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English translation

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declare that I am conversant with the French and
English languages and that to the best of my knowledge
and belief the following is a true translation of the text
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PROCESS TO OBTAIN THE EXPRESSION OF.
OR TO ENHANCE THE LEVEL OF EXPRESSION OF A GENE

5 The present invention relates to a process for the specific replacement of a copy of a gene present in the genome of a receptor eucaryotic organism by integrating a gene that is different to the inactivated gene. Preferably, two copies at least of the receptor gene shall be present in the transfected host cell. The receptor gene is defined as the gene in which the insertion of the different gene is to be made.

10 More particularly, the invention relates to the production of transgenic animals in which the foreign gene was introduced in targeted fashion for the twofold purpose of maintaining the animal's normal genetic functions and of expressing a foreign gene under the control of endogenous promoters.

15 By "different or foreign" gene is meant any nucleotide sequence corresponding in whole or in part to a gene that is "foreign or different" to the receptor gene such as it is normally found in the genome (RNA or DNA), or it may also correspond to an
20 artificially modified sequence of the normal gene or even to a fragment of this sequence.

The invention also relates to the production process of these transgenic animals.

25 In the production of transgenic animals, the conventional methods used for the introduction of heterologous DNA sequences into the germinal cell line do not allow control over the site of integration of the foreign gene into the genome, or over the number of copies thus introduced. The introduction of the foreign
30 gene is made randomly and, in general, several copies of the gene integrate themselves at the same time, sometimes in the form of a head-to-tail tandem , the



site of integration and the number of integrated copies varying from one transgenic animal to another.

It may therefore arise that endogenous cell genes, situated at the point of insertion, thereby become inactivated without this being easily detectable owing to the large number of random insertions. If the product of these genes is important for the development of the animal, such development will be seriously disturbed. Moreover, random insertion of the foreign gene may occur at an inappropriate site for the expression of the gene. Also, the variation in the site and number of insertions between one animal and another, makes the interpretation of expression studies extremely difficult.

A major problem encountered in the production of transgenic animals is obtaining the expression of the foreign gene. In general, two types of experiment have been made in mice.

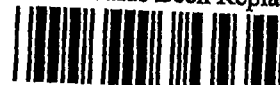
The genes introduced into the germinal line are:

- either "complete" genes, comprising coding sequences flanked by their own regulatory sequences ;
- or compound genes, made up of the coding sequence of a gene fused with the promoter sequence of another gene, both fragments even belonging at times to two different animal species.

In this way confirmation was obtained that the specificity of gene expression in a given tissue is determined by the gene's regulatory sequence(s).

The choice of appropriate promoter for the expression of the foreign gene in the transgenic animal is therefore of primary importance.

Also, the guided mutagenesis of murine genes in embryonic stem cells has recently been conducted with recourse to "gene targeting" (Thomas et al., 1987; Thompson et al., 1989).



In the first case, the HPRT murine gene was muted by insertion and replacement, and in the second case a muted HPRT gene was corrected. Thomson et al. extended their experiments until they obtained chimaera mice and
5 noted the entry of the gene modification into the germinal cell line.

In each of the documents cited, the exact site of integration was targeted by homologous recombination between, firstly exogenous sequences comprising the
10 mutation or correction included in a vector under the control of an exogenous promoter, and secondly their genomic homologue. Nonetheless, it should be noted that the prior authors carried out their experiments, on a specific gene (HPRT) whose activation by mutation was
15 accompanied by a detectable phenotype. The effect of the targeted mutation described by Thomas et al. was to inactivate the HPRT gene and, consequently, to cause to disappear the normally detectable phenotype associated with the HPRT. The Neo^R selection gene, controlled by a
20 TK promoter, was therefore incorporated into the insertion DNA in order to allow the selection of transformants. It is to be observed that the experiments described in the prior art implied a selection either by the receptor gene (e.g. HPRT) or by
25 the insertion gene (e.g. Neo^R). The site of insertion and/or the type of gene inserted is therefore limited to genes which confer a selectable character, a direct selection.

Also, in the prior art the exogenous sequences on
30 the vector therefore serve both to target the integration site and to introduce the modification. After homologous recombination, the modified gene always finds itself in its normal gene environment.

It is recalled that one problem which arises
35 during the production of transgenic animals is the



danger of inactivating an endogenous cell gene which is located at the point of insertion of the foreign gene.

Depending on the function of the product of the inactivated gene, said inactivation may lead to strong physiologic or morphologic disorders in the transgenic animal or could even prevent its survival.

On the other hand, the inactivation of a gene could be considered advantageous if the gene under consideration encoded a virus receptor or other infectious agent.

The inventors have examined the possibility of avoiding the above-described drawbacks, which in certain cases are associated with the possible inactivation of one or more important functioning endogenous cell genes during the production of transgenic animals.

The object of the invention is a process for the replacement or specific insertion of a gene, especially by targeting a DNA, so-called insertion DNA, made up of the entirety of a gene, or part thereof, which may be made functional or whose functioning may be made more effective when it is recombined with a complementary DNA to produce a complete recombinant gene in the genome of an eucaryotic cell, characterized in that :

- the site of insertion is in a chosen gene, so-called receptor gene, and containing the complementary DNA, and in that :

- the eucaryotic cells are transfected with a vector containing an insert itself comprising the insertion DNA and two so-called "flanking" sequences either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired site of insertion in the receptor gene.

- the insertion DNA being heterologous to the receptor gene, and in that :

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- the flanking sequences, chosen from among those which make up said complementary DNA, which, by homologous recombination with corresponding sequences of the receptor gene, permit the reconstitution of a complete recombinant gene in the genome of the eucaryotic cell.

In accordance with the process of the invention, said insertion DNA may contain either a coding sequence or a regulatory sequence, the process then being characterized in that :

- the site of insertion is in a chosen, so-called receptor, gene, and in that :

- the eucaryotic cells are transfected with a vector containing an insert itself comprising the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in the receptor gene,

- the insertion DNA being heterologous to the receptor gene and,

- the flanking sequences being chosen in order to allow, through homologous recombination depending upon the case, either the expression of the coding sequence of the entire insertion DNA under the control of the regulatory sequences of the receptor gene, or the expression of a coding sequence of the receptor gene under the control of regulatory sequences of the insertion DNA.

For example, the insertion DNA may contain a coding sequence deprived of a regulatory element, especially of a promoter that is particular to it.

The invention also relates to a production process of transgenic animals, characterized in that embryonic stem (E.S.) cells are transfected under the above-described conditions and selected for the event of homologous recombination, that is to say the correct



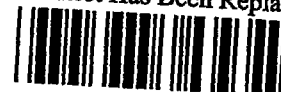
integration of the foreign gene ; the transfected cells are injected into embryos at a stage when they are able to integrate the transfected cells (for example the blastocyst stage), the latter are subsequently
5 reimplanted in a surrogate mother and the chimaera offspring which are obtained at the end of the gestational period, in which colonisation by E.S. cells of the germinal line is observed, are mated. If the E.S. cells have colonised the germinal line of the
10 chimaera animals, transgenic animals which are heterozygous for the replaced gene will be obtained by mating (F₁) of their descendants.

It is also possible to insert the insertion DNA, carried by the vector of the invention, into the egg
15 shortly after fertilisation (i.e. less than 24 hours). In this manner, the insertion is made while the egg is in the unicellular state.

The invention also relates to a plasmid able to carry out the targeted insertion of a recombinant gene, so-called insertion gene, in the genome of an
20 eucaryotic cell, characterized in that it contains an insert, itself comprising the insertion gene and two so-called "flanking" sequences either side of the insertion gene, respectively homologous to the two
25 genomic sequences which are adjacent to the desired insertion site in the receptor gene.

The insertion DNA may be a gene, or part of a gene, heterologous to the transfected species.

The invention also relates to transgenic animals in which at least one endogenous gene, which preferable
30 exists in at least two copies in the genome, was inactivated by insertion of a gene different to the inactivated gene, the insertion gene being inserted at a position which permits the expression of this gene
35 under the control of the regulatory sequences of the inactivated endogenous gene.



Owing to the phenomena of homologous recombination it is therefore possible with the process of the invention to insert foreign genes, in targeted fashion, in particular coding sequences deprived of the promoter normally associated therewith, into the genome of an eucaryotic organism at a site which permits their expression under the control of the endogenous promoter of the gene in which insertion is made, and consequently to inactivate the targeted endogenous gene.

According to one preferred embodiment of the invention, the targeted receptor gene is a gene which exists in at least two copies in the genome. Use of the electroporation method (Ref.11) insures that only one copy of the foreign gene is introduced.

According to this variant of the invention, the effect of the targeted insertion of the gene under consideration (that is to say the so-called insertion gene) is to inactivate the only copy of the endogenous cell gene in which the insertion is made and to leave intact and operational the other copy or copies of this gene.

In this way, the genetic functioning of the transgenic animal is not or only slightly disturbed by the introduction of the foreign gene, even if the insertion inactivates only one copy of a receptor gene that is essential for the animal's development. Either its development is therefore unaffected by the insertion of the foreign gene, or the possible minor disturbances in the event of inactivation of a critical gene are probably not lethal for the animal. The effects of the insertion of a foreign gene in the homozygous state could be of any kind and could be observed in the 2nd generation (F2) after interbreeding between heterozygous individuals (F1).



If, on the other hand, the inactivation of all the copies of a gene is desired, for example in the case of a gene encoding a receptor of an infectious agent, multiple copies of the foreign gene can be introduced. Control over the quantity introduced may be made by making use of known techniques.

The targeted insertion of the foreign gene allows for its introduction into a site where its expression is under the control of the regulatory sequences of the endogenous gene in which the insertion is made.

With the process of the invention it is therefore possible to insert the foreign gene behind an endogenous promoter with the required functions (for example, expression specificity in a given tissue) and, if necessary, without inactivating the other copies of the receptor gene.

According to a preferred embodiment of the invention, the insertion DNA or the vector contains sequences placed between the insertion DNA and the flanking sequences. These sequences may, for example contain a sequence coding for a selective agent allowing the selection of transformants and possibly contain a labelling gene such as LacZ.

According to a particularly preferred embodiment of the invention, the insertion DNA comprises between the flanking sequences, firstly a DNA sequence intended to be recombined with the complementary DNA in the receptor gene to produce a recombinant gene, and secondly a sequence coding for a selective agent to obtain the selection of the transformants and a promoter enabling the expression of the selective agent, the receptor gene and the recombinant gene coding for expression products which do not confer any selectable phenotype. According to this variant the plasmid insert of the invention comprises, between the flanking sequences, firstly a DNA sequence intended to



be recombined with the complementary DNA in the receptor gene, and secondly a sequence coding for a selective agent which allows the selection of the transformants and a promoter which allows the expression of the selective agent, the DNA sequence intended to be recombined with the complementary DNA being other than a gene coding for a selective agent.

In this manner, the selection of the transformants is fully independent from the nature of the receptor gene and of the inserted gene, contrary to the processes described to date in which the inserted gene or the receptor gene necessarily had to code for an expression product allowing the selection of the transformants. The system developed by the inventors provides full flexibility regarding the nature of the receptor gene and of the inserted gene or of the gene formed by the homologous recombination. The inventors have observed in surprising manner that the insertion of sequences of substantial size (for example approximately 7.5 kb) does not affect the frequency of homologous recombination.

The effect which may be produced by the insertion of the DNA sequence according to this aspect of the invention includes for example, depending on the type of sequence inserted, the replacement of a coding sequence, the replacement of a regulatory sequence, the inactivation or the reactivation of a gene by mutation or enhancement of the expression level of a gene. It is possible, according to the invention, to replace a coding phase or part of a coding phase by a heterologous sequence which starts at the initiation codon of the replaced gene so that the expression of the inserted gene fully replaces the expression of the replaced gene. This avoids the formation of proteins which may be undesirable in a transgenic animal.



According to this embodiment of the invention, the insertion DNA may comprise, between the flanking sequences, a heterologous coding sequence deprived of a promoter, the coding sequence being other than a gene coding for a selection agent. The insertion DNA may also comprise, downstream from the coding sequence and still between the flanking sequences, a gene coding for a selection agent associated with a promoter enabling its expression in the target cell.

In this manner, the heterologous coding sequence may be inserted behind an endogenous promoter having the required properties, for example a certain expression specificity, or transcription code etc., the selectability of the transformed cells being fully independent from the expression of the heterologous coding sequence. With this type of construction it is possible, for example, to select the transformants even if the gene replaced by the heterologous coding sequence is not normally expressed in the target cells. This is of particular importance in the production of transgenic animals from E.S. (Embryonic Stem) cells since a high proportion of the genes remains inactive until a more advanced stage in the animal's development is reached. The HOXC8 gene (also known as Hox-3.1) is an example of this type of gene. Also, if the coding sequence codes for an easily detectable protein, for example β -Gal, the development of the transcription code of the replaced endogenous gene can be followed. The pGN vector is an example of this type of construction.

According to another embodiment of the invention, the insertion DNA may comprise a foreign regulatory sequence. The site of insertion, and consequently the flanking sequences, are chosen in relation to the desired objective, namely either the insertion of the foreign regulatory sequence to produce a "double



promoter" effect with the endogenous regulatory sequence, or the replacement of an endogenous promoter by the foreign promoter. The coding sequence under the control of the regulatory sequence may be endogenous.

5 A further possibility is the targeted insertion of a foreign DNA which comprises both a regulatory sequence and a coding sequence. It is possible for the regulatory sequence to be the one which is naturally associated with the coding sequence.

10 The process of the invention uses a vector containing two "flanking" sequences either side of the foreign gene. These flanking sequences have at least 150 base pairs and are preferably of shorter length than the receptor gene. It is essential for these
15 flanking sequences to be homologous to the two genomic sequences which are adjacent to the desired site of insertion. The flanking sequence of the vector upstream from the foreign gene to be introduced is normally homologous to the genomic sequence situated at the 5'
20 end of the insertion site. In the same way, the flanking sequence of the vector downstream from the foreign gene, is normally homologous to the genomic sequence situated at the 3' end of the insertion site.

25 It is possible to introduce "interposing" sequences between either of the flanking sequences and the foreign gene, for example sequences allowing the selection of transformants, labellers, sequences allowing vector cloning etc..

30 The position of these interposing sequences vis-à-vis the foreign gene must nevertheless be chosen so as not to prevent the expression of the foreign gene, in particular of the foreign coding sequence of DNA under the control of the endogenous promoter, or, conversely, the endogenous DNA coding sequence under
35 the control of foreign regulatory elements brought by the insertion sequence.



Despite the presence of the flanking sequences, which promote homologous recombination, it is possible for a certain number of integrations to be made by chance. In order to check that the targeted insertion
5 has taken place in the target site and not elsewhere, the "Polymerase Chain Reaction"(P.C.R.) technique is used (see Ref.10) to amplify the DNA sequence of the locus where insertion is made or should have been made. In this manner, only those clones transformed further
10 to homologous recombination are selected.

The flanking sequences of the vector are, needless to say, chosen in relation to the desired insertion site so that homologous recombination may take place. If necessary, the flanking sequences may
15 comprise replica sequences of the endogenous promoter and/or of the modifications to sequences which precede the initiation codon in order to improve the translation level (upstream sequences) and replica sequences of termination sequences in particular of
20 polyadenylation sites (downstream sequences).

The insertion gene may be any gene of interest. Non-restrictive examples are the lacZ gene (as in the model described below), the genes coding for interleukin or interferon, receptor genes, for example
25 retinoic or beta-3 adrenergic acid or H.I.V., and genes known to be related to certain diseases, for example myopathy etc.

The eucaryotic cells transformed by the process of the invention are also included in the present
30 application.

According to a preferred variant of the invention, the eucaryotic cells are embryonic strain cells (see Ref.14 and 15).

Indeed, a muted E.S. cell may be injected into an
35 early embryo which, after reimplantation, may be borne in chimaera form. If the germinal line is colonised by



the muted cell, the chimaera animal will transmit the mutation to its descendants. Subsequently, the effects of this mutation can be observed in the homozygous state in certain individuals on their development, their behaviour, their metabolism, their pathology etc.

Figure 1 shows the pGN plasmid which is part of the invention.

Figures 2a and b show the molecules pGMA and pGMD respectively constructed from the pGN plasmid in relation to the HOXC8 (Hox-3.1) gene. These plasmids are mutagenesis plasmids. The two parts of the HOXC8 (Hox-3.1) coding phase are represented, on chromosome 15, with the black "homeo" box. The corresponding sequences of HOXC8 (Hox-3.1) were cloned in the pGN plasmid.

(A : polyadenylation signal; Enh/Pro : enhancer-promoter).

07 and 08 show the two oligonucleotides used in P.C.R..

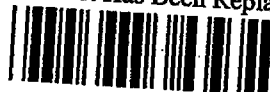
Figures 3 to 6 show the plasmids used for the construction of pGN.

Figure 7 illustrates the detection of homologous recombination with the Polymerase Chain Reaction (P.C.R.) method on transfected E.S. cells.

Figures 8 (a) and (b) show Southern analyses of positive individual clones (L5 and F2) and E.S. cells (C.C.E.)

The process of the invention has wide industrial application and may vary according to the nature of the foreign gene that is introduced, for example for the production of transgenic animals.

The genetics of mammalia will make considerable progress owing to the recent possibility of specifically mutagenising any gene whatsoever, for a better definition of the role they play. Through this technology which uses homologous recombination and E.S. cells, precious information will be obtained on



oncogenes, growth factors, transcription factors etc., genes which concern highly topical subjects of fundamental research or applied research. An important outcome for medical research is the possible reproduction of a human disease of known genetic determination (certain human pathological diseases such as Duchesne's myopathy) so that their mechanisms may be examined more closely and a treatment developed.

By applying the process of the invention, a gene known to be responsible for a certain disease is inserted in targeted fashion into the genome of an E.S. cell. The transgenic animal which is subsequently produced offers a useful model of this disease.

If necessary, and as described above, normal gene functioning may be substantially maintained despite the insertion of a foreign gene.

A further application of the process of the invention is the genetic labelling of animals. This application consists of inserting an insertion gene which is easily detectable, e.g. the lacZ gene which may act as a cell labeller. In this way, filiation studies, e.g. in animals taking part in competitions, may be made easier and follow-up of the race be made.

The insertion of the lacZ gene as insertion gene also provides the possibility of promoter studies. Owing to the possible detection of β -galactosidase activity, the activity and the specificity of different endogenous promoters may be studied by targeting different sites in the same type or different types of cells. Said studies may be conducted in an entire organism, during the development stage or in the adult state, by using the techniques of chimaera or transgenic animals.

The inventors have observed in surprising manner that the frequency of homologous recombination is unaffected by the insertion of fragments of substantial



size, for example the lacZ. For the inventors, this observation suggested that the technique of homologous recombination would be well adapted to the insertion of other heterologous genes of substantial size.

5 The invention also targets the application of the replacement process or specific insertion process to gene therapy.

 Owing to the possible modification of an animal's genome, the process of the invention may therefore be
10 used as "gene therapy". The most evident uses would consist of inactivating receptor genes of infectious agents (virus or bacteria) or toxic agents. If this mutagenesis should prove lethal, the lost function
15 would have to be re-established without re-establishing the sensitivity to the harmful agents. A modified gene coding for such receptor could be reintroduced into the muted cell, unless the modification may be provoked by homologous recombination. This modification of the genetic heritage would confer upon the animal an
20 immunity against the disease under consideration.

 This protocol may also be used in self-graft procedures. Diseased or healthy cells taken from the patient could be treated and immunised and subsequently reimplanted in the same individual.

25 The technique of the invention is also suitable for studies into the activity of pharmaceutical products assumed to have an activity vis-à-vis the expression products of a pathological gene related to a disease. In this case, the insertion gene consists of
30 the pathological gene or fragment thereof, and the pharmaceutical product to be tested is administered to the transgenic animal to evaluate its action on the disease.

 The invention will be illustrated with reference
35 to the pGN plasmid and its use for the targeted insertion of a foreign gene (lacZ, coding for the β -



galactosidase enzyme of E.Coli) into the genome of a mouse E.S. cell. The lacZ gene was chosen because its expression is easily detectable and is simply given by way of illustration.

5 The coding phase of the β -galactosidase enzyme of E.Coli (lacZ; 1-3057), fused with a genomic sequence (7292-3) of the murine HOXC8 (Hox.3-1) gene (Ref.1) starts with the initiation codon of this gene. For the sequence which precedes the initiation codon of HOXC8
10 (Hox.3-1) is identical to the consensus sequence observed in vertebrates (Ref.2), thus giving a better translation level of β -galactosidase in vertebrate cells. The lacZ gene is followed by a polyadenylation signal, for example of the SV 40 virus, like most
15 eucaryotic genes, in order to stabilise the messenger RNAs.

