Generation of a Useful roX1 Allele by Targeted Gene Conversion

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ABSTRACT Methods for altering the sequence of endogenous Drosophila melanogaster genes remain labor-intensive. We have tested a relatively simple strategy that enables the introduction of engineered mutations in the vicinity of existing P-elements. This method was used to generate useful alleles of the roX1 gene, which produces a noncoding RNA involved in dosage compensation. The desired change was first introduced into a genomic clone of roX1 and transgenic flies were generated that carry this sequence in a P-element. Targeted transposition was then used to move the P-element into roX1. Remobilization of the targeted insertion produced large numbers of offspring carrying chromosomes that had precisely introduced the engineered sequences into roX1. We postulate that this occurred by gap repair, using the P-element on the sister chromatid as template. This strategy was used to introduce six MS2 loops into the roX1 gene (roX1MS2-6), enabling detection of roX1 RNA by a MCP-GFP fusion protein in embryos. The roX1MS2-6 remains under the control of the authentic promoter and within the correct genomic context, features expected to contribute to normal roX1 function. The ability to replace relatively large blocks of sequence suggests that this method will be of general use.

The roX1 and roX2 (RNA on the X-1 and -2) are noncoding transcripts that play a central role in sex chromosome dosage compensation. W hit is process ensures a constant ratio of X-linked to autosomal gene products in males that have a single X chromosome. A complex of proteins and roX RNA [the Male Specific Lethal (MSL) complex] is recruited to X-linked genes. This complex directs chromatin modifications that result in increased expression from X-linked genes (Smith et al. 2001; Deng and Meller 2006; Conrad and Akhtar 2011; Larschan et al. 2012). The roX RNAs are essential for X-localization of the intact complex and, despite their lack of sequence similarity, are functionally redundant (Meller and Rattner 2002). Expression of roX1 RNA from an autosomal transgene will rescue roX1 roX2 males. However, both roX genes are X-linked, and both can recruit the MSL complex to chromatin adjacent to sites of roX transcription (Kelley et al. 1999; Kageyama et al. 2001; Park et al. 2003; Oh et al. 2004). This suggests that the function of the roX genes depends, in part, on their situation on the X chromosome.

During P-element–induced mutagenesis of roX1, we observed numerous identical rearrangements. These appear to be produced by a highly favored gene conversion that replaces more than 1 kb of roX1 with sequence contained within a P-element inserted in roX1. Replacement is driven by homology between genomic sequence flanking the insertion site and within the P-element. We tested this as a general strategy for gene engineering by introducing RNA loops from the MS2 virus (MS2 loops) into the endogenous roX1 gene, creating roX1MS2-6. RNAs that contain MS2 loops can be visualized in vivo when a fusion of GFP to the MS2 loop-binding protein (MCP-GFP) is expressed (Bertrand et al. 1998). The roX1MS2-6 allele preserves the normal chromatin context of roX1 and lacks all P-element sequence. Activity of roX1MS2-6 in dosage compensation is indistinguishable from that of wild-type roX1. We have named the
replacement strategy "targeted gene conversion" (TGC). TGC is technically simple and capable of introducing large blocks of non-homologous sequence. It is able to replace sequences that are more than 1 kb from a P-element insertion. The strategy that we tested relies on a P-element near the site to be mutated. However, recently developed methods for directed mutagenesis may enable a modified form of TGC in regions that lack P-elements.

MATERIALS AND METHODS

Fly culture
Flies were raised on a yeast, molasses, and cornmeal diet at room temperature. Mutations are described in citations or Lindley and Zimm (1992).

