Gene Targeting by Homologous Recombination in *Drosophila*

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Drosophila offers many advantages as an experimental organism. However, in comparison with yeast and mouse, two other widely used eukaryotic model systems, *Drosophila* suffers from an inability to perform homologous recombination between introduced DNA and the corresponding chromosomal loci. The ability to specifically modify the genomes of yeast and mouse provides a quick and easy way to generate or rescue mutations in genes for which a DNA clone or sequence is available. A method is described that enables analogous manipulations of the *Drosophila* genome. This technique may also be applicable to other organisms for which gene-targeting procedures do not yet exist.

We have developed a method to carry out gene targeting by homologous recombination in *Drosophila melanogaster*. This technique uses the organism's endogenous machinery of DNA repair and recombination to substitute one allele for another at a targeted gene or to integrate DNA at a target locus, as directed by DNA sequence homology. The method comprises three parts: a transgene that expresses a site-specific recombinase, a transgene that expresses a site-specific endonuclease, and a transgenic donor construct that carries recognition sites for both enzymes and DNA from the locus to be targeted. Flies with all three parts are generated by crossing, and expression of the enzymes is induced by heat-shocking the flies. The concerted action of these two enzymes produces an extrachromosomal recombinogenic donor DNA molecule in the cells of these flies. Progeny with gene-targeting events can be recovered by test-crossing. This gene-targeting technique provides a way to mutate genes that are identified only by sequence, and then to analyze their functions.

Considerable time and effort has been devoted to the development of methods for targeted mutation in *Drosophila*. Very large collections of random P element insertions have been generated and maintained with the hope of recovering mutagenic insertions in a large fraction of genes (*1*). P element–based techniques can be used to extend the utility of P element insertions (*2–6*). Such collections provide a superb genetic resource; however, not all genes are mutated in such collections, and often the mutations that are generated are not null alleles.

Targeting strategy. In organisms in which gene targeting has been achieved, DNA molecules with cut or broken ends have proven to be more recombinogenic than covalently closed circular DNAs (*7, 8*). In *Drosophila*, also, double-strand (ds) breaks in DNA are recombinogenic. X-rays generate ds breaks in DNA and induce both homologous and non-homologous recombination in somatic and germ line cells. DNA breakage produced by P element transposase also induces recombination (*9, 10*).

Accordingly, we constructed a method for gene targeting in *Drosophila* that uses broken-ended extrachromosomal DNA molecules to produce homology-directed changes in a target locus. Two transgenic enzymes were used for this purpose: the FLP site-specific recombinase and the I-SceI site-specific endonuclease. FLP recombinase efficiently catalyzes recombination between copies of the FLP recombination target (FRT) that have been placed in the genome (*11*). When FRTs are in the same relative orientation within a chromosome, FLP excises the intervening DNA from the chromosome in the form of a closed circle. If the FRTs are close to one another, this excision is nearly 100% efficient. We reasoned that these excised DNA molecules would become recombinogenic if they carried a ds break. To generate this break, we also introduced the I-SceI intron-homing endonuclease from yeast into *Drosophila*. I-SceI recognizes and cuts a specific 18–base pair (bp) sequence (*12, 13*).

Inducible double-strand breakage. We constructed a heat-inducible *I-SceI* gene (*70I-SceI*) and used standard P element transformation to generate fly lines carrying the transgene (*14*). Two chromosomally integrated tester constructs were used to assay the efficacy of *70I-SceI*. Each carried a *white+* (*w+*) reporter gene and an adjacent I-SceI cut site (*15*). One of the tester constructs also carried a partial duplication of the *white* reporter gene (Fig. 1, A and B). As a test for cutting, flies that carried *70I-SceI* and a reporter construct were generated by crossing and heat-shocked early in their development. If I-SceI endonuclease were to cut the chromosome at the site adjacent to the *w+* report-

er, we expected that occasional deletions of all or part of the *w+* gene would result, and in a *white*-null background this would show as eye-color mosaicism. The adults that eclosed exhibited frequent mosaicism, indicating that the heat-induced I-SceI can cut its recognition site in the *Drosophila* genome.

We also carried out quantitative assays of I-SceI cutting efficiency by scoring loss of *w+* in the germ line (*16*). The reporter with a cut site adjacent to *w+* exhibited a low frequency of *w+* loss, but the construct that was flanked by a tandem duplication of a portion of *w* showed nearly 90% loss of *w+*; this showed that cutting can be quite efficient. The 60-fold difference in the frequency of *w+* loss probably does not reflect a real difference in cutting efficiencies, but rather a difference in the preferred route of repair. In the second construct, repair with loss of *w+* could occur efficiently either through a single-strand annealing mechanism (*17–19*) or by homologous recombination between the repeats that flank the cut site. These results suggest the possibility that an efficient homologous recombination mechanism exists in germ line cells and that the ds break can provoke that mechanism.

A test of gene targeting. We designed a transgenic targeting construct (the donor) that has an I-SceI cut site placed within a cloned copy of the *Drosophila yellow+* (*y+*) body color gene. This gene was also flanked by FRTs (Fig. 1C) and the entire assembly inserted within a P element for transformation (*20*). In flies that carry this construct, the simultaneous induction of both enzymes should lead to excision of the FRT-flanked DNA and cutting of the excised circle.

