Engineering the Drosophila Genome: Chromosome Rearrangements by Design

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ABSTRACT

We show that site-specific recombination can be used to engineer chromosome rearrangements in *Drosophila melanogaster*. The FLP site-specific recombinase acts on chromosomal target sites located within specially constructed *P* elements to provide an easy screen for the recovery of rearrangements with breakpoints that can be chosen in advance. Paracentric and pericentric inversions are easily recovered when two elements lie in the same chromosome in opposite orientation. These inversions are readily reversible. Duplications and deficiencies can be recovered by recombination between two elements that lie in the same orientation on the same chromosome or on homologues. We observe that the frequency of recombination between *FRT*s at ectopic locations decreases as the distance that separates those *FRT*s increases. We also describe methods to determine the absolute orientation of these *P* elements within the chromosome. The ability to produce chromosome rearrangements precisely between preselected sites provides a powerful new tool for investigations into the relationships between chromosome arrangement, structure, and function.

THE accumulated collection of numerous characterized chromosome rearrangements has contributed significantly to the success of *Drosophila melanogaster* as an experimental organism. Chromosomal inversions are useful as balancers that prevent the recovery of recombinants (STURTEVANT 1926). Regional deficiencies and duplications can be used to investigate dosage effects, to localize genes, and to recover mutations in a defined region (BRIDGES 1917, 1919; MULLER 1935; DEMEREC and HOOVER 1936; JUDD *et al.* 1972). Translocations have been used to investigate the mechanisms of meiotic chromosome segregation, as intermediates in the production of other rearrangements, and in the systematic analysis of aneuploidy in Drosophila (HAWLEY 1980; LYTTLE 1984; LINDSLEY *et al.* 1972). Compound chromosome rearrangements have been used to characterize fundamental aspects of meiosis and recombination (ANDERSON 1925; STURTEVANT and BEADLE 1936; WELSHONS 1955).

Chromosome rearrangements can be produced in Drosophila with the use of ionizing radiation (MULLER 1927; MULLER and PAINTER 1929; PAINTER and MULLER 1929). These rearrangements always involve at least two breakpoints (MULLER and HERSKOWITZ 1954). Since the radiation-induced breakpoints occur more or less randomly throughout the genome, a rearrangement produced by radiation differs from any other, even a very similar rearrangement, at a minimum of four chromosome sites. In many cases it may be difficult to separate phenotypic effects intrinsic to the rearrangement from effects that owe to mutation of genes at the breakpoints or from secondary mutations linked to the rearrangement.

To circumvent these problems we have pursued the use of site-specific recombination as an avenue to the production of chromosome rearrangements. The FLP site-specific recombinase of the yeast 2μ plasmid mediates highly efficient recombination between its target sites that have been integrated in the Drosophila genome. In our experiments FLP is supplied by an *hsp70-FLP* fusion gene (*70FLP*) that can be induced in germline and somatic cells by heat shock. FLP recombination targets (*FRT*s) are short asymmetric sequences that FLP recognizes and recombines directionally (COX 1988). *FRT*s that lie a few kilobases apart on the same chromosome recombine with an efficiency of nearly 100% after the induction of FLP synthesis (GOLIC and LINDQUIST 1989). *FRT*s at allelic sites on homologous chromosomes are also efficiently recombined (GOLIC 1991). *FRT*s separated by several hundred kilobases recombine at a frequency of a few percent (GOLIC 1994). The experiments described here show that *FRT*s separated by several megabases can recombine to produce large scale chromosome rearrangements. Rearrangements that are produced by this method can be easily and precisely reverted by further rounds of FLP synthesis.

MATERIALS AND METHODS

All flies used in this work carry the w^{1118} null mutation on their *X* chromosomes. Other mutations are described by LINDSLEY and ZIMM (1992).

***P* elements:** The *70FLP* gene was constructed by R. PETERSEN (Case Western Reserve University). Its construction will be described elsewhere. It is a heat-inducible *FLP* gene similar to the *hsFLP* construct previously described (GOLIC and LIND-

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QUIST 1989) but shows higher levels of expression after heat shock. It was transformed in a *P* element marked with $rosy^+$ ($P[ry^+, 70FLP]$). In these experiments we used an insertion on the *X* chromosome denoted *70FLP*3F.

To construct the *RS5* and *RS3 P* elements the oligonucleotides 5′-GAGAAAGGATCCAAGCATGCTGCGACGTGAACAGTGAGCTGTA-3′ (which we call BSF1) and 5′-GTTAGAGGATCCCCGCATGCAGCTCGTTACAGTCCGGTGCGTTTTTGGT-3′ (BSF2) were used to add *Bam*HI termini to the *FRT* by PCR amplification of an approximately 200-bp fragment using the plasmid $pP[>w^{hs}>]$ (GOLIC and LINDQUIST 1989) as a template. (These primers also add *Sph*I sites but these were not used in the cloning.) The amplified *FRT* was digested with *Bam*HI and ligated into the *Bcl*I site that lies in the first intron of the w^{hs} gene in either pw6 or pw8 (KLEMENZ *et al.* 1987). Clones in which the *FRT* was oriented in a 3′ to 5′ direction (as diagramed in Figure 1) were selected. The convention used for *FRT* direction is that the *Xba*I site, which lies at one side of the eight base pair spacer of the *FRT* and which determines the directionality of recombination, lies closer to the head of the arrow used to indicate an *FRT*. The resulting plasmids were then cut at the *Bam*HI site in their polylinkers and the same *FRT*-bearing fragment was ligated into these sites. Clones were chosen in which both *FRT*s lay in the same orientation to produce the $pP[RS5]$ and $pP[RS3]$ constructs. These constructs were placed into the chromosomes of flies that carried the w^{1118} null mutation by standard *P*-element transformation techniques (RUBIN and SPRADLING 1982). All *RS*-element insertions were mapped by *in situ* hybridization to polytene chromosomes (PARDUE 1986).

In the experiments to generate inversions on chromosome *2*, the insertion-bearing chromosomes were kept heterozygous with the S^2CyO balancer because the *RS3*-4A insertion is associated with a recessive male-sterile mutation. The insertion-bearing chromosomes *3* were maintained as homozygotes.

PCR confirmation of rearrangements: Genomic DNA was prepared from adult flies carrying each inversion as previously described (GOLIC and LINDQUIST 1989). The oligonucleotides 5′-GTCCGCCTTCAGTTGCACTT-3′ (w7500D) and 5′-TCATCGCAGATCAGAAGCGG-3′ (w11678U) were used to prime the PCR using the genomic DNAs as templates. These oligonucleotides correspond to *white* gene sequences present in *RS5r* and *RS3r*, respectively. They amplify a 1.64-kb fragment in flies where *RS5r* and *RS3r* insertions have recombined at the *FRT*s to reconstitute w^{hs}. The sequence corresponding to the w7500D primer is not present in the w^{1118} deletion allele that is carried by all flies used in this work.

Mapping *FRT* deletions: The altered *P* elements recovered by the screen of Figure 8 were subjected to six PCR procedures to characterize the nature of the alterations. As diagramed in Figure 8A, the reactions used the following primer pairs: 1 used w7500D + w11678U; 2 used w7500D + BSF2; 3 used BSF1 + w11678U; 4 used w12254D (5′-ACAACGGTGAGTGGTTCCAG-3′) + PE5′ (5′-GATAGCCGAAGCTTACCGAAGT-3′); 5 used w12254D + BSF2; 6 used BSF1 + PE5′.

Cytology: Chromosome rearrangements were confirmed by cytological examination of polytene chromosomes from animals heterozygous for the rearrangement and a chromosome of normal sequence as described by LEFEVRE (1976). Images were captured as previously described (GOLIC 1994).

Heat shocks: Heat shocks were performed as previously described (GOLIC and LINDQUIST 1989) in a circulating water bath.

RESULTS

Overview of the method: To recognize the products of recombination between widely separated *FRT*s, we constructed two *P*-element vectors that provide a screen for their recovery. Each carries a functional *white* (w^{hs}) gene (which is required to give the fly pigmented eyes) with an *FRT* placed in the first intron. A second *FRT* was placed downstream of the gene to make $P[RS5]$, or upstream of the gene to make $P[RS3]$ (Figure 1). We refer to these simply as *RS5* and *RS3*, or, more generally, as *RS* (for rearrangement screen) elements. Flies that carried the *RS* insertions were crossed to flies carrying *70FLP* and FLP synthesis was induced with heat shock to catalyze the excision and loss of the *FRT*-flanked portion of each construct. The chromosomal remnants of this excision (designated *RS5r* and *RS3r*) carry reciprocal portions of the *white* gene which, by themselves, are nonfunctional (Figure 1, A and B). FLP-mediated recombination between the remnant-borne *FRT*s will produce a chromosome rearrangement that is marked by a reconstituted *white* gene (Figure 1C). When this occurs in the soma, it can be recognized by the generation of pigmented spots on a white background in the eye; offspring that carry a rearrangement (resulting from germline recombination in their parents) can be distinguished from their siblings that do not carry a rearrangement by their pigmented eyes. Thus, infrequent events can be easily detected.

FLP-mediated exchange between *FRT*s located on the same chromatid will produce a limited number of products that can be predicted based on the orientation and location of the *FRT*s. Recombination between inverted *FRT*s will invert the portion of the chromosome flanked by *FRT*s (Figure 2A). On average, 50% of *RS5r-RS3r* pairs should lie in the correct relative orientation to produce inversions. Recombination between *FRT*s that lie in the same orientation will delete the material between *FRT*s if the *FRT*s are located on the same chromosome arm or will produce circular chromosomes that are deficient for the material outside the *FRT*s if the *FRT*s are on opposite arms (Figure 2B). Other products of FLP-mediated recombination, for instance, dicentric chromosomes or chromosomes carrying large complementary duplications and deficiencies, may result from exchange between *FRT*s on sister chromatids or homologues. We expected to recover mainly inversions in the first set of crosses described below because all the *RS5r-RS3r* combinations that we have used would produce large deficiencies if they were oriented in the same direction in the chromosome. Except in one case (discussed in a subsequent section) the animals carrying such deficiencies are not expected to live (LINDSLEY *et al.* 1972).

