(19)

(11) **EP 1 532 260 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent: **10.12.2008 Bulletin 2008/50**

(21) Application number: **03790868.8**

(22) Date of filing: **06.08.2003**

(51) Int Cl.:
C12N 15/90 (2006.01) *C12N 15/10* (2006.01)
A01K 67/027 (2006.01)

(86) International application number:
PCT/EP2003/008713

(87) International publication number:
WO 2004/020645 (11.03.2004 Gazette 2004/11)

(54) **CHROMOSOMAL LOCI FOR THE STRINGENT CONTROL OF GENE ACTIVITIES VIA TRANSCRIPTION ACTIVATION SYSTEMS**

CHROMOSOMSTELLEN ZUR STRINGENTEN KONTROLLE VON GENAKTIVITÄT DURCH TRANSKRIPTIONSTEUERSYSTEME

LOCI CHROMOSOMIQUES POUR LE CONTROLE STRICT DES ACTIVITES GENIQUES AU MOYEN DE SYSTEMES D'ACTIVATION DE TRANSCRIPTION

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PT RO SE SI SK TR

(30) Priority: **28.08.2002 US 406344 P**

(43) Date of publication of application:
25.05.2005 Bulletin 2005/21

(73) Proprietor: **TET Systems Holding GmbH & Co. KG**
69120 Heidelberg (DE)

(72) Inventors:
- **BUJARD, Hermann**
69120 Heidelberg (DE)
- **SCHÖNIG, Kai**
69115 Heidelberg (DE)

(74) Representative: **Dick, Alexander et al**
Isenbruck Bösl Hörschler Wichmann Huhn Patentanwälte
Theodor-Heuss Anlage 12
68165 Mannheim (DE)

(56) References cited:
EP-A- 1 092 771

- **BARON U ET AL: "CO-REGULATION OF TWO GENE ACTIVITIES BY TETRACYCLINE VIA A BIDIRECTIONAL PROMOTER" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 23, no. 17, 11 September 1995 (1995-09-11), pages 3605-3606, XP000775822 ISSN: 0305-1048**
- **ST-ONGE L. ET AL.: "Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter" NUCL. ACIDS RES., vol. 24, no. 19, 1996, pages 3875-3877, XP002270146**
- **DATABASE EBI DNA SEQUENCES [Online] 2 June 2002 (2002-06-02) "Mus musculus BAC clone RP23-111G16 from chromosome 6" retrieved from EBI Database accession no. AC122874 XP002270147**
- **FESTENSTEIN R. ET AL.: "Locus control region function and heterochromatin-induced position effect variegation" SCIENCE, vol. 271, 1996, pages 1123-1125, XP000876598**
- **HENIKOFF S.: "Conspiracy of silence among repeated transgenes" BIOESSAYS, vol. 207, 1998, pages 532-535,**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Mylan v. Genentech

DOCKET ALARM
Find authenticated court documents without watermarks at docketalarm.com.

Description

[0001] The invention relates to a vector for transgenesis by homologous recombination of a MEO and to the use of such an vector for transgenesis by homologous recombination of a mammalian cell or organism and to the use of such a vector for the preparation of a medicament for somatic gene therapy.

Background of the Invention

[0002] The transgenesis of MEO, that can be e.g. a plant or an animal, comprises the integration of a transcription unit into the MEO's genome that comprises a gene and a regulatory sequence that controls the transcription of said gene. To achieve regulation over a maximum amount of orders of magnitude, it is favorable to employ as a regulatory sequence one or more transcription control sequence(s) susceptible for binding transactivators fused to an enhancerless promotor. The basal activity of a chromosomally integrated minimal (i.e. enhancerless) promotor strongly depends on its chromosomal locus. It is well known that the activity of a transcription unit (consisting of at least a promoter, a gene to be expressed and a polyadenylation site), which is inserted into a chromosomal locus either by random or targeted integration, depends strongly on the context of the surrounding chromatin (Palmiter et al. Annu. Rev. Genet. 29, 465-99). When inserted into heterochromatin, transcription units are usually silenced. In euchromatin, on the other hand, the activity of an inserted transcription unit depends on a number of parameters which may affect transcription of the integrated gene positively or negatively. When the transcriptional unit is located close to an enhancer, it will show an increased activity even in the absence of its cognate activator. This problem can be circumvented, if non-homologous recombination is used, by increasing the number of clones (i.e. different integration events) to be screened, or more actively by shielding the transcriptional unit via transcriptional silencers which may be susceptible to control by ligands. However, it is obvious that the first solution of the problem apart from being time consuming leads to the binding of personal and financial resources. The latter solution of the problem is complicated and often reduces the maximum level of transcription of the gene that can be achieved. Furthermore, also the maximum level of transcription of the gene that can be achieved also varies with the chromosomal locus of integration. The locus may also give rise to position effect variegation ("PEV", Palmiter et al. Annu. Rev. Genet. 29, 465-99) and thus, to mosaic expression of the transgene.

