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Modification of an Existing Chromosomal Inversion to Engineer a Balancer for Mouse Chromosome 15

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ABSTRACT

Chromosomal inversions are valuable genetic tools for mutagenesis screens, where appropriately marked inversions can be used as balancer chromosomes to recover and maintain mutations in the corresponding chromosomal region. For any inversion to be effective as a balancer, it should exhibit both dominant and recessive visible traits; ideally the recessive trait should be a fully penetrant lethality in which inversion homozygotes die before birth. Unfortunately, most inversions recovered by classical radiation or chemical mutagenesis techniques do not have an overt phenotype in either the heterozygous or the homozygous state. However, they can be modified by relatively simple procedures to make them suitable as an appropriately marked balancer. We have used homologous recombination to modify, in embryonic stem cells, the recessive-lethal In(15)21Rk inversion to endow it with a dominant-visible phenotype. Several ES cell lines were derived from inversion heterozygotes, and a keratin-14 (K14) promoter-driven agouti minigene was introduced onto the inverted chromosome 15 in the ES cells by gene targeting. Mice derived from the targeted ES cells carry the inverted chromosome 15 and, at the same time, exhibit lighter coat color on their ears and tails, making this modified In(15)21Rk useful as a balancer for proximal mouse chromosome 15.

INVERSIONS are chromosomal rearrangements that can be quite useful as genetic tools for recovery and maintenance of mutations in model organisms such as Drosophila (Ashburner 1989) and mice (Lyon *et al.* 1982; Roderick 1983; Zheng *et al.* 1999; Rinchik 2000). Progeny arising from single meiotic crossover within the inverted segment in inversion heterozygotes are normally not recovered, which allows one to use a dominant marker carried by the inversion to follow entire nonrecombined blocks of chromosome from generation to generation. This property makes appropriately visible marked inversions useful for recovering and/or maintaining mutations that are not easily recovered or kept as homozygotes (*e.g.*, lethal and fertility mutations or other detrimental mutations). To be usable as this type of balancer chromosome, an inversion (In) must be marked with visible dominant and recessive phenotypes. The recessive phenotype can even be lethality, so that inversion homozygotes are never recovered while In/+ segregants are readily distinguishable from their +/+ counterparts by the dominant phenotypes associated with the inversion.

Investment in the production of inversions as an important component of mouse genetic resources has been proposed (Justice *et al.* 1999; Zheng *et al.* 1999; Rinchik 2000). As gene-knockout, gene-trap, and large-scale ethylnitrosourea-mutagenesis programs worldwide produce a large number of lethal and detrimental mutations throughout the mouse genome, maintaining these mutants in genetic crosses will be a costly and potentially error-prone endeavor. Recently, with advances in embryonic stem (ES) cell technology and the *Cre-lox*P system, it has become possible to create inversions with preset endpoints anywhere in the mouse genome (Zheng *et al.* 1999). Dominant markers (*e.g.*, agouti coat color) can also be incorporated at one end of the inverted segment to endow the inversion with a dominant-visible phenotype. By careful selection of the endpoints, one may also make an inversion recessive lethal or endow it with an easily discerned recessive phenotype.

Gene-targeting technology now also makes it possible to revisit the use of existing mouse inversions to complement the *Cre-lox*P-generated inversion resource in mice. In pre-*Cre-lox*P years, mouse inversions were induced by radiation- or chemical-mutagenesis methods and recovered mainly by screening the progeny of mutagen-treated mice for meiotic anaphase bridges, a cytogenetic hallmark of inversion heterozygotes (Roderick 1983). The largest collection of inversions came from the work of T. Roderick (Roderick and Hawes 1974; Roderick

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1983; Beechey 1994). However, among the many inversions in the collection, only In(4)56Rk shows a dominant phenotype (Roderick *et al.* 1997), although some were recessive lethal, presumably due to the interruption of an essential gene(s) by the inversion breakpoint(s) (Roderick 1983). Thus, these original mutagen-induced mouse inversions were of limited utility as balancers.