The production of inversions: Chromosomes that carry *RS5r* and *RS3r* insertions were produced in one of two ways. In some cases the inheritance of arbitrary marker mutations was followed in order to recombine the *RSr* insertions onto the same chromosome. The second method made use of the fact that the *white* gene carried by these elements often produces flies with or-

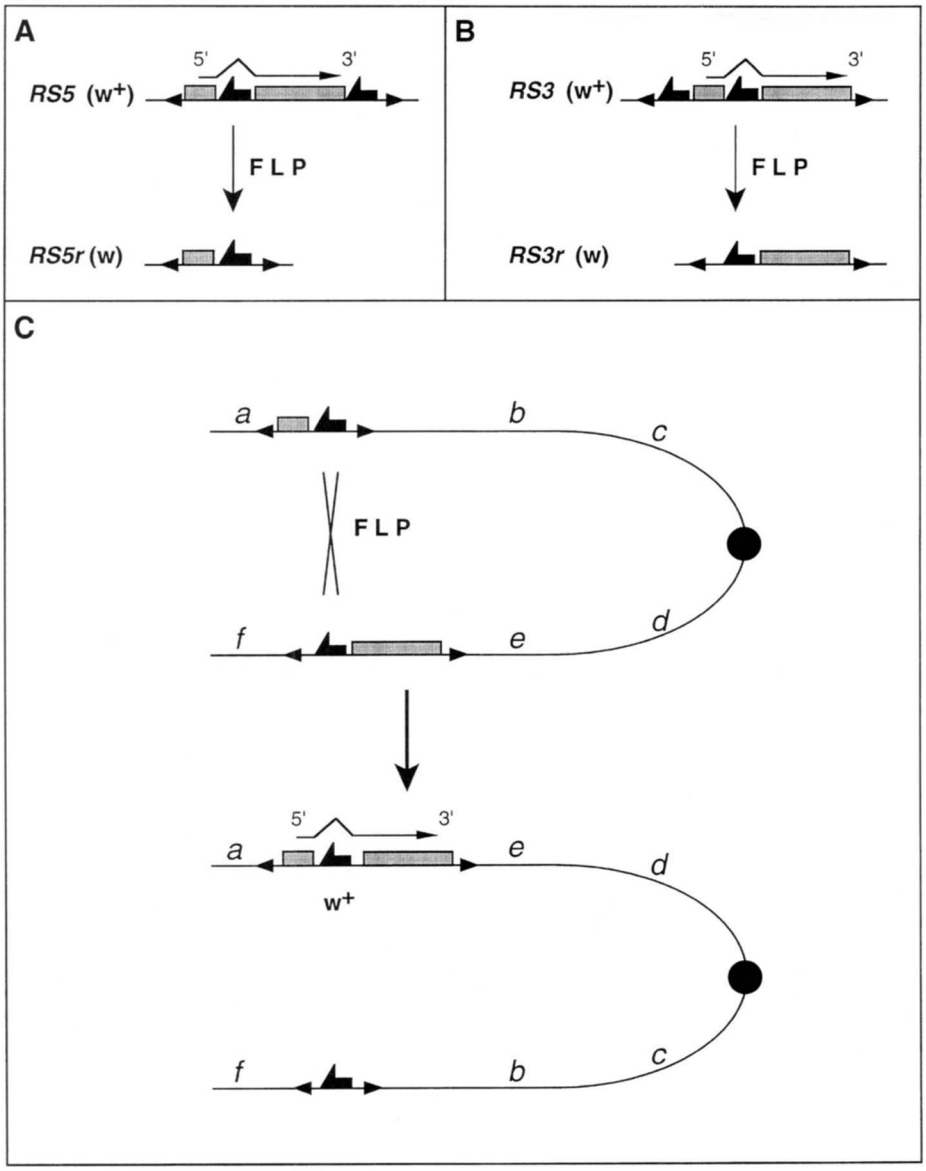

FIGURE 1.—The use of *RS5* and *RS3* elements for recovering rearrangements. (A and B) The chromosomal forms of the original elements and the chromosomal remnant following FLP-mediated excision. The shaded boxes indicate the two portions of the *white* gene (only the first intron is indicated). The messenger RNA is indicated above the gene. Filled half-arrows indicate *FRT*s and their orientation. Small arrowheads indicate the termini of *P* elements. The phenotype that each element confers is indicated in parentheses. Part C indicates how these two elements are used to generate a rearrangement (an inversion in this case) that is marked with w^+. The filled circle denotes the centromere and letters denote arbitrary markers.

ange eyes. In such cases flies with two copies of this gene can be recognized by virtue of their red eyes. Thus, recombinant chromosomes that carried both *RS* insertions were recovered by collecting red-eyed offspring from mothers that were heterozygous for chromosomes carrying one of each insertion. FLP was then used to excise the *FRT*-flanked portion of both constructs in the germline, producing offspring with white eyes. These flies had a chromosome with two *RSr* insertions. Balancers were used to make stocks of flies that carried *70FLP*3F and the *RS5r RS3r* chromosomes.

We produced chromosomes *2* that carried the *RS3* remnant *RS3r*-4A and one of seven different *RS5* remnant insertions (Table 1). Fly stocks were produced that carried one of these seven *RS5r-RS3r* combinations and the *70FLP* gene on their *X* chromosomes. Flies from each line were heat-shocked for 1 hr at 38° during the first 4 days of development. In many cases we observed spots of pigment in the eyes of a fraction of the adults that eclosed (see Figure 7 for an example). The heat-shocked flies were mated *inter se* as three to five pairs per vial, and their progenies were then screened for flies with pigmented eyes. We recovered rearrangements by this method from four of the combinations: three pericentric inversions and one paracentric inversion (Table 1). We also tested the four possible combinations of two *RS5r* and two *RS3r* insertions on chromosome *3* and recovered two inversions, one paracentric and one pericentric, with the *RS3r*-2 site in common (Table 1). The two paracentric inversions [*In(2R)RS3r*-4A;*RS5r*-31B and *In(3L)RS5r*-1A;*RS3r*-2] were recovered with approximately the same frequency: independently-arising inversions were recovered at a rate of one in eight or nine vials on average. The pericentric inversions [*In(2LR)RS5r*-3A;*RS3r*-4A, *In(2LR)RS5r*-8;*RS3r*-4A, *In(2LR)RS5r*-3B;*RS3r*-4A, *In(3LR)RS3r*-2;*RS5r*-2A] were somewhat less frequent, arising once in 25–35 vials (Table 1). In total, we recovered six inversions

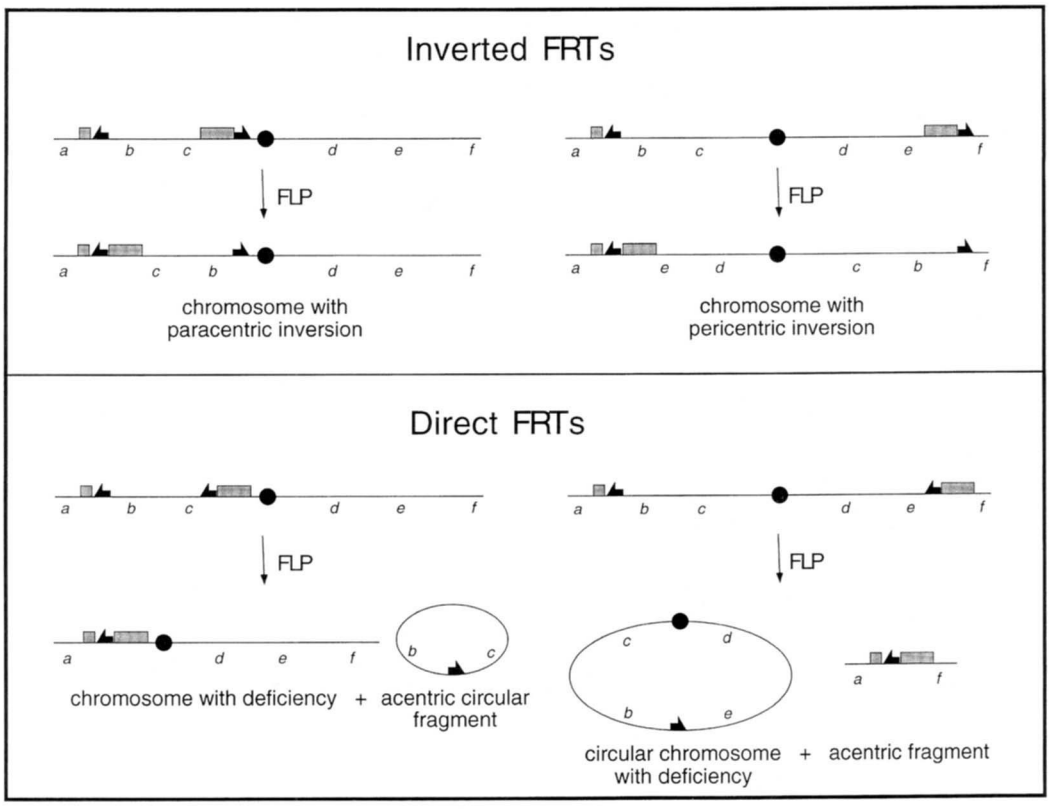

FIGURE 2.—Results of exchange between *RSr* elements. The products of intrachromatid exchange differ depending on the relative orientation of *FRT*s and their disposition about the centromere (indicated by filled circle). If the insertions were oriented in the direction opposite from that shown here, then the other breakpoint would be marked by w^+. In the case of inverted *FRT*s, this would mean that the right breakpoint of the inversion (as drawn) would be marked by w^+, or in the case of direct repeats, the circular product would be marked by w^+.

(designated by the symbol *In*) from 11 *RS5r-RS3r* combinations on chromosomes *2* and *3*. The inversions ranged in size from approximately three to 22 numbered chromosome divisions, with the largest encompassing approximately three-quarters of the mitotic chromosome length.

The cytology of the chromosome *2* inversion series confirmed that all four have one breakpoint at the site of the *RS3*-4A insertion, with the second breakpoint at the location of the particular *RS5* insertion used for that inversion (Figure 3). Cytological examination also confirmed that the breakpoints of the chromosome *3* inversions fell at the sites of the *RS* element insertions (not shown). The conjunction of *RS5r* and *RS3r* elements in each inversion was verified by PCR amplification across the *FRT* (Figure 4).

Each inversion conditions a characteristic level of pigmentation and when multiple isolates of a given inver-

TABLE 1

Recovery of inversions

Combinations[a]		Vials scored	w^+ progeny[b]	Frequency (%)[c]
Chromosome 2				
RS3r-4A(51F)	*RS5r*-3A(30A-B)	145	4 (4)	~0.03
RS3r-4A(51F)	*RS5r*-8(34A1-2)	36	1	~0.03
RS3r-4A(51F)	*RS5r*-15B(34C)	50	0	
RS3r-4A(51F)	*RS5r*-10(35B)	99	0	
RS3r-4A(51F)	*RS5r*-3B(38B)	35	1	~0.03
RS3r-4A(51F)	*RS5r*-7A(54A)	273	0	
RS3r-4A(51F)	*RS5r*-31B(55B)	53	14 (6)	~0.32
Chromosome 3				
RS3r-2(75C-D)	*RS5r*-1A(65B)	309	62 (39)	~0.13
RS3r-2(75C-D)	*RS5r*-2A(88B)	148	7 (6)	~0.03
RS3r-3(82C)	*RS5r*-1A(65B)	628	0	
RS3r-3(82C)	*RS5r*-2A(88B)	224	0	

[a] Insertions are designated by arbitrary isolate numbers. Cytological locations are given in parentheses.
[b] The numbers in parentheses are the number of vials in which w^+ progeny arose.
[c] Based on an estimated 150 progeny per vial. The chromosome *2* frequencies were multiplied by 1.8× to correct for the fact that in these lines the homozygous males are sterile.