The donor construct that we built is designed for "ends-in" targeting (Fig. 2), which appears to be generally more efficient than "ends-out" targeting in both yeast and mammalian cells (*21–25*). Our donor is designed to target the X-linked *y* gene, specifically the *y1* mutant allele, which has a point mutation in the first codon (*26*). In yeast, the likely fate of an ends-in targeting molecule would be integration at the locus of homology, producing a tandem duplication of the targeted gene (Fig. 2) (*27*). Because the I-SceI cut site in the donor is located to the right of the mutation in *y1*, we expected that the right-side copy of *y* in such a tandem duplication should be *y+* and the recessive *y* mutant phenotype would be masked.

We screened for targeted rescue of *y1* by producing flies that carried a heat-inducible *FLP* gene (*70FLP*), *70I-SceI*, and the donor construct of Fig. 1C (*28*). We heat-shocked those flies early in their development, and then test-crossed and screened for progeny that were *y+* but did not carry the chromosome on which the donor construct was originally located (Fig. 3A). Of the 56 indepen-

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dent y^+ rescue events that were recovered, 55 of these mapped to the X chromosome. Molecular analysis using the polymerase chain reaction revealed that in most cases β2 tubulin (β2t) sequences were still present in close proximity to y sequences (29). Therefore, the β2t sequence can serve as a molecular marker for cytological determination of the site of y^+ integration. [The β2t and β3 tubulin (β3t) genes shown in Fig. 1C are part of a selection scheme that was not implemented in these crosses.] Five independently recovered y^+ lines were examined by in situ hybridization to polytene chromosomes. In all five lines, β2t sequences were found at cytological locus 1B, the normal location of y, as well as at the normal site of the β2t gene at 85D (Fig. 3B), confirming that targeted integration had occurred in the y region.

These y rescue events occurred far more efficiently in the female germ line than in the male germ line. Fifty-three independent y^+ progeny (80 total) were recovered from 224 female test vials, for an overall efficiency of about one event per four vials screened. Each vial produced 100 to 150 progeny, so the absolute rate was about one independent y^+ offspring for every 500 gametes. In contrast, only three events were recovered from 201 male test vials. In *Drosophila*, meiotic recombination occurs in females and not in males, but the targeted recombinants that we recovered were probably premeiotic in origin and not directly attributable to this difference. Meiotic events are expected to be independent and exhibit a Poisson distribution. Events that occur in mitotic cells of the germ line can be replicated as cells pass through S phase and may produce multiple y^+ progeny from a single event, leading to clustering of the recovered y^+ events. The female germ line data differed significantly from a Poisson distribution ($P < 0.001$), exhibiting many more clusters than predicted (30).

Molecular analysis. All 56 independent y^+ lines were analyzed by Southern blotting (31). The 55 X-linked lines were all produced by targeted recombination at y. Figure 4 shows the four classes of event that we recovered and the numbers of each. Possible mechanisms for the origins of each type are also indicated, although more complicated origins cannot be excluded.

Class I consists of allelic substitution events that Southern blotting cannot distinguish from the original y^1 allele (Fig. 5). These may have been produced by double crossovers between the donor and y^1 (Fig. 4) or by gene conversion. Class II is equally numerous and consists of tandem duplications of y, with the β2t gene located between the two copies. These duplications almost certainly arose by integrative recombination between the chromosomal y^1 allele and the cut donor, as shown in Fig. 4. (Molecular data are shown in Fig. 5.)

When the donor element was constructed, the I-SceI cut site was cloned into the Sph I site within the intron of y, destroying the Sph I site in the process. Of the 19 class II alleles, 16 had regenerated the Sph I sites in both copies of y (29). This finding demonstrates that the two halves of the I-SceI cut site are readily removed from the cut ends during the recombination reaction and that the region is converted to the sequence of the targeted locus, as predicted by the ds break model of recombination and gap repair (32).

The high frequency of class II tandem duplications suggests another route by which the class I events may have been produced. Recombination between directly repeated y genes at a site to the left of the mutation in y^1 would reduce the duplicate genes to a single copy of y^+. In previous experiments, small tandem duplications that we have generated are very stable [for example, the P element of Fig. 1B; see also (11, 33)]. If a class I event were to occur by this route, it likely would immediately follow the integration event, when nicks or breaks are still present. As Fig. 1 shows, tandem duplications are readily lost when a ds break is introduced between the duplicate copies.