 The activity of the β -galactosidase of E.Coli, which is functional in eucaryotic cells, may be detected in different manners. Cells expressing the
20 lacZ gene take on a blue colour, after fixation, in the presence of X-Gal, which is a substrate of the β -galactosidase (Ref.3). A new substrate, the DGF (di- β -galactopyranoside fluorosceine) permits the detection and determination of the activity of β -gal. while
25 keeping the cells alive (Ref.4). The cells expressing lacZ accumulate a fluorescent product and can be isolated with a cell sorter or FACS (fluorescence-activated cell sorter).

 The transcription unit of the neomycine resisting
30 gene is derived from the most part from the pRSV-neo plasmid (Ref.5). The Long Terminal Repeat (LTR) of the Rous sarcoma virus procures very powerful activating and promoting sequences in numerous eucaryotic cells (Ref.6). From the Tn5 bacterial transposon are derived
35 a promoter that is active in E. Coli and the coding phase of the phosphotransferase enzyme (Ref.7) which is



followed by the polyadenylation signal of the SV40 virus. The same gene under the twin control of the Rsv and Tn5 promoters may confer resistance to neomycine or kanamycine upon bacteria and resistance to G418 upon eucaryotic cells.

Through a simple isolated mutation effect, unit B of activating sequences (enhancer) of the PyEC F9.1 strain of the polyoma virus became much more active in different types of cells, in particular in the cells of embryonic carcinoma (EC) (Ref.8). Two copies of this Py F9.1 enhancer were inserted in tandem into the pGN plasmid upstream from the LTR-RSV, and in the "delayed promoter" orientation of the Polyoma's regulatory region.

In order to enhance the translation level of phosphotransferase, the sequence preceding the initiation codon was modified during oligonucleotide mutagenesis. Therefore sequence T T C G C A U G became G C A C C A U G, which has a much higher correlation with the consensus sequence of translation initiation in vertebrates (Ref.2).

The improvements made to the transcription unit of the neomycine resistant gene could be assessed by transfecting mice embryonic stem cells (ES). Plasmid molarity being equal, a construction with Py F9.1 enhancers produced 7.5-fold more G418 resistant clones than the pRSV-neo, and 2 to 3-fold more than the pMCI-Neo described by Capecchi et al. (Ref.13). Further, the number of clones was increased 60-fold, that is to say 450-fold compared with the pRSV-neo by modifying the translation initiation sequence. Homologous recombination may be a fairly rare event under the applied experimental conditions (e.g. 1/1000 for HPRT, Ref.13). A vector offering high selection efficacy is therefore very useful, especially as electroporation



conditions mainly give rise to the integration of a single copy.

5 The pGN plasmid also contains a bacterial replication origin of *colEI*, pBR322 type, which permits cloning and *E.Coli* preparations.

Finally, a multiple cloning site (M.C.S.) synthesised *in vitro*, which only contains single cutting sites in pGN, was inserted upstream from *lacZ* to facilitate the use of this plasmid.

10 The plasmid "flanking" sequences which provoke homologous recombination are added to the ends of the pGN plasmid after linearisation of the plasmid upstream from *lacZ* by an MCS site (see Fig.2). Under the circumstances, the chosen flanking sequences are
15 homologous to the chromosome sequences derived from HOXC8 (Hox-3.1) which will subsequently take part in the homologous recombination.

Figure 2 situates the molecule constructed from the pGN plasmid in relation to the HOXC8 (Hox-3.1)
20 gene. In this case, a recombination between the plasmid and chromosome sequences of HOXC8 (Hox-3.1) would lead to an insertion at the start of the coding phase of this gene, and therefore to its total inactivation.

The pGN plasmid offers several advantages for
25 this methodology which can be applied to any gene whatsoever. As the event of homologous recombination may be fairly rare (in the region of 1 per 1000 non-homologous integrations), a large number of clones need to be analysed whose G418 resistance is sufficiently
30 strong to express itself in any part of the genome. The changes made to the transcription unit of phosphotransferase bring a perfect solution to these problems. The mutagenesis method by homologous
35 recombination is equivalent to inactivating a gene by insertion or substitution, but the pGN plasmid offers the additional advantage of being able to substitute



the expression of β -galactosidase for that of the muted gene. Finally, MCS facilitates the cloning of genomic fragments.

EXAMPLES :

5 I - Construction of the pGN plasmid

The intermediate plasmids are numbered according to their stage.

Stage 1 :

10 Insertion of a Xho I site in the Bgl I site of pRSV-neo

Insertion of a Xho I linker in the Bgl I site of pRSV-neo, filled with the Klenow fragment of the polymerase DNA of E.Coli.

Stage 2 :

15 Insertion of a Cla I site in the Nde I site of the p1 plasmid.

Insertion of a Cla I linker in the Nde I site of p1, filled with the Klenow polymerase.

Stage 3 :

20 Insertion of an enhancer Py F9.1 in the Cla I site of the p2 plasmid.

25 Insertion of the isolated Py F9.1 Pvu II-Pvu II enhancer by a single site, AccI, in the Cla I site of p2. Selection of a clone containing two enhancers oriented in the direction "delayed promoter".

Stage 4 :

Sma I-HpaI deletion from the p3 plasmid.

30 The two enzymes, giving "free ends" may be ligated directly. This deletion removes the intron of antigen t from SV 40, which offers little use, and substantially reduces the size of the phosphotransferase transcription unit.

Stage 5 :

35 Insertion of a Xho I site in the Bam HI site of pCH110.



Insertion of a Xho I linker in the Bam HI site of the pCH 110 plasmid (Pharmacia), filled with the Klenow polymerase.

Stage 6 :

5 Insertion of 3' lacZ-polyA SV 40 in the p4 plasmid

10 The 3' end of the coding phase of β -galactosidase, followed by the polyadenylation signal of the SV 40 virus, is isolated from plasmid p5 by sites Xho I-Aat II and cloned in plasmid p4 by the same sites.

Stage 7 :

Insertion of 5' lacZ in the KS- vector.

15 The 5' end of the coding phase of β -galactosidase is isolated from the plasmid pMC 1187 (Pharmacia) by sites Pst I-Sac I and cloned in the KS- vector (Stratagene) by the same sites.

Stage 8 :

Fusion of a HOXC8 (Hox-3.1) genomic sequence with the 5' lacZ.

20 A genomic sequence of the HOXC8 (Hox-3.1) gene cloned in vector KS- is purified with successive digestions by the Sac I enzyme, then by the Mung bean nuclease and finally by the Apa I enzyme. This insert is fused with the 5' end of the coding phase of β -galactosidase by cloning in plasmid p7 digested by Apa I-Sma I. The protein thus fused contains the translation initiation codon of the HOXC8 (Hox-3.1) gene followed by the coding phase of β -galactosidase (subsequently checked by sequencing).

25



21

	Met Ser Ser	Ile Pro Gly Asp Pro
	CCAGC ATG AGC TCC	ATT CCC GGG GAT CCC
	GGTCG TAC TCG AGG	TAA GGG CCC CTA GGG
5	↓ Sac I	
	CCAGC ATG AGC T	↓ Sma I
	GGTCG TAC	
	↓ Mung bean nuclease	
	CCAGC ATG	GGG GAT CCC
10	GGTCG TAC	CCC CTG GGG
	↔	
	Met Gly Asp Pro	
	CCAGC ATG GGG GAT CCC	
	GGTCG TAC CCC CTA GGG	

15 Stage 9.

Insertion of HOXC8 (Hox-3.1)-5' lacZ in the p6 plasmid.

20 The fusion HOXC8 (Hox-3.1) - 5' lacZ is isolated from the p8 plasmid by sites Apa I-Sac I and cloned in plasmid p6 by the same sites. This cloning reconstitutes the coding phase of β -galactosidase in its entirety.

Stage 10.

Insertion of the Neo^R gene in the KS+ vector.

25 The neomycine resistant gene (bacterial promoter and coding phase of phosphotransferase) is isolated from pRSV-neo by sites Hind II-Eco RI and cloned in the KS+ vector (Stratagene).

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Stage 11 :

Mutagenesis of the initiation sequence of Neo^R in p10.

5 The initiation sequence of phosphotransferase translation is modified so that it is identical to the consensus sequence observed in vertebrates and to allow a higher level of translation initiation, and therefore an increased resistance of mammal cells to G418. The modification also creates an Apa LI site with which the
10 efficacy of the mutagenesis can be verified.

Apa LI

GTTTCGCATG

⇒

GTGCACCATG

15 An oligonucleotide (CTTGTTC AATCATG GTGCACGATCCTCA) comprising a mismatch region with the pRSV-neo sequence (underlined) is synthesised (Gene Assembler, Pharmacia) then phosphorylated by the kinase polynucleotide of the T4 bacteriophage. A single stranded template of the p10 plasmid is prepared using the f1 origin of the KS+
20 plasmid and hybridized with the oligonucleotide of the mutagenesis. The second strand is synthesised and repaired with the Klenow polymerase and the ligase DNA of the T4 bacteriophage. After transforming the bacteria, the muted clones are sorted with the ³²P-
25 labeled oligonucleotide. The mutagenesis was verified by Apa LI digesting and by sequencing.

Stage 12 :

Replacement of the initiation sequence in the p9 plasmid.

30 A fragment containing the modified sequence of the translation initiation of the neomycin resistant gene is isolated from plasmid p11 by enzymes Hind III-Eag I and cloned in the p9 plasmid by the same sites.

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Stage 13 :

Insertion of the multiple cloning site in plasmid p12.

Two complementary oligonucleotides are synthesised (Gene Assembler, Pharmacia), then phosphorylated. After matching, the MCS is cloned in sites Apa I - Sac II of the p12 plasmid using its cohesive ends

10

```

          XmaI   Asp 718           XmaI
      ApaI   SmaI   KpnI   XbaI   NsiI   SacII
5' CCGCGGGGGGTACCTCTAGAATGCATTCCGC 3'
3' CCGGGGGGGCCCCCATGGAGATCTTACGTAAGG 5'

```

15

The multiple cloning site was also checked by sequencing.

II - Addition of the "flanking" sequences to the end of the pGN plasmid linearised upstream from lacZ' by a M.C.S. site.

The flanking sequences used were chosen in relation to the desired insertion site (for example HOXC8 (Hox-3.1) see Fig.2 (a) and (b) pGMA and pGMD).

In the construction of the pGMD mutagenesis plasmid, two DNA limbs homologous to the HOXC8 (Hox-3.1) locus were cloned to sites Apa I-Nsi I and Nsi I-Sac II of the pGN vector. The 5' limb starts at the Sac II site (CCGCGG) at nucleotide 219 of the cDNA c21 of HOXC8 (Hox-3.1). This fragment extends over 6.8 kb in 5' as far as the first BamHI site. The 3' limb starts at the Apa I site (GGGCCC) at nucleotide 885 of the cDNA c21. This fragment extends over 1.5 kb in 3' as far as the first Pst I site. A NsiI linker was inserted in the BamHI site of fragment 5' and in site PstI of fragment 3'. Limbs 5' and 3' were cloned in vector pGN



at sites Nsi I-Sac II and Apa I-NsiI respectively. The sequence of the cDNA of HOXC8 (Hox-3.1) c21 is published (Ref.1).

5 The mutagenesis plasmid is linearised by digestion with Nsi I before electroporation of E.S. cells. Its ends are made up of two genomic limbs cloned at sites Apa I-Nsi I and Nsi I-Sac II of the pGN vector.

10 The pGMD plasmid shows no polyadenylation signal after the resistance gene but, on the other hand, shows an AU-rich sequence which is responsible for selective mRNA degradation inserted in the sequence of the HOXC8 (Hox-3.1) intron of the plasmid.

15 A further mutagenesis plasmid, pGMA, shows the same structure as the pGMD but contains the polyadenylation and transcription termination signals of SV40 and does not have the AU degradation sequence of mRNA downstream from the Neo^R gene. The purpose of these modifications was to reduce the level of Neo^R transcripts in clones resulting from random
20 integration. On the other hand, clones resulting from homologous recombination events between pGMD and a HOXC8 (Hox-3.1) locus should experience unimpaired growth during G418 selection, the degradation AT
25 sequence of mRNA being eliminated by the recombination process itself, or spliced with the HOXC8 (Hox-3.1) intron.

In the following experimental stages, the protocol described by Thompson et al. 1989 was followed
30 for the production of chimaera animals.

III - Transfection of embryonic mice cells.

The method described by Thompson et al. 1989 was used to transfect embryonic mice cells. Use of the electroporation method insures that only one copy of
35 the foreign gene (lacZ) is introduced per cell. After



transfection, several clones expressing β -galactosidase were isolated.

5 The mutagenesis plasmids pGMD and pGMA were linearised and introduced by electroporation in the E.S. cells to promote the insertion of a single copy in the genome (Ref.11).

The initial transfections were made to compare the targeting efficacy of HOXC8 (Hox-3.1) of the plasmids pGMA and pGMD (see Table I).

10

Homologous recombination in the gene HOXC8
(Hox-3.1)

15

Exp.	Plasmid of muta- genesis	No. of ensemble analysed	No. clones forming the ensemble	No. results P.C.R. positive
I	pGMA	3	600	0(2)
II	pGMD	5	250	3(5)
III	pGMD	84	2-3	5(5)

20

Table I

The E.C. cell line "C.C.E." (Ref. 16) was maintained in continuous manner on nutritious fibroblast layers (Ref.17). For experiments I and II, 1.5 x 10⁷ E.S. cells in 1.5ml HeBS were electroporated (Ref.1.1) at 200 V, with 40 mg of linearised plasmid, then plated over four culture boxes (diameter 100 mm).

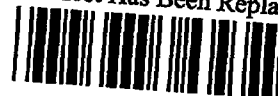
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For experiment III, the shock was performed under the same conditions but one quarter of the cells were plated over four 24-well dishes. The following day, 250 μ g ml⁻¹ G418 were added. Each transfection gave approximately 2400 clones with pGMA and approximately 1000 clones with pGMD.

30

The average number of clones of E.S. cells with G418 resistance in each set of cultures is given in Table I, with the number of sets which gave positive

35



results with the P.C.R. technique. A positive result signifies that a band of 1.6 Kb could be observed on agarose gel stained with ethidium bromide (see Fig.7). The number of culture sets which gave a positive signal after Southern analysis of the P.C.R. mixture and hybridization of a specific probe which did not contain the primer sequences is given between brackets (Fig.8).

Detection of homologous recombination with the P.C.R. method

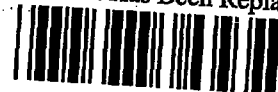
P.C.R. was made on 10^5 cells in a set of 250 clones of transfection II (see lane D of Fig.7). In the other lanes, four sets of transfection III were analysed together by mixing approximately 4 x 5000 cells. Primers 07 and 08 were used in the P.C.R. surrounding the 3'HOXC8 (Hox-3.1) sequence of the mutagenesis plasmid (Fig.2). The fragment of 1.6 Kb covering this 3' sequence can only be amplified in the event of homologous recombination. Lanes 2, 3 and D illustrate positive results.

The DNA of E.S. clones was prepared at the time of filter replication using the "boiling-proteinase K digestion boiling" method (Ref.18). 40 cycles of amplification (40 seconds at 94°C, 1 minute at 60°C, 7 minutes at 72°C) were performed in a reaction mixture of 100 µl, containing 67 mM Tris-HCL (pH 8.6), 16.7 mM $(\text{NH}_4)_2\text{SO}_4$, 5.7 mM MgCl_2 , 10mM 2-mercaptoethanol, 0.01 % (w/v) gelatin, 200 µM dATP, dTTP and dCTP, 100 µM dGTP, 100 µM 7-deaza dGT, 600 ng of each primer (07 : AACTTCCCTCTCTGCTATTC and 08: CAGCAGAAACATACAAGCTG) and 3U polymerase Taq (Perkin Elmer Cetus), covered with 100 µl paraffin. One half of the reaction mixture was applied to 0.7% agarose gel stained with ethidium bromide. The size marker was a Eco RI + Hind III digest of lambda DNA.



Southern analyses

Three clones independent from E.S. cells containing the muted HOXC8 (Hox-3.1) (identified by P.C.R.) were isolated from the positive sets using pipettes. Their DNA was determined by Southern analysis after digestion with the restriction enzymes indicated in Figure 8, in order to confirm the specific targeting and to distinguish between recombined and wild loci. Two different probes were used to analyse the 3' end of the HOXC8 (Hox-3.1) loci in the muted clones and in the non-muted E.S. cells used as controls (Fig.8c). The first "a" probe was contained in the HOXC8 (Hox-3.1) sequences of the mutagenesis plasmid and displayed the number of vector integrations and their physical bindings. One of the three recombined clones also contained a randomly integrated plasmid copy (Fig.8 (a), clone F2). The second "b" probe which was not contained in the mutagenesis vector differentiated between the recombined and wild HOXC8 (Hox-3.1) alleles (Fig. 8b). The recombined HOXC8 (Hox-3.1) locus displayed, with both probes, the hybridization image expected with the restriction sites of the mutagenesis vector and the intact locus. Also, the existence of two areas of recombination in the 3' limb of the vector confirmed the presence or the absence of the AT sequence in the recombined HOXC8 (Hox-3.1) locus (e.g.Fig.8, clone L5). The 5' end of the HOXC8 (Hox-3.1) locus was also analysed for the homologous recombination event. Restriction enzymes which showed no sites in the HOXC8 (Hox-3.1) 5' sequence of 6.8 Kb of the mutagenesis vector were used in the digestion of the DNAs of the recombined clones. These DNAs were subsequently subjected to electrophoresis in a pulsed field to differentiate the fragments of high molecular weight. Southern analysis of this gel also showed the correctly recombined alleles and the wild HOXC8 (Hox-



3.1) alleles, using a probe with a sequence upstream from the mutagenesis plasmid.

Southern analyses showed that an allele of the HOXC8 (Hox-3.1) gene was recombined as planned.

5 Homologous recombination was equivalent to double "crossing-over" between the genomic limbs of the mutagenesis plasmid and the homologous chromosome sequences (Fig.2).

10 In the recombinant clones, the lacZ gene was placed under the control of promoter and regulatory sequences of the HOXC8 (Hox-3.1) upstream from the AUG codon, but the 3' maturation signals of mRNA came from SV40. In these recombined clones, the expression of lacZ was not detectable by staining with β -Gal, which
15 correlates with the absence of HOXC8 (Hox-3.1) transcription in E.S. cells as determined by protection analysis of RNase. The activity of β -Gal could be induced in certain cells after 3 to 4 days' culture in $5 \cdot 10^{-7}$ M retinoic acid, conditions which are known to
20 induce the transcription of HOXC8 (Hox-3.1) (Ref.19).

By using the mutagenesis vector pGMA, which shows total 8.3 Kb DNA homology with the HOXC8 (Hox-3.1) locus, a fragment of 120 bp was replaced by an insertion of 7.2 Kb. The frequency of this targeted
25 replacement (1/900) compares with that recently obtained (1/1000) with HPRT (Ref.13) or with En-2 (1/260) (Ref.20), the inserted heterologous fragments in these latter cases, however, being of much smaller size (1.1 and 1.5 Kb respectively). In surprising
30 manner, it was noted that a very high frequency of homologous recombination (1/40) could be obtained with the pGMD vector. The elimination of the 3' maturation signals of mRNA and the addition of the degradation sequence of mRNA to the gene with neomycine resistance
35 led to reducing the total number of clones with G418 resistance by 2.4 (Table I). The specific targeting

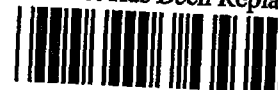


ratio was nearly 10 times higher (900/40). The mechanism of homologous recombination itself must have been affected in the experiments with pGMD. One possible explanation of this results is that the AT sequence of 51 bp could, *in vivo*, provide an open loop in the mutagenesis plasmid on account of its lower fusion temperature. If the HOXC8 (Hox-3.1) sequences which juxtapose the pGMD can be influenced by this opening, either side of the AT region, they could react in much more efficient manner, in the single stranded state, with the HOXC8 (Hox-3.1) chromosome locus. The pattern of mitotic recombination in yeast, suggests that it may be initiated by such strand exchange, even though the mechanism of homologous recombination remains unknown in more complex eucaryotes.

Figure 8 shows the results of Southern analyses made on positive individual clones (L5 and F2) and E.S. cells (C.C.E.).

The probes used only hybridize with the HOXC8 (Hox-3.1) sequences included in the (a) vector or excluded from the mutagenesis vector (b). The hybridization image of the recombined HOXC8 (Hox-3.1) locus (open triangles) clearly distinguishes itself from the wild locus (black triangles). The stars indicate the hybridization bands of a copy of the plasmid which integrated itself randomly. The size marker is a Eco RI + Hind III digest of lambda DNA.