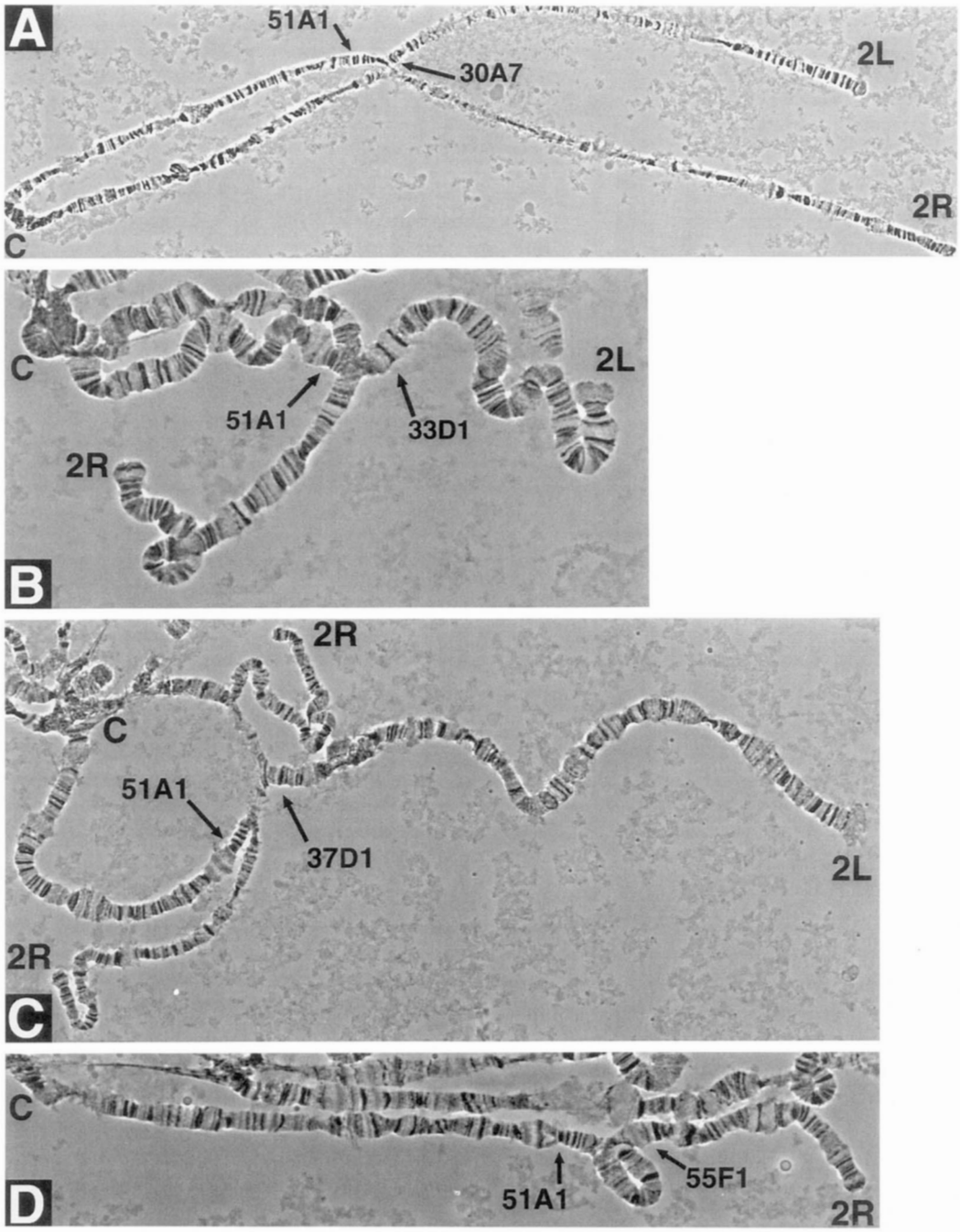

FIGURE 3.—Cytology of the chromosome *2* inversion series. Polytene chromosomes from *In*/+ heterozygotes were examined. An example of each heterozygous inversion is shown. (A) *In*(*2LR*)*RS5r*-3A;*RS3r*-4A; (B) *In*(*2LR*)*RS5r*-8;*RS3r*4A; (C) *In*(*2LR*)*RS5r*-3B;*RS3r*-4A; (D) *In*(*2R*)*RS3r*-4A;*RS5r*-31B. The landmark band 51A1, justproximal to the *RS3*-4A insertion, is indicated in each photograph. In each case a landmark band that lies distal to the *RS5* element is also indicated. C, chromocenter. The tips of left and right arms are indicated as 2L and 2R.

sion were recovered, they exhibited the same degree of pigment. There was a single exception. Among the flies of Table 1, we found one fly in the *RS5r*-1A *RS3r*-2 vials that was notable because it had very light pink eyes, rather than the red eye color that characterized the 61 other w^+ flies that we recovered from this combination. In this fly the w^+ function mapped to chromosome *3*, but PCR revealed that the pigment did not result from the expected *RS5r-RS3r* junction (Figure 4, lane 12). Other experiments have shown that the portion of *white* carried by the *RS3* remnant encodes sufficient protein to make a functional white product if properly expressed (B. STRONACH and K. GOLIC, unpublished results). Normally, *RS3r* is not expressed. We imagine that this exceptional fly carries some form of mutation that causes the *RS3r* portion of the *white* gene to be expressed and to produce this low level of pigment. These exceptional flies are rare and the PCR test quickly distinguishes them from the rearrangements that result from the expected *RS5r-RS3r* recombination.

Reversion of inversions: The inversions produced by FLP, although quite stable in the absence of FLP, can be reverted with further rounds of *70FLP* induction. When FLP was induced in *In*/+ larvae and the adults that eclosed were examined, the somatic reversion of these rearrangements was easily observed as white sectors in otherwise pigmented eyes. We examined all six inversions for somatic reversion, in three cases looking

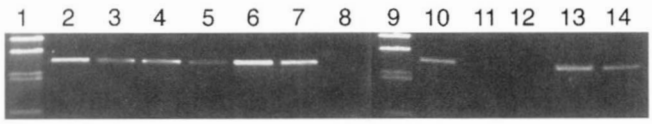

FIGURE 4.—Molecular confirmation of site-specific inversions. PCR was used to amplify across one of the inversion breakpoints. Primer sequences are given in MATERIALS AND METHODS. Lanes 1 and 9 are marker DNAs with sizes (in kilobase pairs) of 2.3, 1.9, 1.4, 1.3, and 0.7 reading from top to bottom. Template DNAs were prepared from these flies: lane 2, w^{1118}; *In(2LR)RS5r-3A;RS3r-4A*; lane 3, w^{1118}; *In(2LR)RS5r-8;RS3r-4A*; lane 4, w^{1118}; *In(2LR)RS5r-3B;RS3r-4A*; lane 5, w^{1118}; *In(2R)RS3r-4A;RS5r-31B*; lane 6, w^{1118}; *In(3L)RS5r-1A;RS3r-2*; lane 7, w^{1118}; *In(3LR)RS3r-2;RS5r-2A*; lane 8, w^{1118}; In^+ *RS3r-2 RS5r-2A*; lane 10 w^{1118}; *In(3LR)RS3r-2;RS5r-2A*; lane 11, w^{1118}; In^+ *RS3r-2 RS5r-2A*; lane 12, progeny of the light pink-eyed fly from the *RS5r-1A RS3r-2* experiments (see text for details); lane 13, w^{1118}; *RS3-2*; lane 14, w^{1118}; *RS5-1A*.

at several independent isolates of the same inversion. All exhibited somatic reversion following the induction of FLP, indicating that the *FRT*s remained intact. In all cases reversion was judged to be more frequent than inversion formation had been. This suggests that in mitotically dividing cells, the chromosomes of an In/In^+ animal may sometimes pair with the inversion loop configuration that is seen in polytene chromosomes. This pairing would have the effect of drawing the *FRT*s closer together, thus facilitating reversion.

We measured the frequencies of inversion formation and reversion in the germlines of flies that carried a chromosome *3* without *RSr* insertions and either the In^+ or the *In* form of the *RS5r-1A RS3r-2* chromosome *3* and confirmed that reversion was more frequent than the formation of inversions. Sixteen of 78 heat-shocked *In*-bearing males transmitted reverted chromosomes; only two of 75 In^+-bearing males transmitted chromosomes with the inversion (Table 2, A and B). The frequency of inversion formation was not measured in females, but reversion was frequent in the germlines of females (Table 2C). In the results of Table 2 there is a slight suggestion that the multiply-inverted balancer homologue *TM6* suppresses reversion. We are exploring the possibility that *TM6* may inhibit formation of the inversion loop configuration. *TM6* does not suppress the expression of the *70FLP* gene.[1]

To confirm that the white-eyed $TM6^+$ or Sb^+ flies were indeed revertants, we measured the frequency of meiotic recombination in the region of the left breakpoint. From the crosses of Table 2 we collected females that carried the reverted chromosome, which is marked with roughoid (*ru*) and *hairy* (*h*), heterozygous with a normal ru^+ h^+ chromosome and test-crossed them to w^{1118}; *ru h* males. The marker *ru* is located to the left of the inverted region; *h* lies inside the inverted region. Thirteen In/In^+ females produced only one recombinant in 1440 offspring (0.07%), while 17 In^+/In^+ control females (*ru RS5r-1A h RS3r-2*/ru^+ h^+) gave an average recombination frequency of 27.2%, SE = 1.0. Six revertant In^+/In^+ females gave an average recombination frequency of 22.2%, SE = 1.6. These data confirm that the flies identified as revertants by their white eyes have lost the inversion.

The production of duplications and deficiencies: The combination *RS3r-4A RS5r-7A* on chromosome *2* undergoes a high frequency of recombination following *70FLP* induction. This recombination can be visualized by the high rate of production of pigmented spots in the eyes (see Figure 7A). However, in spite of screening close to 300 vials no $white^+$ progeny were recovered from this combination. It is likely that cells carrying the w^{hs}-marked product of recombination between these two insertions are viable as somatic clones, but not as a whole animal. This implies that the recombinant product is a deletion rather than an inversion. The $white^+$ clones that are generated by recombination between *RS3r-4A* and *RS5r-7A* can grow to be quite large, encompassing hundreds of cells, if produced early in development. This is true even in genotypes where only recombination in *cis* is possible, excluding the possibility that the clones survive only when the complementary duplication and deficiency are produced by recombination between homologues and that these then segregate together. Because the reconstituted *whs* gene is stably transmitted through several rounds of mitoses, it must be the centric product of recombination that carries w^{hs}. Because the locations of these two elements are known from *in situ* hybridization, their orientation can be deduced (as diagramed in Figure 5).

In the following paragraphs of this section we will refer exclusively to duplication (*Dp*) or deficiency (*Df*) of the segment between *RS3r-4A* and *RS5r-7A*; these are properly specified as *Dp(2;2)51F;54A* and *Df(2R)51F;54A*. To simplify the discussion, we do not specify this in every case, and in some instances use the abbreviations *Dp* and *Df* when referring to the chromosomes that carry these rearrangements.

