Highly Efficient Sex Chromosome Interchanges Produced By I-*Cre*I Expression in Drosophila

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ABSTRACT

The homing endonuclease I-*Cre*I recognizes a site in the gene encoding the 23S rRNA of *Chlamydomonas reinhardtii*. A very similar sequence is present in the 28S *rRNA* genes that are located on the *X* and *Y* chromosomes of *Drosophila melanogaster*. In this work we show that I-*Cre*I expression in Drosophila is capable of causing induced DNA damage and eliciting cell cycle arrest. Expression also caused recombination between the *X* and *Y* chromosomes in the heterochromatic regions where the *rDNA* is located, presumably as a result of a high frequency of double-strand breaks in these regions. Approximately 20% of the offspring of males expressing I-*Cre*I showed exceptional inheritance of *X*- and *Y*-linked markers, consistent with chromosome exchange at *rDNA* loci. Cytogenetic analysis confirmed the structures of many of these products. Exchange between the *X* and *Y* chromosomes can be induced in males and females to produce derivative-altered *Y* chromosomes, attached-*XY*, and attached-*X* chromosomes. This method has advantages over the traditional use of X rays for generating *X-Y* interchanges because it is very frequent and it generates predictable products.

I-*Cre*I is a site-specific homing endonuclease from *Chlamydomonas reinhardtii* (THOMPSON *et al.* 1992). It recognizes a conserved consensus sequence and generates a double-strand break. The 22-bp recognition site for I-*Cre*I occurs in the 23S *rRNA* gene of the Chlamydomonas chloroplast (DURRENBERGER and ROCHAIX 1993). I-*Cre*I can function in human cells (MONNAT *et al.* 1999) or in *Drosophila melanogaster*, where it is used routinely during *in vivo* gene-targeting techniques (RONG *et al.* 2002) when the I-*Cre*I recognition sequences are introduced into a transgene. As we now show, I-*Cre*I also recognizes endogenous sequences in the Drosophila genome. A sequence similar to the conserved Chlamydomonas recognition sequence can be found, with a 17/22-bp match, in the gene for the Drosophila 28S rRNA. None of the differences between the Chlamydomonas and Drosophila sequences prevent cutting by I-*Cre*I *in vitro* (ARGAST *et al.* 1998).

When we first expressed I-*Cre*I in Drosophila, there were strong indications that endogenous genomic sequences were cut. A 1-hr, 37° heat shock that normally does not affect viability (GOLIC and LINDQUIST 1989; RONG and GOLIC 2000) caused almost complete lethality for flies carrying the heat-inducible *70I-CreI* transgene (RONG *et al.* 2002). Two genetic observations

suggested that this lethality was caused by DNA damage to the sex chromosomes. First, females that were heterozygous *white*[+]/*white* displayed frequent eye-color mosaicism after I-*Cre*I expression. Mitotic exchange between the two *X*'s could produce such mosaicism for the *white*[+] gene (STERN 1936), and since double-strand breaks could cause such recombination (FRIESEN 1936), we predicted that an I-*Cre*I recognition site was on the *X* chromosome. Second, *X*/*Y* males were observed to be more sensitive to I-*Cre*I expression than were *X*/*X* females. If exchange occurred between the sex chromosomes, then exchanges between the *X* and *Y* would be more likely to produce sex chromosome aneuploidy than exchanges between two *X*'s. For these reasons, it seemed likely that exchange was occurring between the two sex chromosomes at a genetic element common to both, such as the *rDNA* loci.

The *ribosomal DNA* genes [*rDNA*; genetically defined as the *bobbed* (*bb*) loci] are genetically redundant, being found in clusters on the *X* and on the *Y* chromosomes (LINDSLEY and ZIMM 1992). The *X*-linked *rDNA* locus is within the pericentric heterochromatin of the left (long) arm, while the *rDNA* locus of the *Y* chromosome is within the short arm. Within the *rDNA*, individual transcription units are found in both orientations (ROBBINS and SWANSON 1988). Each cistron contains the genes for the 2S, 5.8S, 18S, and 28S rRNAs, with ~100–200 cistrons found in each cluster. A copy of either the *X*-linked or the *Y*-linked array is typically sufficient for full function in Drosophila.

To confirm that I-*Cre*I lethality and mosaicism were the result of I-*Cre*I-mediated double-strand breaks in the *rDNA*, we investigated whether chromosome rearrangements between sex chromosomes could be recovered after expression of I-*Cre*I. The results of those experiments are reported here. When we induced I-*Cre*I expression in flies carrying marked sex chromosomes, we found that a large fraction of their offspring had rearranged sex chromosomes. Following I-*Cre*I expression, we recovered products of *X-Y* exchange, *Y-Y* exchange, and *X-C(1;Y)* exchange. These chromosomes had the expected genetic properties and cytological structures. These results show that I-*Cre*I expression is an efficient method for producing a variety of experimentally useful rearranged sex chromosomes in Drosophila.

Generation of DNA damage by I-*Cre*I was further confirmed by a strong reduction in the mitotic index in larval neuroblasts after its expression. This result suggests that I-*Cre*I expression can also be a useful method for inducing controlled DNA damage for investigations of cell cycle checkpoints and DNA repair.

MATERIALS AND METHODS

Drosophila stocks and strains: The I-*Cre*I-expressing lines were of genotype $v, P\{v^+, 70I\text{-}CreI\}$ and $w^{1118}; P\{v^+, 70I\text{-}CreI\}$ $Sb/$ *TM6, Ubx*. The *X* chromosomes were either y^1 or $y^1 w^{1118}$. The *Y* chromosomes were $B^SYy^+, y^+ Y$, and $Y,P\{RSw, w^+\}10A$ (referred to as Yw^+ in this work). We used $y w/B^SY$ and $C(1)DX, y f/Y$ during the course of the crosses and for balancing the exchanged chromosomes in stocks. Since we did not use the *forked* marker, we simply refer to it as $C(1)DX, y$ in the text and tables. The *RSw-10A* chromosome was generated in our lab (Golic *et al.* 1998), and all others were obtained from the Bloomington *Drosophila* Stock Center and are described by Lindsley and Zimm (1992).

Heat-shock regimen: Flies of the appropriate genotype (see RESULTS) were allowed to lay eggs for 3 days, after which the adults were transferred to new food. The 0- to 3-day-old embryos and larvae were heat-shocked at 36° by immersing the vial in a circulating water bath for 1 hr. The cotton plugs were pushed down in the vials to prevent the larvae from escaping the heat shock by crawling up the walls of the vial. Progeny were scored on days 14 and 18 after the vial was set.

Cytological analysis: Larvae of the appropriate sex (male for *X-Y* exchanges and female for *X-X* exchanges) were dissected in 0.7% sodium chloride. Brains were hypotonically shocked for 10 min in 0.5% sodium citrate and then were transferred to fresh methanol:acetic acid:water (in 11:11:2 proportions). After 2 min, the brains were moved to dichlorodimethylsilane-treated coverslips containing a drop of fresh 45% acetic acid. The brains were squashed and the slides frozen on dry ice. We removed the coverslips with a razor blade and immediately immersed the slides in 95% ethanol at −20°. After 15 min, we allowed the slides to air dry at room temperature. To stain the neuroblast chromosomes, the slides were rehydrated in 1× PBS with 1 mg/ml 4′,6-diamidino-2-phenylindole. The neuroblasts were mounted, and we used a Zeiss Axiophot microscope and an Axiocam digital camera to visualize and photograph the mitotic neuroblast chromosomes. Images were taken with Axiocam software and postprocessed for brightness and contrast using Adobe Photoshop 7 on a G4 iMac.