Gene conversion using an autosomal template
The p[wmrc] GM roX1[MS2-6/12] transgenes were generated by inserting 6 or 12 MS2 loops into a BglII site in a 4.9-kb genomic EcoRI fragment containing roX1. Males with autosomal insertions of these transgenes were mated to w;roX1[MS2-6]; Df(1)52; Df(y) w; p[wmc] GM roX1[MS2-6/12])+/y males. Df(1)52 removes roX2 and nearby essential genes. Males are rescued by a duplication of the roX2 region on the Y chromosome. These males were mated to C(1)DX y; fl(Dp(1)y) y; p[y]Δ2-399+ females to produce w;roX1[MS2-6]; Df(1)52; p[wmc] GM roX1[MS2-6/12]; p[y]Δ2-399+ dysgenic males that were mated to C(1)DX y; fl males. p[ΔA.3] females. The cosmid p[ΔA.3] restores all essential genes removed by Df2(5)S2, but it is deleted for roX2 and w;wmrc (Meller and Rattner 2002). If the break created by mobilization of roX1[MS2-6] was repaired by copying roX1 sequence within p[wmc] GM roX1[MS2-6/12], then this would result in loss of the w+ marker, restoration of roX1 activity, and incorporation of MS2 loops into roX1. White-eyed sons were mated individually to C(1)DX y; fl; p[ΔA.3] females and MS2 loop incorporation was determined by PCR of single fly squashes.

Targeted transposition
The p[wmrc] GM roX1[MS2-6] transgene was moved into roX1 by targeted transposition, using the roX1[mb720] plArB element as the target site. Dysgenic males (y w;roX1[mb720]; p[wmc] GM roX1[MS2-6])//Sb p[y]Δ2-399+ were mated to C(1)DX y; fl females. Hops p[wmc] Sb sons were collected and individually mated to C(1)DX y; fl females. X-linked insertions were mapped by in situ hybridization. Insertions close to roX1 (3F) were characterized by single fly PCR to verify the presence and orientation, of p[wmc] GM roX1[MS2-6]). Outward-facing primers [plac1(+), pry4(+), and ppy2] (Supporting Information, Table S1) in P-ends were paired with each other or with primers in roX1 (BPR10, BPR15) to determine the arrangement of tandem insertions. Primers are presented in Table S1. Targeted transpositions are denoted as roX1[MS2-6]TXX (tandem insertion) or roX1[MS2-6]RXX (replacement of plArB), followed by the transposition number.

Gene conversion in males
Three independent targeted transpositions of p[wmc] GM roX1[MS2-6] in roX1 were remobilized with p[y]Δ2-399. Lines roX1[MS2-6]T2A and roX1[MS2-6]T4A retain plArB in tandem, and roX1[MS2-6]R3A has replaced plArB with p[wmc] GM roX1[MS2-6]. Dysgenic males were mated to C(1)DX y; fl females. White-eyed sons were individually mated to C(1)DX y; fl females. Introduction of MS2 loops and retention of P-element sequence was determined by PCR. The roX1 primers flanking the MS2 loops (roX1[MS2-6]F and BPR19) amplify 547 bp from roX1+ and 867 bp when MS2 loops are inserted (roX1[MS2-6]).

Gene conversion in females
The targeted transposition roX1[MS2-6]T2A was mobilized in females. A total of 244 dysgenic females (roX1[MS2-6]T2A / Binscisy; Sb p[y]Δ2-3) 99/+) were mated to yw males, with approximately 10 females per vial; 25 out of 26 vials produced at least one white-eyed nonbalancer son, indicating excision. Two hundred sixty-nine excisions were mated individually to C(1)DX y; fl females. A randomly selected subset of these was analyzed by PCR for MS2 loop incorporation and loss of P-element sequences.

DNA blotting
DNA from 100 flies was extracted as described (http://www.fruitfly.org/about/methods/inverse.pcr.html). DNA was suspended in 300 µl DEPC water and treated with RNAase A and proteinase K; 15 µg DNA was digested overnight with EcoRI, concentrated, electrophoresed, and transferred to a charged nylon membrane. Blots were probed with a 32P-labeled, 2.03-kb EcoRI-MblI fragment spanning the promoter and 5’ end of roX1 using previously described methods (Church and Gilbert 1984). Restriction digests of a 4.9-kb roX1 genomic clone served as a molecular weight marker.

