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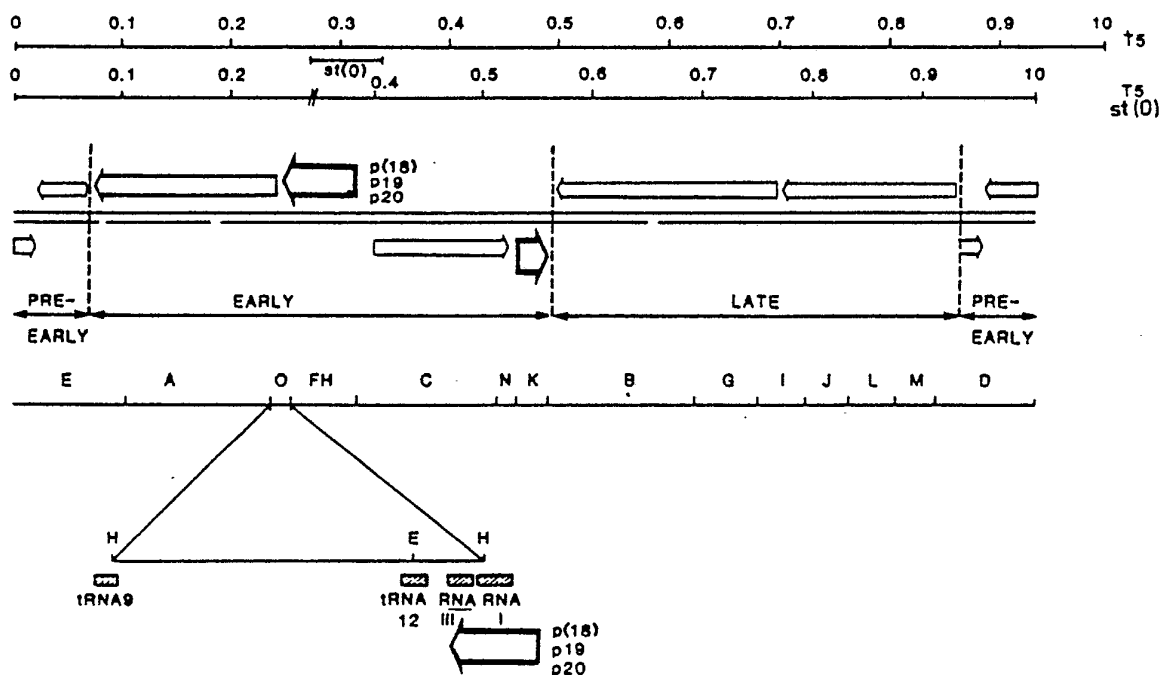
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(54) Title: LYTIC VIRUSES AS EXPRESSION VECTORS, HOST CELL CONTAINING SAME AND PROCESS FOR PROTEIN PRODUCTION



(57) Abstract

A lytic virus having inserted in its chromosome a gene for the production of heterospecific proteins; a host cell infected with such lytic virus; and a process for preparing a heterospecific protein by expression in a host cell having introduced therein such lytic virus. Preferred host cell is E.coli and preferred lytic viruses are phages T5, T5+ and T5 sto.

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Lytic viruses as expression vectors, host cell
containing same and process for protein production

5 The present invention relates to lytic viruses, such as
bacterial phages. The invention also covers host cells infected
by such viruses as well as a process for using such viruses for
the preparation of a heterospecific protein in a host cell.

10 The invention has for an object to provide new biological
techniques enabling integration of episomes in the chromosome
of a lytic virus and maintaining the recombinant characteristic
of the virus through its infectious lifecycle. Although the in-
vention is not to be considered to be so limited it will be
illustrated using E.coli phage T5 which is a known bacterio-
phage.

15 The main object of the present invention is to provide a
lytic virus which in its chromosome has inserted an alien pro-
duction gene, the recombinant virus being stable through its
infectious lifecycle. Such production gene is preferably posi-
tioned in a non-essential region of the chromosome of the virus.

20 The virus of the invention is preferably a bacteriophage,
such as an E.coli phage T5. The phage is preferably selected
from T5+ or its derived stable and autonomously viable deletion
mutants, such as T5 sto. The production gene has preferably been
introduced into the chromosome of the virus by so called homo-
25 logous reciprocal recombination.

The invention also covers host cells infected with such
lytic recombinant virus. The host cell is preferably an E.coli.

30 Another object of the invention is to provide for a pro-
cess for preparing a heterospecific protein which can be re-
covered in an expedient manner. According to this aspect of
the invention a process for preparing a protein by expression
in a host cell comprises the steps:

- a) introducing an alien production gene in the chromosome of
a lytic virus;
- 35 b) infecting a host cell by introducing the resulting re-
combinant virus into same;
- c) allowing expression of the recombinant protein in said
host cell; and

d) recovering the protein thus produced.

It is preferred that the production gene under step a) is introduced by homologous reciprocal recombination. The gene is preferably introduced in a non-essential region of the phage chromosome, and put under control of phage promoters.

In this process for preparing a protein there may be used E.coli bacteria as host cells to be infected by the recombinant phage.

According to the invention there has been developed a phage-based production system taking advantage of three attractive properties of the T5 system, namely the immediate and complete shut-off of host protein synthesis, the degradation of the host chromosome and making use of promoters of extreme signal strength.

The techniques of this invention can be applied in different ways, among which the following two applications are of major importance.

First, the invention enables facile production of recombinant heterospecific proteins by introducing the desired production gene in the virulent phage used. A system based on the lytic phage T5 offers a number of novel features concerning the expression of alien genes, that could be applied in biotechnology: The complete shut-off of the host protein synthesis right after the infection limits the expression of infected cells to phage derived and recombinant proteins. The degradation of the host chromosome to acid solubility reduces problems of DNA viscosity found, when recombinant proteins are purified from E.coli (Fish, N.M. & Lilly, M.D. (1984) Biotechnology 2, 623-627). The insertional activation of a gene after its recombination in the phage genome might allow to express proteins which are lethal to normally growing E.coli cells. Finally the expression is based on a class of promoters, which have been shown to out-compete any other promoters of the E.coli system by their signal strength (von Gabain, A. & Bujard, H. (1979) Proc.Nat.Acad. Sci. USA 76, 189-193).

