G FFITH HACK & CO.
601 ST. KILDA ROAD, MELBOURNE,
VICTORIA 3004, AUSTRALIA
TELEPHONE: (03) 529 8800
FAX: 613 529 6295

Australian Patent Declaration Form

Forms 7 and 8

AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION OR NON-CONVENTION APPLICATION FOR A PATENT OR PATENT OF ADDITION

Name(s) of Applicant(s)

In support of the application no. 81057/87 made by
OXFORD GENE SYSTEMS LIMITED

Title

for a patent for an invention entitled
FUSION PROTEINS AND PARTICLES

Name(s) and address(es) of person(s) making declaration

I/~~We~~, Alan John Kingsman of Greystones, Middle Street, Islip, Oxon OX1 2NP, Great Britain

do solemnly and sincerely declare as follows:—

1. I am/~~we are the applicant(s) for the patent, or~~ authorised by the abovementioned applicant to make this declaration on its behalf.

2. The basic application(s) as defined by Section 141 of the Act ~~was~~/were made in the following country or countries on the following date(s) by the following applicant(s) namely:-

Country, filing date and name of Applicant for the or each basic application

in GREAT BRITAIN on 1 NOVEMBER 1986
by A.J. Kingsman, S.M. Kingsman, S.E. Adams, M.H. Malim and E-J.C. Mellor.
in GREAT BRITAIN on 9 APRIL 1987
by OXFORD GENE SYSTEMS LIMITED
in UNITED STATES on 10 APRIL 1987
by A.J. Kingsman, S.M. Kingsman, S.E. Adams, M.H. Malim and E-J.C. Mellor.

3. The said basic application(s) ~~was~~/were the first application(s) made in a Convention country in respect of the invention the subject of the application.

4. The actual inventor(s) of the said invention ~~is~~/are

Name(s) and address(es) of the or each actual inventor

(SEE ATTACHED SHEET)

5. The facts upon which the applicant(s) is/are entitled to make this application are as follows:-

See reverse side of this form for guidance in completing this part

The applicant is the assignee of the actual inventors and is the assignee of the applicants named in paragraph 2 above in respect of the applications filed on 1st November 1986 and 10th April 1987.

DECLARED at Oxford this 2nd day of June 1989

Alan J. Kingsman

4. The actual inventor(s) of the said invention ~~is~~/are

Alan John KINGSMAN of Greystones, Middle Street, Islip, Oxon OX5 2SF, Great Britain

Susan Mary KINGSMAN of Greystones, Middle Street, Islip, Oxon OX5 2SF, Great Britain

Sally Elizabeth ADAMS of 12 The Phelps, Grovelands, Kidlington, Oxon OX5 1SP, Great Britain

Michael Henry MALIM of Acers, Forge Lane, Upleadon, Glos GL18, Great Britain

Elizabeth-Jane Claire MELLOR of 41 Thackley End, 119 Banbury Road, Oxford OX2 6LB, Great Britain.

(12) PATENT ABRIDGMENT (11) Document No. AU-B-81057/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 606896

(54) Title
FUSION PROTEINS AND PARTICLES

International Patent Classification(s)
(51)[4] C12N 015/00 A61K 037/02 A61K 039/00 A61K 039/21
C07K 015/00 C07K 015/12 C07K 017/00 C12N 001/19
C12N 015/12 C12N 015/48 C12N 015/62 C12P 021/00
G01N 033/53

(21) Application No. : 81057/87 (22) Application Date : 28.10.87

(87) WIPO Number : WO88/03563

(30) Priority Data

(31) Number	(32) Date	(33) Country
8626148	01.11.86	GB UNITED KINGDOM
8708531	09.04.87	GB UNITED KINGDOM
036807	10.04.87	US UNITED STATES OF AMERICA

(43) Publication Date : 01.06.88

(44) Publication Date of Accepted Application : 21.02.91

(71) Applicant(s)
BRITISH BIO-TECHNOLOGY LIMITED

(72) Inventor(s)
ALAN JOHN KINGSMAN; ELIZABETH-JANE CLAIRE MELLOR; MICHAEL HENRY MALIM; SUSAN MARY KINGSMAN; SALLY ELIZABETH ADAMS

(74) Attorney or Agent
GRIFFITH HACK & CO, GPO Box 1285K, MELBOURNE VIC 3001

(57) Claim

1. A fusion protein capable of assembling into a particle, the fusion protein comprising a first amino acid sequence and a biologically active second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a retrotransposon or an RNA retrovirus and wherein, if the second amino acid sequence contains more than thirty amino acid residues, the first thirty residues of the second amino acid sequence do not form an amino acid sequence naturally directly fused to the first amino acid sequence by the said retrotransposon or RNA retrovirus.

12. Nucleic acid comprising a first nucleotide sequence and a second nucleotide sequence, wherein the first nucleotide sequence is substantially homologous with or complementary to genetic material in a retrotransposon or RNA

retrovirus encoding a particle-forming protein, and wherein the second nucleotide sequence encodes, or is complementary to a nucleotide sequence which encodes, another amino acid sequence, the nucleic acid being capable of being expressed in a single reading frame to form a fusion protein which is not naturally produced by the said retrotransposon or RNA retrovirus, and wherein, if the second nucleotide sequence contains more than ninety bases, the first ninety bases do not form a nucleotide sequence expressed as part of a fusion protein naturally produced by the retrotransposon or RNA retrovirus.

AU-AI-81057/87

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C12N 15/00, C12P 21/00, A61K 39/00, C07K 15/00, G01N 33/53

606896

(11) International Publication Number: WO 88/03563

(43) International Publication Date: 19 May 1988 (19.05.88)

(21) International Application Number: PCT/GB87/00764

(22) International Filing Date: 28 October 1987 (28.10.87)

(31) Priority Application Numbers: 8626148, 8708531, 036,807

(32) Priority Dates: 1 November 1986 (01.11.86), 9 April 1987 (09.04.87), 10 April 1987 (10.04.87)

(33) Priority Countries: GB, GB

SECTION 34(4)(a) DIRECTION SEE FOLIO 9

(71) NAME DIRECTED BRITISH BIO-TECHNOLOGY LIMITED Brook House, Watlington Road, Cowley Oxford OX4 5LY ENGLAND

(72) Inventors: KINGSMAN, Alan, John ; KINGSMAN, Susan, Mary ; Greystones, Middle Street, Islip, Oxon OX5 2SF (GB). ADAMS, Sally, Elizabeth ; 12 The Phelps, Grovelands, Kidlington, Oxon OX5 1SP (GB). MALIM, Michael, Henry ; Acers, Forge Lane, Upleadon, Glos. GL18 (GB). MELLOR, Elizabeth-Jane, Claire ; 41 Thackley End, 119 Banbury Road, Oxford OX2 6LB (GB).

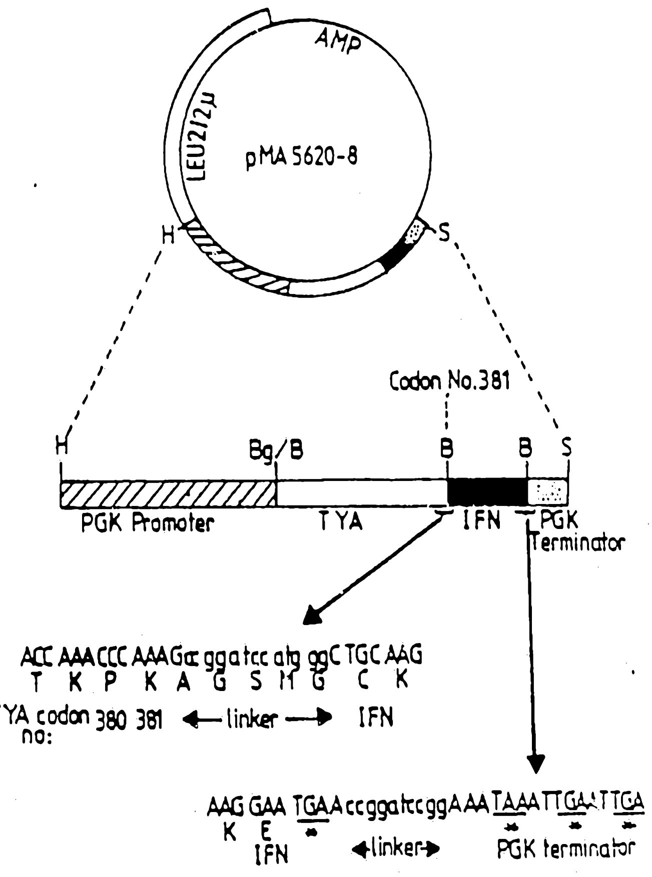
(74) Agent: SHEARD, A., G.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

(54) Title: FUSION PROTEINS AND PARTICLES

(57) Abstract

Fusion proteins comprise a first amino acid sequence and a second amino acid sequence. The first amino acid sequence is derived from a retrotransposon or an RNA retrovirus and confers on the fusion protein the ability to assemble into particles; an example is the product of the *TYA* gene of the yeast retrotransposon Ty. The second amino acid sequence is biologically active; for example it may be antigenic. So particles formed of the fusion proteins may be useful in vaccines or in diagnostic or purification applications.



FUSION PROTEINS AND PARTICLES

TECHNICAL FIELD

The present invention relates to an antigen presentation and purification system. In particular embodiments it relates to particles encoded by the yeast retrotransposon Ty, a vector containing the gene for particle formation, a vector for the high level expression of fusions of the particle protein and any antigen and a method for the production and purification of these fusion proteins in yeast.

BACKGROUND ART

One of the most important applications of the new recombinant DNA technology is in the production of safe vaccines against infectious diseases and the synthesis of defined proteins against which antisera can be raised for experimental, industrial and diagnostic purposes. Theoretically, these goals can be achieved by the synthesis of appropriate antigens in microorganisms such as the yeast Saccharomyces cerevisiae. The antigen would be expressed from an appropriate expression vector such as described in European Patent Application EPA2-0073635.

Correct presentation of the antigen to an animal or human immune system is a key requirement for an effective sub-unit vaccine or immunogen. Presentation has been a major problem with potential vaccines and immunogens made by recombinant DNA as well as for those based on chemically synthesized epitopes. An ideal immunogen is a polymer of multiple antigenic determinants assembled into a high molecular weight carrier. A good immunogen should also have the maximum number of epitopes exposed. These requirements can be difficult to achieve by random chemical coupling of antigens to a carrier. An ideal situation would be where the antigen was presented in the correct conformation on the surface of a large particulate complex. Furthermore the particulate nature of such a system would facilitate the purification of the antigen by simple physical means.

These requirements are rarely achieved by the simple synthesis of monomeric proteins by recombinant DNA technology or chemical synthesis.

Prior to the present invention, the only self-assembling, particulate antigen presentation system for production of immunogens in yeast was based on the fusion of antigens (e.g. the Herpes Simplex Virus I (HSV-I) glycoprotein D or a Poliovirus antigen) to the Hepatitis B surface antigen (HBsAg) protein via recombinant DNA technology (Valenzuela et al 1985 Biotechnology 3, 323). These fusion proteins aggregate to form 22 nm particles. This system has serious disadvantages: 1) yields are very low; 2) particles do not form in the yeast cell but are a by-product of the extraction process (Hitzeman et al 1983 Nucl. Acids Res. 11,27450. This imposes limitations on the production process; 3) some fusions do not form particles; 4) the HBsAg component exerts immunodominance in some cases., i.e. antibodies are made preferentially to HBsAg.

Kniskern et al in Gene 46 135 (1986) have reported that Hepatitis B core antigen (HBcAg) forms particles in yeast at very high levels, but there has been no published report of its use to carry other epitopes. As these particles are reported to be highly immunogenic in mice they may exert immunodominance like the HBsAg particles.

A corresponding system in bacteria has been reported by Haynes et al (Bio/Technology 4 637 1986). Tobacco mosaic virus (TMV) coat protein is the entity which assembles into a particulate structure. When the TMV coat protein is expressed in E. coli and then purified it is possible to assemble the proteins into polyvalent particles in vitro. Fusion proteins made by TMV coat protein and another protein may produce hybrid particles that are immunogenic. A polio epitope of only eight amino acids has been tested and shown to be immunogenic although it was four times less immunogenic than the Salk vaccine. This system has the potential advantages that the size of the particle may be varied experimentally and mixed particles (i.e. carrying more than one type of epitope) could possibly be easy to produce. However, an anticipated disadvantage is that only relatively small antigens will be able to be added to the TMV particle.

Mellor et. al. in Nature 313 243 (1985) disclose a fusion protein comprising the product of a yeast Ty open reading frame ORF1 (now designated the TYA gene), an open reading frame ORF2 (now designated the TYB gene) and nucleic acid coding for interferon. However, there is no disclosure or suggestion in this paper that the fusion proteins produced, or indeed any Ty protein, are or might be capable of assembling into particles.

DISCLOSURE OF THE INVENTION

It has now been discovered that certain proteins expressed by the genetic material of retrotransposons have the ability of self-assembling into particles. It has also been discovered that it is possible to construct fusion proteins, based on such retrotransposon-derived assembling proteins, which can assemble into antigen- (or other biologically active molecule-) presenting particles. A further discovery that has been made is that similar fusion proteins can be constructed based on assembling proteins from RNA retroviruses.

According to a first aspect, the present invention provides a fusion protein capable of assembling into a particle, the fusion protein comprising a first amino acid sequence and a biologically active second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a retrotransposon or an RNA retrovirus and wherein, if the second amino acid sequence contains more than thirty amino acid residues, the first thirty residues of the second amino acid sequence do not form an amino acid sequence naturally directly fused to the first amino acid sequence by the said retrotransposon or RNA retrovirus.

According to a second aspect, the invention provides a particle comprising a plurality of fusion proteins, each fusion protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a retrotransposon or an RNA retrovirus and wherein the second amino acid sequence is biologically active and not naturally fused to the first amino acid sequence by the said retrotransposon or RNA retrovirus.

Such particles will generally be substantially pure, by which is meant at least 5%, 10%, 20%, 50% 80%, 90%, 95% or 99% by weight pure, in increasing order of preference.

A given particle may be composed of a plurality of different fusion proteins; that is to say fusion proteins having different second amino acid sequences from each other. Two, three or even more different second amino acid sequences may be present in a particle.

The first amino acid sequence may be the product of the yeast Ty TYA gene, the product of copia and copia-like elements from insects or the gag gene of RNA retroviruses.

