies of integrated vectors. Classical genetic crosses between these strains and selection of a LEU<sup>-</sup> TRP<sup>-</sup> haploid segregant resulted in a haploid strain containing 4 to 5 copies of the S expression cassette. A TRP<sup>+</sup> revertant of this strain was named Y1295.

#### Example F.6

#### Construction of yeast strains that co-express the S and L antigens

The yeast plasmids pRIT12845 (L protein), pRIT12979 ( $\Delta$ Gly L protein, Example F.1), pRIT13192 (L\* protein, Example F.3), pRIT13193 ( $\Delta$ Gly L\* protein, Example F.3) and pRIT12914 (no hepatitis gene inserted) were each used to transform the yeast strain Y1088 to LEU<sup>+</sup>. The resulting yeast strains obtained were designated Y1301 (S, L), Y1302 (S,  $\Delta$ Gly L), Y1142 (S, L\*), Y1261 (S,  $\Delta$ Gly L\*) and Y1141 (control strain expressing S protein only) respectively.

Likewise, pRIT12845, pRIT12979, pRIT13192, pRIT13193 and pRIT12377 (no hepatitis genes inserted) were introduced into the yeast strain Y1295. The resulting yeast strains obtained were designated Y1304 (S, L), Y1305 (S,  $\Delta$ Gly L), Y1306 (S, L\*), Y1307 (S,  $\Delta$ Gly L\*) and Y1308 (control strain expressing S protein only) respectively.

Co-expression of the S and L proteins was demonstrated by immunoblot analysis of crude cell extracts.

Reaction of the blot with Mab HBS1 specific for an S epitope revealed a band of estimated molecular weight of about 23 kd migrating at the position expected for the S protein in each of the extracts of the above mentioned strains.

Extracts from Y1301, Y1302, Y1304 and Y1305 showed in addition to the 23 kd band, bands of 38 kd and 45 kd migrating like the non-glycosylated and glycosylated forms of the L proteins previously described in U. S. application SN 009,325, and in Example F.2 herein. Treatment of the extracts with endoglycosidase H resulted in the conversion of the 45 kd band into a 41 kd band while the 23 kd and 38 kd bands were not affected.

Extracts from Y1142, Y1261, Y1306 and Y1307 showed in addition to the 23 kd band, bands of 30 kd and 33 kd migrating like the non-glycosylated and glycosylated forms of the L\* and  $\Delta$ Gly L\* proteins described in Example F.4. Treatment of the extracts with endoglycosidase H resulted in the conversion of the 33 kd band into a 30 kd band which is identical to the results described in Example F.4.

Labelling of cells with  $^3\text{H}$  myristic acid and analysis of extracts by SDS-PAGE followed by immunoblot and fluorography demonstrated the presence of hydroxylamine treatment resistant  $^3\text{H}$ -label in the 38 kd (extracts from Y1301 and Y1304) and 30 kd bands (extracts from Y1142 and Y1306) with only minor quantities of  $^3\text{H}$ -label in the 45 kd (extracts from Y1301 and Y1304) and 33 kd (extracts from Y1142 and Y1306) protein bands. The corresponding bands in extracts from Y1302, Y1305, Y1261 and Y1307 contained no detectable  $^3\text{H}$ -label.

The 38 kd and 45 kd forms of the L and Gly L proteins are recognized in immunoblots by the preS1-specific Mab's S1.1 and MA18/7 [W.H. Gerlich, University of Göttingen, FRG], the preS2-specific Mab's S2.4, S2.5, S2.7, S2.8, S2.9 and S2.10 [Smith Kline Biologicals]. The 30 kd and 33 kd forms of the L\* and  $\Delta$ Gly L\* proteins are recognized by Mab S.1.1 (recognizing an epitope present in amino acid sequence 12-32), MA18/7 (recognizing an epitope present in amino acid sequence 28-47) and S2.5 (recognizing an epitope present in amino acid sequence 133-145). Monoclonal MA 18/7 is reported to inhibit binding of virions to liver cell membranes [Pontisso P. et al., Virology, 173:522-530 (1989)]. The 30 kd and 33kd forms of the L\* and  $\Delta$ Gly L\* proteins are not recognized by Mab's S2.4, S2.8, S2.9 which do not react with peptide 120-145 in an ELISA assay nor by Mab's S2.7 and S2.10 which presumably recognize an epitope localised in amino acids 120-137. Monoclonal F35-25 (obtained from M-A Petit, INSERM unité 131, Clamart, France) binds to particles purified from strain Y1307 as shown by an ELISA assay but does not bind to S particles. This monoclonal recognises a peptide sequence (Petit M-A et al., Molec. Immunol., 26:531-537, 1989) shown to be involved in binding of virus to HepG2 hepatoma cells (Neurath et al., Cell 46:429-436, 1986). Furthermore it has been found that (S, L\*) particles bind to HepG2 hepatoma cells and that this binding, and the binding of HB virus particles can be prevented by Monoclonal F35.15 (Petit M-A et al. The 1990 intl. Symposium on Viral Hepatitis and Liver Disease, Abstract 105. Houston, U.S.A., April 4-8, 1990). The Mab Q19/10 [W.H. Gerlich, University of Göttingen, FRG] which recognizes a glycosylation dependent preS2 epitope [Heermann et al. in ''Viral Hepatitis and Liver Disease'', Eds. A. Zuckerman and Alan R. Liss, Inc. New York pp. 697-700, (1988)] and reacts with the 45 kd

form of the L and Gly L proteins does not react with the 30 or 33 kd forms of L\* or  $\Delta$ Gly L\* proteins. These results are in accordance with the amino acid deletions introduced in the L\* and  $\Delta$ Gly L\* proteins.

#### Example F.7

#### Co-expression of S and L or modified L proteins leads to formation of particles of mixed subunit composition

Extracts made from each of the strains described in Example F.6 contain HBsAg related antigenicity as demonstrated by positive results in the AUSRIA assay performed according to the manufacturer's instructions [Abbott Lab.]. Extracts from Y1301, Y1302, Y1142, Y1261, Y1304, Y1305, Y1306 and Y1307 but not those from Y1141 and Y1308 give a positive reaction in the ELISA-S1.1 assay. When subjected to the CsCl equilibrium centrifugation the AUSRIA and ELISA-S1.1 positive material cobanded around a density of 1.2 g/cm<sup>3</sup>. Immunoblots confirmed the antigenicity profiles showing that the protein species found in crude extracts cobanded in the AUSRIA and ELISA-S1.1 positive peak. Sucrose velocity gradient centrifugation of dialyzed peak fractions derived from the CsCl equilibrium gradients showed co-sedimentation of the S and L proteins or modified L proteins as determined by AUSRIA, ELISA-S1.1 and immunoblot. This demonstrates that S and L or modified L proteins are assembled into lipoprotein particles.

Immunoprecipitation reactions with Mab S1.1 were used to demonstrate that the S and L or modified L proteins were associated into one physical entity i.e. particles of mixed subunit composition. Extracts were made from yeast strains expressing the S and L or modified L

01  
02  
03 proteins either alone or together, for instance, from  
04 Y1139 (L\* protein, Example F.3), Y1141 (S protein,  
05 Example F.6) and Y1142 (S and L\* protein, Example  
06 F.6). After semi-purification of the particles by CsCl  
07 banding and dialysis of the peak fractions, the  
08 particles were subjected to the immunoprecipitation  
09 reaction with Mab S.1.1. A mixture of particles from  
10 Y1139 and Y1141 was used as an additional control. The  
11 L\* protein was immunoprecipitated in each case.  
12 Coprecipitation of the S protein with the L\* protein  
13 was observed only from the reaction with particles  
14 derived from Y1142.

15  
16 Identical results were obtained with the other yeast  
17 strains that co-express the S and L or modified L  
18 proteins (Y1301, Y1302, Y1261, Y1304, Y1305, Y1306,  
19 Y1307).

20  
21 Example F.8

22  
23 Stability and pHSA binding of particles containing the  
24 modified L protein

25  
26 The sensitivity to proteolytic degradation by yeast  
27 proteases of the L\* and  $\Delta$ Gly L\* proteins was analysed  
28 by incubating crude cell extracts of Y1142 and Y1161  
29 for either 4 hours at 37°C or 65 hours at 4°C.

30  
31 Extracts of Y1301 and Y1302 served as comparative  
32 source of L and  $\Delta$ Gly L protein. Analysis of extracts  
33 by immunoblot before and after incubation showed an  
34 almost complete disappearance of the L and  $\Delta$ Gly L  
35 proteins after incubation while the L\* and  $\Delta$ Gly L\*  
36 proteins were still readily detectable.  
37

Purified preparations of particles from strains Y1306 and Y1307 were incubated for 7 days at 37°C and examined by SDS-PAGE and silver staining to reveal the polypeptides. No detectable degradation of the L\* or S polypeptides could be observed.

Particles derived from Y1142 by CsCl banding were tested by a modification of the pHSA binding assay described by Pontisso et al. J. Virol. Methods 6, 151-159 (1983). Microplates (Immunoplate I; Nunc, Gibco Europe) were coated with glutaraldehyde polymerized human serum albumin (5 µg/ml pHSA in PBS, 2 hrs 37°C) and non-specific protein binding sites on the plates were saturated by incubation (1 hr 37°C) with PBS containing 1 % bovine serum albumin (BSA). Serial dilutions of the test samples in PBS containing 0.2 % BSA were allowed (1 hr 37°C) to interact with the pHSA coated plates and bound particles were detected (1 hr 37°C) with a non-limiting concentration of horseradish peroxidase coupled anti-S monoclonal antibody (RF1, H.C. Thomas, Royal Free Hospital, London) in PBS containing 0.2 % BSA and revealed with H<sub>2</sub>O<sub>2</sub> and orthophenylenediamine as substrate. Between incubations the plates were washed with 0.15 M NaCl containing 0.05 % Tween 20.