Summary of the invention

[0003] The technical problem underlying the present invention is therefore to provide a chromosomal locus for transgenesis of a mammalian cell or organism which does not influence or hamper the regulation of the transcription units when introduced into the genome by transgenesis, and that allows the expression of the transgene that forms part of the transcription unit at high levels and that does not give rise to any PEV in respect to the transgene.

[0004] The solution to the above technical problem is achieved by providing the embodiment of the invention as set forth in claim 1. Further embodiments of the invention are described in claims 2 to 8.

[0005] A vector for transgenes is according to the present invention comprising a chromosomal locus for transgenesis of a first multicellular eukaryotic organism ("first MEO") by homologous recombination or random integration of a DNA sequence comprising DNA being characteristic for the locus, the locus ensuring both the efficient transcription of the gene that is introduced into the genome at said locus (the "transgene"), and the transcriptional control of said transgene by a transactivator without disturbing interference with other transcription control elements, comprising the steps of:

(a) providing a transgenic line of a second multicellular eukaryotic organism ("second MEO"), comprising:

(a0) a first and a second transcription unit being integrated into the genome of the second MEO;
(a1) at least the first transcription unit being stably transmitted to the progeny;
(a2) the first transcription unit comprising one or more reporter genes and one or more transcription control sequence(s), the transcription of the reporter genes being susceptible to control by the binding of a transactivator to the transcription control sequence(s) ("first ligand");
(a3) the second transcription unit comprising a gene encoding the transactivator,
(a4) the transactivator's affinity for the transcription control sequence(s) being susceptible to control by the binding of a second ligand; and
(a5) the transcription of the reporter genes of a cell of the second MEO being susceptible to be stimulated in dependence on the concentration of the second ligand, provided that the transactivator is expressed in said cell;

(b) identifying a transgenic line of step (a) further comprising the following features:

(b1) the ratio of the amount of the reporter gene(s) product present in a cell in the ON state in which transcription

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

of the reporter gene(s) is maximally stimulated to the amount present in the OFF state in which transcription of the reporter gene(s) is minimally stimulated, is at least 10^2;
(b2) no significant amount of any reporter gene product can be detected during a phase comprising development to adulthood if the second MEO is kept in the OFF state during said phase; and
(b3) no position effect variegation can be observed in respect to the reporter gene(s) in substantially all cells in which the transactivator is expressed.

Detailed description of the invention

[0006] The method usually starts with the preparation of a plurality of transgenic MEO by non-homologous recombination also known as random integration of the tancription unit into the genome of a second MEO used for identification of the locus. The second MEO is preferably a mouse. It is possible to ensure that all transgenic lines obtained by transgenesis exhibit features (a0) to (a5).

[0007] The feature (a2) refers to the first transcription unit that is to be introduced into the genome. The first transcription unit comprises one or more reporter genes and one or more transcription control sequence(s), the transcription of the reporter genes being susceptible to control by the binding of a transactivator to the transcription control sequence(s) (also named 'first ligand' hereinafter). A transactivator is a protein that binds to cognate DNA sequences within a promotor and activates the transcription of this promotor. The affinity of the transactivator for the transcription control sequences are dependent on the concentration of a second ligand (e.g. doxycycline). The binding of the transactivator to both ligands shows positive or negative cooperativity. This means in the context of the invention that the binding of the first ligand positively or negatively influences the binding of the second ligand, and vice versa, depending on the nature of the transactivator. The transcription control sequence or the transcription control sequences are preferably built by a *tet*O sequence that may be multimerized to form e.g. a heptamer of *tet*O sequences. The use of the P_{tet}bi-1 promotor is most preferred (see "Tetracyclines in Biology Chemistry and Medicine" ed. By M. Nelson, W. Hillen and R.A. Greenwald, Birkhauser Verlag Switzerland (2001), p. 139 et seq. and references therein). The reporter genes are preferably genes encoding a luciferase and/or a recombinase like cre recombinase.