Retroviruses include Human Immunodeficiency Virus I and II (HIV-I, HIV-II), SIV, Human T-cell Lymphotrophic Virus I and II (HTLV-I, HTLV-II), Murine Leukaemia Virus, Moloney Murine Leukaemia Virus, Mouse Mammary Tumour Virus, Avian Leukosis Virus, Feline Leukaemia Virus, Human B-cell Lymphotrophic Virus, and Bovine Leukaemia Virus. Retrotransposons, as indicated above, include the Ty element of yeast, the and copia-like copia elements of insects such as Drosophilia melanogaster, VL30 in mice and IAP genes in mice.

Preferred retrotransposons include the yeast retrotransposon Ty. It has previously been shown that Ty directs the synthesis of 60nm virus-like particles (Ty-VLPs) (Mellor et al 1985a Nature 318, 513). It has now been discovered that the pl protein, encoded by the TYA gene is stable and does not appear to require further processing. Therefore the Ty-encoded amino acid sequence is preferably the pl protein encoded by the TYA gene. It is known (Fulton et al NAR 13(11) 1985 4097) that both classes (I and II) of Ty make pl; so either class may be used.

The Ty-encoded amino acid sequence need not be the whole of the pl protein; instead it may be a part of the pl protein encoded by a part of the TYA gene, which part is capable of directing the synthesis of Ty virus-like particles (Ty-VLPs). It has been determined that a portion of the amino acid sequence 286 to 381 (Figure 16) is needed for particle formation. Preferably the Ty-encoded amino acid sequence is derivable from Ty1-15. The stop codon at the end of the TYA gene is preferably not included; if it is included, however, fusion protein may continue to be expressed, albeit at low yield, as it appears that the stop codon may be ignored with a frequency of about 1 in 20 times by the frame shifting mechanism described by Wilson et al (NAR 14(17) 1986 7001).

It is therefore now shown, among other things, that Ty protein p1, the product of the TYA gene (Dobson et. al. 1984 EMBO.J 3, 1115) and portions thereof containing at least a fraction of the 286 to 381 amino acid sequence in Figure 16 is sufficient to produce Ty-VLPs.

The second amino acid sequence (i.e. in particular embodiments the non-Ty protein coding region) could be any amino acid sequence either known to the art or yet to be elucidated. The biological activity may be a single type of activity (for example antigenicity), receptor binding activity, therapeutic activity or targeting activity (i.e. the ability to home to a particular target) or it may have a combination of two or more different biological activities. Equally, different fusion proteins may assemble into a particulate antigen, thereby causing the whole particle to have more than one activity.

As an example the second amino acid sequence may be derived from a lymphokine gene such as an interferon gene, or a gene coding for an antigen of an infectious agent such as a virus, bacterium or other (e.g. protozoal) parasite. For example, it may be an antigen of influenza virus, an HIV-I or HIV-II (i.e. HTLV-III, LAV or AIDS) virus, a rabies virus, FMDV virus, HBsAg, HBcAg, polio virus, influenza virus, parainfluenza virus, respiratory syncytial virus, rhinovirus, coronavirus, adenovirus, Rotaviruses, Norwalkviruses, Arboviruses e.g. Dengue, Herpes simplex, Cytomegalovirus, Epstein Barr virus, Measles virus, Mumps virus, Rubella, Equine influenza virus, Equine rhinopneumonitis, Swine transmissible gastroenteritis virus, Distemper, Parovovirus, FeLV, Venezuelan or Western equine encephalitis, plasmodium trypanosome, Schistosomes, Chlamydia trachomatis or a meningococcus or it may be a fragment of the above antigens or a peptide resulting from a chemically synthesized coding sequence such that the resulting peptide has substantially the same antigenicity of the above antigens or fragments thereof.

The nucleotide sequence for a wide variety of viral and bacterial antigens are known such that the antigen can be prepared by recombinant DNA techniques. These DNA coding sequences can be used to code for the second amino acid sequence of the fusion protein of the present invention. For example, the nucleotide sequence for antigen related to common diseases such as whooping cough and malaria are known. Whooping Cough, Bordatella pertussis, WO 87/03301, June 4, 1987; Malaria Plasmodium vivax and P. falciparum, Dame, et al., Science, 225:593, 1984; Enea, et al., Science, 225:628, 1984; Young, et al., Science, 225:958, 1984. Other common diseses for which antigen nucleotide sequence are known as: Hepatitis, EPO 218,474 and EPO 020,251; Herpes, EPO 216,195 and Japan 61-97630; Rabies, WO 87/00179; Chickenpox, EPO 210,931; and Polio, WO 86/01828 and Japan 61-47585.

A first part of the second amino acid sequence may be a linker sequence, which may in some circumstances be readily cleavable. The remainder of the second amino acid sequence may thus be cleaved off in a purification step.

Particulate antigens in accordance with the invention are therefore useful in the preparation of vaccines, which form a further aspect of the invention. The second amino acid sequence may thus code for an amino acid sequence of an antigen of one of the above infectious agents. The vaccine comprises a particulate antigen and a physiologically acceptable non-toxic carrier, such as sterile physiological saline or sterile PBS. Sterility will generally be essential for parenterally administrable vaccines. One or more appropriate adjuvants may also be present. Examples of suitable adjuvants include muramyl dipeptide, aluminum hydroxide and saponin.

It should be noted that vaccines in accordance with the invention may present more than one antigen. Either a cocktail of different particulate antigens may be used, or a homogeneous population of particulate antigens having more than one epitope could be used (prepared, for example, by allowing a mixture of different hybrid proteins to aggregate into particles or by expressing more than one particulate antigen in the same cell); alternatively, a vaccine could contain a mixture of these sorts of particulate antigens.

In a further aspect, the invention provides nucleic acid comprising a first nucleotide sequence and a second nucleotide sequence, wherein the first nucleotide sequence is substantially homologous with or complementary to genetic material in a retrotransposon or RNA retrovirus encoding a particle-forming protein, and wherein the second nucleotide sequence encodes, or is complementary to a nucleotide sequence which encodes, another, biologically active, amino acid sequence, the nucleic acid being capable of being expressed in a single reading frame to form a fusion protein which is not naturally produced by the said retrotransposon or RNA retrovirus and which fusion protein assembles into a particle.

It will generally be the case that the nucleic acid will be capable of being expressed without splicing or antitermination events. There will generally be no frameshifting.

In certain embodiments of the invention, we provide a <u>TYA</u> gene derivative that can be fused to any non-Ty protein coding sequence to produce a <u>TYA</u> fusion gene. The <u>TYA</u> fusion gene produces a fusion protein that assembles into hybrid Ty-VLPs. These hybrid Ty-VLPs constitute a high molecular weight particulate antigen (or other particle) presentation system that can be produced in very high yields and that can be purified by simple physical procedures.

Further according to the present invention we provide an expression vector including nucleic acid as defined above. An example is pMA5620, which includes the TYA gene derivative, and which directs the high level production of hybrid Ty-VLPs in yeast.

Expression vectors in accordance with the invention will usually contain a promoter. PGK is a preferred promoter, but any other promoter may be used if necessary or desirable. Examples include GAPD, GAL1-10, PHO5, ADH1, CYC1, Ty delta sequence, PYK and hybrid promoters made from components from more than one of these promoters or any other promoter.

The invention also includes host cells for example, bacterial cells, such as *E. coli*, yeast cells such as Saccharomyces Cerevisiae, or animal cells such as Chinese Hamster Ovary cells (CHO) or COS cells containing the above expression vectors that direct production of hybrid particles.

Because of the polyvalent nature of the particulate antigens it is likely that it will be easier to produce antibodies than with conventional antigens and that those antibodies will have specific characteristics. The invention thus further provides antibodies raised against particles of the invention which are antigenic. The antibodies may be polyclonal (obtained for example by injecting antigens into a rabbit) or monoclonal antibodies, produced by hybridoma cells in accordance with the invention. Because of the polyvalent nature of the particulate antigens it is likely that *in vitro* immunization can be achieved more readily than with other forms of antigen; this may facilitate the production of human monoclonal antibodies. Hybridoma cells may be prepared by fusing spleen cells from an immunized animal with a tumor cell. Appropriately secreting hybridoma cells may thereafter be selected. (See Koehler & Milstein *Nature* 1976 296 495).

The invention also provides a suitable technique for purifying biologically active peptides. This aspect of the invention is based on the fact that it is generally relatively easy to separate particles from associated impurities, for example, by filtration or centrifugation. Therefore, there is also provided a method of producing a substantially pure biologically active peptide the method comprising separating particles as described above from associated impurities and subsequently cleaving biologically active peptide from the fusion proteins of the particles.

Fusion protein and particulate antigens of this invention are useful as diagnostic reagents. Particulate antigens for diagnostic purposes are particularly advantageous because they can be physically separated by centrifugation or filtration and can be directly dispersed on solid supports such as glass or plastic slides, dip sticks, macro or micro beads, test tubes, wells of microtiter plates and the like. The particulate antigens of this invention may also be dispersed in fibrous or bibulous materials such as absorbent disc (see U.S. Patent 4,632,901), strips or chromatography columns as the solid support. The particles and fusion proteins readily adhere to solid supports. The particles may after purification be disrupted into fusion proteins and the fusion protein may be dispersed on surfaces as indicated above. These reagents are useful for a variety of diagnostic tests. For example, a test sample suspected of having antibody to the particulate antigen and a fluorescent, enzyme or radio-labeled antibody is competitively reacted with the particulate antigen or fusion protein on a solid support and the amount of labeled antibody which binds to the particulate antigen on the solid support. Particulate antigens of this invention are also useful for agglutination reactions with antibodies. Those skilled in the diagnostic arts will recognize a wide variety of application of particulate antigen and fusion protein of this invention for diagnostic purposes.

The invention is now illustrated by the following Examples, with reference to the accompanying drawings, in which:

FIGURE 1 is a photograph of Ty virus-like particles (Ty-VLPs) purified from MD40-4c transformed with plasmid pMA91-11.

FIGURE 2 is a schematic diagram of the construction of pMA5620 and pMA5620-8.

FIGURE 3 is a schematic diagram of plasmid pMA5620-8, with an expanded diagram of the key components of this example of the invention and the nucleotide sequences of key junctions.

FIGURE 4 shows photographs of SDS-polyacrylamide gel analyses of fractions from a sucrose gradient separation of particles and proteins from MD40-4c containing pMA91-11, pMA91 and pMA5620-8.

FIGURE 5 is a photograph of hybrid Ty:IFN-VLPs purified from MD40-4c containing pMA5620-8.

FIGURE 6 shows photographs of Western blots of the sucrose gradient fractions from extracts of MD40-4c containing pMA5620-8. a) Shows the result using anti Ty-VLP antibody; b) shows the results using anti interferon antibody.

FIGURE 7 shows a photograph of Coomassie stained SDS polyacrylamide gel analyses of proteins in the purified hybrid Ty:IFN-VLP preparation (1) and total extracts of MD40-4c transformed with pMA5620-8 (2) and untransformed MD40-4c (3).

FIGURE 8 shows photographs of Western blots using anti Ty-VLP antibody (b), anti hybrid Ty:IFN-VLP antibody (d) and the corresponding 'prebleed' sera (a + c). Each is used against total extracts of MD40-4c containing pMA91 (1) and pMA91-1 (2).

Plasmid pMA91-1 directs the synthesis of IFN-alpha2. pMA91 is a negative control, producing no interferon.

FIGURE 9 shows the nucleotide sequence of the 262 bp SpeI:SpeI fragment containing HA codons 25-111, referred to in Example 5.

FIGURE 10 shows a diagram of plasmid pMA5620-ha23.

FIGURE 11 shows an SDS-PAGE analysis of sucrose gradient fractions of extracts of MD40-4c transformed with pMA5620 or pMA5620-ha23. Proteins are stained with Coomassie blue.

FIGURE 12 shows an electron micrograph of purified Ty:HA-VLPs.

FIGURE 13 shows Western blots of proteins from sucrose gradient fractions of extracts of MD40-4c transformed with pMA5620-ha23. Blots were probed with anti - Ty-VLP antibody and anti-whole influenza virus antibody.

FIGURE 14 shows Western blots of purified Ty:HA-VLPs, whole influenza virus NT60 (H3) and whole influenza virus PR8 probed, from left to right, with normal 'pre-bled' rabbit serum, anti-Ty-VLP antiserum, normal 'pre-bled' rabbit serum, anti-Ty:HA-VLP antiserum and anti-whole influenza virus serum.

FIGURE 15 shows the strategy for preparing pMA5620-358 from pMA5620. pMA5620-358 contains the first 280 codons of TYA.

FIGURE 16 shows the DNA and amino acid sequence of TYA 1 through 381 amino acids which contains the 286 through 381 segment at least a portion of which is needed for particle formation. Symbols for amino acids are:

Amino Acid	One-letter Symbol
Alanine	A

Arginine	R
Asparagine	N
Aspartic acid	D
Asn and/or Asp	B
Cysteine	C
Glutamine	Q
Glutamic acid	E
Gln and/or Glu	Z
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

EXAMPLE 1

Strains used were E.coli AKEC28 (C600, thrC, thyA, trpC1117, hsdRK, hsdK) and S. cerevisiae MD40-4c (urd2, trp1, leu2-3, leu2-112, his3-11, his3-15). E. coli media were prepared according to Miller (Miller 1972 Experiments in Molecular Genetics, CSH p433) and yeast media were prepared according to Hawthorne and Mortimer (Hawthorne and Mortimer 1960 Genetics 45, 1085).

E. coli was transformed using standard methods (Maniatis et al. 1982 Molecular Cloning - A Laboratory Manual, CSH p199). Yeast was transformed as described by Hinnen et al. (Hinnen et al. 1978 Proc. Natl. Acad. Sci. 75, 1929).

Standard procedures were used for restriction digestion and plasmid constructions (Maniatis et al. 1982 op. cit.).

Restriction enzymes and T4 DNA ligase were used according to the suppliers' instructions. Bal 31 exonuclease digestions were carried out as described by Dobson et al. (Dobson et al. 1982 Nucl. Acids Res. 10, 5463). Deletion end points were determined by DNA sequencing (Sanger et al. 1977 Proc. Natl. Acad. Sci. 74, 5463). BamHI synthetic oligonucleotide linkers were obtained from Pharmacia.

