IN THE UNITED STATES DISTRICT COURT
FOR THE SOUTHERN DISTRICT OF NEW YORK
WHITE PLAINS DIVISION

REGENERON PHARMACEUTICALS, INC., Plaintiff, v. MERUS B.V. Defendant.	Civil Action No. _____ **COMPLAINT FOR PATENT INFRINGEMENT** **DEMAND FOR JURY TRIAL**

JUDGE KARAS

14 CV 1650

Plaintiff Regeneron Pharmaceuticals, Inc. ("Regeneron") hereby alleges a claim for patent infringement against Defendant Merus B.V. ("Merus") as follows:

INTRODUCTION

1. Regeneron brings this action to remedy Merus's infringement of Regeneron's intellectual property concerning genetically engineered animals used in making human biopharmaceutical therapeutics, including human antibodies.

2. Regeneron's groundbreaking work in this field has resulted in the creation of Regeneron's VelocImmune® technology. VelocImmune® is the culmination of the insights of a group of dedicated scientists led by Regeneron's Chief Scientific Officer, Dr. George D. Yancopoulos.

3. Merus is an entity funded by a group of venture capitalists and large pharmaceutical companies, including Pfizer Venture Investments (a member of the Pfizer, Inc. conglomerate (collectively, "Pfizer")) and Johnson & Johnson.

4. Merus's sole business purpose appears to be directed to a genetically modified mouse that infringes Regeneron's intellectual property.

THE PARTIES

5. Regeneron is a corporation organized and existing under the laws of the State of New York, with its principal place of business at 777 Old Saw Mill River Road, Tarrytown, New York 10591. Regeneron was founded in New York in 1988 by a Cornell University professor—Dr. Leonard S. Schleifer—and a prominent research scientist from Columbia University—Dr. George D. Yancopoulos. Drs. Schleifer and Yancopoulos both trained as physician scientists, doctors who focus on finding innovative ways to use science to advance the care of patients. Regeneron is now a global leader in biopharmaceutical research and development with approximately 2,500 employees. Through pioneering science and a commitment to innovation, Regeneron develops, manufactures, and commercializes medicines for the treatment of serious medical conditions, including Neovascular (Wet) Age-Related Macular Degeneration, one of the leading causes of blindness in the world; Cryoprin-Associated Periodic Syndrome (CAPS), a rare and debilitating hereditary condition; and Colorectal Cancer. Regeneron has received numerous honors and awards, including being named the 2012 Biotech Company of the Year.

6. Regeneron has two primary locations, both of which are in the State of New York. Regeneron's research and administrative offices are located in Tarrytown, New York. Regeneron has a manufacturing facility in Rensselaer, New York.

7. Regeneron is informed and believes, and on that basis alleges, that Merus is a corporation organized and existing under the laws of The Netherlands, with its principal place of business at Padualaan 8 (postvak 133), 3584 CH Utrecht, The Netherlands.

JURISDICTION AND VENUE

8. This is an action for patent infringement under the Patent Act, 35 U.S.C. § 100 *et seq.*, including § 271. The Court has subject matter jurisdiction over the matters pleaded herein pursuant to 28 U.S.C. §§ 1331 and 1338(a).

9. Personal jurisdiction is proper over Merus under the laws of New York State, including N.Y.C.P.L.R. § 302(a).

10. Merus transacts business in this state, including with Taconic Farms and Pfizer.

11. Merus has committed acts of infringement in this state by, *inter alia*, making, using, selling, offering to sell, and/or importing infringing products in this state, and has committed tortious acts in this state by making, using, selling, offering to sell, and/or importing infringing products in the United States, with knowledge of Regeneron's intellectual property and presence in this state, and the harm caused to Regeneron by these acts. As its office and labs are located in the Netherlands, Merus also commits acts of infringement in the Netherlands that will be remedied under Regeneron's European intellectual property.

12. Alternatively, personal jurisdiction is proper in this judicial district pursuant to Federal Rule of Civil Procedure 4(k)(2) because Merus has extensive contacts with the United States and exercising jurisdiction over Merus is consistent with the laws of the United States and the United States Constitution. Among other things, Merus has commercial relationships and business dealings with several companies in the United States, including Pfizer, Taconic Farms, Bay City Capital, and Johnson & Johnson. Merus also attends and participates in conferences and trade shows in the United States—including in California, Illinois, Massachusetts, and Washington, D.C. in the past year alone—promoting

its infringing mice, seeking licensing and financial partnerships with United States companies, and soliciting sales and other business in the United States. In addition, Merus has registered at least four trademarks with the United States Patent and Trademark Office ("USPTO"), including a trademark for "MeMo" which it uses in connection with its infringing mice. Each of the applications for those trademarks was filed in compliance with 15 U.S.C. § 1141 *et seq.*, pursuant to which Merus declared its intent to use the marks in commerce in the United States. Merus has also filed several patent applications with the USPTO, including U.S. Serial No. 12/459,285 to Houtzager et al., U.S. Serial No. 12/589,181 to Logtenberg et al., and U.S. Serial No. 13/750,753 to Logtenberg et al.—applications which, based on Regeneron's information and belief, relate to Merus's infringing technology.

13. Venue is proper in this judicial district pursuant to 28 U.S.C. §§ 1391(b) and (d) and 28 U.S.C. § 1400(b), because, among other reasons, Merus is subject to personal jurisdiction because of its activities, including with Pfizer, and has committed acts of infringement by, *inter alia,* making, using, selling, offering to sell, and/or importing infringing products in this state.

INTRA-DISTRICT ASSIGNMENT

14. Assignment to the White Plains Division is proper pursuant to Rule 18 of the Southern District of New York's Rules for the Division of Business Among District Judges because, among other things, Regeneron has its principal place of business in Westchester County, New York.

BACKGROUND FACTS

15. One of the most important biological molecules in nature is the antibody. Antibodies, which are produced by our immune systems, attack foreign organisms such as

viruses and bacteria. The power of antibodies, however, can also be harnessed to treat some of the most serious diseases of our age, including cancers and immune disorders. Creating antibodies that effectively treat disease in humans can be a difficult and inefficient process. To address these problems, Regeneron scientists launched a scientific research program to design a rodent that could create an extraordinary diversity of human antibodies in a rapid and efficient manner. This type of rodent is known as a "genetically modified" animal. Earlier generations of genetically modified animals used to produce antibodies suffered from serious health and functionality issues. Regeneron's inventions led to the creation of Regeneron's VelocImmune® mouse. The VelocImmune® mouse makes part-human and part-mouse antibodies, which allows the VelocImmune® mouse to mount a healthy, functional and diverse immune response to produce antibodies that can be used in making human therapeutics. This work led to the grant of an important family of patents throughout the world. One of these patents, the subject of this action, is United States Patent No. 8,502,018 (the "'018 Patent"). The named inventors include Regeneron's Chief Scientific Officer Dr. George D. Yancopoulos and Senior Vice President of Regeneron Laboratories Dr. Andrew Murphy.

16. After graduating from Bronx High School of Science and Columbia University, Dr. Yancopoulos earned a Ph.D. in Biochemistry and Molecular Biophysics and an M.D. from Columbia University. In 1989, Dr. Yancopoulos joined Regeneron as its founding scientist. Dr. Yancopoulos was the 11th most highly cited scientist in the world in the 1990s and was elected in 2004 to the U.S. National Academy of Sciences. Dr. Yancopoulos is the recipient of numerous additional honors and awards, including Columbia University's Stevens Triennial Prize for Research and Columbia University's Medal of Excellence for Distinguished Achievement.

17. Dr. Murphy earned a B.S. in Molecular Biology from the University of Wisconsin and a Ph.D. in Human Genetics from Columbia University. He joined Regeneron in 1999 as Director of Genomics and Bioinformatics. Dr. Murphy was later named Vice President of Gene Discovery and Bioinformatics in 2001, Vice President of Target Discovery in 2005, and Senior Vice President of Regeneron Laboratories in 2013.

18. Following on the demonstrated success of the VelocImmune® technology, the biopharmaceutical community recognized the power of Regeneron's inventions to create new genetically modified mice useful in making antibody therapeutics. A group of venture capitalists, in concert with some of the largest pharmaceutical companies in the world, including Pfizer and Johnson & Johnson, funded Merus—an entity whose sole apparent purpose is to create and use a genetically modified mouse that copies Regeneron's intellectual property.

COUNT ONE

(Patent Infringement)

19. Regeneron re-alleges and incorporates by reference the allegations contained in paragraphs 1 through 18 above.

20. On August 6, 2013, the United States Patent and Trademark Office issued the '018 Patent, entitled "Methods of modifying eukaryotic cells," to Drs. Andrew Murphy, George Yancopoulos, Margaret Karow, Lynn Macdonald, Sean Stevens, David Valenzuela, and Aris Economides.

21. Regeneron is the owner of the entire right, title, and interest in and to the '018 Patent. A copy of the '018 Patent is attached as Exhibit A.

22. The '018 Patent includes 20 claims. By way of example, claim 9 of the '018 Patent recites:

"[A genetically modified mouse, comprising in its germline human unrearranged variable region gene segments inserted at an endogenous mouse immunoglobulin locus] wherein the mouse produces an antibody that comprises a human variable region and a mouse constant region."

23. The '018 Patent notes:

"A transgenic mouse is created that produces hybrid antibodies containing human variable regions and mouse constant regions. This is accomplished by a direct, in situ replacement of the mouse variable region genes with their human counterparts. The resultant hybrid immunoglobulin loci will undergo the natural process of rearrangements during B-cell development to produce the hybrid antibodies.

Subsequently, fully-human antibodies are made by replacing the mouse constant region with the desired human counterparts. This approach will give rise to therapeutic antibodies much more efficiently than previous methods, e.g. the 'humanization' of mouse monoclonal antibodies or the generation of fully human antibodies . . . Further, this method will succeed in producing therapeutic antibodies for many antigens for which previous methods have failed. This mouse will create antibodies that are human variable region-mouse constant region, which will have the following benefits over the previously available . . . mice that produce totally human antibodies. Antibodies generated by the new mouse will retain murine Fc regions which will interact more efficiently with the other components of the mouse B cell receptor complex, including the signaling components required for appropriate B cell differentiation (such as Iga and Igb). Additionally, the murine Fc regions will be more specific than human Fc regions in their interactions with Fc receptors on mouse cells, complement molecules, etc. These interactions are important for a strong and specific immune response, for the proliferation and maturation of B cells, and for the affinity maturation of antibodies."

24. Regeneron is informed and believes, and on that basis alleges, that Merus has made a genetically modified mouse that comprises, in its germline, at least human unrearranged heavy chain variable region gene segments inserted at an endogenous mouse immunoglobulin locus and that produces an antibody comprising a human variable region and a mouse constant region.

25. Regeneron is informed and believes, and on that basis alleges, that Merus has infringed and is currently infringing one or more claims of the '018 Patent, in violation of 35 U.S.C. § 271, literally and/or under the doctrine of equivalents, by, among other things, making, using, offering for sale, selling, and/or importing within this judicial district and elsewhere in the United States, without license or authority from Regeneron, genetically modified mice and related products and technologies that fall within the scope of one or more claims of the '018 Patent, including for example claim 9. The infringing products include, without limitation, Merus's MeMo mouse and related products and technologies.

26. Regeneron is informed and believes, and on that basis alleges, that Merus is aware of the existence of the '018 Patent. Merus cites U.S. Patent No. 6,596,541, to which the '018 Patent claims priority, as prior art before the United States Patent and Trademark Office. In addition, Merus is participating in European Patent Office Opposition proceedings with respect to Regeneron's European Patent No. 1 360 287. European Patent No. 1 360 287 claims priority to U.S. Application No. 09/784,859, which issued as U.S. Patent No. 6,596,541. The '018 Patent claims priority to this same application.

27. Regeneron is informed and believes, and on that basis alleges, that despite awareness of the '018 Patent, Merus has continued to willfully, wantonly, and deliberately engage in acts of infringement of the '018 Patent, justifying an award to Regeneron of increased damages under 35 U.S.C. § 284, and attorneys' fees and costs incurred under 35 U.S.C. § 285.

28. Regeneron has suffered irreparable injury as a direct and proximate result of Merus's conduct for which there is no adequate remedy at law and will continue to suffer such irreparable injury.

PRAYER FOR RELIEF

WHEREFORE, Regeneron prays for relief against Merus as follows:

A. For a determination that Merus infringes and continues to infringe one or more claims of the '018 Patent;

B. For damages adequate to compensate Regeneron for Merus's infringement of the '018 Patent, but in no event less than either lost profits or a reasonable royalty for the use made of the invention, together with interest and costs under 35 U.S.C. § 284;

C. For a determination that Merus's infringement has been willful, wanton, and deliberate and that the damages against it be increased up to treble on this basis;

D. For an order enjoining the further infringement of the '018 Patent, and enjoining those acts necessary to prevent further infringement of the '018 Patent;

E. For an award of pre- and post-judgment interest on the damages assessed;

F. For an award of supplemental damages to Regeneron, including without limitation interest;

G. For an order providing an accounting;

H. For a determination that this is an exceptional case under 35 U.S.C. § 285 and that an award of attorneys' fees and costs to Regeneron is warranted in this action;

I. For entry of judgment against Merus and in favor of Regeneron in all respects; and

J. For such other and further relief as the Court deems just and proper.

DEMAND FOR JURY TRIAL

Pursuant to Federal Rule of Civil Procedure 38(b), Regeneron hereby demands a trial by jury on all issues triable to a jury.

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March 5, 2014

US008502018B2

(12) **United States Patent**
Murphy et al.

(10) **Patent No.: US 8,502,018 B2**
(45) **Date of Patent: *Aug. 6, 2013**

(54) **METHODS OF MODIFYING EUKARYOTIC CELLS**

(75) Inventors: **Andrew J. Murphy**, Croton-on-Hudson, NY (US); **George D. Yancopoulos**, Yorktown Heights, NY (US)

(73) Assignee: **Regeneron Pharmaceuticals, Inc.**, Tarrytown, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/164,176**

(22) Filed: **Jun. 20, 2011**

(65) **Prior Publication Data**

US 2011/0283376 A1 Nov. 17, 2011

Related U.S. Application Data

(60) Continuation of application No. 13/154,976, filed on Jun. 7, 2011, which is a continuation of application No. 11/595,427, filed on Nov. 9, 2006, which is a continuation of application No. 10/624,044, filed on Jul. 21, 2003, now abandoned, which is a division of application No. 09/784,859, filed on Feb. 16, 2001, now Pat. No. 6,596,541, which is a continuation-in-part of application No. 09/732,234, filed on Dec. 7, 2000, now Pat. No. 6,586,251, application No. 13/164,176, which is a continuation of application No. 11/595,427, filed on Nov. 9, 2006.