Particles derived from Y1141 served as negative control and purified particles from strain 10S44C cir<sup>0</sup> containing pRIT12660 as positive control. pHSA binding was greatly diminished in the Y1142 derived particles (2 % residual activity) compared to the particles derived from 10S44C cir<sup>0</sup> harbouring pRIT12660.

pHSA binding was also not detectable with purified particle preparations from strains Y1306 and Y1307.

Example F.9Immunogenicity of particles containing L\* protein

The immunogenicity of (S, L\*) particles of this invention was studied in Balb/c mice. Particles derived from either Y1142 (S, L\*) or Y1141 (S) were partially purified by CsCl banding, and adsorbed on  $\text{Al}(\text{OH})_3$ . Groups of eight mice were injected with either 50 or 5  $\mu\text{g}$  total protein (corresponding to 1 or 0.1  $\mu\text{g}$  antigen by AUSRIA assay) of each antigen preparation at days 0 and 30. The bleeding of mice was done at day 45 and the different assays performed on individual sera. Anti-HBs antibodies were measured with the AusAb kit (Abbott, USA) and expressed in mIU/ml using the Hollinger formula [Hollinger et al., in 'Viral Hepatitis' Szumness et al. Franklin Institute Press, Philadelphia pp. 451-466 (1982)].

Anti-preS antibodies were measured using a direct ELISA assay in which selected synthetic peptides (peptide 12-32, peptide 32-47 and peptide 120-145) were adsorbed to polystyrene walls of microplate (Immunoplate I; Nunc, Gibco Europe). Adsorption took place overnight at  $4^\circ\text{C}$  from a solution of 0.5  $\mu\text{g}/\text{ml}$  peptide in 0.05 M  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$  buffer pH9.6, 100  $\mu\text{l}$  per well. Non-specific sites on the plates were blocked by incubation with 250  $\mu\text{l}$ , 5 % foetal bovine serum in PBS during 1 hour at  $37^\circ\text{C}$ .

Two fold serial dilutions of the mice sera in PBS containing 1 % foetal bovine serum, 0.05 % Tween 20 were allowed to react with the peptide-coated plate during 2 hrs at  $37^\circ\text{C}$  (100  $\mu\text{l}$  per well). After washing, bound antibodies were detected by a biotinylated anti-mouse Ig from sheep (Amersham, 500 fold dilution

in PBS containing 1 % foetal bovine serum, 0.05 % Tween 20, 100  $\mu$ l per well, incubation for 1 hour at 37°C) and then with a streptavidin - biotinylated horseradish peroxidase complex (Amersham, 1000 fold dilution in PBS containing 1 % foetal bovine serum 0.05 % Tween 20) during 1 hour at 37°C, 100  $\mu$ l per well.

Between incubations, the plates were washed with 0.15 M NaCl, 0.05 % Tween 20. Final revelation was by H<sub>2</sub>O<sub>2</sub> and ortho-phenylenediamine as chromogen (15  $\mu$ l H<sub>2</sub>O and 4 mg ortho-phenylenediamine in 10 ml 0.1 M potassium phosphate buffer, pH6.0, 100  $\mu$ l per well). The reaction was stopped after 20 min by the addition of 25  $\mu$ l 1 N H<sub>2</sub>SO<sub>4</sub> and absorbance at 490 nm measured in an Intermed Immunoreader, NJ 2000, Analis. The titer for each serum was calculated as the reciprocal of the highest dilution giving an optical density of 1.00.

Table 7 shows the respective antibody titers induced in Balb/c mice expressed as Geometric Mean Titer.

Table 7

| Particles<br>derived<br>from | Dose<br>$\mu$ g | $\alpha$ -HBs<br>GMT in<br>mIU/ml | $\alpha$ 120-145<br>GMT* | $\alpha$ 12-32<br>GMT* | $\alpha$ 32-47<br>GMT* |
|------------------------------|-----------------|-----------------------------------|--------------------------|------------------------|------------------------|
| Y1142                        | 50              | 304000                            | 629                      | 5222                   | 2255                   |
| (S, L*)                      | 5               | 234000                            | 212                      | 1490                   | 785                    |
| Y1141                        | 50              | 128000                            | 202                      | 211                    | <200                   |
| (S)                          | 5               | 29000                             | <200                     | 203                    | <200                   |

(\*) The titers were expressed as the inverted dilution for an OD of 1.0.

Particles purified from strain Y1307 (S,  $\Delta$ gly L\*) or from strain RIT4376 [Petre J. et al., Postgrad. Med. J. 63, (Suppl. 2), 73-81 (1987)] were adsorbed onto Al(OH)<sub>3</sub> and injected into groups of 10 Balb/c mice on days 0 and 30. Mice were bled on day 45 and the pooled immune sera examined for the presence of anti-S and anti-preS antibodies as described above. Table 8 shows the respective antibody titers induced in Balb/c mice.

Table 8

| Particles<br>purified from    | Dose<br>mcg | $\alpha$ -HBs<br>mIU/ml | $\alpha$ 12-32* | $\alpha$ 32-47* |
|-------------------------------|-------------|-------------------------|-----------------|-----------------|
| Y1307<br>(S, $\Delta$ gly L*) | 1           | 7335                    | 3128            | 658             |
| RIT4376<br>(S)                | 1           | 5232                    | <100            | <100            |

(\*) Titres are expressed as the inverted dilution giving an OD of 1.0 in the assay.

Example F.10

Integration of an L\* expression cassette in the S. cerevisiae genome by Tyl mediated homologous recombination

It is desirable to integrate both the L\* and S protein expression cassettes of this invention into the host

genome to provide genetically stable strains and to avoid the 20-30% of plasmidless cells generally seen with 2 micron based S. cerevisiae vectors.

Plasmids pRIT13459 and pRIT13460 were constructed as described for the vector pRIT13009-p in co-pending U.S. patent application SN 009,325 and in Jacobs et al. (Gene 80:279-291, 1989). The LEU2 gene, isolated as a 2200 bp XhoI-SalI fragment from the vector pCV9 (Petes et al., Cell 19:765, 1980), was inserted between the SalI sites of the Tyl element on pRIT12927, replacing the original fragment, to give pRIT13144. A 3050 bp HindIII-KpnI fragment carrying the L\* expression cassette from pRIT13220 described in Example F.3 was treated with T4 polymerase and ligated into the T4 polymerase treated SalI site remaining on pRIT13144 and using E. coli strain XL1-Blue as transformation recipient. XL1-Blue is described by Hanahan D. in J.Mol. Biol., 166:557-580 (1983) and is commercially available from Stratagene, 11099 North Torrey Pines Road, La Jolla, California. Both orientations of insertion of the HindIII-KpnI cassette fragment were obtained; pRIT13459 contains the cassette ARG3 terminator region proximal to the LEU2 gene, pRIT13460 contains the cassette TDH3 promoter proximal to the LEU2 gene.

The large XhoI fragments of about 7700 bp from both pRIT13459 and pRIT13460 were purified from 1% agarose gels and used to transform strain EJ cup1 $\Delta$ 3d (see Example F.5 above) to leucine independence by the method of Hinnen et al. (Proc. Natl. Acad. Sci. USA, 75:1929, 1978).

LEU<sup>+</sup> transformants were obtained with both fragments and 12 colonies from each series were analysed by

Western blotting for L\* protein expression and by Southern blotting for the number of integrated cassettes. From the transformation of EJ cup1 $\Delta$ 3d with the fragment from pRIT13459 a strain Y1528 was retained which showed a level of L\* expression equivalent to Y1306 and contained 3-5 copies of the cassette. From the transformation of EJ cup1 $\Delta$ 3d with the fragment from pRIT13460 a strain Y1529 was retained which has similar properties to Y1528.

Both Y1528 and Y1529 were mated with strains Y1295 and its trp<sup>-</sup> parent strain Y1215 (see Example F.5 above) and with strain Y1367 to form diploids as follows :  
Y1528 x Y1295 = Y1586, Y1528 x Y1215 = Y1587, Y1528 x Y1367 = Y1588, Y1529 x Y1295 = Y1589, Y1529 x Y1215 = Y1590, Y1529 x Y1367 = Y1591. Strain Y1367 is  $\alpha$ , leu2, trp1, contains 6-8 copies of the same S-protein cassette as strain Y1295 and was obtained using the strains and methods described in the construction of Y1295 in Example F.5.

The diploid strains listed above are screened for their levels of surface antigen expression and the ratio of L\* and S polypeptides in the particles by Western blotting, AUSRIA assay and epitope specific ELISA tests using the reagents and methods described above, more particularly in Examples F.2 and F.6. The number and stability of the integrated expression cassettes is determined by Southern blotting. If desired the diploid strains may be sporulated to obtain haploid segregants which may then be screened for expression and genetic stability as described above.

Insertion of the 3050 bp HindIII-KpnI fragment from pRIT13222 on pRIT13144 and following of the above described procedures may be carried out to give S.

cerevisiae strains with integrated copies of the  $\Delta$  gly L\* expression cassette and strains expressing S,  $\Delta$  gly L\* particles.