With gene-targeting techniques, however, such existing inversions can be modified to acquire genetic attributes that can make them effective balancers. Here, we report the modification of one of Roderick's recessive-lethal inversions, In(15)21Rk, so that now it carries a dominant coat-color marker, namely the *K14-Agouti* minigene. Heterozygotes carrying this modified In(15)21Rk show light pigmentation/coloration on the tail and back of the ears, whereas homozygotes remain unrecoverable (Roderick 1983). Thus, this modified inversion now possesses the favorable features of a balancer chromosome and should be useful for recovery and maintenance of mutations mapping to the proximal two-thirds of chromosome 15.

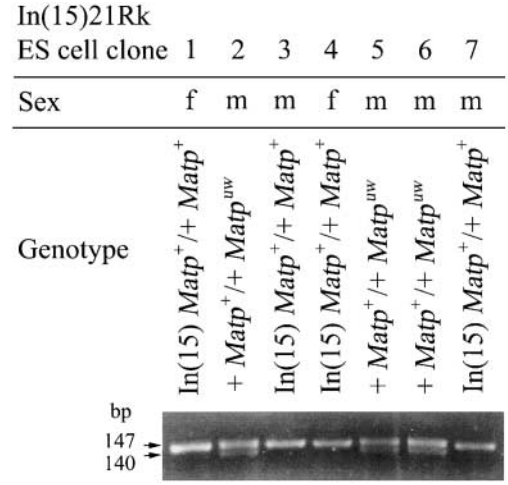

Figure 1.—Characterization of ES cell lines derived from In(15)21Rk +/+ *Matp*^{uw} × 129X1/SvJ. The presence of the inverted chromosome 15 was determined by typing for *Matp* alleles. A single PCR fragment of 147 bp indicates the presence of the In(15)21Rk inversion, which carries *Matp*$^{+}$ and one wild-type (129) chromosome 15, whereas the presence of the *Matp*^{uw} allele (7-bp deletion) indicates the absence of the inversion (double PCR fragments of 147 and 140 bp). The sexes of the ES cell lines are also indicated along with their genotypes. Clone 21Rk-7 was used for targeted insertion of *K14-Agouti* minigene.

MATERIALS AND METHODS

Stock maintenance: Heterozygous In(15)21Rk/+ mice are outwardly normal, whereas homozygous In(15)21Rk/In(15)21Rk mice do not survive to birth. In(15)21Rk was imported from The Jackson Laboratory (Bar Harbor, ME) and was maintained at the Oak Ridge National Laboratory as a closed-colony stock of *a/a*; In(15)21Rk +/+ *Matp*^{uw} compound heterozygotes. *Matp*^{uw} is the prototypic recessive allele at the classical mouse coat-color locus *underwhite* (*uw*); the mutation is a 7-bp deletion identified in exon 3 of the *Matp* (membrane-associated transporter protein) gene (Newton *et al.* 2001; Du and Fisher 2002). Intercrosses of In(15)21Rk +/+ *Matp*^{uw} mice yield three classes of offspring: (i) homozygous In(15)21Rk +/In(15)21Rk + mice, which are nonviable; (ii) compound heterozygous In(15)21Rk +/+ *Matp*^{uw} mice, which have black coat color; and (iii) homozygous + *Matp*^{uw} /+ *Matp*^{uw} mice, which have creamy whitish coat color and eyes lacking pigmentation at birth (but darkening by weaning age).