TABLE 1

Mitotic index without and with I-*Cre*I expression

Genotype	Heat shock	Neuroblasts			
		Mitotic	Nonmitotic	Total	%
X/Y	−	60	1282	1342	4.5
X/Y	+	36	788	824	4.4
X,I-CreI/Y	−	67	1118	1185	5.7
X,I-CreI/Y	+	7	1143	1150	0.6

Mitotic index (metaphase plus anaphase) of cells without (−) and with (+) heat-shock-induced expression of I-*Cre*I (*X*, I-*Cre*I/*Y*). *X/Y* lacks the *70I-CreI* transgene and serves as the control.

Statistical analyses: Statistical analyses were performed using Excel: Mac v.X (Microsoft) or Instat 3.0a (GraphPad Software). Table 1 was analyzed using chi square contingency tables. We compared *X*, I-*Cre*I/*Y* without heat shock to the same genotype with heat shock: $\chi^2 = 48.4$, d.f. = 1, $P < 0.001$; the null hypothesis states that the frequency of mitotic (anaphase plus metaphase) figures are the same. Table 2 was analyzed using chi square contingency tables. We compared the number of progeny expected from meiotic nondisjunction between females outcrossed to *y w/y w* (21 + 14 of 5099, or 0.69%) and those outcrossed to *C(1)DX, y/Y* (8 + 4 of 4064, or 0.3%) after heat shock: $\chi^2 = 6.78$, d.f. = 1, $P = 0.009$; null hypothesis states that the frequencies of nondisjunctional progeny are identical.

RESULTS

Mitotic arrest or delay in response to I-*Cre*I expression: As described in the Introduction, we had several reasons to believe that I-*Cre*I could generate double-strand breaks in the *rDNA*. This type of DNA damage typically produces cell cycle arrest until the damage can be repaired. As confirmation that DNA double-strand breaks were produced by I-*Cre*I expression, we assayed the mitotic index of larval neuroblast cells shortly after heat-shock-induced expression of *70I-CreI*. Cell cycle arrest should be apparent as a reduction in the fraction of cells undergoing mitosis. Third instar larvae of genotype *X, 70I-CreI/Y* were heat-shocked at 36°, returned to room temperature for 1 hr, and then larval brains were dissected, fixed, and stained to look for mitotic figures. As a control, we dissected brains from the same genotype prior to heat shock and from larvae without the *70I-CreI* transgene, both before and after heat shock. The results are shown in Table 1 and clearly indicate that I-*Cre*I expression causes a dramatic drop in mitotic index ($P < 0.001$; see *Statistical analyses* in MATERIALS AND METHODS). The most likely explanation for this phenotype is the rapid induction of I-*Cre*I, followed by efficient DNA cleavage and the induction of DNA damage checkpoints and cell cycle arrest prior to mitosis.

Chromosomes used to examine exchange: The *X* and *Y* chromosomes used in this study are shown in Figure 1.
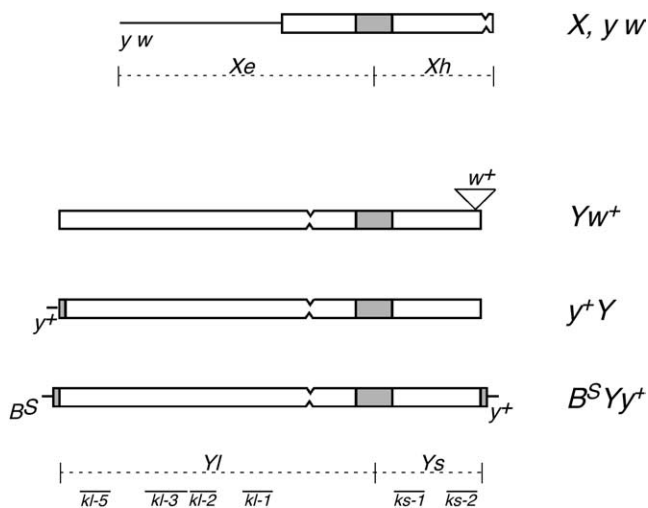
FIGURE 1.—Structures of the chromosomes used in this study. The *X* chromosome has a normal structure and is mutant for *yellow* (*y*) and *white* (*w*). *Yw*⁺ is a normally structured *Y* chromosome that has a *P{white*⁺*}* transposed near the telomere of the short arm ("*w*⁺" triangle). *y*⁺*Y* is a *Y* chromosome of normal structure but has a portion of the *X* chromosome (including *yellow*⁺, *y*⁺) translocated to the telomere of the long arm. *B*ˢ*Yy*⁺ has *X* chromosome material, including *Bar*ˢᵗᵒⁿᵉ (*B*ˢ) and *y*⁺, translocated to the long and short arms, respectively. Centromeres are indicated by constrictions, euchromatin by thin lines, and heterochromatin by rectangles. The blocks of *rDNA* are indicated by the shaded portions of the heterochromatin. *Xe*, *Xh*, *Yl*, and *Ys* are chromosomal segments discussed in the text. The male fertility loci—*kl-1*, *kl-2*, *kl-3*, *kl-5*, *ks-1*, and *ks-2*—are also shown.

Briefly, the *X* chromosome was normal in structure, but carried either recessive *yellow* (*y*) and *white* (*w*) mutations or *y* alone. For the purpose of discussion, we refer to the region between the centromere of the *X* and the *rDNA* locus as *Xh* (*X-heterochromatin*), and the region distal to the *rDNA* locus as *Xe* (*X-euchromatin*). Most genetic information on the *X*, except the centromere and the *rDNA* locus itself, lies within *Xe*.

The *Y* chromosomes were basically normal in structure with the following exceptions. The *Yw*⁺ chromosome carried a *w*⁺-containing *P*-element transposed to a site near the short-arm telomere (Golic *et al.* 1998). The

y⁺*Y* chromosome had the tip of the *X* chromosome (including the *y*⁺ locus) appended to the long arm of the *Y*. The *B*ˢ*Yy*⁺ chromosome contained *X* chromosome-derived material on both the long and the short arm (Williamson 1976; Lindsley and Zimm 1992). The X-ray irradiation that generated this chromosome introduced not only the *B*ˢ and *y*⁺ markers, but also two additional blocks of sex chromosome heterochromatin, presumably containing *rDNA* arrays, on the long arm just proximal to *B*ˢ and on the short arm just proximal to *y*⁺ (Gatti and Pimpinelli 1983). The short-arm fertility factors (*ks-1* and *ks-2*) are located distal to the normal site of *rDNA* on this and on normal *Y* chromosomes. We refer to the region of the *Y* that lies distal to the normal *rDNA* as *Ys* (*Y-short*) and to the remainder of the normal *Y* chromosome as *Yl* (*Y-long*), since it greatly simplifies our discussion. *Yl* includes all the long-arm fertility factors, the centromere, and a small portion of the short arm. This nomenclature differs from the accepted *YS* and *YL* terminology (in uppercase letters) where the boundary of *YS* and *YL* is defined by the centromere, and not by the *rDNA* locus. All three of these *Y* chromosomes are wild type for *rDNA* function and fertility genes (*kl-1*, *kl-2*, *kl-3*, *kl-5*, *ks-1*, *ks-2*) and behave in all respects as normal *Y* chromosomes.

