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TRANSGENIC OR CHIMERIC ANIMAL AND PROCESS FOR SCREENING
PHARMACEUTICAL PRODUCTS

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

More particularly, the invention relates to the
10 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
15 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
20 artificially 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
25 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
30 carried out at random and, in general, several copies of the gene combine at the same time, sometimes under the form of a head-to-tail tandem, the site of



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

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

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

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.

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



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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. Thompson et al. widened their experiments as far as obtaining
5 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
10 the mutation or correction included in the 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
15 activation by mutation was accompanied by a detectable phenotype. The targeted mutation described by Thomas et al. had the effect of inactivating the HPRT gene and, consequently, making the detectable phenotype normally associated with the HPRT disappear. The Neo^R selection
20 gene, under the control of a TK promoter, was thus incorporated into the insertion DNA in order to permit the selection of transformants. It should be noted that the experiments described in the prior art implied a selection either by the receiver 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 thus limited to genes conferring a selectable character, a direct selection.

Besides, in the prior art, the exogenous
30 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.

We should remember that a problem posed during
35 the production of transgenic animals is the danger of



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

The inventors have studied the possibility of avoiding the disadvantages described above and associated, in certain cases, to the possible inactivation of one or several endogenous cell genes with an important function during the production of transgenic animals.

The object of the invention is a process for replacement or specific insertion of a gene, in particular by targeting a DNA, called insertion DNA constituted by the totality of a gene, or by a part of a gene, susceptible to being rendered functional, where 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 receiver gene, and containing the complementary DNA, and in that

- eucaryotic cells are transfected with a vector containing an insert which 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 receiver gene,

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

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

According to the invention, the so-called insertion DNA can contain either a coding sequence or a regulatory sequence, the process then being characterized in that

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

- eucaryotic cells are transfected with a vector containing an insert which 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 receiver gene,

- the insertion DNA being heterologous with respect to the receiver 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 receiver gene, or the expression of a coding sequence of the receiver gene under the control of regulatory sequences of the insertion DNA.

For example, the insertion DNA may contain a coding sequence without the regulation element, notably without its own promoter.

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

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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 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 of the chimeric animal, 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) the progeny.

It is also possible to insert insertion DNA, carried by the vector of the invention, in the egg shortly after fertilization (that is less than 24 hours). In this way, the insertion takes place while the egg is still in the unicellular state.

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 receiver gene.

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

The invention also concerns transgenic animals in which at least one endogenous gene, which is preferably 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 allows the

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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
5 insertion in a targeted manner of foreign genes, in particular coded 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
10 of the gene where the insertion is made, and consequently, the inactivation of the targeted endogenous gene.

In a preferred embodiment of the invention, the targeted receiver gene is a gene which is present in
15 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
20 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
25 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 is not affected by the insertion of the
30 foreign gene, or the possible minor disturbances in the case of inactivation of a critical gene will 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
35 second generation (F2) after crossing heterozygous individuals (F1) among themselves.

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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 the gene codes for a receptor of an infectious agent, multiple copies of the foreign gene 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 receiver gene.

In a preferred embodiment, the insertion DNA, or the vector, contains sequences inserted between the insertion DNA and the flanking sequences. These sequences can for example contain a sequence coding for a selective agent allowing the selection of transformants and eventually a marker gene, for example LacZ.

In a highly preferred embodiment of the invention, the insertion DNA carries, between the flanking sequences, on the one hand a DNA sequence intended to be recombined with the complementary DNA in the receiver gene to provide a recombinant gene and, on the other hand, a sequence coding for a selective agent permitting the selection of transformants and a promoter authorizing the expression of the selective agent, the receiver gene and the recombinant gene coding for expression products not conferring a selectable phenotype. According to this embodiment, the insert of the plasmid of the invention comprises, between the flanking sequences, on the one hand a DNA



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sequence intended to be recombined with the complementary DNA in the receiver gene and, on the other hand, a sequence coding for a selective agent allowing the selection of transformants and a promoter
5 authorizing 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 way, the selection of transformants is
10 entirely independent from the nature of the receiver gene and the inserted gene, contrary to the processes described until now, in which the inserted gene or the receiver gene necessarily had to code for a product of expression allowing the selection of transformants. The
15 system developed by the inventors gives total flexibility regarding the nature of the receiver gene and the inserted gene, or the gene formed by homologous recombination. The inventors have noted that, surprisingly, the insertion of sequences of a
20 significant size (for example about 7.5 kb) does not affect the frequency of homologous recombination.