Derivation and characterization of In(15)21Rk/+ ES cell line: Mouse embryos for derivation of ES cells carrying In(15)21Rk were recovered from superovulated females. Five In(15)21Rk +/+ *Matp*^{uw} females at 6 weeks of age were given 5 units (in 0.1 ml) of pregnant mare's serum (Sigma, St. Louis) and, after 46 hr, given 5 units (in 0.1 ml) of human chorionic gonadotropin (Calbiochem, San Diego), both intraperitoneally. They were mated to 129X1/SvJ males to increase the success rate of ES cell derivation (Kress *et al.* 1998). Females were checked for vaginal plugs the next morning, at which time the embryos were regarded as 0.5 day postcoitum (dpc). Blastocysts were harvested on 3.5 dpc and washed at least five times in ES cell media [Dulbecco's modified Eagle medium (DMEM), high glucose, 15% fetal bovine serum (FBS), 1000 units/ml LIF (Chemicon, Temecula, CA), 0.1 mm nonessential amino acids, 1× nucleosides (Specialty Media, Phillipsburg, NJ), 55 μm β-mercaptoethanol, 25 units/ml penicillin, and 25 μg/ml streptomycin] before they were seeded individually into culture wells of a 96-well plate coated with 0.1% (w/v) gelatin. Blastocysts were then cultured in ES cell medium for 72 hr, and hatched blastocysts with inner-cell-mass (ICM) outgrowth were trypsinized with 0.05% trypsin/EDTA. The cell suspensions were transferred to wells of a 96-well plate containing a feeder-cell monolayer of mitotically inactivated primary murine embryonic fibroblast (PMEF). ES cell lines were identified by observation of their colony morphology and established and expanded by trypsinization and replating of newly formed colonies. The sex of the ES cell line was determined by PCR of two male-specific genes: a Y chromosome-specific DNA fragment (forward primer MJ17, agtgctaagctgc cagtt; reverse primer MJ18, acactgaactgagtgagg) and *Sry* (forward primer 422F, tagtgttcagccctacagcc; reverse primer 422R, cccgaattcgagtacaggtgtgcagctct; King *et al.* 1994; Levy *et al.* 1996). Since the inverted chromosome 15 of In(15)21Rk carries a wild-type *Matp* allele (*Matp*$^{+}$), an ES cell line carrying the inversion should contain only wild-type *Matp* alleles, whereas one lacking the inversion should be heterozygous for *Matp*$^{+}$ and the 7-bp *Matp*^{uw} deletion. The wild-type and 7-bp deletion *Matp* alleles were differentiated by PCR (forward primer UW3-F, ttctctgccctggttctcat; reverse primer UW3-R, cgtattcatgcatcccactg; Figure 1). Male In(15)21Rk ES cell lines thus obtained were injected into B6D2F$_1$ × B6 blastocysts to produce chimeras, which were then crossed to C57BL/6 females to test for germline transmission.

Targeting construct for *Matp*: The complete genomic sequence of the *Matp* wild-type allele was determined from the public databases (*e.g.*, Ensembl; http://www.ensembl.org/Mus_musculus/). We planned to target a *lox*P-*PGK-neo-lox*P cassette and the *K14-Agouti* marker (Kucera *et al.* 1996) into the *Matp*$^{+}$ locus on the inverted chromosome 15 segment of the In(15)21Rk/+ ES cells (see Figure 3C). Since the inverted segment of In(15)21Rk was of DBA/2J origin (Roderick 1983), DBA/2J genomic DNA (purchased from The Jackson Laboratory) was used as template to amplify, by PCR, a 6797-bp DNA fragment from intron 2 of the *Matp*$^{+}$ allele. The PCR reaction (50 μl) contained 0.3 μm of forward primer (MatpF1-C,