X-Y exchange: We first tested whether I-*Cre*I expression in males could cause exchange between the *rDNA* regions of *X* and *Y* chromosomes. For this experiment we made use of the *B*ˢ*Yy*⁺ chromosome. Normally, when a *y/B*ˢ*Yy*⁺ male is mated to *y/y* females, the daughters will be yellow Bar⁺ and the sons will be yellow⁺ Barˢ. In the control experiment (heat-shocked males with no *70I-CreI* transgene), this was observed. There was only one exceptional offspring, a *y*⁺ *B*ˢ female, among nearly 3000 regular offspring (0.03%; Table 2). This most likely arose by meiotic nondisjunction of the sex chromosomes in either a male or a female parent. Such exceptional offspring typically arise at a rate of ∼0.05–0.1% (Bridges 1916a,b).

Crosses to free-X females: To test the ability of I-*Cre*I to induce breakage and exchange in *rDNA*, we crossed *y w/B*ˢ*Yy*⁺; *Sb 70I-CreI/+* males, in which I-*Cre*I expression

TABLE 2

Normal, exceptional, and nondisjunctional progeny without and with I-*Cre*I expression

Mother's genotype	Heat shock	Female progeny				Male progeny				Total	% exceptions
		y B+	y B	y+ B+	y+ B	y+ B	y+ B+	y B	y B+		
y w	−	1500	0	0	1	1371	0	0	0	2872	0.03
y w	+	2316	97	224	21	1868	441	118	14	5099	17.9
C(1)DX, y	−	24	1	5	1401	0	1	0	1830	3262	1.0
C(1)DX, y	+	8	73	304	1181	4	177	424	1893	4064	24.4

Counts and frequencies of exceptional progeny without (−) and with (+) heat-shock-induced expression of I-*Cre*I in *y w/B*ˢ*Yy*⁺ males. Males were outcrossed to either *y w/y w* or *C(1)DX, y/Y* females.
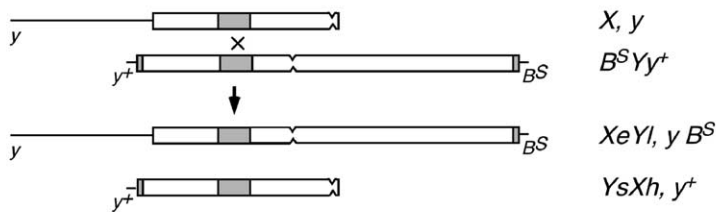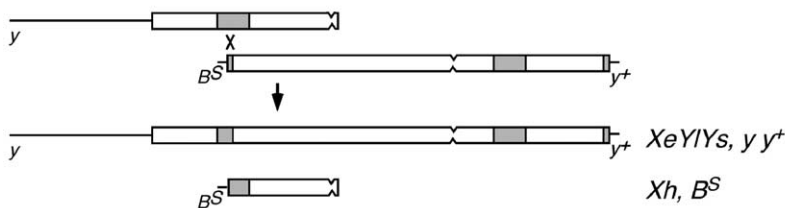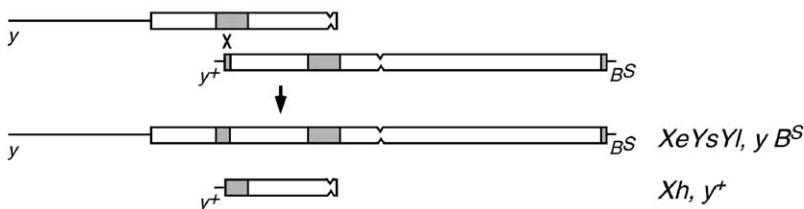
Type 1 exchange:

Type 2 exchange:

Type 3 exchange:

FIGURE 2.—Predicted exchange types. Nine types of simple exchange between regions of *rDNA* are predicted (excluding dyscentric exchanges, *i.e.*, those that generate dicentric chromosomes; LINDSLEY 1955). Types 1–3 involve interchromosomal exchange between *X* and *Y* chromosomes. Types 4–6 involve intrachromosomal exchange between sister chromatids of the marked *Y*. Types 7–9 involve intrachromosomal exchange within one chromatid of the *Y*.

had been induced by heat shock, to *y w/y w* females. The production of progeny with exceptional phenotypes by these males was increased ∼500-fold to 17.9% of all progeny (Table 2). Most of the exceptional progeny inherited only one or the other of the dominant markers from the $B^S Yy^+$ chromosome, and these included daughters that inherited one of these two markers, indicating that some form of chromosomal exchange was responsible for producing most of the exceptional offspring.

A large number of different exchange events are possible with four sites of *rDNA* in the parental males, and at least six different types of exceptional progeny were produced solely on the basis of the inheritance of the B^S and y^+ markers. We considered nine types of exchange that could generate the exceptional progeny: three interchromosomal and six intrachromosomal (types 1–3 and types 4–9, respectively; Figure 2). Excluded from our analyses are multiple exchanges and exchange events without phenotypic consequence, such as exchanges between homologous *rDNA* loci of sister chromatids. Also excluded are exchanges that would generate dicentric or acentric chromosomes, such as exchanges that may occur between *rDNA* subunits lying in opposite orientation. It is likely that such events do

occur, but excluding their consideration at this point simplifies our discussion.

The three interchromosomal exchange types (types 1–3) can produce exceptional Bar^S or yellow$^+$ daughters (*XeYl*, *XeYlYs*, or *XeYsYl*). The Bar^S or yellow$^+$ sons (with *Xh* derivatives) can also be produced by these interchromosomal exchanges or by types 4 and 5 of intrachromosomal exchange. The yellow$^+$ Bar^S daughters and yellow Bar$^+$ sons could result from nondisjunction, which would require an increase of severalfold over the control level. Alternatively, the yellow Bar$^+$ sons might also be produced by intrachromosomal exchanges, such as those designated as types 7 and 8, that could produce ring *Y* chromosomes [$R(Y)$].