(60) Provisional application No. 60/244,665, filed on Oct. 31, 2000.

(51) **Int. Cl.**

A01K 15/00	(2006.01)
A01K 67/027	(2006.01)
C12N 15/63	(2006.01)
C12N 5/00	(2006.01)

(52) **U.S. Cl.**
USPC **800/18**; 800/8; 800/21; 800/22; 435/325; 435/320.1

(58) **Field of Classification Search**
USPC 800/18, 8, 21, 22; 435/325, 320.1
See application file for complete search history.

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(Continued)

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(74) *Attorney, Agent, or Firm* — Foley Hoag LLP

(57) **ABSTRACT**

A method for engineering and utilizing large DNA vectors to target, via homologous recombination, and modify, in any desirable fashion, endogenous genes and chromosomal loci in eukaryotic cells. These large DNA targeting vectors for eukaryotic cells, termed LTVECs, are derived from fragments of cloned genomic DNA larger than those typically used by other approaches intended to perform homologous targeting in eukaryotic cells. Also provided is a rapid and convenient method of detecting eukaryotic cells in which the LTVEC has correctly targeted and modified the desired endogenous gene(s) or chromosomal locus (loci) as well as the use of these cells to generate organisms bearing the genetic modification.

20 Claims, 7 Drawing Sheets

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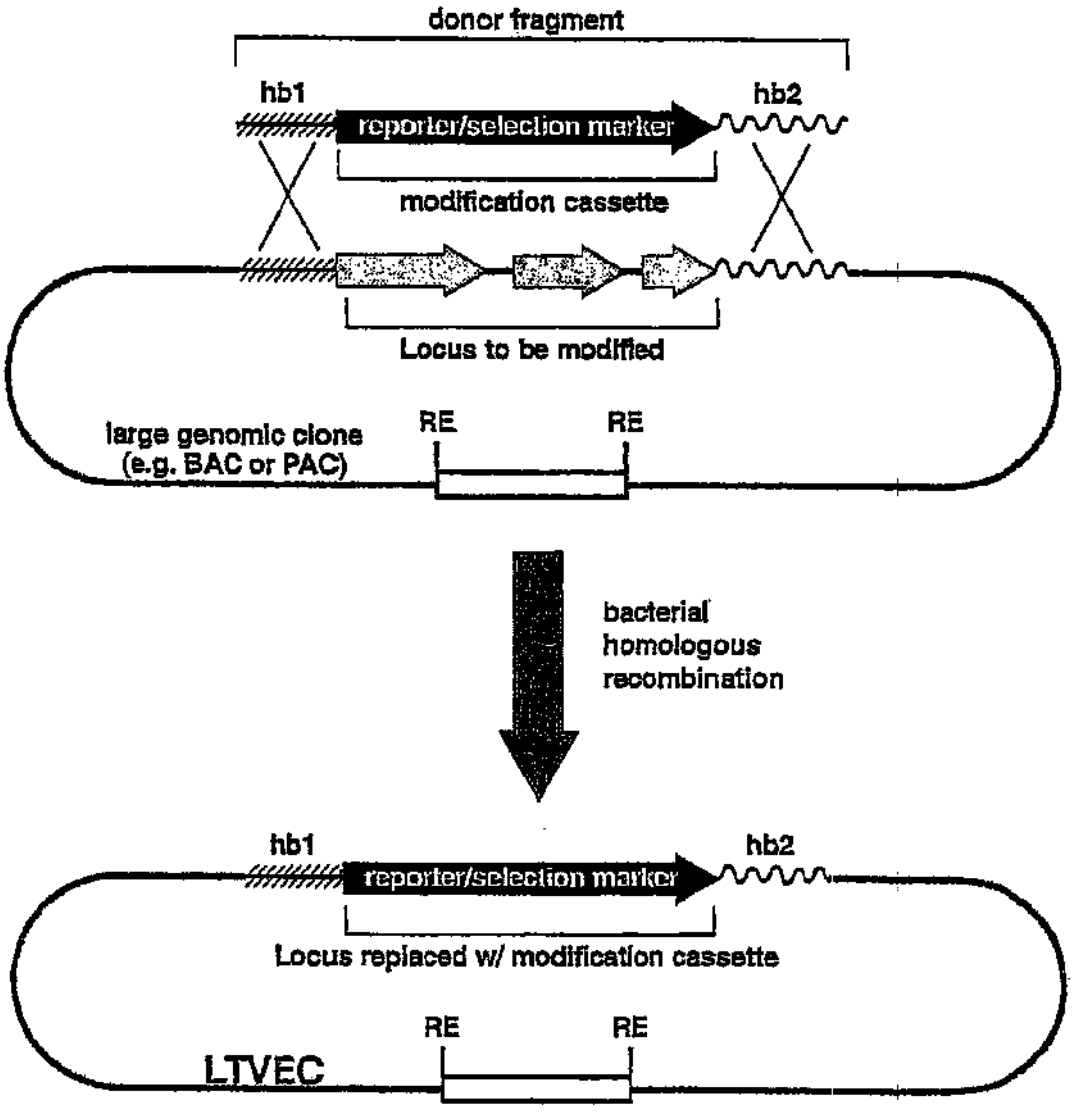
Figure 1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hb2
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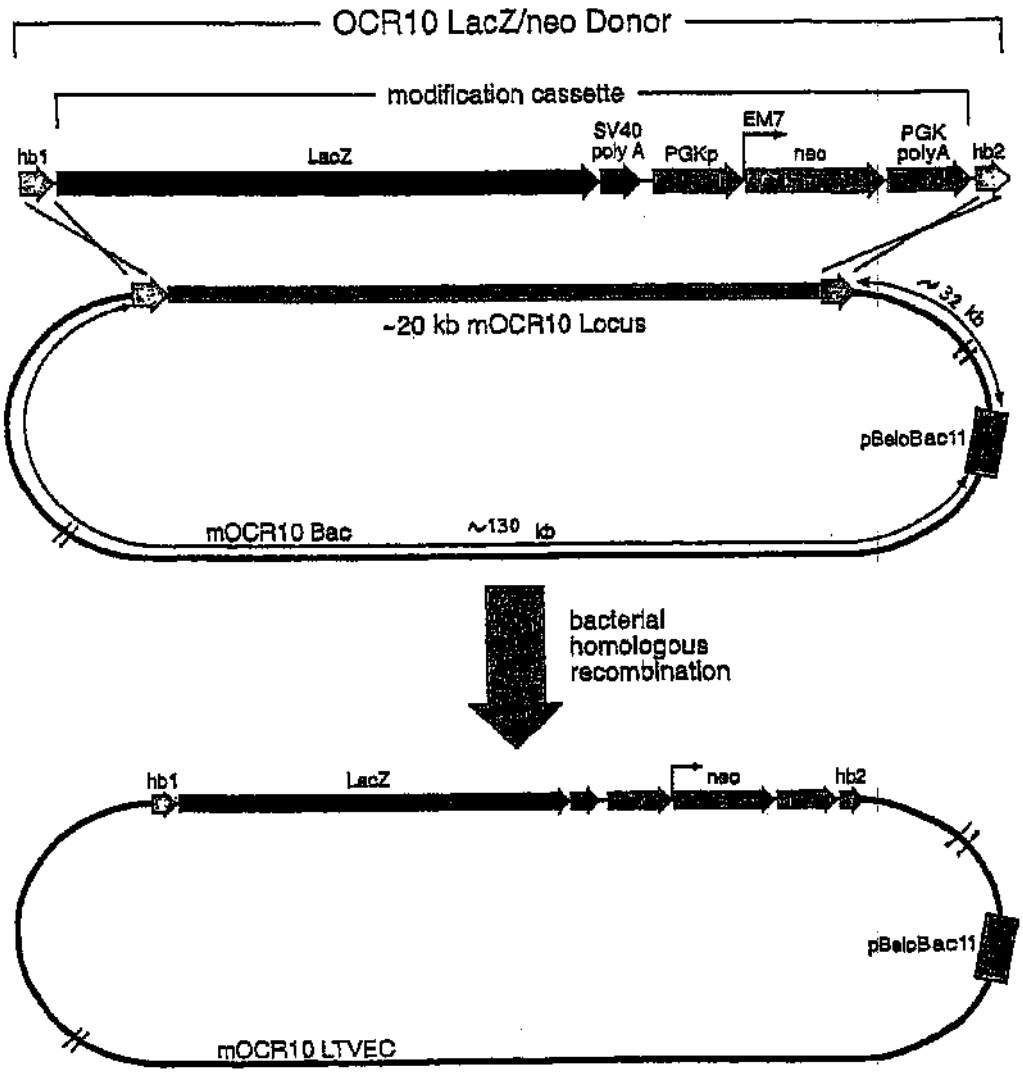
modification cassette
Locus to be modified
large genomic clone
(e.g. BAC or PAC)
RE
RE
bacterial
homologous
recombination
hb1
hb2
reporter/selection marker
Locus replaced w/ modification cassette
RE
RE
LTVEC

Figure 2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modification cassette
hb1
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SV40 poly A
PGKp
EM7
neo
PGK polyA
hb2
~20 kb mOCR10 Locus
~32 kb
pBeloBac11
mOCR10 Bac
~130 kb
bacterial homologous recombination
hb1
LacZ
neo
hb2
pBeloBac11
mOCR10 LTVEC

FIGURE 3A

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        10         20         30         40         50         60
CCCCGGGCTT CCTGTTCTAA TAAGAATACC TCCTAGGTCC CCCATGGGCT AACCTCATCT
GGGGCCCGAA GGACAAGATT ATTCTTATGG AGGATCCAGG GGGTACCCGA TTGGAGTAGA

        70         80         90        100        110        120
TTGGTACTCA ACAGGGGTCT TCTTTATGAG CTTCGGACCA GCTCTTTTGA TGTGGCAGGG
AACCATGAGT TGTCCCCAGA AGAAATACTC GAAGCCTGGT CGAGAAAACT ACACCGTCCC

       130        140        150        160        170        180
ACTGACCCTG GGTGGGGAAG CCACTCAGTG CATGACCCCA GCTGGTTCAC CACATATACC
TGACTGGGAC CCACCCCTTC GGTGAGTCAC GTACTGGGGT CGACCAAGTG GTGTATATGG

       190        200        210            220            230
ACATACTTTT CTTGCAGGTC TGGGACACAG C ATG CCC CGG GGC CCA GTG GCT GCC
TGTATGAAAA GAACGTCCAG ACCCTGTGTC G TAC GGG GCC CCG GGT CAC CGA CGG
                                  Met Pro Arg Gly Pro Val Ala Ala>

   240          250          260          270          280
TTA CTC CTG CTG ATT CTC CAT GGA GCT TGG AGC TGC CTG GAC CTC ACT
AAT GAG GAC GAC TAA GAG GTA CCT CGA ACC TCG ACG GAC CTG GAG TGA
Leu Leu Leu Leu Ile Leu His Gly Ala Trp Ser Cys Leu Asp Leu Thr>

      290          300          310          320          330
TGC TAC ACT GAC TAC CTC TGG ACC ATC ACC TGT GTC CTG GAG ACA CGG
ACG ATG TGA CTG ATG GAG ACC TGG TAG TGG ACA CAG GAC CTC TGT GCC
Cys Tyr Thr Asp Tyr Leu Trp Thr Ile Thr Cys Val Leu Glu Thr Arg>

        340          350          360          370
AGC CCC AAC CCC AGC ATA CTC AGT CTC ACC TGG CAA GAT GAA TAT GAG
TCG GGG TTG GGG TCG TAT GAG TCA GAG TGG ACC GTT CTA CTT ATA CTC
Ser Pro Asn Pro Ser Ile Leu Ser Leu Thr Trp Gln Asp Glu Tyr Glu>

380          390          400          410          420
GAA CTT CAG GAC CAA GAG ACC TTC TGC AGC CTA CAC AAG TCT GGC CAC
CTT GAA GTC CTG GTT CTC TGG AAG ACG TCG GAT GTG TTC AGA CCG GTG
Glu Leu Gln Asp Gln Glu Thr Phe Cys Ser Leu His Lys Ser Gly His>

  430          440          450          460          470
AAC ACC ACA CAT ATA TGG TAC ACG TGC CAT ATG CGC TTG TCT CAA TTC
TTG TGG TGT GTA TAT ACC ATG TGC ACG GTA TAC GCG AAC AGA GTT AAG
Asn Thr Thr His Ile Trp Tyr Thr Cys His Met Arg Leu Ser Gln Phe>

   480          490          500          510          520
CTG TCC GAT GAA GTT TTC ATT GTC AAC GTG ACG GAC CAG TCT GGC AAC
GAC AGG CTA CTT CAA AAG TAA CAG TTG CAC TGC CTG GTC AGA CCG TTG
Leu Ser Asp Glu Val Phe Ile Val Asn Val Thr Asp Gln Ser Gly Asn>

      530          540          550          560          570
AAC TCC CAA GAG TGT GGC AGC TTT GTC CTG GCT GAG AGC ATC AAG CCA
TTG AGG GTT CTC ACA CCG TCG AAA CAG GAC CGA CTC TCG TAG TTC GGT
Asn Ser Gln Glu Cys Gly Ser Phe Val Leu Ala Glu Ser Ile Lys Pro>
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FIGURE 3B

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        580          590          600          610
GCT CCC CCC TTG AAC GTG ACT GTG GCC TTC TCA GGA CGC TAT GAT ATC
CGA GGG GGG AAC TTG CAC TGA CAC CGG AAG AGT CCT GCG ATA CTA TAG
Ala Pro Pro Leu Asn Val Thr Val Ala Phe Ser Gly Arg Tyr Asp Ile>

620          630          640          650          660
TCC TGG GAC TCA GCT TAT GAC GAA CCC TCC AAC TAC GTG CTG AGA GGC
AGG ACC CTG AGT CGA ATA CTG CTT GGG AGG TTG ATG CAC GAC TCT CCG
Ser Trp Asp Ser Ala Tyr Asp Glu Pro Ser Asn Tyr Val Leu Arg Gly>