Figure 8(c) shows the restriction sites of the recombined (rec.) and wild (wt) HOXC8 (Hox-3.1) alleles. The parts of the mutagenesis vector and of the HOXC8 (Hox-3.1) locus are indicated with the same symbols as those used in figure 2. In this case, the AT sequence was integrated by homologous recombination. The vertical arrow indicates the 3' end of the mutagenesis plasmid. The localisation of the "a" and



"b" probes used for the Southern analysis is also shown. The abbreviations used in figure 8 are as follows : B, Bam HI ; D, Dra I ; E, Eco RI ; H, Hind III ; S, Sal I ; X, Xho I.

5 IV - Production of chimaera embryos

A microinjection into blastocysts was made with the recombinant E.S. clones containing an intact HOXC8 (Hox-3.1) allele and a recombined allele; these clones did not contain any other copy of the mutagenesis plasmid. The karyotypes of these cells were normal.

10 Ten to fifteen muted cells were microinjected by blastocyst. After reimplantation in surrogate mothers, the embryos were collected at 9.5, 10.5 and 12.5 days p.c. and analysed for lacZ expression. The transcription code of HOXC8 (Hox-3.1) at these stages had been previously determined by in situ hybridization analysis (Ref.1). The HOXC8 (Hox-3.1) transcripts are detectable for the first time at the delayed gastrulation stage and are found in all tissues in the posterior region of the animal. Later, the distribution progressively becomes limited in space and tissue specific. At the 12.5 days p.c. stage the transcription is localised in the cervical region of the neural tube, about the heart. During embryogenesis, the distribution of the HOXC8 (Hox-3.1) transcription therefore undergoes change. The 10.5 days p.c. stage appears to be a transition period, the transcription taking place both in the two posterior regions and in the cervical neural tube.

30 In chimaera embryos of 9.5 and 10.5 days p.c., the part caudal to the posterior bud showed intense B-Gal activity, while the marker was never detected in the anterior thoracic region or in the head (Fig.9a). In the posterior region, cells stained with B-Gal were observed in all tissues of all embryonic layers. Between the two buds from which the limbs emerge,



stained cells were distributed, in restricted areas, in the superficial ectoderm (Fig. 9b) the posterior regions (Fig. 9c) and, in narrow lines or stripes, in the neural tube (Fig. 9b). These stripes showed irregular, asymmetric distribution on the wall of the neural tube. The transcription of HOXC8 (Hox-3.1) was not detected in the thin layer of cells towards the closed end of the neural tube. It is possible that these cells did not resist against the applied treatments during in situ hybridization. It was noted that the cells of the neural ectoderm form part of different parts of the nervous system at a very early stage and migrate in radial direction in close lateral movement (Ref.21). These results therefore correlate with this observation.

The expression of lacZ therefore correctly illustrated the first part of the transcription of the HOXC8 (Hox-3.1) homeogene, that is to say in all the tissues of the caudal regions of 9.5 and 10.5 days p.c. embryos, and gave new information on the method of transcription of HOXC8 (Hox-3.1).

On the other hand, the expression of lacZ was not observed in the cervical regions of the neural tube of chimaera embryos of 12.5 days, or in the anterior region of embryos of 10.5 days; this was not the result expected after in situ hybridization studies. The later phase of HOXC8 (Hox-3.1) transcription seen from day 10.5 onwards in very localised areas of the neural tube was not evidenced by β -Gal activity. One possible explanation for this result could be that, although the expression of lacZ is under the control of the HOXC8 (Hox-3.1) promoter, the 3' sequences of HOXC8 (Hox-3.1) are absent from the reporting gene. It is possible that the 3' sequences of the AUG initiation codon of HOXC8 (Hox-3.1) have an influence on the delayed expression of HOXC8 (Hox-3.1) in the anterior region. A "gene



proportioning" effect could also account for this result. The self-activation of several homeogenes in Drosophila has been shown genetically or suggested by the formation of complexes between the DNA and the homeobox proteins.

If the delayed component of the HOXC8 (Hox-3.1) transcription in the neural tube is maintained by a similar mechanism, the inactivation of an allele could produce a dominant effect in the cells of the neural ectoderm. Since only one allele may produce the HOXC8 (Hox-3.1) protein, the activation signal may be diluted on the two promoters. The reduced self-inactivation in both loci could therefore lead to total arrest in initiation of the transcription. This could explain why no expression of lacZ was detected in the cervical region of the neural tube of 10.5 and 12.5 day embryos.

V - Entry of the modification into the germinal cell line : production of transgenic animals.

The effects in F₁ and F₂ of the modification made by the insertion of the gene were observed after reproduction of the chimaera animals. Entry of the modification into the germinal cell line was observed.

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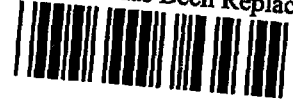


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CLAIMS

1. Process to obtain the expression of, or to enhance the expression level of a gene, or part of a gene, so-called receptor gene, in the genome of an eucaryotic cell, said receptor gene being able to be made functional or more effective when recombined with a complementary DNA, said process comprising :
- 5 - the introduction into the eucaryotic cells of a vector containing an insert itself comprising the complementary DNA, so-called insertion DNA, and two so-called "flanking" sequences either side of the
- 10 insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in relation to the receptor gene ;
- the insertion DNA comprising a gene or part of a gene, and being heterologous to the receptor gene ;
- 15 - the flanking sequences being chosen in order to permit, by homologous recombination, the juxtaposition of the insertion DNA in relation to the receptor gene, in such manner that the insertion DNA makes functional or enhances the functioning of the receptor gene ;
- 20 2. Process according to claim 1 in which the insertion DNA is entirely made up of a gene.
3. Process according to claim 1 in which the insertion DNA is made up of part of a gene, for example
- 25 a regulatory sequence.
4. Process according to claim 1 in which the receptor gene is a gene which is not normally transcribed in the eucaryotic cell.
5. Process according to claim 1 in which the
- 30 receptor gene has no selectable phenotype.
6. Recombined eucaryotic cell, able to express a gene or part of a gene, or of expressing it at an



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enhanced level, said cell being able to be reproduced according to the process of any of claims 1 to 5.

- 5 7. Process for inactivating the gene, so-called receptor gene, in the genome of an eucaryotic cell by the targeted insertion, in the receptor gene, of a DNA, so-called insertion DNA, the process comprising :
- 10 - the introduction into the eucaryotic cell of a vector containing an insert itself comprising the insertion DNA and two sequences, so-called "flanking" sequences, on either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in the receptor gene ;
- 15 - the insertion DNA being heterologous to the receptor gene and comprising a gene or part of a gene able to be made functional or whose functioning may be made more efficient when it is recombined with a complementary DNA in the receptor gene ;
- 20 - the flanking sequences, through homologous recombination, allow the insertion of the insertion DNA at the desired site in the receptor gene ;
- the expression of the insertion DNA subsequently replacing that of the receptor gene.



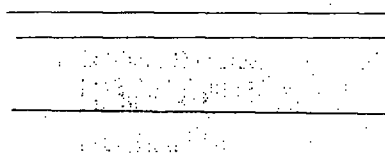
* C L M S *

ABSTRACT OF THE DISCLOSURE**PROCESS TO OBTAIN THE EXPRESSION OF OR TO ENHANCE THE LEVEL OF
EXPRESSION OF A GENE**

The invention relates to a process to obtain the expression of, or to enhance the level of expression of a gene or part of a gene, so-called receptor gene, in the genome of an eucaryotic cell, said receptor gene being able to be made functional or more efficient when it is recombined with a complementary DNA, said process comprising :

- the introduction into eucaryotic cells of a vector containing an insert, itself comprising the complementary DNA, so-called insertion DNA, and two sequences, so-called "flanking" sequences, either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in relation to the receptor gene;
- the insertion DNA comprising a gene or part of a gene, and being heterologous to the receptor gene ;
- the flanking sequences being chosen in order to permit, by homologous recombination, the juxtaposition of the insertion DNA in relation to the receptor gene, in such manner that the insertion DNA makes functional or enhances the functioning of the receptor gene.

Figure 1



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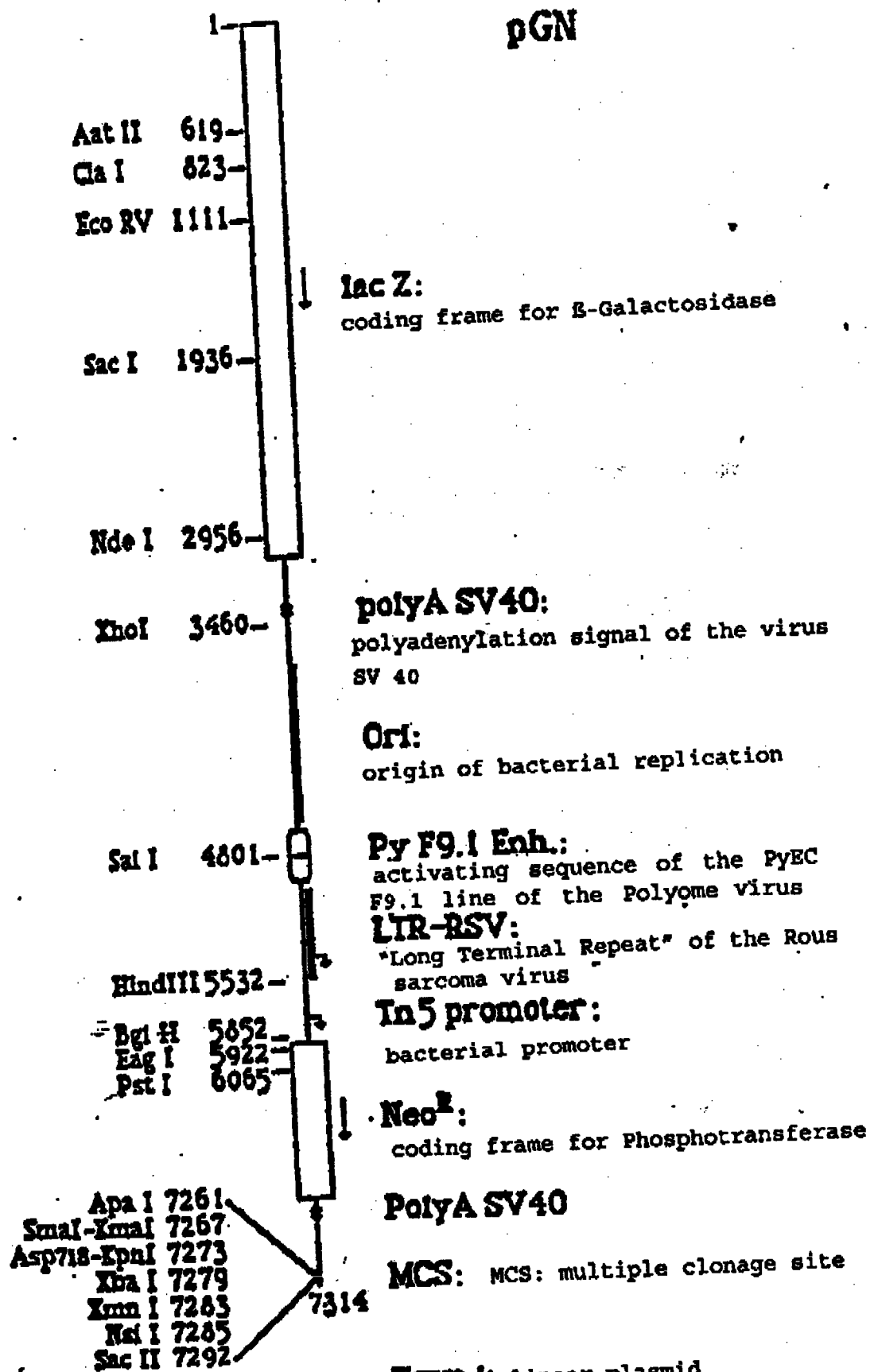
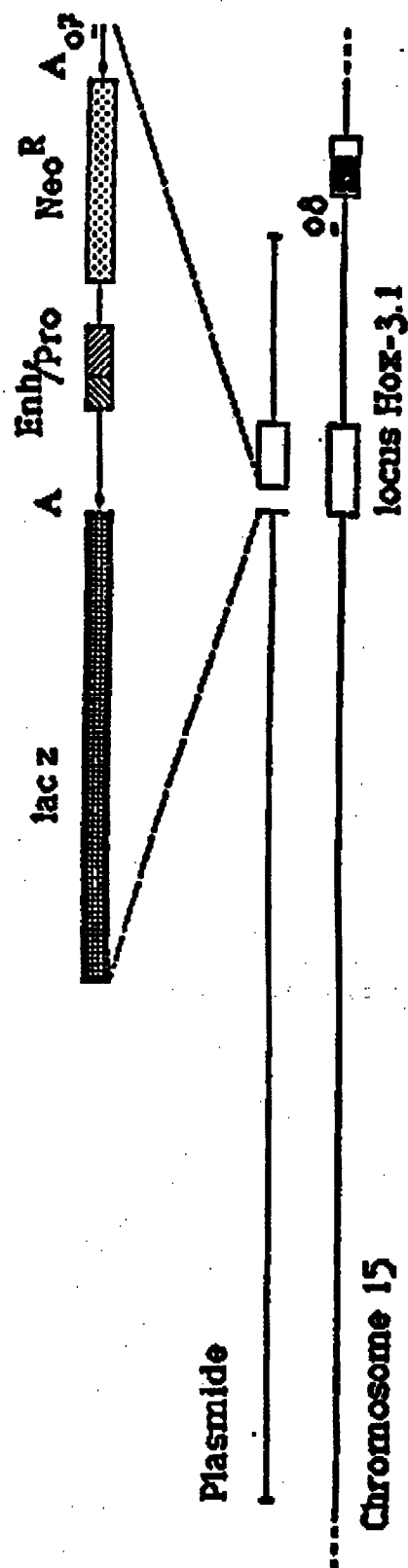


Figure 1: linear plasmid

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**FIGURE 2a**

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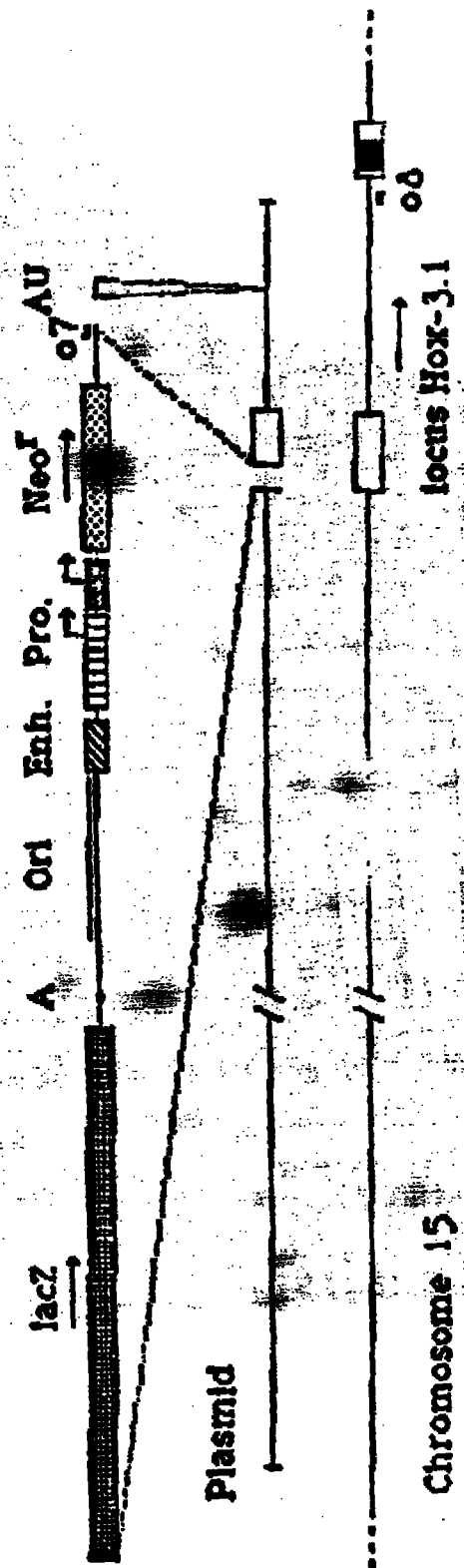


FIGURE 2b

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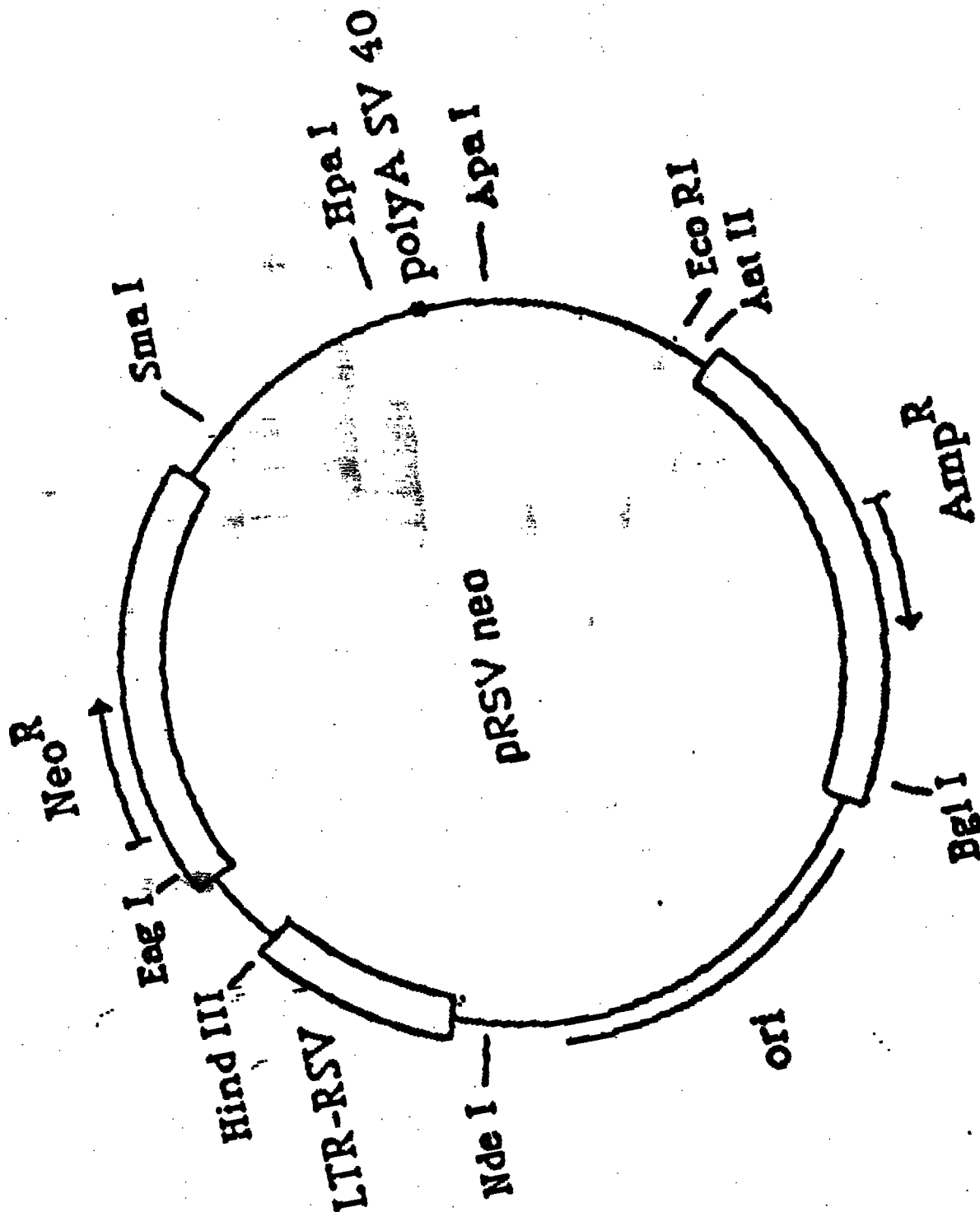