The possible effect of the insertion of the DNA sequence according to this aspect of the invention includes, depending on the type of sequence inserted
25 for example the replacement of a coding sequence, the replacement of a regulatory sequence, the inactivation or reactivation of a gene by mutation or improvement of the expression level of a gene. It is possible, according to the invention, to replace a coding frame
30 or part of a coding frame by a heterologous sequence which begins at the initiation codon of the replaced gene so that the expression of the inserted gene entirely replaces the expression of the replaced gene. This avoids the formation of fusion proteins which
35 could be undesirable in a transgenic animal.

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In this embodiment of the invention, the insertion DNA can carry between the flanking sequences a heterologous coding sequence without promoter, the coding sequence being other than a gene coding for a selection agent. The insertion DNA can also carry, downstream of the coding sequence and still between the flanking sequences, a gene coding for a selection agent, associated with a promoter allowing its expression in the target cell.

10 In this way, the heterologous coding sequence can be inserted after an endogenous promoter which has the desired properties, for example a certain specificity of expression, or transcription pattern etc., the selectability of the transformed cells being entirely independent from the expression of the heterologous coding sequence. This type of construction makes it 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.

15 This is particularly important in the production of transgenic animals from E.S. cells ("Embryonic Stem Cells") since a significant proportion of genes remains inactive until a more advanced stage of the development of the animal. The HOXC8 gene (also known under the name of Hox-3.1) is an example of this type of gene. Moreover, if the coding sequence codes for an easily detectable protein, for example β -Gal, the development of the transcription template of the replaced endogenous gene can be followed. The pGN

20 vector is an example of this type of construction.

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

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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 can be the targeted insertion of a foreign DNA which carries both a regulatory sequence and a coding sequence. It is possible that the regulatory sequence can 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 length of the receiver 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 "intercalated" 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 intercalated 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



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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 amplify the DNA sequence of the locus where the insertion takes place, or should take 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 sequence 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.

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

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

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

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

07 and 08 show the two oligonucleotides used in the PCR.

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

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

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

The process of the invention has a very wide industrial applicability and can vary according to the nature of the foreign gene introduced, for example for the production of transgenic animals.



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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 recombination and E.S. cells, valuable information will be obtained on oncogenes, growth factors, transcription factors etc., genes which concern very actual subjects both in fundamental research and 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 the 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 is the genetic marking of animals. This application 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

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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
5 transgenic animals.

The inventors have observed in a surprising
manner that the frequency of homologous recombination
is not affected by the insertion of fragments of
significant size, for example LacZ. This observation
10 has suggested to the inventors that the technique of
homologous recombination could be well adapted to the
insertion of other heterologous genes which are of
significant size.

The invention is also aimed at the application of
15 the process of replacement or specific insertion for
genic therapy.

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
20 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
25 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
30 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.

35 The technique of the invention is also suitable
for studies of the activity of pharmaceutical products

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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 or a fragment of it, and one
5 administers to the transgenic animal the pharmaceutical product to be tested 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
10 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
15 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 HOXC8 (Hox.3-1) (Ref. 1) begins with the initiation codon of this gene. In fact,
20 the sequence which precedes the initiation codon of HOXC8 (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
25 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
30 in different manners. 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
35 to detect and to dose the β -gal activity, while still

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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).

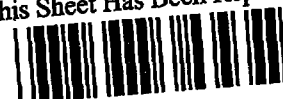
5 The transcription unit of the gene with resistance to neomycin comes, to a large extent, from the plasmid pRSV neo (Ref. 5). The LTR (long terminal repeat) of the virus of Rous-sarcoma provides very powerful activating and promoting sequences in numerous
10 eucaryotic cells (Ref. 6). From the bacterial 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 SV 40. The same gene, under the double
15 control of promoters RSV and Tn5 can confer resistance to neomycin or kanamycin to bacteria and resistance to G418 to eucaryotic cells.