670          680          690          700          710
AAG CTA CAA TAT GAG CTG CAG TAT CGG AAC CTC AGA GAC CCC TAT GCT
TTC GAT GTT ATA CTC GAC GTC ATA GCC TTG GAG TCT CTG GGG ATA CGA
Lys Leu Gln Tyr Glu Leu Gln Tyr Arg Asn Leu Arg Asp Pro Tyr Ala>

   720          730          740          750          760
GTG AGG CCG GTG ACC AAG CTG ATC TCA GTG GAC TCA AGA AAC GTC TCT
CAC TCC GGC CAC TGG TTC GAC TAG AGT CAC CTG AGT TCT TTG CAG AGA
Val Arg Pro Val Thr Lys Leu Ile Ser Val Asp Ser Arg Asn Val Ser>

      770          780          790          800          810
CTT CTC CCT GAA GAG TTC CAC AAA GAT TCT AGC TAC CAG CTG CAG ATG
GAA GAG GGA CTT CTC AAG GTG TTT CTA AGA TCG ATG GTC GAC GTC TAC
Leu Leu Pro Glu Glu Phe His Lys Asp Ser Ser Tyr Gln Leu Gln Met>

        820          830          840          850
CGG GCA GCG CCT CAG CCA GGC ACT TCA TTC AGG GGG ACC TGG AGT GAG
GCC CGT CGC GGA GTC GGT CCG TGA AGT AAG TCC CCC TGG ACC TCA CTC
Arg Ala Ala Pro Gln Pro Gly Thr Ser Phe Arg Gly Thr Trp Ser Glu>

860          870          880          890          900
TGG AGT GAC CCC GTC ATC TTT CAG ACC CAG GCT GGG GAG CCC GAG GCA
ACC TCA CTG GGG CAG TAG AAA GTC TGG GTC CGA CCC CTC GGG CTC CGT
Trp Ser Asp Pro Val Ile Phe Gln Thr Gln Ala Gly Glu Pro Glu Ala>

910          920          930          940          950
GGC TGG GAC CCT CAC ATG CTG CTG CTC CTG GCT GTC TTG ATC ATT GTC
CCG ACC CTG GGA GTG TAC GAC GAC GAG GAC CGA CAG AAC TAG TAA CAG
Gly Trp Asp Pro His Met Leu Leu Leu Ala Val Leu Ile Ile Val>

   960          970          980          990          1000
CTG GTT TTC ATG GGT CTG AAG ATC CAC CTG CCT TGG AGG CTA TGG AAA
GAC CAA AAG TAC CCA GAC TTC TAG GTG GAC GGA ACC TCC GAT ACC TTT
Leu Val Phe Met Gly Leu Lys Ile His Leu Pro Trp Arg Leu Trp Lys>

     1010         1020         1030         1040         1050
AAG ATA TGG GCA CCA GTG CCC ACC CCT GAG AGT TTC TTC CAG CCC CTG
TTC TAT ACC CGT GGT CAC GGG TGG GGA CTC TCA AAG AAG GTC GGG GAC
Lys Ile Trp Ala Pro Val Pro Thr Pro Glu Ser Phe Phe Gln Pro Leu>
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FIGURE 3C

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        1060          1070          1080          1090
TAC AGG GAG CAC AGC GGG AAC TTC AAG AAA TGG GTT AAT ACC CCT TTC
ATG TCC CTC GTG TCG CCC TTG AAG TTC TTT ACC CAA TTA TGG GGA AAG
Tyr Arg Glu His Ser Gly Asn Phe Lys Lys Trp Val Asn Thr Pro Phe>

1100          1110          1120          1130          1140
ACG GCC TCC AGC ATA GAG TTG GTG CCA CAG AGT TCC ACA ACA ACA TCA
TGC CGG AGG TCG TAT CTC AAC CAC GGT GTC TCA AGG TGT TGT TGT AGT
Thr Ala Ser Ser Ile Glu Leu Val Pro Gln Ser Ser Thr Thr Thr Ser>

1150          1160          1170          1180          1190
GCC TTA CAT CTG TCA TTG TAT CCA GCC AAG GAG AAG AAG TTC CCG GGG
CGG AAT GTA GAC AGT AAC ATA GGT CGG TTC CTC TTC TTC AAG GGC CCC
Ala Leu His Leu Ser Leu Tyr Pro Ala Lys Glu Lys Lys Phe Pro Gly>

1200          1210          1220          1230          1240
CTG CCG GGT CTG GAA GAG CAA CTG GAG TGT GAT GGA ATG TCT GAG CCT
GAC GGC CCA GAC CTT CTC GTT GAC CTC ACA CTA CCT TAC AGA CTC GGA
Leu Pro Gly Leu Glu Glu Gln Leu Glu Cys Asp Gly Met Ser Glu Pro>

1250          1260          1270          1280          1290
GGT CAC TGG TGC ATA ATC CCC TTG GCA GCT GGC CAA GCG GTC TCA GCC
CCA GTG ACC ACG TAT TAG GGG AAC CGT CGA CCG GTT CGC CAG AGT CGG
Gly His Trp Cys Ile Ile Pro Leu Ala Ala Gly Gln Ala Val Ser Ala>

1300          1310          1320          1330
TAC AGT GAG GAG AGA GAC CGG CCA TAT GGT CTG GTG TCC ATT GAC ACA
ATG TCA CTC CTC TCT CTG GCC GGT ATA CCA GAC CAC AGG TAA CTG TGT
Tyr Ser Glu Glu Arg Asp Arg Pro Tyr Gly Leu Val Ser Ile Asp Thr>

1340          1350          1360          1370          1380
GTG ACT GTG GGA GAT GCA GAG GGC CTG TGT GTC TGG CCC TGT AGC TGT
CAC TGA CAC CCT CTA CGT CTC CCG GAC ACA CAG ACC GGG ACA TCG ACA
Val Thr Val Gly Asp Ala Glu Gly Leu Cys Val Trp Pro Cys Ser Cys>

1390          1400          1410          1420          1430
GAG GAT GAT GGC TAT CCA GCC ATG AAC CTG GAT GCT GGC AGA GAG TCT
CTC CTA CTA CCG ATA GGT CGG TAC TTG GAC CTA CGA CCG TCT CTC AGA
Glu Asp Asp Gly Tyr Pro Ala Met Asn Leu Asp Ala Gly Arg Glu Ser>

1440          1450          1460          1470          1480
GGT CCT AAT TCA GAG GAT CTG CTC TTG GTC ACA GAC CCT GCT TTT CTG
CCA GGA TTA AGT CTC CTA GAC GAG AAC CAG TGT CTG GGA CGA AAA GAC
Gly Pro Asn Ser Glu Asp Leu Leu Leu Val Thr Asp Pro Ala Phe Leu>

