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A simple and rapid method for constructing ring-X chromosomes in *Drosophila melanogaster*

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Abstract

Ring chromosomes are of basic interest to the geneticist and cell biologist who study their behavior in meiotic and mitotic divisions. In addition, the mitotic instability associated with some ring-X chromosomes has proven useful in *Drosophila* as a means to produce gynandromorphs for developmental studies. We describe a method to construct ring-X chromosomes in *Drosophila* via I-CreI-mediated exchange in *rDNA*, and then rapidly diagnose the recovery of ring chromosomes via FLP-mediated sister chromatid exchange within the ring. The method we describe provides a ready means to tailor the genetic content of ring-X chromosomes, making it suited to produce ring-X chromosomes for a variety of experimental purposes.

Introduction

The transgenic application of site-specific recombinases and homing endonucleases, which can recombine or cut specific target sequences, has opened new avenues for manipulating genes and chromosomes. The production of chromosome rearrangements has, in the past, depended almost entirely on ionizing radiation to produce random chromosome breaks, followed by careful screening to identify those chromosome rejoining events that produced a desired alteration. Repair events that did not restore the normal chromosome constitution could lead to deletions and duplications, inversions, translocations or a variety of more complicated rearrangements (reviewed by Ashburner et al. 2005). Now, through the selective application of transgenic recombinases or endonucleases, a specific rearrangement can often be produced in a much shorter time and with much greater precision than was possible with irradiation (Golic and Golic 1996; Maggert and Golic 2005).

In large part, this success depends on having recombinase target sites at the desired locations in the genome. Two groups have produced large collections of precisely-localized transposon insertions carrying target sites for the FLP recombinase (Ryder et al. 2004, 2007; Parks et al. 2004). However, one limitation is the paucity of elements located in heterochromatic regions. In certain cases it is desirable to have rearrangement breakpoints within heterochromatic regions to limit the aneuploidy that might otherwise be produced in the rearranged chromosome.

One specific example of this is in the production of ring chromosomes. Ring-X chromosomes, designated *R(1)*, have been useful for the production of sex chromosome mosaics, or gynandromorphs, and for basic investigations of chromosome behavior. They have been produced by a variety of methods, most easily by meiotic recombination between

the two arms of a tandem metacentric attached-X chromosome, or *C(1)TM* (Ashburner et al. 2005; Figure 1). In a *C(1)TM* chromosome, one arm is reversed in its normal order with respect to the medially-located centromere, so that the attached X chromosomes are arranged as tandem repeats. When a single meiotic exchange occurs between two arms that are attached to the same centromere, a ring chromosome is produced. Since a chromosome arm generally has one exchange per meiosis, the production of rings is very frequent. The downside of this efficiency is that *C(1)TM* chromosomes are not stable and are not generally maintained in stocks. The challenge then becomes synthesizing a *C(1)TM* chromosome in the easiest possible way. This led us to devise the technique we describe here for the production of *C(1)TM* and *R(1)* chromosomes. This method combines the I-*CreI* homing endonuclease to efficiently synthesize *C(1)TM* chromosomes, and the FLP recombinase to rapidly diagnose the ring-X derivatives that they produce.

Materials and Methods

Induction of *white* mutation on *In(1)EN*: The *In(1)EN*, *y bb* chromosome, which carries *rDNA* sequences at both ends of the chromosome (Lindsley and Zimm 1992; stock #1055 from Bloomington, IN, USA, Drosophila stock center) was mutagenized with Ethyl Nitroso Urea (ENU) to induce a *white* mutation using a standard protocol (Ashburner 1989).

***C(1;Y)30* chromosome:** The *C(1;Y)30* chromosome carries the entire X, from the tip through the *bb* (*rDNA*) locus, attached to a complete Y chromosome. It was produced by I-*CreI*-induced exchange between a normal X and *rDNA* sequences located near the tip of *YL* in *B^S Y y⁺* chromosome (Maggert and Golic 2005). A *P* element insertion at 13E-F (our isolate number number 75B), which carries a mini-*white* gene flanked by directly repeated *FRTs* (Golic and Lindquist 1989), was recombined from a normal X chromosome onto *C(1;Y)30* chromosome by standard meiotic recombination to generate *C(1;Y)30, y w P{>w^{hs}>} • y⁺*.