Further tests confirmed that many, if not all, of the speculated exchanges did occur. Phenotypic observations and genetic behavior revealed at least two types of yellow$^+$ Bar$^+$ males, some of which must have been produced by intrachromosomal exchange. The first type appeared to carry only a portion of the *Y* chromosome. The duplication of the achaete-scute complex (*AS-C*) included with *y$^+$* on the tip of *Ys* produces a Hairy wing phenotype (LINDSLEY and ZIMM 1992). Nine independent yellow$^+$ male progeny that exhibited this Hairy wing phenotype were selected at random, and when mated, all were
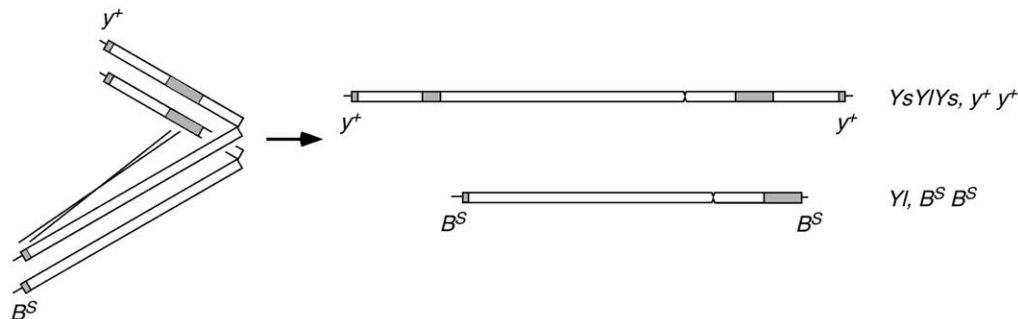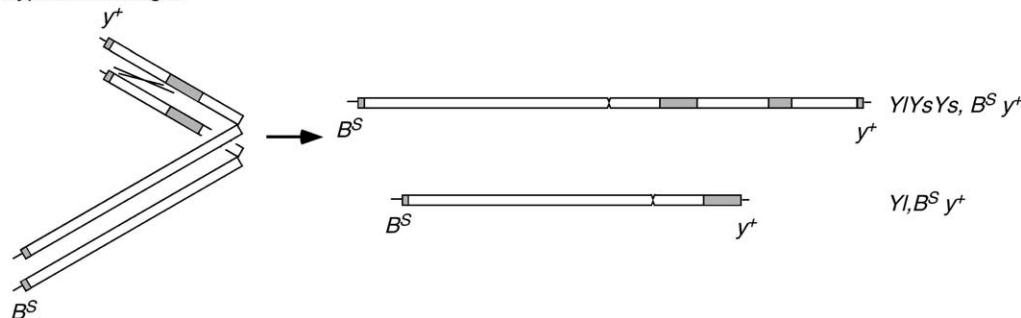
Type 4 Exchange:

$YlYs, y^+ y^+$

$YlYs, B^S B^S$

Type 5 Exchange:

$YsYlYs, y^+ y^+$

$Yl, B^S B^S$
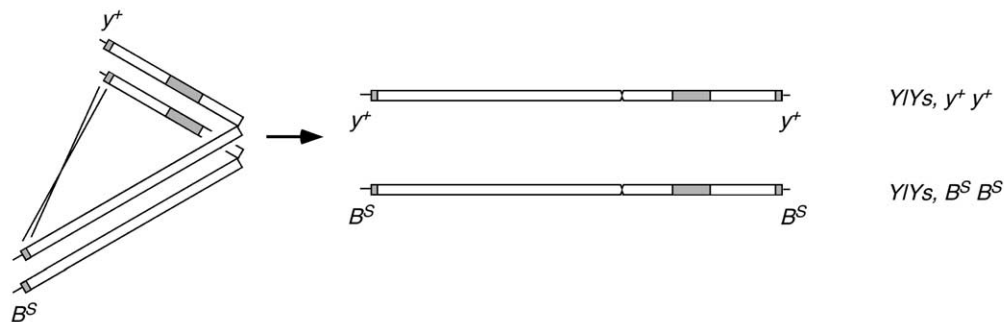
Figure 2.—*Continued.*

Type 6 Exchange:

$YlYsYs, B^S y^+$

$Yl, B^S y^+$

sterile. This was expected for the y^+ derivatives produced by type 1 or type 3 exchange, because such chromosomes lack, at least, the long-arm fertility factors, and males carrying only this *Ys* fragment will be sterile. These sterile males also typically exhibited a variegated yellow/yellow$^+$ phenotype, probably a reflection of position-effect variegation imposed on the y^+ gene because of its proximity to Y heterochromatin. This variegation was not seen with $B^S Yy^+$, but is likely visible in these sterile sons because they lack Yl and, as a consequence, position effects are enhanced because of the reduced heterochromatic content of the genome (Gowen and Gay 1934).

The second type of yellow$^+$ Bar$^+$ son exhibited an extreme Hairy wing phenotype and no y^+ variegation. Three of three randomly selected males were fertile. They probably represent the products of type 4 or type 5 exchanges, with at least one complete complement of fertility factors and two copies of the y^+ duplication, including the *AS*-C.

At least two, possibly three, types of yellow Bar^s sons were recovered. We randomly sampled the progeny of 10 I-*Cre*I-expressing males. Of their pooled yellow Bar^s sons, 20 showed a variegated Bar^s/Bar$^+$ phenotype, 6 showed an extremely strong Bar^s phenotype, and 27 displayed the normal Bar^s phenotype. We crossed 36 of these yellow Bar^s sons, and all were sterile. It is likely that the Bar^s-variegated sterile males carried the smaller product of a type 2 exchange, with Bar^s variegating because a position effect on B^s was revealed owing to a reduction of heterochromatin in the genome of these males. The extreme Bar^s males were sterile and probably carried the $Yl, B^s B^s$ product of a type 5 exchange. Although a chromosome with an extreme Bar^s phenotype could result from type 4 exchange, we expect such events to produce fertile males. It may be that our sampling was insufficient to recover these events: among the yellow Bar^s sons, the extreme Bar^s phenotypes were not frequent and we tested only three such males.
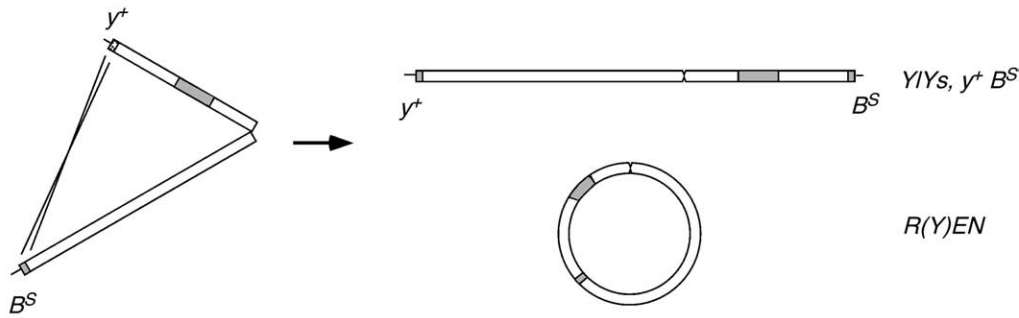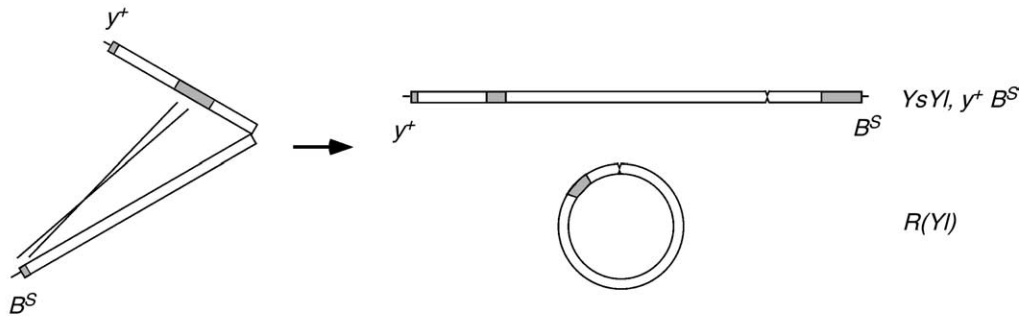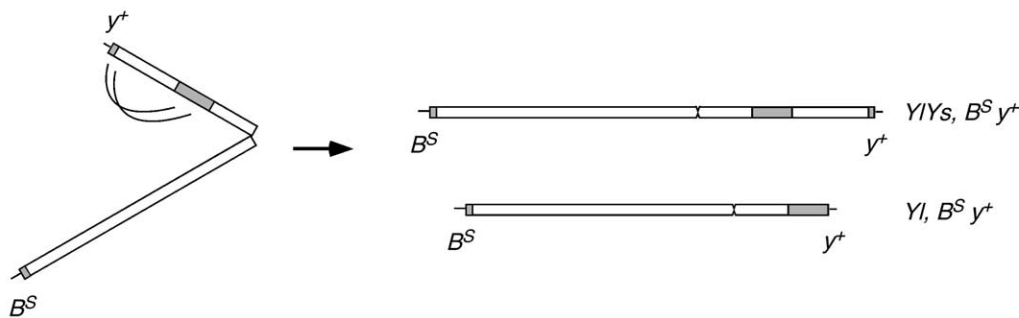
Type 7 Exchange:

Y/Ys, y⁺ B^S

R(Y)EN

Type 8 Exchange:

YsYl, y⁺ B^S

R(Yl)

Figure 2.—*Continued.*

Type 9 Exchange:

Y/Ys, B^S y⁺

Yl, B^S y⁺

All expected phenotypic classes of exceptional females were also recovered. Only one type of yellow⁺ Bar⁺ daughter was expected: that bearing an attached-*XY* produced by a type 2 exchange. Most of the yellow⁺ Bar⁺ daughters had a mild Hairy wing phenotype and did not show variegation of y^+. A few ($7/224 = 3\%$) did exhibit y^+ variegation, although it is not clear that the chromosome that they carry is any different from that of the nonvariegators. In some genetic backgrounds y^+Y chromosomes are known to exhibit y^+ variegation (Hadorn *et al.* 1970), and it is possible that these are simply the visible cases of infrequent variegation. It is also possible that a second round of exchange between the two *rDNA* blocks on the right arm of the attached-*XY* produced by type 2 exchange could delete a portion of the *Y* and alter the context of the appended y^+ gene or the heterochromatic content of the genome, causing variegation of y^+.

The yellow Bar^S daughters were of generally uniform appearance, with a Bar^S phenotype somewhat less severe than that of $X/B^S Yy^+$ males. These were presumably produced by type 1 or type 3 exchange.

Finally, it is worth commenting further on the high frequency of yellow⁺ Bar^S females and yellow Bar⁺ males. These are the expected phenotypes for sex chromosome nondisjunction occurring in either parent. Only two of the yellow Bar⁺ males were mated: one was sterile and one was fertile. Thus, at least some of these exceptional males are fertile and carry a complete *Y* chromosome, likely males carrying a ring-*Y* produced by type 7 exchange. It is also possible that the frequency of $X \leftrightarrow Y$ nondisjunction is elevated in these males. Disjunction of the *X* and *Y* depends on chromosome pairing mediated by elements in the *rDNA* loci (McKee and Karpen 1990). If the *rDNA* complement of the *X* or the *Y* were damaged by I-*Cre*I-mediated double-strand

breaks, it is conceivable that nondisjunction would result because of a decreased pairing fidelity. Thus, we think it likely that males that have expressed I-*Cre*I have increased $X \leftrightarrow Y$ nondisjunction. This issue could be resolved by further testing of the yellow$^+$ Bar^S females, but this has not been done.

Several of the *X-Y* exchange events diagrammed would not produce exceptional progeny and would not have been recognized in this experiment. Type 6 and type 9 exchanges would produce yellow$^+$ Bar^S sons, as would one of the two predicted products from exchanges of types 7 and 8. The *Yl* products from types 6 and 9 exchange might be recovered with appropriate crosses to initially provide the *Ys* fertility factors *in trans*. Recognition and recovery of the other exchange products that differ from the original $B^S Y y^+$ only by the internal arrangement of the arms would be difficult, but would be possible with extensive cytological analysis.

Crosses to attached-X females: We carried out a mating of similarly heat-shocked $y \, w/B^S Y y^+$; *Sb 70I-CreI*/+ males to *C(1)DX, y/Y* females. With this cross, partial *Y* chromosomes should be recoverable in females, and attached-*XY* chromosomes recoverable in males with an extra *Y* to provide any missing fertility factors. In the control cross, using males without *70I-CreI*, 24 yellow Bar$^+$ females were produced. These were probably mostly *XXX* metafemales, as judged by their phenotype of crumpled wings. The five yellow$^+$ Bar$^+$ daughters were almost certainly a result of a single germline mitotic exchange, as they were all offspring of a single male. The two remaining exceptions, a yellow Bar^S female and a yellow$^+$ Bar$^+$ male were also offspring of a single male, apparently representing reciprocal products of spontaneous *X-Y* exchange.

In the crosses with *70I-CreI* expression, we measured 24.4% exceptional progeny. Only a small fraction of these ($12/4064 = 0.3\%$; Table 2) could have resulted from nondisjunction, again showing that breakage-induced exchange is the dominant source of exceptions. As in the previous experiment, all predicted exceptional phenotypes were recovered.

The yellow Bar^S daughters were of at least two types: 10 showed consistent B^S variegation, while 3 showed a very strong Bar^S phenotype, stronger than the typical Bar^S phenotype. These likely represent the *Y*-derivatives produced by type 2 exchange for the *B* variegators or the *Y*-derivatives produced by types 4 and 5 exchange with two copies of B^S for the very strong Bar^S phenotypes. The remaining 60 appeared Bar or weakly variegated for B^S and were not analyzed further. The yellow$^+$ Bar$^+$ daughters were sometimes variegated for y^+, usually showing a very mild Hairy wing phenotype. Since the Hairy wing phenotype produced by the *AS-C*-containing y^+ duplication is reduced in females carrying attached-*X* chromosomes (Lindsley and Zimm 1992), these probably represent flies carrying the *Y*-derivatives with one or two copies of y^+. We did not quantify the frequency of varie-

gating yellow$^+$ or Hairy wing phenotypes in these flies because of the unreliability of these mild phenotypes.

Strikingly, a few percent ($\sim$6%) of both the regular and the exceptional daughters had a bobbed phenotype, as judged by the simultaneous occurrence of both etched tergites and small bristles, likely betraying a deficiency of *rDNA* cistrons. The *C(1)DX* chromosome carries no *rDNA* itself, and these females rely entirely on the *Y* chromosome inherited from their fathers for this function (Ashburner *et al.* 2004). This result indicates that I-*Cre*I expression can result in deletions within the *rDNA* through either intrachromosomal deletion or unequal sister-chromatid exchange. The bobbed females cannot have resulted from a few preexisting *rDNA*-deficient *Y* chromosomes segregating in the stock, because the progeny of a single male were mostly bobbed$^+$, with only occasional bobbed flies.

In these crosses all exceptional types of sons were also observed. There was little or no variation in y^+ or B^S expression in these flies.

We note that the two phenotypes expected as a result of sex chromosome nondisjunction are present at a much lower rate in these crosses than in the crosses to free-*X* females (0.3% *vs.* 0.69%, respectively, $P = 0.009$; see *Statistical analyses* in materials and methods), in spite of a somewhat higher overall recovery of exceptional progeny. This is most likely a consequence of the expected death of three-quarters of the progeny produced by $X \leftrightarrow Y$ nondisjunction in males when mated to *C(1)DX/Y* females, whereas none of the nondisjunction sperm produce lethal zygotic genotypes when crossed to $y \, w/y \, w$ females. In part, this result may also be attributed to the fact that sex chromosome nondisjunction in *C(1)DX/Y* females does not generate progeny of exceptional phenotype, although, on the basis of the control cross to free-*X* females, female nondisjunction accounted for only a small fraction of the yellow$^+$ Bar^S daughters and yellow Bar$^+$ sons in those matings.