1490          1500          1510          1520          1530
TCT TGT GGC TGT GTC TCA GGT AGT GGT CTC AGG CTT GGG GGC TCC CCA
AGA ACA CCG ACA CAG AGT CCA TCA CCA GAG TCC GAA CCC CCG AGG GGT
Ser Cys Gly Cys Val Ser Gly Ser Gly Leu Arg Leu Gly Gly Ser Pro>
```

Figure 3D

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        1540          1550          1560          1570
   GGC AGC CTA CTG GAC AGG TTG AGG CTG TCA TTT GCA AAG GAA GGG GAC
   CCG TCG GAT GAC CTG TCC AAC TCC GAC AGT AAA CGT TTC CTT CCC CTG
   Gly Ser Leu Leu Asp Arg Leu Arg Leu Ser Phe Ala Lys Glu Gly Asp>

1580         1590          1600          1610          1620
   TGG ACA GCA GAC CCA ACC TGG AGA ACT GGG TCC CCA GGA GGG GGC TCT
   ACC TGT CGT CTG GGT TGG ACC TCT TGA CCC AGG GGT CCT CCC CCG AGA
   Trp Thr Ala Asp Pro Thr Trp Arg Thr Gly Ser Pro Gly Gly Gly Ser>

  1630          1640          1650          1660          1670
   GAG AGT GAA GCA GGT TCC CCC CCT GGT CTG GAC ATG GAC ACA TTT GAC
   CTC TCA CTT CGT CCA AGG GGG GGA CCA GAC CTG TAC CTG TGT AAA CTG
   Glu Ser Glu Ala Gly Ser Pro Pro Gly Leu Asp Met Asp Thr Phe Asp>

    1680          1690          1700          1710          1720
   AGT GGC TTT GCA GGT TCA GAC TGT GGC AGC CCC GTG GAG ACT GAT GAA
   TCA CCG AAA CGT CCA AGT CTG ACA CCG TCG GGG CAC CTC TGA CTA CTT
   Ser Gly Phe Ala Gly Ser Asp Cys Gly Ser Pro Val Glu Thr Asp Glu>

       1730          1740          1750          1760          1770
   GGA CCC CCT CGA AGC TAT CTC CGC CAG TGG GTG GTC AGG ACC CCT CCA
   CCT GGG GGA GCT TCG ATA GAG GCG GTC ACC CAC CAG TCC TGG GGA GGT
   Gly Pro Pro Arg Ser Tyr Leu Arg Gln Trp Val Val Arg Thr Pro Pro>

         1780          1790          1800
   CCT GTG GAC AGT GGA GCC CAG AGC AGC TAG
   GGA CAC CTG TCA CCT CGG GTC TCG TCG ATC
   Pro Val Asp Ser Gly Ala Gln Ser Ser ***>
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METHODS OF MODIFYING EUKARYOTIC CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. Ser. No. 13/154,976, filed 7 Jun. 2011, which is a continuation of U.S. Ser. No. 11/595,427, filed 9 Nov. 2006, which is a continuation of U.S. Ser. No. 10/624,044 filed 21 Jul. 2003, which is a divisional of U.S. Ser. No. 09/784,859, filed 16 Feb. 2001, now U.S. Pat. No. 6,596,541, which is a continuation-in-part of U.S. Ser. No. 09/732,234, filed 7 Dec. 2000, now U.S. Pat. No. 6,585,251, which claims the benefit of U.S. Ser. No. 60/244,665, filed 31 Oct. 2000, now abandoned; this application is also a continuation of U.S. Ser. No. 11/595,427, filed 9 Nov. 2006, which is a continuation of U.S. Ser. No. 10/624,044 filed 21 Jul. 2003, which is a divisional of U.S. Ser. No. 09/784,859, filed 16 Feb. 2001, now U.S. Pat. No. 6,596,541, which is a continuation-in-part of U.S. Ser. No. 09/732,234, filed 7 Dec. 2000, now U.S. Pat. No. 6,585,251, which claims the benefit of U.S. Ser. No. 60/244,665, filed 31 Oct. 2000, now abandoned; each of which is incorporated by reference herein.

FIELD OF THE INVENTION

The field of this invention is a method for engineering and utilizing large DNA vectors to target, via homologous recombination, and modify, in any desirable fashion, endogenous genes and chromosomal loci in eukaryotic cells. The field also encompasses the use of these cells to generate organisms bearing the genetic modification, the organisms, themselves, and methods of use thereof.

BACKGROUND

The use of LTVECs provides substantial advantages over current methods. For example, since these are derived from DNA fragments larger than those currently used to generate targeting vectors, LTVECs can be more rapidly and conveniently generated from available libraries of large genomic DNA fragments (such as BAC and PAC libraries) than targeting vectors made using current technologies. In addition, larger modifications as well as modifications spanning larger genomic regions can be more conveniently generated than using current technologies. Furthermore, the present invention takes advantage of long regions of homology to increase the targeting frequency of "hard to target" loci, and also diminishes the benefit, if any, of using isogenic DNA in these targeting vectors.

The present invention thus provides for a rapid, convenient, and streamlined method for systematically modifying virtually all the endogenous genes and chromosomal loci of a given organism.

Gene targeting by means of homologous recombination between homologous exogenous DNA and endogenous chromosomal sequences has proven to be an extremely valuable way to create deletions, insertions, design mutations, correct gene mutations, introduce transgenes, or make other genetic modifications in mice. Current methods involve using standard targeting vectors, with regions of homology to endogenous DNA typically totaling less than 10-20 kb, to introduce the desired genetic modification into mouse embryonic stem (ES) cells, followed by the injection of the altered ES cells into mouse embryos to transmit these engineered genetic modifications into the mouse germline (Smithies et al., Nature, 317:230-234, 1985; Thomas et al., Cell, 51:503-512, 1987; Koller et al., Proc Natl Acad Sci USA, 86:8927-8931, 1989; Kuhn et al., Science, 254:707-710, 1991; Thomas et al., Nature, 346:847-850, 1990; Schwartzberg et al., Science, 246:799-803, 1989; Doetschman et al., Nature, 330:576-578, 1987; Thomson et al., Cell, 5:313-321, 1989; DeChiara et al., Nature, 345:78-80, 1990; U.S. Pat. No. 5,789,215, issued Aug. 4, 1998 in the name of GenPharm International) In these current methods, detecting the rare ES cells in which the standard targeting vectors have correctly targeted and modified the desired endogenous gene(s) or chromosomal locus (loci) requires sequence information outside of the homologous targeting sequences contained within the targeting vector. Assays for successful targeting involve standard Southern blotting or long PCR (see for example Cheng, et al., Nature, 369:684-5, 1994; U.S. Pat. No. 5,436,149) from sequences outside the targeting vector and spanning an entire homology arm (see Definitions); thus, because of size considerations that limit these methods, the size of the homology arms are restricted to less than 10-20 kb in total (Joyner, The Practical Approach Series, 293, 1999).

The ability to utilize targeting vectors with homology arms larger than those used in current methods would be extremely valuable. For example, such targeting vectors could be more rapidly and conveniently generated from available libraries containing large genomic inserts (e.g. BAC or PAC libraries) than targeting vectors made using current technologies, in which such genomic inserts have to be extensively characterized and trimmed prior to use. In addition, larger modifications as well as modifications spanning larger genomic regions could be more conveniently generated and in fewer steps than using current technologies. Furthermore, the use of long regions of homology could increase the targeting frequency of "hard to target" loci in eukaryotic cells, since the targeting of homologous recombination in eukaryotic cells appears to be related to the total homology contained within the targeting vector (Deng and Capecchi, Mol Cell Biol, 12:3365-71, 1992). In addition, the increased targeting frequency obtained using long homology arms could diminish any potential benefit that can be derived from using isogenic DNA in these targeting vectors.

The problem of engineering precise modifications into very large genomic fragments, such as those cloned in BAC libraries, has largely been solved through the use of homologous recombination in bacteria (Zhang, et al., Nat Genet, 20:123-8, 1998; Yang, et al., Nat Biotechnol, 15:859-65, 1997; Angrand, et al., Nucleic Acids Res, 27:e16, 1999; Muyrers, et al., Nucleic Acids Res, 27:1555-7, 1999; Narayanan, et al., Gene Ther, 6:442-7, 1999), allowing for the construction of vectors containing large regions of homology to eukaryotic endogenous genes or chromosomal loci. However, once made, these vectors have not been generally useful for modifying endogenous genes or chromosomal loci via homologous recombination because of the difficulty in detecting rare correct targeting events when homology arms are larger than 10-20 kb (Joyner supra). Consequently, vectors generated using bacterial homologous recombination from BAC genomic fragments must still be extensively trimmed prior to use as targeting vectors (Hill et al., Genomics, 64:111-3, 2000). Therefore, there is still a need for a rapid and convenient methodology that makes possible the use of targeting vectors containing large regions of homology so as to modify endogenous genes or chromosomal loci in eukaryotic cells.

In accordance with the present invention, Applicants provide novel methods that enables the use of targeting vectors containing large regions of homology so as to modify endogenous genes or chromosomal loci in eukaryotic cells via

homologous recombination. Such methods overcome the above-described limitations of current technologies. In addition, the skilled artisan will readily recognize that the methods of the invention are easily adapted for use with any genomic DNA of any eukaryotic organism including, but not limited to, animals such as mouse, rat, other rodent, or human, as well as plants such as soy, corn and wheat.

SUMMARY OF THE INVENTION

In accordance with the present invention, Applicants have developed a novel, rapid, streamlined, and efficient method for creating and screening eukaryotic cells which contain modified endogenous genes or chromosomal loci. This novel methods combine, for the first time: 1. Bacterial homologous recombination to precisely engineer a desired genetic modification within a large cloned genomic fragment, thereby creating a large targeting vector for use in eukaryotic cells (LTVECs); 2. Direct introduction of these LTVECs into eukaryotic cells to modify the endogenous chromosomal locus of interest in these cells; and 3. An analysis to determine the rare eukaryotic cells in which the targeted allele has been modified as desired, involving an assay for modification of allele (MOA) of the parental allele that does not require sequence information outside of the targeting sequence, such as, for example, quantitative PCR.

A preferred embodiment of the invention is a method for genetically modifying an endogenous gene or chromosomal locus in eukaryotic cells, comprising: a) obtaining a large cloned genomic fragment containing a DNA sequence of interest; b) using bacterial homologous recombination to genetically modify the large cloned genomic fragment of (a) to create a large targeting vector for use in the eukaryotic cells (LTVEC); c) introducing the LTVEC of (b) into the eukaryotic cells to modify the endogenous gene or chromosomal locus in the cells; and d) using a quantitative assay to detect modification of allele (MOA) in the eukaryotic cells of (c) to identify those eukaryotic cells in which the endogenous gene or chromosomal locus has been genetically modified. Another embodiment of the invention is a method wherein the genetic modification to the endogenous gene or chromosomal locus comprises deletion of a coding sequence, gene segment, or regulatory element; alteration of a coding sequence, gene segment, or regulatory element; insertion of a new coding sequence, gene segment, or regulatory element; creation of a conditional allele; or replacement of a coding sequence or gene segment from one species with an homologous or orthologous coding sequence from a different species. An alternative embodiment of the invention is a method wherein the alteration of a coding sequence, gene segment, or regulatory element comprises a substitution, addition, or fusion, wherein the fusion comprises an epitope tag or bifunctional protein. Yet another embodiment of the invention is a method wherein the quantitative assay comprises quantitative PCR, comparative genomic hybridization, isothermic DNA amplification, or quantitative hybridization to an immobilized probe, wherein the quantitative PCR comprises TAQMAN® technology or quantitative PCR using molecular beacons. Another preferred embodiment of the invention is a method wherein the eukaryotic cell is a mammalian embryonic stem cell and in particular wherein the embryonic stem cell is a mouse, rat, or other rodent embryonic stem cell. Another preferred embodiment of the invention is a method wherein the endogenous gene or chromosomal locus is a mammalian gene or chromosomal locus, preferably a human gene or chromosomal locus or a mouse, rat, or other rodent gene or chromosomal locus. An additional preferred embodiment is one in which the LTVEC is capable of accommodating large DNA fragments greater than 20 kb, and in particular large DNA fragments greater than 100 kb. Another preferred embodiment is a genetically modified endogenous gene or chromosomal locus that is produced by the method of the invention. Yet another preferred embodiment is a genetically modified eukaryotic cell that is produced by the method of the invention. A preferred embodiment of the invention is a non-human organism containing the genetically modified endogenous gene or chromosomal locus produced by the method of the invention. Also preferred in a non-human organism produced from the genetically modified eukaryotic cells or embryonic stem cells produced by the method of the invention.

A preferred embodiment is a non-human organism containing a genetically modified endogenous gene or chromosomal locus, produced by a method comprising the steps of: a) obtaining a large cloned genomic fragment containing a DNA sequence of interest; b) using bacterial homologous recombination to genetically modify the large cloned genomic fragment of (a) to create a large targeting vector (LTVEC) for use in embryonic stem cells; c) introducing the LTVEC of (b) into the embryonic stem cells to modify the endogenous gene or chromosomal locus in the cells; d) using a quantitative assay to detect modification of allele (MOA) in the embryonic stem cells of (c) to identify those embryonic stem cells in which the endogenous gene or chromosomal locus has been genetically modified; e) introducing the embryonic stem cell of (d) into a blastocyst; and f) introducing the blastocyst of (e) into a surrogate mother for gestation.

An additional preferred embodiment of the invention is a non-human organism containing a genetically modified endogenous gene or chromosomal locus, produced by a method comprising the steps of: a) obtaining a large cloned genomic fragment containing a DNA sequence of interest; b) using bacterial homologous recombination to genetically modify the large cloned genomic fragment of (a) to create a large targeting vector for use in eukaryotic cells (LTVEC); c) introducing the LTVEC of (b) into the eukaryotic cells to genetically modify the endogenous gene or chromosomal locus in the cells; d) using a quantitative assay to detect modification of allele (MOA) in the eukaryotic cells of (c) to identify those eukaryotic cells in which the endogenous gene or chromosomal locus has been genetically modified; e) removing the nucleus from the eukaryotic cell of (d); f) introducing the nucleus of (e) into an oocyte; and g) introducing the oocyte of (f) into a surrogate mother for gestation.

Yet another preferred embodiment is a non-human organism containing a genetically modified endogenous gene or chromosomal locus, produced by a method comprising the steps of: a) obtaining a large cloned genomic fragment containing a DNA sequence of interest; b) using bacterial homologous recombination to genetically modify the large cloned genomic fragment of (a) to create a large targeting vector for use in eukaryotic cells (LTVEC); c) introducing the LTVEC of (b) into the eukaryotic cells to genetically modify the endogenous gene or chromosomal locus in the cells; d) using a quantitative assay to detect modification of allele (MOA) in the eukaryotic cells of (c) to identify those eukaryotic cells in which the endogenous gene or chromosomal locus has been genetically modified; e) fusing the eukaryotic cell of (d) with another eukaryotic cell; f) introducing the fused eukaryotic cell of (e) into a surrogate mother for gestation.

A preferred embodiment of the invention is a method for genetically modifying an endogenous gene or chromosomal locus of interest in mouse embryonic stem cells, comprising:

a) obtaining a large cloned genomic fragment greater than 20 kb which contains a DNA sequence of interest, wherein the large cloned DNA fragment is homologous to the endogenous gene or chromosomal locus; b) using bacterial homologous recombination to genetically modify the large cloned genomic fragment of (a) to create a large targeting vector for use in the mouse embryonic stem cells, wherein the genetic modification is deletion of a coding sequence, gene segment, or regulatory element; c) introducing the large targeting vector of (b) into the mouse embryonic stem cells to modify the endogenous gene or chromosomal locus in the cells; and d) using a quantitative assay to detect modification of allele (MOA) in the mouse embryonic stem cells of (c) to identify those mouse embryonic stem cells in which the endogenous gene or chromosomal locus has been genetically modified, wherein the quantitative assay is quantitative PCR. Also preferred is a genetically modified mouse embryonic stem cell produced by this method; a mouse containing a genetically modified endogenous gene or chromosomal locus produced by this method; and a mouse produced from the genetically modified mouse embryonic stem cell.

Another preferred embodiment is a mouse containing a genetically modified endogenous gene or chromosomal locus of interest, produced by a method comprising the steps of: a) obtaining a large cloned genomic fragment greater than 20 kb which contains a DNA sequence of interest, wherein the large cloned DNA fragment is homologous to the endogenous gene or chromosomal locus; b) using bacterial homologous recombination to genetically modify the large cloned genomic fragment of (a) to create a large targeting vector for use in the mouse embryonic stem cells, wherein the genetic modification is deletion of a coding sequence, gene segment, or regulatory element; c) introducing the large targeting vector of (b) into the mouse embryonic stem cells to modify the endogenous gene or chromosomal locus in the cells; and d) using a quantitative assay to detect modification of allele (MOA) in the mouse embryonic stem cells of (c) to identify those mouse embryonic stem cells in which the endogenous gene or chromosomal locus has been genetically modified, wherein the quantitative assay is quantitative PCR; e) introducing the mouse embryonic stem cell of (d) into a blastocyst; and f) introducing the blastocyst of (e) into a surrogate mother for gestation.