Class III consists of tandem duplications of y with insertions or deletions of material in one of the two copies (Fig. 4). These alter-

Ends-in targeting

marker gene

target gene

Ends-out targeting

marker gene

target gene

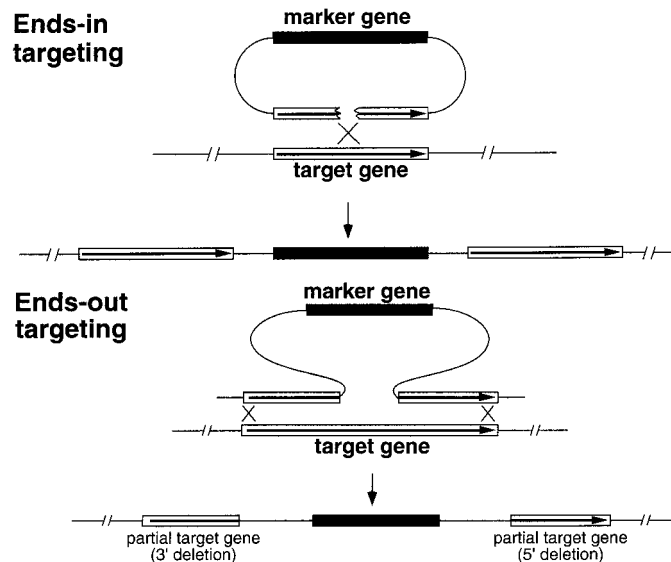partial target gene (3' deletion)   partial target gene (5' deletion)

Fig. 2. Gene-targeting configurations. Two typical forms of gene-targeting constructs are shown, with the results of their recombination with the target locus.

Fig. 1. Gene-targeting components. (**A** and **B**) Testing I-SceI efficiency (16). The reporter constructs were transformed via P elements (indicated by small arrowheads) and carried the I-SceI cut site (as indicated) either (A) adjacent to a shortened version of the wild-type w^+ gene (indicated by the large solid arrow), or (B) flanked by a complete copy and a nonfunctional partial copy of that w^+ gene. The complete gene is ~4.5 kb in length, and the nonfunctional partial gene is ~3.5 kb. (**C**) The construct for *yellow* targeting. At the top is the donor construct ($P[y$-donor]) as it would appear in the chromosome when initially transformed via P elements. Below is the form of the extra-chromosomal donor DNA after FLP-mediated excision and I-SceI cutting. The arrow indicates the transcriptional direction of *yellow*. The cut site is the 18-bp I-SceI recognition sequence; the β2t gene and the coding region of the β3t gene are indicated. S, Sal I restriction site. Distances between Sal I sites are given in kilobases. Locations of the DNAs used as probes for chromosome in situ hybridization and Southern blot analyses are shown.
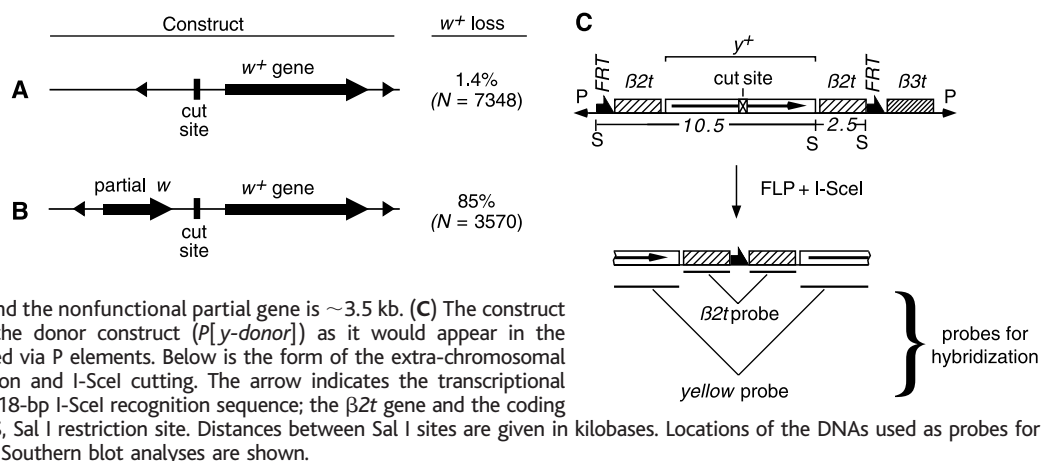
ations occur about the location at which the I-SceI cut site was placed. Although we have not identified the additional DNA that is present in the insertion alleles, the stronger hybridization signal exhibited by the upper band in lane 6 (Fig. 5) suggests that in at least some cases it is from the y gene. The class III events may arise by imprecise initiation or resolution of the recombination reaction.

Class IV, the least frequent class, consists of y^1 rescue events resulting from the inte-

gration of two additional copies of y (Fig. 4). Five such events were recovered; four were targeted to *yellow* and produced a triplication of the gene, and one occurred on chromosome 3. Although our experiments used flies with only a single donor transgene, two copies of the donor will be present when a cell is in G_2 phase. The two copies on sister chromatids might dimerize through FLP-mediated unequal sister chromatid exchange (*11*) or by end joining of two independently excised and