The results disclosed herein show the feasibility of a T5 based expressions system. A number of modifications can be considered to ease the applications and to optimize the system. A set of deletion mutants and plasmids designed for insertion could be constructed that are suitable for different sized

inserts and optimal expression concerning the juxtaposition of promoters and inserts. Furthermore, plasmids designed for insertion could be furnished with an indicator gene, like the one encoding β -galactosidase, allowing to identify recombinant phages by a colorimetric plaque test described for other phages (Messing, J., Gronenborn, B., Muller-Hill, B. & Hofschneider, P.M. (1977) Proc.Natl.Acad.Sci. USA 3642-3646). A further modification of this strategy is the use of T5-mutants, defective in the "early"- "late" switch, which can be amplified on suppressor host strains. Those phages give rise to abortive infections stagnating at the "early" stage when non permissive cells are infected (McCorquodale, D.J. (1975) CRC Critical Reviews in Microbiology 4, 213-234). The advantage of an abortive infection system is the long period expression of "early" proteins without entering the stage of phage DNA replication and lysis of the cells.

Second, such a system can be applied to analyze recombinant gene products without interference of cellular protein synthesis, as it is done by using mini cells (Frazer, A.C. & Curtiss III, R. (1975) Curr.Top.Microbiol.Immunol. 69, 1-84) or maxi cells (Sancar, A., Hack, A.M. & Rupp, D. (1979) J. Bacteriol. 137, 692-693). The approach is based on inserting the genes of interest in phages, as it has been demonstrated for the CAT gene, and analyzing the short term radio-labeled extracts of infected cells for recombinant proteins. The expression of those cells is in accordance with this invention constrained to phage derived proteins.

The present invention will now be further illustrated by non-limiting examples with reference to the appended drawings, wherein:

Fig. 1 shows an autoradiogram of DNA-blotting;

Fig. 2 shows by autoradiogram the activity of chloramphenicol acetyl transferase in infected cells;

Fig. 3 is a model of the physical structure of the T5 phage; and

Fig. 4 is a map of one integration plasmid used.

Fig. 5 is a map of another integration plasmid used.

As previously indicated the invention will be non-limitationally illustrated in relation to E.coli phage T5.

As indicated above T5 has a number of unique properties making the organism attractive to study the integration of episomes in its chromosome. The phage is organized in a linear double stranded DNA molecule exhibiting a length of 121284 basepairs (Rhoades, M. (1982) J.Virol. 43, 566-573). Stable deletions in a non-essential region of the chromosome (see McCorquodale, D.J. (1975) CRC Critical Reviews in Microbiology 4, 213-234) may allow insertion of genetic material up to a size of 14,000 base pairs.

Finally the assignment of roughly 100 recognition sites for restriction enzymes along the genome simplifies the analysis of genetic rearrangements. Furthermore the phage exhibits a number of unique functions and features during its life cycle that could be more easily studied when episomes could be inserted as a probe in the viral genome.

During the infectious cycle three sets of genes, "pre-early", "early" and "late" are activated in cascades. The three classes of genes are clustered along the chromosome in the order "preearly", "early" and "late". The "preearly" genes are injected first in the host cell and only after their expression the remaining chromosome is actively moved in the host. Thereafter the "early" and "late" genes are activated. The expression of the "early" genes prepares the replication of the phage's chromosome and the synthesis of the phage capsids, which both begin with the onset of "late" genes. Immediately after the first step injection (FST) of the chromosome host, protein synthesis is completely blocked and the DNA of the host is degraded within the first minutes to acid solubility. The efficiency of T5 directed expression is partly explained by the unusual strength of its promoters. The promoters of the E.coli T5 have been shown to be by far the strongest within E.coli and its related extrachromosomal replicons and phages.

The yield of phage-directed biosynthesis during the infection is remarkable: with a burst size of about 200 phage particles per cell the infected cells replicate within the infectious cycle of 45 minutes about $2.4 \cdot 10^7$ base pairs. This is five times the equivalence of the host's chromosome. The rate of protein synthesis increases also by two and a

half times in the infected cells (Chinnadurai, G. & MacCorquodale, D.J. (1973) Proc.Nat.Acad.Sci. USA 70 3502-3505). The dramatic efficiency of T5-directed expression is partly explained by the unusual strength of its promoters (von Gabain, A. & Bujard, H. (1977) Molec.gen.Genet. 157, 301-311 and von Gabain, A. & Bujard, H. (1979) Proc.Nat.Acad.Sci. USA 76, 189-193).

EXAMPLES

Materials and methods used.

Enzymes. Restrictionendonucleases, T4 DNA ligase, S1 nuclease and Alkaline phosphatase were obtained from Boehringer Mannheim; the conditions for using the enzymes were those suggested by the manufacturer or those described by Maniatis, T., Fritsch, E.F. & Sambrook, J. in Molecular Cloning edited by Cold Spring Harbor Laboratory 1982.

Phage and plasmid constructions. T5⁺ and T5 sto were kindly provided by H. Bujard. The plasmid pACYC 177 was a kind gift of S.N. Cohen. pPL 1 is derivative of pPL 603 containing a Chloramphenicol-acetyl-transferase (CAT) gene from B.Pumilis (see Williams, D.M., Duvall, E.J. & Lovett, P.S. (1981) J. Bacteriol. 146, 1162-1165); it was donated by L. Rutberg. The restriction fragment HindIII 0 derived from the T5 chromosome was inserted in the unique HindIII site of pBR 322 or pACYC 177; the constructs were named pBRO and pACYCO, respectively. The distance between the EcoRI site assymmetrically mapping in the phage derived insert and the next EcoRI site on pBR322 (see Rodriguez, R.L.F., Bolivar, R.J., Greene, M.C., Betlach, H.L., Heynecker, H.L., Boyer. H.W., Crosa, J.H. & Falkow, S. (1977) Gene 2, 95-113) and the next XhoI site on pACYC 177 (see Chang, A.C.Y. & Cohen, S.N. (1978) J.Bacteriol. 134, 1141-1156) were determined in order to figure out the orientation of the inserts in the two vectors (see also Figure 5). pBRAO was derived from pBRO by removing the 249 basepair EcoRI fragment spanning from the phage insert into the pBR 322 vector. pPRAOCAT finally was constructed by inserting the BglIII - BamHI fragment isolated from pPL 1 in the unique BamHI site of pPRAO as described below in the result section and as indicated in Figure 5.

