Transgene Coplacement and High Efficiency Site-Specific Recombination With the Cre/*loxP* System in Drosophila

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ABSTRACT

Studies of gene function and regulation in transgenic Drosophila are often compromised by the possibility of genomic position effects on gene expression. We have developed a method, called transgene coplacement, in which any two sequences can be positioned at exactly the same site and orientation in the genome. Transgene coplacement makes use of the bacteriophage P1 system of Cre/*loxP* site-specific recombination, which we have introduced into Drosophila. In the presence of a *cre* transgene driven by a dual *hsp70-Mos1* promoter, a *white* reporter gene flanked by *loxP* sites is excised with virtually 100% efficiency both in somatic cells and in germ cells. A strong maternal effect, resulting from Cre recombinase present in the oocyte, is observed as white or mosaic eye color in F_1 progeny. Excision in germ cells of the F_1 yields a strong grand-maternal effect, observed as a highly skewed ratio of eye-color phenotypes in the F_2 generation. The excision reactions of Cre/*loxP* and the related FLP/*FRT* system are used to create Drosophila lines in which transgenes are at exactly allelic sites in homologous chromosomes.

THE practical benefits of an efficient targeted-integration system in Drosophila would be extensive. Because of position effects on transgenes, expression can differ quantitatively and qualitatively among transformants of the same construct (LAURIE-AHLBERG and STAM 1987; BRENNAN and DICKINSON 1988). If it were possible to compare transgenes at the same position in the genome, fewer transformants would need to be analyzed in experiments in which subtle differences in expression between transgenes are to be detected. Indeed, some experiments demand that the analyzed transgenes be present in the same position in the genome. For example, when it is the magnitude of position effect itself that is of interest (HENIKOFF 1994; REUTER and SPIERER 1992), it would be of great use to be able to target genes modified *in vitro* to various genomic positions. Likewise, transvection effects and other pairing-dependent phenomena (WU 1993; HENIKOFF 1994) could also be studied more effectively with the ability to generate allelic transgenes. Finally, in evolutionary genetic studies in which relative fitness of genotypes is assayed by competition in experimental populations, it is an absolute requirement that genes segregate from one another. Therefore, these competition experiments have been limited to studies of preexisting intraspecific variation (ÁRNASON 1991) or of mitochondrial haplotypes introduced by introgression (HUTTER and RAND 1995). The ability to generate allelic transgenes would open up such experiments to comparisons between interspecific homologues and between genes modified *in vitro*. It would also make easier the implementation and interpretation of studies of intraspecific variation, by eliminating the confounding effect of initial linkage disequilibrium. We report here a novel method to create allelic transgenes in Drosophila that relies only on *P*-element-mediated germline transformation and two site-specific intramolecular recombination reactions. This method is termed transgene coplacement.

One component of transgene coplacement is the Cre/*loxP* site-specific recombination system from bacteriophage P1. The 38-kD Cre ("*c*auses *re*combination") recombinase and its 34-bp *loxP* ["*lo*cus of crossing over(*x*), *P*1"] target sequence provide the circular bacteriophage P1 with an efficient means of maintaining unit-copy replicons in *Escherichia coli* (AUSTIN *et al.* 1981). After replication of the phage DNA, dimerization by homologous recombination of daughter copies would preclude faithful partitioning of the virus were it not for the efficient Cre-catalyzed intramolecular recombination that breaks down phage plasmid dimers into monomers each containing a single *loxP* site. The *loxP* site consists of 13-bp inverted repeats separated by an asymmetric 8-bp spacer region, which gives the site its directionality. Cre-mediated recombination excises intervening DNA between *loxP* sites in direct orientation and inverts DNA between inverted *loxP* sites. All recombination events reconstitute intact *loxP* sites and are therefore reversible. Despite its prokaryotic source, the Cre/*loxP* system has been shown to be active in eukaryotic systems (SAUER 1987; SAUER and HENDERSON 1988; ODELL *et al.* 1990). In yeast, plant, and mamma-

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lian cells, intermolecular integration as well as intramolecular excision events have been observed (SAUER and HENDERSON 1990; ALBERT *et al.* 1995).