cut donor molecules. Integration of such a dimer could produce the observed results. Although all three bands detected with a y probe should hybridize with equal efficiency, the class IV event shown in Fig. 5 (lane 9) shows a stronger hybridization signal on the 8.0-kb band than on the 10.5- and 12.5-kb bands. This particular event may carry yet a fourth copy of y. The remaining four class IV recombinants appear to be the simpler events shown in Fig. 4.
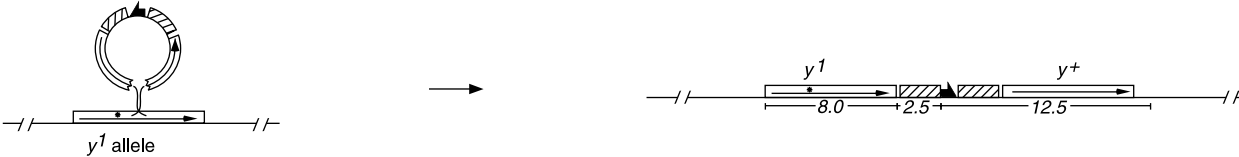
Fig. 3. Targeting the y gene. (**A**) Crossing schemes for *yellow* rescue (*28*). (**B**) Cytological localization of a targeted insertion. The cytological positions of $\beta 2t$ hybridization are indicated on the chromosomes of this y^1/y^+ class III female.
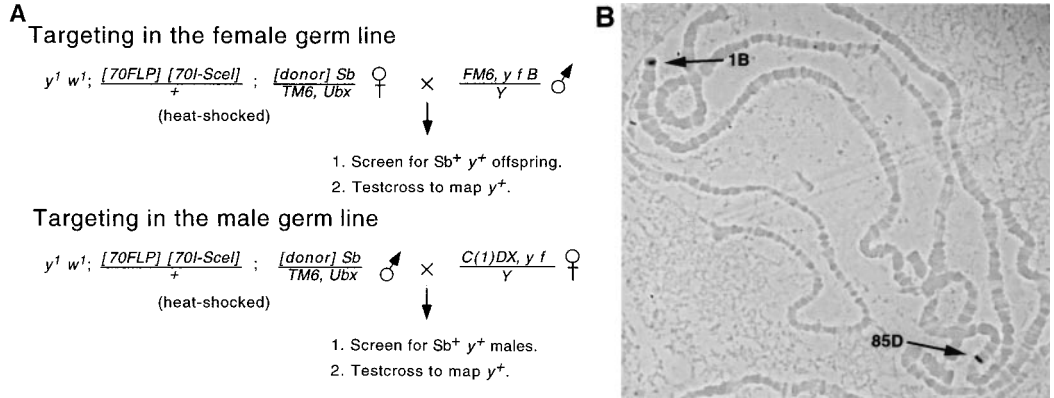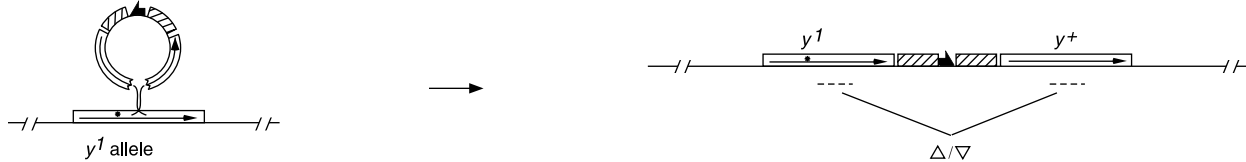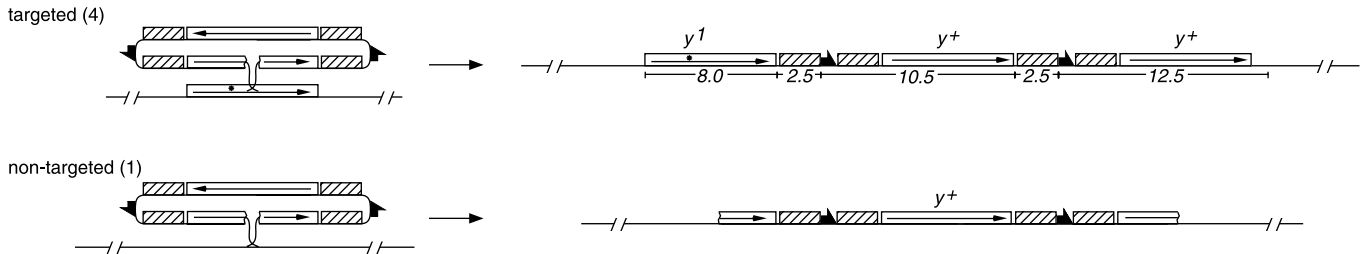

Fig. 4. Types of targeting events. The four classes of recovered targeting events are shown, with the likely mechanism of origin for each indicated at the left and the product of each event at the right. Recovered numbers of each class are indicated in parentheses. The targeting construct is shown in Fig. 1C. The approximate position of the point mutation in y^1 is indicated by a small asterisk. The expected sizes (in kilobases) of the DNA fragments produced by Sal I digestion are shown below each product at the right. The presumed allelomorphs of y are indicated above each copy of the gene. The approximate locations of the insertions (∇) and deletions (Δ) found in class III events are indicated.