Plasmid DNA was isolated from *E. coli* preparatively as described by Chinault and Carbon (Chinault and Carbon 1979 Gene 5, 111) and for rapid analysis by the method of Holmes and Quigley (Holmes and Quigley 1981 Anal. Biochem. 114, 193).

Ty-VLPs were purified as follows: Yeast cells were grown selectively at 30°C to a density of 8×10^6 cells.ml^{-1}. The cells were then collected by low speed centrifugation, washed once in ice-cold water and resuspended in TEN buffer (10mM Tris, pH 7.4; 2mM EDTA; 140mM Nacl) at 1ml per 1 litre of cells. The cells were disrupted by vortexing with glass beads (40-mesh;BDH) at 4°C until >70% were broken. The beads were pelleted by low speed centrifugation, then the supernatant was collected and the debris removed by centrifugation in a microfuge for 20 minutes. The Ty-VLPs were then pelleted from the supernatant by centrifugation at 100,000g for 1 hour at 4°C and by resuspended overnight in TEN buffer. The resuspended Ty-VLPs were centrifuged in a microfuge for 15 minutes at 4°C to remove cell debris prior to loading the supernatant onto a 15-45% (w/v) sucrose gradient in 10mM Tris, pH 7.4; 10nM NaCl and spinning at 76,300g for 3 hours at 15°C. Fractions were collected through the bottom of the tube and the peak fractions were identified by running aliquots of the fractions on SDS-PAGE gels and Coomassie blue staining. VLPs were concentrated by centrifugation of the peak fractions at 100,000g for 1 hour at 4°C.

Protein extracts of whole yeast cells were prepared as previously described (Mellor et al 1983 Gene 24, 1). Gel procedures were those of Laemmli (Laemmli 1970 nature 227, 68). Protein concentrations were measured by a dye-binding assay (Bradford 1976 Anal. Biochem. 72, 248) obtained from Bio-Rad Laboratories.

A polyclonal horse anti-interferon (IFN)-alpha antibody was purchased from Boehringer Mannheim. Anti-Ty-VLP antisera were prepared in rabbits by standard procedures.

Western blotting was carried out as described by Towbin et al. (Towbin et al. 1979 Proc. Natl. Acad. Sci. 76 4350). Bound antibody was detected using either a rabbit anti-horse second antibody conjugated to horseradish peroxidase (Miles Scientific) or a goat anti-rabbit second antibody followed by a peroxidase-anti-peroxidase (PAP) complex raised in rabbit (Sigma). The enzymatic reaction was developed as described by De Blas and Cherwinski (De Blas and Cherwinski 1983 Anal. Biochem. 133, 214).

Plasmid, pMA91-11 has been described previously (Dodson et al 1984 EMBO.J. 3, 1115): it contains the first 1450 nucleotides of the major transcriptional unit of the Ty element, Ty1-15, inserted into the high efficiency expression vector pMA91 (Mellor et al. 1983 op. cit; Kingsman and Kingsman 1985 Biotech. and Genet. Eng. Rev. 3, 377). The Ty component was derived from pKT40b as described by Dobson et al. (op. cit); pKT40b was deposited on 8 April 1987 in the National Collection of Industrial Bacteria, Aberdeen, U.K. under accession number NCIB 12427. In turn, the expression vector pMA91 consists of plasmid pBR322 sequences which allow replication and selection in *E. coli*, the yeast 2 micron plasmid origin of replication, which allows efficient autonomous replication in yeast, the yeast LEU2 gene as a selectable marker in both yeast leu2 and *E. coli* leuB mutants and a BgIII expression site which separates the upstream non-coding region of the yeast PGK gene from -1500 to -1 from the 3' region of PGK which contains all the signals for yeast -

transcription termination. Plasmid pMA91 is also described in USA-4615974, although it should be carefully noted that the plasmid designated as pMA3013 in Figure 15 of this US patent is what is now known as plasmid pMA91. the plasmid shown in the lower part of Figure 1 of USA-4615974 has since been renamed.

Ty expression is driven, therefore, from the promoter of the highly efficient yeast phosphoglycerate kinase gene (PGK) and yeast extracts of strains containing pMA91-11 overproduce massive amounts of pl protein, the primary translation product of the TYA gene (Dobson *et al.* 1984 op. cit; Mellor *et al.* 1985a op. cit). We now demonstrate that extracts of yeast transformants containing pMA91-11 contain Ty-VLPs in large quantities (Figure 1). Therefore, TYA alone contains sufficient information to make Ty-VLPs and the pl protein found in extracts of MD40-4C containing pMA91-11 is assembled into particles (Figures 1 and 4). This information provides the basis for the present invention.

The constructions of a plasmid vector, pMA5620, that would direct the synthesis of any hybrid Ty-VLP particle is shown schematically in Figure 2. This required the construction of a vector containing a convenient restriction endonuclease site within the TYA gene such than any coding sequence can be inserted into that site to create a TYA hybrid gene. However, it is essential that within such a hybrid there is sufficient TYA coding sequence to direct the synthesis of Ty-VLPs.

Plasmid pMA91-11 was cleaved with BglII, digested with Bal 31 exonuclease for various times and re-ligated in the presence of excess Bam HI linkers (CCGGATCCGG). The deletion end points of the resulting plasmids were determined by DNA sequencing. Plasmid pMA91-357 is a deletion derivative in which 265bp have been removed. This places the BamHI linker one nucleotide beyond codon 381 of TYA (Figure 3).

In order to provide both transcription termination sequences and translation stop codons in all three reading frames the deleted PGK 3' terminator sequences of pMA91-357 were replaced with a 287 bp BamHI-SalI DNA fragment isolated from plasmid pDT86. This DNA fragment is a modified 3' transcription terminator fragment from the yeast PGK gene which contains translation stop codons in all three reading frames downstream of the BamHI site (Figures 2 and 3). This terminator fragment starts with a BamHI linker (CCGGATCCGG) linked to the last sense codon of the PGK coding sequence and extends to the HindIII site 279 nucleotides beyond the PGK coding sequence (Hitzeman et al. 1982 Nucl. Acids Res. 10, 7791). In these constructions the HindIII site has been converted to a SalI site using a synthetic linker. The terminator fragment is not critical and any fragment containing termination codons in all three reading frames followed by yeast transcription terminator would suffice. The resulting plasmid, pMA5620, contains a unique BamHI site into which any suitable sequence can be inserted to produce a hybrid protein which will be assembled into hybrid Ty-VLPs.

In order to test pMA5620 an interferon-alpha2 (IFN) cDNA was used as a model antigen coding sequence. A 540bp BamHI IFN-alpha2 cDNA fragment, designated fragment 8 (Mellor et al. 1985b Gene 33, 215) was inserted into the unique BamHI site of plasmid pMA5620. The resulting plasmid is designated pMA5620-8 (Figures 2 and 3).

VLPs were prepared from yeast strain MD40-4c transformed with plasmid pMA5620-8 and fractions from a 15-45% sucrose gradient were run on an SDS-PAGE gel. The proteins in the gradient were visualized by Coomassie blue staining (Figure 4). The position in the gradient of the predicted 70 kd TYA-IFN fusion protein demonstrated conclusively the particulate nature of the protein. Electron microscopy of the fractions confirmed that particles were present (Figure 5). In order to establish that this 70 kd particulate protein was indeed a Ty(50kd):IFN(20kd) fusion protein fractions from sucrose gradients were again run on SDS-PAGE gels and the separated proteins were transferred to nitrocellulose filters. One filter was probed with a Ty-VLP antibody known to react with Ty proteins and another was probed with an anti-IFN-alpha antibody (Figure 6). In both cases the 70kd protein reacted strongly, indicating that this protein contained both Ty and IFN epitopes.

The efficiency of the method for purification of hybrid Ty:IFN-VLPs is illustrated in Figure 7. The 70 kd hybrid protein is by far the major species in the preparation.

In order to test the efficacy of these hybrid Ty:IFN-VLPs in eliciting an immune response to the model antigen, interferon, an antiserum was raised in rabbits against concentrated hybrid Ty:IFN-VLPs purified from yeast extracts transformed with pMA5620-8. An extract of MD40-4c transformed with plasmid pMA91-1 which over produces IFN-alpha2 (Mellow et al. 1985b op. cit.) was run on an SDS-PAGE gel along side an extract of MD40-4c transformed with pMA91 which produces no interferon. The separated proteins were transferred to nitrocellulose and probed with either Ty-VLP antisera or Ty:IFN-VLP antisera (Figure 8). Whereas both antisera reacted with Ty proteins present in the extracts only the anti hybrid Ty:IFN-VLP antisera reacted with the 20kd IFN protein in the extract containing plasmid pMA91-1.

These data show: 1) pMA5620-8 directs the synthesis of a hybrid fusion protein composed of the first 381 amino acids of Ty protein p1 and a non-Ty protein. In this case interferon-alpha2; 2) this fusion protein forms hybrid Ty:IFN-VLPs; 3) the hybrid Ty:IFN-VLPs can be isolated easily; 4) the hybrid Ty:IFN-VLPs present the model antigen, interferon-alpha2, to the rabbit immune system. It is reasonable, therefore, to expect that pMA5620 will direct the synthesis of any hybrid Ty-VLP and that any antigen present in such a particle will be presented to any mammalian immune system e.g. rat, hamster, dog, cat, cattle, sheep, pigs and human. Clearly the interferon cDNA used as an example here could be replaced with any other piece of DNA that would encode a complete protein or part of a protein.

The particulate nature of this antigen presentation system and the ease with which the hybrid Ty-VLPs can be purified make this invention useful as a means of stimulating the production of antibodies to defined antigens, as a means of creating new vaccines, as a means of preparing defined antigens for monoclonal antibody production, as a means of preparing antigens for use in diagnostic assay methods.

Hybrid Ty:IFN-VLPs can therefore be used to raise anti-IFN antibodies (either monoclonal or polyclonal, but preferably monoclonal), which may then be used for the purification of IFN.

EXAMPLE 2

The copia element of Drosophilia melanogaster is remarkably similar to the Ty element of yeast in both genetic organization and virus-like particle formation (Mount & Rubin 1985 Mol. Cell. Biol. 5 1630). The region of copia that is equivalent to TYA is called gag by analogy with retrovirus organization. Therefore expression at high levels of the gag region of a copia element in an insect cell expression system may be found to lead to proteins which assemble into particles similar to the Ty-VLPs. If the gag is fused to the coding sequence of another protein the resulting fusion protein may also be found to assemble into a hybrid copia particle analogous to the Ty:IFN hybrid particles described above.

In order to establish copia particles as an antigen presentation and purification system gag from plasmid pPW220 (Potter & Brosein 1979 Cell 17 415) is manipulated by standard recombinant DNA procedures for insertion into a typical high efficiency Baculovirus expression vector such as pAc373 (Smith et al. 1983 J. Virol. 46, 584) to produce plasmid pAc373-gag. Expression is driven by the polyhedrin promoter. An interferon cDNA is then inserted, in frame, into the 3′ end of gag to produce plasmid pAc373-gag-IFN. This plasmid is directly analogous to pMA5620-8 (see Example 1 above). pAc373-gag-IFN is then introduced into the Baculovirus, Autographa californica nuclear polyhedrosis virus (AcNPV) by in vivo recombination in Spodoptera frugiperda cells (Smith et al. 1983 Mol. Cell. Biol. 4 2156). Recombinant viruses are harvested from occlusion-minus plaques and used to reinfect fresh S. frugiperda cells. Hybrid copia:IFN particles are harvested at 72-96 hours post-infection and fractionated on a sucrose gradient to separate them from Baculovirus contamination. Particles are identified by electron microscopy and Western blotting as described for Ty-IFN hybrid particles (see Example 1 above).

EXAMPLE 3

The general procedure of Example 2 is followed, except that a recombinant plasmid containing the gag-IFN fusion expressed by the Drosophila ADH gene promoter (Benyajati et al. 1983 Cell 33 125) is introduced into any insect cell (e.g. Drosophila Schneider 2 cells) by simple DNA mediated transfection.

EXAMPLE 4

The TYA gene of the yeast Ty element is directly analogous to the gag genes of avian and mammalian retroviruses. As the product of TYA alone is capable of forming particles it is likely that any gag protein will do the same and could therefore act as the basis for an antigen presentation and purification system.

In order to establish retroviral gag proteins as antigen purification and presentation systems the gag gene from HIV-I is manipulated by standard recombinant DNA procedures to be inserted into the yeast expression vector pMA91 and a derivative of the mammalian expression vector pSV2 (Southern and Berg 1982 J. Mol. Appl. Genet. 1 327), which contains a mammalian dhfr cDNA (Kaufman et al. 1985 Mol. Cell. Biol. 5

1750), to produce pMA91-HIVgag and pSV25-HIVgag respectively. An interferon cDNA is then inserted, in frame, into both of these molecules at the 3 end of gag to produce pMA91-HIVgag-IFN and pSV25-HIVgag-IFN respectively. These molecules are analogous to pMA5620-8 (see Example 1 above). pMA91-HIVgag-IFN is then used to transform yeast strain MD40-4c and pSV25-HIVgag-IFN is used to transfect CHO-dhfr- cells. Stable transformants are propagated and cell extracts prepared and fractionated on sucrose gradients. Hybrid HIVgag:IFN particles are identified by electron microscopy and Western blotting. As described for Ty:IFN hybrid particles (see Example 1 above).

EXAMPLE 5

The procedure of Example 4 is followed, except that pSV25-HIVgag-IFN is introduced into COS cells (Gluzman et al. 1977 J. Virol. 24 534) and hybrid particles are harvested between 3 and 9 days post transfection.

EXAMPLE 6

In this example we demonstrate that hybrid Ty-VLPs containing part of an influenza virus hemagglutinin (HA) can be produced and that these hybrid Ty:HA-VLPs induce the formation of anti-HA antibodies in rabbits.