Growth and Purification of Phages. T5⁺ and T5 sto were grown as described by Bujard, H. & Hendrickson H.E. (1973) Europ. J.Biochem. 33, 517-528. Recombinant phages were cultivated the same way but grown and purified in media, solutions and buffers containing 10mM MgSO₄, and 1mM Spermine (Labedan, F.T.B. & Legault-Démare, J. (1984) J.Virol. 50, 213-219.) CsCl gradients were performed in a VTi 50 rotor (Beckman), 33,000 rpm, 40 hrs at 20°C. The initial density was adjusted to 1.56 g/ml. Sometimes phages were first purified in a CsCl step gradient performed in a swing out rotor (SW 40) 30,000 rpm, 1hr at 20°C; the phages were overlaid on a preformed step gradient containing equal volume (2.5 ml) of the following densities: 1.5 g/ml, 1.0 g/ml, 0.5 g/ml.

Preparation of Plasmid- and Phage-DNA. Plasmid DNA was prepared by following the experimental procedure described by Maniatis, T., Fritsch, E.F. & Sambrook, J. Molecular Cloning edited by Cold Spring Harbor Laboratory 1982. The extraction of phage DNA was as described by von Gabain, A., Hayward, G. & Bujard, H. (1976) Molec.gen.Genet. 143, 279-290 and Bujard, H. & Hendrickson, H.E. (1973) Europ.J.Biochem. 33, 517-528 with the following modifications: The phageparticles were incubated with DNaseI (10ug/ml, Worthington) for 1 hr at 37°C prior to loading them on the CsCl step gradient; the incubation was performed in phagesuspensionbuffer (Bujard, H. & Hendrickson, H.E. (1973) Europ.J.Biochem. 33, 517-528) containing 10mM MgSO₄.

Plaque lift and Blotting techniques. The number of plaque forming units was determined with the soft agar technique as described by Maniatis, T., Fritsch, E.F. & Sambrook, J. Molecular Cloning edited by Cold Spring Harbor Laboratory 1982 for e.g. the λ phage with the following modifications the soft agar contained always 2mM CaCl₂ and additionally 10mM MgCl₂ and 1mM Spermine when recombinant phages were plated. Plaque lifts were performed with Nitrocellulose filters following the procedure as it is described for recombinant λ phages (loc.cit.); hybridization and washing of filters were

persued according to the same protocol; the filters were finally exposed to X-ray film (Kodak X-OMAT) for periods of 2-24 hrs. Blotting of electrophoretically fractionated RNA or DNA samples was carried out following procedures previously described (loc.cit.). For all hybridizations plasmid DNA or in some cases isolated DNA fragments were nicktranslated using ^{32}P -labeled deoxyribonucleotid-triphosphates as radioisotopes (loc.cit.).

Chloramphenicol acetyl transferase (CAT) assay. Cells were synchronously infected with recombinant phages (multiplicity of infection (M.O.I) was two) containing the CAT gene integrated in the chromosome; aliquots of 2ml were withdrawn at several timepoints during the infectious cycle and probed for their content of CAT enzyme as described by Nilsson, G., Belasco, J.G., Cohen. S.N. & von Gabain, A. (1984) Nature 312, 75-77.

Analysis of RNA. RNA was extracted from T5 infected cells and control cells as described in detail by von Gabain, A., Belasco, J.G., Schottel, J., Chang, A.C.Y. & Cohen, S.N. (1983) Proc.Nat.Acad.Sci.USA 80, 653-657. The RNA was analyzed either by blotting techniques or according to Berk and Sharp (Berk, A.J. & Sharp, P.A. (1977) Cell 12, 721-723) as described in detail by Nilsson, G., Belasco, J.G., Cohen, S.N. & von Gabain, A. (1984) Nature 312, 75-77 and von Gabain, A., Belasco, J.G., Schottel, J., Chang, A.C.Y. & Cohen, S.N. (1983) Proc.Nat.Acad.Sci. USA 80, 653-657.

EXPERIMENTAL RESULTS:

To enable full understanding of the experiments a short explanation of the drawing figures will now be made.

Figure 1 shows the autoradiogram of a plaquehybridization. Cells harboring pBR OCAT were infected with T5sto (see Materials and Methods and notes to table below). After lysis 2000 phages were plated on agar plates (Panel A); the plaques were analyzed by the plaque-lift technique using Nitrocellulose as described in Materials and Methods. The filter was hybridized with radio-labeled pPL 1 and further treated as described by Maniatis, T., Fritsch, E.F. & Sambrook, J. Molecular Cloning edited by Cold Spring Harbor Laboratory 1982. An X-ray film (Kodak X-OMAT) was exposed to the filter for 4 hrs and afterwards processed. The area on the agarplate corresponding to a positive plaque was punched out; the phages were isolated as described by Maniatis, T., Fritsch, E.F. & Sambrook, J. Molecular Cloning edited by Cold Spring Harbor Laboratory 1982. The eluated phages were expanded as described in Results and Materials and Methods and purified over a CsCl-gradient. The band of higher density corresponding to the recombinant phages was harvested. 300 phages were plated and analyzed as described above (Panel B). In the experiment shown in Panel A one out of 2000 plaques reacted with the probe, whereas in the one shown in Panel B 98% of the plaques hybridized to the probe.

Figure 2 shows an autoradiogram of a DNA blotting according to Southern (see Maniatis, T., Fritsch, E.F. & Sambrook, J. Molecular Cloning edited by Cold Spring Harbor Laboratory 1982). Cells harboring the plasmid pACYCO were infected with T5 sto the phages were harvested as described above and DNA was extracted and digested by the restriction endonucleases HindIII and PstI as indicated. As control DNA isolated from T5 sto was digested by both enzymes and DNA from pACYC 177 by PstI (see Chang, A.C.Y. & Cohen, S.N. (1978) J. Bacteriol. 134, 1141-1156). The DNA fragments were fractionated on an 0.8% agarose gel as described by Maniatis, T., Fritsch, E.F. & Sambrook, J. Molecular Cloning edited by Cold Spring Harbor Laboratory 1982 and transferred to Nitrocellulose. Hyb-

ridization was performed according to loc.cit; DNA from the plasmid pACYC 177 was used as probe. X-ray film (X-OMAT) was exposed to the filter for 6 days. (a) DNA from pACYC 177 digested with PstI; (b) DNA from the Ecoliphage λ digested with HindIII; (c) DNA from T5 sto digested with HindIII; (d) DNA from T5 sto digested with PstI; DNA from T5 sto grown on E.coli containing pACYCO was digested with Hind III (e) and with PstI (f), respectively. The sizes in kilobases are indicated at the corresponding positions of the signals.