Visualization, photography, and image processing
Immunodetection of MSL1 on polytene preparations was performed as previously described (Kelley et al. 1999). MCP-GFP is removed by acetic acid fixation, preventing visualization on polytene chromosomes. To visualize MCP-GFP recruitment in embryo nuclei, homozygous roX1[MS2-6]; roX2Δ; [wmc] MCP-GFP females were mated to males carrying a p[wmc] sqh-mCherry insertion on the X chromosome. Male embryos are distinguished by lack of mCherry signal. The 3-h to 12-h embryo collections were dechorionated, fixed in 4% paraformaldehyde with 0.1% TWEEN-20, DAPI-stained, and mounted with DABCO anti-fade agent in 50% glycerol. Z-stacks were recorded for individual embryos using an Olympus Fluoview FV10i scanning confocal microscope with a 60x water/oil immersion lens. Images were processed by converting to 8-bit format and importing individual Z-stacks into ImageJ. Because mCherry signal was weak and diffuse, the brightness of this channel was uniformly enhanced for reproduction (Figure 4, C, H, and M). Consistent patterns of GFP localization were observed in images of more than 30 embryos from three collections.

RESULTS

An autosomal roX1[MS2-6] transgene restores X chromosomal MSL1 localization
RNA accumulation can be visualized in tissues or chromosome preparations by in situ hybridization. Although useful, this method is time-consuming and incompatible with living tissue. RNAs that contain stem loops from the MS2 virus can be visualized in vivo when a fusion of MCP-GFP is expressed (Figure 1A) (Bertrand et al. 1998). A roX1 transgene was constructed with six MS2 loops (roX1[MS2-6]) inserted in a region previously shown to be nonessential (Figure 1B) (Stuckenholz et al. 2003; Deng et al. 2005). An autosomal copy of this transgene, p[wmc] GM roX1[MS2-6], rescues X-localization of a key member of the MSL complex, Male-Specific Lethal 1 (MSL1), in roX1 roX2A males (Figure 1C). However, ectopic recruitment surrounding the site of transgene insertion is also observed (arrow, Figure 1C). Fully wild-type behavior of roX1 is consequently expected to require expression from the X chromosome, possibly from the roX1 locus itself.

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Gene conversion by repair using a sister chromatid template

During P-element mutagenesis of roX1, we obtained a series of mutations that suggested a strategy for inducing precise changes in target genes. A reporter construct containing the roX1 promoter fused to LacZ (p[w+mc roX1P-βgal]) was moved into roX1 in an effort to capture enhancers in the vicinity. This was accomplished by targeted transposition to the pArB element in roX1Δb710 (Figure S1A, B) (Gloor et al. 1991; Heslip and Hodgetts 1994). The resulting insertion, roX1[w+tandem], retained pArB and is marked with w+mc, facilitating subsequent mutagenesis. Hybrid element insertion was used to generate roX1Δb91, deleted for the pArB element and 891 bp flanking the insertion site, but retained p[w+mc roX1P-βgal] (Preston and Engels 1996; Preston et al. 1996) (Figure S1C). Remobilization of roX1Δb91 produced numerous white-eyed offspring from virtually every dysgenic parent. However, only a few imprecise excisions were identified (Figure S2A). Instead, almost 70% of excisions carried molecularly identical rearrangements exemplified by the severe roX1ΔSNC17A allele (Figure S1D). These appeared to be produced by a gene conversion that occurs when the double-stranded break produced by P-element mobilization undergoes gap repair using a sister chromatid template (Figure S2B). The rearrangement generated is consistent with repair driven by homology between the roX1 promoter on the broken chromosome more than 1 kb from the break site and in p[w+mc roX1P-βgal]. Homology is also shared by terminal inverted repeats at the 5′ and 3′ P-element ends (P-ends). In all 38 flies recovered with this rearrangement, the 3′ P-end has been precisely replaced by the 5′ P-end, a structure consistent with the proposed mechanism of repair. These chromosomes lost 1.2 kb of flanking Xchr sequence fused to the roX1 promoter. This mechanism is thus capable of efficiently replacing large regions close to P-elements.