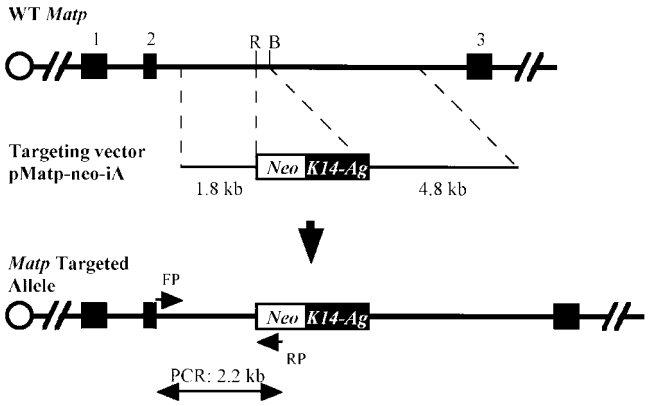

FIGURE 2.—Targeted insertion of *PGK-neo-K14-Agouti* minigene cassette onto the *Matp* (*underwhite*; *uw*) locus. The first three exons (nos. 1–3) of the mouse *Matp* gene are shown as black blocks, and the thick line represents the introns. The targeting vector pMatp-neo-iA with left and right homology arms of 1.8 and 4.8 kb, respectively, was targeted between the second and the third exons of *Matp* by homologous recombination in ES cells heterozygous for In(15)21Rk. Correctly targeted insertions of the *PGK-neo-K14-Agouti* minigene cassette were identified by PCR using a primer pair specific to the upstream sequence of the left arm (FP) and the *neo* marker (RP), respectively, as indicated by the arrowheads. The expected PCR product is 2.2 kb. The overall targeting frequency was 3.6%. Restriction enzyme sites: R, *Eco*RV; B, *Bam*HI.

tacatcgatacagtgaaactgtggagggaca), 0.3 μM of reverse primer (MatpR1-N, tattgcggccgcctcccatgaggcagtaagacct), 1× *Pfu* turbo polymerase buffer, 0.5 mM dNTPs, 2.5 mM $MgCl_2$, 2.5 units *Pfu* turbo polymerase (Stratagene, La Jolla, CA), and 200 ng DBA/2J genomic DNA using cycling parameters as follows: 95° initial denaturation for 2 min, 35 cycles of 95° denaturation for 30 sec, 58° annealing for 30 sec, 72° extension for 10 min, and a final extension at 72° for 10 min. The primer pair was designed such that the amplicon contained a *Cla*I site and a *Not*I site at the 5′- and 3′-end, respectively, which facilitated subcloning. The PCR product was gel purified using a gel-extraction kit (QIAGEN, Valencia, CA) and cloned into pBluescript SK+ to form pMatp. A *lox*P-*PGK-neo-lox*P cassette released from p1338 (a gift from T. J. Ley) by *Bam*HI and *Eco*RV digestion was subcloned into the pMatp (digested with *Bam*HI and *Eco*RV) to form pMatp-neo. Consequently, the left and right homology arms are 1796 and 4824 bp, respectively. Finally, a 4-kb *K14-Agouti* minigene (K14iA; KUCERA *et al.* 1996) was subcloned by blunt-end ligation into the vector pMatp-neo at the *Bam*HI site between the PGK-neo marker and the right homology arm. The resulting targeting construct was designated as pMatp-neo-iA (Figure 2).

Ten million In(15)21Rk/+ ES cells were electroporated with 15 μg of linearized targeting construct pMatp-neo-iA at 230 V and 500 μF. They were plated onto two 100-mm culture dishes with PMEF, cultured, and selected with G418 (300 μg/ml) for 7 days. A total of 192 neomycin-resistant clones were screened for homologous recombination events by PCR. The sequence of the forward primer was derived from the region outside of the left homology arm, with the reverse primer derived from within the *neo* marker. Genomic DNAs were isolated from the ES cells in 96-well plate format as described (RAMIREZ-SOLIS *et al.* 1993) and were used as DNA templates for PCR screening. The expected amplicon from a targeted recombinant should be 2.2 kb (Figure 2). Targeted ES cell clones were injected into $B6D2F_1$ × B6 blastocysts to produce chimeras. Subsequently, male chimeras were mated to C57BL/6J females to produce mice carrying the In(15)21Rk inversion marked by *K14-Agouti*. These mice are designated as In(15)21Rk-*Matp*$^{+tm(K14\text{-}Agouti)1R}$ and abbreviated as LE21Rk (LE stands for light ear).

Genotyping LE21Rk: Progeny from germline chimeras were genotyped with sequence-tagged site marker *D15Mit6* (see Figure 3C), which is polymorphic among the mouse strains DBA/2J, C57BL/6J, and 129X1/SvJ, yielding PCR products 141, 137, and 111 bp, respectively. Mice carrying the DBA/2J allele of *D15Mit6* should contain the inversion whereas those carrying the 129 allele should not. PCR reactions were performed as described (DIETRICH *et al.* 1994) and analyzed on 3.5% Metaphor agarose gels (Cambrex Bio Science Rockland, Rockland, ME).