Figure 3 is an autoradiogram showing the activity of chloramphenicol acetyltransferase in recombinant T5 infected cells. 10 min after infection the different forms of mono- and diacetylated chloramphenicol appear. Cell extracts were made by taking aliquots of cells before infection 2, 4, 10 and 20 min after infection, and disrupted by lysozyme treatment and sonication. This cell extract was assayed by incubation with ^{14}C -labelled chloramphenicol and analysed by ascending TLC (see Nilsson, G., Belasco, J.G., Cohen, S.N. & von Gabain, A. (1984) Nature 312, 75-77).

Figure 4 is a model of the physical structure of T5st(0) DNA (113 kb) with its "nickpattern". Alongside the physical model of the DNA, the different transcriptional regions and directions of transcription are indicated by arrows. The map of the HindIII restriction sites is shown below the physical map. The region where the plasmid is integrated (by homologous recombination) is blown up, showing a physical map of the section of the T5 genome encoding the phage-specific stable RNAs (see Ksenzenko, V.N., Kamynina, T.P., Kazantsev, S.I., Shlyapnikov, M.G., Krykov, V.M. & Bayev, A.A. (1982) Biochimica et Biophysica Acta 697, 235-242.) The direction of transcription of these genes are indicated by an arrow below and the probable promoters initiating the transcription of the recombinant proteins are marked (see Stueber, D., Delius, H. & Bujard, H. (1978) Molec. gen.Genet. 166, 141-149).

Figure 5 is a map of the integration plasmid carrying a 906 bp EcoRI-Hind III fragment from T5. The CAT-86 gene ob-

tained from pPL1 (Rutberg personal communication), a derivative of pPL 603 (see Williams, D.M., Duvall, E.J. & Lovett, P.S. (1981) J.Bacteriol. 146, 1162-1165), is inserted in the BamHI site. The CAT gene is deprived of its promotor (loc.cit.).

In the appended table there is displayed the frequency of inserting plasmids of different background in the viral genomes. Cells harboring the respective plasmids or not containing any episome were infected with T5⁺ or T5 sto (M.O.I. varied between three and five). After lysis the titers of the phages were determined and roughly 20,000 phages were plated out on four agar plates and submitted to a plaquelift as described above. The sizes of the homologous regions between the respective phage and plasmid are indicated in basepairs. The sizes of the plasmids are given in kilobases.

a) Construction of recombinant phages.

The HindIII fragment O (1200 basepairs length) mapping in the non essential region of the T5 chromosome was inserted in the plasmids pBR322 (Rodriquez, R.L.F., Bolivar, P.J., Greene, M.C., Betlach, H.L., Heynecker, H.L., Boyer, H.W., Crosa, J.H. & Falkow, S. (1977) Gene 2, 95-113) and pACYC 177 (Chang, A.C.Y. & Cohen, S.N. (1978) J.Bacteriol. 134, 1141-1156) as described above. E.coli containing either the chimeric plasmid pACYCO or pBRO and its derivatives were infected with T5 st.o, a deletion mutant of the wild-type phage missing 8570 base pairs in the deletable region next to the region spanning the HindIII fragment O. The descending phages were plated on agar plates in order to determine the number of plaque forming units. The indicator strain did not contain the respective plasmids. Plates containing between 10^2 and 10^4 plaques were submitted to plaquelift (see Maniatis, T., Fritsch, E.F. & Sambrook, J. Molecular Cloning edited by Cold Spring Harbor Laboratory 1982) employing nicktranslated pBR 322 and pACYC 177 as probes. Plaques reacting positively with the probes were indentified with a frequency between 0.5 and 2×10^{-3} (see the appended table). The number of positively reacting plaques was not significantly reduced, when 20% of the HindIII fragment O were deleted in the plasmid (see Materials

and Methods used and the appended table). No positive plaques were identified when cells were infected with the wildtype phage T5⁺ or when cells were infected containing the respective plasmids without the viral insert (see the appended table).

5 The experiments were repeated using an E.coli strain as host which was recA minus: the same results were obtained and the frequency of positive plaques was not changing significantly.

Replating phages isolated from positive plaques or restriking them on indicator cells allowed to enrich and to
10 expand the number of plaques that reacted with the probe (see Figure 1). The same result was obtained, when those phages were expanded in fluid culture. The ratio between T5 sto and recombinant phage did not change when mixtures of both were amplified over several passages in host cells
15 not containing the respective plasmids.

b) Characterisation of recombinant phages.

Phages derived from plaques giving positive signal were expanded in fluid cultures as described above. After purifying them over a CsCl-step gradient, DNA was extracted
20 and dissected in parallel with several restriction endonucleases. The DNA fragment were fractionated on agarose gels and subsequently transferred on to nitrocellulose. The filters were probed with the respective plasmids not carrying the phage insert and with the HindIII fragment O. Figure
25 2) shows the analysis of the recombinant phages obtained when E.coli was infected carrying e.g. pACYC O; the DNA was dissected with the restriction enconucleases HindIII and PstI. HindIII cuts twice pACYC O as a result of inserting the page segment in the unique HindIII site of the vec-
30 tor, whereas PstI cuts only once in the vector part of pACYC O. The HindIII digest disclosed an unique fragment (4.1 kb) lightening up exclusively with the plasmid-specific probe, whereas the PstI digest disclosed three fragments (20 kb, 6.8 kb, and 5.2 kb, respectively) hybridizing to both pro-
35 bes. The result is compatible with a multiple insertion of the plasmid by homologous recombination: The HindIII fragment corresponds to the excised pACYC O (Figures 2 and 4), whereas the smallest PstI fragment (Figures 2 and 4) reflects

the repeat of pACYC 177 and HindIII O, which is excised when plasmid and the phage fragment are arranged in duplications as a consequence of multiple insertions, the two largest PstI fragments (see Figure 2) correspond exactly to the predicted sizes expected when the distance to adjacent PstI sites in the phage chromosome is calculated (see Figure 4). The results do not allow to determine the exact number of inserted plasmids per recombinant phage; however, the relative signal strength of the PstI fragments points to three inserts per chromosome in the average. The same results were obtained, when chimeric phages were analyzed, which were amplified on E.coli harboring the other types of plasmids, such as pBRΔOCAT.