In a further embodiment the L\* expression cassette is inserted on a vector similar to pRIT13134-P [Jacobs et al., Gene 80; 279-291 (1989)] and which carries the URA3 and CUP1 genes as selective markers inserted within the Tyl element of pRIT12927. Such a vector is identified as pRIT13501 from which a BglII fragment of about 6500 bp with the above elements can be recovered for transformation and integration into an appropriate yeast host by selection for URA<sup>+</sup> transformants. Following transformation the number of integrated cassettes may be advantageously increased by selection for increased levels of copper resistance as disclosed in copending U.S. application SN 07/368,401.

#### Example F.11

#### Expression of S, L\* mixed particles with ad and ay subtype determinants

An integrative vector with an expression cassette for an L\* protein of ay subtype was constructed by exchanging the XbaI-SacII fragment encoding the C-terminal part of an ad subtype S protein and part of the ARG transcription terminator with a corresponding fragment from an S-gene of ay subtype. Plasmid pRIT13459 (Example F.10) was digested with XbaI and SacII endonucleases and the largest fragment was purified from a 1% agarose gel and ligated to a purified 1000 bp XbaI-SacII fragment from pRIT10780 to give pRIT13500. pRIT10780 is disclosed in De Wilde et al., Develop. Biol. Standard, 59:99-107, (1985).

01  
02  
03 The L\* coding sequence on pRIT13500 consists of DNA  
04 with sequences identical to those from a virus of ad  
05 specificity from the ATG codon to the XbaI site and of  
06 DNA derived from a virus of ayw specificity from the  
07 XbaI site to the termination codon. The expressed L\*  
08 polypeptide will have ay subtype determinants.

09  
10 The XhoI fragment of about 7700 bp from pRIT13500 can  
11 be integrated into the yeast genome and the resulting  
12 transformants mated with strains such as Y1295 and  
13 Y1367 by the methods described above so as to obtain  
14 strains expressing mixed particles in which the L\*  
15 polypeptide is of ay subtype and the S polypeptide of  
16 ad subtype.

17  
18 The above description and examples are illustrative and  
19 not limiting of the invention. For example, a variety  
20 of additional modifications to the L protein may be  
21 made, e.g., partial deglycosylation or a combination of  
22 various of the modifications made herein, in addition  
23 to those mentioned in the examples to generate modified  
24 L proteins and particles of mixed or homogeneous  
25 subunit composition containing these modified L  
26 proteins of appropriate composition for vaccine use.

27  
28 Additional modifications that may be made include  
29 reintroduction of preS sequences, introduction of  
30 coding sequences derived from HBV protein coding  
31 sequences other than from the envelope gene, e.g.  
32 sequences from the HBV core gene, or sequences coding  
33 for immunological important amino acid regions from  
34 other pathogens. Additional known vector components  
35 may be employed to replace the specific marker genes,  
36 promoters and linker sequences and the like employed in  
37 the examples. Other types of host cells may be

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employed. The host cells, vectors and vector components employed in these examples are illustrative and may be replaced with other strains or modifications by one of skill in the art. This invention encompasses all improvements and modifications falling within the scope of the following claims.

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40

1. A protein, the amino acid sequence of which consists of parts of the amino acid sequence of the hepatitis B virus large (L) protein (ad or ay subtype), characterised in that the amino acid sequence of the protein consists of either:

- (a) residues 12 - 52, followed by residues 133 - 145, followed by residues 175 - 400 of the said L protein; or
- (b) residue 12, followed by residues 14 - 52, followed by residues 133 - 145, followed by residues 175 - 400 of the said L protein.

2. A particle comprising a multimeric assembly, of a protein according to claim 1.

3. A composite particle comprising a multimeric assembly of the small (S) protein of Hepatitis B surface antigen and a protein according to claim 1.

4. A composite particle as claimed in claim 3, said particle being expressed in *S. cerevisiae* or *H. polymorpha*.

5. A particle as claimed in claim 3 or claim 4 in which the S protein is of ad subtype and the protein according to claim 1 comprises residues from an L protein of ay subtype or vice versa.

6. A vaccine comprising an immunoprotective amount of a protein according to claim 1 or of a particle according to any one of claims 2 to 5.

7. A recombinant DNA molecule comprising a DNA coding sequence for expression of a protein according to claim 1 operatively linked to a regulatory sequence.

8. A recombinant vector comprising the DNA molecule of claim 7.

9. A host cell transformed with a recombinant DNA molecule of claim 8.

10. A cell according to claim 9 selected from the group of yeasts consisting of *Saccharomyces*, *Hansenula*, *Pichia*, *Candida*, *Kluyveromyces* and *Schizosaccharomyces*.

11. A cell as claimed in claim 9 or claim 10 which is additionally transformed with a recombinant DNA vector capable of expressing the S protein of Hepatitis B surface antigen.

12. A method of producing a protein according to claim 1 which comprises culturing a cell according to claim 9 or claim 10 in appropriate culture media and isolating said protein from the cell lysate or extract of said culture media.

13. A method of producing simultaneously a protein according to claim 1 and a Hepatitis B surface antigen S protein comprising culturing the cell of claim 11 in appropriate culture media and isolating from the cell lysate or extract of said culture media protein particles of mixed subunit composition.

14. The use of a protein according to claim 1 or of a particle according to any one of claims 2 to 5 in the preparation of a vaccine composition for the prevention or for the treatment of hepatitis B infection in humans.

15. The use of a protein according to claim 1 or of a particle according to any one of claims 2 to 5 in the preparation of a reagent or test kit for the detection of HBV or HBV-derived proteins or anti-HBV antibodies in a biological sample.

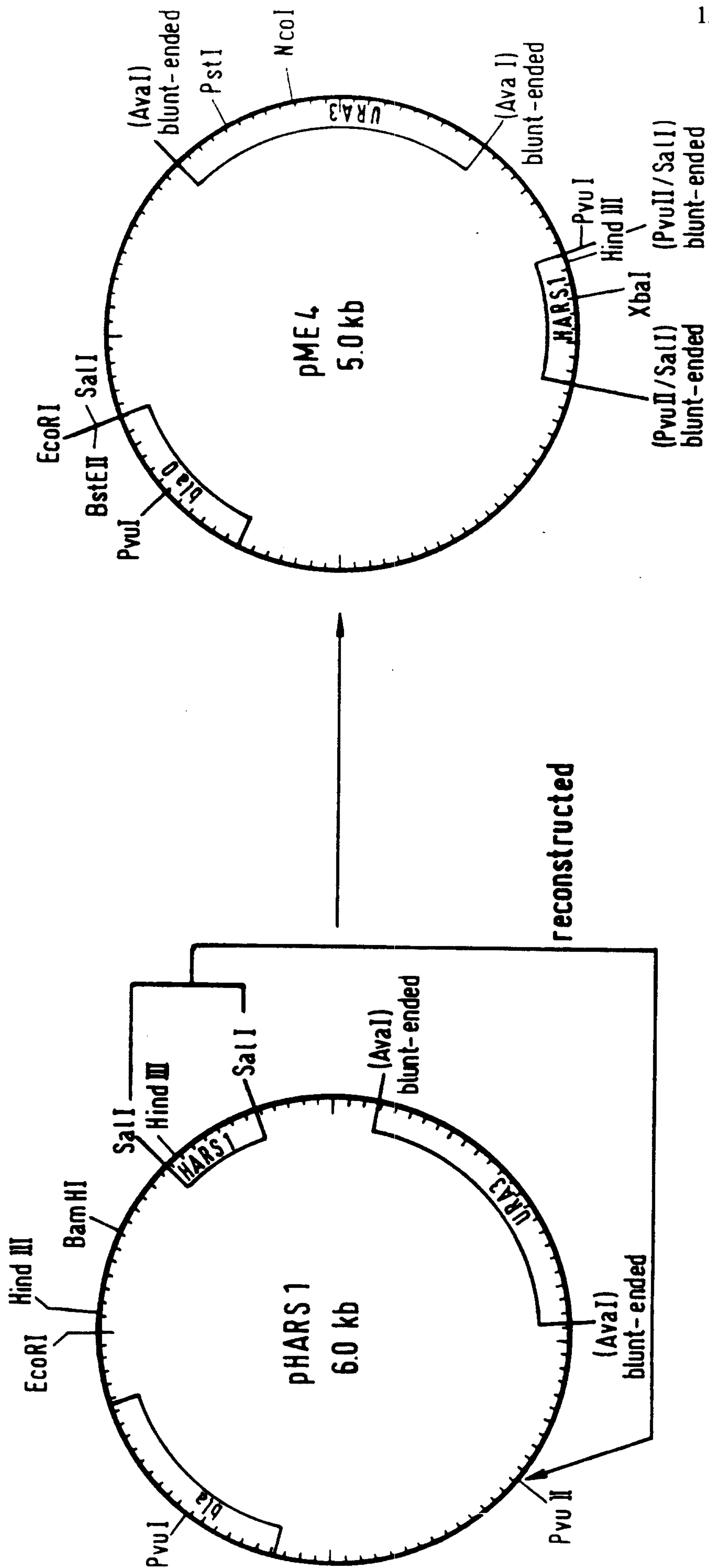
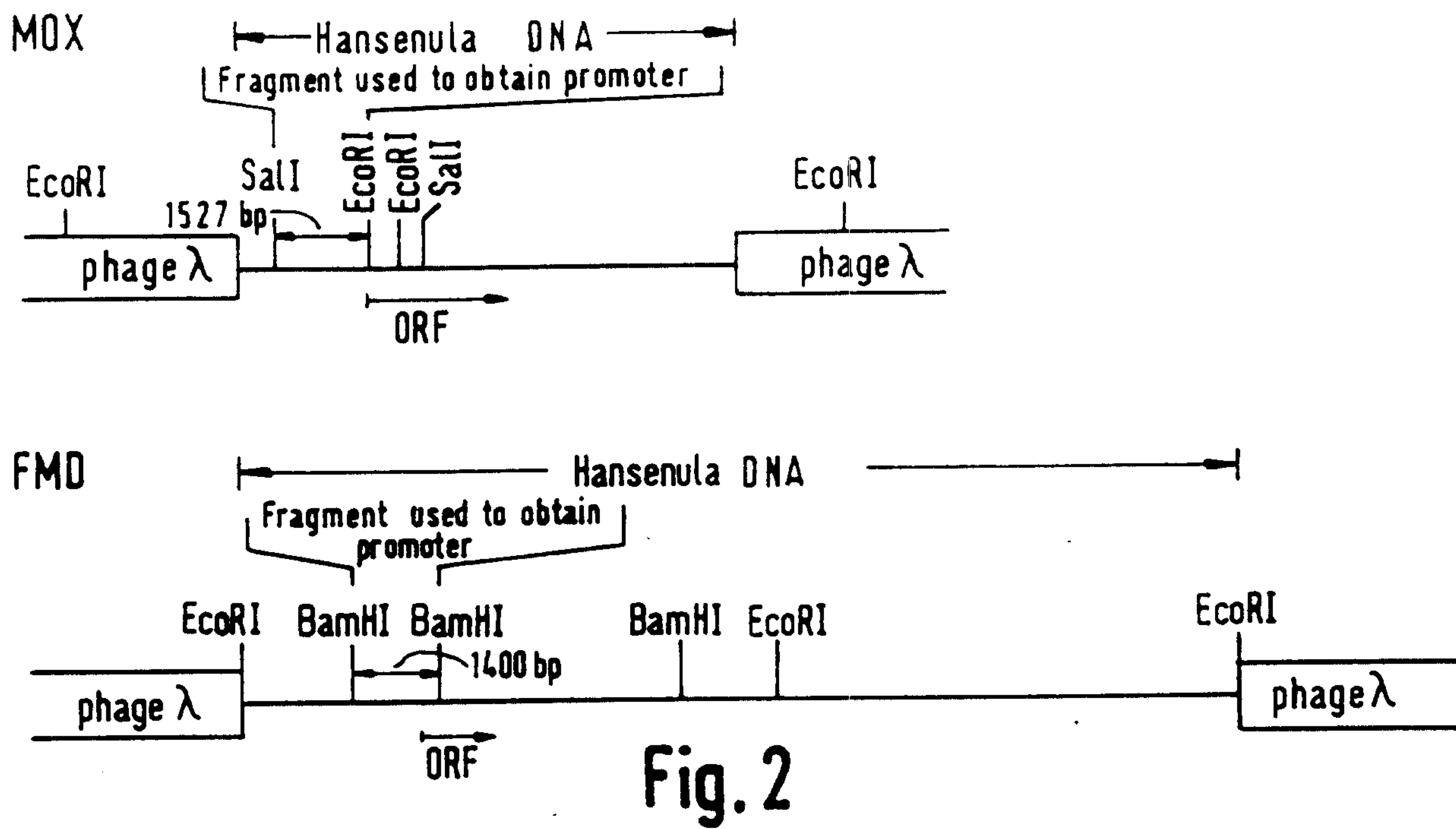