Fluorescent *in situ* hybridization: Metaphase chromosome spreads were prepared from mouse splenocytes as described (FOX and POVEY 2000) with minor modifications. In brief, spleen cells were dissociated in Hank's balanced salt solution and were cultured in DMEM supplemented with 10% FBS, concanavalin A (3 μg/ml), and phytohemagglutinin (6 μg/ml) for 48 hr. The spleen culture was then treated with colchicine at a final concentration of 0.25 μM and incubated for 30 min prior to making chromosome spreads. Bacterial artificial chromosome (BAC) clone RP24-96K14 (BACPAC Resources Center, http://bacpac.chori.org/), which contains the *Matp* that mapped within the inversion, was fluorescein labeled with the Prime-It kit (Stratagene), and fluorescent *in situ* hybridization (FISH) was performed as described in the instruction manuals. The FISH results were observed using a Zeiss Axioskop 2 inverted fluorescent microscope with a Spot 100 digital camera linked to a Macintosh G4 computer. The images were processed using Adobe Photoshop v.5.5.

RESULTS

ES cell lines from embryos generated from the cross In(15)21Rk +/+ *Matp*^{uw} × 129X1/SvJ: ES cell lines were generated from embryos of the cross In(15)21Rk +/+ *Matp*^{uw} × 129X1/SvJ. The strain-129 genome was introduced because it is known to result in a higher success rate of deriving ES cell lines from the ICM of 3.5-dpc blastocysts (KRESS *et al.* 1998). Among 19 blastocysts harvested and cultured from five superovulated females, 15 gave rise to ICM outgrowths. From these ICMs, seven ES cell lines were established, of which four (two male, two female) contained In(15)21Rk (Table 1) as indicated by PCR genotyping of the *Matp* allele (see Figure 1 and MATERIALS AND METHODS). Clone 21Rk-7, a male inversion-bearing ES cell clone, was confirmed to transmit to the germline and used for subsequent gene-targeting experiments.

Insertion of the *K14-Agouti* minigene at the *Matp*$^{+}$ locus in the inversion In(15)21Rk: It has been documented that preferential homologous recombination occurs best when the homology arms of the targeting vector and the ES cell genome are isogenic (TE RIELE *et al.* 1992). Consequently, we generated homology arms of the agouti-minigene targeting vector from DBA/2J genomic DNA by PCR so that it would recombine preferentially with the inverted chromosome 15 (of DBA/2J