To improve the likelihood of efficiently producing translatable *cre* mRNA in Drosophila, we constructed a chimeric *cre* gene in which the translation initiation and polyadenylation signal sequences were donated by the active *mariner* transposable element, *Mos1* (MEDHORA *et al.* 1988), and the promoter was a fusion of the *hsp70* promoter (MCGARRY and LINDQUIST 1985) and the *Mos1* promoter. The *hsp70-Mos1* fusion promoter has been shown to be active in both somatic and germline tissues without heat shock (LOHE *et al.* 1995). In addition, *Mos1* contains an exact match to the Drosophila translation initiation consensus sequence (CAVENER 1987).

To assay the function in Drosophila of the chimeric *cre* gene, we constructed a mini-*white* gene flanked by direct *loxP* repeats. This target gene serves as a sensitive and easily scored reporter both because of its cell-autonomous action and because the intensity of pigmentation differs with the number of gene doses present in a given cell. The *cre* gene and the *loxP*-flanked reporter gene were transformed separately into the germline of *Drosophila melanogaster* by *P*-element-mediated transformation. Lines derived from each of these transformations were crossed together to bring the recombinase and its target together. If, in the presence of active Cre, the *loxP*-flanked mini-*white* gene is excised, the resulting pigment cells will lack the reporter gene and therefore be white. If Cre is active in the germline, excision of the reporter gene will result in white-eyed progeny. Using this mini-*white* reporter system, and confirming site-specific recombination by molecular analysis, we demonstrate that Cre is active with extremely high efficiency in the Drosophila soma and germline.

The second component of transgene coplacement is the FLP/*FRT* site-specific recombination system from the 2μ plasmid of *Saccharomyces cerevisiae,* which has been introduced into Drosophila and shown to mediate site-specific excisions, duplications, inversions, and sister-chromatid exchanges (GOLIC and LINDQUIST 1989; GOLIC 1991, 1994). The vector for transgene coplacement includes two alternating pairs of *loxP* and *FRT* sites, a mini-*white* marker for transformation, and two strategically placed cloning sites for introducing the transgenes of interest. Once introduced into the genome by *P*-element-mediated transformation, passage through a Cre-producing strain eliminates one transgene and passage through a FLP-producing strain eliminates the other. In both cases, the mini-*white* marker is eliminated as well, thereby providing an easily scored reversion phenotype that accompanies transgene excision. The vector is therefore called a "waffle" vector because it allows one to choose either of two possibilities (and also for its component parts: *white, FRT,* and *loxP*). The end result is the creation of a pair of homologous chromosomes with the alternative transgenes present in exactly allelic sites and in the same orientation. A practical application of the method is demonstrated in transgene coplacement of the alcohol dehydrogenase (*Adh*) genes of *D. melanogaster* and *D. affinidisjuncta.*