Lack of repair utilizing a template on a different chromosome

To determine if efficient gene conversion was an intrinsic property of the roX1 locus that is independent of template location, we attempted to generate a useful allele of roX1 by introducing sequence from an engineered roX1ΔSNC17A transgene situated on an autosome. Gene conversion at white (w) occurs in a small percentage of excisions when a P-element is mobilized from w and a template with homology to insertion site is present in the genome (Banga and Boyd 1992; Johnson-Schlitz and Engels 1993; Nassif et al. 1994; Lankenau et al. 1996). We attempted to introduce MS2 loops into roX1 from an autosomal p[w+mc roX1P-βgal] donor on the third chromosome and the roX1Δb91 target site on the X chromosome were generated. To enable phenotypic detection of gene conversion, the target X chromosome was also deleted for roX2 (see Materials and Methods for full description of genotypes and matings). The roX1Δb91 is a severe loss-of-function mutant. Conversion to roX1ΔSNC17A will restore male viability and eliminate the w+mc marker in roX1Δb91. Approximately 100 white-eyed sons were recovered and tested by PCR for incorporation of MS2 loops, but only wild-type roX1 sequences were detected. Although
a gene conversion strategy utilizing a template situated on another chromosome may be productive in some situations, it was not useful in this instance.

**Targeted transposition of p[w\textsuperscript{mc} GM roX1\textsuperscript{MS2-6}]**

To determine if p[w\textsuperscript{mc} GM roX1\textsuperscript{MS2-6}] would be utilized for gap repair if situated in roX1, targeted transposition was used to move it to the plArB insertion site in roX1\textsuperscript{mb1210} (Figure 2). Mobilization produced abundant hops to the X chromosome, 68% of which (34/50 insertions) were in roX1. The reason for the unusually high efficiency of targeting is unknown, but an interaction of roX genes in the male germ line, where transposition occurred, is suggested. Insertions on the X chromosome were characterized by in situ hybridization and PCR. The plArB was retained in tandem with 32 of the insertions. However, two precise replacements of plArB with p[w\textsuperscript{mc} GM roX1\textsuperscript{MS2-6}] were recovered.

**Mobilization of targeted insertions to create roX1\textsuperscript{MS2-6}**

Three targeted insertions in roX1 were remobilized: a replacement line (roX1\textsuperscript{[MS2-6]R36A}) and two tandem insertions with different orientations (roX1\textsuperscript{[MS2-6]T2A} and roX1\textsuperscript{[MS2-6]T4B}) (Figure 2). Dysgenic males (roX1\textsuperscript{[MS2-6]XX}; Sb\textsuperscript{py+D2-3}99/+) were mated to C(1)DX\textsubscript{y1 f1} females. Mobilization is very frequent, with more than 90% of dysgenic males producing white-eyed sons, which comprise ~20% of male offspring. White-eyed sons were mated individually to C(1)DX\textsubscript{y1 f1} females and analyzed by PCR for repair of roX1 and inclusion of MS2 loops. Amplicons spanning the MS2 loop insertion site produce products characteristic of both wild-type roX1 (547 bp) and roX1\textsuperscript{MS2-6} (869 bp) from targeted transpositions, but almost 99% of white-eyed offspring produced a single amplicon. A total of 352 excisions were analyzed (169 for roX1\textsuperscript{[MS2-6]T2A}, 103 for roX1\textsuperscript{[MS2-6]T4B}, 80 for roX1\textsuperscript{[MS2-6]R36A}). Regardless of the starting line, more than 10% of white-eyed sons had incorporated MS2 loops into the repaired chromosome (Figure 3, B–E and Table 1). Amplicons from representative flies containing MS2 loops were sequenced, confirming faithful copying. The MS2 loops are 322 bp of nonhomologous sequence situated 430 bp from the point of P-element insertion (Figure 3A). Incorporation of MS2 loops therefore requires a gene conversion tract more

