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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 of European Patent Application n° 95 107 952.4

Signature

Paris, February 21, 1996

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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 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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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).

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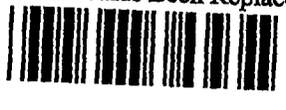

In the first case, the HPRT murine gene was muted by insertion and replacement, and in the second case a muted HPRT gene was corrected. Thomson et al. extended their experiments until they obtained chimaera mice and noted the entry of the gene modification into the germinal cell line.

In each of the documents cited, the exact site of integration was targeted by homologous recombination between, firstly exogenous sequences comprising the 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 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 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 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 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 during the production of transgenic animals is the

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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 en 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

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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 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 chimaera animals, transgenic animals which are heterozygous for the replaced gene will be obtained by mating (F_1) of their descendants.

It is also possible to insert the insertion DNA, carried by the vector of the invention, into the egg 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 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 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 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 under the control of the regulatory sequences of the inactivated endogenous gene.

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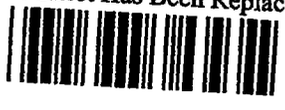

Owing to the phenomena of homologous recombination it is therefore possible with the process of the invention to insert foreign genes, in targeted fashion, in particular coding sequences deprived of the promoter normally associated therewith, into the genome of an eucaryotic organism at a site which 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).

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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 selectionable phenotype. According to this variant the plasmid insert of the invention comprises, between the flanking sequences, firstly a DNA sequence intended to

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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 initiatio codon of the replaced gene so that the expression c the inserted gene fully replaces the expression of tl replaced gene. This avoids the formation of protei which may be undesirable in a transgenic animal.

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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 ß-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

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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.

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.

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 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' 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.

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..

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 the control of foreign regulatory elements brought by the insertion sequence.

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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 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 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 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 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 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 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 early embryo which, after reimplantation, may be borne in chimaera form. If the germinal line is colonised by

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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

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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 ß-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

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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.

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 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 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 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.

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 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 to the pGN plasmid and its use for the targeted insertion of a foreign gene (lacZ, coding for the ß-

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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.

The coding phase of the ß-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 (Hox.3-1) is identical to the consensus sequence observed in vertebrates (Ref.2), thus giving a better translation level of ß-galactosidase in vertebrate cells. The lacZ gene is followed by a polyadenylation signal, for example of the SV 40 virus, like most eucaryotic genes, in order to stabilise the messenger RNAs.

The activity of the ß-galactosidase of E.Coli, which is functional in eucaryotic cells, may be detected in different manners. Cells expressing the lacZ gene take on a blue colour, after fixation, in the presence of X-Gal, which is a substrate of the ß-galactosidase (Ref.3). A new substrate, the DGF (di-ß-galactopyranoside fluorosceine) permits the detection and determination of the activity of ß-gal. while 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 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 a promoter that is active in E. Coli and the coding phase of the phosphotransferase enzyme (Ref.7) which is

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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

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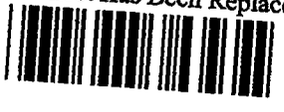

conditions mainly give rise to the integration of a single copy.

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.

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 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) 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 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 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 recombination is equivalent to inactivating a gene by insertion or substitution, but the pGN plasmid offers the additional advantage of being able to substitute

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

EXAMPLES :

I - Construction of the pGN plasmid

The intermediate plasmids are numbered according to their stage.

Stage 1 :

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 :

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, filed with the Klenow polymerase.

Stage 3 :

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

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.

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 :

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 the pCH 110 plasmid (Pharmacia), filled with the Klenow polymerase.

Stage 6 :

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

The 3' end of the coding phase of ß-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.

The 5' end of the coding phase of ß-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.

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 ß-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 ß-galactosidase (subsequently checked by sequencing).

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          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
        ⇓ 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
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Stage 9.

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

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.

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.

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 efficacy of the mutagenesis can be verified.

		Apa LI
GTTTCGCATG	⇒	GTGCACCATG

An oligonucleotide (CTTGTTCAATCATGGTGCACGATCCTCA) 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+ plasmid and hybridized with the oligonucleotide of the mutagenesis. The second strand is synthesised and repaired with the Klenow polymcrase and the ligase DNA of the T4 bacteriophage. After transforming the bacteria, the muted clones are sorted with the ^{32}P-labeled oligonucleotide. The mutagenesis was verified by Apa LI digesting and by sequencing.

Stage 12 :

Replacement of the initiation sequence in the p9 plasmid.

A fragment containing the modified sequence of the translation initiation of the neomycine 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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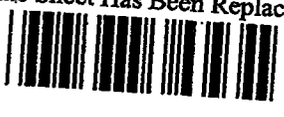
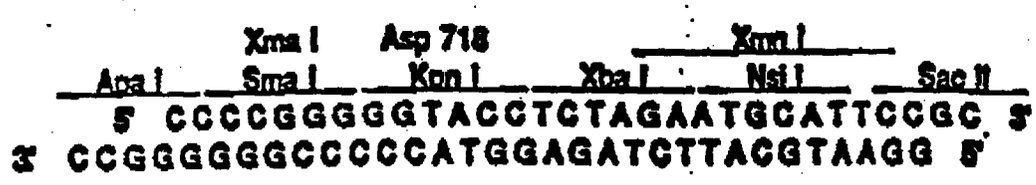


Staqe 13 :

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



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

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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).

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.

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.

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 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 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 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 the foreign gene (lacZ) is introduced per cell. After

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transfection, several clones expressing ß-galactosidase were isolated.

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).

Homologous recombination in the gene HOXC8 (Hox-3.1)

Exp.	Plasmid of mutagenesis	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)

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, 1.5×10^7 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).

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 $\mu g\ ml^{-1}$ G418 were added. Each transfection gave approximately 2400 clones with pGMA and approximately 1000 clones with pGMD.

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

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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 $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10mM 2-mercaptoethanol, 0.01 % (w/v) gelatin, 200 µm dATP, dTTP and dCTP, 100 µm dGTP, 100 µM7-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.

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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-

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

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 ß-Gal, which correlates with the absence of HOXC8 (Hox-3.1) transcription in E.S. cells as determined by protection analysis of RNase. The activity of ß-Gal could be induced in certain cells after 3 to 4 days' culture in 5.10^{-7} M retinoic acid, conditions which are known to 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 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 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 led to reducing the total number of clones with G418 resistance by 2.4 (Table I). The specific targeting

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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

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"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 1.

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.

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 beon 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.

In chimaera embryos of 9.5 and 10.5 days p.c., the part caudal to the posterior bud showed intense ß-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 ß-Gal were observed in all tissues of all embryonic layers. Between the two buds from which the limbs emerge,

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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 ß-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

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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 F_1 and F_2 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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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

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

- 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 ;
- 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 ;
- 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.