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In these mutation-rescue experiments, the donor DNA was cut in the middle of the wild-type rescuing allele. For generation of a chromosomal y^+ gene to occur, recombination that is stimulated by the cut must almost inevitably occur with the y^1 allele. If a single copy of the donor were to integrate elsewhere, it seems highly unlikely that a functional copy of y^+ would be produced. Thus, our screen practically demands that only integration events targeted to y would be detected. Whereas class I, II, and III events give no information on the relative frequencies of targeted events versus random insertions, class IV events allow us to examine this issue because when a dimer is generated before integration, the middle copy of y^+ should be functional even when the donor molecule integrates at a site other than the y gene. We recovered five class IV events, and four of the five had integrated at the normal location of y on the X chromosome. Therefore, even in cases where it was possible to detect integration at sites other than y, the majority of recombinants were targeted to y. The single nontargeted class IV integrant was located on chromosome 3 but did not appear (by Southern blotting) to be targeted to the $\beta 2t$ gene.

Gene targeting: Strategies and designs. We have shown that P element–mediated, randomly inserted transgenes can be converted to targeted insertions through the use of a site-specific recombinase and site-specific endonuclease. Targeting events were identified by a genetic linkage screen and arose at a rate of about one targeted recombinant for every 500 progeny (in the female germ line). Our screen detected events that used a donor DNA to convert a mutant allele to the wild type, but changing wild-type alleles to mutant alleles is likely to be of more general interest, especially with the recent completion of the *Drosophila* genome sequence (*34*). The technique we developed should be readily adapt-able to this need. The targeted recombinants that we detected are very similar to the recombinants that would be recovered in yeast when transforming with linearized donor DNA. Accordingly, many of the techniques that have been developed for disrupting genes in yeast are likely to have analogous applications in *Drosophila* (*27*).

One possible scheme for targeted mutagenesis is shown in Fig. 6. A fragment of the gene to be mutated would have an I-SceI cut site placed within it. This donor DNA and a marker gene would be placed between FRTs and then into a transposon vector for transformation. After induction of FLP and I-SceI in females, targeting events could be detected by altered linkage of the marker gene, then verified by genetic or molecular techniques. In this example, a class II integration event should produce two truncated mutant alleles.

Many of the targeted events that we recovered were not produced by precise recombination. The class III events had alterations in the targeted locus that would not be predicted by homologous exchange. Some of the class II events may also have very small alterations that are not detectable by Southern blotting. It is likely that there were many additional class III targeted events that were not recovered in our screen because they carried deletions that destroyed the y^+ allele. So, although gene targeting often resulted from precise recombination, there are also many imprecise and potentially mutagenic events. This suggests that it may not be necessary for the donor to carry a mutant form of the target locus (such as the truncated gene of Fig. 6). Mutant alleles may be produced at a reasonable rate simply by imprecise targeting events. This result has precedence in the examination of stably transformed *Drosophila* cell lines. Cherbas and Cherbas (*35*) observed that in many cases, DNA transfected into cell lines had integrated near the chromosomal locus with homology to that DNA, and that rearrangements were often produced that in some cases generated mutations of the chromosomal locus. This phenomenon, which they termed parahomologous targeting, may be closely related to the processes that are responsible for the class III events that we recovered.

It should also be possible to introduce point mutations and a variety of other changes with this technique. Moreover, the frequent occurrence of class I events suggests that it will be possible to develop methods for producing allelic substitutions at other loci. Finally, the frequent replacement of the I-SceI cut site sequences at the termini of the donor with the wild-type genomic sequence suggests that it should be possible to carry out targeting with an I-SceI cut site placed within a gene's coding sequence, without necessar-

Fig. 5. Southern blot analyses of targeting events. Genomic DNA from males with an X-linked targeting event was digested with Sal I and, after blotting, hybridized with the *y* gene probe (**A**) and then stripped and rehybridized with the β*2t* probe (**B**). See Fig. 1C for locations of probes. The two outside lanes are markers with sizes (in kilobases) indicated next to each band. Lanes 1 and 13 are controls: C1 is DNA from y^1 males; C2 is DNA from y^1 males that also carry the donor construct shown in Fig. 1C. In (A), the hybridization signal of C2 consists of 10- and 10.5-kb bands that are not well separated. Class I events are indistinguishable from y^1 with either probe. Class II events exhibit two bands of hybridization with the *y* probe and two bands of hybridization with the β*2t* probe in addition to the ~15-kb band that represents the endogenous β*2t* locus. Lanes 4, 8, and 11 represent class III events having deletions within the y^1 copy. Lane 6 is a class III event with an insertion in the y^+ copy. Lane 9 is a targeted class IV event that is atypical in that, although it shows the expected bands in (A) and (B), hybridization to the y^1 band is more intense than expected.
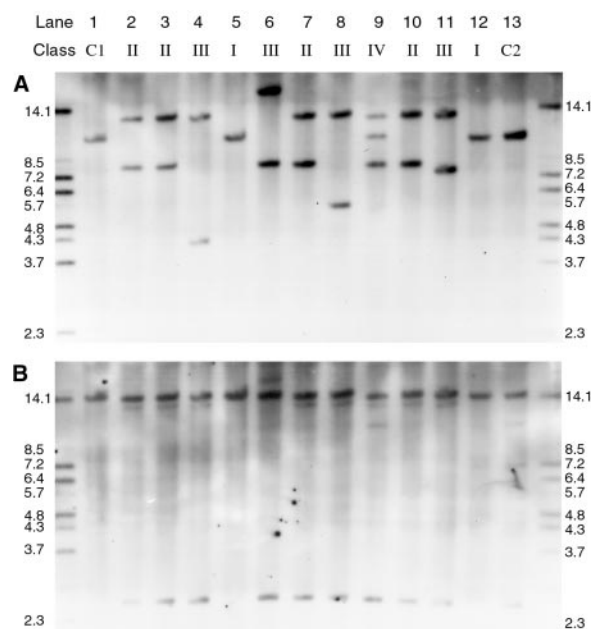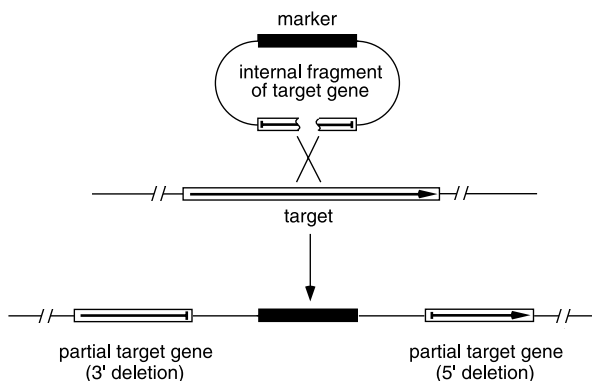
Fig. 6. Gene knockout by targeting with a truncated gene. The donor DNA used for targeting consists of a truncated gene, missing portions at both the 5′ and 3′ ends. Donor integration would disrupt the endogenous gene by splitting it into two pieces, each having a deletion of a different part of the gene.