The strategy for these experiments was essentially the same as for the production and analyses of the hybrid Ty:IFN-VLPs described in Example 1. MRC12/80 anti-whole influenza virus antibody was obtained from the World Influenza Centre at NIMR, Mill Hill, U.K. and was raised against influenza virus strain A/Scotland/840/74 x A/PR/8/34 (H3N2). A region of the HA coding sequence corresponding to codons 25-111 of the HA1 domain of influenza virus A/Memphis/102/72 (H3N2) was chosen for the formation of hybrid Ty:HA-VLPs on the basis of the data of Wilson et al. (1984 Cell 37, 767). A 262 bp SpeI:SpeI (Figure 9), corresponding to codons 25-111, was purified from an appropriate digest of plasmid MX29. MX29 is plasmid pAT153 (Twigg and Sherratt, 1980 Nature 283, 216) with an H3 HA cDNA derived from the HA RNA from influenza virus strain A/Memphis/102/72 G:C tailed into the PstI site. The sticky-ends of the SpeI:SpeI fragment were filled-in using DNA polymerase I and then the fragment was blunt-end ligated into the HincII site of plasmid pSP46 to produce pSP46-ha23. pSP46 is a derivative of pSP64 (Promega Biotec) in which the HindIII site of pSP64 has been converted into a BglII site. A BamHI:BglII fragment, designated ha23, was purified from pSP46-ha23. ha23 contains the filled-in 262 bp SpeI:SpeI fragment. ha23 was then inserted into the BamHI site of pMA5620 to produce pMA5620-ha23 (Figure 10). This plasmid was then used to transform yeast strain MD40-4C to leucine independence.

VLPs were prepared from yeast strain MD40-4c transformed with pMA5620-ha23 or pMA5620 and fractionated on a 15-45% sucrose gradient. Fractions were run on an SDS-PAGE gel and proteins were visualized with Coomassie blue (Figure 11). The position in the gradient of the predicted TYA-HA fusion protein demonstrated the particulate nature of the protein. Electron microscopy of the fractions confirmed that particles were present (Figure 12).

In order to confirm that the particles contained HA antigens fractionated extracts of MD40-4c containing pMA5620-ha23 from similar gradients were again run on SDS-PAGE gels and then separated proteins were electroblotted to nitrocellulose filters. One filter was probed with anti-Ty-VLP antibody known to react with Ty proteins and another was probed with anti-HA antibody. In both cases the putative Ty:HA fusion protein reacted strongly indicating that this protein contained both Ty and HA epitopes (Figure 13).

In order to test the efficacy of these hybrid Ty:HA-VLPs in eliciting an immune response to HA an antiserum was raised in rabbits against concentrated hybrid Ty-HA-VLPs purified from extracts of MD40-4c transformed with pMA5620-ha23. This antiserum was then used to probe proteins from disrupted whole influenza virus by Western blotting. Three protein samples were used. The first was purified Ty:HA-VLPs used to raise the antiserum. The second was whole influenza virus PR8 (H3). The third was whole influenza virus NT60 (H1). Both PR8 and NT60 were obtained from Professor George Brownlee, Sir William Dunn School of Pathology, Oxford, U.K. Figure 14 shows that the anti-Ty:HA-VLP antibody reacts with the HA1 region of the H3 HA but not with the H1 HA. No such reaction is seen with pre-bled serum nor with anti-Ty-VLP antibody. As a positive control the same samples were probed with anti-whole virus antibody. In this case both H1 HA and H3 HA reacted with the antiserum. The hybrid Ty:HA-VLPs induce the production of anti-HA antibody specific for an epitope of H3 antigen.

These data show that pMA5620-ha23 directs the synthesis of a hybrid fusion protein composed of the first 381 amino acids of Ty protein p1 and a small region of a viral protein, influenza virus HA. This fusion protein forms hybrid Ty:HA-VLPs. The hybrid Ty:HA-VLPs can be isolated easily and present the small HA component to the rabbit immune system in such a way that anti-HA antibodies are produced. These antibodies can distinguish between H1 HA and H3 HA in a Western blot. Clearly the HA component of the hybrid Ty:HA-VLPs could be replaced with any other influenza virus component or any other virus component and it is reasonable to expect that similar results would be obtained.

EXAMPLE 7

In order to create a series of truncated TYA genes that contained progressively less sequence at the 3′ end, a standard Bal 31 digestion was carried out using pMA5620 as the starting molecule. Plasmid pMA 5620 as cleaved with BamHI, digested with Bal 31 for various times and religated in the presence of excess BamHI linkers (CCGGATCCGG). the deletion end points of the resulting plasmids were determined by DNA sequencing. One of these deletions contains only the first 286 codons of TYA and is designated 358. The deleted PGK terminator sequences of 358 were replaced by ligating the large PvuII-BamHI fragment from pMA5620 with the 680 bp PvuII-BamHI fragment from 358. The resulting plasmid is designated pMA5620-358 (Figure 15).

Interferon alpha-2 cDNA BamHI fragments with two slightly different 3' termini (IFN-8 and IFN-2; Mellor et. al. 1985 Gene 33, 215; Mellor et. al. 1985 Nature 313, 243) were inserted, in frame, into the BamHI sites for pMA5620 and pMA5620-358 to produce plasmids pMA5620-8 and pMA5620-358-2.

Plasmids pMA91-11, pMA5620 and pMA5620-358 were used to transform yeast strain MD40-4c to leucine independence. The resulting transformants were compared with respect to TYA RNA produced from the plasmids and with respect to the amounts of p1 or truncated p1 proteins, as determined by Western blotting using anti Ty-VLP antibody. The amounts of RNA and protein were the same in each transformant.

Extracts from each transformant were then analyzed by fractionation on 15-45% sucrose gradients follows by Coomassie blue staining for both pMA91-11 and pMA5620 there are p1 or truncated p1 proteins in the region of the gradient that is characteristic of particulate structures. However, in the case of pMA5620-358 no particulate material is observed.

An interferon alpha-2 cDNA was fused, in frame, to each of the plasmids pMA5620 and pMA5620-358 to produce plasmids pMA5620-8 and pMA5620-358-2. These plasmids were used to transform yeast strain MD40-4c to leucine independent and Ty homologous RNA and p1 and p1:interferon fusion proteins were measured as described above. The levels of RNA and protein are the same for both transformants and also the same as pMA5620. When extracts of yeast transformants containing pMA5620-8 were analyzed on 15-45% sucrose gradients a 70 kD p1:interferon fusion protein was seen in the region of the gradient characteristic of particulate structures. However, no particulate material was seen when extracts containing pMA5620-358-2 were analyzed.

Thus Ty-VLP formation requires at least a portion of the sequence between amino acids 286 and 381. The DNA and amino acid sequence of pl is shown in (Figure 16) which also shows the DNA and amino acid sequence of amino acids 286 to 381.

EXAMPLE 8

ENZYME IMMUNOASSAY PROCEDURE WITH VLP ANTIGEN

96-well microtitre plates are coated with VLP antigen (virus like particle with antigen) by incubating 50μl of 20μg/ml of VLPs in 50mM sodium carbonate buffer, pH 9.5, in each well for two hours at room temperature. Excess VLPs antigen are washed out of the wells by three, five minute washes with phosphate buffered saline (PBS), pH 7.4. In order to minimize background reactions, the wells are blocked with 100μl of 2% casein in PBS for one hour at room temperature, followed by three, five minute washes with PBS containing 0.1% Tween-20 (PBS-T). Primary antibody in a test sample is suitably diluted in PBS-T containing 0.5% casein (PBS-CT). A suitable dilution may be a three-fold dilution series from 1/10 to 1/7,290. Primary antibody reactive to the non-Ty component of any hybrid Ty-VLPs. 50μl of diluted primary antibody is added to the appropriate wells and incubated for two hours at room temperature. Excess primary antibody is removed by three, five minute washes with PBS-T. Secondary antibody is horseradish peroxidase-labeled anti-species IgG, and is diluted 1/1,500 in PBS-CT. 50μl of diluted secondary antibody is added to each well and incubated for two hours at room temperature, followed by five, five minute washes with PBS-T. The substrate is 3,3',5,5'-tetramethylbenzidine at a concentration of 0.1mg/ml in 0.1M sodium acetate, adjusted to pH6.0 with 0.5M citric acid, plus 0.03% hydrogen peroxide. 50μl of substrate is added to each well and the color reaction developed for 10 minutes. The reaction is terminated by the addition of 25μl of 0.5M sulfuric acid to each well. Color development is assessed by measurement at 450nm using a microplate reader. In this way a direct assay of the primary antibody in the test sample is performed.

EXAMPLE 9

CLEAVAGE OF EXOGENOUS PROTEIN FROM HYBRID TY-VLPS

In situations where hybrid Ty-VLPs are used to purify pl fusion proteins it is advantageous to remove the pl component of the fusion protein to leave the exogenous or non-pl component. This requires cleavage at the junction of the pl and non-pl sequence. This provide a means of using Ty-VLPs to produce authentic proteins. One way of achieving this end is to introduce a specific proteolytic cleavage site at the pl/non-pl junction.

In order to produce non-fusion interferon from Ty-VLPs a derivative of pMA5020, designated pMA5623, was constructed. This is identical to pMA5620 except that at codon 381 of TYA four additional codons are added before the BamHI linker. These codons encode the amino acid sequence Ile-Glu-Gly-Arg which is a cleavage site for the blood coagulation factor Xa (Nagai & Thogersen, 1984, Nature, 309, 810). The junction region for a construction analogous to pMA5620-8, pMA5623-8, is as follows:

```
CCC AAA ATC GAG GGT AGG gga tcc atg ggC TGC AAG
 P   R   I   B   G   R   G   S   M   G   C   K
380 381     Factor Xa                    ' IFN
 TYA                   -BamHI
```

When pMA5623-8 is transformed into yeast strain MD40-4c a pl:IFN fusion protein is produced that assembles into hybrid IFN:Ty VLPs. However, in contrast to transformants containing pMA5620-8, incubation of the particulate fusion protein with Bovine Blood faction Xa cleaves the interferon from the particles because of the presence of the cleavage site at the protein fusion junction.

Hybrid IFN-Ty-VLPs are therefore purified from non-particulate proteins by a first round of sucrose density gradient fractionation, as described previously. The IFN is then cleaved off and purified from the particulate p1 protein by a second round of sucrose density gradient fractionation to yield pure "authentic" interferon.

The interferon coding sequence illustrated in this example can be replaced by any other coding sequence and so this strategy can be applied to the expression and purification of any protein.

What is claimed is:

1. A fusion protein capable of assembling into a particle, the fusion protein comprising a first amino acid sequence and a biologically active second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a retrotransposon or an RNA retrovirus and wherein, if the second amino acid sequence contains more than thirty amino acid residues, the first thirty residues of the second amino acid sequence do not form an amino acid sequence naturally directly fused to the first amino acid sequence by the said retrotransposon or RNA retrovirus.

2. A fusion protein as claimed in claim 1, which is substantially pure.

3. A fusion protein as claimed in claim 2, wherein the retrotransposon is the yeast retrotransposon Ty or a copia-like element.

4. A fusion protein as claimed in claim 3, wherein the Ty-encoded amino acid sequence is the pl protein encoded by the TYA gene.

5. A fusion protein as claimed in claim 3 wherein the Ty-encoded amino acid sequence is part of the pl protein encoded by a part of the TYA gene, which part is capable of directing the synthesis of Ty virus-like particles (Ty-VLPs).

6. A fusion protein as claimed in claim 2, wherein the Ty-encoded amino acid sequence is derivable from Tyl-15.

7. A particle comprising a plurality of fusion proteins, each fusion protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence is substantially homologous with a particle-forming protein encoded by a retrotransposon or an RNA retrovirus and wherein the second amino acid sequence is biologically active and not naturally fused to the first amino acid sequence by the said retrotransposon or RNA retrovirus.

8. A particle as claimed in claim 7, which is substantially pure.

9. A particle comprising a plurality of fused proteins as claimed in claim 1.

10. A particle as claimed in claim 7, wherein the second amino acid sequences in the fusion proteins are not all the same as each other.

11. A vaccine comprising a particle as claimed in claim 8, wherein the particle is a particulate antigen.

12. Nucleic acid comprising a first nucleotide sequence and a second nucleotide sequence, wherein the first nucleotide sequence is substantially homologous with or complementary to genetic material in a retrotransposon or RNA retrovirus encoding a particle-forming protein, and wherein the second nucleotide sequence encodes, or is complementary to a nucleotide sequence which encodes, another amino acid sequence, the nucleic acid being capable of being expressed in a single reading frame to form a fusion protein which is not naturally produced by the said retrotransposon or RNA retrovirus, and wherein, if the second nucleotide sequence contains more than ninety bases, the first ninety bases do not form a nucleotide sequence expressed as part of a fusion protein naturally produced by the retrotransposon or RNA retrovirus.

13. Nucleic acid coding for a fusion protein as claimed in claim 1.

14. Nucleic acid as claimed in claim 12, wherein the second sequence contains no linker sequence.

15. Nucleic acid as claimed in claim 12, wherein the second sequence includes linker sequence which is adjacent to the first sequence.

16. An expression vector comprising nucleic acid as claimed in claim 12.

17. An expression vector as claimed in claim 16, which an expression vector selected from the class consisting of bacterial, yeast, mammalian and insect expression vectors.

18. An expression vector as claimed in claim 17 containing a derivative of the <u>TYA</u> gene with a restriction enzyme cleavage site, within the <u>TYA</u> gene, in which is

inserted the second nucleotide sequence.

19. An expression vector as claimed in claim 17, which contains the first 381 codons of the TYA gene.

20. A cell selected from the class consisting of bacterial, yeast, mammalian and insect cells including an expression vector as claimed in claim 16.

21. An antibody raised against a particle as claimed in claim 7.

22. An antibody as claimed in claim 21 which is a monoclonal antibody.

23. An antibody as claimed in claim 22 which is a human antibody.

24. A method of producing a substantially pure biologically active peptide, the method comprising separating particles as claimed in claim 7 from associated impurities and subsequently cleaving biologically active peptide from the fusion proteins of the particles.

25. A diagnostic reagent comprising a particle or fusion protein of claim 7 dispersed on a solid support which particle or fusion protein is immunologically reactive to an antibody produced in response to infectious agents in mammals.

26. An intermediate in a diagnostic assay comprising the particle or fusion protein of claim 7 immunologically bound to an antibody from mammalian body fluid.

27. The fusion protein of claim 1 wherein the second amino acid sequence is immunologically reactive to an antibody produced in response to an infectious agent in mammals.