Fig.1



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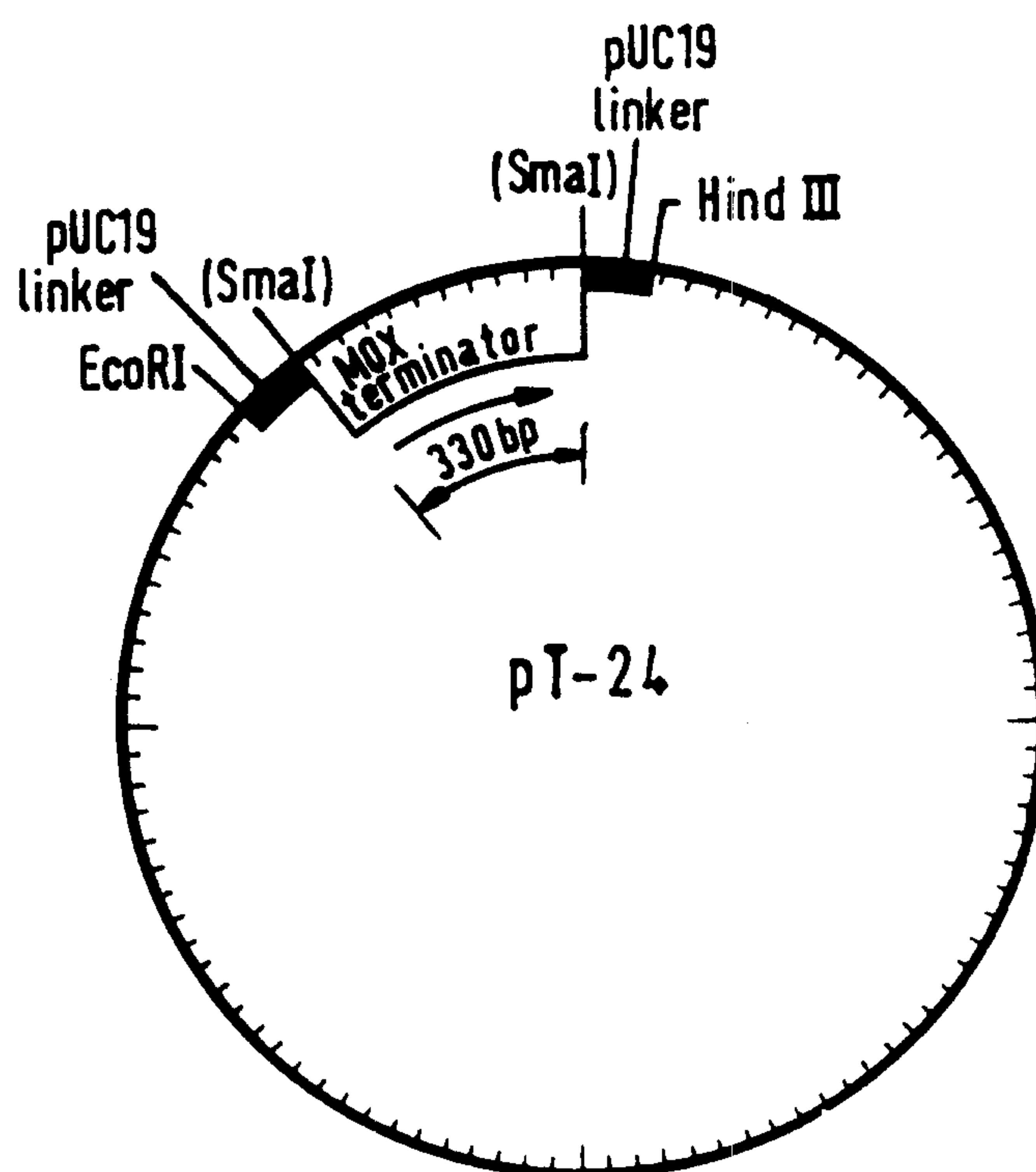