It should be possible to recover the *RS3r-4A RS5r-7A* deficiency chromosome if the corresponding duplication could be generated. The deficiency could then be recovered over the duplication in what would be a euploid animal. Duplications of a region flanked by *FRT*s can be produced by unequal sister-chromatid exchange (GOLIC and LINDQUIST 1989; GOLIC 1994), but

[1] The efficiency of *70FLP* induction was measured by scoring the frequency of recombination between the directly repeated *FRT*s of an *RS5* construct. w^{1118}; *RS5-1A* males were crossed to w^{1118} *70FLP; cu* kar^2 *Sb/TM6*. The progeny of this cross were heat-shocked for 1 hr at 34° during the first 1 to 2 days of development. Adult males of the genotypes w^{1118} *70FLP; RS5-1A/TM6* and w^{1118} *70FLP; RS5-1A/Sb* were mated individually to two w^{1118} female virgins, and the $TM6^+$ or Sb^+ progeny were scored for eye color. Twenty-seven *Sb* males exhibited an average recombination frequency of 0.83, SE = 0.04 (1339 Sb^+ progeny); 24 *TM6* males showed an average recombination frequency of 0.97, SE = 0.016 (1037 $TM6^+$ progeny). Although the difference between these results is statistically significant, the difference is in the opposite direction from what would be expected if *TM6* suppressed *70FLP*. Whether this difference is of biological significance is a matter for further investigation.

TABLE 2

Inversion and reversion of the *RS5r*-1A *RS3r*-2 chromosome

Parental genotype	HS[b]	*n*	Progeny[a]		
			In	In^+	*Sb or TM6*
A. Inversion in males					
In^+/Sb	+	38	18 (2)	1894	2052
	−	17	0	928	1248
$In^+/TM6$	+	37	0	2216	1832
	−	19	0	1241	953
Parental genotype	*HS*[b]	*n*	Progeny[a]		
			In^+	*In*	*Sb* or *TM6*
B. Reversion in males					
In/Sb	+	39	49 (9)	1804	2140
	−	15	0	726	851
In/TM6	+	39	18 (7)	2152	1944
	−	18	0	1249	882
Parental genotype	*HS*[c]	*n*	Progeny[c]		
			In^+	*In*	*TM6*
C. Reversion in females					
In/TM6	+	20	19 (10)	999	1022

HS, heat shock; n, number of individuals mated.

[a] Progeny were scored as *In* if they were w^+; In^+ if they were w. Numbers in parentheses are the number of parents that produced any progeny with a newly inverted or reverted chromosome.

[b] Males were heat-shocked as first-instar larvae at 38.5° for 1 hr. Adults were mated individually to two w^{1118} females. Males carried *70FLP*3F.

[c] Females were heat-shocked at the mid-pupal stage at 38° for 1 hr and then mated individually to w^{1118} males. Females carried a single copy of *70FLP*3F.

their recovery in this instance would be difficult because the junction of *FRT*s in this duplication chromosome would not be marked by a reconstituted w^{hs} gene. Instead, we generated males that were heterozygous for the two insertions and then screened for FLP-mediated exchange between homologues. One product of this exchange should carry the duplication (see Figure 5). The markers *aristaless* (*al*), *curved* (*c*), and *speck* (*sp*) were used to screen for the desired recombinant. Males of the genotype w^{1118} *70FLP;* al^+ *RS3r*-4A c^+ sp^+/ *al c*

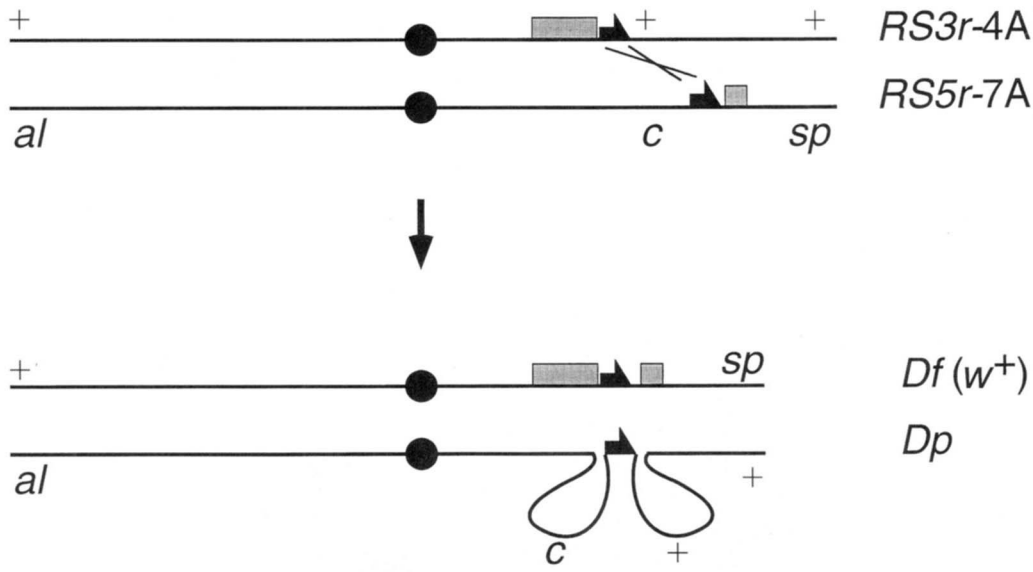

FIGURE 5.—Duplication and deficiency formation. At the top, the orientation and relative locations of the *RS3r*-4A and *RS5r*-7A insertions are diagramed along with the locations of the mutations used as markers. The expected products of FLP-mediated recombination between homologues that carry the two insertions are shown below.

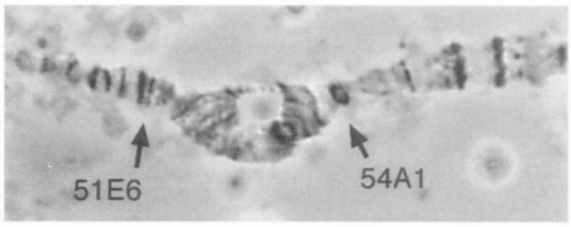

FIGURE 6.—Duplication cytology. Polytene chromosomes from animals that carried a duplication of the *RS3r*-4A *RS5r*-7A chromosome segment (*Dp(2;2)*51F;54A) were examined. This photograph shows the heterozygous duplication. Landmark bands are indicated.

RS5r-7A *sp* were heat-shocked for 1 hr at 38° during the first 2 days of development and then crossed to w^{1118}; *al c sp* females. Four of the 43 tested males produced al c^+ sp^+ recombinants, which should carry the duplication. These recombinants occurred in clusters: one male produced two al c^+ sp^+ male recombinants; in a second instance three such recombinants were recovered. Both were kept by crossing to w^{1118}; *al c sp* females. The other two recombinants were a female that died before reproducing and a male that was sterile. The reciprocal recombinant, presumably bearing the deficiency, was not recovered, nor were any w^+ flies recovered.

The presence of the expected duplication in the first isolate was verified cytologically (Figure 6). We verified the duplication in the second isolate by showing that the al c^+ sp^+ recombinant chromosome suppresses the *Minute(2)S-7* mutation, which lies in this interval (LINDSLEY and ZIMM 1992). *Minute* (*M*) genes are haplo-insufficient and their mutant phenotype is suppressed by a duplication of the wild-type allele. We crossed w^{1118}; *M(2)S-7/SM1, Cy* males to w^{1118}; *Dp, al c* c^+ sp^+*/al c sp* females. From this cross there were Cy^+ progeny that were M and Cy^+ progeny that were M^+. We collected eight Cy^+ M^+ males and crossed them individually to w^{1118}; *al c sp* females. From each of these crosses we recovered only two classes: al progeny that were M^+, and al^+ progeny that were M. This confirms that this al c+ sp+ recombinant chromosome carries a duplication of $M(2)S\text{-}7^+$. In this particular combination of RS insertions the acquisition of a duplication could have been monitored by screening for a chromosome that suppressed *M(2)S-7*. We chose to screen by looking for flanking marker recombination because we wished to demonstrate a more general method. Screening for suppression of a *Minute* could, in many cases, be used as the method of choice (GRELL 1969). With the particular combination of *RS* elements used here the duplication was not marked by w^+, but it is expected that 50% of *RSr* pairs that lie in the same orientation will mark the duplication with a reconstituted w^{hs} and simplify its recovery.

To recover the deficiency of the *RS3*-4A *RS5*-7A interval we crossed the w^{1118}; *Dp*/+ males to w^{1118} *70FLP; RS3r*-4A/*RS5r*-7A females (as three males by two females per vial). These females were heat-shocked by two protocols: in the larval stage and again as adults immediately prior to mating, each time for 1 hr at 37°; or, only as adults at 37° for 45 min or 1 hr. We then screened for progeny with pigmented eyes. These should have the *Df/Dp* genotype. One such w^+ fly was recovered from four vials using the first heat-shock protocol and two more were recovered from three vials with the second heat-shock protocol. All were crossed individually to w^{1118}; *Dp*/+ flies. The w^+ progeny of these crosses were mated *inter se* and their w^+ progeny selected to establish w^{1118}; *Dp/Df* stocks. These stocks may carry a segregating copy of *70FLP*, but have, nevertheless, been very stable. Only w^+ M^+ flies appear each generation. Both chromosomes appear to be lethal when homozygous.

Once these *Dp/Df* flies were established as a stock we were surprised to discover that *Df*/+ flies do survive when *Dp/Df* flies are outcrossed. We crossed w^{1118}; *Dp/Df* males to w^{1118} female virgins. Forty 1 × 1 matings produced 2844 w progeny and 171 w^+ M progeny. The deficient flies obviously have a greatly reduced viability relative to the duplicated flies. We also found that *Df*/+ males and *Df*/+ females are sterile (data not shown). However, the fact that the deficient flies do survive leads to the question of why, in spite of extensive testing, we were not able to recover the *Df*-bearing chromosome in our original screen? The deficient cells have a Minute phenotype, and it is likely that they divide more slowly than do the euploid cells (FERRUS 1975; MORATA and RIPOLL 1975). Further, if the deficient flies are sterile because of a germline defect, then it is likely that deficient cells do not make gametes when they are generated early in development. Finally, if a *Df*-bearing zygote is produced, it would most likely be Minute and relatively inviable and have little chance of completing development and being recovered as a $white^+$ adult.

Recombination in cis and trans: Flies from the *Dp/Df* stock were used in an experiment to measure the relative frequencies of *cis* and *trans* recombination between widely spaced *RSr* elements. Flies that carried *70FLP*3F and *RS3r*-4A and *RS5r*-7A, either on the same chromosome or on homologues, were heat-shocked and mated individually to w^{1118}; *Dp, al/Df, al*. When a gamete with a new duplication or deficiency is produced, half of the time it will unite with a gamete carrying the complementary rearrangement and produce a w^+ M^+ offspring. These were scored as new duplication or deficiency chromosomes. The results of these crosses are shown in Table 3. There is one obvious difference in the tests of Table 3—the recombinant products were transmitted at a much higher rate through the female germline than the male germline. This may, at least in part, be attributable to a higher production of FLP in the females. The females had two copies of *70FLP* and were given two heat shocks.