[0008] The second transcription unit may be introduced into the genome of the transgenic line of the second MEO by breeding with a transgenic MEO line expressing a transactivator in substantially all cells of the MEO or in a subset of cells that can be histologically identified. This means that the transgenic line of the second MEO of feature (a) will be generally heterozygous in respect to both transcription units. The transactivator encoded by the gene which forms a part of the second transcription unit is preferably selected from a group consisting of tTA and rtTA and derivatives thereof binding to the *tet*O sequence as a first ligand and to doxycycline as a second ligand with either negative or positive cooperativity. For the transactivators which can be preferably used see "Tetracyclines in Biology Chemistry and Medicine" ed. By M. Nelson, W. Hillen and R.A. Greenwald, Birkhäuser Verlag Switzerland (2001), p. 139 ff. and references therein; PNAS 89:5547-5551 (1992); Annu. Rev. Genet. 36:153-173 (2002).

[0009] After providing a plurality of transgenic MEOs each individual is tested for the following features (b1) to (b3):

(b1) the ratio of the amount of the reporter gene(s) product present in a cell in the ON state in which transcription of the reporter gene(s) is maximally stimulated to the amount present in the OFF state in which transcription of the reporter gene(s) is minimally stimulated is at least 10^2;
(b2) no significant amounts of any reporter gene product can be detected during a phase comprising development to adulthood, if the second MEO is kept in the OFF state during said phase; and
(b3) no position effect variegation, PEV can be observed in respect to the reporter gene(s) in all cells in which the transactivator is expressed.

[0010] In feature (b1) the ability of the reporter genes to be regulated is defined. A ratio of the reporter gene present in a cell in the ON state in which transcription of the reporter gene(s) is maximally stimulated to the amount present in the OFF state in which transcription of the reporter gene(s) is minimally stimulated of 10^2 will ensure that the cross talk of other factors that elevate the transcription background in the OFF state due to enhancers in the vicinity of the integration site of the first transcription unit is limited. However, preferably the ratio is at least 10^4-10^5, more preferably about 10^5 or even higher. It is clear that the ratio can only be determined for the cells of the second MEO expressing the reporter genes whose expression level is to be tested. This requires that the transctivator is expressed in these cells, too. The expression pattern of the transactivator can be influenced by the choice of the promotor governing the transcription of the gene encoding for the transactivator which forms a part of the second transcription unit.

[0011] The OFF and the ON state can be selected by adjusting the concentration of the second ligand in the cells. If the transactivator is tTA or rtTA and the second ligand is a tetracyline, e.g. doxycycline, then the concentration of said ligand can be adjusted by offering a liquid nutrient containing the second ligand to the MEOs, preferably being mice. If one of the reporter genes is luciferase the amount of protein can be detected by conventional testing of the enzymatic

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

activity of the protein homogenate of the tested cells. If one of the reporter genes is cre recombinase the amount of protein can be detected by using R26R transgenic mice as starting material for the production of transgenic mice that have a *lox*P-flanked DNA insert integrated into their genome that allows simple testing of recombination events as described in the examples.

5 **[0012]** If the first transcription unit comprises more than one reporter gene all of the reporter genes are preferably coregulated.

[0013] In feature (b2) the ability of the reporter genes is defined to be kept silent during a phase comprising development to adulthood including embryogenesis. The regulation of the reporter genes inserted into the genome at a specific chromosomal locus should not vary during development of the MEO. Especially it is important to select for transgenic 10 MEO that do not show significant amounts of any reporter gene during a phase comprising the development to adulthood of the MEO if held in the OFF state. An amount of a reporter gene product is not significant if the amount is not significantly elevated compared to amounts of individuals of the same genotype in the OFF state in any developmental stage. More precisely, the amount of a reporter gene product is not significant if it is 10-fold or less above the detection level of individuals with a null genotype. If a reporter gene is a recombinase (e.g. cre recombinase) the amounts of the reporter 15 gene in the cells of the second MEO are not significant if no recombination activity in the cells is observed during said phase. If the MEO is a mouse the recombination activity is preferably tested using the mouse line R26R. R26R can be used as starting material for the construction of the mouse line trangenic in respect to the first transcription unit or the *lox*P-flanked gene can be introduced into the genome by breeding.