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**Figure 3** All predicted products of homology-dependent gene conversion are recovered. (A) The roX1\textsuperscript{[MS2-6]T2A} is a tandem insertion of p[w\textsuperscript{mc} GM roX1\textsuperscript{MS2-6}] at the 3’ end of plArB. Alignment of the engineered roX1\textsuperscript{MS2-6} (gray line) is shown collinear to and below the corresponding genomic sequence. The MS2 loops are 430 bp from the plArB insertion site. (B and C) Predicted products of homology-dependent gap repair and gene conversion. Left panels depict short repair tracts that do not incorporate MS2 loops; right panels depict longer tracts incorporating MS2 loops into the repaired chromosome. (B) Homology in roX1 precisely substitutes a portion of roX1\textsuperscript{[MS2-6]T2A} (thick gray line) at the plArB insertion site. (C) Homology in roX1 and at P-ends leads to retention of the 3’ P-end and duplication of 5’ roX1 sequence. (D) An imprecise excision removing w\textsuperscript{mc} from roX1\textsuperscript{[MS2-6]T2A}. (E) MS2 loop incorporation was detected by PCR using primers (arrows) flanking the MS2 loop insertion site (top). The roX1\textsuperscript{MS2-6} produces an 869-bp amplicon and roX1\textsuperscript{+} produces a 547-bp amplicon. Three representative excisions in each category are shown. Contraction of the MS2 loop array in excision 36A.1 was detected by a reduction of the amplicon to 800 bp (right). (F) Blot of EcoR1-digested DNA probed with the roX1 promoter (black bar, E). Hybridization to a single 4.9-kb roX1 fragment is seen in wild-type (WT) flies and in a gene conversion that did not incorporate MS2 loops or retain a P-end (roX1\textsuperscript{+}). A single 5.2-kb band is detected in two precise conversions incorporating MS2 loops (lines 2A.1 and 4B.1). Hybridization to a single 5.1-kb band is observed in excision 36A.1, consistent with the reduced MS2 loop array observed by PCR. Line 2.5 is the imprecise excision depicted in (D). A 5.2-kb band from p[w\textsuperscript{mc} GM roX1\textsuperscript{MS2-6}] and a 2.5-kb band produced by disruption of genomic roX1 by insertion of plArB are present.
than 750 bp in length. However, three flies generated by mobilization of roX1[MS2-6]R36A produced 800 bp PCR amplicons, consistent with contraction of the MS2 loop array during gene conversion (Figure 3E).

Our aim was to engineer roX1 without leaving vector or P-element sequence behind. However, homology at P-ends can support gap repair, leading to predictable rearrangements. When the tandem insertion roX1[MS2-6]T2A is mobilized, homology-dependent gap repair can restore roX1 with no P-element sequences or with a 3’ P-end retained (Figure 3, B and C). Flies that retain the 3’-P-end also duplicate the 5’ end of roX1 and, depending on the length of repair tract, have full-length wild-type roX1 (roX1P3) or roX1 with MS2 loops (roX1P3-MS2-6) (Figure 3C). Retention of the 3’ P-end is also possible following mobilization of the replacement line roX1[MS2-6]R36A (Figure S5). When the tandem insertion roX1[MS2-6]T2A is mobilized, the 3’ end of p[w+mC]GM roX1[MS2-6] as well as the entire plArB element may be retained (Figure S4). All of these alternative outcomes were readily identified by PCR (Table 2). Eight out of 18 MS2 loop-containing excisions of roX1[MS2-6]T2A retained a 3’ P-end. However, one of these is an imprecise excision that is mutated for w+mC but retains both P-elements in tandem (Figure 3D). In agreement with the structure determined by PCR, this line also produced both 547-bp and 869-bp PCR amplicons when tested for presence of MS2 loops. As expected, a single 4.9-kb band is detected in this line. Line 2.1 retains no MS2 loops into the 4.9-kb genomic hybridizing band at 5.1 kb (Figure 3 E and F). Line 2.1 retains no P-end; 103 out of 125 (82.4%) retained the 3’ end; 103 out of 125 (82.4%) retained the 3’ end; 103 out of 125 (82.4%) retained the 3’ end. We then selected white-eyed sons incorporated MS2 loops into the roX1 locus. This efficiency, 2.3%, contrasts with more than 10% MS2 loop incorporation in the offspring of dysgenic males. Two of the three lines contained a 3’ P-end, and thus represent an alternative rearrangement.

Reduced efficiency of MS2 loop incorporation could result from use of roX1 on the balancer chromosome as the repair template. Alternatively, it could reflect differences in the repair process in the male and female germ lines. For example, if repair tracts tend to be shorter in females, then inclusion of MS2 loops would be less frequent. To address these possibilities, we searched for P-element sequences on the repaired chromosomes. Retention of P-ends is expected when a sister chromatid template is utilized. We examined 125 randomly selected white-eyed offspring (including three with MS2 loops) for the presence of a 3’ end; 103 out of 125 (82.4%) retained the 3’ end. We then selected 29 flies at random (out of 125) and tested for the junction between the 3’ end of p[w+mC]GM roX1[MS2-6] and plArB. Twenty-one (72.4%) retained the junction. These findings are consistent with the idea that template-directed gap repair in females strongly favors copying of the sister chromatid.