Through the effect of a simple single mutation, unit B of the activating (enhancer) sequences of the
20 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
25 LTR-RSV, and in the "late 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
30 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 the initiation of translation in vertebrates (Ref.2).

The improvements made to the transcription unit
35 of the gene of resistance to neomycin could be estimated by transfecting embryonic cell strains (ES)

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of mice. With equal molarity in plasmid, a construction with the enhancer Py F9.1 produced 7.5 times more clones resistant to G418 than pRSV neo and 2 to 3 times more than the pMC1 neo described by Capecchi et al. (Ref. 13). Again, the number of clones was raised by 60x, that is 450x compared with pRSV neo, by modifying the sequence of initiation of translation. Homologous recombination may be a fairly rare event, according to the experimental conditions applied (e.g. 1/1000 for HPRT, Ref. 13). A vector presenting a high selection efficiency is thus very useful, especially since the conditions of electroporation result mainly in the integration of a single copy.

Moreover, the pGN plasmid contains an origin of bacterial replication of the type colE1, pBR322, which allows cloning 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.

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 HOXC8 (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 HOXC8 (Hox-3.1). In this case, recombination between the plasmid and chromosomal sequences of HOXC8 (Hox-3.1) would result in an insertion at the beginning of the coding phase of this gene, thus in its total inactivation.

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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 analyze 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 genetic fragments.

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



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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 "late promoter".

5 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
10 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

15 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
20

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
25 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
30 the sites Pst I-Sac I and cloned in the vector KS- (Stratagene) through the same sites.

8th stage:Fusion of a genomic sequence HOXC8 (Hox-3.1) with 5' LacZ

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A genomic sequence of the gene HOXC8 (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
5 insert is fused with the 5' end of the coding phase of
 β -galactosidase by cloning in the p7 plasmid digested
by Apa I-Sma I. The protein thus fused contains the
initiation codon of the translation of the gene HOXC8
(Hox-3.1) followed by the coding phase of β -
10 galactosidase (then verified by sequencing).

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 I

15	CCAGC ATG AGC T	↓ Sma I
	GGTCG TAC	

↓ Mung bean nuclease

CCAGC ATG	GGG GAT CCC
GGTCG TAC	CCC CTG GGG

20	Met Gly Asp Pro
	CCAGC ATG GGG GAT CCC
	GGTCG TAC CCC CTA GGG

9th stage:

25 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 through the sites Apa I-SacI and
cloned in the p6 plasmid through the same sites. The
30 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+

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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
5 neomycin is isolated from the p11 plasmid through the enzymes Hind III-Eag I and cloned in the p9 plasmid through the same sites.

13th stage:

10 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
15 sticky ends.

XmaI Asp 718 XmnI
ApaI SmaI KpnI XbaI NsiI SacII
5' CCCCAGGGGTACCTCTAGAATGCATTCCGC 3'
20 3' CCGGGGGGCCCCCATGGAGATCTTACGTAAGG 5'

The multiple cloning site was also verified by sequencing.

25 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, HOXC8 (Hox-3.1), see Fig. 2a and 2b pGMA and pGMD).

30 In the construction of the mutagenesis plasmid pGMD, two fragments of DNA homologous to the locus HOXC8 (Hox-3.1) were cloned at the sites Apa I-Nsi I and Nsi I-Sac II of the pGN vector. The 5' fragment begins at the site Sac II (CCGCGG) at nucleotide 219 of
35 the DNAC c21 of HOXC8 (Hox-3.1). This fragment extends over 6.8 kb in 5' up to the first BamHI site. The 3'



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fragment begins at the site Apa 1 (GGGCCC) at nucleotide 885 of the cDNA c21. This fragment extends over 1.5 kb in 3' up to the first PstI site. A NsiI linker was inserted in the BamHI site of the 5' fragment and in the PstI site of the 3' fragment. The 5' and 3' fragments were cloned in the pGN vector in the sites Nsi I-Sac II and Apa I-Nsi I, respectively. The sequence of the cDNA of HOXC8 (Hox-3.1) c21 has been published (Ref. 1).