FIG.1

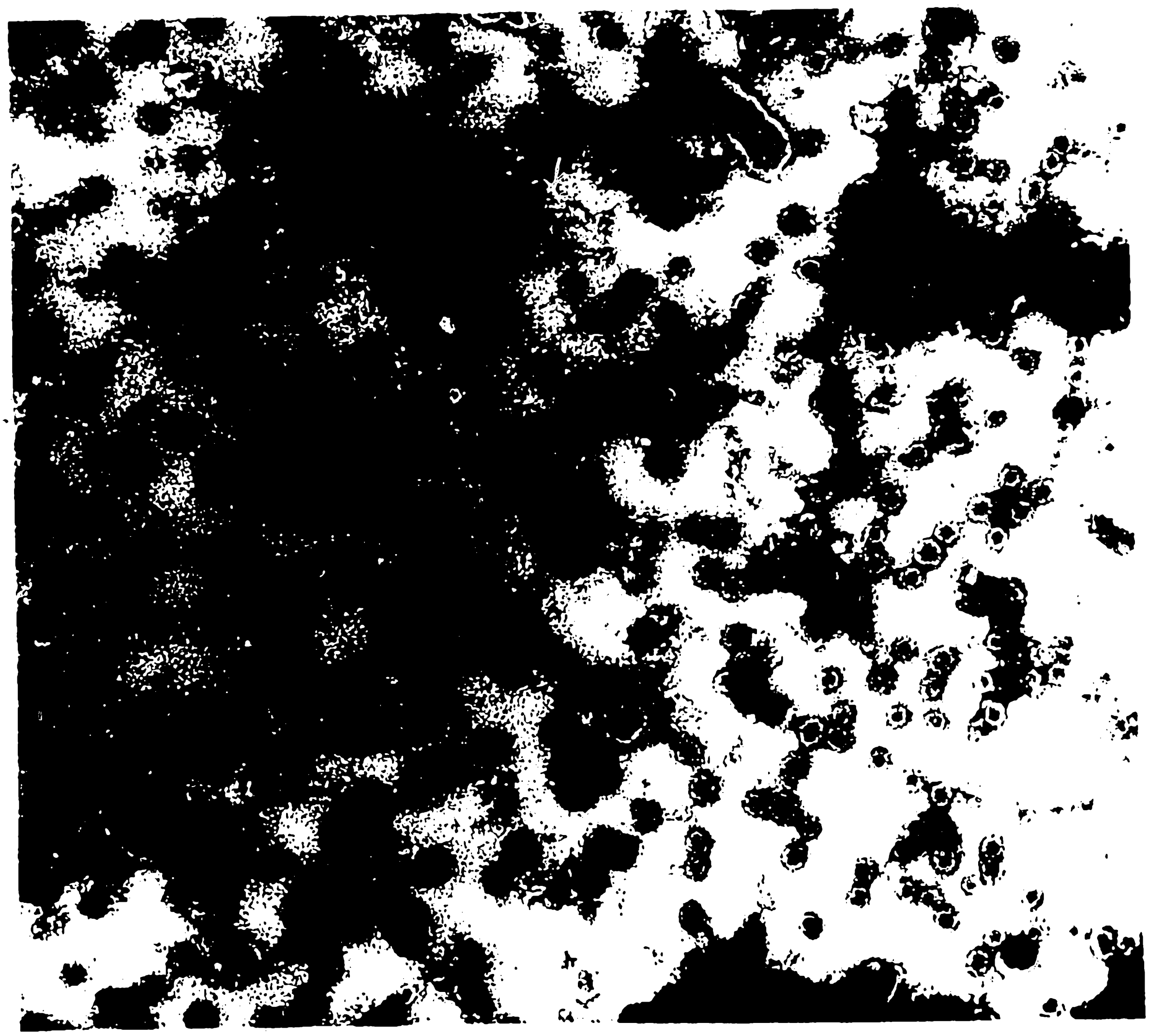


FIG.2

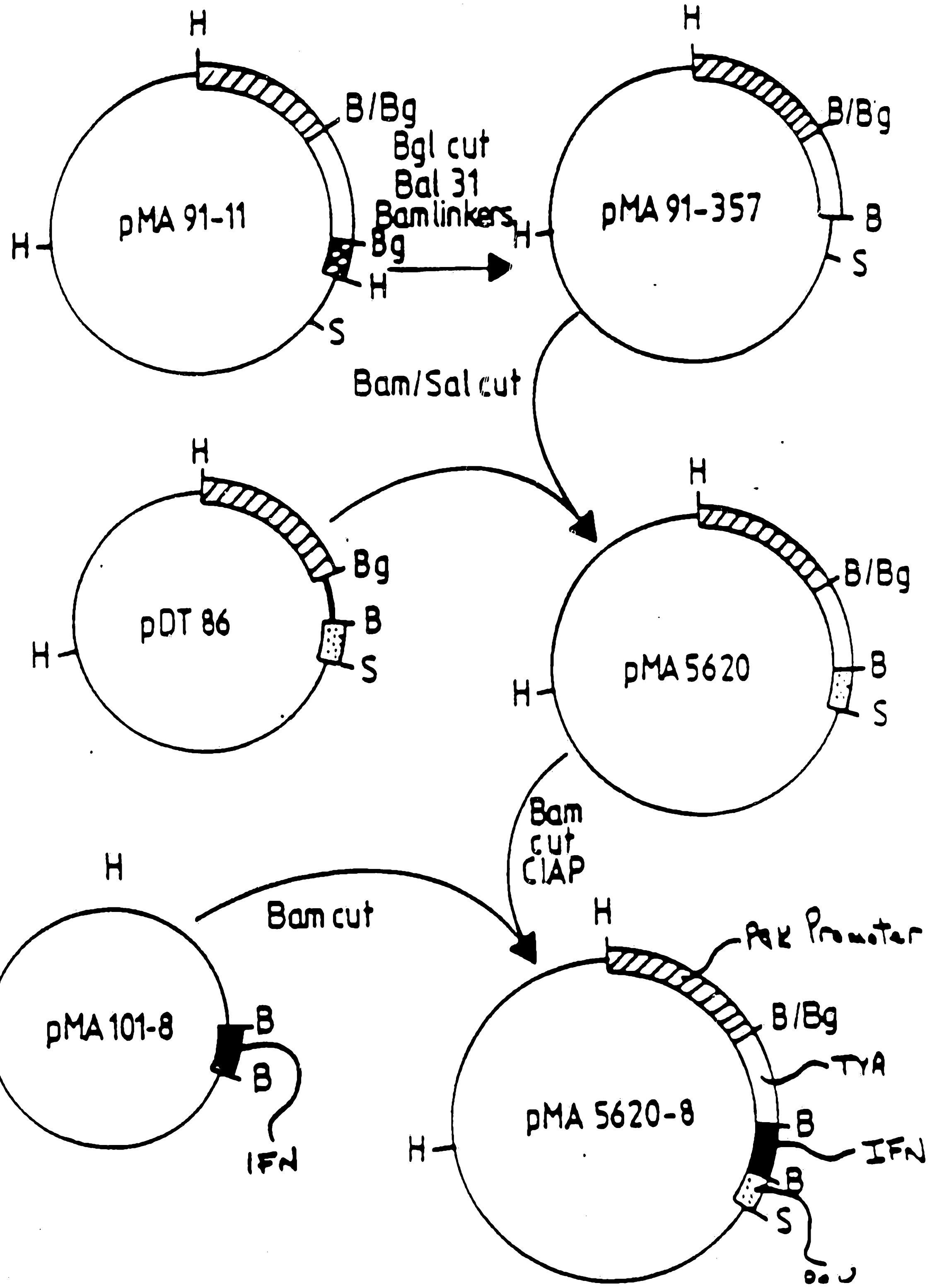
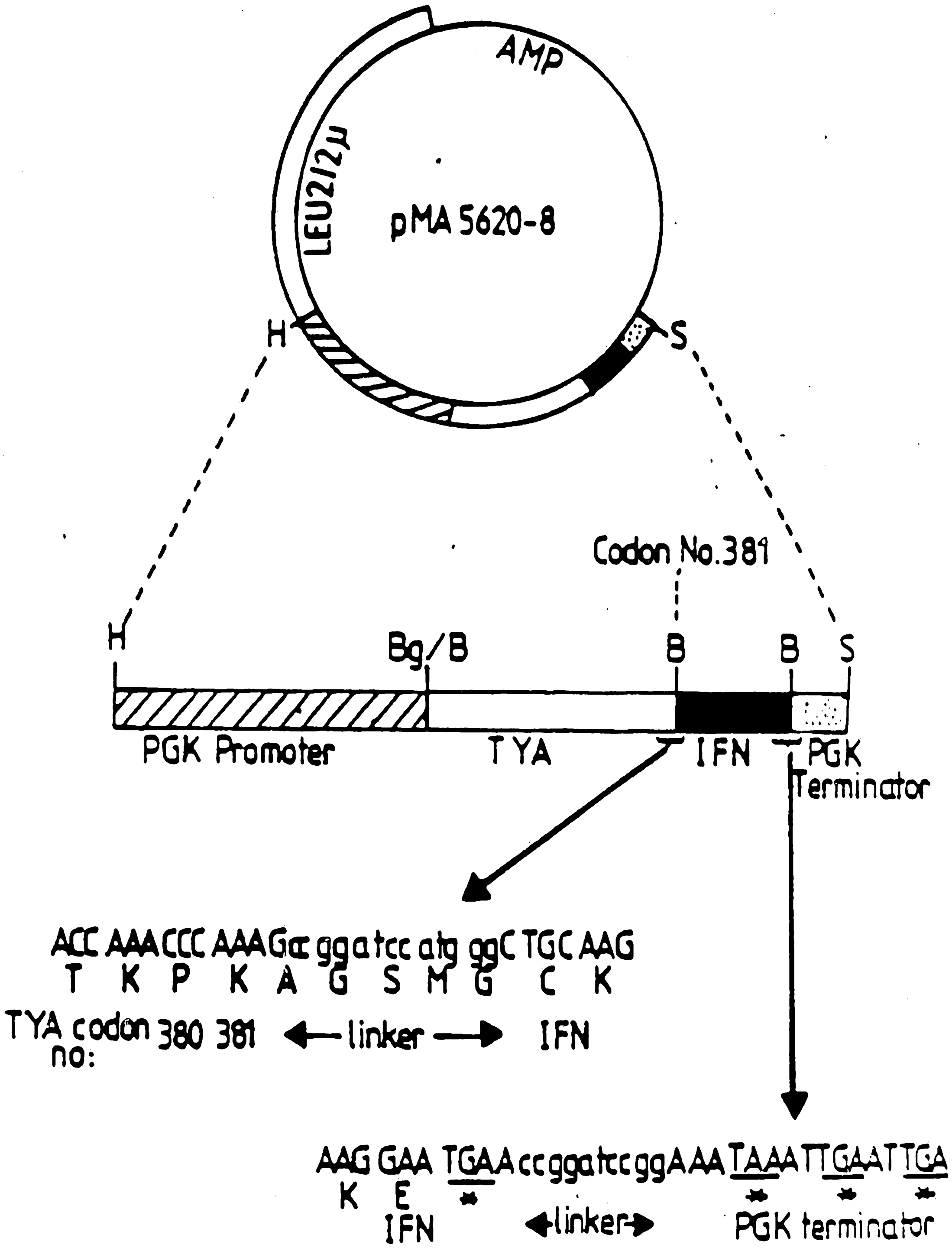

H
B/Bg
Bgl cut
Bal 31
Bamlinkers
pMA 91-11
H
Bg
H
S
H
B/Bg
pMA 91-357
H
B
S
Bam/Sal cut
H
Bg
pDT 86
H
B
S
H
B/Bg
pMA 5620
H
B
S
Bam
cut
CIAP
H
Bam cut
pMA 101-8
B
B
IFN
H
PGK Promoter
B/Bg
TYA
pMA 5620-8
H
B
IFN
B
S

FIG.3


AMP
LEU2/2μ
pMA 5620-8
H
S
Codon No.381
H
Bg/B
B
B
S
PGK Promoter
TYA
IFN
PGK Terminator
ACC AAA CCC AAA Gcc gga tcc atg ggC TGC AAG
T K P K A G S M G C K
TYA codon no: 380 381
linker
IFN
AAG GAA TGAA ccggatccggAAA TAAATTGAATTGA
K E
IFN
linker
PGK terminator