The phages were further characterized by fractionating recombinant phages and their predecessors on CsCl density gradients as described above. Typically two bands were observed, the upper and corresponded to a boyant density of 1.54 g/cm^3 we found for T5sto, whereas the lower one disclosed a boyant density of 1.56 g/cm^3 when e.g. pBRΔO CAT (see Figure 5) was inserted in the phage genome (see Figure 2).

The boyant density of the lower band is larger than that determined for wild type phages (see Hertel, R., Marchi, L. & Mueller, K. (1963) Virology 18, 576-581). Analyzing phages, which were obtained from the two respective bands, showed that the upper band contained exclusively T5sto, whereas the lower band reflected to 99% recombinant phages with the expected insert. Because the increment of boyant density is larger than the one observed when comparing the phages T5sto and T5⁺ which differ about 8 kb in their genomic size, the increment of boyant density agrees well with insertion of three respective plasmids. When CsCl purified recombinant phages were employed to reinfect cells containing again the inserted plasmids, phages were harvested which banded in addition to the "normal" position at densities in the gradient which could reflect multiple inserts.

c) Expression of phage directed recombinant proteins.

The integration of plasmids in the phage genome could mediate expression of heterospecific genes, by reinfecting cells with recombinant phages. In order to test the possibi-

lity an easy assayable gene, Chloroamphenicol transferase (CAT) derived from B.pumilis was inserted in pBR322 (see Figure 5 and materials and methods used). The gene was taken from a plasmid constructed previously to measure promotor activity (cf. Williams, D.M., Duvall, E.J. & Lovett, P.S. (1981) J-Bacteriol. 146, 1162-1165) and therefore deprived of any endogenous promotor. Furthermore, the gene was orientated in the plasmid in such a way that homologous recombination would insert it downstream of "early" phage promoters which control expression of phage specific tRNAs (see Figures 4 and 5). Cells containing the construct disclosed some basic level of CAT activity. As neither the BgIII - BamHI fragment containing the CAT gene, nor the upstream mapping HindIII fragment O show any promotor activity in vivo and in vitro (see Williams, D.M., Duvall, E.J. & Lovett, P.S. (1981) J. Bacteriol. 146, 1162-1165 and Ksenzenko, V.N., Kamynina, T.P., Kazantsev, S.I., Shlyapnikov, M.G., Krykov, V.M. & Bayev, A.A. (1982) Biochimica et Biophysica Acta 697, 235-242 and Stueber, D., Delius, H. & Bujard, H. (1978) Molec.gen.Genet. 166, 141-149), the expression must originate from a promotor mapping on pBR322 that has been described previously (see Chang, A.C.Y., Nunberg, J.H., Kaufman, R., Erlich, H.A., Schimke, R.T. & Cohen, S.N. (1982) Nature 275, 617-624 and Stueber, D. & Bujard, H. (1981) Proc.Nat.Acad.Sci.USA 78, 167-171). However, integration of the plasmid in the phage genome by homologous recombination separated promotor and CAT gene (see Figure 4 and 5). Recombinant phages were isolated and enriched over CsCl gradient as described above.

E.coli cells MM 294 were synchronously infected with recombinant phages and the CAT activity was monitored during the course of the infections cycle by analyzing aliquots of infected cells as described previously (see Nilsson, G., Belasco, J.G., Cohen, S.N. & von Gabain, A. (1984) Nature 312, 75-77). The results are shown in Figure 3. Expression is not detectable before the "early" stage of infection, namely after about 10 minutes, and continues until the "late" stage of infection. Another way of proving phage

mediated expression of the CAT gene was demonstrated as follows: Cells not containing pPRAOCAT were infected with the recombinant phages (M.O.I. was 5); 10 minutes after infection Chloramphenicol was added to the culture (100 g/ml). The infected cells lysed after the typical time period of the infectious cycle (see McCorquodale, D.J. (1975) CRC Critical Reviews in Microbiology 4, 213-234). The same experiment was repeated using T5 sto for infection; no lysis was obtained. Comparing the titers obtained for recombinant phages and T5 sto disclosed, that recombinant phages amplify 10^5 times more phages per ml than T5 sto when they are grown in presence of the translational inhibitor; the titer obtained for T5 sto was 10^4 and could reflect the number of phages of the inoculum not adsorbing the host.

E.coli cells (MM 294) containing the chimeric plasmid pBRAOCAT have been deposited with Deutsche Sammlung von Mikroorganismen, Göttingen, West Germany, on July 16, 1985, the accession number being DSM 3386.

d) Expression of β -galactosidase in a T5 based expression system

For this experiment the β -galactosidase gene deprived of any promoter was inserted - in homologous way to the cat gene as described above - in a pBR322 derivative containing the HindIII fragment O of T5 (see Figure 5). The orientation of the insert was designed in a way that the gene would be recombined under the control of T5 specific promoter after its integration in the phage genome. Recombinant phages containing the β -galactosidase gene were identified by a plaque lift, as described above.

The phages were further amplified and then used to synchronously infect E.coli cells. The multiplicity of infections was 5 phages per bacterial cell.

Aliquots of cells (2.5×10^9) were withdrawn from the culture before and after infection at indicated times (see the Table below) and the β -galactosidase content was determined using an enzyme assay (reference). Enzyme activity was detectable 10 minutes after infection and steadily increased until the lysis of the cells. It should be noted that the fraction of recombinant phages was not determined for the phagestock used in the experiment. Therefore the number of units per cell can not be taken

as measure for the amount of β -galactosidase, which can be produced in such system, when it is adusted to optimal condition. (For experimental details see Experiments in Molecular Biology by J.H. Miller, Cold Spring Harbor Lab. 1972.

The experiment shows the general applicability of a T5 based expression system and furthermore the approach made it possible to identify recombinant phages, as blue peagues, in a colorimetric assay.