### Table 1 Rearrangements recovered during generation of roX1[MS2-6]

| Targeted Transposition | Excisions Analyzed | No MS2 Loop Incorporation | MS2 Loop Incorporation | Imprecise Excisions |
|------------------------|-------------------|---------------------------|-----------------------|---------------------|
| roX1[MS2-6]T2A         | 169               | 150 (88.75%)              | 18 (10.65%)           | 2 (1.18%)           |
| roX1[MS2-6]T4B         | 103               | 90 (87.37%)               | 12 (11.65%)           | 1 (0.97%)           |
| roX1[MS2-6]R36A        | 80                | 71 (88.75%)               | 8 (10%)               | 1 (1.25%)           |

### Table 2 Retention of P-element sequences

| Parent Line            | Flies with MS2 Loops | 3’ P-End | P-Element Junction |
|------------------------|----------------------|----------|--------------------|
| roX1[MS2-6]T2A         | 18                   | 8        | 1\(^a\)            |
| roX1[MS2-6]T4B         | 12                   | 2\(^b\)  | 2\(^b\)            |
| roX1[MS2-6]R36A        | 8                    | 0        | 0                  |

| Parent Line            | Flies Without MS2 Loops | 3’ P-End | P-Element Junction |
|------------------------|-------------------------|----------|--------------------|
| roX1[MS2-6]T2A         | 13 (out of 150)         | 12       | 0                  |
| roX1[MS2-6]T4B         | 10 (out of 90)          | 6        | 0                  |
| roX1[MS2-6]R36A        | 12 (out of 71)          | 0        | 0                  |

\(^a\) Imprecise excision.

\(^b\) Two rearrangements retained plArB and the 3’ P-end of p[w+mC] GM roX1[MS2-6].
Table 3  roX1\textsuperscript{MS2\textendash6} retains roX1 activity

| roX1\textsuperscript{MS2\textendash6} Line | Mother | Father | Daughters | Sons |
|---|---|---|---|---|
| 2A.1 | roX1\textsuperscript{MS2\textendash6} roX2D | ++/Y | 100% (1048) | 96% (1001) |
| 4B.1 | roX1\textsuperscript{MS2\textendash6} roX2D | ++/Y | 100% (480) | 99% (474) |
| 36A.1 | roX1\textsuperscript{MS2\textendash6} roX2D | ++/Y | 100% (661) | 99% (654) |

**DISCUSSION**

The roX RNAs occupy a central position in fly dosage compensation. Full upregulation of X-linked genes does not occur in male roX1 roX2 mutants, and the MSL proteins mislocalize to ectopic autosomal sites (Meller and Rattner 2002; Deng and Meller 2006). Although autosomal roX transgenes rescue roX1 roX2 males, these transgenes also recruit MSL proteins to flanking autosomal chromatin, which is then modified in a manner similar to that at compensated X-linked genes (Kelley et al. 1999; Henry et al. 2001; Kelley and Kuroda 2003; Oh et al. 2004; Larschan et al. 2007). These observations suggest that position of roX genes on the X chromosome contributes to their normal function. More generally, the presence of complex or distal regulatory elements, or a requirement for a specific chromatin context, may contribute to deficiencies in the function of transgenes. Our objective was to generate an allele of roX1 that would function normally yet be readily visualized by GFP. The engineered allele roX1\textsuperscript{MS2\textendash6} supports full male viability in a roX2\textsuperscript{D} background. Visualization of roX1\textsuperscript{MS2\textendash6} RNA with MCP-GFP reveals punctate labeling of a subnuclear domain in male embryos and does not require lengthy histological protocols, making roX1\textsuperscript{MS2\textendash6} a new resource for detection of roX1 localization.