Results

The method we developed to produce ring-X chromosomes involves three steps: (1) I-*CreI* is used to induce exchange between *rDNA* repeats of an inverted-X chromosome and an attached-XY chromosome to generate a *C(1)TM* chromosome; (2) Meiotic exchange in females carrying the newly-generated *C(1)TM* produces ring chromosomes; and (3) Ring-X production is confirmed cytologically or by a rapid phenotypic test.

We used the homing endonuclease I-*CreI* to induce heterochromatic exchange between a compound XY chromosome, *C(1;Y)30*, and *In(1)EN*. *C(1;Y)30* is marked with *yellow* (*y*) and *white* (*w*) mutant alleles, carries a *P* element with an *FRT*-flanked *white⁺* gene (*w^{hs}*) at 13E-F, and also carries *y⁺* on the tip of the short arm of the Y. The second X chromosome carried an inversion of all euchromatin (*In(1)EN*) and was marked with *y*, and in the second series of experiments, also carried a mutant allele of *w*. We generated females that carried these two X chromosomes and a heat shock-inducible I-*CreI* transgene on chromosome 3. They were heat-shocked at 36° for 1 hr during the first five days of development, as previously described (Maggert and Golic 2005), and crossed to the indicated males (Figure 2).

The *I-CreI* endonuclease cuts within the *rDNA* repeats of the *X* and *Y* chromosomes, and this is frequently followed by exchange between these chromosomes (Maggert and Golic 2005). The desired *C(1)TM* chromosomes are recognized, in the first scheme, as exceptional daughters that are yellow and Bar^S , and in the second scheme as exceptional daughters that are yellow and white^+ (Figure 2). In addition to *C(1)TM* (Figure 3A), alternative single *rDNA* exchanges can produce exceptional daughters that have other attached-*X* chromosomes (Figure 3B-D). These alternative attached-*X* chromosomes do not give rise to ring chromosomes, though *C(1)RA* can generate dicentric chromosomes and *C(1)TA* will generate single rod *X* chromosomes via meiotic exchange. Our second scheme is unlikely to recover *C(1)TA* chromosomes, since it required that the exceptional females receive the w^{hs} -marked *P* element. Some progeny with apparently stable attached-*X* chromosomes were recovered in our schemes — these may have been *C(1)RM* or *C(1)RA* chromosomes. Other schemes to produce *C(1)TM* chromosomes also generate attached-*X* variants (Lindsley and Sander 1965; Ganetzky and Figenshow 1973). To recover ring-*X* chromosomes, the putative attached-*X* bearing females were crossed as indicated (Figure 2). A single exchange of the type shown in region I will produce a ring-*X* that does not carry the *P* element; such an exchange in region II will produce one that does carry the *P* element (Figure 1).

In the first scheme we set up 341 single female crosses, of which 252 were fertile. We recovered 24 independent potential *C(1)TM* females, confirmed by cytology in one case (Figure 4A), which were then crossed to recover ring-bearing sons. For ten females that produced sons of the expected ring phenotype, cytological examination showed that seven had ring chromosomes (Figure 4B). At least 3/24 of the females may have carried one of the other attached-*X* chromosomes diagrammed in Figure 3 because they did not yield any sons with the phenotype expected for a ring.

In the second crossing scheme we set up 66 single female crosses, of which 51 were fertile, and identified six independent potential attached-*X* daughters. Mass matings yielded another 24 potential attached-*X* females. Of these 30, 27 were fertile and 16/27 yielded potential ring-bearing sons. We carried out cytological examination of five, and verified that three were rings.

Since some of the ring chromosomes that are generated by this scheme will carry the *FRTs* of the $P\{>w^{hs}>\}$ transgene (recognized in the second scheme by virtue of carrying the w^+ transgene) it should be possible to catalyze sister-chromatid exchange in the ring by expression of the *FLP* recombinase (Golic and Lindquist 1989). Such an exchange is expected to produce a dicentric chromosome (Figure 4C). Cytological examination revealed the expected double-bridge dicentrics in anaphase and telophase figures after heat-shock induction of *FLP* expression (Figure 4D). Since dicentric formation is often followed by cell death (Ahmad and Golic 1999; Titen and Golic 2008) we thought it might be possible to detect a phenotypic consequence of dicentric formation, and thus diagnose the presence of ring chromosomes. We crossed ring males to females carrying an *eyFLP* transgene, which expresses *FLP* specifically in the eye-antennal disc. Daughters that carried a ring-*X* and *eyFLP* had a distinct phenotype of small rough eyes (Figure 4E). We tested two independently produced rings that had been previously verified cytologically, and both

showed this phenotype. Thus, when a ring carries $P\{>w^{hs}>\}$, it may be diagnosed by crossing to $eyFLP$ and examining the progeny for the small rough eye phenotype.