TABLE

	Minutes before/after infection	Number of β -galactosidase units
	0	0
	2	0
15	10	55
	20	105
	45	237
	after lysis	275

It should be observed that this invention is by no means limited to the specific embodiment described above. Thus, other genes than the CAT gene as described above can be introduced in the chromosome of a suitable lytic virus to express other proteins of commercial interest. Among such embodiments there may be mentioned genes for expressing for example insulin, interferons, insulin-like growth factors, antitrombin, etc.

CLAIMS

1. A lytic virus having inserted in its chromosome a gene for the production of heterospecific proteins.
2. A virus according to claim 1, wherein said gene is positioned in a non-essential region of the chromosome of the virus.
3. A virus according to claim 1 or 2, wherein the gene has been introduced by homologous reciprocal recombination.
4. A virus according to any preceding claim which is a bacterio-phage.
5. A virus according to claim 4 which is an E.coli phage T5.
6. A virus according to claim 5 selected from T5+ and T5 sto.
7. A host cell infected with a lytic virus according to claim 1.
8. A host cell according to claim 7, which is an E.coli.
9. A host cell according to claim 7 or 8, wherein the virus is a bacterio-phage, such as an E.coli phage T5.
10. A host cell according to claim 9, wherein the bacterio-phage is selected from T5+ and its derived stable and autonomously viable deletion mutants, such as T5 sto.
11. A process for preparing a protein by expression in a host cell comprising the steps:
 - a) introducing an alien production gene in the chromosome of a lytic virus;
 - b) infecting a host cell by introducing the resulting recombinant virus into same;
 - c) allowing expression of the recombinant protein in said host cell; and
 - d) recovering the protein thus produced.
12. A process according to claim 11, wherein under step a) the production gene is introduced by homologous reciprocal recombination.
13. A process according to claim 11 or 12, wherein the gene is introduced in a non-essential region of the virus chromosome.

14. A process according to any of claims 11-13, wherein the production gene under step a) is introduced in the chromosome of a bacterial phage, such as E.coli, T5+ or its derived stable and autonomously viable deletion mutants, such as T5 sto.

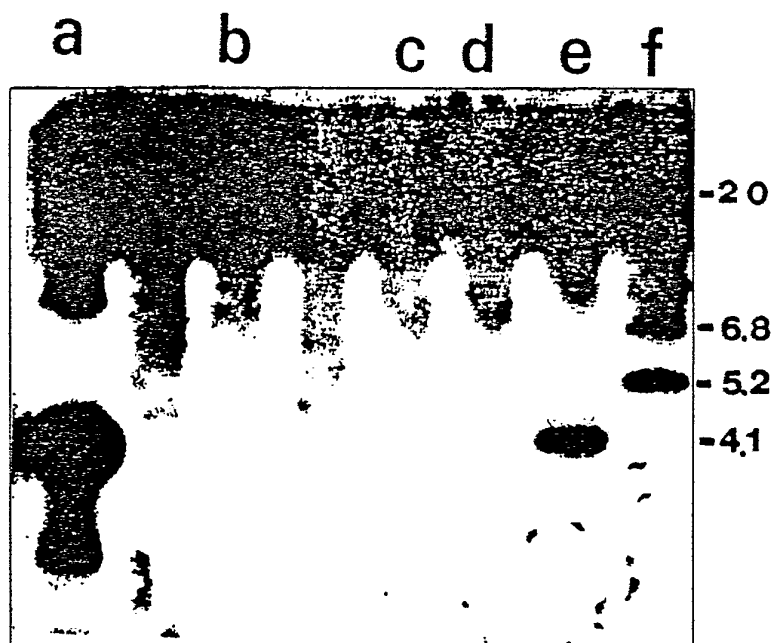
15. A process according to claim 14, wherein under step b) an E.coli is infected.

16. A process according to claim 14 or 15, wherein the gene is introduced in a non-essential region of the phage chromosome and put under control of phage promoters.

17. A process for analyzing recombinant gene products comprising the steps:

- a) inserting an alien gene in the chromosome of a lytic virus;
- b) infecting a host cell by introducing the recombinant virus from step a) above; and
- c) analyzing the proteins expressed in said cell.