FIG. 3

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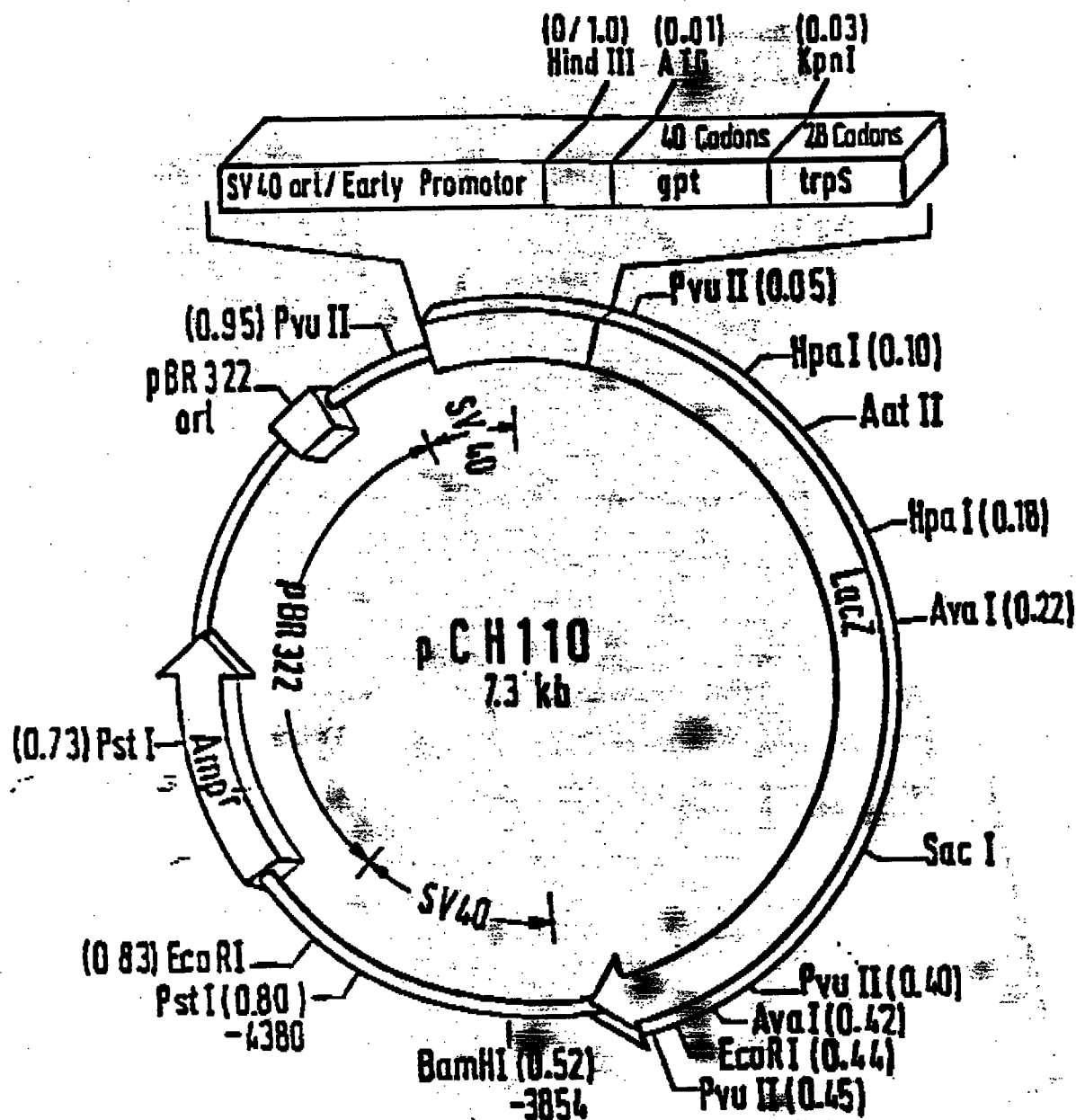


FIG. 5

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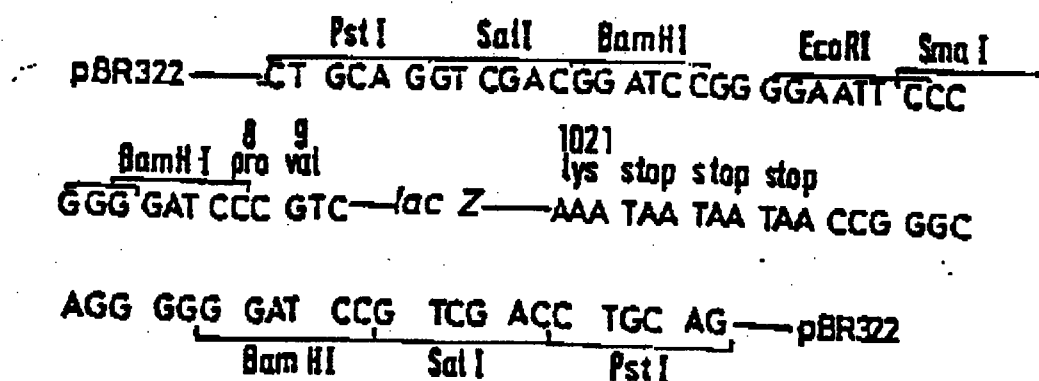
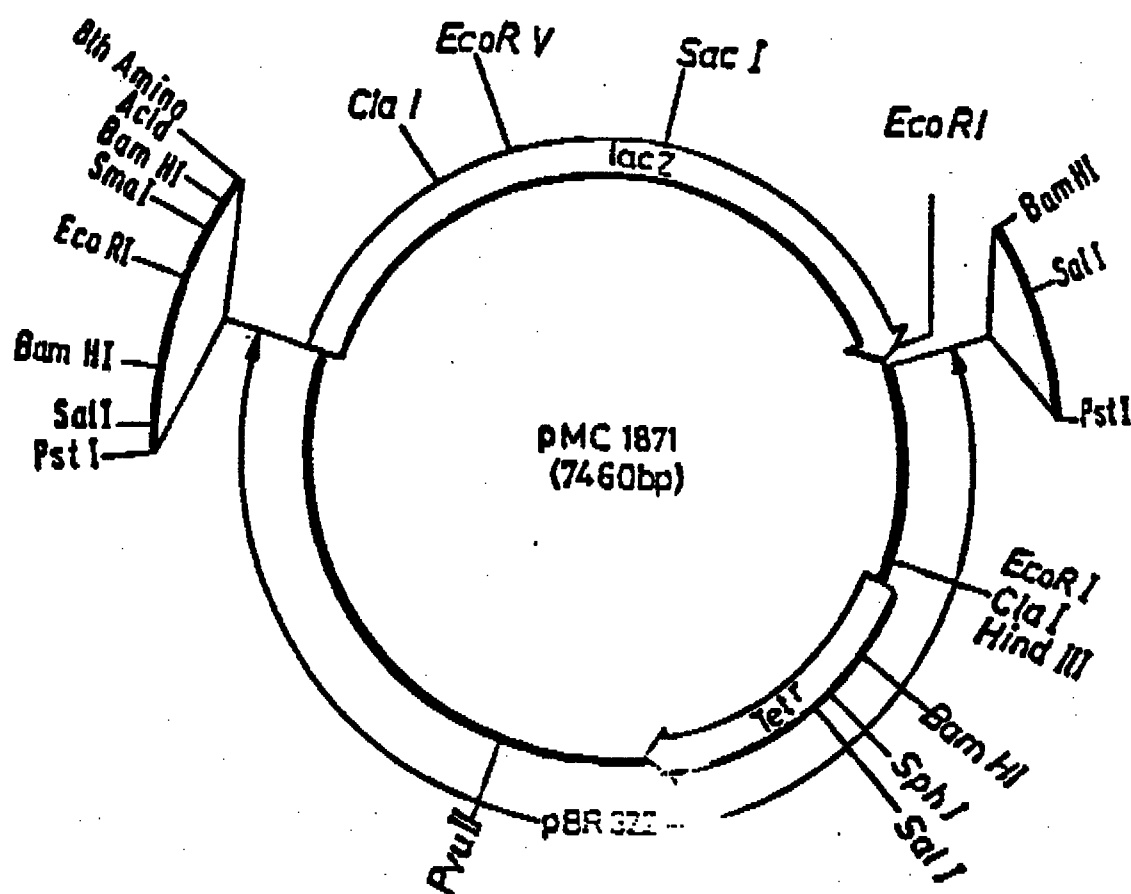


FIG.6

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1 2 3 4 5 6 7 8 9 10 M D

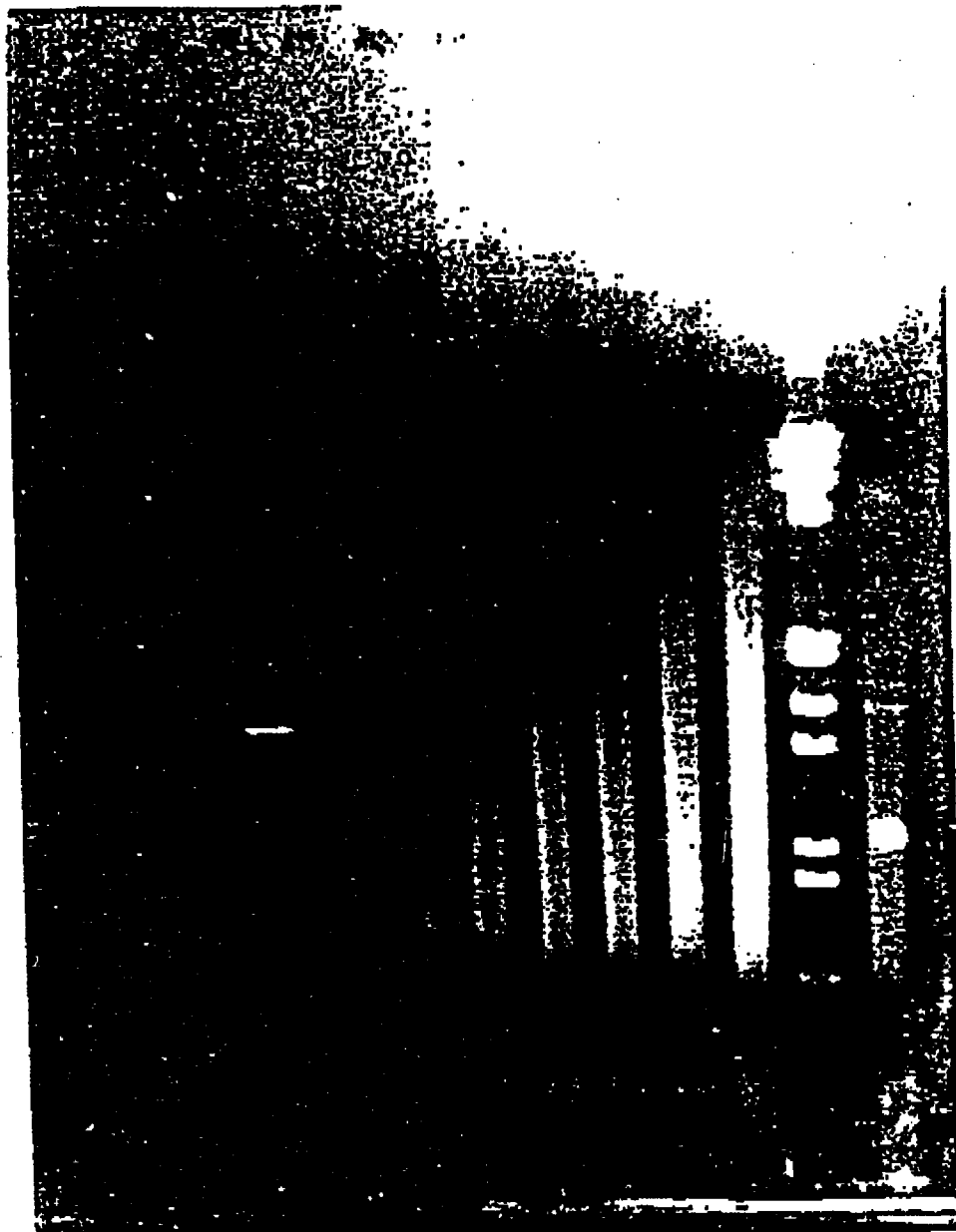


FIG. 7

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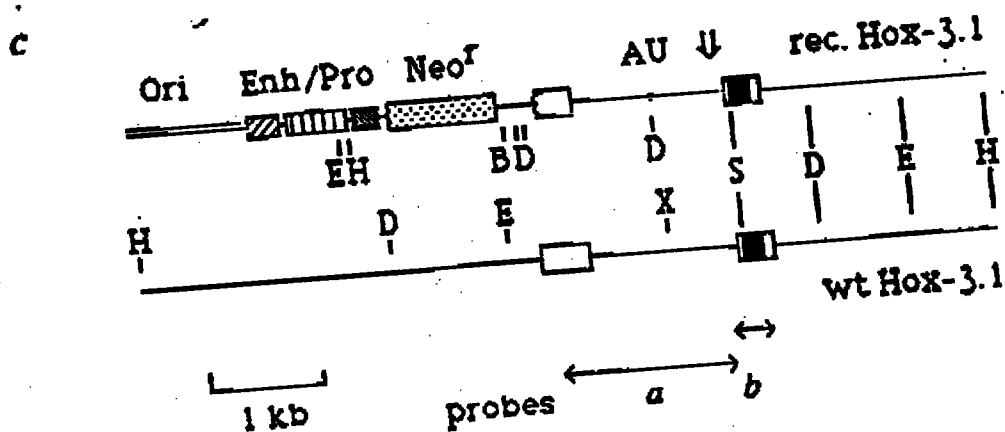
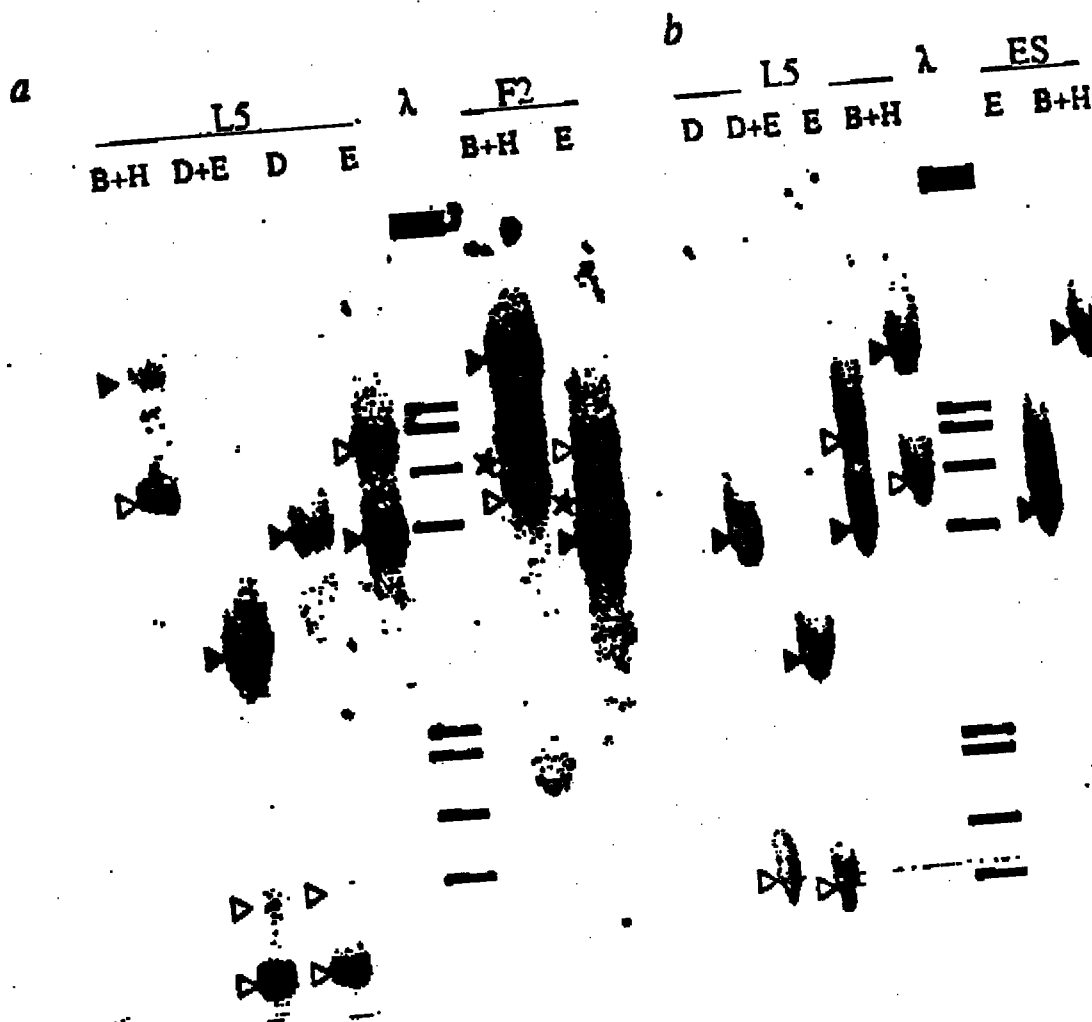


FIG. 8

GUTMANN-PLASSERAUD

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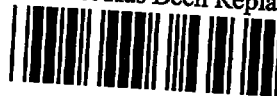
Fig. 9A

GUTMANN-FLASSERAUD

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Fig. 9B

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Fig. 9C

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PROCESS FOR SPECIFIC REPLACEMENT - A COPY OF A GENE PRESENT IN THE RECIPIENT GENOME BY INTEGRATION OF A GENE DIFFERENT FROM THAT WHERE THE INTEGRATION OCCURS

The present invention relates to a process for specific replacement of a copy of a gene present in the genome of a recipient eucaryotic organism by the integration of a gene different from the inactivated gene. The recipient gene will preferably be present in at least 2 examples in the transfected host cell. The recipient gene is defined as being the gene where the insertion of the different gene will be made.

More particularly, the invention relates to the production of transgenic animals in which the foreign gene has been introduced in a targeted manner to allow both the maintenance of the normal genetic functions of the animal and the expression of the foreign gene under the control of endogenous promoters.

By "different or foreign gene" is meant any nucleotide sequence corresponding to the totality or to a part of a "foreign or different" gene of the receptor gene as it is found normally in the genome (RNA or DNA), where it corresponds also to an artificially

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modified sequence of the normal gene or again to a fragment of this sequence.

The invention also concerns the process of production of these transgenic animals.

In the production of transgenic animals, the conventional methods used for the introduction of heterologous DNA sequences in the germ cell line do not allow control of the site of integration of the foreign gene in the genome, nor the number of copies thus introduced. The integration of the foreign gene is carried out at random and, in general, several copies of the gene combine at the same time, sometimes in the form of a head-to-tail tandem, the site of integration and the number of copies integrated varying from one transgenic animal to another.

The genes introduced in the germ line are:

- either "complete" genes, including coding sequences flanked by their own regulatory sequences;
- or composite genes, formed from the coding sequence of a gene fused with the promoter sequence of another gene, the two fragments sometimes even belonging to two different animal species.

It was thus possible to confirm that the specificity of the expression of genes in such and such a tissue is determined by their regulatory sequence(s).

The choice of the appropriate promoter for the expression of the foreign gene in the transgenic animal is thus of primordial importance.

It can thus happen that endogenous cellular genes, situated at the point of insertion, may thereby be inactivated, without this being easily detectable

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because of the numerous insertions made at random. If the product of these genes is important for the development of the animal, this development will be seriously disturbed. Moreover, the aleatory insertion of the foreign gene may take place at a site which is not appropriate for the expression of the gene. In addition, the fact that there is a variation in the site and in the number of insertions from animal to animal makes interpretation of studies of expression extremely difficult.

Besides, the directed mutagenesis of murine genes in embryonic stem cells has recently been carried out using a technique of gene targeting (Thomas et al., 1987; Thompson et al., 1989).

In the first case, the HPRT murine gene was mutated by insertion and replacement and, in the second case, a mutated HPRT gene was corrected. Thomson et al. widened their experiments as far as obtaining chimeric mice and recorded the passage of the genetic modification into the germ cell line.

In each of the documents quoted, the precise site of integration was targeted by homologous recombination between, on the one hand, exogenous sequences carrying the mutation or correction included in a vector, under the control of an exogenous promoter and, on the other hand, their genomic homologue. This being so, it must be pointed out that earlier authors have carried out their experiments on a specific gene (HPRT) whose mutation was accompanied by a detectable phenotype.

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The exogenous sequences on the vector thus serve both to target the site of integration and to introduce the modification. Following homologous recombination, the modified gene always finds itself in its normal genetic environment.

It should be recalled that a problem posed during the production of transgenic animals is the danger of inactivating an endogenous cellular gene existing at the insertion point of the foreign gene.

According to the function of the product of the inactivated gene, such an inactivation can lead to significant physiological or morphological disorders in the transgenic animal, or could even prevent its survival.

On the other hand, the inactivation of a gene could be considered as advantageous if the gene in question coded for a virus receptor or other infectious agent.

A major problem encountered in the production of transgenic animals is the obtaining of the expression of the foreign gene. In general, two types of experiments have been performed in mice.

The inventors have studied the possibility of avoiding the disadvantages described above, and associated in some cases with the possible inactivation of one or more endogenous cellular genes having an important function during the production of transgenic animals.