Fig. 3

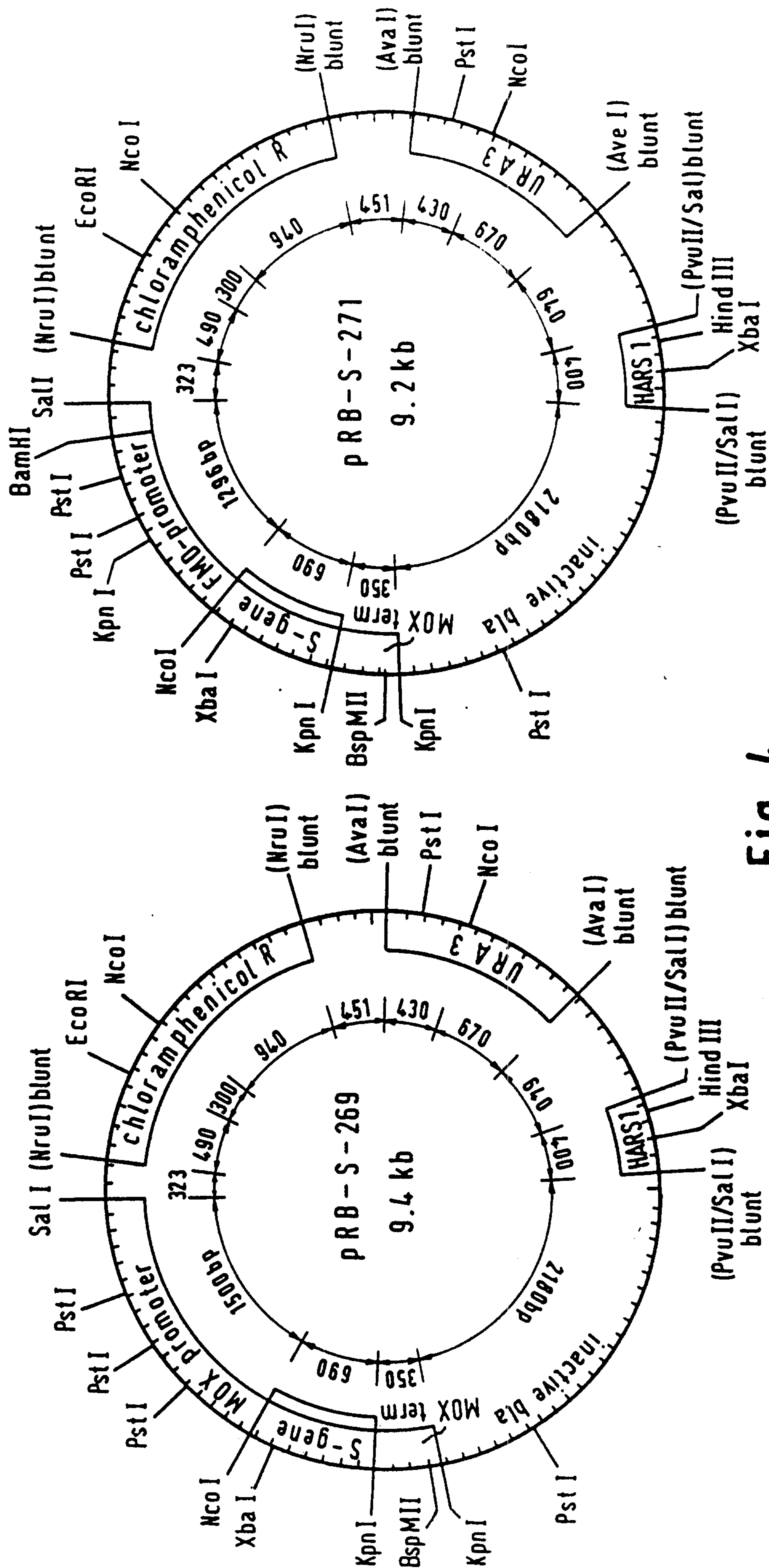


Fig. 4

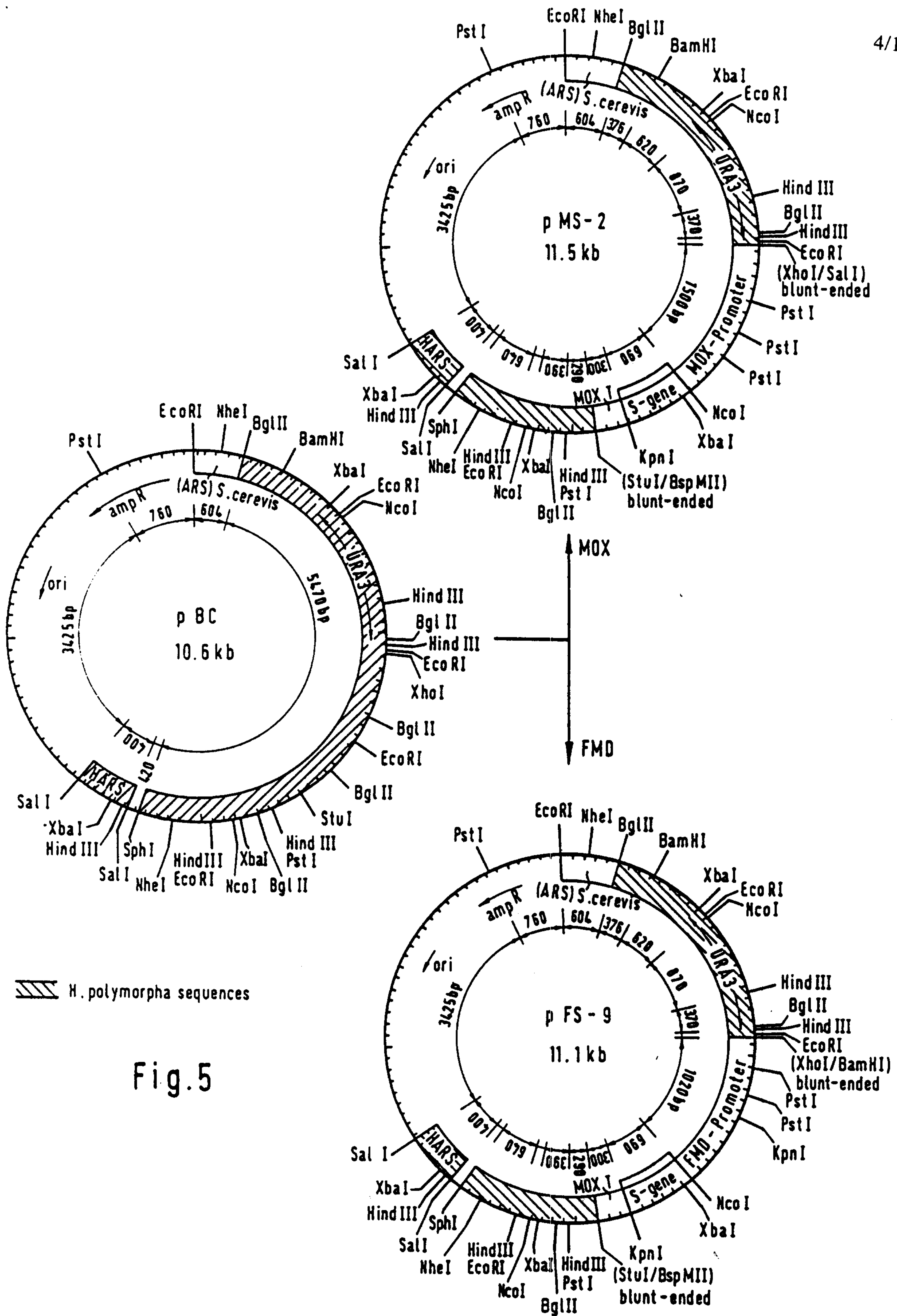


Fig.5

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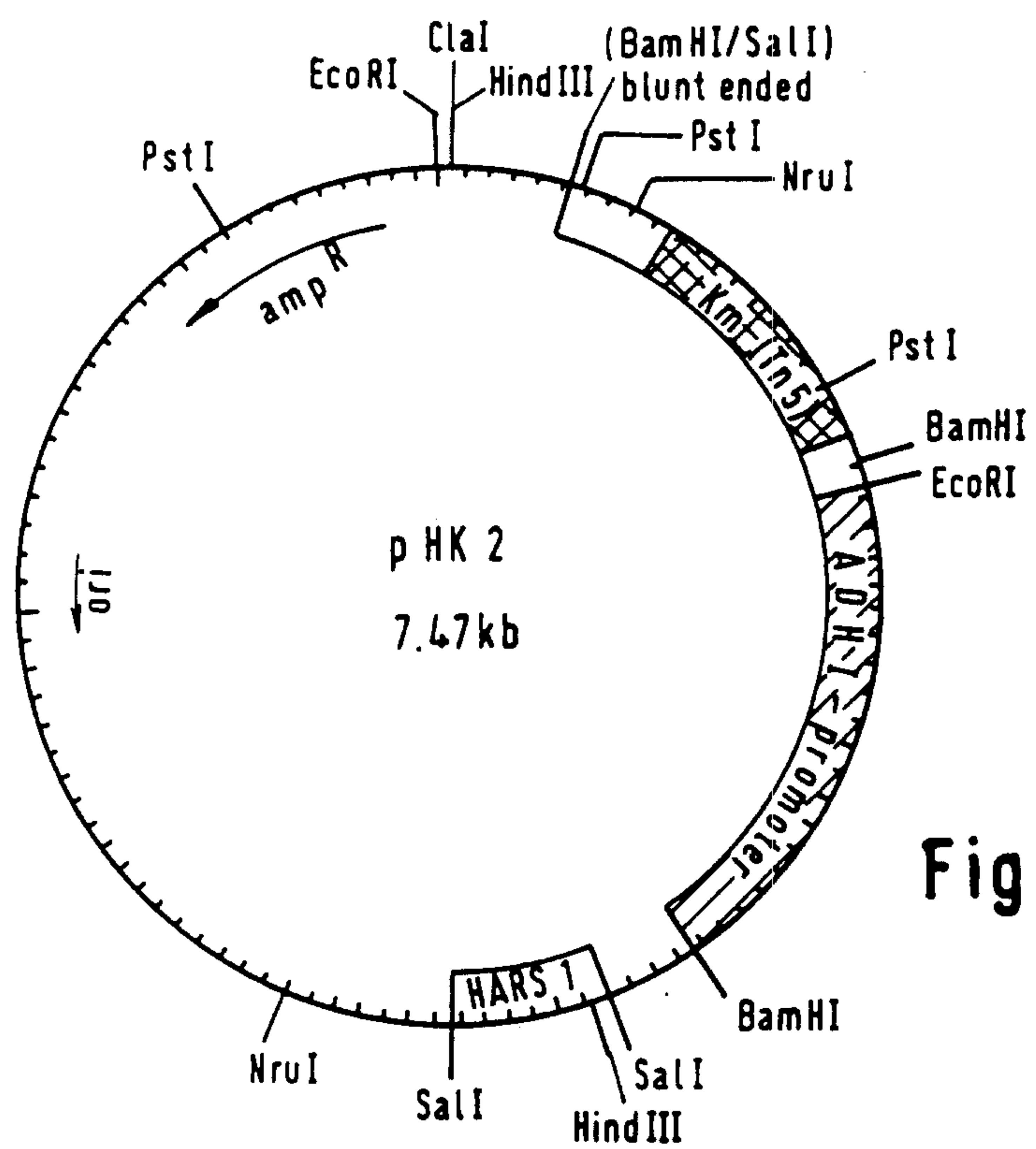