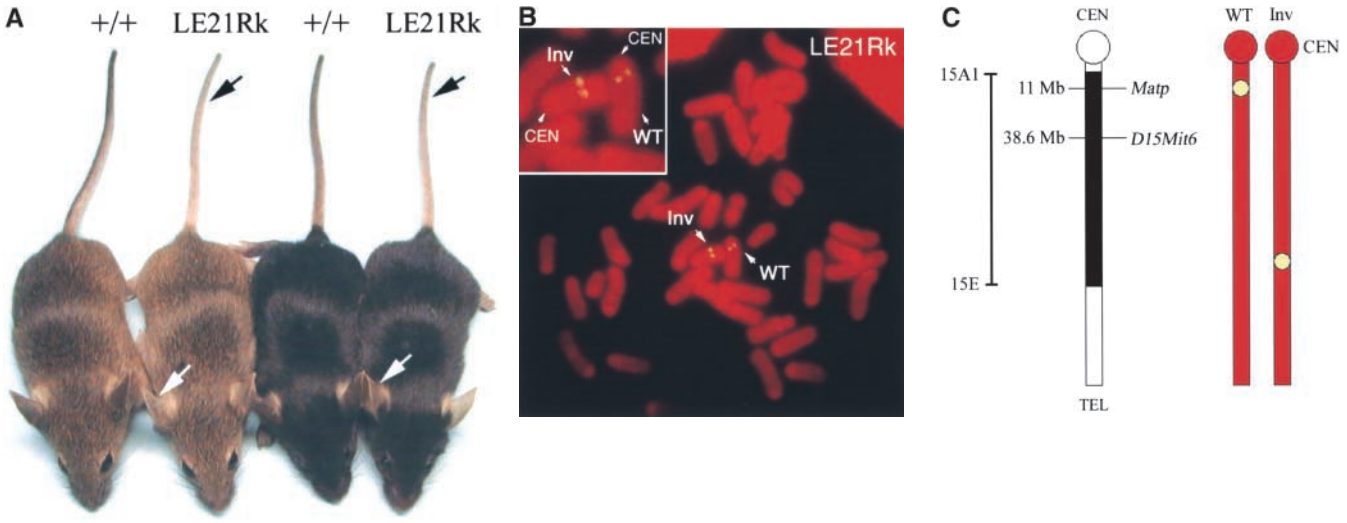

FIGURE 3.—*K14-Agouti* minigene-marked inversion mice. (A) Agouti and nonagouti (black) progeny from a male chimeric for ES cell contribution carrying the *K14-Agouti* minigene targeted to In(15)21Rk. The genotype of In(15)21Rk-*Matp*$^{+tm(K14\text{-}Agouti)1R}$/+ + is abbreviated as LE21Rk. The inversion mice have lighter pigmentation on their tails (solid arrows) as well as on the backs of their ears (open arrows). Agouti progeny heterozygous for the marked inversion also have lighter agouti coat color than the wild-type agouti-colored littermate. (B) FISH analysis of the LE21Rk inversion. Spleen-cell metaphase chromosome spreads from marked inversion mice, LE21Rk, were hybridized with fluorescently labeled BAC RP24-96K14 from the *Matp* locus. The fluorescent signal from the probe is close to the centromere (CEN) in the wild-type (WT) chromosome 15, but farther away in the inverted (Inv) chromosome 15. (C) Schematic of the chromosome 15 FISH results. The inverted segment of chromosome 15 spans cytogenetic bands 15A1 and 15E, as depicted in the black block, covering more than half of chromosome 15. The *Matp* (formerly *uw*) and *D15Mit6* loci are located at 11.0 and 38.6 Mb, respectively, according to the UCSC Mouse Genome Browser (http://genome.ucsc.edu/; mouse genome assembly frozen in February 2003). The fluorescent signal from the probe, which contains *Matp*, is represented as a yellow dot in the schematic of the FISH result. WT, wild type; Inv, inversion; CEN, centromere; TEL, telomere.

origin) rather than with the noninverted 129 allele. Among 192 ES clones screened after electroporation of targeting vector pMatp-neo-iA and G418 selection, seven homologous recombinants (3.6%) were recovered that showed the PCR fragment (2.2 kb) expected for a successful targeting event. These targeted ES cell clones were expanded and frozen as stocks. One clone (D8) was injected into B6D2F$_1$ × B6 blastocysts, resulting in four male germline chimeras that were subsequently used to generate the *K14-Agouti*-marked In(15)21Rk mice.

Production of *K14-Agouti*-marked In(15)21Rk mice: Male germline chimeras derived from the targeted D8 ES cell lines were bred with C57BL/6J females. About 50% (25/52) of their progeny showed lighter pigmentation of the ears and tails, indicating expression of the agouti gene (Figure 3A; ZHENG *et al.* 1999). Genotyping of *D15Mit6* for all the 52 progeny showed that each of the 25 lighter pigmented mice carried the DBA/2 and B6 alleles of chromosome 15. On the other hand, each of their wild-type littermates (27 genotyped) carried the 129 and B6 alleles at *D15Mit6* (data not shown). These genotyping results clearly indicated that *K14-Agouti* had been successfully targeted into the DBA/2J allele of *Matp* in In(15)21Rk/+ ES cells, that is, into the inverted chromosome 15. FISH analysis of a mouse with light ears and tail, using the *Matp*-containing BAC clone RP24-96K14 as probe, confirmed the presence of a chromosome 15 inversion (Figure 3, B and C): the fluorescent signal (greenish dots) from the probe on the inverted chromosome 15 (Figure 3, B and C, Inv) is farther away from the centromere (CEN) compared to the signal on the wild-type chromosome 15 (WT).