partial target gene (3′ deletion)   partial target gene (5′ deletion)

ily destroying that portion of the gene.

One aspect of gene targeting that is likely to strongly influence targeting efficiency is the extent of homology or nonhomology between donor and target. Many reports show that increased donor-target homology increases the absolute targeting frequency in mammalian cells [e.g., (22, 36, 37)]. In *Drosophila*, investigators have examined the effect of homology in the context of P transposon break–induced gene conversion. The ds break that is left behind when a P element transposes is a substrate for repair by gene conversion, and it may use ectopically located homologous sequences as template. Dray and Gloor (38) found that as little as 3 kb of total template-target homology sufficed to copy a large nonhomologous segment of DNA into the target with reasonable efficiency. In prior work on FLP-mediated DNA mobilization, we observed that when the donor and target shared 4.1 kb of homology, FLP-mediated integration at a target FRT was about 10 times as efficient as when they shared 1.1 kb (39). An extended stretch of homology may transform transient contacts between extrachromosomal DNA molecules and chromosomal sequences into relatively long-lived associations, and hence may promote recombination. Reduced donor-target homology may specifically reduce the frequency of targeting, causing a shift in the ratio of targeted to nontargeted events. The limited data available from *Drosophila* lead us to surmise that 3 to 4 kb of donor-target homology [but possibly much less (40, 41)] may suffice for efficient targeting, although the donor and target shared 8 kb of homology in the present experiments.

Bellaiche *et al.* (42) attempted to use a similar system to carry out gene targeting in *Drosophila*, but failed. One obvious difference between their experiments and ours was that we found targeting to be much more efficient in females than in males, but they looked only in males. Their experiments also differed in other ways: I-SceI cutting was far more efficient in our experiments; we used an ends-in targeting strategy, whereas they used an ends-out strategy; and our construct was completely homologous to the target locus on either side of the cut ends (excepting the dozen or so base pairs that made up the I-SceI cut site), whereas theirs carried a large stretch of nonhomology at one end. Further work will be required to determine which of these differences are significant.

The gene-targeting technique that we describe is efficient enough that chemical or genetic selection methods were not needed, but these could certainly be implemented as part of the scheme if it were useful. Furthermore, the procedure that we describe does not require special lines of cultured cells, as does mouse gene targeting. Because the technique is carried out in the intact organism, it might be used for gene targeting in many other species of animals or plants, with the only requirement being the existence of a method of transformation.