10 The mutagenesis plasmid is linearized by digesting with Nsi I before electroporation of E.S. cells. Its extremities are formed by the two genomic fragments cloned at the sites Apa I-Nsi I and Nsi I-Sac II of the pGN vector.

15 The pGMD plasmid does not present any signal of polyadenylation after the gene of resistance but, on the other hand, presents a sequence rich in AU responsible for selective degradation of mRNA, inserted in the sequence of the intron of HOXC8 (Hox-3.1) of the plasmid.

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

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

III - Transfection of embryonic mouse cells

5 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
10 expressing β -galactosidase were isolated.

The mutagenesis plasmids pGMD and pGMA were linearized and introduced by electroporation into E.S. cells in order to favor the insertion of only one copy in the genome (Ref. 11).

15 The initial transfections were carried out to compare the targeting efficiency of the HOXC8 (Hox-3.1) of the plasmids pGMA and pGMD (see table I).

Homologous recombination in the gene HOXC8

20 (Hox-3.1)

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

30 Table I

The E.S. cell line "C.C.E." (Ref. 16) was maintained in a continuous manner on fibroblast feeding layers (Ref. 17). For experiments I and II, 1.5×10^7 E.S. cells in 1.5 ml HeBS were electroporated (Ref. 11)
35 at 200 V, with 40mg of linearized plasmid, and then

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spread over four culture trays (diameter 100 mm). For experiment III, the shock was carried out under the same conditions but a quarter of the cells were spread over four 24-well plates. The next day, 250 $\mu\text{g ml}^{-1}$ of G418 were added. Each transfection produced about 2400 clones with pGMA and about 1000 clones with pGND.

The average number of clones of E.S. cells resistant to G418 in each group is indicated in table I, as well as the number of groups giving a positive result with the P.C.R. technique. A positive result means that a band of 1.6 kb could be observed on an agarose gel coloured with ethidium bromide (see Fig. 7). The number of groups giving a positive signal after a Southern analysis of the P.C.R. mixture and hybridization of a specific probe which does not contain the primer sequences is indicated between brackets (Fig. 8).

Detection of homologous recombination with P.C.R.
P.C.R. was carried out on 10^5 cells of a group of 250 clones of transfection II (see lane D of Figure 7). In the other lanes, four groups of transfection III were analysed together by mixing about 4 x 5000 cells. The primers 07 and 08 used in the P.C.R. surround the sequence 3'HOXC8 (Hox-3.1) of the mutagenesis plasmid (Fig. 2). The fragment of 1.6 Kb covering this 3' sequence cannot be amplified except in the case of homologous recombination. Lanes 2, 3 and D illustrate these positive results.

The DNA of E.S. clones was prepared at the moment of replica on filter by utilizing the method "boiling-proteinase K digestion boiling" (Ref. 18). 40 amplification cycles (40 seconds at 94°C, 1 minute at 60°C, 7 minutes at 72°C) were carried out in a reaction mixture of 100 μl , containing 67 mM Tris-HCl (pH 8.6), 16.7 mM $(\text{NH}_4)_2\text{SO}_4$, 6.7 mM MgCl_2 , 10 mM 2-mercaptoethanol, 0.01% (p/v) gelatine, 200 μM dATP,

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dTTP and dCTP, 100 μ M dGTP, 100 μ M 7-deaza dGT, 600 ng of each primer (07: AACTTCCCTCTCTGCTATTC and 08: CAGCAGAAACATACAAGCTG) and 3U polymerase Taq (Perkin Elmer Cetus), covered by 100 μ l paraffin. Half of the
5 reaction mixture was applied on an agarose gel 0.7% coloured with ethidium bromide. The main marker is an Eco RI + Hind III digest of lambda DNA.