pMA 91-11

Bottom -Gradient fractions - Top

97K

68K

pI →

43K

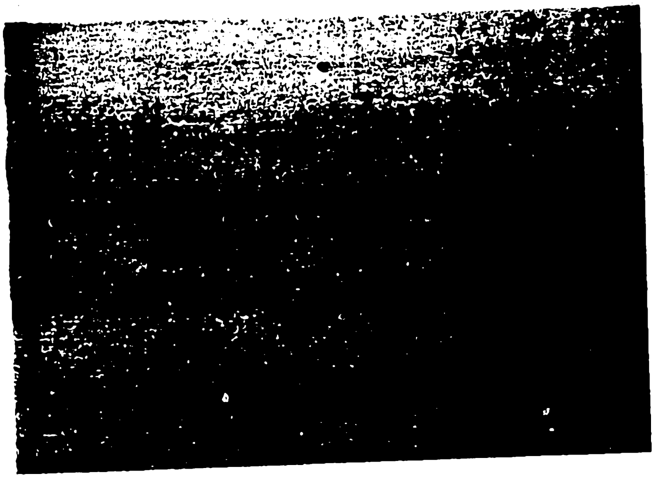


FIG.4a

pMA 91

Bottom - Gradient fractions - Top

97K

68K

43K

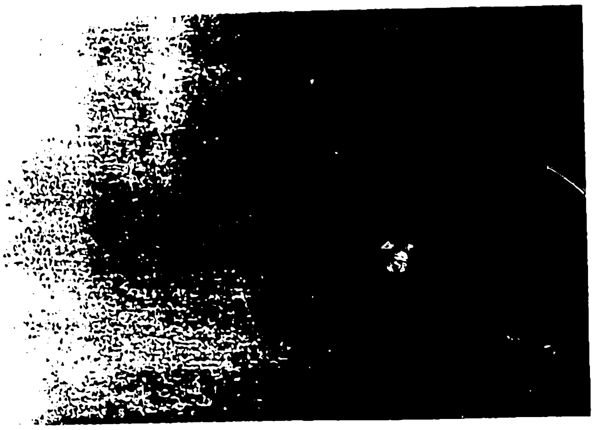


FIG.4b

pMA 5620-8

Bottom - Gradient fractions - Top

97 K

pI-IFN → 68 K

43K

FIG.4c

FIG.5

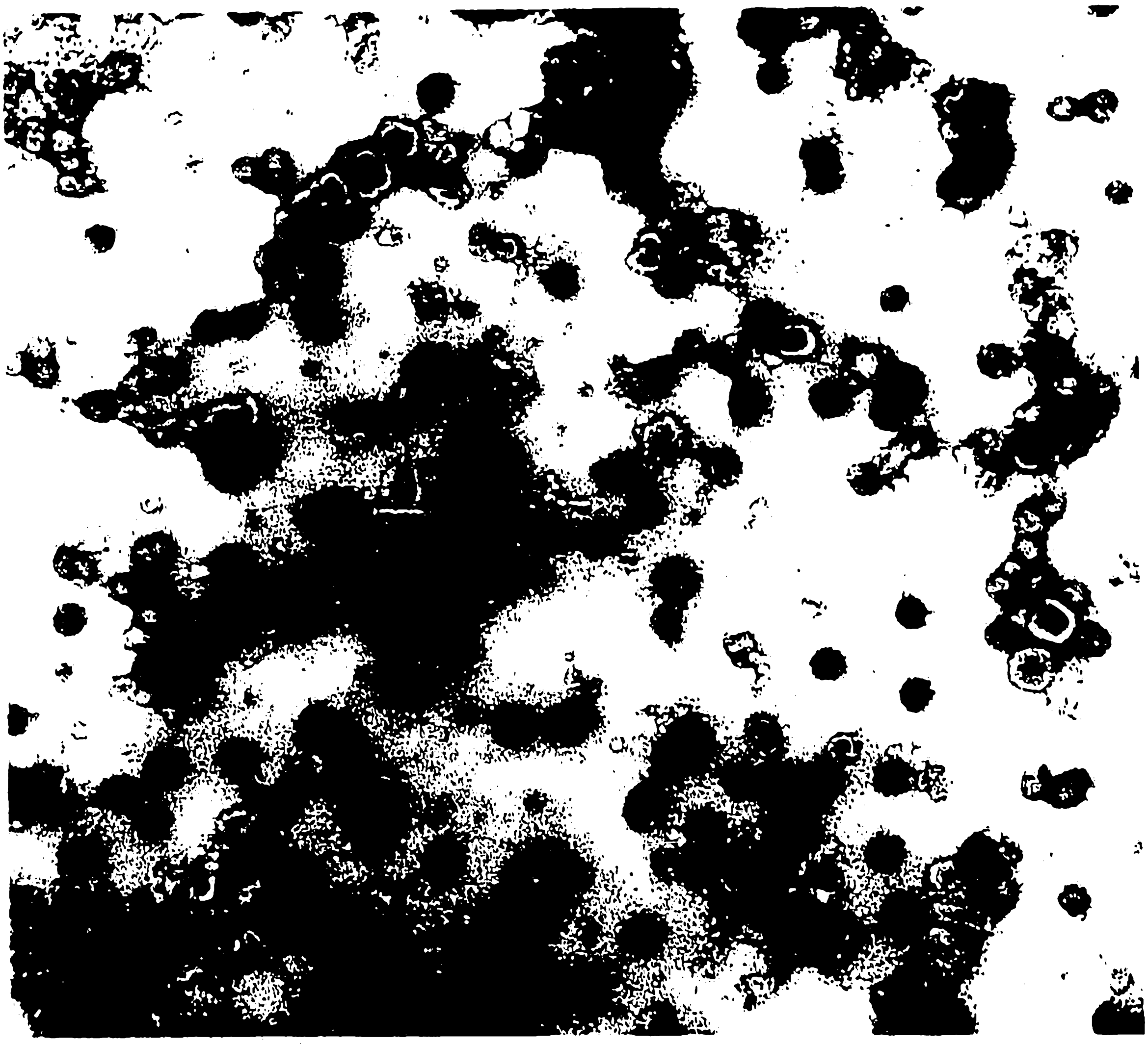


FIG.6a

Ty-VLP antibody

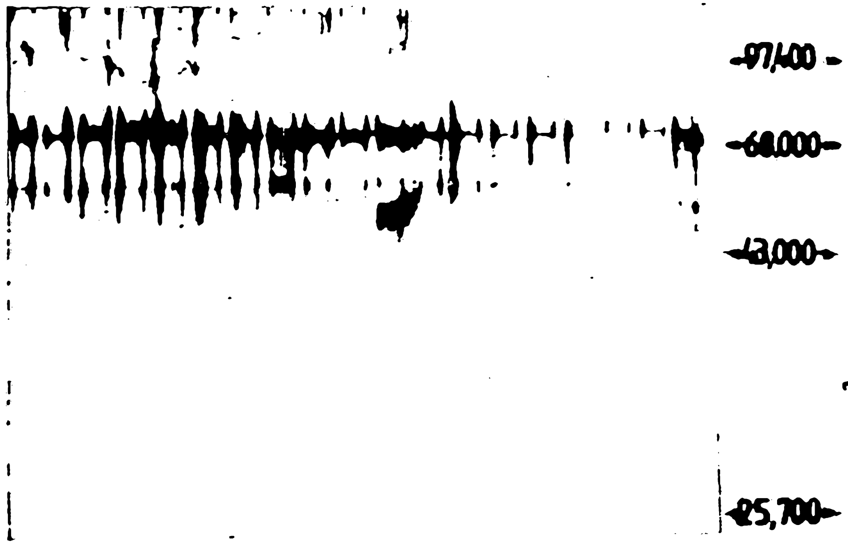


FIG.6b

IFN antibody

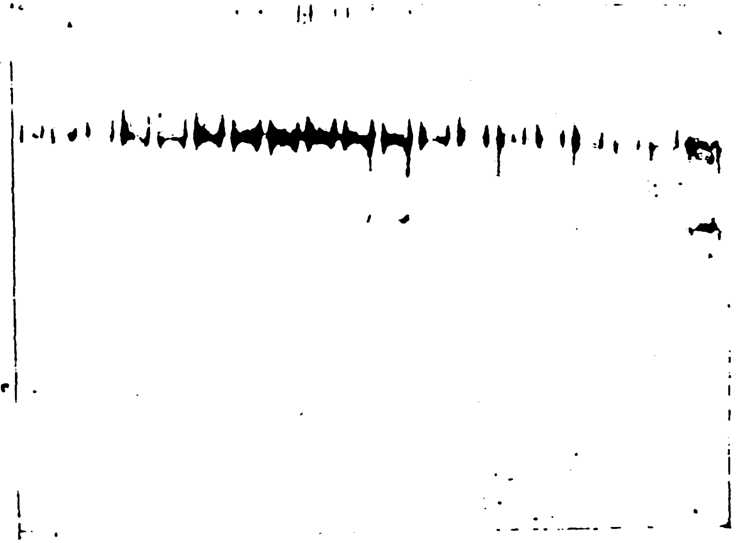


FIG.7

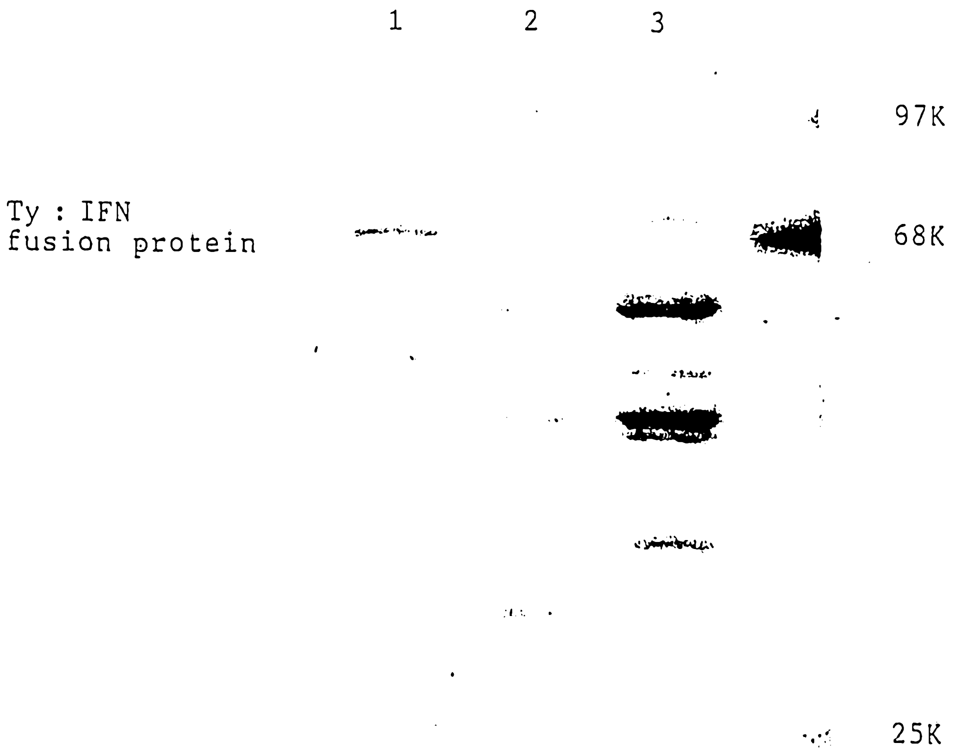


FIG.8a pre-bleed 91 91-1

FIG.8b Ty-VLP antibody 91 91-1

FIG.8c pre-bleed 91 91-1

FIG.8d Ty:IFN-VLP antibody 91 91-1

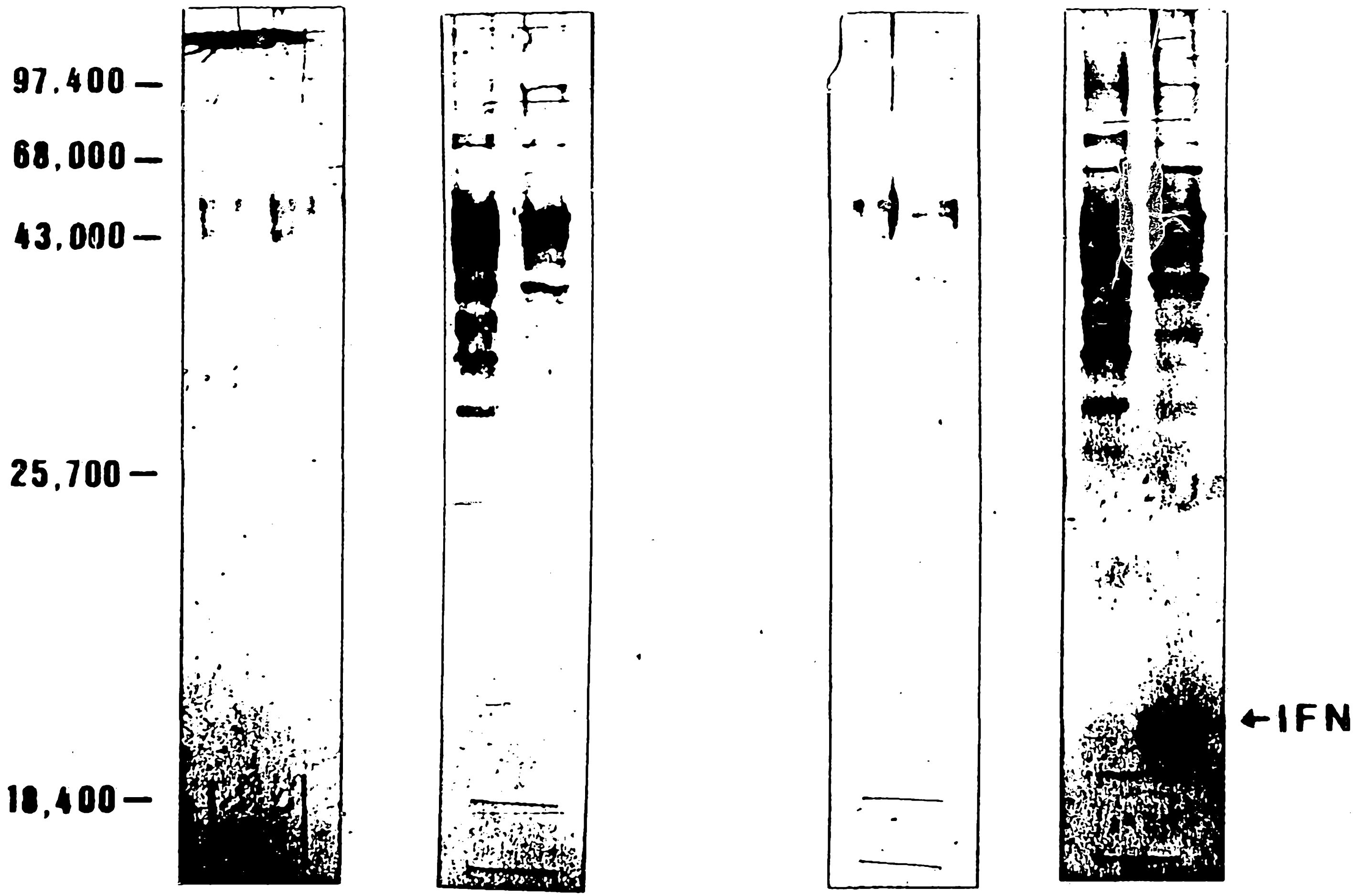


FIG.9

```
  SpeI
A CTA GTG AAA ACA ATC ACA AAT GAT CAG ATT GAA GTG ACT AAT GCT ACT
  25                  30                  35                  40

  GAG CTG GTT CAG AGT TCC TCA ACG GGG AAA ATA TGC AAC AAT CCT CAT
                  45                  50                  55

  CGA ATC CTT GAT GGA ATA GAC TGC ACA CTG ATA GAT GCT CTA TTG GGG
              60                  65                  70

  GAC CCT CAT TGT GAT GGC TTT CAA AAT GAG ACA TGG GAC CTT TTC GTT
          75                  80                  85

  GAA CGC AGC AAA GCT TTC AGC AAC TGT TAC CCT TAT GAT GTG CCA GAT
      90                  95                  100
                          SpeI
  TAT GCC TCC CTT AGG TTA CTA GT
  105                 110
```