[0014] Feature (b3) prescribes that no position effect variegation, PEV can be observed in respect to the reporter 20 gene(s) in all cells in which the transactivator is expressed and in which therefore the reporter genes can be transcribed and expressed. Generally PEV is presumed to be caused by plasticity of the promotor structure in the surrounding of a transcription unit. This plasticity may result in mosaic expression in populations of identical cells.

[0015] In step (c) sequence information of a sequence flanking the first transcription unit is obtained that is sufficient to determine the chromosomal locus on the genome of said second MEO. Preferably step (c) further comprises the 25 following steps:

(c1) cloning of genome fragments of the second MEO in bacterial artificial chromosomes ("BACs") or yeast artificial chromosomes ("YACs");
(c2) testing the clones of step (c1) for the presence of the first transcription unit; and
30 (c3) obtaining sequence information of one or both sequence regions that flank the sequence of the first transcription unit in clones tested positive in step (c2) sufficient to determine the chromosomal locus on the genome of said second MEO.

[0016] Cloning of genome fragments in bacterial or yeast artificial chromosomes is known to a person skilled in the 35 art (see examples for references). If an artificial chromosome is tested positive for the presence of the first transcription unit then the flanking sequences are determined until sufficient information is gained to determine the chromosomal locus of the first transcription unit of the respective transgenic line.

[0017] The invention relates to a vector for transgenesis of a mammalian cell or organism by homologous recombination, the vector comprising at least one transcription unit comprising the gene to be introduced into the genome and 40 sequences flanking the transcription unit(s), characterized in that the flanking sequences are selected so that homologous recombination at a chromosomal LC-1 locus obtainable by an above method and characterized in mouse by any one of SEQ ID Nos: 1 to 4 is ensured. Such a vector can also be used for somatic gene therapy.

[0018] The invention further relates to a vector for transgenesis of a mammalian cell or organism by random integration of a DNA sequence comprising DNA being characteristic for a chromosomal LC-1 locus, the vector comprising at least 45 one transcription unit containing the gene to be introduced into the genome (the "transgene") and sequences flanking the transcription unit(s), characterized in that the flanking sequences comprise a sequence being characteristic for a chromosomal LC-1 locus obtainable by an above method and characterized in mouse by any one of SEQ ID Nos: 1 to 4. This vector is also useful for the transgenesis of species for which no ES technology is available. Such a vector can also be used for somatic gene therapy.

50 **[0019]** The sequences flanking the transcription unit(s) of this vector will generally be much larger than the flanking sequences necessary for ensuring homologous recombination. The length of the flanking sequences is selected so that even if the transgenesis is not effected by homologous recombination but by random integration the transcription of the transgene is regulated as it would be if transgenesis was effected by homologous recombination. That means that the flanking sequences are large enough to emulate the influence of the chromosomal locus obtainable by an above method 55 on the transcription of the transgene. The flanking sequences will have for example a length of 5 kbp to 150 kbp.

[0020] If a chromosomal locus is identified, sequence information can be obtained that allows the design of the sequences that flank the transcription unit to be introduced into the genome so that homologous recombination at a chromosomal locus obtainable by an above method is ensured or that allow random integration as described above.

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

The insertion of the transcription unit at the chromosomal locus on the genome of the MEO has the consequence that the regulation of said transcription unit is not hampered by enhancers or other sequences in the vicinity of the integration site, that the transcription up to high levels is possible provided that the transcription unit contains a suitable gene the transcription of which is controlled by a suitable promotor, and that no PEV is observed in respect to the genes of said transcription unit.

[0021] A preferred embodiment of such a vector is characterized in that the vector comprises a first and a second transcription unit as defined above spaced by a sequence of sufficient length to prevent any influence of transcription factors that bind to one of the transcription units and the transcription of the respective other transcription unit. Such a vector allows the generation of a transgenic MEO line into which reporter genes encoding e.g. luciferase and/or cre recombinase or any other genes of interest and the transactivator gene encoding the transcativator that controls the transcription of the reporter gene(s) are introduced by one step. If the vector comprises more than one transcription units the transcription units define a cassette that is flanked by sequences being characteristic for a chromosomal locus obtainable by an above method so that homologous recombination at a chromosomal locus obtainable by an above method is ensured if homologous recombination is chosen.