TABLE 3

Rates of *cis* and *trans* recombination

	Female recombination			Male recombination		
	Tested	Germline events[c]	Total recovery[d]	Tested	Germline events[c]	Total recovery[d]
cis[a]	134	47 (35.1)	60/6267 (1.9)	81	2 (2.5)	4/3958 (0.2)
trans[b]	96	27 (28.1)	48/5893 (1.6)	134	8 (6.0)	24/6867 (0.7)

[a] w^{1118} *70FLP*3F; *RS3r*-4A *RS5r*-7A/S^2*CyO* females were mated to w^{1118} *70FLP*3F; *cu kar*^{2} *Sb*/*TM6, Ubx* e^s males. Progeny were heat-shocked at 38° for 1 hr during the first 1–2 days of development. Females were given a second heat shock of 37° for 1 or 2 hr as adults before mating. The different lengths of heat shock did not produce significantly different results and the two groups have been pooled. The Cy^+ sons or daughters of this cross were individually mated to w^{1118}; *Dp, al*/*Df, al* flies to score for new duplication or deficiency formation.

[b] w^{1118} *70FLP*3F; *RS3r*-4A/S^2*CyO* females were mated to w^{1118} *70FLP*3F; *RS5r*-7A/S^2*CyO* males. Their progeny were heat-shocked and mated as described for the *cis* experiment.

[c] The number of individuals that produced one or more w^+ M^+ (*Dp*/*Df*) offspring is given with percentage in parentheses. This represents the minimum number of germline events.

[d] Reported as number of w^+ M^+/total M^+ progeny with percentage in parentheses. Excluding the w^+ M progeny from the analysis corrects for the fact that only half of the gametes with new duplicated or deficient chromosomes give rise to surviving M^+ progeny. Frequencies were multiplied by 2× to correct for the fact that the insertions are hemizygous.

The difference between recombination frequencies when elements were in *cis* or in *trans* is not statistically significant in either males or females (when considering independent events). This suggests that *FRT*s on homologous chromosomes are as easily recombined by FLP as are *FRT*s on the same chromosome, at least when those *FRT*s are separated by a substantial distance (in this case approximately 2.3×10^6 bp; estimated from SORSA 1988). But, because the deficiency is Minute and causes sterility in males and females, these results are no doubt complicated by reduced fitness in the germline of cells with the rearrangements. To clarify this issue, we examined recombination in cells of the eye that were at or near their final mitotic division when *70FLP* was induced. We looked at the frequency of w^+ spots induced in the eyes of flies that were heat-shocked in late third instar. In these eyes each pigmented spot is an independent event, and variation in spot frequency is easily visualized. In this experiment recombination in *cis* was clearly more frequent than recombination in *trans,* Flies that carried *RS3r*-4A and *RS5r*-7A as hemizygous insertions in *trans* showed fewer spots of pigment after *70FLP* induction than flies with the insertions in *cis,* which in turn showed fewer spots than flies carrying both insertions in the homozygous condition (Figure 7). We believe that this experiment gives a more accurate depiction of the actual rates of recombination than the germline results because deleterious effects of the rearrangements should be minimized in somatic cells at this late stage of development.

The unequal recoveries of recombinants: When the duplicated and deficient chromosomes are generated by *trans* recombination, they are produced as reciprocal products of the same event. However, neither females nor males transmitted equal numbers of the *Df* and *Dp* chromosomes (Table 4). We first consider the results of recombination in females.

In the experiment of Table 3 three-fourths of the recombinant chromosomes recovered from the females with the *trans* arrangement were cases of newly formed deficiencies rather than duplications ($P < 0.05$). This suggests that the *Dp*-bearing cells (at least) are at a selective disadvantage in the female germline. This is a rather surprising result considering that *Df*/+ flies are sterile, whereas *Dp*/+ flies are fertile. We suggest two possible explanations.

One mechanism that may operate to favor transmission of the deficiency from females with the *trans* arrangement is meiotic drive. NOVITSKI (1951) showed that when meiotic exchange generates a meiosis II dyad of unequally sized chromatids, the smaller chromatid of the dyad is recovered at a higher rate than the larger. Other examples are reviewed by ASHBURNER (1989). If, in our experiment, the duplication and deficiency come mainly from FLP-mediated exchange in meiotic prophase, then their recoveries may be unequal owing to meiotic drive operating at the second meiotic division. In the present case the two dyads produced after meiotic recombination would be *Df*/+ and +/*Dp*. The deficiency should be recovered preferentially from the *Df*/+ pair, and the + chromosome from the +/*Dp* pair, leading to an overall deficit of the *Dp*-bearing chromosome. However, it seems somewhat unlikely that the difference in size conferred by deletion or duplication of the region between *RS3r*-4A and *RS5r*-7A would be sufficient to confer a recovery differential of the magnitude observed here.

As an alternative, we suppose that recombination between homologues occurs almost entirely in G2 of mitosis and that the reciprocal products of a non-sister exchange usually segregate to opposite daughter cells when the cell divides (PIMPINELLI and RIPOLL 1986; K. BEUMER and K. G. GOLIC, unpublished data). If this recombination occurs during the final four mitotic divi-

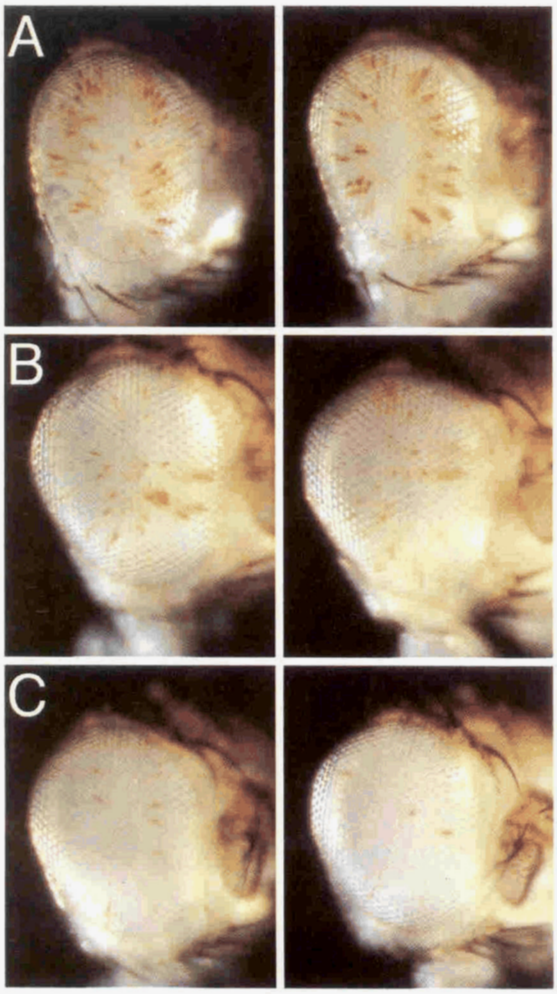

FIGURE 7.—Comparison of somatic recombination between *RSr* elements in *cis* and *trans*. Males that carried a single copy of *70FLP*3F and the *RS3r*-4A and *RS5r*-7A insertions on chromosome *2* were heat-shocked as late third-instar larvae at 38° for 1 hr. Eyes of the adults that eclosed were photographed, and examples that typify each class are shown here. (A) Spot frequencies when both insertions were homozygous. (B) Spot frequencies in the *cis* hemizygotes. (C) Spot frequencies in the *trans* hemizygotes. The pattern of vertical stripes that is slightly apparent in A appears reproducibly. It can be explained, at least partly, by the nonuniform response to heat shock of cells at different positions in the eye at this stage of development (AHMAD and GOLIC 1996).

sions that generate the 16 cystocytes and if the duplication winds up in the cell that becomes the oocyte, then the nurse cells will, on balance, be hypoploid. Since the nurse cells perform the majority of biosynthesis for the oocyte, this may result in oocyte inviability. On the other hand, if a deficient chromosome segregates to a cell that becomes the oocyte, then the nurse cells will, on balance, be hyperploid and the oocyte may survive.

When *Dp* and *Df*-bearing cells are generated at earlier stages in the germline, it is possible that both types of aneuploid cells are at a selective disadvantage and infrequently reach maturity as a functional egg. Two aspects of the experimental results add support to this

TABLE 4

Relative production of deficiencies and duplications

	Deficiency	Duplication	Both
trans female	14	4	1
cis female	17	2	0
trans male	1	4	2

The w^+ M^+ males that were recovered in the experiment of Table 3 were crossed to their w female sibs, which were w^{1118}; *Dp, al/+*. The w^+ male carried a newly formed deficiency (and was therefore w^{1118}; *Dp, al/Df,* al^+) if the w^+ progeny of this cross were never al and some of the w progeny were al. If some w^+ progeny were al and no w progeny were al then the original male carried a newly formed duplication. In some instances a single parent in the experiments of Table 3 produced multiple w^+ M^+ offspring. When more than one w^+ M^+ male was tested from a single parent, they were scored as though a single event gave rise to all tested males.

notion. First, when the progeny of heat-shocked females were scored (the experiment of Table 3), they were counted twice: once between the 12th and 14th day after the cross was started and again on the 18th day. Two-thirds of the w^+ M^+ progeny were recovered in the first count. (The totals for both the *cis* and *trans* tests were 70 w^+ M^+ recovered in the first count and 38 in the second while the numbers of w M^+ progeny recovered in the two counts were 6019 and 6033, respectively, $P = 0.002$.) Second, the average number of w^+ M^+ progeny produced by a female that produced any such offspring is small (less than two in both *trans* and *cis* combinations). We conclude that when cells with recombinant chromosomes are formed they rarely persist to form large clones. Both results suggest that cells with the deficient or duplicated chromosome are at a selective disadvantage in the female germline.

When the *RSr* elements were located on the same chromosome, we again recovered a significant excess of the *Df*-bearing chromosome from the heat-shocked females. With this arrangement the deficiency and the duplication can be produced as reciprocal products of an unequal sister-chromatid exchange and would always segregate at mitosis. However, in this case the deficiency alone can be produced by simple excision. When *FRT*s are very near one another, excision is the predominant event (GOLIC and LINDQUIST 1989). When elements with *FRT*s separated by approximately 5 kilobase pairs or less were used, the data of GOLIC and GOLIC (1996) show a germline excision frequency of 99.7% with over 45,000 progeny scored. If this also applies to elements separated by much greater distances, then it is not surprising that the *Df* is recovered in excess. If excision predominates, it also provides an explanation for the fact that the rearranged chromosomes are not recovered at a greater rate through the germline when *FRT*s are in *cis*—the product that is produced with the greatest frequency is also selected against most strongly, with a net effect of no increased germline transmission. If

there are cells where unequal sister-chromatid exchange does occur, the two models that were proposed to explain the preferential recovery of *Df*-bearing gametes from females with the *trans* arrangement may also apply here. When insertions are in *cis*, the effect of meiotic drive should be even more pronounced, because the asymmetric dyad has a greater size disparity.

It is worth noting that the duplication produced by the *cis* combination (presumably by unequal sister chromatid exchange) is different than the duplication that we recovered after *trans* recombination: it carries three *FRT*s that bracket the repeated chromosomal segments, while the duplication produced by *trans* recombination carries only one *FRT* that lies between the repeats. The first should be readily reversible by intrachromosomal recombination with FLP; the latter can be reverted by FLP only by exchange with a homologue that carries the deficiency.