Cytology of rearranged chromosomes: To confirm the existence of the expected chromosome rearrangements, we examined the mitotic metaphase cytology of several such rearrangements. We did not attempt to generate stable stocks of all derivatives generated in this study, but chose to retain a subset of derivatives with phenotypes expected for some exchange products. After selecting chromosomes that behaved genetically as expected for products of type 1, 2, or 3 exchanges (Figure 2), we prepared mitotic figures to observe the structure of both *X*- and *Y*-derivatives. In some cases (*e.g.*, Figure 3, C and E) we performed crosses to generate males that carried reciprocal exchange chromosomes, enabling the analysis of both in the same preparation. Normal *X* (arrow) and *Y* (arrowhead) chromosomes are shown in Figure 3A, and the $B^S Y y^+$ chromosome is shown in Figure 3B with small triangles indicating the material appended to the tip of each arm during its construction.
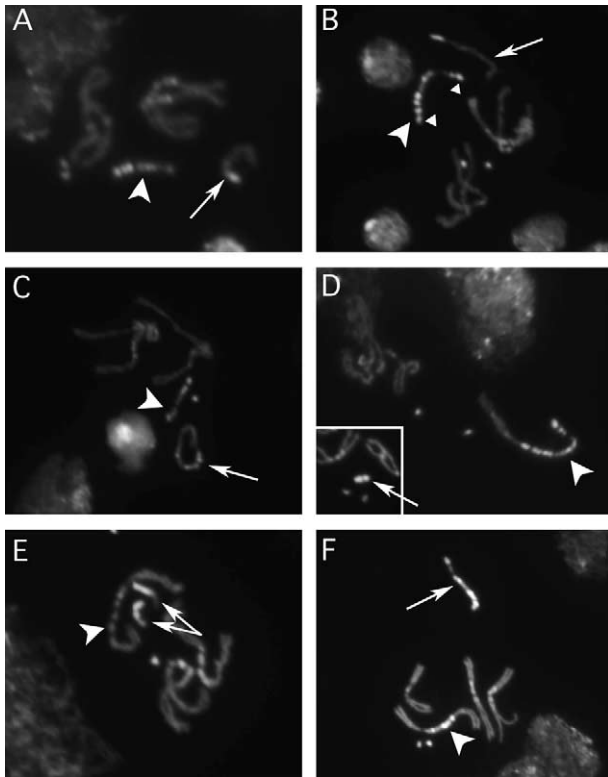
FIGURE 3.—Cytology of recombinant chromosomes. Mitotic chromosomes from larval neuroblasts. (A) Normal-structure *X* (arrow) and *Y* (arrowhead) chromosomes. (B) Karyotype containing a normal *X* chromosome (arrow) and the *B*ˢ*Yy*⁺ (arrowhead) chromosome. Triangles indicate chromatin added to the *B*ˢ*Yy*⁺ chromosome during its construction. (C) An attached-*XY* chromosome product of a type 1 exchange. The arrow marks the *YsXh* chromosome and the arrowhead marks the *XeYl* chromosome. (D) An attached-*XY* chromosome product of a type 2 exchange. An arrowhead marks the *XeYlYs* chromosome; the *Xh* chromosome [prepared from a *C(1)DX/Xh, B*ˢ female] is indicated in the inset by an arrow. (E) A *C(1;Y)* chromosome product of a type 3 exchange. Two *Xh* chromosomes are marked with arrows (one is likely from a neighboring nucleus), and the *C(1;Y)* chromosome is marked with an arrowhead. (F) A *C(1)RM* chromosome generated as diagrammed in Figure 4. The *C(1)RM* chromosome is indicated by an arrowhead, and the normal *Y* is indicated by an arrow. In A–F, the autosomal karyotype is normal.

A type 1 exchange was expected to generate a short *Y* chromosome derivative, consisting of *Ys* marked with *y*⁺ attached to *Xh*. We selected 11 chromosome stocks that could represent either type 1 or type 3 exchanges. Of those, 4 were confirmed to be products of type 1 exchanges cytologically, and the remainder were products of type 3 exchanges. A chromosome with a structure indicative of a type 1 exchange is indicated by an arrowhead in Figure 3C. The complementary exchange product, *Xe* attached to *Yl* and marked by *B*ˢ, is indicated by an arrow. Since males with the karyotype shown in Figure 3C possess an entire *Y* chromosome, albeit split into two different chromosomes, we expected them to be fertile and all were.

Type 2 exchange was expected to generate two chromosome derivatives: one very short one consisting of just *Xh* and the *B*ˢ duplication from the tip of *Yl* and the other consisting of the entire *Y* chromosome plus *Xe*. We cytologically analyzed chromosomes from five chromosome stocks that behaved like products of type 2 exchanges, and all had this structure. The short chromosome, taken from a Barˢ female, is indicated with an arrow in the inset in Figure 3D (the small dots are chromosomes *4*), and the longer *XeYlYs* chromosome, taken from a fertile yellow⁺ male, is indicated by an arrowhead.

The type 3 exchanges were expected to generate chromosomes similar to those of type 2: a small chromosome consisting of *Xh* and the end of *Ys* and another consisting of the *Y* chromosome and *Xe*. In the type 3 exchange, the *Xh* chromosome is marked with the *yellow*⁺ duplication and the *XeYsYl* chromosome with *B*ˢ. Since the *yellow*⁺ end of the *B*ˢ*Yy*⁺ is longer than the *B*ˢ fragment, we expected that the *Xe y*⁺ chromosome would be slightly longer than the *B*ˢ chromosome produced by type 1 exchange (Figure 3E, arrows). Of 11 chromosomes that behaved genetically as type 1 or 3 exchanges, 7 were demonstrated to be products of type 3 exchanges cytologically. The expected structure of these chromosomes (Figure 2) was further confirmed by the fertility of yellow males, indicating that they contain all the normal *Y* sequence on their large *XeYsYl* chromosomes.