The absence of readily accomplished homologous recombination in *Drosophila* is a notable drawback in a powerful model organism. Groundbreaking studies more than a decade ago established a technique for homologous recombination in flies, but this process remains labor-intensive (Rong and Golic 2000; Gao et al. 2008; Huang et al. 2009; Wesolowska and Rong 2010). More recently, a strategy for reinsertion of large clones that has been modified by recombinase has been shown to be quite efficient (Bateman et al. 2013). This and similar strategies that use site-specific recombination leave vector remnants or recombination sites within the genome (Crown and Sekelsky 2013). In contrast, we have introduced an engineered change with no residual vector sequences. Alternative rearrangements that retain a P-end can be predicted and easily detected by PCR.

We have named this new strategy Targeted Gene Conversion (TGC) to reflect the two-step process required: targeted transposition followed by gene conversion. TGC is a variation of older techniques that utilized repair-mediated gene conversion to engineer *Drosophila* genes. These relied on transposon mobilization to generate double-stranded breaks that were then repaired using a template supplied by the homolog (Gloor et al. 1991; Johnson-Schlitz and Engels 1993), by a transposon at another position in the genome (Nassif et al. 1994; Lankenau et al. 1996; Merli et al. 1996), or by DNA injected into dysgenic embryos (Bang and Boyd 1992). The efficiency of this process, typically not exceeding a few percent of excised chromosomes, has limited its use. In contrast, almost all excisions of targeted insertions containing the template are repaired using the template, and 10% of these incorporated MS2-6 loops into roX1.

Directed mutagenesis has been improved by the use of zinc finger nucleases (ZFEN) and, more recently, TALENs and CRISPR/Cas9 nucleases, to introduce double-stranded breaks at specific sites (Bibikova et al. 2002; Christian et al. 2010; Bassett et al. 2013; Gratz et al. 2013). When repair templates with the desired changes are present, these sequences may be introduced by gene conversion (Gaj et al. 2013). The ability to rapidly generate mutations at a specific site is a clear strength of these methods. However, the efficiency of gene conversion decreases sharply with an increased distance from the break site (Moehle et al. 2007).

The potential for efficient replacement of longer sequences is anticipated to be a primary strength of TGC. Generation of roX1\textsuperscript{MS2\textendash6} \_\_\_\_ required resection of more than 1.2 kb from the break site, followed by copying more than 3 kb of sequence, including the
entire LacZ gene, into the break. Because this precise rearrangement accounted for 68% of excisions, TGC readily replaced large blocks of sequence. Gene conversion was also easily achieved on mobilization of roX1[MS2-6] or roX1[MS2-6] but, in this instance, no resection of broken ends was necessary to uncover homology with [w+]GM roX1[MS2-6]. Instead, incorporation of MS2 loops requires a repair tract to extend at least 750 bp from the break and to accommodate 322 bp of nonhomologous sequence. Ten percent of excisions incorporate MS2 loops, consistent with a previous study that documented conversion tracts extending almost 2 kb (Nassif and Engels 1993).

The roX1[MS2-6] and three targeted roX1[MS2-6] insertions are readily mobilized by transposase, with more than 90% of dysgenic males producing white-eyed offspring. This is not a general feature of P-element insertions in roX1, because only 20% of dysgenic roX1[MS2-6] males produce ry offspring (V. H. Meller, unpublished results). Despite high mobility, recovery of imprecise excisions was remarkably low. Four out of 56 excisions of roX1[MS2-6] and 4 out of 352 excisions of targeted roX1[MS2-6] insertions were imprecise. The apparent high mobility and bias against imprecise excision are likely attributable to the presence of an alternative template for gap repair that excludes w+[+].

A clear limitation of our strategy is the need to move the template sequence close to the target site. We have accomplished this by targeted transposition, but targeted transpositions typically comprise a few percent of new insertions and require a P-element at the target site. The exceptionally rich coverage of P-element insertions in Drosophila makes this feasible in many instances. Alternatively, recently developed techniques that use engineered nucleases, such as TALENs, CRISPR/Cas9, or ZFNs, could be used to introduce a landing site, such as attP, at the desired location (Groth et al. 2004; Gaj et al. 2013). Integration of a selectable marker and template flanked by P-ends would generate a mutagenic precursor for TGC without the need for a preexisting P-element (Figure S6).

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