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14. The coding region of I-SceI was excised from pCMV/SCE1XNLS [P. Rouet, F. Smih, M. Jasin, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6064 (1994)] as a 900-bp Eco RI–Sal I fragment. The Eco RI overhang was blunted by Klenow treatment. This fragment was cloned between the blunted Bam HI and the Sal I sites of p70ATG→Bam [R. B. Petersen and S. L. Lindquist, *Cell Regul.* **1**, 135 (1989)]. The resulting plasmid has the *I-SceI* gene inserted between the *Drosophila hsp70* promoter and its 3′ untranslated region (UTR). This *70I-SceI* transgene was cloned as a 2.6-kb Sal I–Not I fragment into the P element vector pYC1.8 [Y.-W. C. Fridell and L. L. Searles, *Nucleic Acids Res.* **19**, 5082 (1991)]. This gave rise to plasmid pP[v+,70I-SceI].
15. The 18-bp I-SceI cut site (termed I-site here) (13) was synthesized as two oligonucleotides, GGCCGGTAGG-GATAACAGGGTAATGTAC and ATTACCCTGTTTATC-CCTAGC, that were allowed to anneal to each other and cloned between Not I and Kpn I of plasmid pw8 [R. Klemenz, U. Weber, W. J. Gehring, *Nucleic Acids Res.* **15**, 3947 (1987)]. This generated pP[w8, I-site], the tester construct of Fig. 1A. The same synthetic I-site was cloned between the Not I and Kpn I sites of pP[X97] (39) to generate pP[X97, I-site]. Each of these constructs was transformed by standard P element–mediated techniques. The FRT-flanked portion of *P[X97, I-site]* was mobilized to the *RS3r*-4A element on chromosome 2 and to the *RS3r*-2 element on chromosome 3 by FLP-mediated DNA mobilization (39), generating the tester construct of Fig. 1B in two different locations.
16. At 0 to 3 days of development, flies that carried a transformed copy of *70I-SceI* and a reporter construct were given a single heat shock (38°, 1 hour). The reporter-bearing chromosome or its homolog also carried a dominant genetic marker. The heat-shocked males that received were test-crossed individually, and their progeny were scored for eye color. The frequency of w^+ loss is measured as the fraction of progeny receiving the reporter chromosome that were white-eyed. For the reporter *P[w8, I-site]*, the results of Fig. 1A are the summed results of testing five independent insertions of the reporter that were located on chromosome X, 2, or 3. For the reporter of Fig. 1B, two independent insertions were tested.
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20. The 5-kb genomic fragment of β2t was as described [H. D. Hoyle and E. C. Raff, *J. Cell Biol.* **111**, 1009 (1990)]. A Sal I site followed by the 34-bp FRT was synthesized by annealing of two oligonucleotides, ACCCGGGTCGACGAAGTTCCTATACTTTCTAGAGAA-TAGGAACTTCGGGCCC and GATCGGGCCCGAAGT-TCCTATTCTCTAGAAAGTATAGGAACTTCGTCGA-CCCGGGTGGCC, and then cloning into the Apa I site upstream of the ATG codon of β2t. This generated the β2tFRT gene with an FRT between the promoter and the coding region. The p[β3*]+3′UTR plasmid has a β3t gene controlled by the promoter and 3′ UTR of β2t [H. D. Hoyle, J. A. Hutchens, F. R. Turner, E. C. Raff, *Dev. Genet.* **16**, 148 (1995)]. The same FRT was cloned into Apa I upstream of its ATG codon in the same direction as the FRT in the β2tFRT gene, which is Sal I–FRT-ATG. The unique Not I in the β3t coding region was mutated without changing codon specificity by site-directed mutagenesis. This generated the β2FRTβ3* gene. These two genes were then assembled into the vector pSE280 (Invitrogen). First, the entire β2FRTβ3* gene was put between Not I and Kpn I. The promoterless β2tFRT gene was then cloned between the Xho I and Not I sites. The 8-kb Xho I–Kpn I fragment was cloned into the P element vector Carnegie3 [G. M. Rubin and A. C. Spradling, *Nucleic Acids Res.* **11**, 6341 (1983)]. This produced a vector with a unique Not I flanked by the β2tFRT and β2FRTβ3* genes. This Not I site was used as a site to insert an 8-kb genomic fragment of the *yellow* gene with an I-site cloned into the intron. This was done by synthesizing the I-site as two oligonucleotides (TACGCTAGGGATAACAGGGTAATCATG and ATTA-CCCTGTTATCCCTAGCGTACATG), allowing them to anneal, and ligating into the unique Sph I site in plasmid pS/G, which contains the *yellow* genomic region [P. K. Geyer and V. G. Corces, *Genes Dev.* **1**, 996 (1987)]. The Sph I was mutated in the cloning process. The y^+ gene served as a transformation marker for the completed construct, pP[y-donor].
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29. Y. S. Rong and K. G. Golic, data not shown.
30. The distribution of targeting events in females was as follows: 178 vials with 0 targeted recombinants, 26 vials with 1, 12 vials with 2, 3 vials with 3, 4 vials with 4, and 1 vial with 5 [χ^2 (2 df) = 25.6]. In some cases, events from the same vial were molecularly distinct and are reported as independent events.
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Negative Poisson's Ratios for Extreme States of Matter

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Negative Poisson's ratios are predicted for body-centered-cubic phases that likely exist in white dwarf cores and neutron star outer crusts, as well as those found for vacuumlike ion crystals, plasma dust crystals, and colloidal crystals (including certain virus crystals). The existence of this counterintuitive property, which means that a material laterally expands when stretched, is experimentally demonstrated for very low density crystals of trapped ions. At very high densities, the large predicted negative and positive Poisson's ratios might be important for understanding the asteroseismology of neutron stars and white dwarfs and the effect of stellar stresses on nuclear reaction rates. Giant Poisson's ratios are both predicted and observed for highly strained coulombic photonic crystals, suggesting possible applications of large, tunable Poisson's ratios for photonic crystal devices.