MATERIALS AND METHODS

Plasmid construction: The construction of the chimeric *cre* gene was as follows. An *Afl*III site was created at the initiation codon of *cre* coding sequence by PCR amplification of the *cre*-encoding plasmid pRH200 (MACK *et al.* 1992; thanks to RON HOESS) with a mutagenic upper primer that created an A-to-C substitution at the nucleotide position immediately upstream of the initiation codon (A**ATGT** → A**CATGT**). The 148-bp product was rendered blunt-ended with T4 polymerase and ligated into *Hin*cII-cut pK19 (PRIDMORE 1987; thanks to ANDY COCKBURN), creating plasmid pMLS201. Sequencing confirmed that no changes were made in the sequence except the desired one. A 149-bp fragment generated by complete *Sph*I and partial *Afl*III digestion of pMLS201 was ligated with a 2.5-kb fragment generated by complete *Sph*I and partial *Afl*III digestion of plasmid pMLS100, creating pMLS101. pMLS100 had been created by subcloning the 1-kb *Eco*RI-*Sph*I fragment from pAd31-*hsp70:Mos1* (LOHE *et al.* 1995) into pSP72 (Promega Corporation, Madison, WI), thus reducing the number of nuisance *Afl*III sites to deal with in subsequent steps. This 1-kb fragment contains the 5′ end of the *Mos1* element, including the *Mos1* promoter and most of the *Mos1* coding sequence but neither inverted repeat. The 260-bp *Eco*RI-*Sph*I fragment from pMLS101 was inserted into *Eco*RI/*Sph*I-cut pAd31-*hsp70:Mos1,* thus reconstituting the fusion *hsp70/Mos1* promoter upstream of the 5′ portion of the *cre* coding sequence, creating plasmid pMLS102. The remainder of the *cre* coding sequence was inserted into pMLS102 as an *Age*I-*Sph*I fragment from pMLS200 (a plasmid created by inserting a stuffer fragment containing a *Sph*I site into the *Eco*RI/*Mlu*I sites of pRH200), creating plasmid pMLS103. To bring the putative polyadenylation signal sequence of *Mos1* adjacent to the stop codon of *cre,* pMLS103 was digested completely with *Mlu*I and partially with *Nhe*I, then rendered blunt-ended with the Klenow fragment of DNA polymerase I, and religated to itself. The desired plasmid, designated pMLS104, was verified by a series of test digests including *Nhe*I, which cut both at the intact *Nhe*I site 3′ to the polyadenylation signal and at the *Nhe*I site 5′ to the polyadenylation signal, a site generated by ligation of blunt-ended *Nhe*I and *Mlu*I sites. In pMLS104, the polyadenylation signal is 33 bp downstream of the *cre* stop codon. Finally, two *P*-element constructs, shown in Figure 1, were made, joining the chimeric *cre* gene with either the intronless *yellow* or mini-*white* selectable markers. p*P*[y^+, *cre*] was constructed by cloning the *Not*I-*Xho*I fragment from pMLS104 into *Not*I/*Xho*I-cut pCar-y, a derivative of Carnegie 4 with the intronless *yellow* gene as a marker (PATTON *et al.* 1992; kindly provided by PAM GEYER). p*P*[w^+, *cre*] was constructed by cloning the *Not*I-*Kpn*I fragment from pMLS104 into *Not*I/*Kpn*I-cut pCaSpeR4, a derivative of pCaSpeR (PIRROTTA 1988).

p*P*[$\hat{}\,w^+\,\hat{}$] was constructed by inserting two *loxP* sites in direct orientation into pCaSpeR4, flanking the mini-*white* gene, as shown in Figure 1. The first site was introduced as a *loxP*-containing *Xba*I-*Eco*RI fragment from pRH560 (courtesy of RON HOESS) into pCaSpeR4, creating plasmid p*P*[$\hat{}\,w^+$]. The second site was placed between the end of the mini-*white* gene and the 5′ *P* element sequences in p*P*[$\hat{}\,w^+$] at the unique *Nsi*I site, as a *loxP*-containing *Pst*I fragment. Proper orientation was confirmed by *Hin*dIII/*Xba*I and *Hin*dIII/*Eco*RI double digests. Presence of the directly-repeated *loxP* sites was also confirmed by transforming p*P*[$\hat{}\,w\,\hat{}$] into *E. coli* strain NS2114