Southern analyses

Three independent clones of E.S. cells containing
10 the mutated HOXC8 (Hox-3.1) (identified by P.C.R.) were isolated from positive groups using pipettes. Their DNA was examined by Southern analysis after digestion with the restriction enzymes indicated in Figure 8, so as to confirm the specific targeting and to make the
15 distinction between recombined and wild-type loci. Two different probes were used in the analysis of the 3' end of loci HOXC8 (Hox-3.1) in the mutated clones and in the non-mutated E.S. used as controls (Figure 8c). The first probe "a" was contained in the HOXC8 (Hox-
20 3.1) sequences of the mutagenesis plasmid and showed the number of vector integrations and their physical links. One of the three recombined clones also contained a copy of the plasmid integrated at random (Fig. 8a, clone F2). The second "b" probe which was
25 not in the mutagenesis vector made the distinction between the recombined and wild-type alleles HOXC8 (Hox-3.1) (Fig. 8b). The recombined locus HOXC8 (Hox-3.1) presented, with the two probes, the image of hybridization expected from the restriction maps of the
30 mutagenesis vector and of the intact locus. In addition, the existence of two fields of recombination in the 3' fragment of the vector was confirmed by the presence or the absence of the AT sequence in the recombined locus HOXC8 (Hox-3.1), (for example Fig. 8, clone L5). The 5' end of the locus HOXC8 (Hox-3.1) was
35 also analyzed for a homologous recombination event.

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Restriction enzymes not presenting sites in the sequence HOXC8 (Hoc-3.1) 5' of 6.8 kb of the mutagenesis vector were utilized in the digestion of the DNAs of recombined clones. These DNAs were then submitted to electrophoresis in a pulsed field to differentiate the fragments of high molecular weight. A Southern analysis of this gel also indicated the correctly recombined alleles and the wild-type HOXC8 (Hoc-3.1) alleles, using a sensor presenting a sequence upstream from the mutagenesis plasmid.

Southern analyses have shown that an allele of the gene HOXC8 (Hoc-3.1) was recombined as predicted. The homologous recombination was equivalent to a double "crossing-over" between the genomic arms of the mutagenesis plasmid and the homologous chromosomal sequences (Fig. 2).

In the recombinant clones, the gene LacZ was placed under the control of promoter and regulatory sequences of HOXC8 (Hox-3.1) upstream from the AUG codon, but the 3' signals of maturation of mRNA came from the SV 40. In these recombined clones, the expression of LacZ was not detectable by colouring with β -Gal, which is coherent with the absence of transcription of HOXC8 (Hoc-3.1) in E.S. cells determined by analysis of RNase protection. The activity of β -Gal could be induced in certain cells after 3 or 4 days of culture in the presence of $5 \cdot 10^{-7}M$ retinoic acid, conditions known for inducing the transcription of HOXC8 (Hoc-3.1) (Ref. 19).

By using the mutagenesis vector pGMA, which presents a total homology of 8.3 Kb DNA with the locus HOXC8 (Hoc-3.1), a fragment of 120 pb was replaced by an insertion of 7.2 Kb. The frequency of this targeted replacement (1/900) is comparable to that obtained recently (1/1000) with HPRT (Ref. 13) or with En-2

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(1/260) (Ref. 20), the heterologous fragment inserted being nonetheless in these latter cases of a much less significant size (1.1 and 1.5 Kb respectively). In a surprising manner, it was noted that a very high homologous recombination frequency (1/40) could be obtained with the pGMD vector. The elimination of 3' signals of maturation of mRNA and the addition of the sequence of degradation of mRNA to the gene of resistance to neomycin had the effect of reducing the total number of clones resistant to G418 by 2.4 (table I). The specific targeting result was almost ten times higher (900/40). The mechanism of homologous recombination was probably affected in the experiments with pGMD. A possible explanation for these results could be that an AT sequence of 51 pb could provide, *in vivo*, an open loop in the mutagenesis plasmid because of its lower fusion temperature. If the HOXC8 (Hoc-3.1) sequences close to the pGMD can be influenced by this opening, on each side of the AT region, they could react in a more efficient manner, in the single-strand state, with the chromosomal locus HOXC8 (Hoc-3.1). The model of mitotic recombination in yeast suggests that it could be initiated by such an exchange of strands, even though the mechanism of homologous recombination remains unknown in the more complex eucaryotes.