Discussion

The first $C(1)TM$ chromosome was produced by double exchange between a ring- X , an inverted X and a normal X in triploid females (Novitski and Lindsley 1950). A much easier method for generating $C(1)TM$ chromosomes is by exchange between a normal X and the tandem metacentric X known as $Dp(1;1)TMG, B^S$, with a recovery of 13 $C(1)TM$ among 6305 progeny (Lindsley and Sandler 1963). Unfortunately, we have not found this chromosome in stock. Therefore we devised an alternate scheme to produce $C(1)TM$ chromosomes and then ring- X chromosomes.

The work described here demonstrates an efficient method for producing tandem metacentric attached- X chromosomes, which will then generate ring- X chromosomes through normal meiotic recombination. Approximately 10% of females with induced $I-CreI$ expression produced candidate attached- X daughters, and approximately one-third of these daughters produced offspring with verified ring- X chromosomes.

The ring- X chromosomes that we generated were frequently filicidal, that is, they were lethal to a significant portion of progeny that received them, whether those were daughters or sons. In several cases, progeny with the phenotype indicative of a ring were recovered from putative $C(1)TM$ females, but could not be maintained because of complete or near-complete lethality during propagation. We confirmed that this was a result of zygotic lethality, and not meiotic drive, by egg-to-adult viability counts. The viability of offspring of $R(1)$ males was greatly reduced relative to the control, and this reduction was almost entirely a result of reduced survival of $R(1)$ -bearing daughters (Table 1).

This lethality is most likely a consequence of the presence of Y material within the ring (Oster 1964; Novitski and Puro 1978; Stone 1982), and in fact, the rings that we produced are more properly called $R(1;Y)$ chromosomes because they carry significant Y chromosome material (Figure 4A, B). By starting this scheme with chromosomes that have different heterochromatic content, such as $X\cdot YS$ (Lindsley and Zimm 1992) in place of $C(1;Y)30$ that we used here, ring- X chromosomes with minimal Y content might be generated. We also note that there are two distinctly different $C(1)TM$ chromosomes that could be produced by our scheme, one carrying YS material proximal to the $rDNA$, and the other carrying, in addition, the YS fertility factors that lie distal to the $rDNA$. This may account for differences in the filicidal character of different rings. As an alternate approach, a $C(1;Y)$ chromosome such as $YSX\cdot YL, B^S$ (Lindsley and Zimm 1992), which carries $rDNA$ sequences near the left and right tips, might also be a usable progenitor of a ring via intrachromosomal $I-CreI$ -induced exchange.

The primary purpose of our second crossing scheme, in which we selected for ring-bearing progeny that carried a w^+ -marked P element, was to recover rings that carried the $FRTs$ of that element. Then, FLP -mediated recombination could be used to generate dicentric chromosomes, and the resulting phenotype could be used for diagnosis of the ring. This

required that the rings derive from exchange that occurred to one side of the element (region II of Figure 1). By the use of appropriately selected mutations or marked *P* elements on the starting *X* chromosomes it would be possible to readily screen for ring-producing crossovers that occur within a specific region and thereby carry portions derived from one or the other of the two original rod chromosomes. Because it is somewhat difficult to alter the genetic content of ring chromosomes an advantage of building a ring chromosome *de novo* is that the rod chromosomes used as starting material are more easily designed to have the desired genotype. Furthermore, if a *P* element retained in the ring carries at least one *FRT*, then it will lend to rapid diagnosis using *eyFLP* to produce the small and rough eye phenotype.