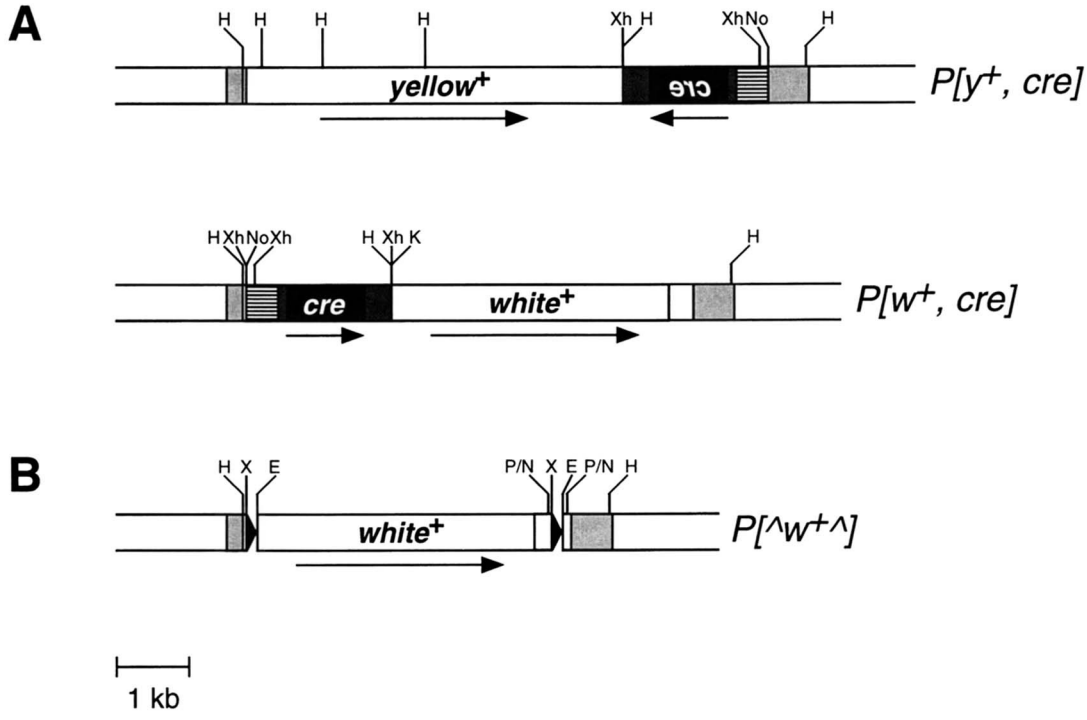


FIGURE 1.—Cre, *loxP* constructs to assay expression in Drosophila. (A) The chimeric *cre* gene, consisting of Cre coding sequence (black, arrow shows direction of transcription), *Mos1* promoter and downstream sequences (dark shading), and *hsp70* promoter (horizontal bars). In p*P*[y^+, *cre*], this chimeric gene is inserted into the *Not*I and *Xho*I sites of pCar-y. In p*P*[w^+, *cre*], the chimeric gene is inserted into the *Not*I and *Kpn*I sites of pCaSpeR4. *P*-element sequences are shown in light shading. (B) *P*[$\hat{}\, w^+ \hat{}$], showing *loxP* sites (solid, not to scale with rest of diagram), and *P*-element sequences (light shading). Directionality of *loxP* sites is indicated by representation as arrowheads. Restriction sites indicated are No, *Not*I; X, *Xba*I; E, *Eco*RI; P, *Pst*I; N, *Nsi*I; H, *Hin*dIII; Xh, *Xho*I; and K, *Kpn*I.

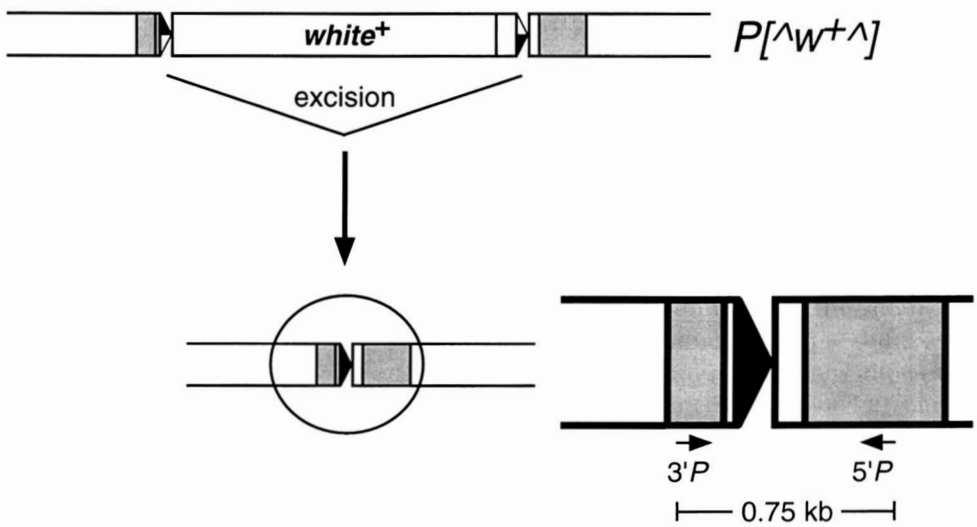
(STERNBERG *et al.* 1986; thanks to RON HOESS), which constitutively expresses Cre, extracting plasmid DNA, and observing the deletion product on an agarose gel. This method was also used to generate positive control DNA for PCR.