FIG.10

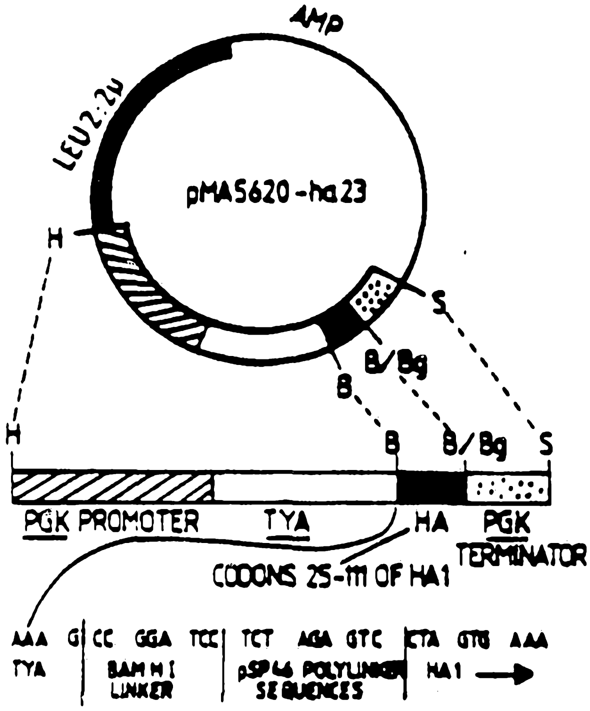
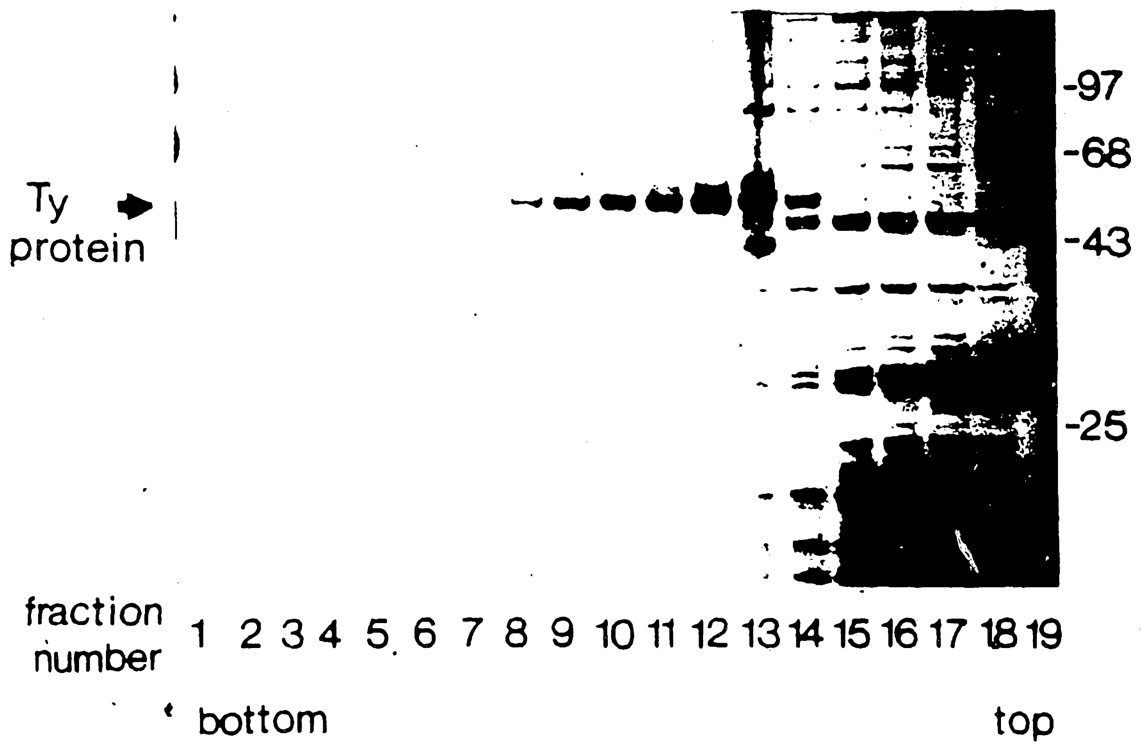

AMP
LEU2:2u
pMAS620-ha23
H
S
B/Bg
B
H
B
B/Bg
S
PGK PROMOTER
TYA
HA
PGK
TERMINATOR
CODONS 25-111 OF HA1
AAA G CC GGA TCC TCT AGA GTC CTA GTG AAA
TYA
BAM H I
LINKER
pSP46 POLYLINKER
SEQUENCES
HA1