The object of the invention is a process for specific replacement, in particular by targeting a DNA, called insertion DNA constituted by part of a gene

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susceptible to being rendered functional, or this functioning can be made more efficient, when it is recombined with a complementary DNA to then provide a complete recombinant gene in the genome of a eucaryotic cell, characterized in that

- the insertion site is in a chosen gene, called a recipient gene, and containing the complementary DNA, and in that

- eucaryotic cells are transfected with a vector containing an insert which itself comprises the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene,

- the insertion DNA being heterologous vis-à-vis the recipient gene, and

- the flanking sequences, being chosen from those which make up the abovementioned complementary DNA and which allow, through homologous recombination with corresponding sequences of the recipient gene, the reconstitution of a complete recombinant gene in the genome of the eucaryotic cell.

The invention also relates to a process for the production of transgenic animals, characterized in that E.S. cells are transfected in the conditions described above and selected for the occurrence of homologous recombination, that is the correct integration of the foreign gene, the transfected cells are injected into embryos at a stage where they are able to integrate the transfected cells (for example, at the blastocyst

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stage), and these are then reimplanted in a surrogate mother and the chimeric individuals obtained at the end of gestation are mated. If the E.S. cells have colonized the germinal line of the chimeric animal, heterozygous transgenic animals for the replaced gene will be obtained by mating (F1) in the progeny.

The invention also concerns a plasmid able to effect the targeted insertion of a recombinant gene called the insertion gene in the genome of a eucaryotic cell, characterized in that it contains an insert which itself comprises the insertion gene and two so-called "flanking" sequences on either side of the insertion gene, respectively homologous to the two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene.

The invention also concerns transgenic animals in which at least one endogenous gene, has been inactivated by the insertion of a gene which is different from the inactivated gene, the insertion gene being inserted in a position which allows the expression of this gene under the control of regulatory sequences of the inactivated endogenous gene.

Thus, the process of the invention allows, thanks to the phenomenon of homologous recombination, the insertion in a targeted manner of foreign genes, in particular coding sequences without the promoter with which they are usually associated, in the genome of a eucaryotic organism at a site which allows their expression under the control of the endogenous promoter of the gene where the insertion is made, and

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consequently, the inactivation of the targeted endogenous gene.

In a preferred embodiment of the invention, the targeted recipient gene is a gene which is present in the genome in at least two copies. Utilization of the electroporation technique (ref. 11) ensures the introduction of only one copy of the foreign gene.

In this embodiment of the invention, the targeted insertion of the gene of interest (i.e. the so-called insertion gene) has the effect of inactivating the single copy of the endogenous cellular gene where the insertion takes place and leaves intact and operational the other copy (copies) of this gene.

In this way, the genetic functioning of the transgenic animal is not or is little disturbed by the introduction of the foreign gene, even if the insertion inactivates a single copy of a receiving gene essential for the development of the animal. Either its development would not be affected by the insertion of the foreign gene, or the possible minor disturbances in the case of inactivation of a critical gene would probably not be lethal for the animal. The effects of the insertion of the foreign gene in the homozygous state could be of any kind and would be observed in the 2nd generation (F2) after crossing heterozygous individuals (F1) among themselves.

If, on the other hand, the inactivation of all the copies of a gene is desired, for example in the case where the gene codes for a receptor of an infectious agent, multiple copies of the foreign gene

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are introduced. Control of the quantity introduced can be ensured by using known techniques.

The targeted insertion of the foreign gene thus allows its introduction in a site where its expression is under the control of regulatory sequences of the endogenous gene where the insertion takes place.

The process of the invention also allows insertion of the foreign gene behind an endogenous promoter which has the desired functions (for example, specificity of expression in a particular tissue) and this, in the relevant case, without inactivating the other copies of the recipient gene.

In another embodiment of the invention, the insertion DNA can carry a foreign regulatory sequence. The site of insertion and, consequently, the flanking sequences are chosen in relation with the desired aim, that is either the insertion of the foreign regulatory sequence to give an effect of "double promoter" with the endogenous regulatory sequence, or the replacement of an endogenous promoter by the foreign promoter. The coding sequence which comes under the control of the regulatory sequence can be endogenous.

Another possibility would be the targeted insertion of a foreign DNA which carries both a regulatory sequence and a coding sequence. It is possible for the regulatory sequence to be that which is naturally associated with the coding sequence.

The process of the invention activates a vector containing two "flanking" sequences on either side of the foreign gene. These flanking sequences have at least 150 base pairs and are preferably inferior to the

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length of the recipient gene. It is essential that these two flanking sequences be homologous to the two genomic sequences which are immediately adjacent to the desired insertion site. The flanking sequence of the vector which is located upstream of the foreign gene to be introduced is normally homologous to the genomic sequence which is located at the 5' end of the insertion site. In the same way, the flanking sequence of the vector located downstream of the foreign gene is normally homologous to the genomic sequence which is located at the 3' end of the site of insertion.

It is possible to introduce "intercalating" sequences between either one of the flanking sequences and the foreign gene, for example sequences permitting the selection of transformants (markers) sequences permitting cloning of the vector, etc.

The position of these intercalating sequences with respect to the foreign gene must however be chosen so as not to hinder the expression of the foreign gene, in particular the coding sequence of the foreign DNA under the control of the endogenous promoter or, inversely, the coding sequence of the endogenous DNA under the control of foreign elements of regulation carried by the insertion sequence.

Despite the presence of flanking sequences, which encourages homologous recombination, it is possible that a certain number of integrations take place at random. In order to verify that the targeted insertion has actually taken place in the targeted site and not in another place, the technique of "Polymerase Chain Reaction" (P.C.R.) (see Ref. 10) is used to

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amplify the DNA sequence of the locus where the insertion should have taken place. In this way, only the clones transformed following a homologous recombination are selected.

The flanking sequences of the vector are evidently chosen in relation to the desired site of insertion so that homologous recombination can take place. Thus the flanking sequences can carry duplicated sequences of the endogenous promoter and/or modifications to the sequences which precede the initiation codon to raise the translation level (upstream sequences) and duplicated sequences of the termination sequences particularly of the sites of polyadenylation (downstream sequences).

The insertion gene can be any gene of interest. One will cite as non-limiting examples the gene LacZ (as in the model described below), the genes coding for interleukin or interferon, receptor genes for example of retinoic acid or beta-3 adrenergic or H.I.V., and genes known to be linked with certain diseases, for example myopathy etc.

In a preferred embodiment of the invention, the eucaryotic cells are embryonic stem cells (see Ref. 14 and 15).

In fact, a mutated E.S. cell can be injected into an early embryo which, after reimplantation, can be born under a chimeric form. If the germ line is colonized by the mutated cell, the chimeric animal will transmit the mutation to its progeny. After this, it will be possible to observe the effects of this mutation, in the homozygous state in some individuals,

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on their development, their behaviour, their metabolism, their pathology etc.

Figure 1 shows the pGN plasmid.

Figure 2 shows the molecule constructed from the pGN plasmid in relation to the gene Hox-3.1.

Figures 3 to 6 show the plasmids used in the construction of the pGN.

The process of the invention has a very wide industrial applicability and can vary according to the nature of the foreign gene introduced.

The genetics of mammals will progress considerably in view of the recent possibility of specifically mutating any gene, thus making it possible to define its role more accurately. Through this technology which involves homologous recombinations and E.S. cells, valuable information will be obtained on oncogenes, growth factors, transcription factors etc., genes which concern very actual subjects in fundamental research or in applied research. An important outcome for medical research is the possibility of reproducing a human disease whose genetic determination is known (certain pathological human diseases, such as the myopathy of Duchesne) so as to study its mechanisms better and search for a therapy.

By applying the process of the invention, a gene known to be responsible for a certain disease is inserted in a targeted manner into the genome of an E.S. cell. The transgenic animal which results from this process presents a useful model of this disease.

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If necessary, and as described above, normal genetic functions can be significantly maintained, despite the insertion of the foreign gene.

Another application of the process of the invention consists in inserting an insertion gene which is easily detectable e.g. the LacZ gene which can then play the role of a cell marker. In this way, filiation studies e.g. in competition animals are facilitated, and the breed can be followed up.

Insertion of the LacZ gene as insertion gene also makes promoter studies possible. In view of the possibility of identifying the activity of β -galactosidase, the activity and specificity of different endogenous promoters can be studied by targeting different sites in the same type or different types of cells. The same studies could be carried out on an entire organism, during development or at the adult stage, by using the techniques of chimeric or transgenic animals.

In view of the possibility of modifying the genome of an animal, the process of the invention can thus also be used as "gene therapy". The most obvious utilizations would consist in inactivating the genes of receptors of toxic or infectious agents (viruses or bacteria). If this mutagenesis proved to be lethal, it would be necessary to re-establish the lost function without re-establishing the sensitivity to the harmful agents. A modified gene coding for such a receptor could be reintroduced into the mutated cell, as long as the modification can be provoked by homologous recombination. This modification of the genetic

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character would confer on the animal an immunity against the disease under consideration.

This protocol can also intervene in the framework of auto-grafting. Cells, sick or healthy, taken from a patient, could be treated and immunized, then reimplanted back into the same individual.

The technique of the invention is also suitable for studies of the activity of pharmaceutical products presumed to have an activity with regard to products of expression of a pathological gene linked to a disease. In this case, the insertion gene is constituted by the pathological gene and one administers to the transgenic animal the pharmaceutical product with a view to evaluating its action on the disease.

The invention will be illustrated by referring to the pGN plasmid and its utilization in the targeted insertion of a foreign gene (LacZ, coding for the enzyme β -galactosidase of E.coli) in the genome of a mouse E.S. cell. The gene LacZ was chosen because of the fact that its expression can easily be detected and is used simply to demonstrate the feasibility of the technique.

The coding frame of the enzyme β -galactosidase of E. Coli (LacZ; 1-3057), fused with a genomic sequence (7292-3) of the murine gene Hox.3-1 (Ref. 1) begins with the initiation codon of this gene. In fact, the sequence which precedes the initiation codon of Hox-3.1 is identical to the consensus sequence observed in vertebrates (Ref.2), thus allowing a better translation rate of β -galactosidase into the cells of vertebrates. The gene LacZ is followed by a signal of

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polyadenylation for example of the virus SV 40, like most of the eucaryotic genes, so as to stabilize the messenger RNAs.

The activity of β -galactosidase of E. Coli, which is functional in the eucaryotic cells, can be detected in different ways. Cells expressing the LacZ gene take on a blue colouring, after fixation, in the presence of X-Gal, which is a substrate of β -galactosidase (Ref. 3). A new substrate, FDG (fluorescein di- β -galactopyranoside), makes it possible to detect and to dose the β -gal activity, while still keeping the cells alive (Ref. 4). The cells expressing LacZ accumulate a fluorescent product and can be isolated with the aid of a cell sorter or FACS (fluorescence-activated cell sorter).

The transcription unit of the gene with resistance to neomycin comes, to a large extent, from the plasmid pRSV neo (Ref. 5). The LTR (long terminal repeat) of the virus of Rous-sarcoma provides very powerful activating and promoting sequences in numerous eucaryotic cells (Ref. 6). From the bacterial transposon Tn5 comes a promoter active in E. Coli and the coding phase of the enzyme phosphotransferase (Ref. 7), which is followed by the polyadenylation signal of the virus SV40. The same gene, under the double control of promoters RSV and Tn5 can confer resistance to neomycin or kanamycin on bacteria and resistance to G418 on eucaryotic cells.

Through the effect of a single point mutation, unit B of the activating (enhancer) sequences of the strain PyEC F9.1 of the Polyoma virus has become much

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more active in different types of cells, and in particular in the cells of embryonic carcinoma (EC) (Ref. 8). Two copies of this enhancer Py F9.1 have been inserted in tandem in the pGN plasmid, upstream of the LTR-RSV, and in the "later promoter" orientation of the regulatory region of the Polyoma.

In order to improve the translation rate of phosphotransferase, the sequence preceding the initiation codon was modified during a mutagenesis by oligonucleotide. Thus the sequence T T C G C A U G became G C A C C A U G, corresponding much better to the consensus sequence of initiation of translation in vertebrates (Ref. 2).

The improvements made to the transcription unit of the gene of resistance to neomycin could be estimated by transfecting embryonic cell strains (ES) of mice. With equal molarity in plasmid, a construction with the enhancer Py F9.1 produced 7.5x more clones resistant to G418 than pRSV neo. Again, the number of clones was raised by 60x, that is 450x compared with pRSV neo, by modifying the sequence of initiation of translation.

Moreover, the pGN plasmid contains an origin of bacterial replication of the type colE1, pBR322, which allows clonings and preparations in E. Coli.

Finally, a multiple cloning site (M.C.S.), synthesized in vitro, which only contains unique restriction sites in pGN, was inserted upstream of LacZ, so as to facilitate the utilizations of this plasmid.

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The plasmid "flanking" sequences which provoke homologous recombination are added to the extremities of the pGN plasmid after linearization of the plasmid upstream from LacZ, by an MCS site (see Fig. 2). In this case, the flanking sequences chosen are homologous to the chromosome sequences taken from Hox-3.1 which intervene later in the homologous recombination.

Figure 2 provides the location of the molecule built from the pGN plasmid with respect to the gene Hox-3.1. In this case, recombination between the plasmid and chromosomal sequences of Hox-3.1 would result in an insertion at the beginning of the coding frame of this gene, thus in its total inactivation.

The pGN plasmid comprises several advantages for this methodology, which is applicable to any gene. As the event of homologous recombination can be fairly rare (of the order of 1 per 1000 non-homologous integrations), it is necessary to be able to analyse a large number of clones whose resistance to G418 is sufficiently strong to be expressed in any part of the genome. The modifications made to the transcription unit of phosphotransferase answer these problems perfectly. The method of mutagenesis by homologous recombination is equivalent to inactivating a gene by an insertion or a substitution, but the pGN plasmid presents the supplementary advantage of being able to substitute the expression of β -galactosidase to that of the mutated gene. Finally, the MCS facilitates the cloning of genomic fragments.

EXAMPLES:

I - Construction of the pGN plasmid

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The intermediate plasmids are numbered according to their stage.

1st stage:

Insertion of a Xho I site in the Bgl I site of pRSV neo

Insertion of a Xho I linker in the Bgl I site of pRSV neo, filled by the Klenow fragment of the DNA polymerase of E. Coli.

2nd stage:

Insertion of a Cla I site in the Nde I site of the p1 plasmid

Insertion of a Cla I linker in the Nde I site of p1, filled by Klenow polymerase.

3rd stage:

Insertion of Py F9.1 enhancer in the Cla I site of the p2 plasmid

Insertion of enhancer Py F9.1 Pvu II-Pvu II isolated by a unique Acc I site, in the Cla I site of p2. Selection of a clone containing two enhancers oriented in the direction "later promoter".

4th stage:

Deletion Sma I-Hpa I of the p3 plasmid

The two enzymes, presenting "blunt end" extremities, can be bonded directly. This deletion removes the intron of the t antigen of SV 40, which is not very useful, and reduces appreciably the size of the transcription unit of phosphotransferase.

5th stage:

Insertion of a Xho I site in the Bam HI site of pCH110

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Insertion of a Xho I linker in the Bam HI site of plasmid pCH 110 (Pharmacia), filled by the Klenow polymerase.

6th stage:

Insertion of 3' LacZ-polyA SV 40 in the p4 plasmid

The 3' end of the coding phase of β -galactosidase, followed by the signal of polyadenylation of the virus SV 40 is isolated from the p5 plasmid through sites Xho I-Aat II and cloned in the p4 plasmid through the same sites.

7th stage:

Insertion of 5' LacZ in the KS vector

The 5' end of the coding phase of β -galactosidase is isolated from plasmid pMC 1871 (Pharmacia) through the sites Pst I-Sac I and cloned in the vector KS- (Stratagene) through the same sites.

8th stage:

Fusion of a genomic sequence Hox-3.1 with 5' LacZ

A genomic sequence of the gene Hox-3.1, cloned in the vector KS-, is purified by successive digesting with the enzyme Sac I, then with the Mung bean nuclease and finally with the enzyme Apa I. This insert is fused with the 5' end of the coding phase of β -galactosidase by cloning in the p7 plasmid digested by Apa I-SmaI. The protein thus fused contains the initiation codon of the translation of the gene Hox-3.1 followed by the coding phase of β -galactosidase (then verified by sequencing).

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Met Ser Ser	Ile Pro Gly Asp Pro
CCAGC <u>ATG AGC TCC</u>	ATT <u>CCC GGG</u> GAT CCC
GGTCG TAC TCG AGG	TAA GGG CCC CTA GGG

↓ Sac I

CCAGC ATG AGC T

↓ Sma I

GGTCG TAC

↓ Mung bean nuclease

CCAGC ATG GGG GAT CCC

GGTCG TAC ⇔ CCC CTG GGG

Met Gly Asp Pro

CCAGC ATG GGG GAT CCC

GGTCG TAC CCC CTA GGG

9th stage:

Insertion of Hox-3.1-5' LacZ in the p6 plasmid

The fusion Hox-3.1-5' LacZ is isolated from the p8 plasmid through the Apa I-Sac I sites and cloned into the p6 plasmid through the same sites. The effect of this cloning is to reconstitute the coding phase of β -galactosidase in its totality.

10th stage:

Insertion of the gene Neo^R in the vector KS+

The gene of resistance to neomycin (bacterial promoter and coding phase of phosphotransferase) is isolated in the pRSV neo through the sites Hind III-Eco RI and cloned in the vector KS+ (Stratagene).

11th stage:

Mutagenesis of the initiation sequence of Neo^R in p10

The sequence of the initiation of translation of phnosphotransferase is modified to be identical to

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the consensus sequence observed in vertebrates and thus to allow a higher level of initiation of translation and therefore an increased resistance to G418 for the cells of mammals. The modification also creates an Apa LI site which makes it possible to control the efficiency of the mutagenesis.

Apa LI

GTTCGCGATG ⇒ GTGCACCATG

An oligonucleotide

(CTTGTTCAATCATGGTGCACGATCCTCA)

comprising a region of mismatch pairing with the sequence of pRSV neo (underlined) is synthesized (Gene Assembler, Pharmacia) then phosphorylated by the polynucleotide kinase of the T4 bacteriophage. A single-strand template of the p10 plasmid is prepared with the f1 origin of the KS+ plasmid and hybridized with the mutagenesis oligonucleotide. The second strand is synthesized and repaired by the Klenow polymerase and the DNA ligase of the T4 bacteriophage. After transformation of bacteria, the mutated clones are screened with the aid of the oligonucleotide marked with ³²P. The mutagenesis was verified by digesting with Apa LI and also by sequencing.

12th stage:

Replacement of the initiation sequence in the p9 plasmid

A fragment containing the modified sequence of initiation of translation of the gene of resistance to neomycin is isolated from the p11 plasmid through the

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enzymes Hind III-Eag I and cloned in the p9 plasmid through the same sites.

13th stage:

Insertion of the multiple cloning site in the p12 plasmid

Two complementary oligonucleotides are synthesized (Gene Assembler, Pharmacia) then phosphorylated. After pairing, the MCS is cloned in the sites Apa I-Sac II of the p12 plasmid through its sticky ends.

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      XmaI   Asp 718           XmnI
  ApaI   SmaI   KpnI   XbaI   NsiI   SacII
5' C C C C G G G G G T A C C T C T A G A A T G C A T T C C G C 3'
3' C C G G G G G G C C C C C A T G G A G A T C T T A C G T A A G G 5'

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The multiple cloning site was also verified by sequencing.

II - Addition of "flanking sequences" at the extremities of the linearized pGN plasmid upstream from LacZ' by an M.C.S. site

The flanking sequences used were chosen in relation to the desired site of insertion (for example, Hox-3.1, see Fig. 2)

In the experimental stages which follow, the protocol described by Thomson et al. 1989, was followed for the production of chimeric animals.

III - Transfection of embryonic mouse cells

The method described by Thompson et al. 1989, was used to transfect embryonic mouse cells. Utilization of the technique of electroporation ensures the introduction of a single copy of the foreign gene

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(LacZ) per cell. After transfection, several clones expressing β -galactosidase were isolated.

IV - Production of chimeric mice

The clones expressing β -galactosidase were injected into the blastocysts and colonized normal individuals and made them chimeric.

V - Passage of the modification into the germ cell line: production of transgenic animals

The effects in F_1 and in F_2 of the modification brought by targeted insertion were observed after reproduction of the chimeric animals. Passage of the modification into the germ cell line was observed.

VI - Use of the P.C.R. technique

ES cells were also transfected with the Hox-3.1 mutagenesis plasmid (Figure 2). Several homologous recombination events were identified by the "polymerase chain reaction" (PCR).

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CLAIMS

1. Process for specific replacement, in particular by targeting a DNA, called insertion DNA constituted by a part of a gene, susceptible to being rendered functional, or this functioning can be made more efficient, when it is recombined with a complementary DNA to then provide a complete recombinant gene in the genome of a eucaryotic cell, characterized in that

- the insertion site is in a chosen gene, called a chosen recipient gene, and containing the complementary DNA, and in that

- eucaryotic cells are transfected with a vector containing an insert which itself comprises the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene,

- the insertion DNA being heterologous vis-à-vis the recipient gene, and

- the flanking sequences, being chosen from those which make up the abovementioned complementary DNA and which allow, through homologous recombination with corresponding sequences of the recipient gene, the reconstitution of a complete recombinant gene in the genome of the eucaryotic cell.