One embodiment of the invention is a method of replacing, in whole or in part, in a non-human eukaryotic cell, an endogenous immunoglobulin variable region gene locus with an homologous or orthologous human gene locus comprising: a) obtaining a large cloned genomic fragment containing, in whole or in part, the homologous or orthologous human gene locus; b) using bacterial homologous recombination to genetically modify the cloned genomic fragment of (a) to create a large targeting vector for use in the eukaryotic cells (LTVEC); c) introducing the LTVEC of (b) into the eukaryotic cells to replace, in whole or in part, the endogenous immunoglobulin variable gene locus; and d) using a quantitative assay to detect modification of allele (MOA) in the eukaryotic cells of (c) to identify those eukaryotic cells in which the endogenous immunoglobulin variable region gene locus has been replaced, in whole or in part, with the homologous or orthologous human gene locus.

Another embodiment is a method of replacing, in whole or in part, in a non-human eukaryotic cell, an endogenous immunoglobulin variable region gene locus with an homologous or orthologous human gene locus further comprising the steps: e) obtaining a large cloned genomic fragment containing a part of the homologous or orthologous human gene locus that differs from the fragment of (a); f) using bacterial homologous recombination to genetically modify the cloned genomic fragment of (e) to create a second LTVEC; g) introducing the second LTVEC of (f) into the eukaryotic cells identified in step (d) to replace, in whole or in part, the endogenous immunoglobulin variable gene locus; and h) using a quantitative assay to detect modification of allele (MOA) in the eukaryotic cells of (g) to identify those eukaryotic cells in which the endogenous immunoglobulin variable region gene locus has been replaced, in whole or in part, with the homologous or orthologous human gene locus.

Another embodiment of the above method is a method wherein steps (e) through (h) are repeated until the endogenous immunoglobulin variable region gene locus is replaced in whole with an homologous or orthologous human gene locus.

Another embodiment of the method is one in which the immunoglobulin variable gene locus is a locus selected from the group consisting of a) a variable gene locus of the kappa light chain; b) a variable gene locus of the lambda light chain; and c) a variable gene locus of the heavy chain.

A preferred embodiment is a method wherein the quantitative assay comprises quantitative PCR, FISH, comparative genomic hybridization, isothermic DNA amplification, or quantitative hybridization to an immobilized probe, and in particular wherein the quantitative PCR comprises TAQMAN®. technology or quantitative PCR using molecular beacons.

Yet another preferred embodiment is a method of replacing, in whole or in part, in a mouse embryonic stem cell, an endogenous immunoglobulin variable region gene locus with its homologous or orthologous human gene locus comprising: a) obtaining a large cloned genomic fragment containing, in whole or in part, the homologous or orthologous human gene locus; b) using bacterial homologous recombination to genetically modify the large cloned genomic fragment of (a) to create a large targeting vector for use in the embryonic stem cells; c) introducing the large targeting vector of (b) into mouse embryonic stem cells to replace, in whole or in part, the endogenous immunoglobulin variable gene locus in the cells; and d) using a quantitative PCR assay to detect modification of allele (MOA) in the mouse embryonic stem cells of (d) to identify those mouse embryonic stem cells in which the endogenous variable gene locus has been replaced, in whole or in part, with the homologous or orthologous human gene locus.

In another embodiment, the method further comprises: e) obtaining a large cloned genomic fragment containing a part of the homologous or orthologous human gene locus that differs from the fragment of (a); f) using bacterial homologous recombination to genetically modify the cloned genomic fragment of (e) to create a large targeting vector for use in the embryonic stem cells; g) introducing the large targeting vector of (f) into the mouse embryonic stem cells identified in step (d) to replace, in whole or in part, the endogenous immunoglobulin variable gene locus; and h) using a quantitative assay to detect modification of allele (MOA) in the mouse embryonic stem cells of (g) to identify those mouse embryonic stem cells in which the endogenous immunoglobulin variable region gene locus has been replaced, in whole or in part, with the homologous or orthologous human gene locus.

Another preferred embodiment is a genetically modified immunoglobulin variable region gene locus produced by the methods described above; a genetically modified eukaryotic cell comprising a genetically modified immunoglobulin variable region gene locus produced by the methods described above; a non-human organism comprising a genetically

modified immunoglobulin variable region gene locus produced by the methods described above; and a mouse embryonic stem cell containing a genetically modified immunoglobulin variable region gene locus produced by the methods described above.

Also preferred is an embryonic stem cell wherein the mouse heavy chain variable region locus is replaced, in whole or in part, with a human heavy chain variable gene locus; an embryonic stem cell of claim wherein the mouse kappa light chain variable region locus is replaced, in whole or in part, with a human kappa light chain variable region locus; an embryonic stem cell wherein the mouse lambda light chain variable region locus is replaced, in whole or in part, with a human lambda light chain variable region locus; and an embryonic stem cell wherein the heavy and light chain variable region gene loci are replaced, in whole, with their human homologs or orthologs.

Yet another preferred embodiment is an antibody comprising a human variable region encoded by the genetically modified variable gene locus of described above; an antibody further comprising a non-human constant region; and an antibody further comprising a human constant region.

Also preferred is a transgenic mouse having a genome comprising entirely human heavy and light chain variable region loci operably linked to entirely endogenous mouse constant region loci such that the mouse produces a serum containing an antibody comprising a human variable region and a mouse constant region in response to antigenic stimulation; a transgenic mouse having a genome comprising human heavy and/or light chain variable region loci operably linked to endogenous mouse constant region loci such that the mouse produces a serum containing an antibody comprising a human variable region and a mouse constant region in response to antigenic stimulation; a transgenic mouse containing an endogenous variable region locus that has been replaced with an homologous or orthologous human variable locus, such mouse being produced by a method comprising: a) obtaining one or more large cloned genomic fragments containing the entire homologous or orthologous human variable region locus; b) using bacterial homologous recombination to genetically modify the cloned genomic fragment(s) of (a) to create large targeting vector(s) for use in mouse embryonic stem cells; c) introducing the large targeting vector(s) of (b) into mouse embryonic stem cells to replace the entire endogenous variable region locus in the cells; and d) using a quantitative PCR assay to detect modification of allele (MOA) in the mouse embryonic stem cells of (c) to identify those mouse embryonic stem cells in which the entire endogenous variable region locus has been replaced with the homologous or orthologous human variable region locus; e) introducing the mouse embryonic stem cell of (d) into a blastocyst; and f) introducing the blastocyst of (e) into a surrogate mother for gestation.

Still yet another preferred embodiment of the invention is a method of making a human antibody comprising: a) exposing the mouse described above to antigenic stimulation, such that the mouse produces an antibody against the antigen; b) isolating the DNA encoding the variable regions of the heavy and light chains of the antibody; c) operably linking the DNA encoding the variable regions of (b) to DNA encoding the human heavy and light chain constant regions in a cell capable of expressing active antibodies; d) growing the cell under such conditions as to express the human antibody; and e) recovering the antibody. In another preferred embodiment, the cell described above is a CHO cell. Also preferred is a method of wherein the DNA of step (b) described above is isolated from a hybridoma created from the spleen of the mouse exposed to antigenic stimulation in step (a) described above.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**: Schematic diagram of the generation of a typical LTVEC using bacterial homologous recombination. (hb1=homology box 1; hb2=homology box 2; RE=restriction enzyme site).

FIG. **2**: Schematic diagram of donor fragment and LTVEC for mouse OCR10. (hb1=homology box 1; lacZ=β-galactosidase ORF; SV40 polyA=a DNA fragment derived from Simian Virus 40, containing a polyadenylation site and signal; PGKp=mouse phosphoglycerate kinase (PGK) promoter; EM7=a bacterial promoter; neo=neomycin phosphotransferase; PGK polyA=3' untranslated region derived from the PGK gene and containing a polyadenylation site and signal; hb2=homology box 2)

FIGS. **3**A-3D: Sequence of the mouse OCR10 cDNA (upper strand, SEQ ID NO:5), homology box 1 (hb1), homology box 2 (hb2), and TAQMAN® probes and primers used in a quantitative PCR assay to detect modification of allele (MOA) in ES cells targeted using the mOCR10 LTVEC. hb1: base pairs 1 to 211; hb2: base pairs 1586 to 1801; TAQMAN® probe and corresponding PCR primer set derived from mOCR10 exon 3: TAQMAN® probe: nucleotides 413 to 439—upper strand; Primer ex3-5': nucleotides 390 to 410—upper strand; Primer ex3-3': nucleotides 445 to 461—lower strand; TAQMAN® probe and corresponding PCR primer set derived from mOCR10 exon 4: TAQMAN® probe: nucleotides 608 to 639—upper strand; Primer ex4-5': nucleotides 586 to 605—upper strand; Primer ex4-3': nucleotides 642 to 662—lower strand.

FIG. **4**A-4D: (SEQ ID NO:5-6) Schematic diagram of the two LTVECs constructed to replace the mouse VDJ region with human VDJ region. A: Large insert (BAC) clones spanning the entire VDJ region of the human heavy chain locus are isolated. B: In this example, large insert (BAC) clones are isolated from the ends of the mouse VDJ region as a source of homology arms which are used to direct integration via homologous recombination of the human VDJ sequences in a two step process. C-D: In the first step, LTVEC1 is constructed by bacterial homologous recombination in *E. coli*. LTVEC1 contains, in order: a large mouse homology arm derived from the region upstream from the mouse DJ region, but whose absolute endpoints are not important; a cassette encoding a selectable marker functional in ES cells (PGK-neomycinR); a loxP site; a large human insert spanning from several V gene segments through the entire DJ region; and a mouse homology arm containing the region immediately adjacent to, but not including, the mouse J segments. In the second step, LTVEC2 is constructed by bacterial homologous recombination in *E. coli*. LTVEC2 contains, in order: a large mouse homology arm containing the region adjacent to the most distal mouse V gene segment, but not containing any mouse V gene segments; a large insert containing a large number of distal human V gene segments; a mutant loxP site called lox511 in the orientation opposite to that of the wild type loxP sites in LTVEC2 and LTVEC1 (this site will not recombine with wild type loxP sites but will readily recombine with other lox511 sites); a wild type loxP site; a second selectable marker (PGK-hygromycinR); and a mouse homology arm derived from the V region, but whose absolute endpoints are not important.

DETAILED DESCRIPTION

A "targeting vector" is a DNA construct that contains sequences "homologous" to endogenous chromosomal

nucleic acid sequences flanking a desired genetic modification(s). The flanking homology sequences, referred to as "homology arms", direct the targeting vector to a specific chromosomal location within the genome by virtue of the homology that exists between the homology arms and the corresponding endogenous sequence and introduce the desired genetic modification by a process referred to as "homologous recombination".

"Homologous" means two or more nucleic acid sequences that are either identical or similar enough that they are able to hybridize to each other or undergo intermolecular exchange.

"Gene targeting" is the modification of an endogenous chromosomal locus by the insertion into, deletion of, or replacement of the endogenous sequence via homologous recombination using a targeting vector.

A "gene knockout" is a genetic modification resulting from the disruption of the genetic information encoded in a chromosomal locus. A "gene knockin" is a genetic modification resulting from the replacement of the genetic information encoded in a chromosomal locus with a different DNA sequence. A "knockout organism" is an organism in which a significant proportion of the organism's cells harbor a gene knockout. A "knockin organism" is an organism in which a significant proportion of the organism's cells harbor a gene knockin.

A "marker" or a "selectable marker" is a selection marker that allows for the isolation of rare transfected cells expressing the marker from the majority of treated cells in the population. Such marker's gene's include, but are not limited to, neomycin phosphotransferase and hygromycin B phosphotransferase, or fluorescing proteins such as GFP.

An "ES cell" is an embryonic stem cell. This cell is usually derived from the inner cell mass of a blastocyst-stage embryo. An "ES cell clone" is a subpopulation of cells derived from a single cell of the ES cell population following introduction of DNA and subsequent selection.

A "flanking DNA" is a segment of DNA that is collinear with and adjacent to a particular point of reference.

"LTVECs" are large targeting vectors for eukaryotic cells that are derived from fragments of cloned genomic DNA larger than those typically used by other approaches intended to perform homologous targeting in eukaryotic cells.

"Modification of allele" (MOA) refers to the modification of the exact DNA sequence of one allele of a gene(s) or chromosomal locus (loci) in a genome. This modification of allele (MOA) includes, but is not limited to, deletions, substitutions, or insertions of as little as a single nucleotide or deletions of many kilobases spanning a gene(s) or chromosomal locus (loci) of interest, as well as any and all possible modifications between these two extremes.

"Orthologous" sequence refers to a sequence from one species that is the functional equivalent of that sequence in another species.

General Description

Applicants have developed a novel, rapid, streamlined, and efficient method for creating and screening eukaryotic cells which contain modified endogenous genes or chromosomal loci. In these cells, the modification may be gene(s) knockouts, knockins, point mutations, or large genomic insertions or deletions or other modifications. These cells may be embryonic stem cells which are useful for creating knockout or knockin organisms and in particular, knockout or knockin mice, for the purpose of determining the function of the gene(s) that have been altered, deleted and/or inserted.

The novel methods described herein combine, for the first time: 1. Bacterial homologous recombination to precisely engineer a desired genetic modification within a large cloned genomic DNA fragment, thereby creating a large targeting vector for use in eukaryotic cells (LTVECs); 2. Direct introduction of these LTVECs into eukaryotic cells to modify the corresponding endogenous gene(s) or chromosomal locus (loci) of interest in these cells; and 3. An analysis to determine the rare eukaryotic cells in which the targeted allele has been modified as desired, involving a quantitative assay for modification of allele (MOA) of the parental allele.

It should be emphasized that previous methods to detect successful homologous recombination in eukaryotic cells cannot be utilized in conjunction with the LTVECs of Applicants' invention because of the long homology arms present in the LTVECs. Utilizing a LTVEC to deliberately modify endogenous genes or chromosomal loci in eukaryotic cells via homologous recombination is made possible by the novel application of an assay to determine the rare eukaryotic cells in which the targeted allele has been modified as desired, such assay involving a quantitative assay for modification of allele (MOA) of a parental allele, by employing, for example, quantitative PCR or other suitable quantitative assays for MOA.