Y-Y exchanges: It should be possible to detect I-*Cre*I-induced exchange between two *Y* chromosomes by using males with two *Y* chromosomes that differ by markers on either side of a region of *rDNA* and screening for recombinants. To investigate the possibility of *Y-Y* exchange, we heat-shocked *y w/y*⁺*Y/Yw*⁺; *70I-CreI/*+ males and crossed them to *y w/y w* females. We were interested in exchange events that generated *y*⁺*Yw*⁺ chromosomes (for other work) and so collected yellow⁺ white⁺ sons that represented potential *Y-Y* exchange events. Some of these progeny were expected to carry recombinant *Y* chromosomes with both markers (*y w/y*⁺*Yw*⁺), while others were expected to be males that received two paternal *Y* chromosomes and a maternal *X* chromosome (*y w/y*⁺*Y/Yw*⁺). Progeny with the desired recombinant chromosomes could be distinguished by three criteria. First, since the *white*⁺ gene on the *Yw*⁺ variegates (GOLIC *et al.* 1998; MAGGERT and GOLIC 2000), a *y w/Yw*⁺ male has very little *white*⁺ expression. In contrast, a *y w/y*⁺*Y/Yw*⁺ male shows greater *w*⁺ expression as a result of suppression of position-effect variegation by his extra *Y* chromosome. Second, a *y w/y*⁺*Y/Yw*⁺ male mated to *y w* females produces sons of three types: yellow⁺ white, yellow white⁺, and yellow⁺ white⁺, but a *y w/y*⁺*Yw*⁺ male produces only yellow⁺ white⁺ sons. Third, the *y w/y*⁺*Y/Yw*⁺ male also frequently transmits the marked *Y* chromosomes to daughters, giving female progeny with yellow⁺ or white⁺
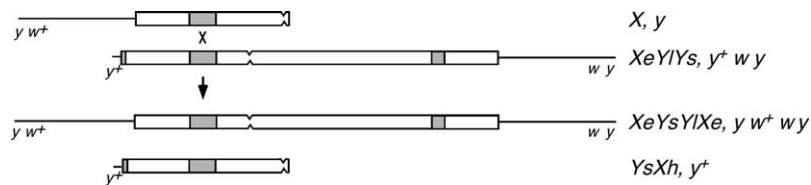
FIGURE 4.—Construction of an attached-*X* chromosome. Exchange between a normal-sequence *X* chromosome and *C(1;Y)* to generate *C(1)RM* is diagrammed.

phenotypes. In contrast, a *y w/y⁺Yw⁺* male produces only yellow white female progeny. Thus, the desired *y w/y⁺Yw⁺* male has relatively low expression of *white⁺*, exhibits linkage between *y⁺* and *w⁺*, and shows sex-limited inheritance of these genes. We crossed 36 yellow⁺ white⁺ males with low levels of *white⁺* expression to *y w/y w* females. Of these, two gave only yellow white daughters and yellow⁺ white⁺ sons. We concluded that these carried the desired recombinant *y⁺Yw⁺* chromosomes. This result demonstrates that I-*Cre*I is also useful for mediating exchange between two *Y* chromosomes.

I-*Cre*I-mediated exchange in females: To test whether I-*Cre*I-mediated exchange can occur in females, we chose to generate a new attached-*X* chromosome by exchange between a normal-sequence *X* chromosome (marked with *y*) and an attached-*XY* or *C(1;Y)* (Figure 3D). On the basis of its genetic and cytological properties, this *C(1;Y)* has the structure expected for the chromosome produced by a type 2 exchange and is marked *y w·y⁺* as diagrammed in Figure 4.

To generate the attached-*X* chromosome, *y w⁺/ C(1;Y), y w·y⁺; I-CreI/+* females were heat-shocked and mated to *y w/BˢY* males. Progeny with a new attached-*X* chromosome were expected to appear as exceptional Barˢ daughters, with the reciprocal product producing exceptional Bar⁺ sons. We scored the offspring of 53 individual females. Among 1570 daughters, we recovered 10 Barˢ daughters. Cytological analysis of one such chromosome revealed the structure of *XeYlXe* (Figure 3F, Figure 4). Another attached-*X* with the structure *XeYlYsXe* was also possible, but we did not specifically screen for this chromosome. We also recovered 6 Bar⁺ sons that were sterile and not investigated further.

These reverse-metacentric attached-*X* chromosomes [*C(1)RM*] were initially *w/w⁺*. Since they were generated, they all have produced only matroclinous daughters and patroclinous sons, confirming that they are attached-*X* chromosomes. However, a fraction of the female progeny were white, indicating that recombination occurred between the two *Xe* arms to generate *w/w* homozygotes, a normal behavior for *C(1)RM* chromosomes with heterozygous arms (ANDERSON 1925).

I-*Cre*I-induced exchange is limited to the sex chromosomes: Although I-*Cre*I consensus sites were not found in the Drosophila genome at any site other than at the *rDNA* loci, we investigated genetically whether chromosome exchange could occur elsewhere in the genome in response to DNA damage induced by expression of I-*Cre*I. Since meiotic recombination does not normally

occur in males in Drosophila (ASHBURNER *et al.* 2004), we monitored whether I-*Cre*I-mediated exchange would occur on autosomes *2* and *3* in males.

The test for recombination on chromosome *2* was carried out by crossing *y w; 70I-CreI/TM3, Sb* virgins to *alˡ sp²* males and heat-shocking the progeny at 36° for 1 hr. *aristaless* (*al*) and *speck* (*sp*) are located at the tips of opposite arms of chromosome *2*, so recombination anywhere along chromosome *2* was expected to produce *al +* and *+ sp* recombinant chromosomes. Daughters were heterozygous for *yellow* and *white* on the *X* chromosome, allowing us to confirm that 70I-*Cre*I was induced by the presence of yellow patches on the abdomen and white sectors in the eyes of females. The *al sp/+ +*; I-*Cre*I/+ heterozygous sons were outcrossed to females that were heterozygous for *Sternopleural/SM1, Cy al sp*. We scored the *SM1*-bearing progeny and observed 1063 nonrecombinant (*al sp* or *+ +*) progeny. We recovered no recombinant progeny, indicating that I-*Cre*I was unable to induce exchange on chromosome *2* between *aristaless* and *speck*.

We independently tested chromosome *3* exchange in a similar fashion, using a chromosome that carried recessive mutations for *roughoid* (*ru*), *hairy* (*h*), *Kinked* (*Ki*), *rotund* (*rn*), *pink* (*p*), *rough* (*ro*), and *claret* (*ca*). Progeny of *ru h Ki rn p ro ca/70I-CreI* heterozygous males crossed to *ruˡ hˡ Kiˡ rnʳᵒᵉ⁻¹ pᵖ roˡ caˡ* females fell into two classes exclusively: 1606 wild-type flies and 959 that exhibited all recessive phenotypes. We conclude that neither chromosome *2* nor chromosome *3* possesses sequences recognized by the I-*Cre*I enzyme.

DISCUSSION

Compound chromosomes, like translocations, are rearrangements involving genetic elements joined to separate centromeres. Compound chromosomes are distinct in that they involve whole arms of homologous chromosomes. The generation of compounds of the *X* and *Y* relies critically upon exchanges that occur in heterochromatin. The reasons for this are straightforward. The only genes that are clearly located in *X* heterochromatin are the multicopy *rDNA*, which are also found on the *Y*. Thus, rearrangement breakpoints in *X* heterochromatin are readily tolerated and move essentially the entire chromosome as one piece. Rearrangements involving the *Y* are, by necessity, a result of breaks in heterochromatin because the *Y* is entirely

heterochromatic. Furthermore, because the *Y* is entirely dispensable except for male fertility, and because *Y* aneuploidy is well tolerated, segments of the *Y* linked to the *X* have often been used to provide regions in which rearrangement breakpoints may occur to generate more complex compound chromosomes (NOVITSKI 1954).

Exchanges to generate rearrangements between the *X* and *Y* have been achieved classically in one of two ways: spontaneously or by induction with ionizing radiation. Spontaneous exchanges between the *X* and the *Y* tend to occur in regions of shared homology, specifically between *X* heterochromatin and the base of the short arm of the *Y*, where the *rDNA* is located on each chromosome (NEUHAUS 1937; LINDSLEY 1955; MADDERN 1981; WILLIAMS *et al.* 1989). Induced exchanges also occur in these regions (LUCCHESI 1965) but can occur in other regions with appreciable frequency (ASHBURNER *et al.* 2004). The frequencies of *X-Y* exchange in males typically range from $\sim$0.01% occurring spontaneously up to $\sim$2% occurring after irradiation.