2. Process according to Claim 1, for targeted insertion of a DNA, termed insertion DNA containing either a coding sequence or a regulatory sequence into the genome of an eucaryotic cell, characterized in that

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- the insertion site is in a chosen gene, called a recipient gene, and in that

- eucaryotic cells are transfected with a vector containing an insert which itself comprises the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene,

- the insertion DNA being heterologous with respect to the recipient gene, and

- the flanking sequences being chosen so as to permit by homologous recombination depending on the case, either the expression of the coding sequence of the entire insertion DNA under the control of the regulatory sequences of the recipient gene, or the expression of a coding sequence of the recipient gene under the control of regulatory sequences of the insertion DNA.

3. Process according to Claim 1 or 2, characterized in that the insertion DNA contains a coding sequence without the regulatory element, especially a promoter which is specific to it.

4. Process according to any one of Claims 1 to 3, characterized in that the recipient gene is present in the genome of the eucaryotic cell in at least two copies.

5. Process according to any one of Claims 1 to 4, characterized in that each of the flanking sequences has a length greater than 150 base pairs, and less than the length of the recipient gene.

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6. Process according to any one of Claims 1 to 5, characterized in that the eucaryotic cells are embryonic stem (E.S.) cells.

7. Process according to one of the preceding claims, characterized in that the insertion gene is a gene heterologous to the transfected species.

8. Process according to one of the preceding claims, characterized in that the vector contains sequences intercalated between the insertion gene and the flanking sequences.

9. Process according to Claim 8, characterized in that the intercalating sequences contain a marker permitting the selection of the transformants and/or the cloning of the vector.

10. Process according to one of the preceding claims, characterized in that the transfection is performed by electroporation.

11. Process according to one of the preceding claims, characterized in that the Polymerase Chain Reaction (P.C.R.) technique is used to amplify the DNA sequence of the locus where the insertion occurs in order to verify that the insertion took place at the desired site.

12. Process for the production of transgenic animals, characterized in that E.S. cells are transfected by the process according to one of Claims 1 to 11 and selected for the occurrence of homologous recombination, that is the correct integration of the foreign gene, the cells are injected into embryos at a stage where they are able to integrate the transfected cells (for example, at the blastocyst stage), and these

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are then reimplanted in a surrogate mother and the chimeric individuals obtained at the end of gestation, and in which is noted the colonization through E.S. cells of the germ line, are mated in order to obtain heterozygous transgenic animals for the replaced gene.

13. Plasmid capable of carrying out the targeted insertion of a so-called insertion gene into the genome of an eucaryotic cell, characterized in that it contains an insert which itself comprises the insertion gene and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are adjacent to the desired site of insertion in the recipient gene.

14. Plasmid pGN as illustrated in Figure 1.

15. Eucaryotic cells transformed by the process of Claim 1.

16. Cells according to Claim 15, characterized in that they are E.S. cells.

17. Transgenic animal in which a single copy of a gene which is present in the genome in at least two copies, has been inactivated by the insertion of a gene which is different from the inactivated gene, the insertion gene being inserted in a position which permits the expression of this gene under the control of regulatory sequences of the inactivated endogenous gene.

18. Application of the process of Claim 1 for gene therapy.

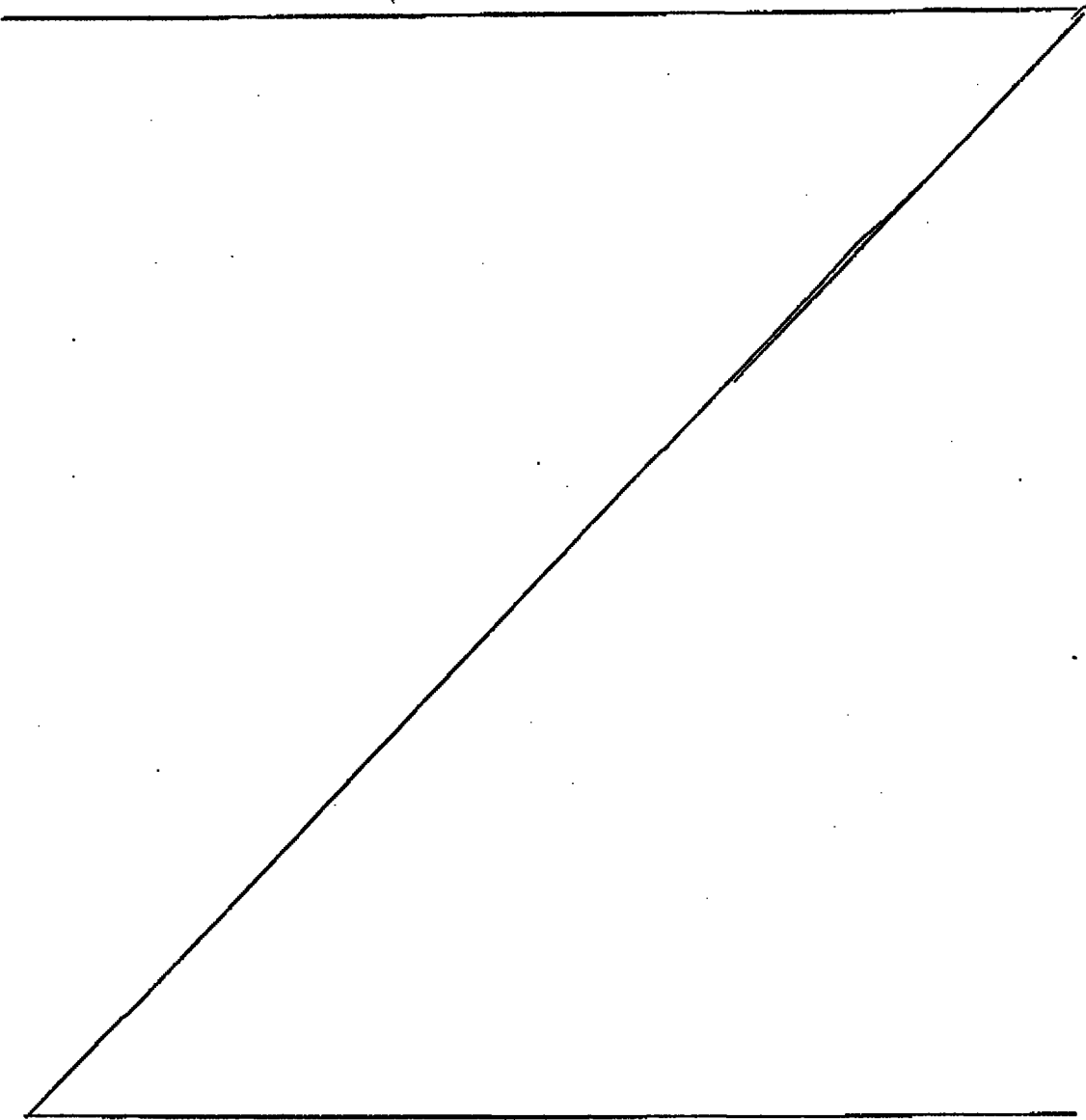
19. Application of the process of Claim 1 for the production of transgenic animals.

20. Application of the process of Claim 16 for genetic marking of animals.

21. Application of the process of Claim 1 for screening pharmaceutical products presumed to have an activity with regard to the expression products of a pathological gene linked to a disease, characterized in that the insertion gene consists of the pathological gene or a fragment thereof and in that the pharmaceutical product to be tested is administered to the transgenic animal in order to evaluate its activity on the disease.

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In a preferred embodiment of the invention, the eucaryotic cells are embryonic stem cells (see Ref. 14 and 15).

In fact, a mutated E.S. cell can be injected into an early embryo which, after reimplantation, can be born under a chimeric form. If the germ line is colonized by the mutated cell, the chimeric animal will transmit the mutation to its progeny. After this, it will be possible to observe the effects of this mutation, in the homozygous state in some individuals,

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on their development, their behaviour, their metabolism, their pathology etc.

Figure 1 shows the pGN plasmid.

Figure 2 shows the molecule constructed from the pGN plasmid in relation to the gene Hox-3.1. This plasmid is a mutagenesis plasmid. The two parts of the coding frame of the Hox-3.1 gene are represented, on chromosome 15, with the "homeo" box in black. The corresponding sequences of Hox-3.1 were cloned into the pGN plasmid.

(A: polyadenylation signal; Enh/Pro: enhancer-promoter). 07 and 08 represent the two oligonucleotides used in the PCR.

Figures 3 to 6 show the plasmids used in the construction of the pGN.

The process of the invention has a very wide industrial applicability and can vary according to the nature of the foreign gene introduced.

The genetics of mammals will progress considerably in view of the recent possibility of specifically mutating any gene, thus making it possible to define its role more accurately. Through this technology which involves homologous recombinations and E.S. cells, valuable information will be obtained on oncogenes, growth factors, transcription factors etc., genes which concern very actual subjects in fundamental research or in applied research. An important outcome for medical research is the possibility of reproducing a human disease whose genetic determination is known (certain pathological human diseases, such as the myopathy of Duchesne) so as to study its mechanisms better and search for a therapy.

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By applying the process of the invention, a gene known to be responsible for a certain disease is inserted in a targeted manner into the genome of an E.S. cell. The transgenic animal which results from this process presents a useful model of this disease.

If necessary, and as described above, normal genetic functions can be significantly maintained, despite the insertion of the foreign gene.

Another application of the process of the invention consists in inserting an insertion gene which is easily detectable e.g. the LacZ gene which can then play the role of a cell marker. In this way, filiation studies e.g. in competition animals are facilitated, and the breed can be followed up.

Insertion of the LacZ gene as insertion gene also makes promoter studies possible. In view of the possibility of identifying the activity of β -galactosidase, the activity and specificity of different endogenous promoters can be studied by targeting different sites in the same type or different types of cells. The same studies could be carried out on an entire organism, during development or at the adult stage, by using the techniques of chimeric or transgenic animals.

In view of the possibility of modifying the genome of an animal, the process of the invention can thus also be used as "gene therapy". The most obvious utilizations would consist in inactivating the genes of receptors of toxic or infectious agents (viruses or bacteria). If this mutagenesis proved to be lethal, it would be necessary to re-establish the lost function without re-establishing the sensitivity to the harmful

agents. A modified gene coding for such a receptor could be reintroduced into the mutated cell, as long as the modification can be provoked by homologous recombination. This modification of the genetic character would confer on the animal an immunity against the disease under consideration.

This protocol can also intervene in the framework of auto-grafting. Cells, sick or healthy, taken from a patient, could be treated and immunized, then reimplanted back into the same individual.

The technique of the invention is also suitable for studies of the activity of pharmaceutical products presumed to have an activity with regard to products of expression of a pathological gene linked to a disease. In this case, the insertion gene is constituted by the pathological gene and one administers to the transgenic animal the pharmaceutical product with a view to evaluating its action on the disease.

The invention will be illustrated by referring to the pGN plasmid and its utilization in the targeted insertion of a foreign gene (LacZ, coding for the enzyme β -galactosidase of E.coli) in the genome of a mouse E.S. cell. The gene LacZ was chosen because of the fact that its expression can easily be detected and is used simply to demonstrate the feasibility of the technique.

The coding frame of the enzyme β -galactosidase of E. Coli (LacZ; 1-3057), fused with a genomic sequence (7292-3) of the murine gene Hox.3-1 (Ref. 1) begins with the initiation codon of this gene. In fact, the sequence which precedes the initiation codon of Hox-3.1 is identical to the consensus sequence observed

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in vertebrates (Ref.2), thus allowing a better translation rate of β -galactosidase into the cells of vertebrates. The gene LacZ is followed by a signal of polyadenylation for example of the virus SV 40, like most of the eucaryotic genes, so as to stabilize the messenger RNAs.

The activity of β -galactosidase of E. Coli, which is functional in the eucaryotic cells, can be detected in different ways. Cells expressing the LacZ gene take on a blue colouring, after fixation, in the presence of X-Gal, which is a substrate of β -galactosidase (Ref. 3). A new substrate, FDG (fluorescein di- β -galactopyranoside), makes it possible to detect and to dose the β -gal activity, while still keeping the cells alive (Ref. 4). The cells expressing LacZ accumulate a fluorescent product and can be isolated with the aid of a cell sorter or FACS (fluorescence-activated cell sorter).

The transcription unit of the gene with resistance to neomycin comes, to a large extent, from the plasmid pRSV neo (Ref. 5). The LTR (long terminal repeat) of the virus of Rous-sarcoma provides very powerful activating and promoting sequences in numerous eucaryotic cells (Ref. 6). From the bacterial transposon Tn5 comes a promoter active in E. Coli and the coding phase of the enzyme phosphotransferase (Ref. 7), which is followed by the polyadenylation signal of the virus SV40. The same gene, under the double control of promoters RSV and Tn5 can confer resistance to neomycin or kanamycin on bacteria and resistance to G418 on eucaryotic cells.

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Through the effect of a single point mutation, unit B of the activating (enhancer) sequences of the strain PyEC F9.1 of the Polyoma virus has become much more active in different types of cells, and in particular in the cells of embryonic carcinoma (EC) (Ref. 8). Two copies of this enhancer Py F9.1 have been inserted in tandem in the pGN plasmid, upstream of the LTR-RSV, and in the "later promoter" orientation of the regulatory region of the Polyoma.

In order to improve the translation rate of phosphotransferase, the sequence preceding the initiation codon was modified during a mutagenesis by oligonucleotide. Thus the sequence T T C G C A U G became G C A C C A U G, corresponding much better to the consensus sequence of initiation of translation in vertebrates (Ref. 2).

The improvements made to the transcription unit of the gene of resistance to neomycin could be estimated by transfecting embryonic cell strains (ES) of mice. With equal molarity in plasmid, a construction with the enhancer Py F9.1 produced 7.5x more clones resistant to G418 than pRSV neo. Again, the number of clones was raised by 60x, that is 450x compared with pRSV neo, by modifying the sequence of initiation of translation.

Moreover, the pGN plasmid contains an origin of bacterial replication of the type colE1, pBR322, which allows clonings and preparations in E. Coli.

Finally, a multiple cloning site (M.C.S.), synthesized in vitro, which only contains unique restriction sites in pGN, was inserted upstream of

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LacZ, so as to facilitate the utilizations of this plasmid.

The plasmid "flanking" sequences which provoke homologous recombination are added to the extremities of the pGN plasmid after linearization of the plasmid upstream from LacZ, by an MCS site (see Fig. 2). In this case, the flanking sequences chosen are homologous to the chromosome sequences taken from Hox-3.1 which intervene later in the homologous recombination.

Figure 2 provides the location of the molecule built from the pGN plasmid with respect to the gene Hox-3.1. In this case, recombination between the plasmid and chromosomal sequences of Hox-3.1 would result in an insertion at the beginning of the coding frame of this gene, thus in its total inactivation.

The pGN plasmid comprises several advantages for this methodology, which is applicable to any gene. As the event of homologous recombination can be fairly rare (of the order of 1 per 1000 non-homologous integrations), it is necessary to be able to analyse a large number of clones whose resistance to G418 is sufficiently strong to be expressed in any part of the genome. The modifications made to the transcription unit of phosphotransferase answer these problems perfectly. The method of mutagenesis by homologous recombination is equivalent to inactivating a gene by an insertion or a substitution, but the pGN plasmid presents the supplementary advantage of being able to substitute the expression of β -galactosidase to that of the mutated gene. Finally, the MCS facilitates the cloning of genomic fragments.

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EXAMPLES:

I - Construction of the pGN plasmid

The intermediate plasmids are numbered according to their stage.

1st stage:

Insertion of a Xho I site in the Bgl I site of pRSV neo

Insertion of a Xho I linker in the Bgl I site of pRSV neo, filled by the Klenow fragment of the DNA polymerase of E. Coli.

2nd stage:

Insertion of a Cla I site in the Nde I site of the p1 plasmid

Insertion of a Cla I linker in the Nde I site of p1, filled by Klenow polymerase.

3rd stage:

Insertion of Py F9.1 enhancer in the Cla I site of the p2 plasmid

Insertion of enhancer Py F9.1 Pvu II-Pvu II isolated by a unique Acc I site, in the Cla I site of p2. Selection of a clone containing two enhancers oriented in the direction "later promoter".

4th stage:

Deletion Sma I-Hpa I of the p3 plasmid

The two enzymes, presenting "blunt end" extremities, can be bonded directly. This deletion removes the intron of the t antigen of SV 40, which is not very useful, and reduces appreciably the size of the transcription unit of phosphotransferase.

5th stage:

Insertion of a Xho I site in the Bam HI site of pCH110

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Insertion of a Xho I linker in the Bam HI site of plasmid pCH 110 (Pharmacia), filled by the Klenow polymerase.

6th stage:

Insertion of 3' LacZ-polyA SV 40 in the p4 plasmid

The 3' end of the coding phase of β -galactosidase, followed by the signal of polyadenylation of the virus SV 40 is isolated from the p5 plasmid through sites Xho I-Aat II and cloned in the p4 plasmid through the same sites.

7th stage:

Insertion of 5' LacZ in the KS vector

The 5' end of the coding phase of β -galactosidase is isolated from plasmid pMC 1871 (Pharmacia) through the sites Pst I-Sac I and cloned in the vector KS- (Stratagene) through the same sites.

8th stage:

Fusion of a genomic sequence Hox-3.1 with 5' LacZ

A genomic sequence of the gene Hox-3.1, cloned in the vector KS-, is purified by successive digesting with the enzyme Sac I, then with the Mung bean nuclease and finally with the enzyme Apa I. This insert is fused with the 5' end of the coding phase of β -galactosidase by cloning in the p7 plasmid digested by Apa I-SmaI. The protein thus fused contains the initiation codon of the translation of the gene Hox-3.1 followed by the coding phase of β -galactosidase (then verified by sequencing).

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Met Ser Ser	Ile Pro Gly Asp Pro
CCAGC <u>ATG AGC TCC</u>	ATT <u>CCC GGG</u> GAT CCC
GGTCG TAC TCG AGG	TAA GGG CCC CTA GGG
↓ Sac 1	
CCAGC ATG AGC T	↓ Sma 1
GGTCG TAC	
↓ Mung bean nuclease	
CCAGC ATG	GGG GAT CCC
GGTCG TAC	CCC CTG GGG
	↔
Met Gly Asp Pro	
CCAGC ATG GGG GAT CCC	
GGTCG TAC CCC CTA GGG	

9th stage:

Insertion of Hox-3.1-5' LacZ in the p6 plasmid

The fusion Hox-3.1-5' LacZ is isolated from the p8 plasmid through the Apa I-Sac I sites and cloned into the p6 plasmid through the same sites. The effect of this cloning is to reconstitute the coding phase of β -galactosidase in its totality.

10th stage:

Insertion of the gene Neo^R in the vector KS±

The gene of resistance to neomycin (bacterial promoter and coding phase of phosphotransferase) is isolated in the pRSV neo through the sites Hind III-Eco RI and cloned in the vector KS+ (Stratagene).

11th stage:

Mutagenesis of the initiation sequence of Neo^R in p10

The sequence of the initiation of translation of phnosphotransferase is modified to be identical to the consensus sequence observed in vertebrates and thus

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to allow a higher level of initiation of translation and therefore an increased resistance to G418 for the cells of mammals. The modification also creates an Apa LI site which makes it possible to control the efficiency of the mutagenesis.

Apa LI

GTTTCGCATG ⇒ GTGCACCATG

An oligonucleotide

(CTTGTTCAATCATGGTGCACGATCCTCA)

comprising a region of mismatch pairing with the sequence of pRSV neo (underlined) is synthesized (Gene Assembler, Pharmacia) then phosphorylated by the polynucleotide kinase of the T4 bacteriophage. A single-strand template of the p10 plasmid is prepared with the fl origin of the KS+ plasmid and hybridized with the mutagenesis oligonucleotide. The second strand is synthesized and repaired by the Klenow polymerase and the DNA ligase of the T4 bacteriophage. After transformation of bacteria, the mutated clones are screened with the aid of the oligonucleotide marked with ³²P. The mutagenesis was verified by digesting with Apa LI and also by sequencing.

12th stage:

Replacement of the initiation sequence in the p9 plasmid

A fragment containing the modified sequence of initiation of translation of the gene of resistance to neomycin is isolated from the p11 plasmid through the enzymes Hind III-Eag I and cloned in the p9 plasmid through the same sites.