The ability to utilize targeting vectors with homology arms larger than those used in current methods is extremely valuable for the following reasons: 1. Targeting vectors are more rapidly and conveniently generated from available libraries containing large genomic inserts (e.g. BAC or PAC libraries) than targeting vectors made using previous technologies, in which the genomic inserts have to be extensively characterized and "trimmed" prior to use (explained in detail below). In addition, minimal sequence information needs to be known about the locus of interest, i.e. it is only necessary to know the approximately 80-100 nucleotides that are required to generate the homology boxes (described in detail below) and to generate probes that can be used in quantitative assays for MOA (described in detail below). 2. Larger modifications as well as modifications spanning larger genomic regions are more conveniently generated and in fewer steps than using previous technologies. For example, the method of the invention makes possible the precise modification of large loci that cannot be accommodated by traditional plasmid-based targeting vectors because of their size limitations. It also makes possible the modification of any given locus at multiple points (e.g. the introduction of specific mutations at different exons of a multi-exon gene) in one step, alleviating the need to engineer multiple targeting vectors and to perform multiple rounds of targeting and screening for homologous recombination in ES cells. 3. The use of long regions of homology (long homology arms) increase the targeting frequency of "hard to target" loci in eukaryotic cells, consistent with previous findings that targeting of homologous recombination in eukaryotic cells appears to be related to the total homology contained within the targeting vector. 4. The increased targeting frequency obtained using long homology arms apparently diminishes the benefit, if any, from using isogenic DNA in these targeting vectors. 5. The application of quantitative MOA assays for screening eukaryotic cells for homologous recombination not only empowers the use of LTVECs as targeting vectors (advantages outlined above) but also reduces the time for identifying correctly modified eukaryotic cells from the typical several days to a few hours. In addition, the application of quantitative MOA does not require the use of probes located outside the endogenous gene(s) or chromosomal locus (loci) that is being modified, thus obviating the need to know the sequence flanking the modified gene(s) or locus (loci). This is a significant improvement in the way the screening has been performed in the past and makes it a much

less labor-intensive and much more cost-effective approach to screening for homologous recombination events in eukaryotic cells.

Methods

Many of the techniques used to construct DNA vectors described herein are standard molecular biology techniques well known to the skilled artisan (see e.g., Sambrook, J., E. F. Fritsch And T. Maniatis. Molecular Cloning: A Laboratory Manual, Second Edition, Vols 1, 2, and 3, 1989; Current Protocols in Molecular Biology, Eds. Ausubel et al., Greene Publ. Assoc., Wiley Interscience, NY). All DNA sequencing is done by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.).

Step 1. Obtain a Large Genomic DNA Clone Containing the Gene(s) or Chromosomal Locus (Loci) of Interest.

A gene(s) or locus (loci) of interest can be selected based on specific criteria, such as detailed structural or functional data, or it can be selected in the absence of such detailed information as potential genes or gene fragments become predicted through the efforts of the various genome sequencing projects. Importantly, it should be noted that it is not necessary to know the complete sequence and gene structure of a gene(s) of interest to apply the method of the subject invention to produce LTVECs. In fact, the only sequence information that is required is approximately 80-100 nucleotides so as to obtain the genomic clone of interest as well as to generate the homology boxes used in making the LTVEC (described in detail below) and to make probes for use in quantitative MOA assays.

Once a gene(s) or locus (loci) of interest has been selected, a large genomic clone(s) containing this gene(s) or locus (loci) is obtained. This clone(s) can be obtained in any one of several ways including, but not limited to, screening suitable DNA libraries (e.g. BAC, PAC, YAC, or cosmid) by standard hybridization or PCR techniques, or by any other methods familiar to the skilled artisan.

Step 2. Append Homology Boxes 1 and 2 to a Modification Cassette and Generation of LTVEC.

Homology boxes mark the sites of bacterial homologous recombination that are used to generate LTVECs from large cloned genomic fragments (FIG. 1). Homology boxes are short segments of DNA, generally double-stranded and at least 40 nucleotides in length, that are homologous to regions within the large cloned genomic fragment flanking the "region to be modified". The homology boxes are appended to the modification cassette, so that following homologous recombination in bacteria, the modification cassette replaces the region to be modified (FIG. 1). The technique of creating a targeting vector using bacterial homologous recombination can be performed in a variety of systems (Yang et al. supra; Muyrers et al. supra; Angrand et al. supra; Narayanan et al. supra; Yu, et al., Proc Natl Acad Sci USA, 97:5978-83, 2000). One example of a favored technology currently in use is ET cloning and variations of this technology (Yu et al. supra). ET refers to the recE (Hall and Kolodner, Proc Natl Acad Sci USA, 91:3205-9, 1994) and recT proteins (Kusano et al., Gene, 138:17-25, 1994) that carry out the homologous recombination reaction. RecE is an exonuclease that trims one strand of linear double-stranded DNA (essentially the donor DNA fragment described infra) 5' to 3', thus leaving behind a linear double-stranded fragment with a 3' single-stranded overhang. This single-stranded overhang is coated by recT protein, which has single-stranded DNA (ssDNA) binding activity (Kovall and Matthews, Science, 277:1824-7, 1997). ET cloning is performed using *E. coli* that transiently express the *E. coli* gene products of recE and recT (Hall and Kolodner, Proc Natl Acad Sci USA, 91:3205-9, 1994; Clark et al., Cold Spring Harb Symp Quant Biol, 49:453-62, 1984; Noirot and Kolodner, J Biol Chem, 273:12274-80, 1998; Thresher et al., J Mol Biol, 254:364-71, 1995; Kolodner et al., Mol Microbiol, 11:23-30, 1994; Hall et al., J Bacteriol, 175: 277-87, 1993) and the bacteriophage lambda (λ) protein λgam (Murphy, J Bacteriol, 173:5808-21, 1991; Poteete et al., J Bacteriol, 170:2012-21, 1988). The kgam protein is required for protecting the donor DNA fragment from degradation by the recBC exonuclease system (Myers and Stahl, Annu Rev Genet, 28:49-70, 1994) and it is required for efficient ET-cloning in recBC$^+$ hosts such as the frequently used *E. coli* strain DH10b.

The region to be modified and replaced using bacterial homologous recombination can range from zero nucleotides in length (creating an insertion into the original locus) to many tens of kilobases (creating a deletion and/or a replacement of the original locus). Depending on the modification cassette, the modification can result in the following: (a) deletion of coding sequences, gene segments, or regulatory elements; (b) alteration(s) of coding sequence, gene segments, or regulatory elements including substitutions, additions, and fusions (e.g. epitope tags or creation of bifunctional proteins such as those with GFP); (c) insertion of new coding regions, gene segments, or regulatory elements, such as those for selectable marker genes or reporter genes or putting new genes under endogenous transcriptional control; (d) creation of conditional alleles, e.g. by introduction of loxP sites flanking the region to be excised by Cre recombinase (Abremski and Hoess, J Biol Chem, 259:1509-14, 1984), or FRT sites flanking the region to be excised by Flp recombinase (Andrews et al., Cell, 40:795-803, 1985; Meyer-Leon et al., Cold Spring Harb Symp Quant Biol, 49:797-804, 1984; Cox, Proc Natl Acad Sci USA, 80:4223-7, 1983); or (e) replacement of coding sequences or gene segments from one species with orthologous coding sequences from a different species, e.g. replacing a murine genetic locus with the orthologous human genetic locus to engineer a mouse where that particular locus has been 'humanized'.

Any or all of these modifications can be incorporated into a LTVEC. A specific example in which an endogenous coding sequence is entirely deleted and simultaneously replaced with both a reporter gene as well as a selectable marker is provided below in Example 1, as are the advantages of the method of the invention as compared to previous technologies.

Step 3 (Optional). Verify that Each LTVEC has been Engineered Correctly.

Verify that each LTVEC has been engineered correctly by: a. Diagnostic PCR to verify the novel junctions created by the introduction of the donor fragment into the gene(s) or chromosomal locus (loci) of interest. The PCR fragments thus obtained can be sequenced to further verify the novel junctions created by the introduction of the donor fragment into the gene(s) or chromosomal locus (loci) of interest. b. Diagnostic restriction enzyme digestion to make sure that only the desired modifications have been introduced into the LTVEC during the bacterial homologous recombination process. c. Direct sequencing of the LTVEC, particularly the regions spanning the site of the modification to verify the novel junctions created by the introduction of the donor fragment into the gene(s) or chromosomal locus (loci) of interest.

Step 4. Purification, Preparation, and Linearization of LTVEC DNA for Introduction Into Eukaryotic Cells.

a. Preparation of LTVEC DNA:

Prepare miniprep DNA (Sambrook et al. supra; Tillett and Neilan, Biotechniques, 24:568-70, 572, 1998; of the selected LTVEC and re-transform the miniprep LTVEC DNA into *E.*

coli using electroporation (Sambrook et al. supra). This step is necessary to get rid of the plasmid encoding the recombinogenic proteins that are utilized for the bacterial homologous recombination step. It is useful to get rid of this plasmid (a) because it is a high copy number plasmid and may reduce the yields obtained in the large scale LTVEC preps; (b) to eliminate the possibility of inducing expression of the recombinogenic proteins; and (c) because it may obscure physical mapping of the LTVEC. Before introducing the LTVEC into eukaryotic cells, larger amounts of LTVEC DNA are prepared by standard methodology; Sambrook et al. supra; Tillett and Neilan, Biotechniques, 24:568-70, 572, 1998). However, this step can be bypassed if a bacterial homologous recombination method that utilizes a recombinogenic prophage is used, i.e. where the genes encoding the recombinogenic proteins are integrated into the bacterial chromosome (Yu, et al. supra), is used.

b. Linearizing the LTVEC DNA:

To prepare the LTVEC for introduction into eukaryotic cells, the LTVEC is preferably linearized in a manner that leaves the modified endogenous gene(s) or chromosomal locus (loci) DNA flanked with long homology arms. This can be accomplished by linearizing the LTVEC, preferably in the vector backbone, with any suitable restriction enzyme that digests only rarely. Examples of suitable restriction enzymes include NotI, PacI, SfiI, SrfI, SwaI, FseI, etc. The choice of restriction enzyme may be determined experimentally (i.e. by testing several different candidate rare cutters) or, if the sequence of the LTVEC is known, by analyzing the sequence and choosing a suitable restriction enzyme based on the analysis. In situations where the LTVEC has a vector backbone containing rare sites such as Cos N sites, then it can be cleaved with enzymes recognizing such sites, for example λ terminase (Shizuya et al., Proc Natl Acad Sci USA, 89:8794-7, 1992; Becker and Gold, Proc Natl Acad Sci USA, 75:4199-203, 1978; Rackwitz et al., Gene, 40:259-66, 1985).

Step 5. Introduction of LTVEC into Eukaryotic Cells and Selection of Cells where Successful Introduction of the LTVEC has Taken Place.

LTVEC DNA can be introduced into eukaryotic cells using standard methodology, such as transfection mediated by calcium phosphate, lipids, or electroporation (Sambrook et al. supra). The cells where the LTVEC has been introduced successfully can be selected by exposure to selection agents, depending on the selectable marker gene that has been engineered into the LTVEC. For example, if the selectable marker is the neomycin phosphotransferase (neo) gene (Beck, et al., Gene, 19:327-36, 1982), then cells that have taken up the LTVEC can be selected in G418-containing media; cells that do not have the LTVEC will die whereas cells that have taken up the LTVEC will survive (Santerre, et al., Gene, 30:147-56, 1984). Other suitable selectable markers include any drug that has activity in eukaryotic cells, such as hygromycin B (Santerre, et al., Gene, 30:147-56, 1984; Bernard, et al., Exp Cell Res, 158:237-43, 1985; Giordano and McAllister, Gene, 88:285-8, 1990), Blasticidin S (Izumi, et al., Exp Cell Res, 197:229-33, 1991), and other which are familiar to those skilled in the art.

Step 6.

Screen for homologous recombination events in eukaryotic cells using quantitative assay for modification of allele (MOA). Eukaryotic cells that have been successfully modified by targeting the LTVEC into the locus of interest can be identified using a variety of approaches that can detect modification of allele within the locus of interest and that do not depend on assays spanning the entire homology arm or arms. Such approaches can include but are not limited to: (a) quantitative PCR using TAQMAN® (Lie and Petropoulos, Curr Opin Biotechnol, 9:43-8, 1998); (b) quantitative MOA assay using molecular beacons (Tan, et al., Chemistry, 6:1107-11, 2000); (c) fluorescence in situ hybridization FISH (Laan, et al., Hum Genet, 96:275-80, 1995) or comparative genomic hybridization (CGH) (Forozan, et al., Trends Genet, 13:405-9, 1997; Thompson and Gray, J Cell Biochem Suppl, 13943, 1993; Houldsworth and Chaganti, Am J Pathol, 145:1253-60, 1994); (d) isothermic DNA amplification (Lizardi et al., Nat Genet, 19:225-32, 1998; Mitra and Church, Nucleic Acids Res, 27:e34, 1999); and (e) quantitative hybridization to an immobilized probe(s) (Southern, J. Mol. Biol. 98: 503, 1975; Kafatos et al., Nucleic Acids Res 7(6):1541-52, 1979).

Applicants provide herein an example in which TAQMAN® quantitative PCR is used to screen for successfully targeted eukaryotic cells. For example, TAQMAN® is used to identify eukaryotic cells which have undergone homologous recombination wherein a portion of one of two endogenous alleles in a diploid genome has been replaced by another sequence. In contrast to traditional methods, in which a difference in restriction fragment length spanning the entire homology arm or arms indicates the modification of one of two alleles, the quantitative TAQMAN® method will detect the modification of one allele by measuring the reduction in copy number (by half) of the unmodified allele. Specifically, the probe detects the unmodified allele and not the modified allele. Therefore, the method is independent of the exact nature of the modification and not limited to the sequence replacement described in this example. TAQMAN® is used to quantify the number of copies of a DNA template in a genomic DNA sample, especially by comparison to a reference gene (Lie and Petropoulos, Curr. Opin. Biotechnol., 9:43-8, 1998). The reference gene is quantitated in the same genomic DNA as the target gene(s) or locus (loci). Therefore, two TAQMAN® amplifications (each with its respective probe) are performed. One TAQMAN® probe determines the "Ct" (Threshold Cycle) of the reference gene, while the other probe determines the Ct of the region of the targeted gene(s) or locus (loci) which is replaced by successful targeting. The Ct is a quantity that reflects the amount of starting DNA for each of the TAQMAN® probes, i.e. a less abundant sequence requires more cycles of PCR to reach the threshold cycle. Decreasing by half the number of copies of the template sequence for a TAQMAN® reaction will result in an increase of about one Ct unit. TAQMAN® reactions in cells where one allele of the target gene(s) or locus (loci) has been replaced by homologous recombination will result in an increase of one Ct for the target TAQMAN® reaction without an increase in the Ct for the reference gene when compared to DNA from non-targeted cells. This allows for ready detection of the modification of one allele of the gene(s) of interest in eukaryotic cells using LTVECs.

As stated above, modification of allele (MOA) screening is the use of any method that detects the modification of one allele to identify cells which have undergone homologous recombination. It is not a requirement that the targeted alleles be identical (homologous) to each other, and in fact, they may contain polymorphisms, as is the case in progeny resulting from crossing two different strains of mice. In addition, one special situation that is also covered by MOA screening is targeting of genes which are normally present as a single copy

in cells, such as some of the located on the sex chromosomes and in particular, on the Y chromosome. In this case, methods that will detect the modification of the single targeted allele, such as quantitative PCR, Southern blottings, etc., can be used to detect the targeting event. It is clear that the method of the invention can be used to generate modified eukaryotic cells even when alleles are polymorphic or when they are present in a single copy in the targeted cells.