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

The probes used only hybridize with HOXC8 (Hoc-3.1) sequences included in the vector (a) or excluded from the mutagenesis vector (b). The image of hybridization of the recombined locus HOXC8 (Hoc-3.1) (open triangles) is clearly distinguished from the wild-type locus (black triangles). The stars indicate the hybridization bands of a copy of the plasmid which



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was integrated at random. The size marker is a digest Eco RI + Hind III of Dna lambda.

Figure 8(c) shows the restriction maps of recombined (rec.) and wild-type (wt) HOXC8 (Hoc-3.1) alleles. The parts of the mutagenesis vector and of the locus HOXC8 (Hoc-3.1) are indicated by the same symbols as those used in Figure 2. In this case, the sequence AT was integrated by homologous recombination. The vertical arrow indicates the 3' end of the mutagenesis plasmid. The localization of probes "a" and "b" used in the Southern analysis is also indicated. The abbreviations used in Figure 8 are the following: B, Bam HI; D, Dra I; E, Eco RI, H, Hind III; S, Sal I; X, Xho I.

15 IV - Production of chimeric embryos

A micro-injection was made in blastocysts with two recombinant E.S. clones containing an intact allele HOXC8 (Hoc-3.1) 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 mutated cells were micro-injected per blastocyst. After reimplantation in surrogate mothers, the embryos were taken at 9.5, 10.5 and 12.5 days p.c. and analyzed for LacZ expression. The transcription template of HOXC8 (Hoc-3.1) at these stages had been determined beforehand by hybridization analysis *in situ* (Ref. 1). The HOXC8 (Hoc-3.1) transcripts are detectable for the first time at the stage of late gastrulation and are spread throughout all the tissues of the posterior part of the animal. Later, the distribution becomes progressively limited in space and specific to the tissue. At the stage of 12.5 days p.c. the transcription is localized in the cervical region of the neural tube, at the level of the heart. During embryogenesis, the repartition of the transcription of HOXC8 (Hoc-3.1) thus undergoes

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modifications. The stage 10.5 days p.c. seems to be a period of transition, the transcription taking place both in the two rear regions and in the cervical neural tube.

5 In chimeric embryos of 9.5 and 10.5 days p.c., the caudal part to the posterior bud presented intense β -Gal activity, while the marker was never detected in the anterior thoracic region or the head (Fig. 9a). In the posterior region, the cells coloured by β -Gal were
10 observed in all the tissues and all the embryonic layers. Between the two buds which form the members, coloured cells were distributed in the restricted zones, in the superficial ectoderm (Fig. 9b), as in the rear regions (Fig. 9c) and, in straight lines or rays,
15 in the neural tube (Fig. 9b). These rays showed an irregular and asymmetric distribution on the wall of the neural tube. The transcription of HOXC8 (Hoc-3.1) was not detected in the thin layer of cells near the closure of the neural tube. Perhaps these cells did not
20 resist the treatments applied during the hybridization in situ. It was observed that the cells of the neural ectoderm very soon become part of the different parts of the nervous system and migrate in a radial direction, following narrow lateral movements (Ref.
25 21). These results are thus in line with this observation.

The expression of LacZ thus illustrated correctly the first part of the transcription of the homeogene HOXC8 (Hoc-3.1), that is to say in all the tissues of
30 the caudal regions of embryos of 9.5 and 10.5 days p.c., and provided new information concerning the mode of transcription of HOXC8 (Hoc-3.1).