18. A process according to claim 17, wherein said alien gene is inserted downstream of a ribosomal binding site.

Fig. 1

SUBSTITUTE SHEET

Fig. 2

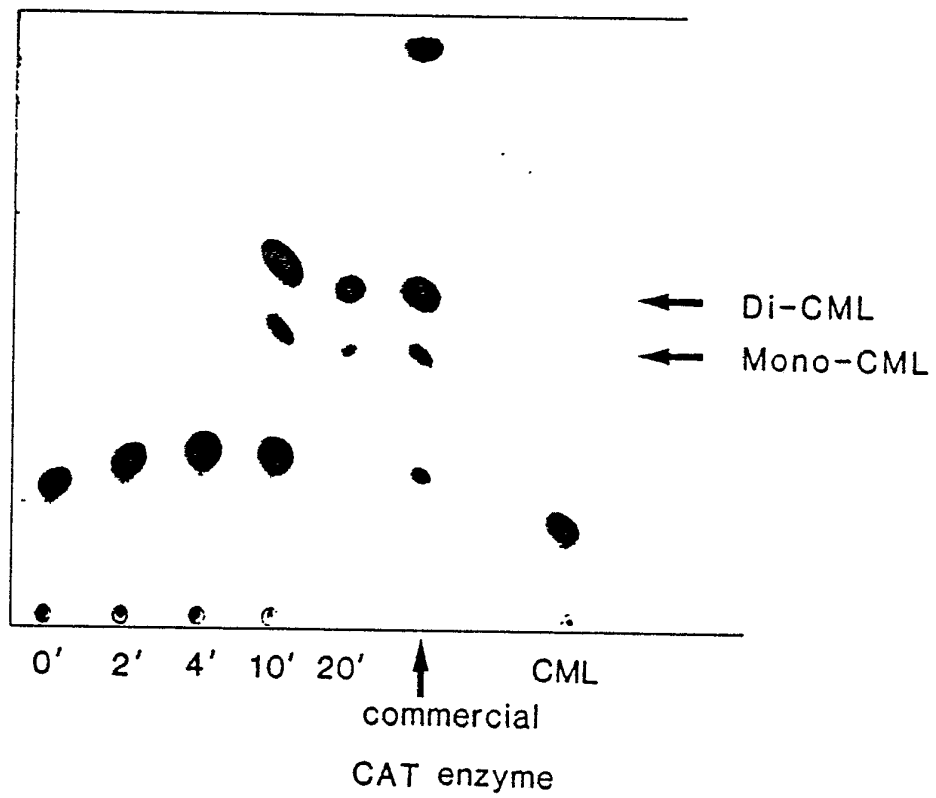


Fig. 3

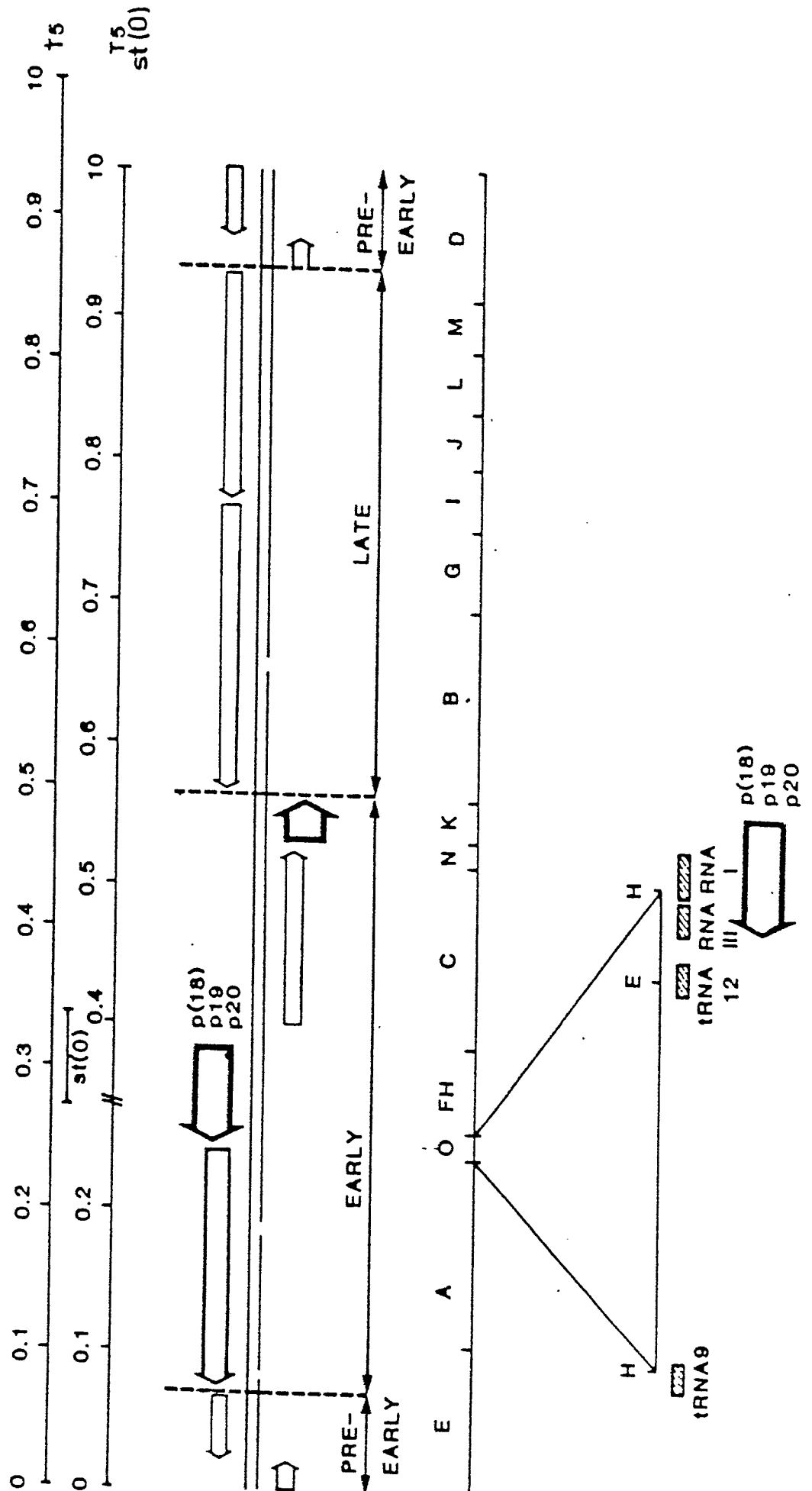


Fig. 4

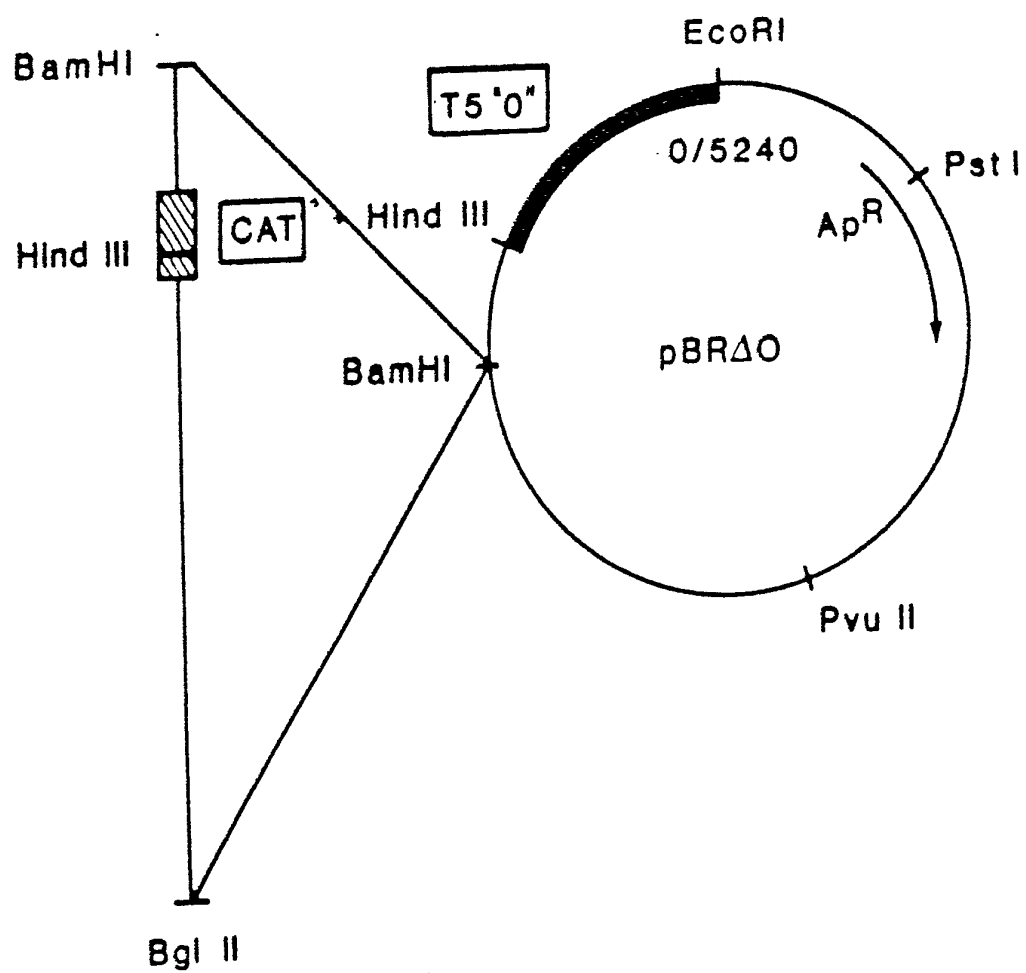
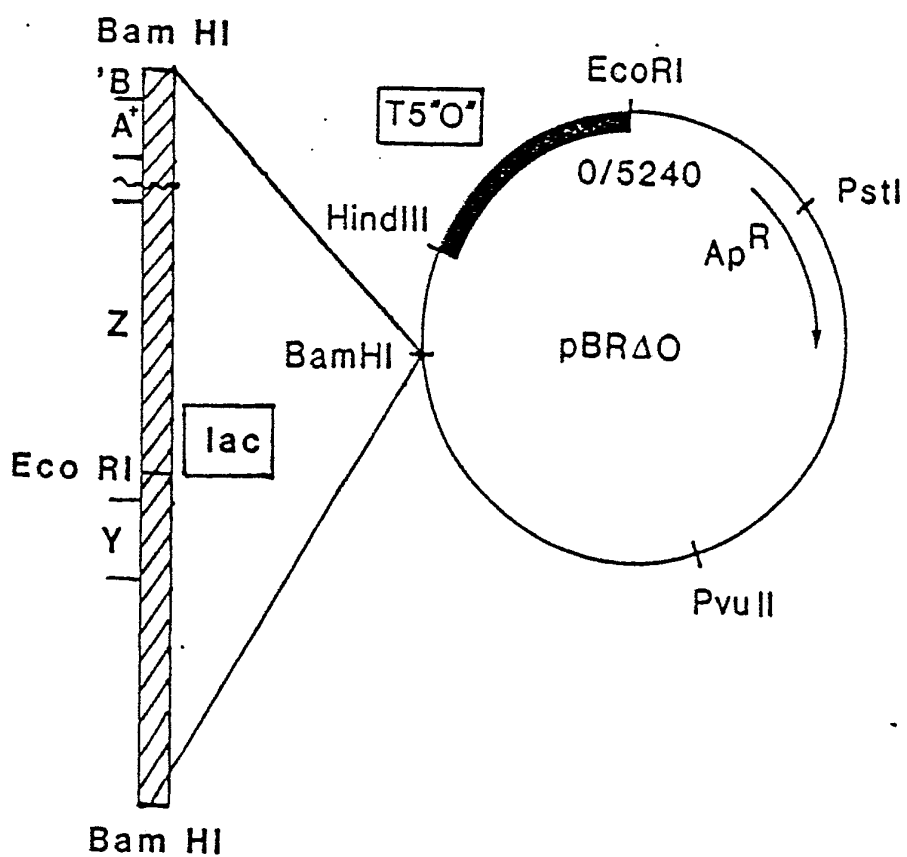


Fig. 5



INTERNATIONAL SEARCH REPORT

International Application No

PCT/SE86/00477

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 ⁴		
C 12 N 7/00, 15/00, C 12 P 21/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC US C1	C 12 N 7/00, 15/00 435:6, 172-172.3, 235, 239, 259, 317	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
SE, NO, DK, FI classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X, Y	US, A, 4 469 791 (COLSON C.A. ET AL) 4 September 1984 See in particular col. 3, 1 34-42 & EP, 0034470 GB, 2069503 JP, 56128796 CA, 1183090	1-4, 7, 8, 11-13, 17
Y	US, A, 4 415 660 (GOLDSTEIN R.N) 15 November 1983 See in particular col. 2. & US, 4332901	1-4, 7, 8, 11-13, 17
A	US, A, 4 506 013 (HERSHBERGER C.L. ET AL) 19 March 1985	1