The lighter ear and tail color of mice heterozygous for the modified In(15)21Rk are readily observable around weaning age, starting from the 16th day after birth. The *K14-Agouti*-marked In(15)21Rk inversion is designated as In(15)21Rk-*Matp*$^{+tm(K14\text{-}Agouti)1R}$ and is abbreviated as LE21Rk.

DISCUSSION

This report describes genetic modification via an ES cell intermediate of an existing chromosomal inversion in the mouse. We engineered the recessively lethal In(15)21Rk inversion to carry a minigene that codes for a dominant coat-color phenotype. Success in derivation of ES cell lines from this (or any) mutant stock is a prerequisite for this type of subsequent genetic modification of an existing resource. Here we showed that ES cell lines could be efficiently derived from this inversion stock. It is known that genetic background will affect the permissibility of deriving ES cells (KRESS *et al.* 1998): introducing the genome of inbred strain 129 is known to enhance the establishment of ES cell

TABLE 1

Embryonic stem cell lines derived from the cross In(15)21Rk +/+ *Matp*^{uw} × 129X1/SvJ

No. of blastocysts harvested and cultured	19
No. of ICM outgrowths	15
No. of ES cell lines established	7
No. of ES cell lines carrying the 21Rk inversion (see Figure 1)	4 (2 male; 2 female)
No. of inversion ES cell lines tested for germline competency (no. of germline competent)	1 (1)

lines. In addition, the presence of 129 DNA introduced allelic polymorphisms to facilitate initial molecular genotyping of the offspring for proving concordance of the introduced coat-color phenotype with the presence of the inversion in carriers. Four ES cell lines (two male and two female) containing In(15)21Rk were established from 19 cultured blastocysts. The first of the four ES lines carrying the inversion went germline (the other three were not tested), and it was the one used to modify the inversion with the *K14-Agouti* coat-color minigene.

Available genetic data indicate that the *Matp* (formerly, *uw*) locus is very close to both the proximal inversion breakpoint and the centromere (Figure 3C; Searle and Beechey 1989). We therefore targeted the *K14-Agouti* minigene into *Matp* in the inverted chromosome 15 to minimize double-crossing-over-mediated loss of the resulting coat-color marker from the inversion. By using isogenic DBA/2J DNA to develop the targeting construct, we increased the odds of successful insertion of the *K14-Agouti* minigene into the wild-type *Matp* allele carried within the inversion. The targeting frequency was 3.6%, which is comparable to other reports using PCR methods to generate the targeting vector (Sharp *et al.* 1996). While we did not molecularly test directly whether the *K14-Agouti* minigene had been targeted into the inverted chromosome 15 in the ES cells, segregation analysis of pups from the resulting chimeras unambiguously showed this to be the case. About 50% of pups from chimeric males derived from the targeted ES cells showed lighter ears and tails, a phenotype that cosegregated, without exception, with a DBA/2J allele of *D15Mit6*. On the other hand, all littermates lacking this lighter ear and tail color carried the 129 allele. FISH analysis of a LE21Rk mouse confirmed that the mouse does indeed carry a chromosome 15 inversion while its wild-type littermate does not (Figure 3B; the FISH result for a wild-type littermate is not shown). These FISH results also confirm that, as expected, *Matp* is indeed located within the inversion.

We have shown that an existing, unmarked inversion can be modified to carry a visible phenotype appropriate for its use as a balancer chromosome. Although it was not necessary for the inversion that we modified, which is already recessive lethal, one can readily place the dominant marker into an essential locus or into one whose disruption would result in a readily detectable recessive phenotype, thus simultaneously endowing the unmarked inversion with both independent dominant and recessive phenotypes. Unless an inversion with specific breakpoints is the goal, one may consider the modification of a subset of the existing, unmarked inversions (such as the Roderick/Jackson Laboratory inversion resource, which covers almost all the mouse chromosomes) to complement the marked-inversion resource created by chromosome-engineering techniques.