The work presented here shows that I-*Cre*I may also be used to generate rearrangements between the *X* and *Y* chromosomes, and with nearly 10-fold greater efficiency than has been previously achieved. Rearrangements occurring between *rDNA* regions of the *X* and $B^S Y y^+$ arose in $\sim$20% of the progeny of an I-*Cre*I-expressing male. Special schemes were not required to avoid aneuploidy of *X* chromosome segments because exchange occurred in heterochromatin. Many of the predicted *X* and *Y* exchange events were recovered and verified genetically and/or cytologically. I-*Cre*I is known to generate double-strand breaks in the *23S rRNA* gene of Chlamydomonas, and it is almost certainly the case that the sex chromosome rearrangements that we produced result from double-strand breaks in the closely related *28S* gene of Drosophila. We confirmed that I-*Cre*I causes DNA damage since its expression results in a large reduction in the mitotic index, an expected response to double-strand breaks.

New compound-*XY* and compound-*X* chromosomes were produced in these experiments. For instance, we designed and generated a novel *C(1)RM* chromosome that shows the properties of typical reverse-metacentric compound-*X* chromosomes (NOVITSKI 1954). Attached-*X* chromosomes that exist and are in use typically have been kept in stock for decades. Some experiments may require the generation of attached-*X* chromosomes with heterozygous markers or with specific markers. Attached-*X* chromosomes are especially useful in meiotic studies, because they allow half-tetrad analysis of meiotic exchanges. However, to be useful in studies of exchange, such chromosomes should be heterozygous for markers along their length so that recombination can be assayed. It is difficult to maintain heterozygosity on attached-*X* chromosomes, and this has limited their use for this purpose. This work shows that, with I-*Cre*I, it is possible

to build new heterozygous attached-*X* chromosomes with relative ease as needed. The efficient generation of exchanges with I-*Cre*I makes it quite feasible to routinely produce compound sex chromosomes.

Several types of exchange products were predicted and confirmed cytologically. It is likely that chromosome derivatives other than those described in Figure 2 were also produced. Dicentric chromosomes may be generated by unequal sister-chromatid exchange between *rDNA* repeats in opposite directions or by nonhomologous end-joining. Such dicentric chromosomes break in mitosis and, in the male germline, are subject to *de novo* telomere addition (AHMAD and GOLIC 1998). Thus, some of the exceptional progeny that we recovered may carry the products of dicentric breakage. The high frequency of exchange products suggests that multiple exchange events probably also occurred and were transmitted, but we did not specifically screen for such chromosomes.

Although our *X-Y* exchange experiments used a *Y* chromosome with three regions of *rDNA*, I-*Cre*I exchanges should also occur between normal *X* and *Y* chromosomes. Exchange events could be recovered if, for instance, the *Y* chromosome was appropriately marked, perhaps with *P*-element insertions. In this fashion we produced a doubly marked *Y* by using I-*Cre*I to mediate exchange between two singly marked *Y* chromosomes. Although our sample size was limited, the efficiency of this exchange appears to be on the order of 5%. *Y* chromosomes normally do not undergo meiotic recombination, and I-*Cre*I represents the most efficient way of generating recombinant *Y* chromosomes.

The primary limitation on the variety of *X-Y* rearrangements that could be recovered in our experiments was the location of *rDNA* regions where I-*Cre*I makes breaks. It may be possible to expand the range of chromosome rearrangements that can be recovered through the use of *P*-element insertions carrying the canonical recognition site for I-*Cre*I (RONG *et al.* 2002) or carrying Drosophila *rDNA* (KARPEN *et al.* 1988; MCKEE and KARPEN 1990).

As an alternative to either spontaneous recombination or radiation-induced exchange, the maternal-effect mutation *Rex* (*Ribosomal exchange*) may be used to induce *X-Y* recombination at rates up to $\sim$3% (RASOOLY and ROBBINS 1991). *Rex* also causes chromosomal exchange between regions containing *rDNA* and can produce expansion and contraction of the *rDNA* repeats (ROBBINS 1981). The *rDNA* arrays of Drosophila include repeated retrotransposon elements—the R1 and R2 elements; the origin, function, and regulation of the R2 element in the *rDNA* genes has been reviewed recently (EICKBUSH and EICKBUSH 2003). It is possible that *Rex* represents a mutation that derepresses these elements, allowing for an increased incidence of DNA damage in these loci, as has been proposed (HAWLEY and MARCUS 1989; ROBBINS and PIMPINELLI 1994). We note that I-*Cre*I has

effects that are very similar to those of *Rex*, and it is likely that these effects arise from the same cause—the generation of double-strand breaks within the *rDNA*.

A large number of homing endonucleases are known (Burt and Koufopanou 2004). Among these, there are likely to be others that recognize sequences normally found in the Drosophila genome, and they might be similarly used to generate rearrangements between endogenous sites. Other techniques that might also produce chromosome rearrangements at specific loci are being developed. Double-strand breaks can be generated at many specific sites in the genome with engineered site-specific nucleases. Carroll and co-workers (Bibikova *et al.* 2002) have designed novel zinc-finger nucleases that can target specific sequences, and have used these to generate double-strand breaks within the *yellow* gene. Another approach has been to alter the specificity of existing nucleases that recognize complex sites. Stoddard and co-workers (Chevalier *et al.* 2002) have altered the DNA recognition of a chimeric homing endonuclease (I-*Cre*I and I-*Dmo*I), creating a synthetic enzyme, E-*Dre*I, that has altered DNA specificity.

The use of nucleases designed to recognize existing sites has the potential to generate specific chromosome rearrangements between normal genomic sequences. Another approach has been to use transposons, or sequences carried by transposons, to generate rearrangements between the sites of transposon insertion. In Drosophila, chromosome rearrangements between defined sites in euchromatin have also been generated during the mobilization of transposons (Lim 1979, 1988; Berg *et al.* 1980; Engels and Preston 1984; Gray *et al.* 1996) by FLP-*FRT*-mediated site-specific recombination (Golic and Golic 1996; Ryder *et al.* 2004; Thibault *et al.* 2004), by expression of I-*Sce*I to make double-strand breaks simultaneously at two different sites (Egli *et al.* 2004; Y. S. Rong, personal communication), and by Cre-*lox* site-specific recombination (Egli *et al.* 2004). However, these methods all rely on the insertion of foreign sequences and, as yet, do not fulfill a need for the efficient generation of rearrangements in heterochromatin. It may be possible to extend the use of site-specific recombinases or I-*Sce*I to produce rearrangements between heterochromatic sites by generating collections of elements that carry those target sites at heterochromatic locations (Konev *et al.* 2003). Until that time, I-*Cre*I expression serves for the efficient production of compound sex chromosomes.

Beyond the demonstrated utility of this technique in generating chromosome translocations, I-*Cre*I may also be used as a tool for studying the responses to DNA damage (Royou *et al.* 2005). Because the induction of I-*Cre*I by heat shock causes a large number of breaks at a defined location, it causes rapid and efficient cell cycle arrest. I-*Cre*I expression provides an alternative to the standard use of X rays for the induction of damage, and in some respects is preferable, because the nature of the cellular damage is more precisely defined than when ionizing radiation is used.

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