A	US, A, 4 508 896 (FOOR F. ET AL) 2 April 1985	1
A	US, A, 4 460 689 (FOOR F. ET AL) 17 July 1984	1
A	US, A, 4 495 280 (BUJARD H.G.) 22 January 1985	1, 5, 6
¹⁰ * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
1986-12-19	1986 -12- 2 9	
International Searching Authority	Signature of Authorized Officer	
Swedish Patent Office	Carl-Olof Gustafsson	

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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
A	Chemical Abstracts Vol 101, (1984), abstract 207 450w, Diss. Abstr. Int, B 1984, 45(3), 754.	1
A	Chemical Abstracts Vol 102 (1985), abstract 143 902f, J. Embryol. Exp. Morphol. 1984, 83 (Suppl. Programmes Dev) 75-88.	1
X,P	EP, A1, 176 170 (INSTITUT MERIEUX) 2 April 1986 See p. 4-5, 14-17. & JP, 61001390	1-3,7, 11-13
X	Eukaryotic Viral Vectors (conf.) 1981, p 55-60, published 1982, Ed. Gluzman Y. (LIU C.-C. et al) "Expression of Hepa- titis B Surface Antigen Using Lytic and Nonlytic SV-40 Based Vectors."	1-3,7, 11-13
X	GB, A, 2 105 344 (GENENTECH INC.) 23 March 1983 & EP, 0073656 AU, 87798/82 AU, 555575 JP, 58056685	1-3,7, 11-13