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13th stage:

Insertion of the multiple cloning site in the p12 plasmid

Two complementary oligonucleotides are synthesized (Gene Assembler, Pharmacia) then phosphorylated. After pairing, the MCS is cloned in the sites Apa I-Sac II of the p12 plasmid through its sticky ends.

```

      Xma I   Asp 718           Xmn I
    _____
  Apa I   Sma I   Kpn I   Xba I   Nsi I   Sac II
5' C C C C G G G G G T A C C T C T A G A A T G C A T T C C G C 3'
3' C C G G G G G G C C C C C A T G G A G A T C T T A C G T A A G G 5'
  
```

The multiple cloning site was also verified by sequencing.

II - Addition of "flanking sequences" at the extremities of the linearized pGN plasmid upstream from LacZ' by an M.C.S. site

The flanking sequences used were chosen in relation to the desired site of insertion (for example, Hox-3.1, see Fig. 2)

In the experimental stages which follow, the protocol described by Thomson et al. 1989, was followed for the production of chimeric animals.

III - Transfection of embryonic mouse cells

The method described by Thompson et al. 1989, was used to transfect embryonic mouse cells. Utilization of the technique of electroporation ensures the introduction of a single copy of the foreign gene (LacZ) per cell. After transfection, several clones expressing β -galactosidase were isolated.

30 IV - Production of chimeric mice

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The clones expressing β -galactosidase were injected into the blastocysts and colonized normal individuals and made them chimeric.

V - Passage of the modification into the germ cell line: production of transgenic animals

The effects in F_1 and in F_2 of the modification brought by targeted insertion were observed after reproduction of the chimeric animals. Passage of the modification into the germ cell line was observed.

VI - Use of the P.C.R. technique

ES cells were also transfected with the Hox-3.1 mutagenesis plasmid (Figure 2). Several homologous recombination events were identified by the "polymerase chain reaction" (PCR).

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CLAIMS

1. Process for specific replacement, in particular by targeting a DNA, called insertion DNA constituted by a part of a gene, susceptible to being rendered functional, or this functioning can be made more efficient, when it is recombined with a complementary DNA to then provide a complete recombinant gene in the genome of a eucaryotic cell, characterized in that

- the insertion site is in a chosen gene, called a chosen recipient gene, and containing the complementary DNA, and in that

- eucaryotic cells are transfected with a vector containing an insert which itself comprises the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene,

- the insertion DNA gene being heterologous vis-à-vis the recipient gene, and

- the flanking sequences, being chosen from those which make up the abovementioned complementary DNA and which allow, through homologous recombination with corresponding sequences of the recipient gene, the reconstitution of a complete recombinant gene in the genome of the eucaryotic cell.

2. Process according to Claim 1 for targeted insertion of a DNA, termed insertion DNA containing either a coding sequence or a regulatory sequence into the genome of an eucaryotic cell, characterized in that

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- the insertion site is in a chosen gene, called a recipient gene, and in that

- eucaryotic cells are transfected with a vector containing an insert which itself comprises the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene,

- the insertion DNA being heterologous with respect to the recipient gene, and

- the flanking sequences being chosen so as to permit by homologous recombination depending on the case, either the expression of the coding sequence of the entire insertion DNA under the control of the regulatory sequences of the recipient gene, or the expression of a coding sequence of the recipient gene under the control of regulatory sequences of the insertion DNA.

3. Process according to Claim 1 or 2, characterized in that the insertion DNA contains a coding sequence without the regulatory element, especially a promoter which is specific to it.

4. Process according to any one of Claims 1 to 3, characterized in that the recipient gene is present in the genome of the eucaryotic cell in at least two copies.

5. Process according to any one of Claims 1 to 4, characterized in that each of the flanking sequences has a length greater than 150 base pairs, and less than the length of the recipient gene.

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6. Process according to any one of Claims 1 to 5, characterized in that the eucaryotic cells are embryonic stem (E.S.) cells.

7. Process according to one of the preceding claims, characterized in that the insertion gene is a gene heterologous to the transfected species.

8. Process according to one of the preceding claims, characterized in that the vector contains sequences intercalated between the insertion gene and the flanking sequences.

9. Process according to Claim 8, characterized in that the intercalating sequences contain a marker permitting the selection of the transformants and/or the cloning of the vector.

10. Process according to one of the preceding claims, characterized in that the transfection is performed by electroporation.

11. Process according to one of the preceding claims, characterized in that the Polymerase Chain Reaction (P.C.R.) technique is used to amplify the DNA sequence of the locus where the insertion occurs in order to verify that the insertion took place at the desired site.

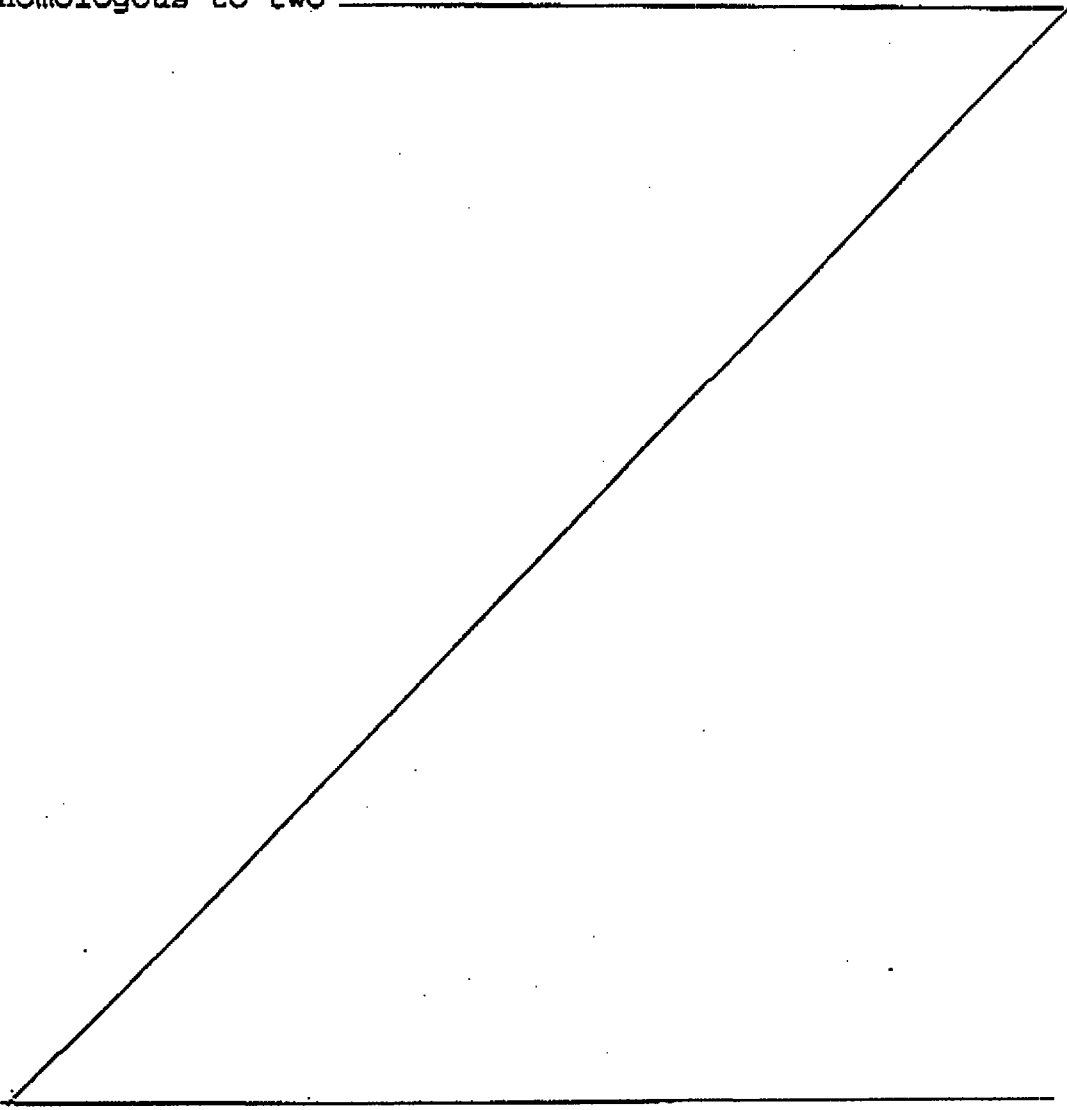
12. Process for the production of transgenic animals, characterized in that E.S. cells are transfected by the process according to one of Claims 1 to 11 and selected for the occurrence of homologous recombination, that is the correct integration of the foreign gene, the cells are injected into embryos at a stage where they are able to integrate the transfected cells (for example, at the blastocyst stage), and these

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are then reimplanted in a surrogate mother and the chimeric individuals obtained at the end of gestation, and in which is noted the colonization through E.S. cells of the germ line, are mated in order to obtain heterozygous transgenic animals for the replaced gene.

13. Plasmid capable of carrying out the targeted insertion of a so-called insertion gene into the genome of an eucaryotic cell, characterized in that it contains an insert which itself comprises the insertion gene and the two so-called "flanking" sequences on either side of the insertion gene, respectively homologous to two _____



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—genomic sequences which are adjacent to the desired site of insertion in the recipient gene.

14. Plasmid pGN as illustrated in Figure 1.

15. Eucaryotic cells transformed by the process of Claim 1.

16. Cells according to Claim 15, characterized in that they are E.S. cells.

17. Transgenic animal in which a single copy of a gene which is present in the genome in at least two copies, has been inactivated by the insertion of a gene which is different from the inactivated gene, the insertion gene being inserted in a position which permits the expression of this gene under the control of regulatory sequences of the inactivated endogenous gene.

18. Application of the process of Claim 1 for gene therapy.

19. Application of the process of Claim 1 for the production of transgenic animals.

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20. Application of the process of Claim 16 for genetic marking of animals.

21. Application of the process of Claim 1 for screening pharmaceutical products presumed to have an activity with regard to the expression products of a pathological gene linked to a disease, characterized in that the insertion gene consists of the pathological gene or a fragment thereof and in that the pharmaceutical product to be tested is administered to the transgenic animal in order to evaluate its activity on the disease.

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18. Application of the process of Claims 1 to 11 for the introduction of a targeted modification in a recipient gene.

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19. Application of the process of Claim 1 for the production of transgenic animals.

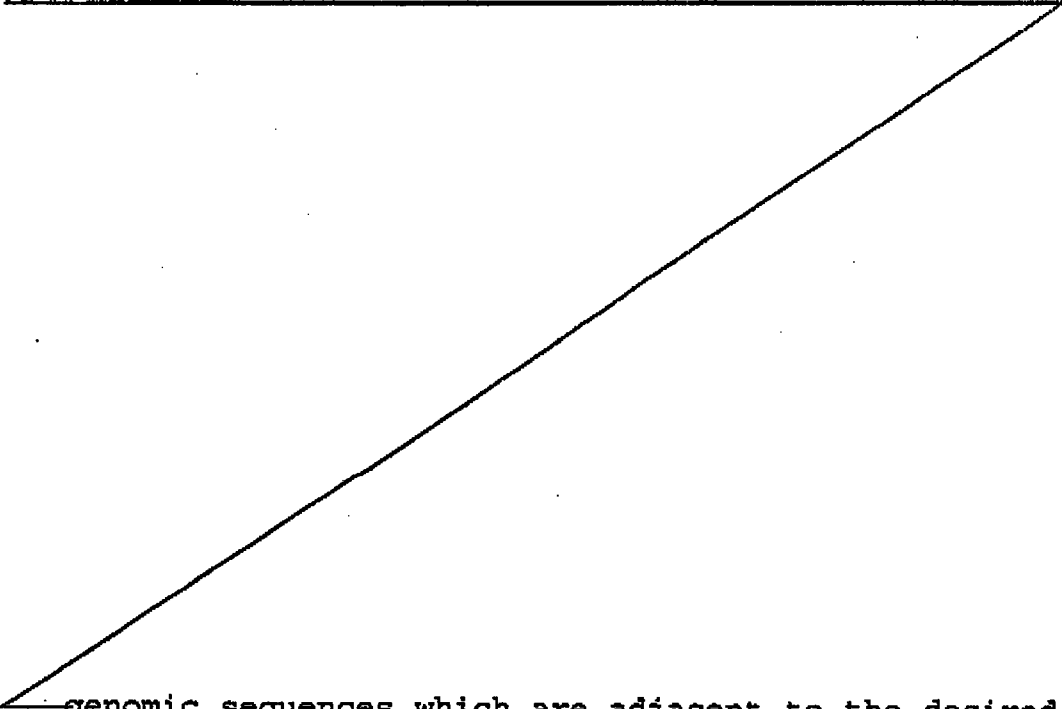
20. Application of the process of Claim 16 for genetic marking of animals.

21. Application of the process of Claim 1 for screening pharmaceutical products presumed to have an activity with regard to the expression products of a pathological gene linked to a disease, characterized in that the insertion gene consists of the pathological gene or a fragment thereof.

22. Process for screening pharmaceutical products . presumed to have an activity with regard to the expression products of a pathological gene linked to a disease, characterized in that an insertion DNA is inserted into the genome of an eucaryotic cell according to the process of Claim 1, the insertion DNA consisting of the pathological gene or a fragment thereof, and the activity of the pharmaceutical product with regard to it is then evaluated.

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genomic sequences which are adjacent to the desired site of insertion in the recipient gene.

14. Plasmid pGN as illustrated in Figure 1.

15. Eucaryotic cells transformed by the process of Claim 1.

16. Cells according to Claim 15, characterized in that they are E.S. cells.

17. Transgenic animal obtained in particular by the process of any one of Claims 1 to 12 and in which a single copy of a gene which is present in the genome in at least two copies, has been inactivated by the insertion of a gene which is different from the inactivated gene, the insertion gene being inserted in a chosen position which permits the expression of this gene under the control of regulatory sequences of the inactivated endogenous gene.

18. Application of the process of Claims 1 to 11 for the introduction of a targeted modification in a recipient gene.

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CLAIMS

1. Process for specific replacement, in particular by targeting a DNA, called insertion DNA comprising a part of a gene, susceptible to being rendered functional, or this functioning can be made more efficient, when it is recombined with a complementary DNA to then provide a complete recombinant gene in the genome of a eucaryotic cell, characterized in that

- the insertion site is in a chosen gene, called a chosen recipient gene, and containing the complementary DNA, and in that

- eucaryotic cells are transfected with a vector containing an insert which itself comprises the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene,

- the insertion DNA gene being heterologous vis-à-vis the recipient gene and comprising, besides the gene part, a sequence coding for a selective agent and a promoter allowing the expression of the selective agent,

- the flanking sequences, being chosen from those which make up the abovementioned complementary DNA and which allow, through homologous recombination with corresponding sequences of the recipient gene, the reconstitution of a complete recombinant gene in the genome of the eucaryotic cell.



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2. Process according to Claim 1, in which the insertion DNA contains either a coding sequence, characterized in that

- the insertion site is in a chosen gene, called a recipient gene, and in that

- eucaryotic cells are transfected with a vector containing an insert which itself comprises the insertion DNA and two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are immediately adjacent to the desired site of insertion in the recipient gene,

- the insertion DNA being heterologous with respect to the recipient gene, and

- the flanking sequences being chosen so as to permit by homologous recombination depending on the case, either the expression of the coding sequence of the entire insertion DNA under the control of the regulatory sequences of the recipient gene, or the expression of a coding sequence of the recipient gene under the control of regulatory sequences of the insertion DNA.

3. Process according to Claim 1 or 2, characterized in that the insertion DNA contains a coding sequence without the regulatory element, especially a promoter which is specific to it.

4. Process according to any one of Claims 1 to 3, characterized in that the recipient gene is present in the genome of the eucaryotic cell in at least two copies.

5. Process according to any one of Claims 1 to 4, characterized in that each of the flanking sequences



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has a length greater than 150 base pairs, and less than the length of the recipient gene.

6. Process according to any one of Claims 1 to 5, characterized in that the eucaryotic cells are embryonic stem (E.S.) cells.

7. Process according to one of the preceding claims, characterized in that the insertion gene is a gene heterologous to the transfected species.

8. Process according to one of the preceding claims, characterized in that the vector contains sequences intercalated between the insertion gene and the flanking sequences.

9. Process according to Claim 8, characterized in that the intercalating sequences contain at least one sequence permitting the selection of the transformants and/or the cloning of the vector.

10. Process according to one of the preceding claims, characterized in that the transfection is performed by electroporation.

11. Process according to one of the preceding claims, characterized in that the Polymerase Chain Reaction (P.C.R.) technique is used to amplify the DNA sequence of the locus where the insertion occurs in order to verify that the insertion took place at the desired site.

12. Process for the production of transgenic animals, characterized in that E.S. cells are transfected by the process according to one of Claims 1 to 11 and selected for the occurrence of homologous recombination, that is the correct integration of the foreign gene, the cells are injected into embryos at a stage where they are able to integrate the transfected





cells (for example, at the blastocyst stage), and these are then reimplanted in a surrogate mother and the chimeric individuals obtained at the end of gestation, and in which is noted the colonization through E.S. cells of the germ line, are mated in order to obtain heterozygous transgenic animals for the replaced gene.

13. Plasmid capable of carrying out the targeted insertion of a so-called insertion DNA into the genome of an eucaryotic cell, characterized in that it contains an insert which itself comprises the insertion DNA and the two so-called "flanking" sequences on either side of the insertion DNA, respectively homologous to two genomic sequences which are adjacent to the desired site of insertion in the recipient gene, the insertion DNA comprising a part of a gene and a sequence coding for a selective agent with a promoter allowing the expression of the selective agent.

14. Plasmid pGN as illustrated in Figure 1.

15. Eucaryotic cells transformed by the process of Claim 1.

16. Cells according to Claim 15, characterized in that they are E.S. cells.

17. Transgenic animal which can be obtained by the process of any one of Claims 1 to 12 and in which a single copy of a gene which is present in the genome in at least two copies, has been inactivated by the insertion of a gene which is different from the inactivated gene, the insertion gene being inserted in a chosen position which permits the expression of this gene under the control of regulatory sequences of the inactivated endogenous gene.



18. Application of the process of Claims 1 to 11 for the introduction of a targeted modification in a recipient gene.

19. Application of the process of Claim 1 for the production of transgenic animals.

20. Application of the process of Claim 16 for genetic marking of animals.

21. Application of the process of Claim 1 for screening pharmaceutical products presumed to have an activity with regard to the expression products of a pathological gene linked to a disease, characterized in that the insertion gene consists of the pathological gene or a fragment thereof.

22. Process for screening pharmaceutical products presumed to have an activity with regard to the expression products of a pathological gene linked to a disease, characterized in that an insertion DNA is inserted into the genome of an eucaryotic cell according to the process of Claim 1, the insertion DNA consisting of the pathological gene or a fragment thereof, and the activity of the pharmaceutical product with regard to it is then evaluated.