Fig. 6



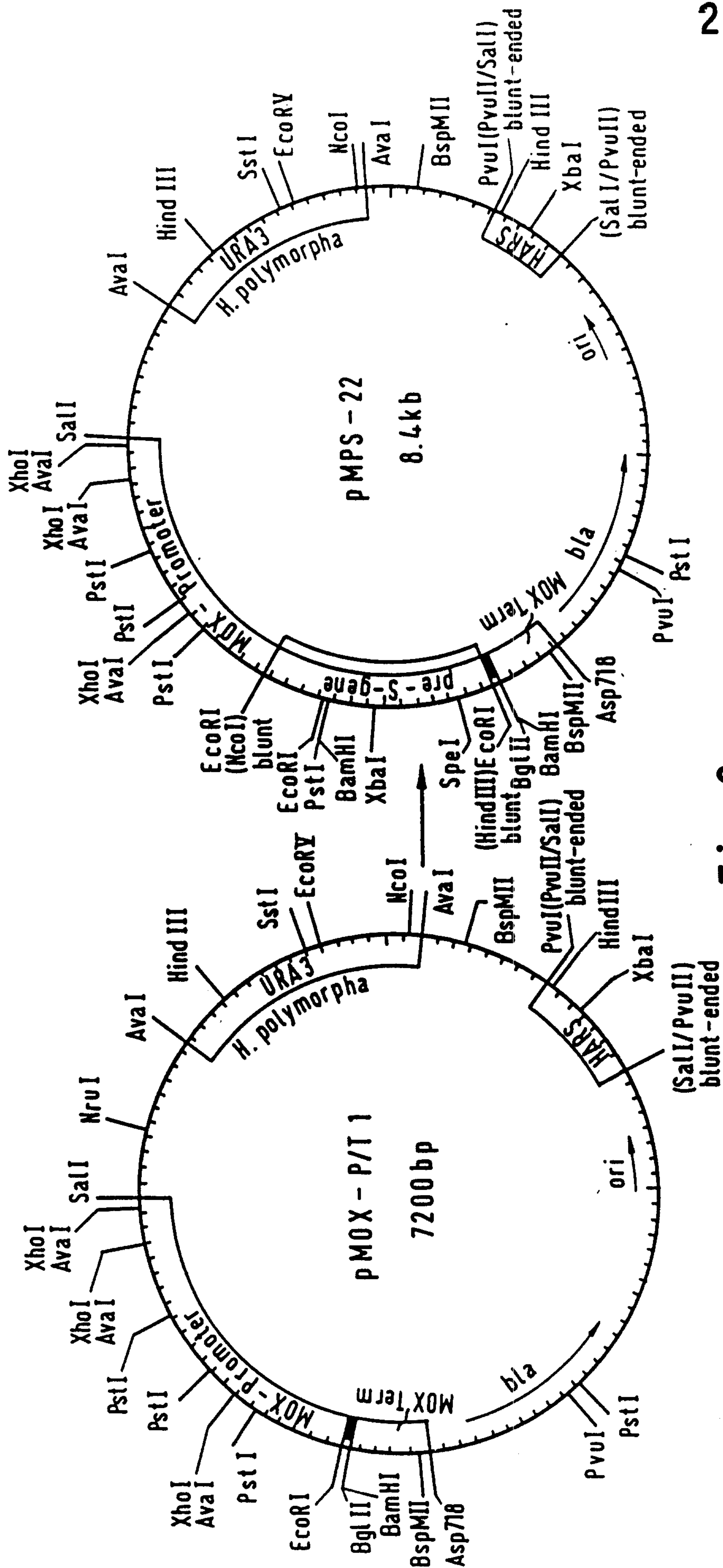


Fig. 8

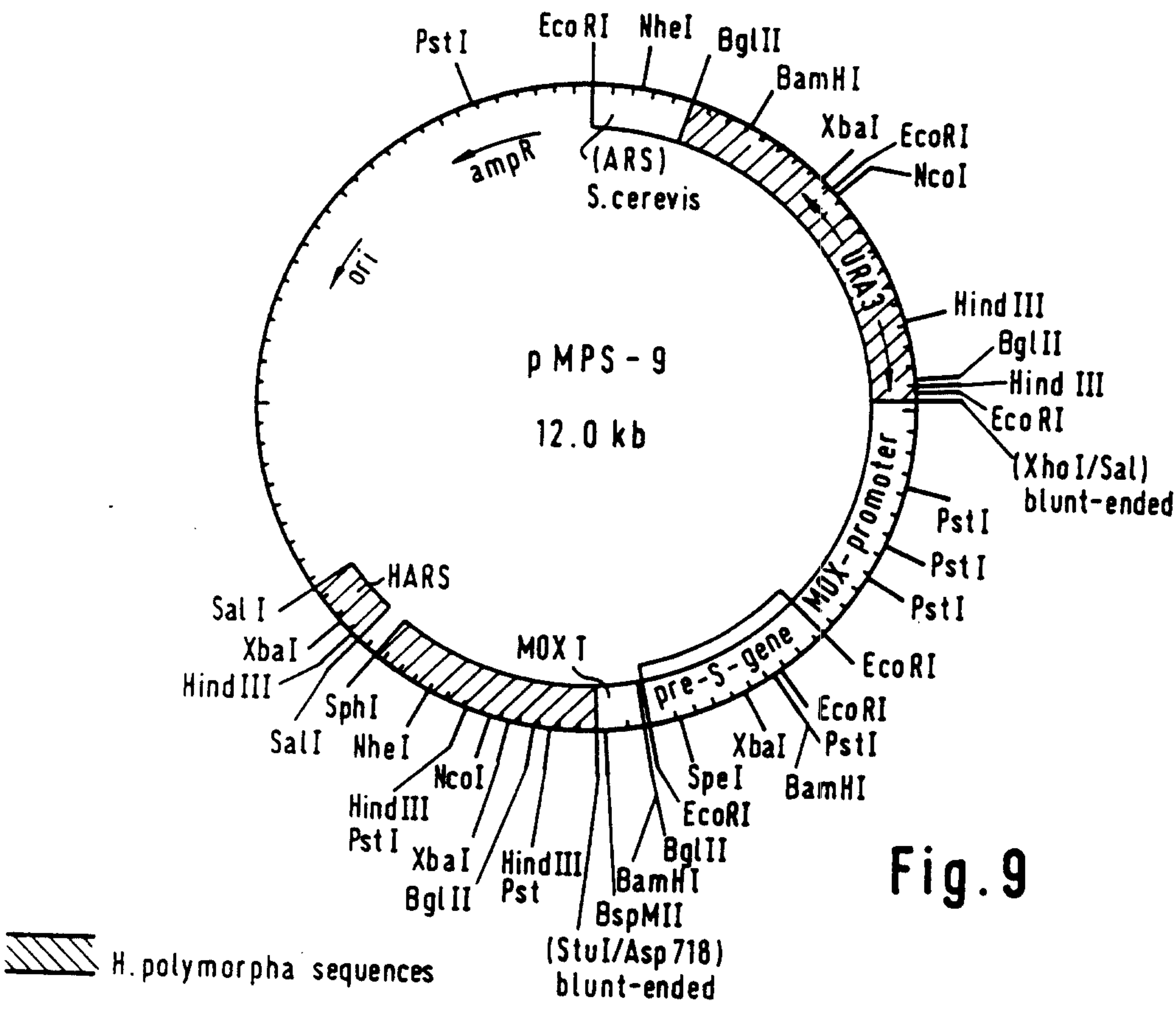
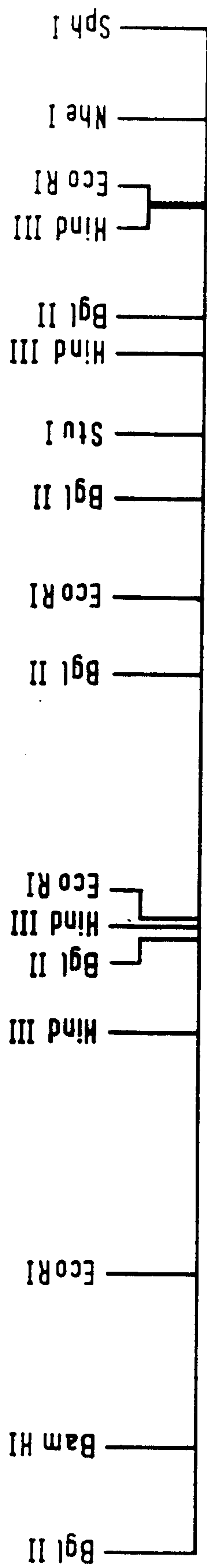