On the other hand, the expression of LacZ was not observed in the cervical regions of the neural tube of
35 chimeric embryos of 12.5 days, nor in the anterior



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region of embryos of 10.5 days; this was not the result expected from studies of hybridization in situ. The ulterior phase of transcription of HOXC8 (Hoc-3.1) observed from day 10.5 in the very localized zones of the neural tube were not made evident by the activity of β -Gal. A possible explanation for this result could be that, even though the expression of LacZ was under the control of the promoter HOXC8 (Hoc-3.1), the 3' sequences of HOXC8 (Hoc-3.1) are absent in the reporter gene. It is possible that the 3' sequences of the AUG initiation codon of HOXC8 (Hoc-3.1) have an influence on the late expression of HOXC8 (Hoc-3.1) in the anterior domain. An effect of "gene dosing" could also explain this result. Auto-activation of several homeogenes of *Drosophila* was demonstrated genetically or suggested by the formation of complexes between the DNA and the proteins of the homeoboxes.

If the late component of transcription of HOXC8 (Hoc-3.1) in the neural tube is maintained by a similar mechanism, the inactivation of an allele could have a dominating effect in the cells of the neural ectoderm. Since one allele only would produce the protein HOXC8 (Hoc-3.1), the signal of activation would be diluted on the two promoters. The reduction of auto-inactivation in the two loci could thus lead to a total interruption of the transcription initiation. This could explain why no expression of LacZ was detected in the cervical region of the neural tube of embryos of 10.5 and 12.5 days.

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

The effects in F₁ and in F₂ 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.

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CLAIMS

1. Transgenic or chimeric animal comprising cells whose genome comprises:

- a heterologous DNA, called insertion DNA, and being constituted by the totality of a gene or by a part of a gene;

- the insertion DNA being positioned in the genome in relationship to an endogenous gene, called the receiver gene, comprising a complementary DNA, in such a way that the insertion DNA or the complementary DNA is rendered functional, or its functioning is rendered more efficient due to the association of the insertion DNA and the complementary DNA.

2. Animal according to claim 1, characterized in that the insertion DNA contains either a coding sequence, whose expression takes place under the control of regulatory sequences of the receiver gene, or a regulatory sequence permitting the expression of a coding sequence of the receiver gene.

3. Animal according to claim 2, characterized in that the insertion DNA is constituted by a coding sequence other than the coding sequence of a gene coding for a selection agent.

4. Animal according to claim 1, characterized in that the insertion DNA comprises the totality of a gene.

5. Animal as claimed in any one of the preceding claims characterized in that the receiver gene is a gene which normally is not transcribed in the cell.

6. Animal as claimed in any one of the preceding claims characterized in that the receiver gene is inactivated following the insertion of the insertion DNA, the expression of the insertion DNA substituting that of the receiver gene.

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7. Animal as claimed in any one of the preceding claims characterized in that the insertion DNA replaces a part of the receiver gene.

5 8. Animal as claimed in any one of the preceding claims characterized in that the receiver gene is, apart from the presence of the insertion DNA, in its natural genetic environment.

9. Animal as claimed in any one of the preceding claims characterized in that the genome contains,
10 adjacent to the insertion DNA, a so-called "intercalated" sequence comprising for example a sequence coding for a selection agent with a promoter authorizing the expression of the agent of selection, or a marker.

15 10. 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
20 permits the expression of this gene under the control of regulatory sequences of the inactivated endogenous gene.

11. Animal as claimed in any one of the preceding claims utilized for a genetic marking.

25 12. Process for screening pharmaceutical products presumed to have an activity with regard to expression products of a pathological gene linked to a disease, characterized by the administration of the product to a transgenic animal according to any one of the claims 1
30 to 11, the insertion DNA being constituted by the pathological gene or a fragment of it.

13. Nucleic acid obtained from the homologous recombination between an insertion DNA and a receiver gene, said nucleic acid comprising:

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- an insertion DNA, heterologous with respect to the receiver gene, the insertion DNA being constituted by the totality of a gene or by a part of a gene;
- a receiver gene;
- the insertion DNA being positioned in relationship to the receiver gene comprising a complementary DNA in such a way that the insertion DNA or the complementary DNA is rendered functional, or its functioning is rendered more efficient due to the association of the insertion DNA and the complementary DNA.
14. Cell of a transgenic animal as claimed in any one of claims 1 to 10.
15. Cell line issuing from a transgenic animal as claimed in any one of claims 1 to 10.

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