When males with the *trans* arrangement of elements were heat-shocked and tested, the recoveries were skewed in the opposite direction: there was an excess of *Dp*-bearing gametes. However, this excess is not statistically significant. Further experimentation would be required to determine if cell-cell competition favors the *Dp*-bearing cells over the *Df*-bearing cells in the male germline, but it is probable that this is the case. In the male germline, the induction of *hsp70*-promoted fusion genes (such as *70FLP*) is limited to stem cells (BONNER *et al.* 1984; GOLIC and GOLIC 1996). Any cells that possess a recombinant chromosome must survive through at least four rounds of mitoses plus meiosis in order to produce gametes that have the rearrangement. As is the case with females, *Df*/+ males are sterile but *Dp*/+ males are fertile. It therefore seems likely that deficient cells would be at a severe disadvantage in the male germline. This explanation is consistent with the observed excess recovery of *Dp*-bearing gametes from *trans* males. It may be that *Df*-bearing gametes are transmitted by males only infrequently, when the two recombinant chromatids segregate together at mitosis. In support of this notion, two of the three males that transmitted the deficiency also transmitted the duplication.

Finally, if the *Df*/+ cells cannot make sperm, then, we can predict that the only recombinant chromosome that would be recoverable from males with the *cis* arrangement would be the duplication. Unfortunately so few recombinants were recovered from *cis* males that we could not test this hypothesis.

Determining *RS* orientation: Because recombination between *FRT*s is directional, the ability to design rearrangements is hampered by a lack of knowledge about the orientation of the *RS5* and *RS3* insertions. Up to this point, we knew the relative orientation of two *RS* insertions only after they were successfully used to produce a rearrangement. Further examination of the rearranged chromosomes provides information that allows a determination of the absolute orientation of the *RS* elements.

If the rearrangement is an inversion, then *in situ* hybridization to polytene chromosomes of the *In/In* homozygote can be used to determine absolute orientation of the involved elements. A probe made from w^{hs} sequence should hybridize to the breakpoint that has the reconstituted w^{hs} gene. For instance, Figure 1C illustrates the case where the *RS5r* element is located at what is to become the left breakpoint (between *a* and *b*) and has its *FRT* oriented toward the left. In this case hybridization to chromosomes of the *In/In* homozygote would be detected at the $a|e$ junction breakpoint. If the orientation of both insertions were reversed then hybridization would instead be detected at the $b|f$ junction. If the *RS5r* was initially located at what would become the right-hand breakpoint (between *e* and *f*), and *RS3r* at the left, the orientations can be determined in like fashion.

If recombination generates a deficiency, then the rearrangement breakpoints will segregate. This occurs either because the acentric product is lost after recombination in *cis* or because the reciprocal products of recombination in *trans* segregate at mitosis or meiosis. The orientation of the insertions can be deduced by determining which of the two products carries the w^{hs} gene, as in the previous section. We concluded that *RS3*-4A and *RS5*-7A lie in the chromosome with their *FRT*s pointing to the right as indicated in Figure 5. (See MATERIALS AND METHODS for the convention on the designation of *FRT* orientation.) It follows that any *RS5* insertions that can be used to make inversions in combination with *RS3*-4A must lie in the opposite orientation. Therefore, *RS5*-3A, *RS5*-3B, *RS5*-8, and *RS5*-31B must lie in the chromosome with their *FRT*s pointing to the left. It is tempting to assign the opposite orientation to *RS5*-15B and *RS5*-10, but without positive evidence upon which to base that assignment, we consider such inference unwarranted, especially considering the relatively low number of vials examined for *RS5*-15B.

Finally, a "bootstrap" method may be used to determine the absolute orientation of *RS* element insertions without their involvement in a rearrangement. This method comprises two steps: (1) a screen to recover deletions of one of the two *FRT*s from *RS* element insertions; and (2) a test-cross to detect the results of FLP-mediated recombination between the altered *RS* element and the allelic *RSr* on a marked chromosome. An *RS5* insertion is used as an example in the following discussion; a similar logic is applicable to *RS3* insertions.

Screening for FRT loss: Many investigators have observed that *P*-element transposase produces partial internal deletions of *P* elements *in situ* (DANIELS *et al.* 1985; reviewed by ENGELS 1989). We utilized this property to attempt to recover deletions of one of the two *FRT*s of *RS5* and *RS3* element insertions. Figure 8A diagrams the scheme. First, males that carry an intact

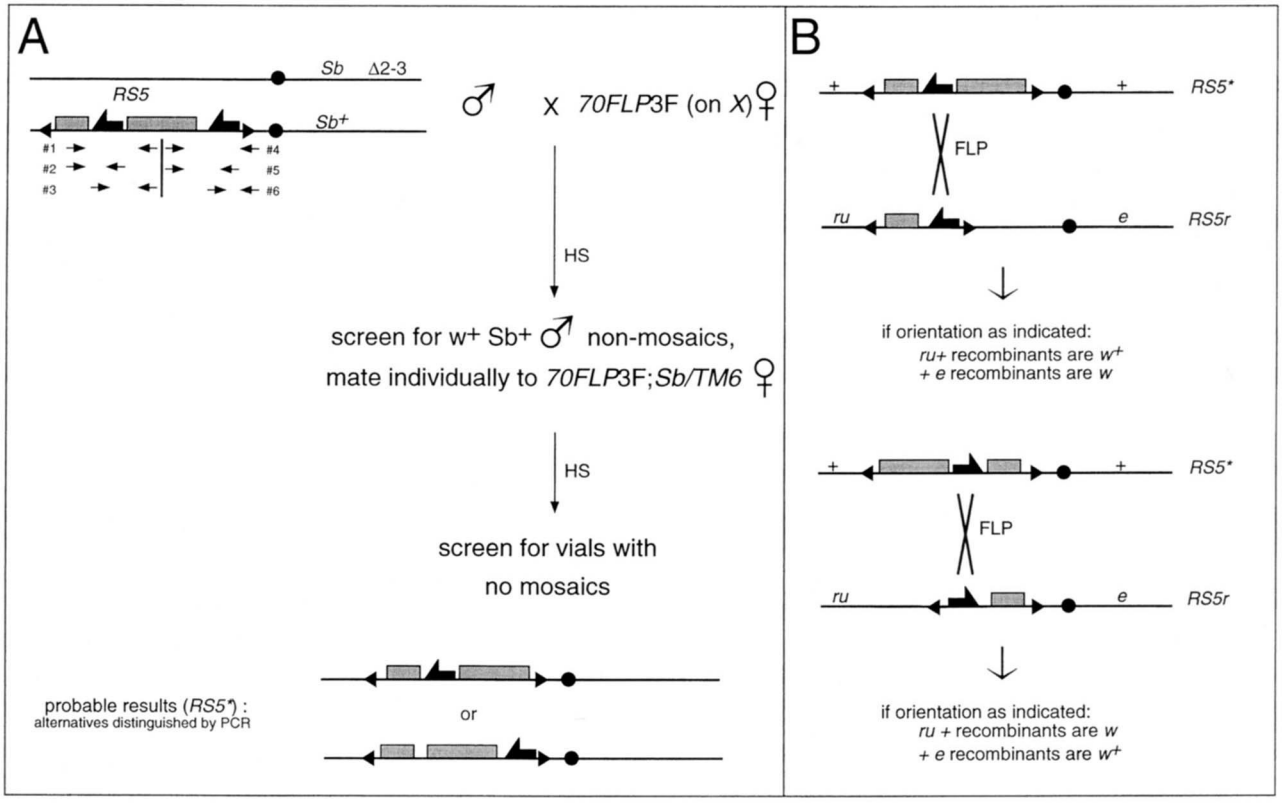

FIGURE 8.—A method for determination of the orientation of *RS* element insertions. All flies carry the w^{1118} mutation on their *X* chromosomes. (A) Diagram of the method used to recover altered *RS* elements (*RS**) that lack one of the two *FRT*s. In this case an *RS5* element is used as an example. The locations of primer pairs that were used to characterize the *RS5** elements are indicated beneath the *RS5* element. The numbers correspond to the results of PCR tests given in Table 6. Part B shows how the *RS** element can be used in combination with the allelic *RSr* element on a marked chromosome to determine *FRT* orientation. Recombination is induced in the male germline and assayed by test-crossing. This example uses an *RS5* element located between *ru* and *e* on chromosome *3*.

RS element and the transposase source *Δ2-3* (99B) on a chromosome marked with *Sb* (ROBERTSON *et al.* 1988) are generated by crossing. These males are crossed to females that carry an *X*-linked insertion of the *70FLP* gene. Progeny from this cross are collected in vials for 4–5 days and then heat-shocked at 38° for 1 hr. The Sb^+ adults are collected (in order to eliminate *Δ2-3*) and examined, looking for w^+ nonmosaics. When *70FLP* is induced under these conditions in animals that carry a normal *RS* insertion, the cellular excision rate is very near 100%, leading to flies with almost entirely white eyes. If exposure to transposase has produced an altered *RS* element (denoted *RS**) in which one of the two *FRT*s has been deleted, FLP will no longer catalyze excision, and an animal with solidly pigmented eyes should result. Flies that are selected through this first round are retested by crossing them back to w^{1118} *70FLP* flies that carry the appropriate balancer chromosome and their progeny are heat-shocked as larvae (as in the previous generation). If approximately half the progeny are w^+ and these are all nonmosaic, the chromosome is kept for the next step.

The four *RS* insertions on chromosome *3* were examined by this method and the results are given in Table 5. The two *RS3* insertions did not produce derivatives that gave germline recombinants and were not examined further. The PCR was used to determine which of the two *FRT*s has been deleted from the *RS5** derivatives. The locations of the primer pairs are shown in Figure 8 (see MATERIALS AND METHODS for primer sequences). Results of the PCR tests are given in Table 6.

Male recombination test: Males that carried *70FLP*, the *RS** element, and the allelic *RSr* insertion were heat-shocked early in development to induce mitotic recombination. The heat-shocked males were test-crossed and the progeny that received recombinant chromosomes were scored as w^+ or w (Table 6). Figure 8B depicts the results expected if the *RS** element had retained only the *FRT* located in the first intron. The *RS5*-1A derivative A, and the *RS5*-2A derivatives H-1, H-3, I, and J are examples of this *RS** type. If the *FRT* located in the intron was lost, and the downstream *FRT* retained, then the expected results are reversed: the *RS5*-2A derivatives G-1, G-2 and L-1 are examples. The PCR tests of *RS5*-2A derivative E indicated that there might still be two *FRT*s present, with the right hand (as drawn in Figure 8A) *FRT* separated from the w^{hs} gene. Although crossing results were consistent with other derivatives that car-

TABLE 5

Recovery of *RS elements**

RS insertion	No. of vials of *RS*/Δ*2-3* tested[a]	No. of *RS** recovered	Sterile or died before testing	Produced recombinants?	
				No	Yes
RS5-1A	16	6	5	0	1
RS5-2A	43	20	7	1	12
RS3-2	3	5	1	4	0
RS3-3	16	6	4	2[b]	0

[a] Crosses were set up as two to three pairs per vial.
[b] One of these was a transposition to another chromosome.

ried only the left *FRT*, we did not rely on this derivative to assign orientation.