FIG.11a

T 5620



T 5620-HA23

FIG.11b

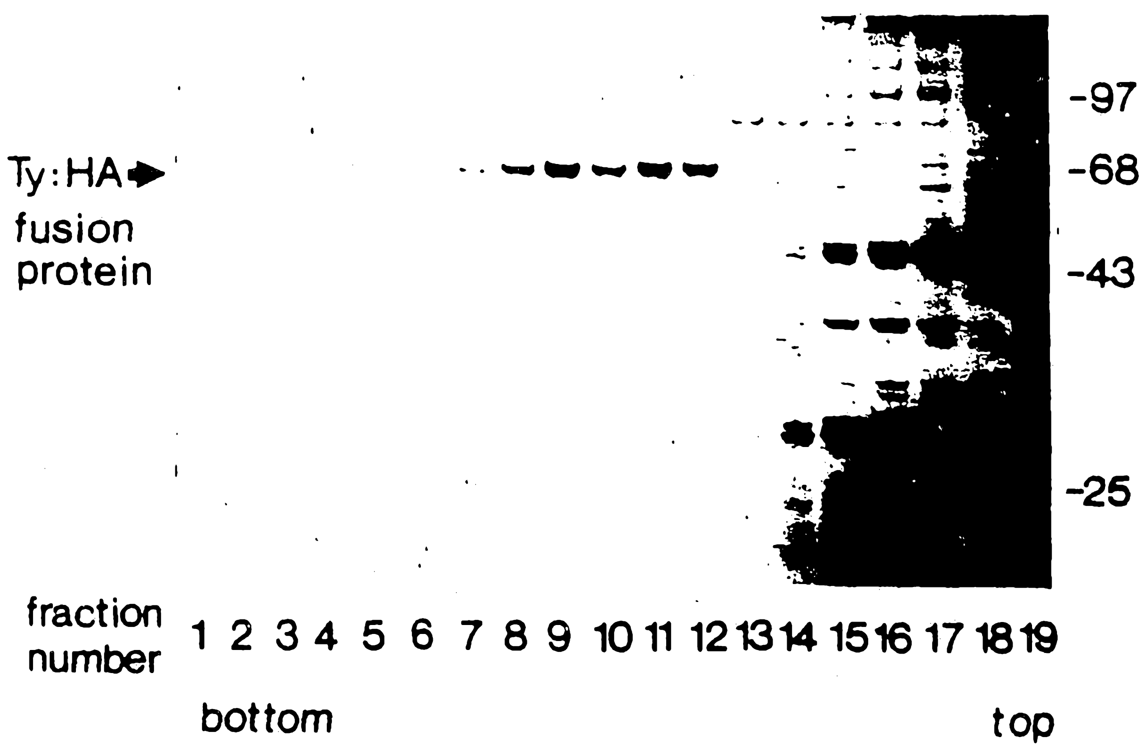


FIG.12

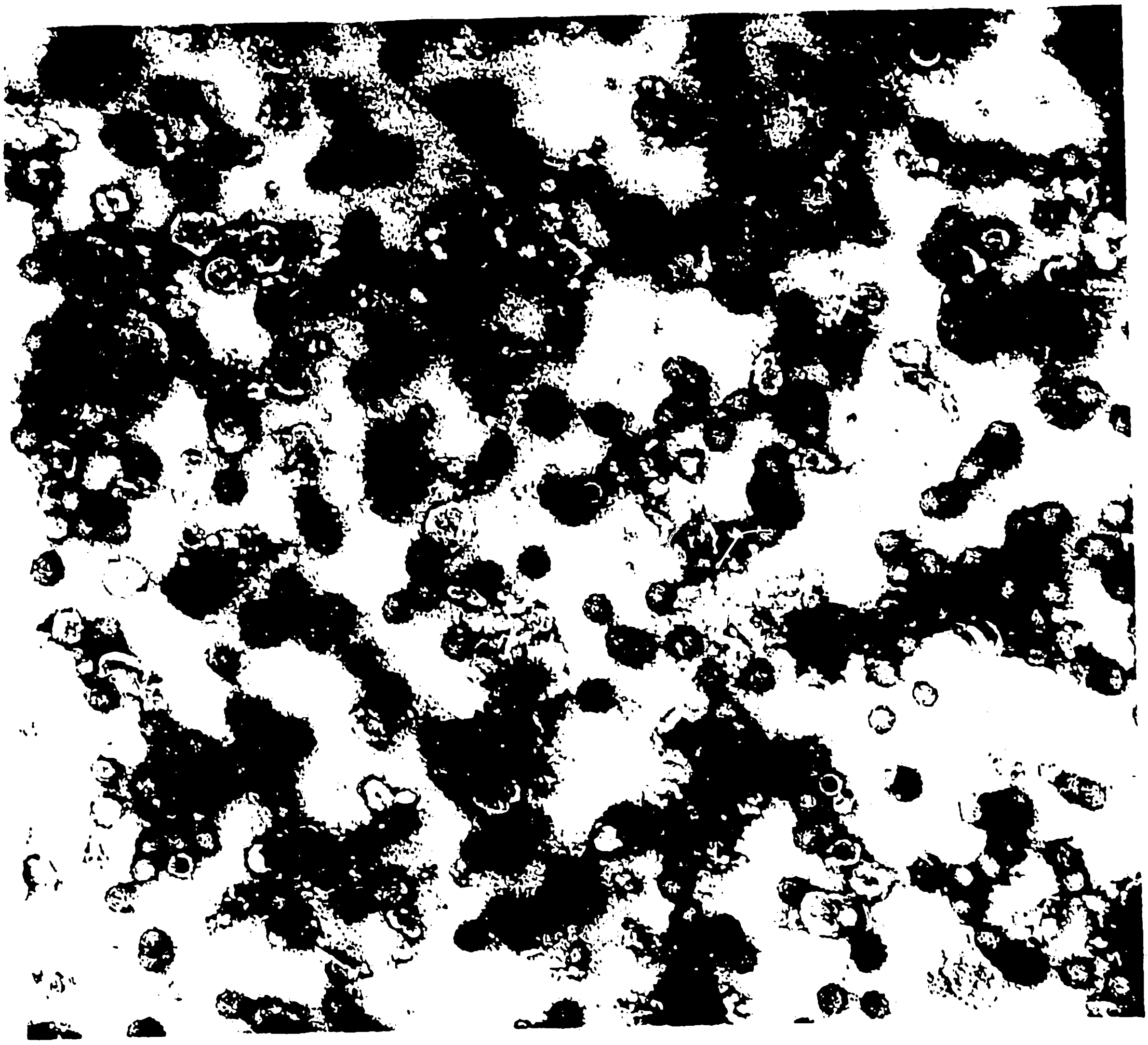


FIG.13a

T 5620-HA23 Anti-Ty antibody

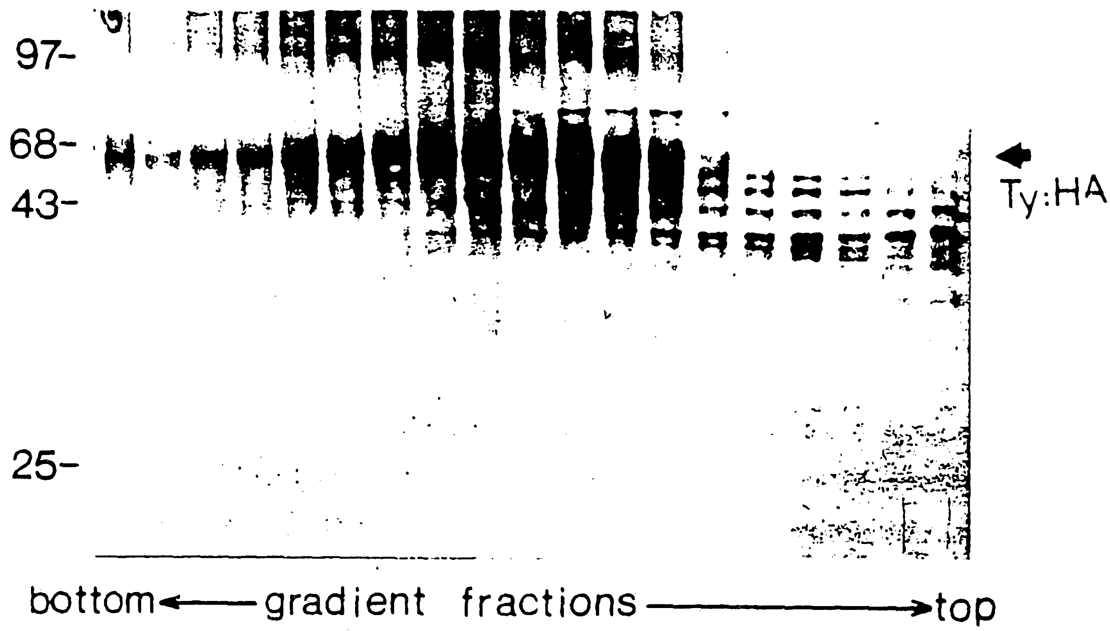


FIG.13b

T 5620-HA23 Anti-flu antibody

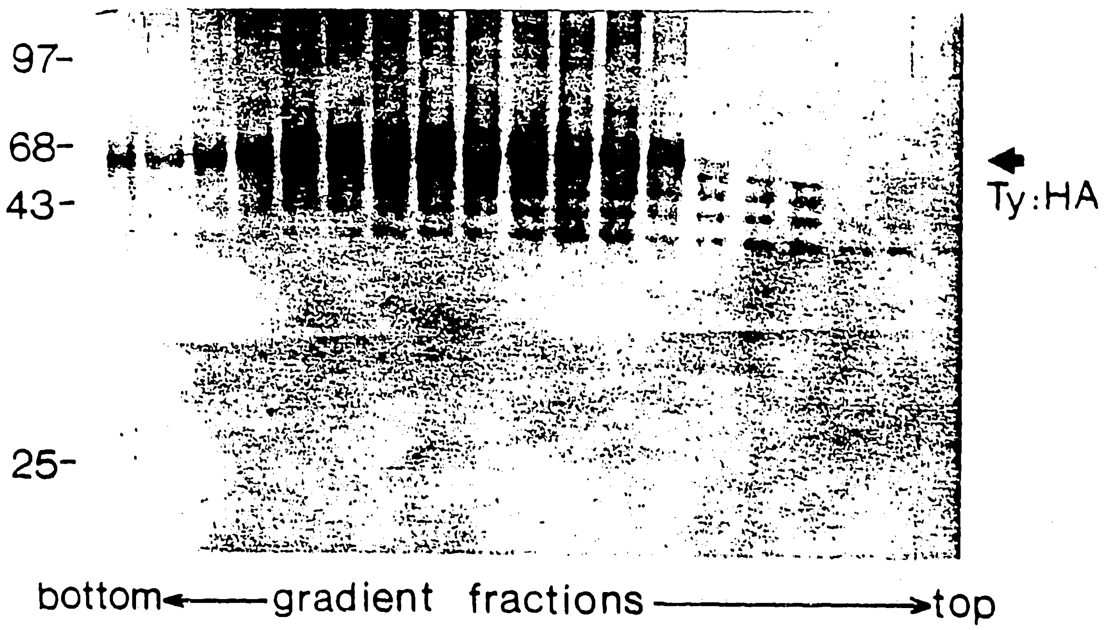


FIG.14a FIG.14b FIG.14c FIG.14d FIG.14e

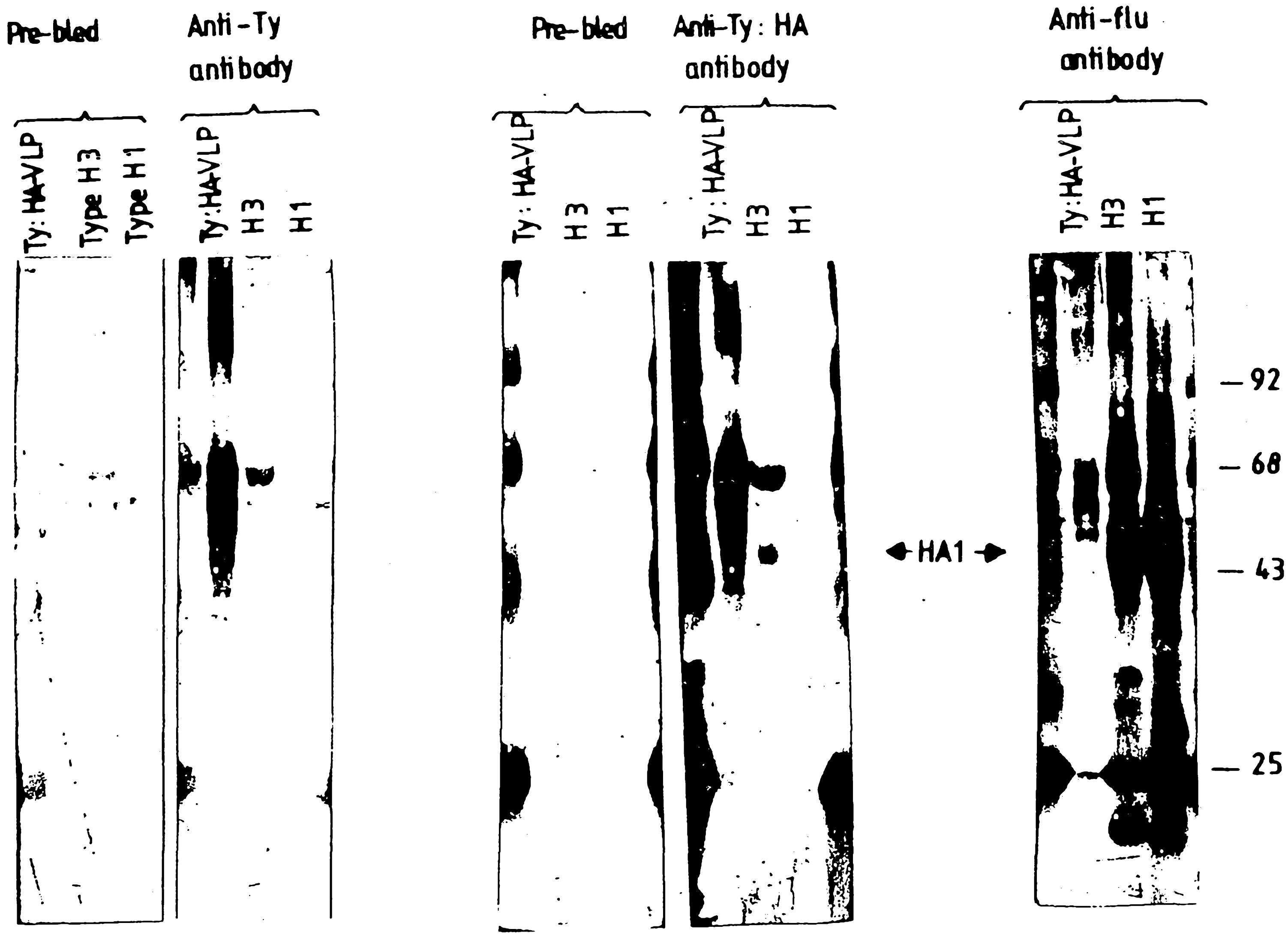


FIG.15

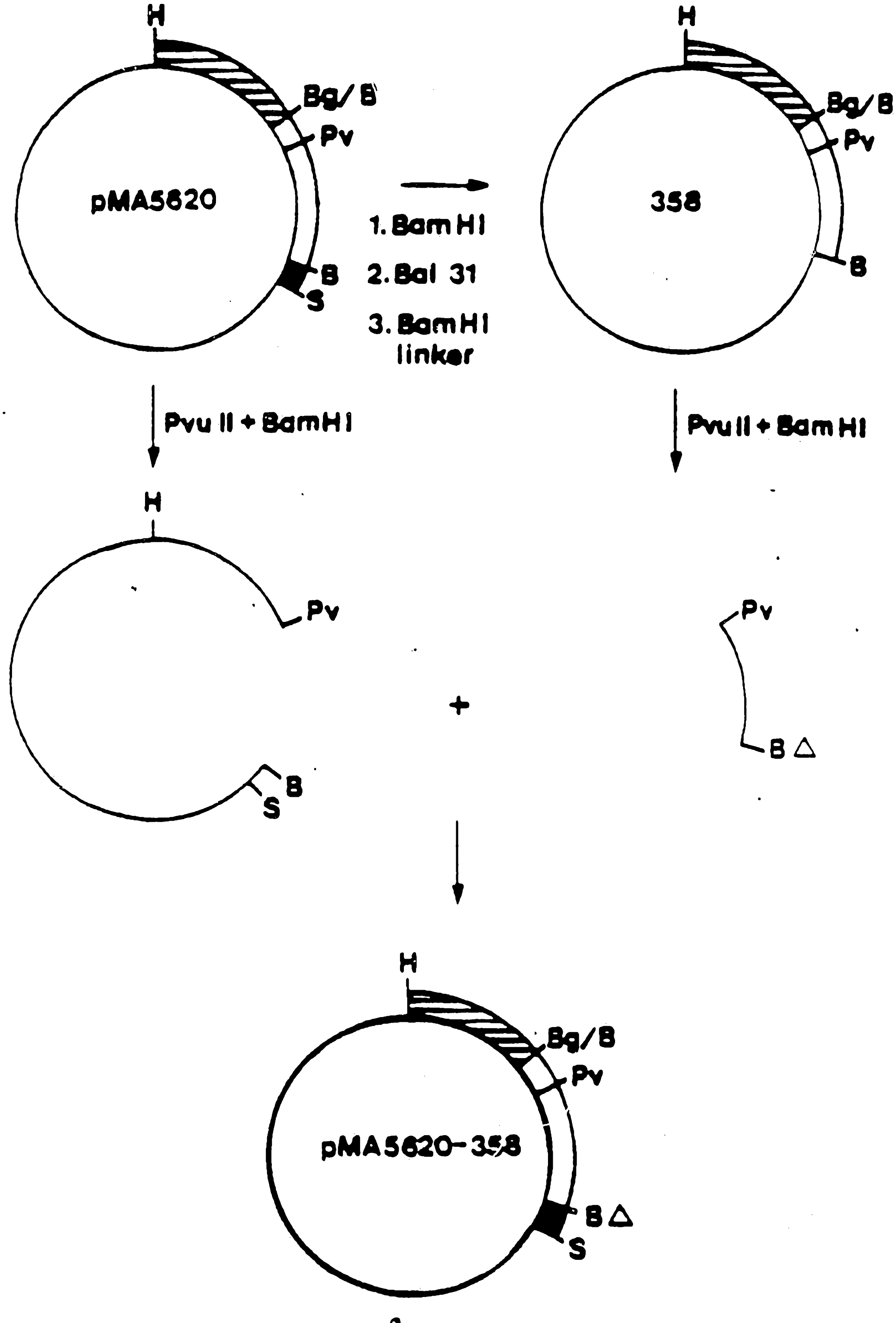

H
Bg/B
Pv
pMA5620
B
S
1. BamHI
2. Bal 31
3. BamHI linker
H
Bg/B
Pv
358
B
Pvu II + BamHI
Pvu II + BamHI
H
Pv
B
S
+
Pv
B △
H
Bg/B
Pv
pMA5620-358
B △
S

FIG.16

```
ATGGAATCCCAACAATTATCTCAACATTCACCCATTTCTCATGGTAGCGCCTGTGCTTCGGTTACTTCTA
 M  E  S  Q  Q  L  S  Q  H  S  P  I  S  H  G  S  A  C  A  S  V  T  S

AGGAAGTCCACACAAATCAAGATCCGTTAGACGTTTCAGCTTCCAAAACAGAAGAATGTGAGAAGGCTTC
K  E  V  H  T  N  Q  D  P  L  D  V  S  A  S  K  T  E  E  C  E  K  A  S

CACTAAGGCTAACTCTCAACAGACAACAACACCTGCTTCATCAGCTGTTCCAGAGAACCCCCATCATGCC
  T  K  A  N  S  Q  Q  T  T  T  P  A  S  S  A  V  P  E  N  P  H  H  A

TCTCCTCAAACTGCTCAGTCACATTCACCACAGAATGGGCCGTACCCACAGCAGTGCATGATGACCCAAA
 S  P  Q  T  A  Q  S  H  S  P  Q  M  G  P  Y  P  Q  Q  C  M  M  T  Q

ACCAAGCCAATCCATCTGGTTGGTCATTTTACGGACACCCATCTATGATTCCGTATACACCTTATCAAAT
N  Q  A  N  P  S  G  W  S  F  Y  G  H  P  S  M  I  P  Y  T  P  Y  Q  M

GTCGCCTATGTACTTTCCACCTGGGCCACAATCACAGTTTCCGCAGTATCCATCATCAGTTGGAACGCCT
  S  P  M  Y  F  P  P  G  P  Q  S  Q  F  P  Q  Y  P  S  S  V  G  T  P

CTGAGGACTCCATCACCTGAGTCAGGTAATACATTTACTGATTCATCCTCAGCGGACTCTGATATGACAT
 L  R  T  P  S  P  E  S  G  N  T  F  T  D  S  S  S  A  D  S  D  M  T

CCACTAAAAAATATGTCAGACCACCACCAATGTTAACCTCACCTAATGACTTTCCAAATTGGGTTAAAAC
S  T  K  K  Y  V  R  P  P  P  M  L  T  S  P  N  D  F  P  N  W  V  K  T

ATACATCAAATTTTTACAAAACTCGAATCTCGGTGGTATTATTCCGACAGTAAACGGAAAACCCGTACGT
  Y  I  K  F  L  Q  N  S  N  L  G  G  I  I  P  T  V  N  G  K  P  V  P

CAGATCACTGATGATGAACTCACCTTCTTGTATAACACTTTTCAAATATTTGCTCCCTCTCAATTCCTAC
 Q  I  T  D  D  E  L  T  F  L  Y  N  T  F  Q  I  F  A  P  S  Q  F  L

CTACCTGGGTCAAAGACATCCTATCCGTTGATTATACGGATATCATGAAAATTCTTTCCAAAAGTATTGA
P  T  W  V  K  D  I  L  S  V  D  Y  T  D  I  M  K  I  L  S  K  S  I  E

AAAAATGCAATCTGATACCCAAGAGGCAAACGACATTGTGACCCTGGCAAATTTGCAATATAATGGCAGT
  K  M  Q  S  D  T  Q  E  A  N  D  I  V  T  L  A  N  L  Q  Y  N  G  S

ACACCTGCAGATGCATTTGAAACAAAAGTCACAAACATTATCGACAGACTGAACAATAATGGCATTCATA
 T  P  A  D  A  F  E  T  K  V  T  N  I  I  D  R  L  N  N  N  G  I  H

TCAATAACAAGGTCGCATGCCAATTAATTATGAGAGGTCTATCTGGCGAATATAAATTTTTACGCTACAC
I  N  M  R  V  A  C  Q  L  I  M  R  G  L  S  G  E  Y  K  F  L  R  Y  T

ACGTCATCGACATCTAAATATGACAGTCGCTGAACTGTTCTTAGATATCCATGCTATTTATGAAGAACAA
  R  H  R  H  L  N  M  T  V  A  E  L  F  L  D  I  H  A  I  Y  E  E  Q

CAGGGATCGAGAAACAGTAAACCTAATTACAGGAGAAATCCGAGTGATGAGAAGAATGATTCTCGCAGCT
Q  G  S  R  N  S  K  P  N  Y  R  R  N  P  S  D  E  K  N  D  S  R  S

ATACGAATACAACCAAACCCAAAGcc[illegible]tc
```

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00764

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

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

IPC[4]: C 12 N 15/00, C 12 P 21/00; A 61 K 39/00; C 07 K 15/00; G 01 N 33/53

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC[4]	C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Nature, volume 313, 17 January 1985, J. Mellor et al.: "A retrovirus-like strategy for expression of a fusion protein encoded by yeast transposon Ty1", pages 243-246 see page 245 cited in the application --	1-27
X	Proc. Natl. Acad. Sci. USA, volume 82, May 1985, J. Clare et al.: "Nucleotide sequence of a yeast Ty element: Evidence for an unusual mechanism of gene expression", pages 2829-2833 see the whole article --	1-27
X	M. Inouye (Ed.): "Experimental Manipulation of Gene Expression", 1983, Academic Press, Inc., (New York, US), R.C. Mulligan: "Construction of highly transmissible mammalian cloning vehicles derived from murine retro-viruses", pages 155-173: chapter 8 see pages 159-164 --	1,2

./.

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19th January 1988	26 FEB 1988
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

International Application No. PCT/GB 87/00764

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	Nucleic Acids Research, volume 15, no. 18, 25 September 1987, M.H. Malim et al.: "The production of hybrid Ty:IFN virus-like particles in yeast", pages 7571-7581 see the whole article	1-27