[0022] A mammalian cell or organism can be obtained by a method of manufacture a transgenic non-human multicellular eukaryotic organism by transgenesis via homologous recombination using a vector as described above.

[0023] The figures show:

Figure 1: Outline of the Tet regulatory principle. Left upper part shows the mode of action of the Tc controlled transactivator (tTA). tTA binds in absence of the effector molecule Dox to the *tet*O sequence within P_{tet} and activates transcription of gene x. Addition of Dox prevents tTA from binding and, thus, the initiation of transcription. Left lower part depicts the dose response of Dox on tTA dependent gene expression. Gene activity is maximal in the absence of the antibiotic but as effector concentrations increase transcription gradually decreases to background levels at Dox concentration > 5 ng/ml. Right upper part illustrates the mechanism of action of the reverse Tc controlled transactivator (rtTA). rtTA is identical to tTA with the exception of 4 amino acid substitutions in the TetR moiety. rtTA requires Dox for binding to *tet*O sequences within P_{tet} in order to activate transcription of gene y. Right lower part outlines the dose response of Dox on the rtTA dependent transcription activation. By increasing the effector concentration beyond 20 ng/ml of Dox, rtTA dependent gene expression is gradually stimulated. P_{tet} is a minimal promoter fused downstream of an array of 7 *tet* operators (7). It interacts with tTA as well as with rtTA.

Figure 2: Topography of tTA/rtTA responsive promoters (P_{tet}). P_{tet}-1 is composed of a minimal promoter derived from the human cytomegalovirus promoter IE of which the sequence between -53 and +75 (+1 being the transcriptional start site) was fused to an array of 7 equally spaced *tet* operator sequences (5). In P_{tet}bi-1. two minimal promoters flank either side of the array of *tet* operators as described in ref. 9.

Figure 3: Structure of the bidirectional *luc*/*cre* transcription unit and of BAC E11. A. The genes of the firefly luciferase and of Cre recombinase, respectively, are coregulated by P_{tet}bi-1. The *luc* gene is flanked by a SV40, the *cre* gene by the human growth hormone (hGH) polyadenylation site (A_n). The expression cassette can be retrieved via unique NotI cleavage sites indicated. B. BAC E11 contains three tandemly integrated *luc*/*cre* transcription units and at the left border a fragment of the hGH polyA site. The insert is flanked at the left side by a 25 kb and at the right side by a 50 kb fragment of mouse DNA. Sequence analysis revealed that the two regions stem from mouse chromosome 6. The size of the insert in BAC E11 is 95 kb, it is flanked by NotI sites as indicated. The cloning vehicle pBeloBAC-HD has been described previously (10).

Figure 4: Localization of the E11 region in C1 of mouse chromosome 6. A. Partial sequences of the E11 insert were obtained by probing respective E11 I DNA with primers initiating DNA synthesis from sequences within the vector pBeloBAC (primer 3) or pBluescript (T7 sequencing primer), respectively. B. Blast results of the 4 sequences shown using the ENSEMBL mouse genomic library (http://www.ensembi.org) C. Position of the E11 region within C1 of mouse chromosome 6.

Figure 5: Analysis of E11 transgenic mice. Six mouse lines. E11-1 to E11-6, stably transmitting the intact E11 fragment were crossed with mice of the TA^{LAP}-2 line expressing tTA specifically in hepatocytes. Luciferase activity in presence and absence of Dox was determined in extracts of the liver and various other tissues and then compared with respective values obtained with the parent LC-1 mouse line. Luciferase activity measured in liver extracts is shown. The values given are the means from 4 to 5 animals.

Figure 6: Transcription units incorporated in the LC-1 and rTA^{LAP}-1 mouse line, respectively. (A) The bidirectional tTA/rtTA responsive promoter P_{tet}bi-1, present in LC-1 animals, contains an array of seven tet operator sequences

DOCKET ALARM Find authenticated court documents without watermarks at docketalarm.com.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.

WHAT WILL YOU BUILD? | sales@docketalarm.com | 1-866-77-FASTCASE

fastcase®
Smarter legal research.