Step 8. Uses of genetically modified eukaryotic cells. (a) The genetically modified eukaryotic cells generated by the methods described in steps 1 through 7 can be employed in any in vitro or in vivo assay, where changing the phenotype of the cell is desirable. (b) The genetically modified eukaryotic cell generated by the methods described in steps 1 through 7 can also be used to generate an organism carrying the genetic modification. The genetically modified organisms can be generated by several different techniques including but not limited to: 1. Modified embryonic stem (ES) cells such as the frequently used rat and mouse ES cells. ES cells can be used to create genetically modified rats or mice by standard blastocyst injection technology or aggregation techniques (Robertson, Practical Approach Series, 254, 1987; Wood, et al., Nature, 365:87-9, 1993; Joyner supra), tetraploid blastocyst injection (Wang, et al., Mech Dev, 62:137-45, 1997), or nuclear transfer and cloning (Wakayama, et al., Proc Natl Acad Sci USA, 96:14984-9, 1999). ES cells derived from other organisms such as rabbits (Wang, et al., Mech Dev, 62:137-45, 1997; Schoonjans, et al., Mol Reprod Dev, 45:439-43, 1996) or chickens (Pain, et al, Development, 122: 2339-48, 1996) or other species should also be amenable to genetic modification(s) using the methods of the invention. 2. Modified protoplasts can be used to generate genetically modified plants (for example see U.S. Pat. No. 5,350,689 "*Zea mays* plants and transgenic *Zea mays* plants regenerated from protoplasts or protoplast-derived cells", and U.S. Pat. No. 5,508,189 "Regeneration of plants from cultured guard cell protoplasts" and references therein). 3. Nuclear transfer from modified eukaryotic cells to oocytes to generate cloned organisms with modified allele (Wakayama, et al., Proc Natl Acad Sci USA, 96:14984-9, 1999; Baguisi, et al., Nat Biotechnol, 17:456-61, 1999; Wilmut, et al., Reprod Fertil Dev, 10:639-43, 1998; Wilmut, et al., Nature, 385:810-3, 1997; Wakayama, et al., Nat Genet, 24:108-9, 2000; Wakayama, et al., Nature, 394:369-74, 1998; Rideout, et al., Nat Genet, 24:109-10, 2000; Campbell, et al., Nature, 380:64-6, 1996). 4. Cell-fusion to transfer the modified allele to another cell, including transfer of engineered chromosome(s), and uses of such cell(s) to generate organisms carrying the modified allele or engineered chromosome(s) (Kuroiwa, et al., Nat Biotechnol, 18:1086-1090, 2000). 5. The method of the invention are also amenable to any other approaches that have been used or yet to be discovered.

While many of the techniques used in practicing the individual steps of the methods of the invention are familiar to the skilled artisan, Applicants contend that the novelty of the method of the invention lies in the unique combination of those steps and techniques coupled with the never-before-described method of introducing a LTVEC directly into eukaryotic cells to modify a chromosomal locus, and the use of quantitative MOA assays to identify eukaryotic cells which have been appropriately modified. This novel combination represents a significant improvement over previous technologies for creating organisms possessing modifications of endogenous genes or chromosomal loci.

EXAMPLES

Example 1

Engineering Mouse ES Cells Bearing a Deletion of the OCR10 Gene

a. Selection of a Large Genomic DNA Clone Containing mOCR10.

A Bacterial Artificial Chromosome (BAC) clone carrying a large genomic DNA fragment that contained the coding sequence of the mouse OCR10 (mOCR10) gene was obtained by screening an arrayed mouse genomic DNA BAC library (Incyte Genomics) using PCR. The primers employed to screen this library were derived from the mOCR10 gene cDNA sequence. Two primer pairs where used: (a) OCR10.RAA (SEQ ID NO:1) and OCR10.PVIrc (SEQ ID NO:2) which amplifies a 102 bp DNA; and (b) OCR10.TDY (SEQ ID NO:3)) and OCR10.QETrc (SEQ ID NO:4)) which amplifies a 1500 bp DNA. This mOCR10 BAC contained approximately 180 kb of genomic DNA including the complete mOCR10 coding sequence. This BAC clone was used to generate an LTVEC which was subsequently used to delete a portion of the coding region of mOCR10 while simultaneously introducing a reporter gene whose initiation codon precisely replaced the initiation codon of OCR10, as well as insertion of a selectable marker gene useful for selection both in *E. coli* and mammalian cells following the reporter gene (FIG. 2). The reporter gene (LacZ), encodes the *E. coli* β-galactosidase enzyme. Because of the position of insertion of LacZ (its initiating codon is at the same position as the initiation codon of mOCR10) the expression of lacZ should mimic that of mOCR10, as has been observed in other examples where similar replacements with LacZ were performed using previous technologies (see "Gene trap strategies in ES cells", by W Wurst and A. Gossler, in Joyner supra). The LacZ gene allows for a simple and standard enzymatic assay to be performed that can reveal its expression patterns in situ, thus providing a surrogate assay that reflects the normal expression patterns of the replaced gene(s) or chromosomal locus (loci).

b. Construction of Donor Fragment and Generation of LTVEC.

The modification cassette used in the construction of the mOCR10 LTVEC is the lacZ-SV40 polyA-PGKp-EM7-neo-PGK polyA cassette wherein lacZ is a marker gene as described above, SV40 polyA is a fragment derived from Simian Virus 40 (Subramanian, et al., Prog Nucleic Acid Res Mol Biol, 19:157-64, 1976; Thimmappaya, et al., J Biol Chem, 253:1613-8, 1978; Dhar, et al., Proc Natl Acad Sci USA, 71:371-5, 1974; Reddy, et al., Science, 200:494-502, 1978) and containing a polyadenylation site and signal (Subramanian, et al., Prog Nucleic Acid Res Mol Biol, 19:157-64, 1976; Thimmappaya, et al., J Biol Chem, 253:1613-8, 1978; Dhar, et al., Proc Natl Acad Sci USA, 71:371-5, 1974; Reddy, et al., Science, 200:494-502, 1978), PGKp is the mouse phosphoglycerate kinase (PGK) promoter (Adra, et al., Gene, 60:65-74, 1987) (which has been used extensively to drive expression of drug resistance genes in mammalian cells), EM7 is a strong bacterial promoter that has the advantage of allowing for positive selection in bacteria of the completed LTVEC construct by driving expression of the neomycin phosphotransferase (neo) gene, neo is a selectable marker that confers Kanamycin resistance in prokaryotic cells and G418 resistance in eukaryotic cells (Beck, et al., Gene, 19:327-36, 1982), and PGK polyA is a 3' untranslated region derived

from the PGK gene and containing a polyadenylation site and signal (Boer, et al., Biochem Genet, 28:299-308, 1990).

To construct the mOCR10 LTVEC, first a donor fragment was generated consisting of a mOCR10 homology box 1 (hb1) attached upstream from the LacZ gene in the modification cassette and a mOCR10 homology box 2 (hb2) attached downstream of the neo-PGK polyA sequence in the modification cassette (FIG. 2), using standard recombinant genetic engineering technology. Homology box 1 (hb1) consists of 211 bp of untranslated sequence immediately upstream of the initiating methionine of the mOCR10 open reading frame (mOCR10 ORF) (FIG. 3A-3D). Homology box 2 (hb2) consists of last 216 bp of the mOCR10 ORF, ending at the stop codon (FIG. 3A-3D).

Subsequently, using bacterial homologous recombination (Zhang, et al. supra; Angrand, et al., supra; Muyrers, et al. supra; Narayanan et al. supra; Yu et al. supra), this donor fragment was used to precisely replace the mOCR10 coding region (from initiation methionine to stop codon) with the insertion cassette, resulting in construction of the mOCR10 LTVEC (FIG. 2). Thus, in this mOCR10 LTVEC, the mOCR10 coding sequence was replaced by the insertion cassette creating an approximately 20 kb deletion in the mOCR10 locus while leaving approximately 130 kb of upstream homology (upstream homology arm) and 32 kb of downstream homology (downstream homology arm).

It is important to note that LTVECs can be more rapidly and conveniently generated from available BAC libraries than targeting vectors made using previous technologies because only a single bacterial homologous recombination step is required and the only sequence information required is that needed to generate the homology boxes. In contrast, previous approaches for generating targeting vectors using bacterial homologous recombination require that large targeting vectors be "trimmed" prior to their introduction in ES cells (Hill et al., Genomics, 64:111-3, 2000). This trimming is necessary because of the need to generate homology arms short enough to accommodate the screening methods utilized by previous approaches. One major disadvantage of the method of Hill et al., is that two additional homologous recombination steps are required simply for trimming (one to trim the region upstream of the modified locus and one to trim the region downstream of the modified locus). To do this, substantially more sequence information is needed, including sequence information spanning the sites of trimming.

In addition, another obvious advantage, illustrated by the above example, is that a very large deletion spanning the mOCR10 gene (approximately 20 kb) can be easily generated in a single step. In contrast, using previous technologies, to accomplish the same task may require several steps and may involve marking the regions upstream and downstream of the coding sequences with loxP sites in order to use the Cre recombinase to remove the sequence flanked by these sites after introduction of the modified locus in eukaryotic cells. This may be unattainable in one step, and thus may require the construction of two targeting vectors using different selection markers and two sequential targeting events in ES cells, one to introduce the loxP site at the region upstream of the coding sequence and another to introduce the loxP site at the region downstream of the coding sequence. It should be further noted that the creation of large deletions often occurs with low efficiency using the previous targeting technologies in eukaryotic cells, because the frequency of achieving homologous recombination may be low when using targeting vectors containing large deletion flanked by relatively short homology arms. The high efficiency obtained using the method of the invention (see below) is due to the very long homology arms present in the LTVEC that increase the rate of homologous recombination in eukaryotic cells.

c. Verification, Preparation, and Introduction of mOCR10 LTVEC DNA into ES Cells.

The sequence surrounding the junction of the insertion cassette and the homology sequence was verified by DNA sequencing. The size of the mOCR10 LTVEC was verified by restriction analysis followed by pulsed field gel electrophoresis (PFGE) (Cantor, et al., Annu Rev Biophys Biophys Chem, 17:287-304, 1988; Schwartz and Cantor, Cell, 37:67-75, 1984). A standard large-scale plasmid preparation of the mOCR10 LTVEC was done, the plasmid DNA was digested with the restriction enzyme NotI, which cuts in the vector backbone of the mOCR10 LTVEC, to generate linear DNA. Subsequently the linearized DNA was introduced into mouse ES cells by electroporation (Robertson, Practical Approach Series, 254, 1987; Joyner supra; Sambrook, et al. supra). ES cells successfully transfected with the mOCR10 LTVEC were selected for in G418-containing media using standard selection methods.

d. Identification of Targeted ES Cells Clones Using a Quantitative Modification of Allele (MOA) Assay.

To identify ES cells in which one of the two endogenous mOCR10 genes had been replaced by the modification cassette sequence, DNA from individual ES cell clones was analyzed by quantitative PCR using standard TAQMAN® methodology as described (Applied Biosystems, TAQMAN® Universal PCR Master Mix, catalog number P/N 4304437). The primers and TAQMAN® probes used are as described in FIG. 3A-3D (SEQ ID NO:5-6). A total of 69 independent ES cells clones where screened and 3 were identified as positive, i.e. as clones in which one of the endogenous mOCR10 coding sequence had been replaced by the modification cassette described above.

Several advantages of the MOA approach are apparent: (i) It does not require the use of a probe outside the locus being modified, thus obviating the need to know the sequence flanking the modified locus. (ii) It requires very little time to perform compared to conventional Southern blot methodology which has been the previous method of choice, thus reducing the time for identifying correctly modified cells from the typical several days to just a few hours. This is a significant improvement in the way screening has been performed in the past and makes it a much less labor-intensive and more cost-effective approach to screening for homologous recombination events in eukaryotic cells. Yet another advantage of the method of the invention is that it is also superior to previous technologies because of its ability to target difficult loci. Using previous technologies, it has been shown that for certain loci the frequency of successful targeting may by as low as 1 in 2000 integration events, perhaps even lower. Using the method of the invention, Applicants have demonstrated that such difficult loci can be targeted much more efficiently using LTVECs that contain long homology arms (i.e. greater than those allowed by previous technologies). As the non-limiting example described above demonstrates, the Applicants have targeted the OCR10 locus, a locus that has previously proven recalcitrant to targeting using conventional technology. Using the method of the invention, Applicants have shown that they have obtained successful targeting in 3 out of 69 ES cells clones in which the mOCR10 LTVEC (containing more than 160 kb of homology arms, and introducing a 20 kb deletion) had integrated, whereas using previous technology for ES cell targeting using a plasmid-based vector with homology arms shorter than 10-20 kb while also introducing a deletion of less than 15 kb, no targeted events were identified among more than 600

integrants of the vector. These data clearly demonstrate the superiority of the method of the invention over previous technologies.

Example 2

Increased Targeting Frequency and Abrogation of the Need to Use Isogenic DNA when LTVECs are Used as the Targeting Vectors

As noted above, the increased targeting frequency obtained using long homology arms should diminish the benefit, if any, derived from using genomic DNA in constructing LTVECs that is isogenic with (i.e. identical in sequence to) the DNA of the eukaryotic cell being targeted. To test this hypothesis, Applicants have constructed numerous LTVECs using genomic DNA derived from the same mouse substrain as the eukaryotic cell to be targeted (presumably isogenic), and numerous other LTVECs using genomic DNA derived from mouse substrains differing from that of the eukaryotic cell to be targeted (presumably non-isogenic). The two sets of LTVECs exhibited similar targeting frequencies, ranging from 1-13%, indicating that the rate of successful targeting using LTVECs does not depend on isogenicity.

The approach of creating LTVECs and directly using them as targeting vectors combined with MOA screening for homologous recombination events in ES cells creates a novel method for engineering genetically modified loci that is rapid, inexpensive and represents a significant improvement over the tedious, time-consuming methods previously in use. It thus opens the possibility of a rapid large scale in vivo functional genomics analysis of essentially any and all genes in an organism's genome in a fraction of the time and cost necessitated by previous methodologies.

Example 3

Use of LTVECs to Produce Chimeric and Human Antibodies

a. Introduction.

The rearrangement of variable region genes during the initial development of B cells is the primary mechanism whereby the immune system produces antibodies capable of recognizing the huge number of antigens that it may encounter. Essentially, through DNA rearrangements during B cell development, a huge repertoire of variable (V) region sequences are assembled which are subsequently joined to a constant (C) region to produce complete heavy and light chains which assemble to form an antibody. After functional antibodies have been assembled, somatic hypermutation which occurs in the secondary lymphoid organs, introduces further diversity which enables the organism to select and optimize the affinity of the antibody.

The production of antibodies to various antigens in non-human species initially provided great promise for the large scale production of antibodies that could be used as human therapeutics. Species differences, however, leads to the production of antibodies by humans which inactivate the foreign antibodies and cause allergic reactions. Attempts were subsequently made to "humanize" the antibodies, thus making them less likely to be recognized as foreign in humans. Initially, this process involved combining the antigen binding portions of antibodies derived from mice with the constant region of human antibodies, thereby creating recombinant antibodies that were less immunogenic in humans. A second approach which was developed was phage display, whereby human V regions are cloned into a phage display library and regions with the appropriate binding characteristics are joined to human constant regions to create human antibodies. This technology is limited, however, by the lack of antibody development and affinity maturation which naturally occurs in B cells.

More recently, endogenous genes have been knocked out of mice, and the genes replaced with their human counterparts to produce entirely human antibodies. Unfortunately, the use of these constructs has highlighted the importance of an endogenous constant region in the development and optimization of antibodies in B cells. Human antibodies produced by transgenic mice with entirely human constructs have reduced affinity as compared to their mouse counterparts. Accordingly, the much acclaimed methods of producing humanized antibodies in mice and other organisms, wherein endogenous variable and constant regions of the mice are knocked out and replaced with their human counterparts, has not resulted in optimal antibodies.

The use of chimeric antibodies, which utilize human variable regions with mouse constant regions through B cell maturation, followed by subsequent engineering of the antibodies to replace the mouse constant regions with their human counterparts, has been suggested (U.S. Pat. No. 5,770,429). However, the only methodology that has existed to date for making such chimeras has been trans-switching, wherein the formation of the chimeras is only a rare event which occurs only in heavy chains. Heretofore, there has been no mechanism to produce, in transgenic animals, large scale replacement of the entire variable gene encoding segments with human genes, thereby producing chimeras in both the heavy and light chains. Utilizing Applicants' technology, as disclosed herein, chimeric antibodies are generated which can then be altered, through standard technology, to create high affinity human antibodies.

b. Brief Description.