It should be noted that most existing radiation- or chemical-induced inversions are large, most likely a consequence of the way in which they were first identified in the progeny of mutagenized mice (*i.e.*, by the presence of anaphase bridges in newly induced inversion heterozygotes, which would likely result in a bias toward ascertaining larger inversions since the longer the inverted segment, the higher the anaphase bridge frequency, and therefore the better it can be distinguished from the background noise). Whereas large inversions can cover a larger region as a balancer, they are also likely to permit an increased frequency of double crossing over with the wild-type chromosome, particularly in the midportion of the inversion, compared to smaller inversions. Because of this, larger inversions might, at first, appear to be unsuitable for marker modification and use as balancers. However, very little direct data still exist in mice to resolve the issue of inversion length *vs.* utility as a balancer. Analysis of a three-generation homozygosity screen using the In(1)1Rk inversion (~35–50 cM long) showed that only 3.6% presumed double crossovers had occurred (54 of 1503 cases; Roderick 1983). A gross estimation suggested that an inversion 50 cM long may have a maximum double-crossover frequency of 6.25% for loci that map to the middle of the inverted segment; the double-crossover frequency for most other loci within the inversion should be lower (Rinchik 2000). This gross estimate, however, was made on the assumption of no crossover interference. Recent genome-wide analysis of 188 meioses from interspecific crosses (Broman *et al.* 2002) appears to validate the long-standing belief that there is considerable positive double-crossover interference in the mouse, which should decrease the occurrence of clustered double crossovers, which in turn should also decrease the overall double-crossover frequency per chromosome, even in large inversions. Particularly cogent in the Broman *et al.* (2002) study was the finding, in 188 meioses, of double-crossover frequencies ranging from 0 to 4.2% in chromosomes 10 and 12–19. Thus, if one assumes these frequencies will be valid for inversion heterozygotes as they are for interspecific backcrosses, even large inversions covering most of the length of each of these

chromosomes may be worthy of modification for use as balancers.

Modification of a long, existing inversion could also serve as an important intermediate step in the creation of a "true" balancer—*i.e.*, the type of multiply inverted chromosome, such as that used routinely in Drosophila, that will virtually eliminate recovery of any recombinant progeny, including double crossovers. For example, one could envision applying chromosome-engineering techniques to introduce smaller inversions into existing larger inversions that have been captured in ES cell lines. In this way, multiply inverted, marked balancers could be created with fewer of the ES cell manipulation steps that might otherwise compromise their germline pluripotency. These types of multiply inverted true balancers would be the tools of choice for phenotype-driven regional mutagenesis, as well as providing indispensable tools for maintaining all lethal or otherwise detrimental mutations induced anywhere in the mouse genome by any technique.

Genetic background often drastically affects phenotype of a mutation due to modifier genes (Nadeau 2001). Therefore, one must take genetic background of a balancer into consideration before using it to maintain a specific mutation. Ideally, a balancer should already be a congenic strain in the desired genetic background before it is used to maintain a specific mutation. Otherwise it should be bred into a desired genetic background first and then used as a balancer so that a specific phenotype can be maintained and studied later.

Chromosome 15 is now virtually covered by two well-marked inversions: In(15)21Rk-*Matp*$^{+tm(K14\text{-}Agouti)1R}$, described here and covering the proximal two-thirds of the chromosome, and In(15)*Eh*2Rl (Bangham 1965, 1968; Davisson *et al.* 1990; Rinchik 2000), which covers the distal one-half to one-third. Thus, any chromosome 15 mutation that cannot be maintained in homozygotes can now be easily and cost effectively maintained using one of these inversions. As the use of mutant mice becomes even more widespread to analyze gene function and complex biological systems in the postgenome era, newly available balancer chromosomes (Zheng *et al.* 1999; Rinchik 2000; Nishijima *et al.* 2003) will serve as important tools for recovering new mutations and for maintaining lethal and otherwise detrimental as well as currently difficult-to-maintain genetic mutations, such as unmarked lethal deletions (Kushi *et al.* 1998; Thomas *et al.* 1998; You *et al.* 1997a,b).

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