Fig.9



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Fig.10

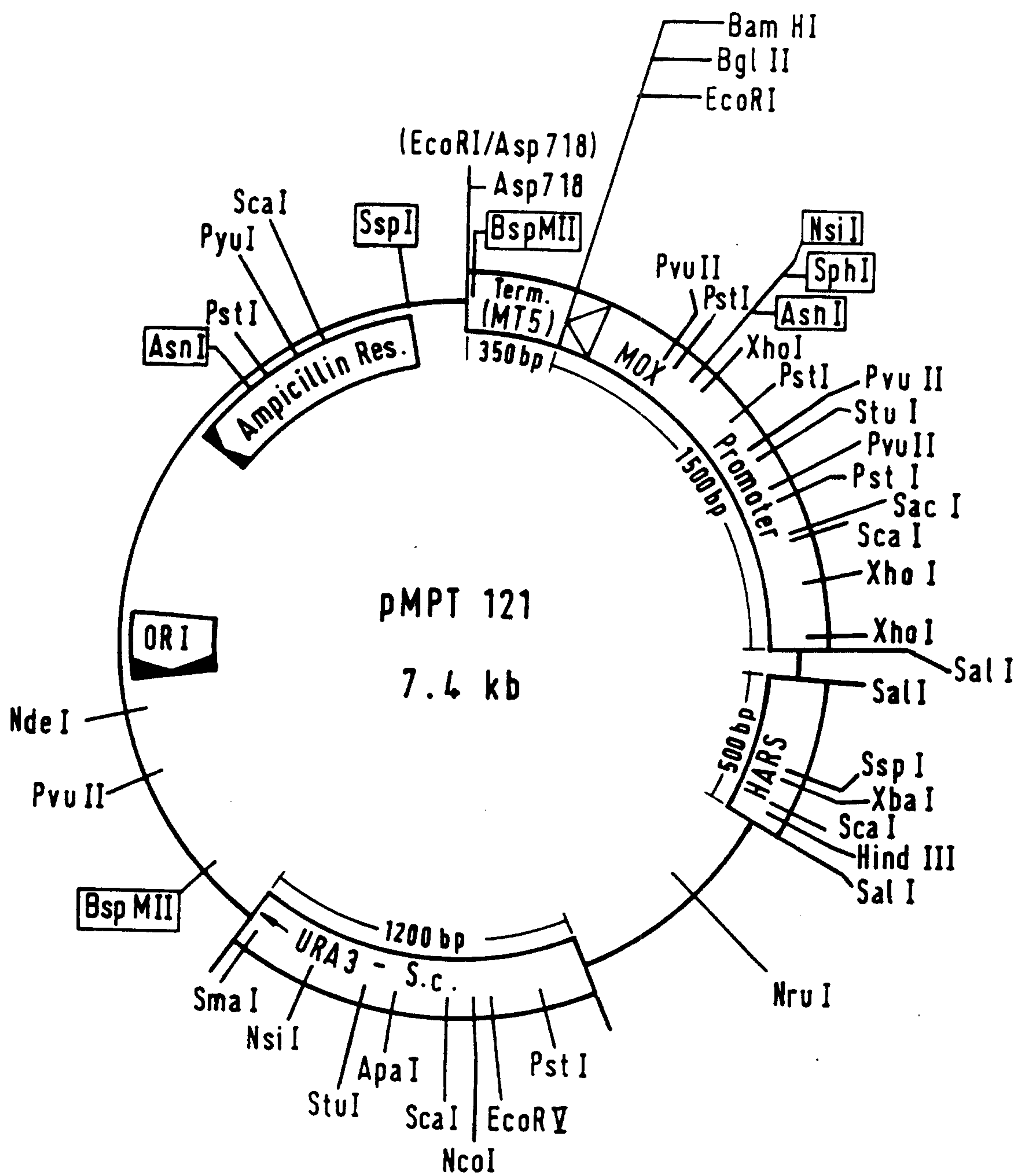


Fig.11

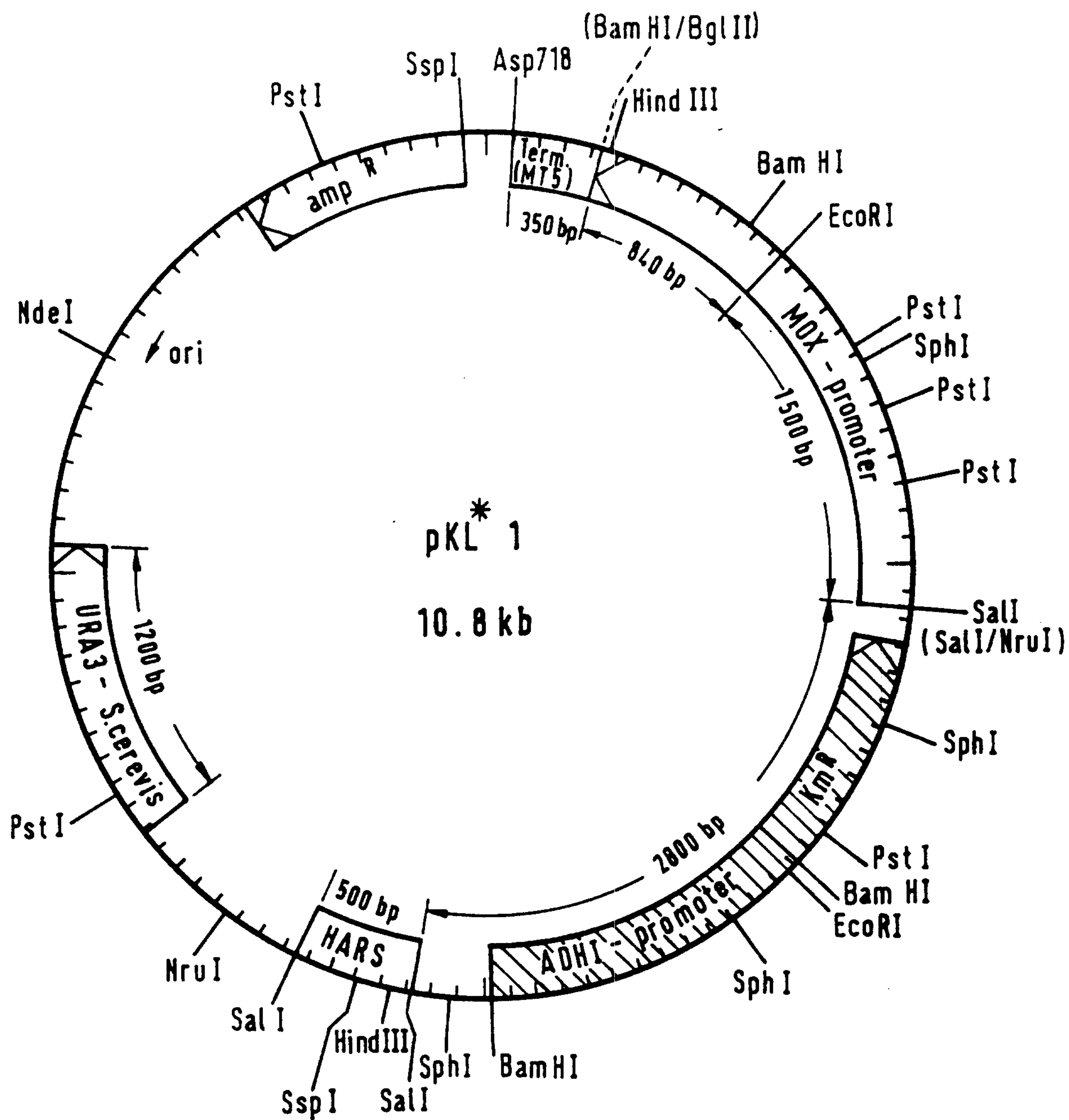