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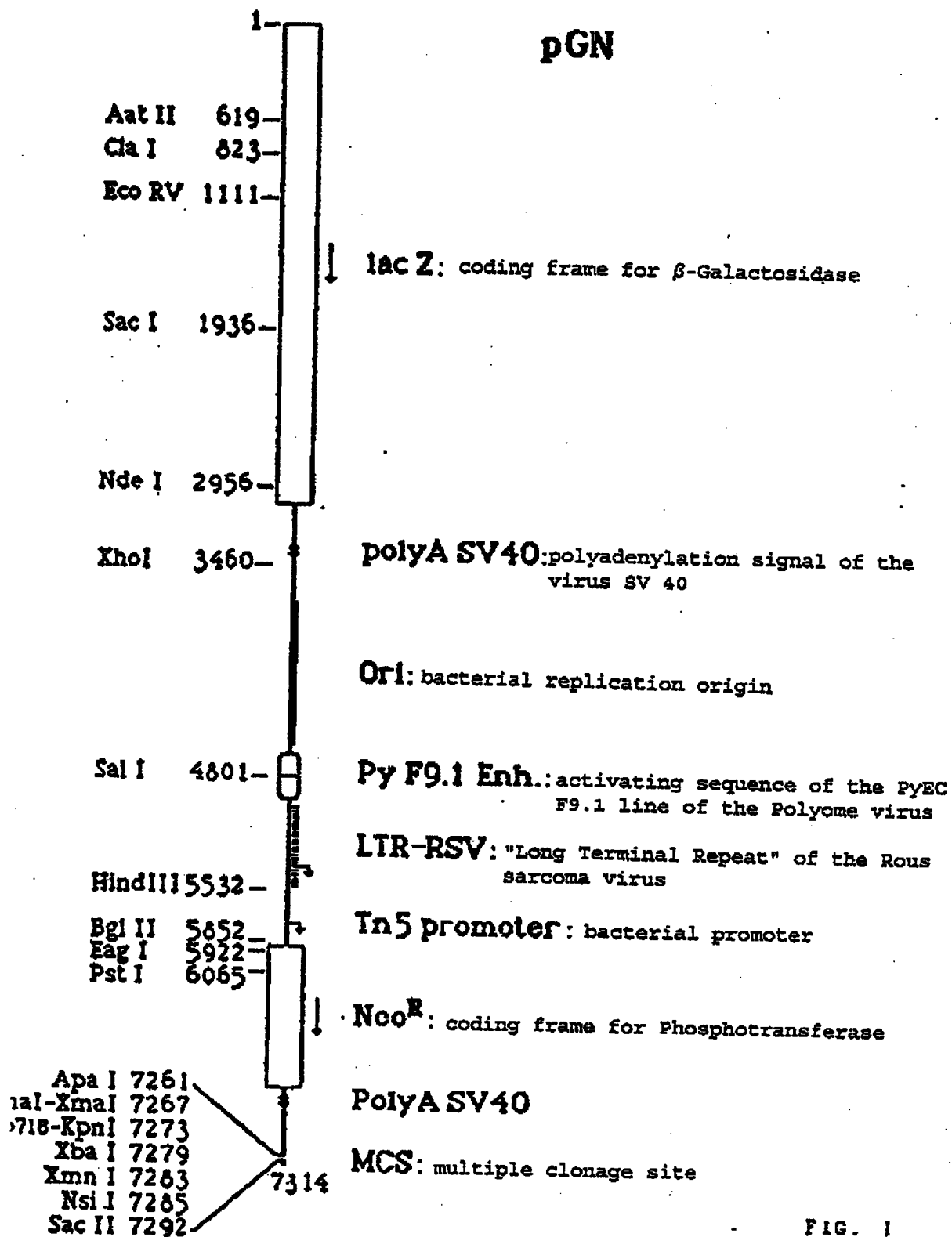


FIG. 1

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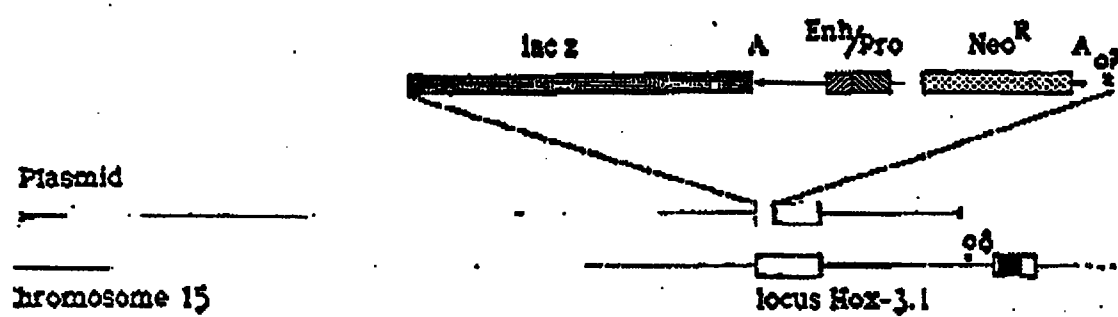


FIGURE 2

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FIG. 3

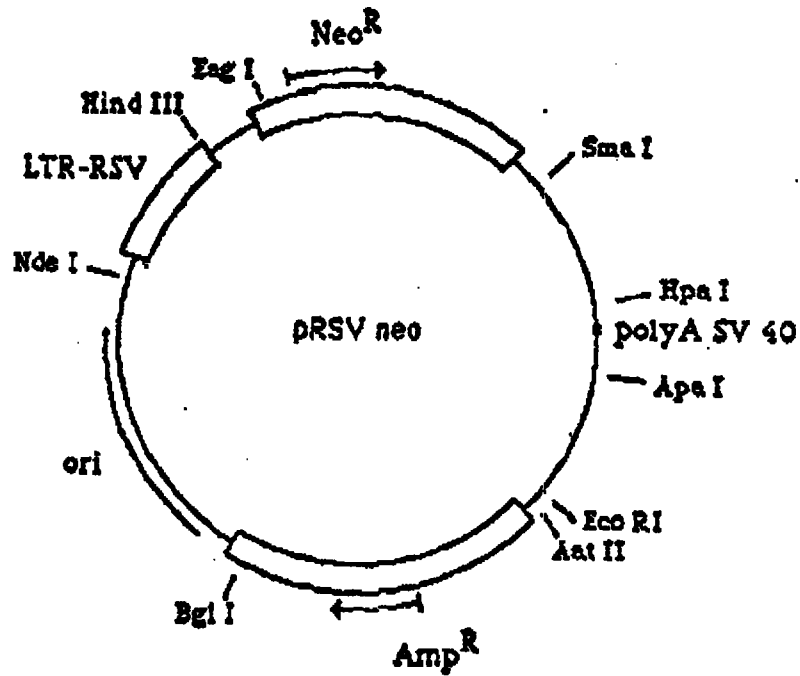
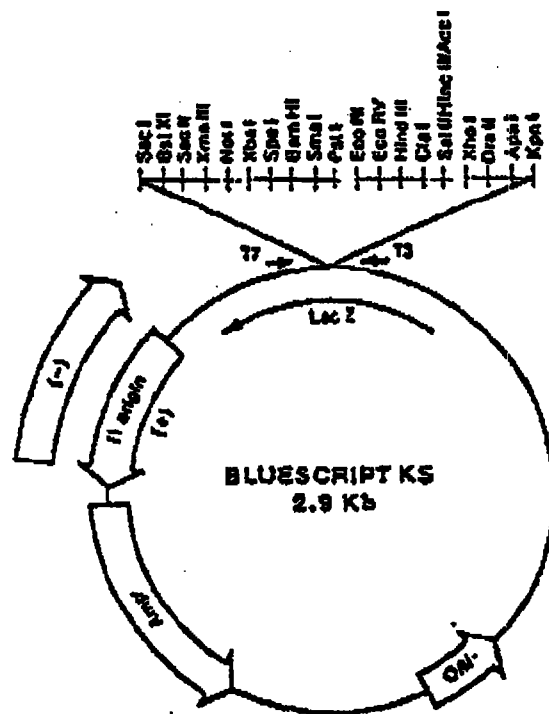


FIG. 4



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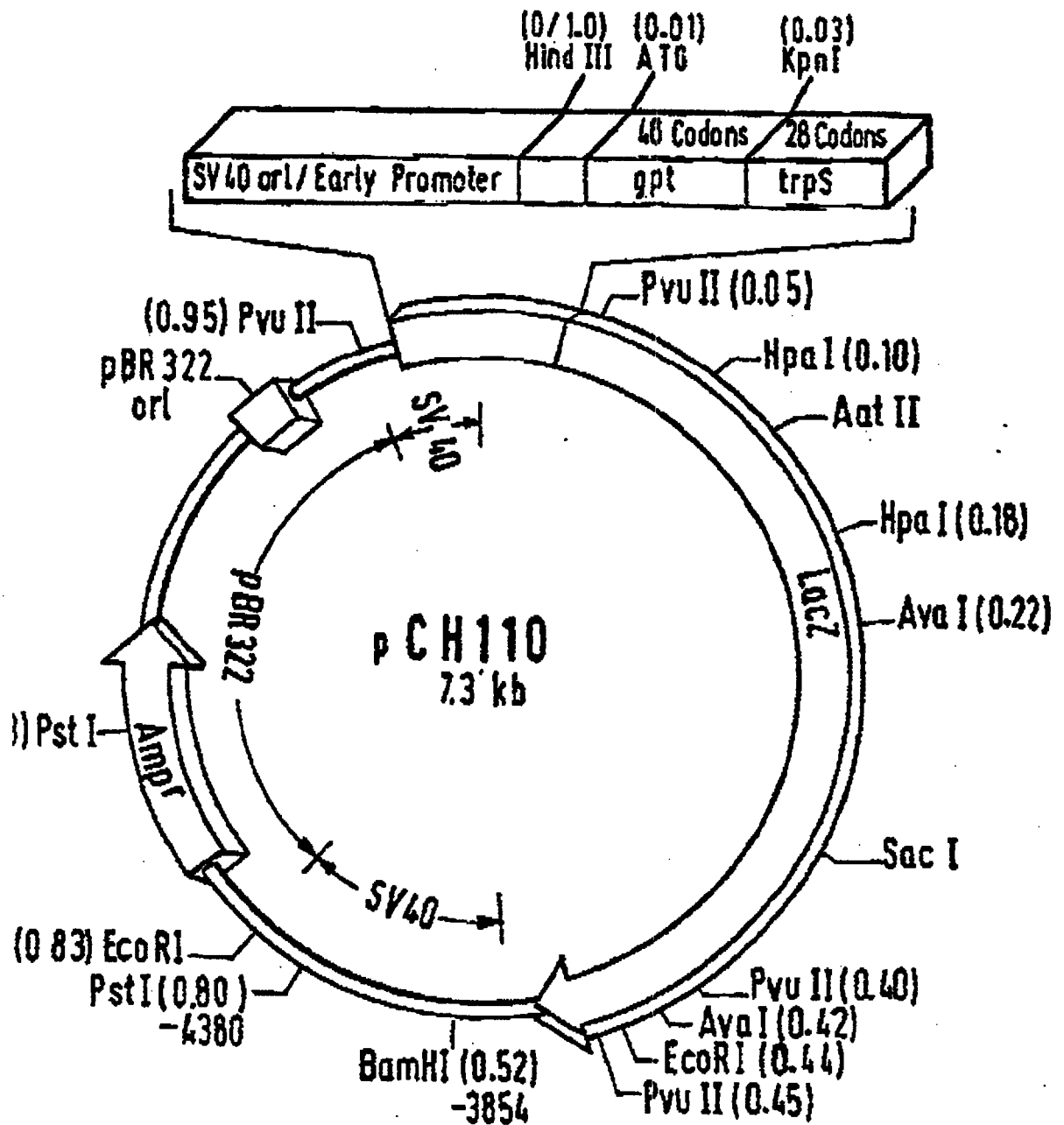


FIG. 5

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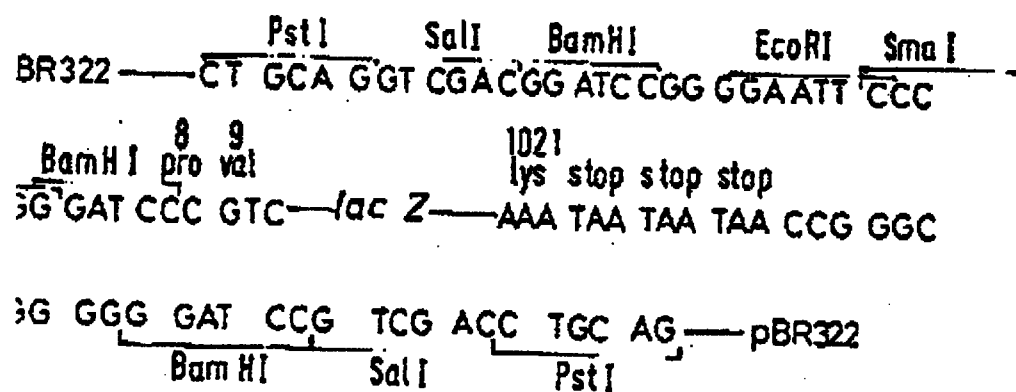
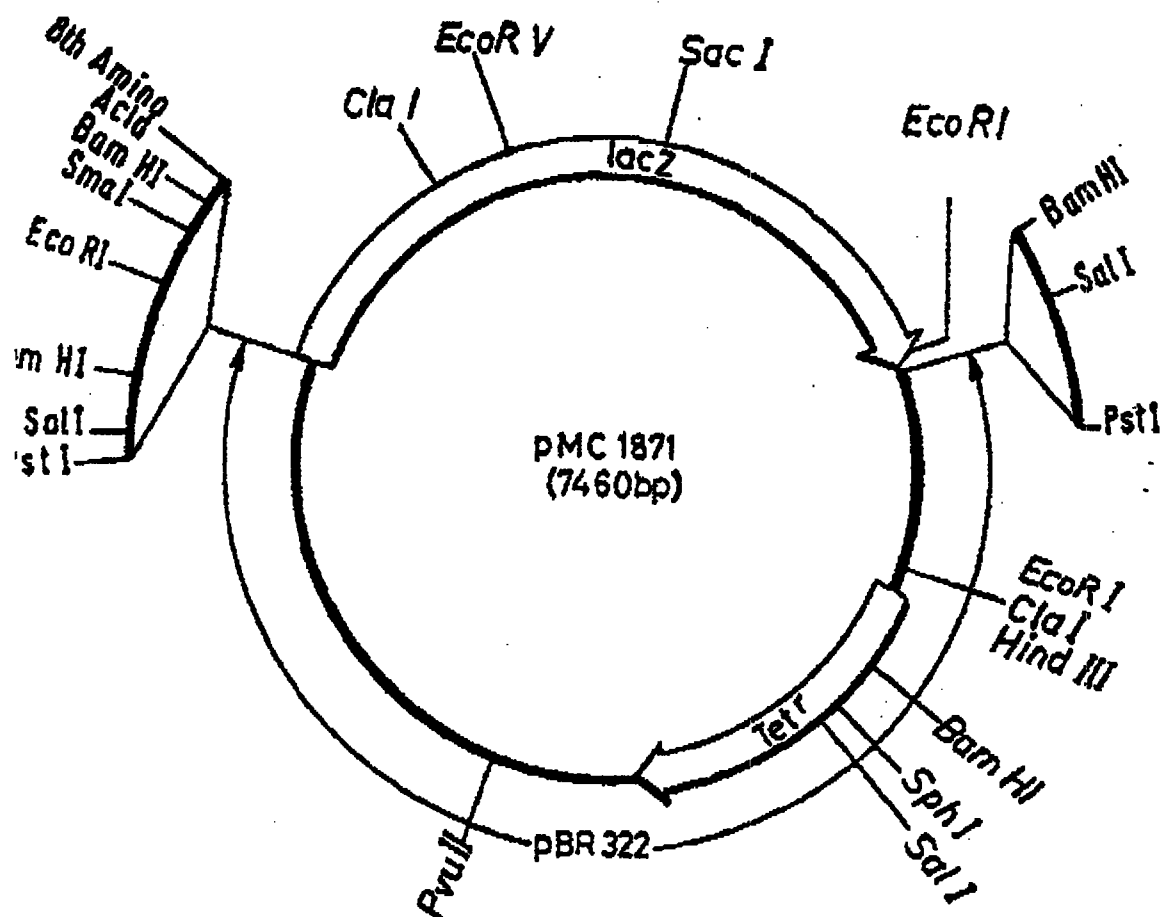


FIG.6

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CLAIMS

1. Process to obtain the expression of, or to enhance the expression level of a gene, or part of a gene, so-called receptor gene, in the genome of an eucaryotic cell, said receptor gene being able to be made functional or more effective when recombined with a complementary DNA, said process comprising :
- the introduction into the eucaryotic cells of a vector containing an insert itself comprising the complementary DNA, so-called insertion DNA, and two so-called "flanking" sequences either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in relation to the receptor gene ;
 - the insertion DNA comprising a gene or part of a gene, and being heterologous to the receptor gene ;
 - the flanking sequences being chosen in order to permit, by homologous recombination, the juxtaposition of the insertion DNA in relation to the receptor gene, in such manner that the insertion DNA makes functional or enhances the functioning of the receptor gene ;
2. Process according to claim 1 in which the insertion DNA is entirely made up of a gene.
3. Process according to claim 1 in which the insertion DNA is made up of part of a gene, for example a regulatory sequence.
4. Process according to claim 1 in which the receptor gene is a gene which is not normally transcribed in the eucaryotic cell.
5. Process according to claim 1 in which the receptor gene has no selectable phenotype.

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6. Recombined eucaryotic cell, able to express a gene or part of a gene, or of expressing it at an enhanced level, said cell being able to be reproduced according to the process of any of claims 1 to 5.

5. 7. Process for inactivating a gene, so-called receptor gene, in the genome of an eucaryotic cell by the targeted insertion, in the receptor gene, of a DNA, so-called insertion DNA, the process comprising :

10 - the introduction into the eucaryotic cell of a vector containing an insert itself comprising the insertion DNA and two sequences, so-called "flanking" sequences, on either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in the receptor gene ;

15 - the insertion DNA being heterologous to the receptor gene and comprising a gene or part of a gene able to be made functional or whose functioning may be made more efficient when it is recombined with a complementary DNA in the receptor gene ;

20 - the flanking sequences, through homologous recombination, allow the insertion of the insertion DNA at the desired site in the receptor gene ; .

25 - the expression of the insertion DNA subsequently replacing that of the receptor gene ;

- provided that the receptor gene and the insertion DNA code for expression products which do not confer a selectable phenotype.

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PROCESS TO OBTAIN THE EXPRESSION OF OR TO THE
ENHANCE THE LEVEL OF EXPRESSION OF A GENE

The present invention relates to a process for the specific replacement of a copy of a gene present in the genome of a receptor eucaryotic organism by integrating a gene that is different to the inactivated gene. Preferably, two copies at least of the receptor gene shall be present in the transfected host cell. The receptor gene is defined as the gene in which the insertion of the different gene is to be made.

More particularly, the invention relates to the production of transgenic animals in which the foreign gene was introduced in targeted fashion for the twofold purpose of maintaining the animal's normal genetic functions and of expressing a foreign gene under the control of endogenous promoters.

By "different or foreign" gene is meant any nucleotide sequence corresponding in whole or in part to a gene that is "foreign or different" to the receptor gene such as it is normally found in the genome (RNA or DNA), or it may also correspond to an artificially modified sequence of the normal gene or even to a fragment of this sequence.

The invention also relates to the production process of these transgenic animals.

In the production of transgenic animals, the conventional methods used for the introduction of heterologous DNA sequences into the germinal cell line do not allow control over the site of integration of the foreign gene into the genome, or over the number of copies thus introduced. The introduction of the foreign gene is made randomly and, in general, several copies of the gene integrate themselves at the same time, sometimes in the form of a head-to-tail tandem, the

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**PROCESS TO OBTAIN THE EXPRESSION OF, OR TO ENHANCE THE
LEVEL OF EXPRESSION OF A GENE.**

The present invention relates to a process for specific replacement of a copy of a gene present in the genome of a recipient eucaryotic organism by the integration of a gene different from the inactivated gene. The recipient gene will preferably be present in at least 2 examples in the transfected host cell. The recipient gene is defined as being the gene where the insertion of the different gene will be made.

More particularly, the invention relates to the production of transgenic animals in which the foreign gene has been introduced in a targeted manner to allow both the maintenance of the normal genetic functions of the animal and the expression of the foreign gene under the control of endogenous promoters.

By "different or foreign gene" is meant any nucleotide sequence corresponding to the totality or to a part of a "foreign or different" gene of the receptor gene as it is found normally in the genome (RNA or DNA), where it corresponds also to an artificially

CLAIMS

1. Process to obtain the expression of, or to enhance the expression level of a gene, or part of a gene, so-called receptor gene, in the genome of an eucaryotic cell, said receptor gene being able to be made functional or more effective when recombined with a complementary DNA, said process comprising :
- the introduction into the eucaryotic cells of a vector containing an insert itself comprising the complementary DNA, so-called insertion DNA, and two so-called "flanking" sequences either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in relation to the receptor gene ;
 - the insertion DNA comprising a gene or part of a gene, and being heterologous to the receptor gene ;
 - the flanking sequences being chosen in order to permit, by homologous recombination, the juxtaposition of the insertion DNA in relation to the receptor gene, in such manner that the insertion DNA makes functional or enhances the functioning of the receptor gene ;
2. Process according to claim 1 in which the insertion DNA is entirely made up of a gene.
3. Process according to claim 1 in which the insertion DNA is made up of part of a gene, for example a regulatory sequence.
4. Process according to claim 1 in which the receptor gene is a gene which is not normally transcribed in the eucaryotic cell.
5. Process according to claim 1 in which the receptor gene has no selectionable phenotype.
6. Recombined eucaryotic cell, able to express a gene or part of a gene, or of expressing it at an



enhanced level, said cell being able to be reproduced according to the process of any of claims 1 to 5.

5 7. Process for inactivating the gene, so-called receptor gene, in the genome of an eucaryotic cell by the targeted insertion, in the receptor gene, of a DNA, so-called insertion DNA, the process comprising :

10 - the introduction into the eucaryotic cell of a vector containing an insert itself comprising the insertion DNA and two sequences, so-called "flanking" sequences, on either side of the insertion DNA, respectively homologous to two genomic sequences adjacent to the desired insertion site in the receptor gene ;

15 - the insertion DNA being heterologous to the receptor gene and comprising a gene or part of a gene able to be made functional or whose functioning may be made more efficient when it is recombined with a complementary DNA in the receptor gene ;

20 - the flanking sequences, through homologous recombination, allow the insertion of the insertion DNA at the desired site in the receptor gene ;

- the expression of the insertion DNA subsequently replacing that of the receptor gene.



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