A transgenic mouse is created that produces hybrid antibodies containing human variable regions and mouse constant regions. This is accomplished by a direct, in situ replacement of the mouse variable region genes with their human counterparts. The resultant hybrid immunoglobulin loci will undergo the natural process of rearrangements during B-cell development to produce the hybrid antibodies.

Subsequently, fully-human antibodies are made by replacing the mouse constant regions with the desired human counterparts. This approach will give rise to therapeutic antibodies much more efficiently than previous methods, e.g. the "humanization" of mouse monoclonal antibodies or the generation of fully human antibodies in HUMAB™ mice. Further, this method will succeed in producing therapeutic antibodies for many antigens for which previous methods have failed. This mouse will create antibodies that are human variable region-mouse constant region, which will have the following benefits over the previously available HUMAB™ mice that produce totally human antibodies. Antibodies generated by the new mouse will retain murine Fc regions which will interact more efficiently with the other components of the mouse B cell receptor complex, including the signaling components required for appropriate B cell differentiation (such as Iga and Igb). Additionally, the murine Fc regions will be more specific than human Fc regions in their interactions with Fc receptors on mouse cells, complement molecules, etc. These interactions are important for a strong and specific immune response, for the proliferation and maturation of B cells, and for the affinity maturation of antibodies.

Because there is a direct substitution of the human V-D-J/V-J regions for the equivalent regions of the mouse loci all of

the sequences necessary for proper transcription, recombination, and/or class switching will remain intact. For example, the murine immunoglobulin heavy chain intronic enhancer, Em, has been shown to be critical for V-D-J recombination as well as heavy chain gene expression during the early stages of B cell development (Ronai et al. Mol Cell Biol 19:7031-7040 (1999)], whereas the immunoglobulin heavy chain 3' enhancer region appears to be critical for class switching (Pan et al. Eur J Immunol 30:1019-1029 (2000)) as well as heavy chain gene expression at later stages of B cell differentiation (Ong, et al. J Immunol 160:4896-4903 (1998)). Given these various, yet crucial, functions of the transcriptional control elements, it is desirable to maintain these sequences intact.

The required recombination events which occur at the immunoglobulin loci during the normal course of B cell differentiation may increase the frequency of aberrant, non-productive immunoglobulin rearrangements when these loci are inserted at improper chromosomal locations, or in multiple copies, as in currently available mice. With reductions in productive immunoglobulin rearrangement and, therefore, appropriate signaling at specific steps of B cell development the aberrant cells are eliminated. Reductions of B cell numbers at early stages of development significantly decreases the final overall B cell population and greatly limits the immune responses of the mice. Since there will be only one, chimeric, heavy or light chain locus (as opposed to mutated immunoglobulin loci and with human transgenic loci integrated at distinct chromosomal locations for heavy and light chains in the currently available mice) there should be no trans-splicing or trans-rearrangements of the loci which could result in non-productive rearrangements or therapeutically irrelevant chimeric antibodies (Willers et al. Immunobiology 200:150-164 (2000); Fujieda et al. J. Immunol. 157:3450-3459 (1996)).

The substitutions of the human V-D-J or V-J regions into the genuine murine chromosomal immunoglobulin loci should be substantially more stable, with increased transmission rates to progeny and decreased mosaicism of B cell genotypes compared with the currently available mice (Tomizuka et al Proc Natl Acad Sci (USA) 97:722-727 (2000)). Furthermore, introduction of the human variable regions at the genuine murine loci in vivo will maintain the appropriate global regulation of chromatin accessibility previously shown to be important for appropriately timed recombination events (Haines et al. Eur J Immunol 28:4228-4235 (1998)).

Approximately ⅓ of human antibodies contain lambda light chains, as compared to mice in which only 1/20 of murine antibodies contain lambda light chains. Therefore, replacing murine lambda light chain V-J sequences with lambda light chain V-J sequences derived from the human locus will serve to increase the repertoire of antibodies as well as more closely match the genuine human immune response, thus increasing the likelihood of obtaining therapeutically useful antibodies.

An additional benefit of integrating the human sequences into the genuine murine immunoglobulin loci is that no novel integration sites are introduced which might give rise to mutagenic disruptions at the insertion site and preclude the isolation of viable homozygous mice. This will greatly simplify the production and maintenance of a breeding mouse colony.

c. Materials and Methods:

Precise replacement of the mouse heavy chain locus variable region (VDJ) with its human counterpart is exemplified using a combination of homologous and site-specific recombination in the following example, which utilizes a two step process. One skilled in the art will recognize that replacement of the mouse locus with the homologous or orthologous human locus may be accomplished in one or more steps. Accordingly, the invention contemplates replacement of the murine locus, in whole or in part, with each integration via homologous recombination.

Large insert (BAC) clones spanning the entire VDJ region of the human heavy chain locus are isolated (FIG. 4A). The sequence of this entire region is available in the following GenBank files (AB019437, AB019438, AB019439, AB019440, AB019441, X97051 and X54713). In this example, large insert (BAC) clones are isolated from the ends of the mouse VDJ region as a source of homology arms (FIG. 4B) which are used to direct integration via homologous recombination of the human VDJ sequences in a two step process.

In the first step, LTVEC1 (FIG. 4D) is constructed by bacterial homologous recombination in *E. coli*. LTVEC1 contains, in order: a large mouse homology arm derived from the region upstream from the mouse DJ region, but whose absolute endpoints are not important; a cassette encoding a selectable marker functional in ES cells (PGK-neomycinR); a loxP site; a large human insert spanning from several V gene segments through the entire DJ region; and a mouse homology arm containing the region immediately adjacent to, but not including, the mouse J segments. Mouse ES cells will be transformed by standard techniques, for example, electroporation, with linearized LTVEC1, and neomycin resistant colonies will be screened for correct targeting using a MOA assay. These targeted ES cells can give rise to mice that produce antibodies with hybrid heavy chains. However, it will be preferable to proceed with subsequent steps that will eliminate the remainder of the mouse variable segments.

In the second step, LTVEC2 (FIG. 4C) is constructed by bacterial homologous recombination in *E. coli*. LTVEC2 contains, in order: a large mouse homology arm containing the region adjacent to the most distal mouse V gene segment, but not containing any mouse V gene segments; a large insert containing a large number of distal human V gene segments; a mutant loxP site called lox511 (Hoess et al. Nucleic Acids Res. 14:2287-2300 (1986)), in the orientation opposite to that of the wild type loxP sites in LTVEC2 and LTVEC1 (this site will not recombine with wild type loxP sites but will readily recombine with other lox511 sites); a wild type loxP site; a second selectable marker (PGK-hygromycinR); and a mouse homology arm derived from the V region, but whose absolute endpoints are not important. Mouse ES cells that were correctly targeted with LTVEC1 will then be transformed by standard techniques with linearized LTVEC2, and hygromycin resistant colonies will be screened for correct targeting using a MOA assay. Correctly targeted ES cells resulting from this transformation will hereafter be referred to as "double targeted ES cells".

Subsequent transient expression of CRE recombinase in the double targeted ES cells will result in deletion of the remainder of the mouse V region. Alternatively, the double targeted ES cells can be injected into host blastocysts for the production of chimeric mice. Breeding of the resultant chimeric mice with mice expressing CRE recombinase early in development will result in deletion of the remainder of the mouse V region in the progeny F1. This later alternative increases the likelihood that the hybrid heavy chain locus will be passed through the germline because it involves culturing the ES cells for fewer generations.

The inclusion of lox511 in LTVEC2 will allow for the insertion of additional human V gene segments into the hybrid locus. One approach would be to use bacterial homologous recombination to flank a large genomic DNA clone containing many additional human V gene segments

with lox511 and loxP sites. Co-transformation of such a modified large genomic DNA clone into double targeted ES cells with a plasmid that transiently expresses CRE recombinase will result in the introduction of the additional V gene segments by cassette exchange (Bethke et al. Nucleic Acids Res. 25:2828-2834 (1997)).

A second approach to the incorporation of additional V gene segments is to independently target a large genomic DNA clone containing many additional human V gene segments into the mouse locus using, for instance, the same mouse homology arms included in LTVEC2. In this case, the additional human V gene segments would be flanked by lox511 and loxP sites, and the targeted ES cells would be used to create a mouse. The mice derived from double targeted ES cells and the mice derived from the ES cells containing the additional V gene segments would be bred with a third mouse that directs expression of CRE recombinase during meiosis. The close proximity of the two recombinant loci during meiotic pairing would result in a high frequency of CRE induced inter-chromosomal recombination as has been seen in other systems (Herault et al. Nature Genetics 20: 381-384 (1998)).

The final steps in creating the human variable/mouse constant monoclonal antibody producing-mouse will be performing the equivalent variable region substitutions on the lambda and kappa light chain loci and breeding all three hybrid loci to homozygocity together in the same mouse. The resultant transgenic mouse will have a genome comprising entirely human heavy and light chain variable gene loci operably linked to entirely endogenous mouse constant region such that the mouse produces a serum containing an antibody comprising a human variable region and a mouse constant region in response to antigenic stimulation. Such a mouse may then be used as a source of DNA encoding the variable regions of human antibodies. Using standard recombinant technology, DNA encoding the variable regions of the heavy and light chains of the antibody is operably linked to DNA encoding the human heavy and light chain constant regions in cells, such as a CHO cells, which are capable of expressing active antibodies. The cells are grown under the appropriate conditions to express the fully human antibodies, which are then recovered. Variable region encoding sequences may be isolated, for example, by PCR amplification or cDNA cloning. In a preferred embodiment, hybridomas made from transgenic mice comprising some or all of the human variable region immunoglobulin loci (Kohler et al. Eur. J. Immunol., 6:511-519 (1976) are used as a source of DNA encoding the human variable regions.

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Ser
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We claim:

1. A genetically modified mouse, comprising in its germline human unrearranged variable region gene segments inserted at an endogenous mouse immunoglobulin locus.

2. The mouse of claim 1, wherein the human unrearranged variable region gene segments are heavy chain gene segments, and the mouse immunoglobulin locus is a heavy chain locus.

3. The mouse of claim 1, wherein the human unrearranged variable region gene segments are light chain gene segments, and the mouse immunoglobulin locus is a light chain locus.

4. The mouse of claim 3, wherein the light chain gene segments are human kappa light chain gene segments.

5. The mouse of claim 1, wherein the unrearranged variable region gene segments are contained on a genomic DNA fragment larger than 20 kb.

6. The mouse of claim 1, wherein the human variable region gene segments are capable of rearranging to form a functional V region gene.

7. The mouse of claim 1, where following rearrangement the mouse expresses a functional antigen-binding molecule encoded by the human gene segments.

8. The mouse of claim 1, wherein rearrangement of the human variable gene segments in the mouse results in a variable region gene that comprises a rearranged human variable region gene linked to a mouse constant region gene.

9. The mouse of claim 1, wherein the mouse produces an antibody that comprises a human variable region and a mouse constant region.

10. The mouse of claim 1, wherein the mouse does not comprise a human immunoglobulin constant gene.

11. A genetically modified mouse, comprising in its germline human unrearranged variable region gene segments linked to a mouse constant region gene, wherein the mouse lacks a human constant region gene, and wherein the mouse constant region gene is at an endogenous mouse immunoglobulin locus.

12. The mouse of claim 11, wherein the human unrearranged variable region gene segments are heavy chain gene segments.

13. The mouse of claim 11, wherein the human unrearranged variable region gene segments are light chain gene segments.

14. The mouse of claim 13, wherein the light chain gene segments are human kappa light chain gene segments.

15. The mouse of claim 11, wherein the unrearranged variable region gene segments are contained on a genomic DNA fragment larger than 20 kb.

16. The mouse of claim 11, wherein the human variable region gene segments are capable of rearranging to form a functional variable region gene.

17. The mouse of claim 11, where following rearrangement the mouse expresses a functional antigen-binding molecule encoded by the human gene segments.

18. The mouse of claim 11, wherein rearrangement of the human variable gene segments in the mouse results in a variable region gene that comprises a rearranged human variable region gene operably linked to a mouse constant region gene.

19. The mouse of claim 11, wherein the mouse produces an antibody that comprises a human variable region and a mouse constant region.

20. A mouse, comprising a modification in the germline of the mouse, wherein the modification comprises

(a) a hybrid heavy chain locus comprising an insertion of human immunoglobulin heavy chain V, D, and J gene segments, wherein the human heavy chain immunoglobulin V, D, and J gene segments are linked to a mouse immunoglobulin heavy chain gene, wherein the mouse immunoglobulin heavy chain gene is at an endogenous mouse immunoglobulin locus;

(b) a hybrid light chain locus comprising an insertion of human immunoglobulin light chain V and J gene segments, wherein the human V and J gene segments are linked to a mouse immunoglobulin light chain constant region gene sequence;

wherein (a) rearranges to form a hybrid heavy chain sequence comprising a human variable region linked to a mouse constant region, and (b) rearranges to form a hybrid light chain sequence comprising a human variable region linked to a mouse constant region, and wherein the mouse is incapable of forming an antibody that comprises a human variable region and a human constant region.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

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APPLICATION NO. : 13/164176
DATED : August 6, 2013
INVENTOR(S) : Andrew J. Murphy et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title page at item number (75) Inventors: replace:

"Andrew J. Murphy, Croton-on-Hudson, NT (US);
George D. Yancopoulos, Yorktown Heights, NY (US)" with

-- Andrew J. Murphy, Croton-on-Hudson, NT (US);
George D. Yancopoulos, Yorktown Heights, NY (US);
Margaret Karow, Santa Rosa Valley, CA (US);
Lynn Macdonald, White Plains, NY (US);
Sean Stevens, San Francisco, CA (US) --

Signed and Sealed this
Tenth Day of September, 2013

Teresa Stanek Rea
Acting Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,502,018 B2
APPLICATION NO. : 13/164176
DATED : August 6, 2013
INVENTOR(S) : Andrew J. Murphy et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title page at item number (75) Inventors: replace:

"Andrew J. Murphy, Croton-on-Hudson, NY (US);
George D. Yancopoulos, Yorktown Heights, NY (US)" with

-- Andrew J. Murphy, Croton-on-Hudson, NY (US);
George D. Yancopoulos, Yorktown Heights, NY (US);
Margaret Karow, Santa Rosa Valley, CA (US);
Lynn Macdonald, White Plains, NY (US);
Sean Stevens, San Francisco, CA (US) --

This certificate supersedes the Certificate of Correction issued September 10, 2013.

Signed and Sealed this
Seventeenth Day of December, 2013

Margaret A. Focarino

Margaret A. Focarino
Commissioner for Patents of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE
Certificate

Patent No. 8,502,018 B2 Patented: August 6, 2013

On petition requesting issuance of a certificate for correction of inventorship pursuant to 35 U.S.C. 256, it has been found that the above identified patent, through error and without any deceptive intent, improperly sets forth the inventorship.

Accordingly, it is hereby certified that the correct inventorship of this patent is: Andrew J. Murphy, Croton-on-Hudson, NY (US); George D. Yancopoulos, Yorktown Heights, NY (US); Margaret Karow, Santa Rosa Valley, CA (US); Lynn Macdonald, White Plains, NY (US); Sean Stevens, San Francisco, CA (US); Aris N. Economides, Tarrytown, NY (US); and David M. Valenzuela, Yorktown Heights, NY (US).

Signed and Sealed this Fourth Day of March 2014.

PETER PARAS, JR.
Supervisory Patent Examiner
Art Unit 1632
Technology Center 1600