One highly desirable property of some ring-*X* chromosomes is their mitotic instability, leading to the generation of gynandromorphs during development. However, such chromosomes are difficult to maintain and tend to become stable over generations (Ashburner et al. 2005). The two most often used unstable ring-*X* chromosomes are probably no longer extant, at least in their unstable forms. At least some of the rings that we recovered do generate gynandromorphs at a frequency above controls ($3/75 = 4\%$ in one case where gynandromorphs were scored). Although this frequency is not high, it may be usable for mosaic analysis. It is also quite possible that rings with different heterochromatic content might produce higher frequencies of gynandromorphs.

The relative predictability and efficiency with which I-*CreI* can be used to induce exchange between regions of *rDNA* makes it feasible to construct a variety of different ring-*X* chromosomes, starting with different *X* and *Y* chromosome variants. It may be possible to devise a scheme to repeatedly build unstable ring-*X* chromosomes, eliminating the tedious job of maintaining them in stock. In addition, ring-*X* variants could be used to explore fundamental aspects of ring chromosome behavior, such as lethality and mitotic instability (Hinton 1955, 1957, 1959). We note that such abnormalities are not restricted to *Drosophila*, but are also characteristic of ring chromosomes in humans (Kosztolanyi 1987).

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Figure 1.

Meiotic exchange within a tandem metacentric compound X chromosome. Exchange between the two arms attached to the same sister centromere (arbitrarily chosen as the top centromere in this figure) will generate a single-X that is circular in form, a ring-X. In this figure we have diagramed the $C(1)TM$ chromosome that our schemes are designed to recover. Exchange at site I or site II will generate chromosomes that respectively either do not, or do, have the P element shown.

Scheme 1:

Scheme 2:

Figure 2.

The crossing schemes we used to recover attached-X bearing females and screen for ring-X bearing sons. Putative ring-X bearing daughters are also phenotypically recognizable, though we chose to recover the chromosome in sons. In scheme 2 we specifically screened only for the sons that received the w^{hs} -marked *P* element.

Figure 3.

Four types of attached-X chromosome that might be produced by single exchange. A, B indicate chromosomes produced by exchange between homologs in regions of *rDNA*; C, D indicate chromosomes produced by exchange between sister chromatids in regions of *rDNA*. Centromeres are indicated as solid circles; heterochromatin as thick solid line; euchromatin as thin solid line; *rDNA* segments as grey shaded blocks; *FRT*s as hatched arrowheads; the w^{hs} transgene as a solid rectangle.

Figure 4.

Verification of ring chromosomes. (A) Mitotic figure of a recovered *C(1)TM* chromosome (arrow). The lower euchromatic arm derives from *In(1)EN*, as can be seen by the pairing of the tips, a result of the heterochromatin found at that location. Note that this chromosome carries substantial *Y* chromatin, which appears as brightly-staining blocks in this DAPI-stained preparation. The portion of the *Y* that is carried by this *C(1)TM* is indicated by the bar next to the normal *Y* in this *C(1)TM/Y* female. (B) Mitotic figure of a ring chromosome derived by meiotic recombination between the two arms of a *C(1)TM* chromosome. This chromosome also carries substantial *Y* chromatin. The normal *X* is marked for comparison. (C) Schematic diagram of sister chromatid exchange in a ring chromosome leading to the production of a double-bridge dicentric chromosome. (D) Mitotic figure of double-bridge dicentric chromosome connecting two telophase nuclei, generated by FLP-mediated sister chromatid exchange (see text for details). (E) The phenotype produced by an *eyFLP* transgene in females that are heterozygous for a ring-*X* carrying *FRTs* and a normal *X*. For this experiment an *eyFLP* transgene with a high level of expression was used. Lower levels of expression produce correspondingly reduced phenotypes.

Table 1

Egg-to-adult viability of *R(1)*-bearing progeny.

Cross	eggs	adults		adult recovery	
		male	female	male	female
$y^w \sigma^R \times y^w \bar{Q}$	309	112	124	0.36	0.40
$R(1)15N \sigma^R \times y^w \bar{Q}$	454	152	68	0.33	0.15

Eggs were collected for 2-3 hours from the indicated crosses, and adults that eclosed were scored through day 18 (at 25°). *R(1)15N* is a ring chromosome recovered in the screens described. Adult recovery is calculated as male or female adult offspring divided by total eggs.