These tests indicate that the *RS5*-1A and *RS5*-2A insertions lie in the chromosome with their *FRT*s pointing to the left. An independent experiment (unpublished data) confirms that the *RS3*-2 element lies with its *FRT*s pointing toward the right. This result is wholly concordant with the observation that *RS3r*-2 can be used to make inversions with either *RS5r*-1A or *RS5r*-2A.

The success of this test depends on the recovery of either of the two products shown in Figure 8A. Chromosomes that are substantially different than those bearing simple deletions of an *FRT* may also be produced by transposase. The combination of the genetic and molecular screens should eliminate from consideration any derivatives that have an *FRT* in more than one location relative to w^{hs}. It is possible that an altered element may have also transposed. If the element moved to a nearby site, then recombinants may still be recovered if the transposed element lies in the same orientation as the original element. These recombinants will give the correct determination of orientation, although one of the reciprocal recombinants may not be recovered because it carries a deficiency. If the transposed element lies in the opposite orientation, recombination between it and the original *RSr* element will produce dicentrics (Golic 1994) and we do not expect to recover recombinants—no answer will be given using such an inverted *RS** element. Transposase may also produce double insertions. The occurrence of white$^+$

TABLE 6

***RS* orientation results**

RS element	*RS** isolate[b]	Parentals		Recombinants				PCR tests[a]							
		ru e w	*ru$^+$ e$^+$ w$^+$*	*ru e$^+$ w$^+$*	*ru$^+$ e w*	*ru e$^+$ w*	*ru$^+$ e w$^+$*	1	2	3	4	5	6	SM[c]	*FRT*[d]
RS5-1A	Control							1.6	0.7	1.1	3.0	2.4	0.8		
RS5-2A	Control							1.6	0.7	1.1	3.0	2.4	0.8		
RS5-1A	A	76	89	54	48	0	0	1.6	0.7	1.1	2.7	—	—	—	←
RS5-2A	E	98	114	21	33	0	0	1.6	0.7	1.1	—	—	0.8	NE	×
	G-1	60	77	0	0	20	24	—	—	—	3.0	2.4	0.8	—	←
	G-2	12	37	0	0	8	11	—	—	—	3.0	2.4	0.8	—	←
	H-1[e]	51	81	15	20	0	0	1.6	0.7	1.1	—	—	—	+	←
	H-3[f]	70	84	8	14	0	0	1.6	0.7	1.1	—	—	—	+	←
	I	90	132	10	20	0	0	1.6	0.7	1.1	—	—	—	+	←
	J	89	130	22	52	0	1[g]	1.6	0.7	1.1	—	—	—	+	←
	L-1[h]	30	62	0	0	8	17	—	—	—	3.0	2.4	0.8	–	←

[a] The sizes of the amplified fragments (in kilobase pairs) are indicated.—, no amplification. The locations of primer pairs are shown in Figure 8.
[b] When two or more *RS** isolates were obtained from the same set of parents they were given the same initial letter designation.
[c] Somatic mosaicism (SM) was scored as + if the heat-shocked parents exhibited obious white sectors on a pigmented background in the eye. The derivatives A, G-1, and G-2 had rare small red spots on a lighter background; white spots were not seen. The basis for this mosaicism is uncertain. NE, not examined.
[d] *FRT*, *FRT* orientation: ←, FRTs of parental insertion face left; ×, no orientation assigned from this cross.
[e] One other *RS** derivative was produced by this same set of parents and gave an identical pattern of recombinants but was not characterized by PCR.
[f] This cross also produced one ru$^+$ e$^+$ w and three ru e w$^+$.
[g] Occasionally, recombinants arise from recombination at sites other than *FRT*s (Golic and Golic 1996).
[h] Three other *RS** derivatives were isolated from the same set of parents and gave identical patterns of recombinants but were not characterized by PCR.

or white flies in both recombinant classes is an indicator that two insertions are present. In some cases it might still be possible to deduce orientation from the results, but it is probably wiser to generate several *RS** derivatives and rely on those that can be interpreted without complication.

In half the cases, when the difference between *RSr* and *RS** is distal to the *FRT,* the recombination that is measured in the germline should be accompanied by somatic white mosaicism (Table 6). This mosaicism might also be used to determine orientation. Although the somatic results were in accord with expectations, we feel that somatic mosaicism should not be relied upon to determine orientation. For instance, an absence of mosaicism could result simply from a lack of recombination, as with many of the *RS** derivatives (Table 5). The possibility that transposed or duplicate elements are present on the *RS** derivative also complicates the interpretation of somatic mosaicism. The test we describe relies on the recovery of viable progeny. This provides an additional level of assurance that the recombinants derive from the expected exchanges, and it is unlikely that the test will give an answer that leads to the assignment of an incorrect orientation.

DISCUSSION

We have shown that FLP-*FRT* site-specific recombination can be used to efficiently generate large-scale chromosome rearrangements in *D. melanogaster.* Paracentric and pericentric inversions, duplications and deficiencies were produced in germline tissue, transmitted, and stably maintained. This method of producing chromosome rearrangements does not depend upon the expected properties of the desired rearrangement for their recovery. By using two target sites that generate a dominant genetic signal when recombined, predetermined rearrangements can be recovered in the absence of any other phenotype.

The use of site-specific recombination to generate rearrangements obviates the need for the labor-intensive steps that are often required to recover and characterize multiple chromosome rearrangements, in order that rearrangements that have desired characteristics can be found. After irradiation, inversions have been detected by the reduction in meiotic exchange that they cause: deficiencies by loss of genes and duplications by their suppression of haplo-insufficient phenotypes. [These methods and others are discussed by ASHBURNER (1989).] The rearrangements that are recovered must then be confirmed and characterized cytologically. Methods have been developed that can aid the recovery of rearrangements with breakpoints in a limited region (DUBININ and SIDOROV 1934; LEWIS 1954; GREEN 1967; LIM 1979; BERG *et al.* 1980; LYTTLE 1984; GUBB *et al.* 1988, 1990). However, these methods still rely on the somewhat unpredictable action of irradiation or transposable elements to produce the rearrangements and are typically of limited applicability. One problem that also arises when intrachromosomal rearrangements are desired is that the majority of rearrangements recovered after irradiation have interchromosomal interchanges. The rearrangements that are recovered also have variable endpoints and potentially undetected secondary mutations. When using site-specific recombination, the breakpoints of the rearrangement can be chosen in advance, and the likelihood of an additional mutation occurring coincident with the formation of a rearrangement should be quite low. Because the rearrangements made by site-specific recombination are reversible, the reverted chromosomes can serve as controls to deal with this concern.

Previous work showed that FLP could catalyze recombination between *FRT*s separated by approximately 0.5 Mb to produce a deficiency or duplication of a defined region (GOLIC 1994). In this work we showed that FLP-mediated intrachromosomal rearrangements could be readily recovered between *FRT*s that are separated by tens of megabases. We also showed that a chromosomal region lying between *FRT*-bearing *P* elements oriented in the same direction could be deleted or duplicated by recombination between those elements in *cis* or in *trans.* In the latter case we screened for flanking marker recombination in the male germline to first recover a duplication, because the deficient animals survived very poorly. The deficiency was then recovered in a duplication/deficiency heterozygote. The deficiency that we generated in this work is quite large nearing the limit of what can be tolerated in Drosophila (LINDSLEY and SANDLER *et al.* 1972). Smaller (and typically more useful) deficiencies could, in most cases, be recovered directly.

The production of deficiencies and duplications by site-specific recombination is made easier by the fact that the two *FRT*-bearing elements need not be placed on the same chromosome. This contrasts with deletions that can be made by exposing two separated *P* elements to P transposase. Such deletions were observed only when the two elements were located in *cis* (COOLEY *et al.* 1990).

In order to recover a desired rearrangement between a complementary pair of *RS* insertions at chosen sites, the insertions must be properly oriented. In some cases there may be a pair of insertions located at the desired sites, but in the wrong relative orientation to produce the desired rearrangement. In those cases it may be possible to reorient one of the insertions by transposition. It is a relatively easy matter to recover double *P*-element insertions at a site. When the original insertion does not produce full red eyes, as is typically the case with the *RS* elements, the majority of dark red-eyed progeny that arise after exposure to transposase are double insertions at or near the original site. When the two elements lie in opposite orientation, FLP can

generate dicentric chromosomes at a high frequency by unequal sister chromatid exchange. The formation of dicentric chromosomes is betrayed by a set of characteristic developmental defects that can be used to diagnose the presence and orientation of the two elements (GOLIC 1994). When such a double-insertion is identified, then a second round of transposition could be used to remove the original element, leaving behind an element in the opposite orientation. The orientation of the remaining single element might be determined by assaying for the phenotypes that result from the production of dicentric chromosomes following FLP-induced recombination with the original insertion on the homologue. The inverted element could then be used to generate the desired rearrangement.

Chromosome rearrangements have been produced in other organisms through the use of site-specific recombination. In *Saccharomyces cerevisiae* a recombination system from *Zygosaccharomyces rouxii* was used to engineer intra- and interchromosomal rearrangements with very high efficiency (MATSUZAKI *et al.* 1990). The Cre-*lox* system has been used to generate rearrangements in mice (RAMIREZ-SOLIS *et al.* 1995; SMITH *et al.* 1995) and in plants (QIN *et al.* 1994; MEDBERRY *et al.* 1995; OSBORNE *et al.* 1995). In mouse ES cells, transient Cre expression yielded a relatively low efficiency of rearrangement production, but because these cells are readily amenable to chemical selection, the rearrangements were easily recovered. In both tobacco and Arabidopsis, chromosome rearrangements could be produced at a fairly high frequency when using constitutive synthesis of the Cre recombinase. It is likely that continuous high levels of FLP synthesis could be used to increase the efficiency of rearrangement production in Drosophila. It might be possible to approach this goal through the application of multiple heat shocks throughout development. It should also be possible to use FLP to produce chromosome rearrangements in other experimental organisms. FLP requires no yeast host factors to function and has been shown to work in bacteria, other yeasts, insects, mammals, and plants (COX 1983; CREGG and MADDEN 1989; MORRIS *et al.* 1991; KONSOLAKI *et al.* 1992; O'GORMAN *et al.* 1991; LYZNIK *et al.* 1993, 1995; LLOYD and DAVIS 1994; KILBY *et al.* 1995; DYMECKI 1996).