Rubber bands, Jell-O, and soft biological tissues share an important and unusual property with ultradense crystals in neutron stars and white dwarfs (*1–3*), vacuumlike ion crystals in electromagnetic traps (*4–6*), and crystallized colloids (*7–16*). Each can behave as if they are incompressible when stretched. This means that the fractional volume change, $\Delta V/V$, produced by a fractional elongation, $\Delta L/L$, is so small that the derivative $\gamma \equiv (L/V)(dV/dL)$ approaches zero. This behavior is described with the Poisson's ratio, which is defined as $\nu_{ij} = -\varepsilon_j/\varepsilon_i$, where ε_j is the lateral strain in the *j*-axis direction arising from a strain of ε_i applied in a longitudinal *i*-axis direction. The sum of Poisson's ratios for a stretch direction must approach unity for an effectively incompressible solid. Hence, isotropic rubbers and soft tissues have Poisson's ratios of ~0.5 (*17, 18*).

In contrast, negative Poisson's ratios are known for reentrant foams and related structures (*17–19*), microporous polymers (*20*), polymer laminates (*21*), hinged phases (*22, 23*), the noncubic phases of arsenic, bismuth, and α-cristobalite (*24, 25*), and certain cubic metals (*26, 27*). These materials have the counterintui-

tive property of expanding laterally when stretched, but none behaves as incompressible when stretched. For example, the sum of Poisson's ratios is between 1.15 and 1.59 for the cubic metallic elements (*27*), so they provide large values of $(L/V)(dV/dL)$.

Here, we investigate whether effectively incompressible crystal phases of white dwarf stars, neutron stars, and colloids can have negative Poisson's ratios. The search for materials with negative Poisson's ratios has focused on materials with large bending or torsional interactions and a large ratio of shear modulus to bulk modulus (*17, 18*), which are on the opposite extreme from these unusual states of matter with two-body, central-force interactions and a small ratio of shear modulus to bulk modulus. We predict the effects of coulombic screening, stretch-induced density changes, temperature, large crystal strains, and ion mixing on the extremal Poisson's ratios. Finally, we obtain experimental evidence of negative Poisson's ratios for one of these unusual states of matter.

The minimum and maximum Poisson's ratios for cubic crystals are usually for a stretch along a [110] direction, which is a cube-face diagonal, and resulting lateral deformations measured along the orthogonal [1$\bar{1}$0] and [001] directions, respectively. These Poisson's ratios can be expressed as functions of the Poisson's ratio, ν_{12}, and the tensile and shear elastic compliances, S_{11} and S_{44}, for the cube-axis directions (*27*):

$$\nu(1\bar{1}0) = -[1 - \nu_{12} - (S_{44}/2S_{11})]/[1 - \nu_{12} + (S_{44}/2S_{11})] \quad (1)$$

and

$$\nu(001) = 2\nu_{12}/[1 - \nu_{12} + (S_{44}/2S_{11})] \quad (2)$$

We use these equations to predict the allowable range of $\nu(1\bar{1}0)$ and $\nu(001)$ for a cubic crystal that behaves as incompressible when stretched. Stability requires that S_{11} and S_{44} are both positive. Substituting $\nu_{12} = 0.5$ into Eqs. 1 and 2 shows (Fig. 1A) that $1 \geq \nu(1\bar{1}0) \geq -1$, $2 \geq \nu(001) \geq 0$, and $\nu(1\bar{1}0) < 0$ when $S_{44}/S_{11} < 1$, which corresponds to high elastic anisotropy. A negative Poisson's ratio means that stretching causes a lateral expansion, which contradicts common experience for ordinary materials that act incompressible—like rubber bands.

Star crystals: Effectively incompressible crystals with unscreened charges. White dwarf cores and the outer crusts of neutron stars have been long proposed to consist of ultradense metallic crystals (*1–3*). These body-centered-cubic (bcc) crystals would have Fermi energies and electron densities that can be orders of magnitude higher than those of conventional metals. One might expect only positive Poisson's ratios for ultradense crystals, because the minimum Poisson's ratios of ordinary bcc metals become positive when the Fermi energy and electron density are large. The difference in bonding explains why this result for conventional metals is not valid for ultradense metals, as well as why ultradense metals behave as incompressible when stretched. The structure of an ordinary bcc metal can be approximated as arising from the balance between an attractive interaction between atom cores and the repulsive volume-dependent electron gas energy (*28*). At high pressures the atom cores shrink by loss of electrons to the electron gas. Eventually, at ultrahigh pressures the solid consists of well-separated nuclei in a free-electron sea. The electrostatic interactions between nuclei then dominate crystal shape, and the opposing contributions from the Fermi energy and pressure determine the crystal volume. Any significant volume change would enormously increase the energy change required for elongation, and tensile elongations therefore proceed at essentially constant volume. Hence, ultradense matter behaves like an anisotropic type of Jell-O (*29*), elongating and shearing much more readily than changing volume. These arguments pertain for densities between ~10^4 g/cm³ and ~10^{11} g/cm³, which are much lower than nuclear densities (~2×10^{14} g/cm³). Outside this range, either the atom cores are incompletely ionized (when density and pressure are too

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