Fig.12

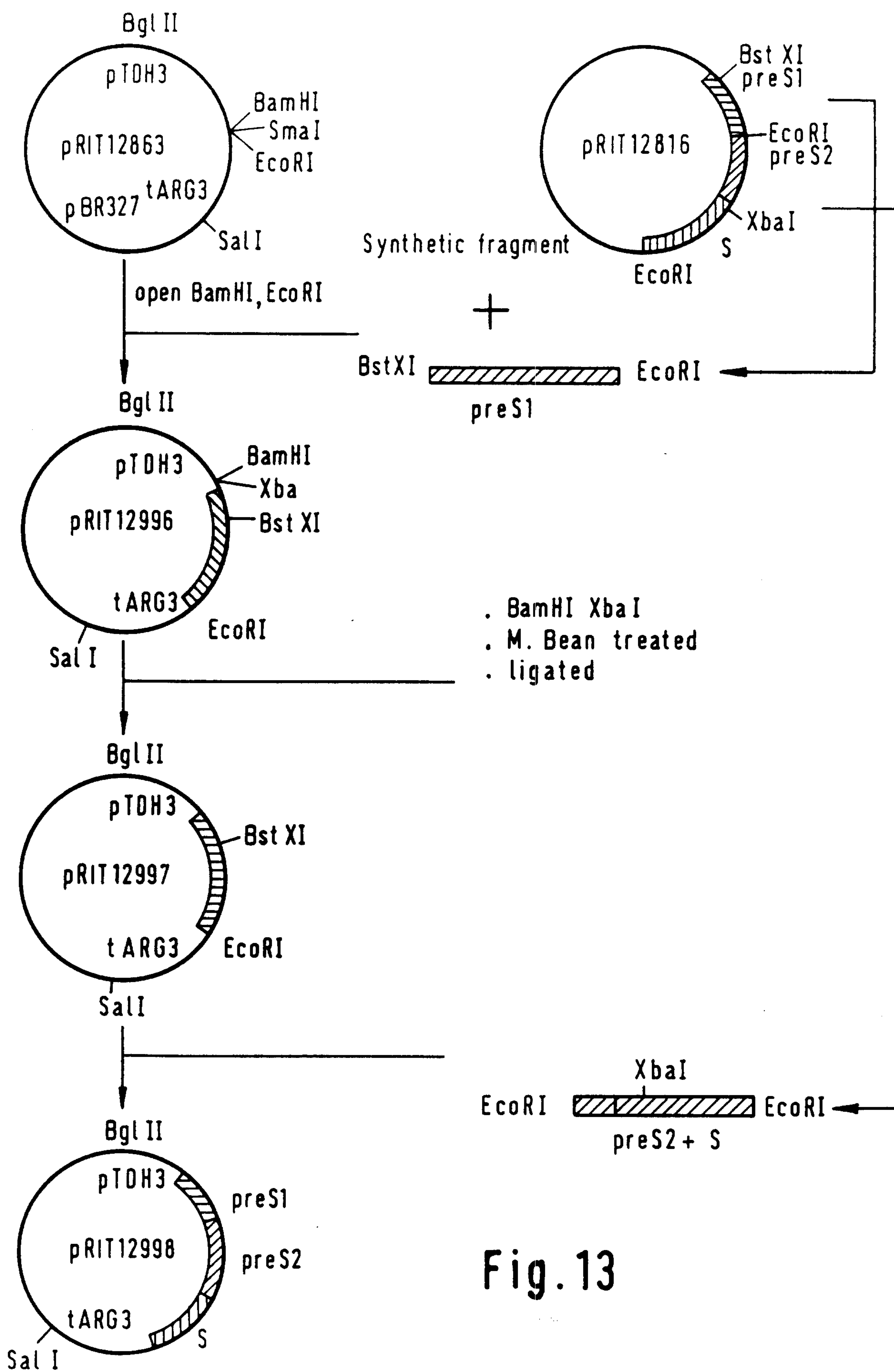


Fig. 13

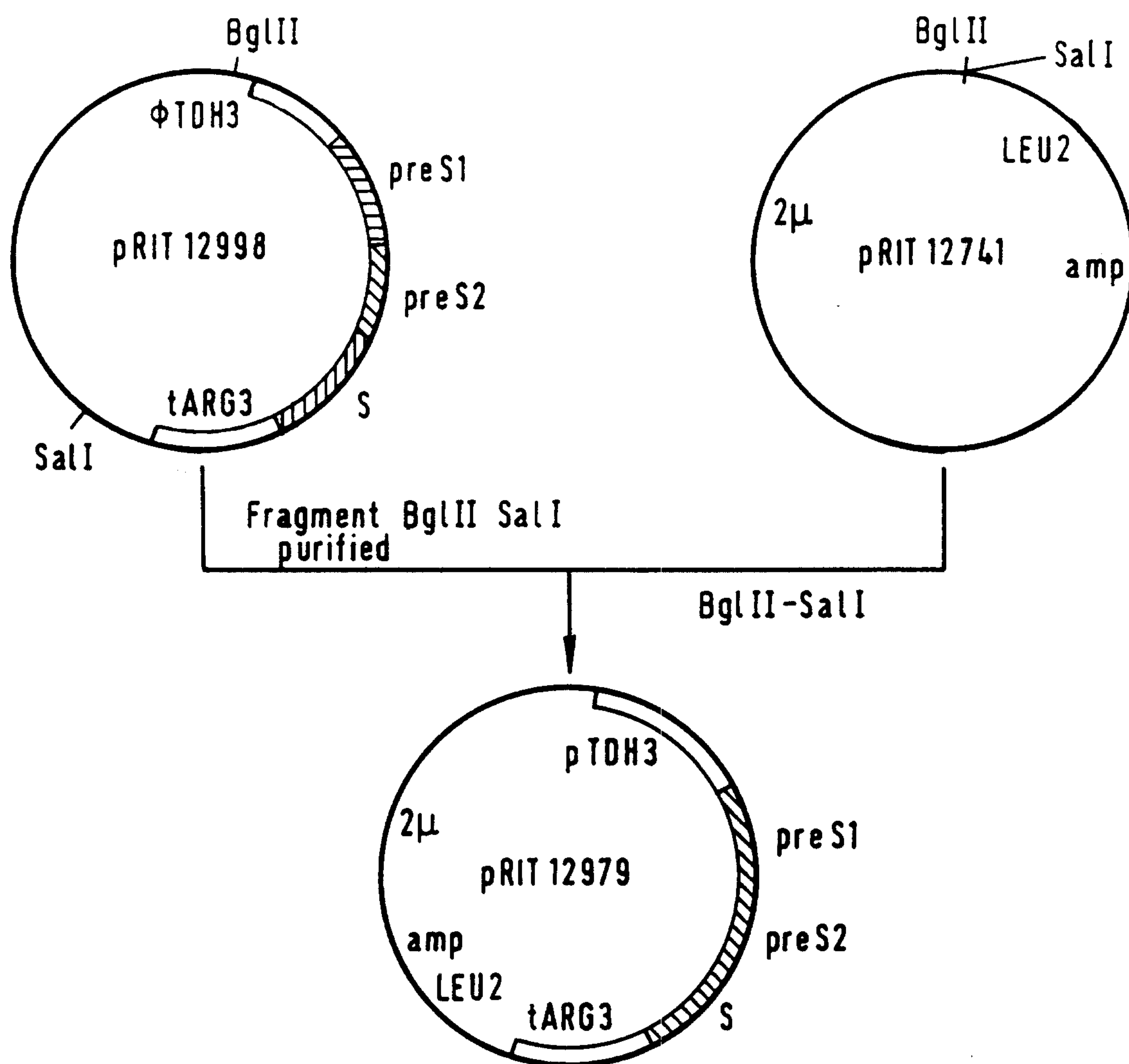


Fig. 14

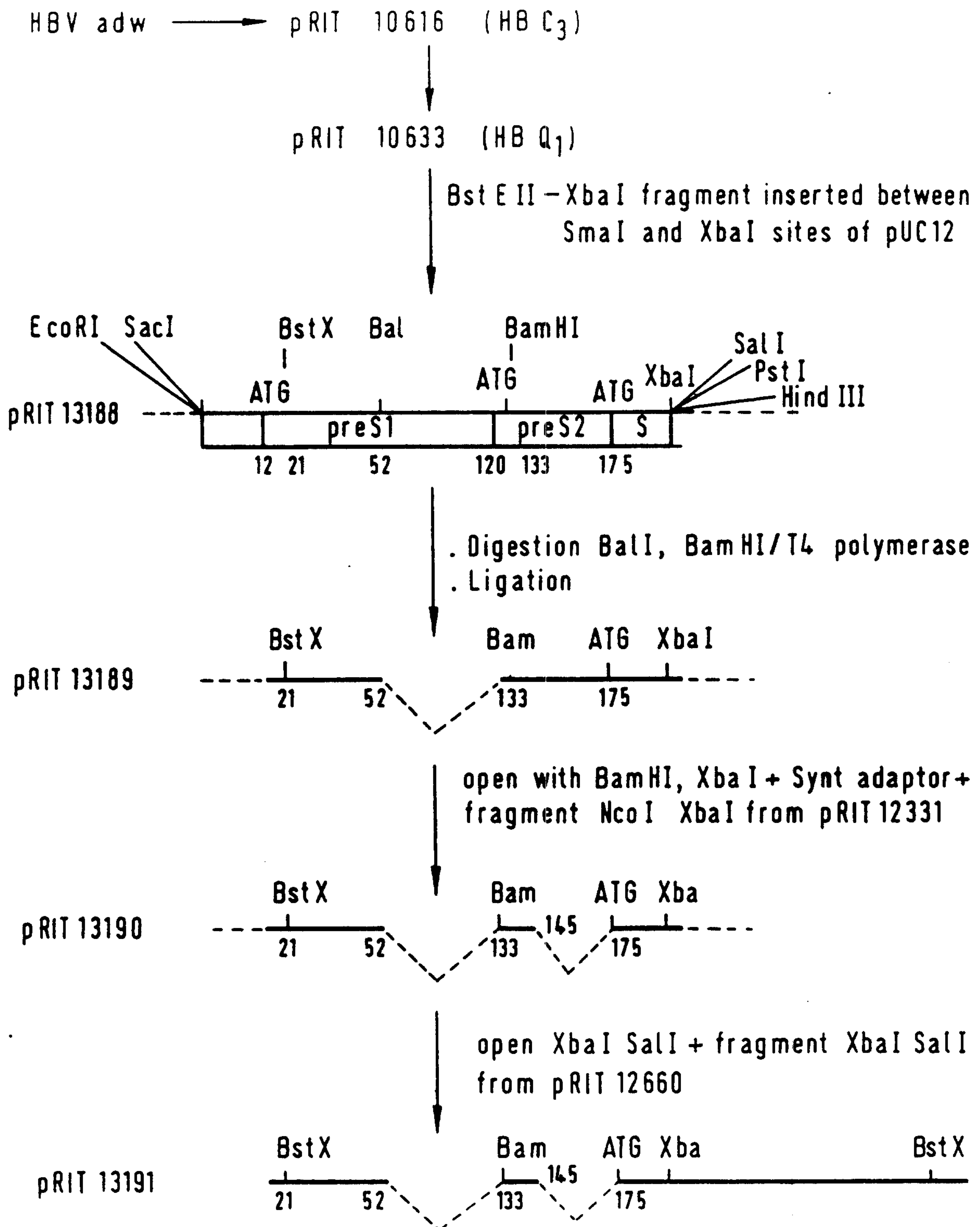


Fig. 15

Fig.16