In both reports of intrachromosomal rearrangements in plants there were rearrangements that were generated somatically but could not be transmitted germinally (MEDBERRY *et al.* 1995; OSBORNE *et al.* 1995). Molecular evidence was presented to show that, after recombination, both rearrangement breakpoints could still be detected in somatic tissue. In both cases it was concluded that certain inversions could not be transmitted through germ cells. The underlying assumption is that if a deletion were to occur, the rearrangement breakpoint on the extrachromosomal circle would be lost. It is not unreasonable to suppose that, on occasion, the novel conjunction of genetic elements at a rearrangement breakpoint might not be tolerated. This might be especially true in plants because of the necessity for genetic activity in the haploid gametophytes. However, we believe that the evidence presented does not exclude the possibility that the nontransmissible rearrangements were in fact deletions, rather than inversions. We have found that an excised circular DNA can be maintained in nondividing cells in Drosophila (AHMAD and GOLIC 1996). If this were also true in plants, it could account for the detection of both breakpoints by molecular methods. This question arises because the orientation of the insertions in question could be deduced only from the rearrangements themselves. In yeast and in mice this problem was solved by using homologous recombination to place target sites into sequences whose orientation within the chromosome was known or to produce insertions in each orientation at a site. This is not yet an option in Drosophila or in plants, nor is it a general solution to the problem in mice.

We describe several methods that can be used to determine the absolute orientation of insertions within the chromosome. The combined knowledge of insert location and orientation allows for the production of chromosome rearrangements by design. As more insertions of *RS* elements or similar constructs are collected and localized this technique will become progressively more useful.

The ability to generate chromosome rearrangements with predetermined endpoints provides a powerful tool for study of the relationships between chromosome arrangement, structure and function. One area that particularly lends itself to study with these rearrangements is the examination of chromosome pairing. The frequency of FLP-mediated recombination between *FRT*s at the breakpoints of heterozygous rearrangements could be assayed in order to deduce pairing relationships in mitotic cells. For instance, the results we presented suggest that homologues of an inversion heterozygote sometimes pair in the form of an inversion loop in mitotically dividing cells. Another approach might be to assay constructs of reporter genes joined to pairing-sensitive sequences that are inserted within or adjacent to the rearranged segments (KASSIS *et al.* 1991; FAUVARQUE and DURA 1993; KAPOUN and KAUFMAN 1995).

Cytological assays have been used to detect the association of homologous sequences in mitotic cells (KOPCZYNSKI and MUSKAVITCH 1992; HIRAOKA *et al.* 1993). These associations are very frequent when the homologous sequences are alleles. Several studies have suggested that highly repetitive DNA can associate with homologous sequences at nonallelic sites (BARR and ELLISON 1972; LEE 1975; YOON and RICHARDSON 1978). Such ectopic associations have been detected cytologically in the case of the $brown^{Dominant}$ (bw^{D}) mutation, and can be quite frequent (CSINK and HENIKOFF 1996;

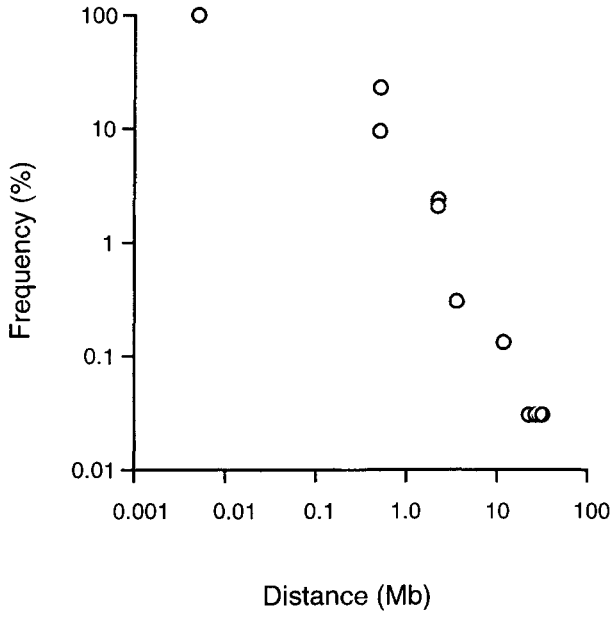

FIGURE 9.—The variation of FLP-mediated recombination frequency with distance of *FRT* separation. The data for this graph are taken from Tables 1 and 3 of this paper, from GOLIC (1994), and from GOLIC and GOLIC (1996). Distances are estimated from SORSA (1988). The data of Table 3 were collected separately for males and females; we added the two frequencies in order to make them comparable to the results of Table 1. The data from GOLIC (1994) were corrected for viability. Because recombination occurred only in males in those experiments, the frequencies were then doubled to place them on par with most of the data presented in this paper, where recombination could occur in both males and females.

DERNBURG *et al.* 1996). However, these assays lack the resolution to tell us whether such an association is precise or intimate; the interaction that leads to site-specific recombination must be both. One great advantage of the genetic approach for such studies is that rare or fleeting pairing interactions can be detected and quantitated. It would likely be very difficult to obtain convincing cytological evidence of a pairing interaction between specific sequences that occurs only briefly or that is limited to a particular tissue.

Ectopic association in mitotic cells between sequences that are not highly repetitive has also been examined. ENGELS and co-workers obtained extensive data on ectopic interactions that lead to *P*-element induced gene conversion. In one aspect their findings parallel ours: they found that *cis* interactions were more frequent than *trans* interactions (ENGELS *et al.* 1994). However, in other respects their data differ significantly. They found no relationship between the distance of separation and the frequency of gene conversion. We find that the frequency of recombination between two *FRT*s is strongly dependent on their proximity (Figure 9). Studies of other types of pairing-dependent interactions concur, in suggesting that ectopic interactions between homologous sequences in mitotic cells are strongly dependent on their proximity (GUBB *et al.* 1986; KASSIS *et al.* 1991). NASSIF and ENGELS (1993) also argue that very short stretches of homology, perhaps less than 115 base pairs, suffice for highly efficient ectopic interactions. The *RSr* elements that we used provide approximately 1000 base pairs of homology (not entirely isosequential) at ectopic sites, but do not exhibit the efficient recombination of *FRT*s located at precisely allelic sites, where homology extends the length of the chromosome (GOLIC 1991). This recombination is sensitive to disruptions in homology that are several megabases distant (GOLIC and GOLIC 1996).

The difference between the results from ENGELS and co-workers and our results suggests the possibility that different mechanisms are involved. When double-strand breaks occur, as when a *P* element excises for transposition, the cell's survival typically depends on repairing those breaks. Perhaps a special highly efficient mechanism exists to search for homologous sequences that can be used as a template to repair that break, ensuring the cell's survival. On the other hand, a special mechanism may not be needed to facilitate pairing in cells with a double-strand break. Previous experiments provided evidence that a cell's ability to pair homologous sequences is enhanced when the length of the cell cycle is extended (GOLIC and GOLIC 1996). If the double-strand break causes cell-cycle arrest until it is mended (HARTWELL and WEINERT 1989), then pairing might be more efficient because the cells have an extended period to find homology.

Normal mitotic pairing in Drosophila may have evolved so as to minimize ectopic associations, where the proximity of ectopic enhancers might cause inappropriate gene regulation. (This does not exclude the possibility that some selective advantage is provided by the mitotic pairing of allelic sequences.) Ectopic pairing might then occur solely as a result of chance encounters. Our results are consistent with the notion that the ectopic contacts that lead to recombination and the formation of rearrangements stem from chance encounters between *RSr* insertions located at different sites. The correlation that we see between proximity and recombination frequency follows from the fact that the two sequences are, in effect, tethered by the length of chromosome between them. The shorter the tether, the more likely they are to encounter each other. DERNBURG *et al.* (1996) proposed a mechanism whereby such chance encounters may occur; they suggested that ectopic pairing arises by a random three-dimensional walk of a flexible chromosome within the nucleus. This would bring about pairing of homologous sequences with increasing frequency as the distance between those sequences decreased. If two regions of ectopic homology do come into close proximity, a stable pairing interaction could result, just as with allelic homology.

The data of HILLIKER (1985) on radiation-induced interchanges in somatic cells also reveal a preference

for short range interactions. The average span between the breakpoints of autosomal rearrangements was less than one-fifth the length of a chromosome arm. HILLIKER additionally observed a very pronounced preference for interchanges to be limited to a single arm. We observed more frequent recombination between *RSr* elements on the same arm than on opposite arms, but our data are not extensive enough to rule out the possibility that this is simply a result of the greater distance between the opposite-arm insertions that we used.

One instance of ectopic association that occurs with a high frequency is the case of bw^D. The bw^D mutation is caused by an insertion of more than 2 Mb of heterochromatin at the *bw* locus (HENIKOFF *et al.* 1995). The highly repetitive nature of heterochromatin and the extensive stretch of homology may facilitate ectopic association with centric heterochromatin in this instance. Even so, HENIKOFF's group has presented compelling evidence that the frequency of this association depends on the proximity of bw^D and centric heterochromatin (TALBERT *et al.* 1994; HENIKOFF *et al.* 1995; CSINK and HENIKOFF 1996).

Finally, a comparison of our results, which derive mainly from recombination in mitotic cells, with measures of ectopic pairing in meiotic cells, serves to point out similarities and differences in chromosome behavior in the two cell types. A frequent method for the study of ectopic recombination in meioses has been the examination of recombination between repeated sequences located at slightly staggered sites (LEWIS 1941; JUDD 1965; GREEN 1966). STURTEVANT (1925) provided the original demonstration of unequal crossing over. In that work he utilized a tandem duplication, the *Bar* mutation, and showed that meiotic pairing could occur when sequences were out of register. This generated further amplification or reduction of the originally duplicated segment that caused variation in the Bar phenotype. It is probable that the original *Bar* duplication itself arose by ectopic recombination between transposons inserted at slightly different locations on the *X* chromosome (TSUBOTA *et al.* 1989). It is clear that ectopic recombination must occur naturally and has the potential to provide genetic duplications that can be acted on by natural selection to produce further variation. In fact, it has been suggested that the typically deleterious consequences of rearrangements generated by ectopic recombination serve to limit the spread of transposable elements (MONTGOMERY *et al.* 1987). Thus, ectopic recombination is likely to be of fundamental importance in evolution.

It would be of interest to know whether the frequency of meiotic ectopic exchange varies with the distance between the elements of homology as we find for mitotic cells. Several workers have suggested that this is the case (GOLDBERG *et al.* 1983; DAVIS *et al.* 1987; HIPEAU-JACQUOTTE *et al.* 1989), but convincing evidence is lacking in Drosophila. However, GOLDMAN and LICHTEN (1996) have recently shown that the efficiency of ectopic recombination in yeast is correlated with the proximity of the recombining sequences.

One very interesting difference between our mitotic results and the meiotic results in Drosophila comes from the work of MONTGOMERY *et al.* (1991). They screened for ectopic exchanges in the vicinity of the *white* locus. The majority of such exchanges occurred between dispersed copies of the transposon *roo*. The frequency of such exchange was much higher in flies that were heterozygous for two *X* chromosomes with copies of *roo* at different locations than it was in flies that were homozygous for either of those chromosomes. They suggested that ectopic exchange is low if an insertion is homozygous because it will then pair preferentially with the allelic insertion. If an insertion is hemizygous then it is available for ectopic exchange. If this were true for FLP-mediated mitotic exchange, then the experiment of Figure 7 should show more recombination in the hemizygotes than in the homozygotes, but the opposite is true. Perhaps this reflects an allowance in mitotic cells for the multiple association of more than two copies.

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