The vector p*P*[*wFl*], shown in Figure 7, was constructed by adding two *loxP* sites and two *FRT* sites to pCaSpeR4, such that two unique cloning sites remain for the addition of two alleles. Details of construction of the vector are available from the authors and on our World Wide Web site at http://www.oeb.harvard.edu/hartl/lab/. p*P*[$\hat{}\, a{>}w^+ \hat{}\, m{>}$], the derivative of p*P*[*wFl*] used for the polytene chromosome *in situ* hybridizations, carries a Slow allele of the *Adh* gene from *D. melanogaster* (LAURIE-AHLBERG and STAM 1987; kindly provided by the authors) cloned into the *Xho*I site and the *Adh* gene from *D. affinidisjuncta* (BRENNAN and DICKINSON 1988; kindly provided by MARK BRENNAN) cloned into the *Not*I/*Spe*I sites. All restriction enzymes were supplied by New England Biolabs, Inc. (Beverly, MA).

Drosophila transformation and fly stocks: *P*-element-mediated transformation of Drosophila was carried out by standard means (RUBIN and SPRADLING 1982; SPRADLING and RUBIN 1982) using helper plasmid pπ25.7wc (KARESS and RUBIN 1984). The host strain was w^{1118} for *P*[w^+, *cre*] and *P*[$\hat{}\, w^+ \hat{}$], and was *Df(1)w, y* w^{67c23} for *P*[y^+, *cre*] and *P*[$\hat{}\, a{>}w^+ \hat{}\, m{>}$]. Surviving G_0 adults were backcrossed individually to host-strain partners, and G_1 flies were scored for w^+ *or* y^+ phenotype. Single-insertion transformants were assigned to chromosomes based upon segregation against dominantly marked balancers and were maintained either as homozygous or balanced stocks. The second-chromosome insertion of *P*[$\hat{}\, w^+ \hat{}$] used in this study ($\hat{}\, w^+ \hat{}\, 2$) was made homozygous on a *y w* background by crossing with *y w;Cyo/Sco* females, mating *Cy* F_1, and selecting *y* Cy^+ F_2 to found the line. The *TM6B, cre*w3 insertion of *P*[w^+, *cre*] was generated by mobilizing the *cre*w2 insertion with the stable genomic source of *P* transposase, Δ2-3(99B) (ROBERTSON *et al.* 1988). The *MKRS, hsFLP* genotype was obtained from the Bloomington Stock Center. Flies were maintained on standard cornmeal-molasses medium and at 25°. Sixty-minute heat shocks for FLP expression were performed on first-instar larvae in glass vials in a 38° water bath. Descriptions of mutants and special chromosomes are found in LINDSLEY and ZIMM (1992).

PCR amplification and sequencing: Single flies were prepared for PCR amplification by the method of GLOOR and ENGELS (1991). PCR for the excision assay was carried out under standard conditions in 20-μl volumes with *Taq* polymerase (Boehringer Mannheim Corporation, Indianapolis, IN) and 1× buffer with Mg^{2+} provided by the supplier. Thirty cycles of 15 sec at 94°, 15 sec at 57°, and 2 min at 72°, followed by 5 min at 72° and storage at 4° were performed in a Perkin Elmer (Foster City, CA) DNA Thermal Cycler 480. A pair of oligonucleotide primers complimentary to the 3′ and 5′ *P*-element ends was used in all reactions. The sequences of the primers are: 5′-AGCATGTCCGTGGGGTTTGA-3′ ("3′*P*") and 5′-CTTGGGTGCAGCCTTGGTGA-3′ ("5′*P*"). GIBCO BRL (Gaithersburg, MD) supplied the 1-kb ladder. Amplified products were cloned into the vector pCRII (Invitrogen Corporation, San Diego, CA) according to manufacturer's instructions. DNA sequencing of plasmid inserts was performed using DyeDeoxy terminators and an ABI 373A automated sequencing machine (Applied Biosystems, Inc., Foster City, CA).

Polytene chromosome fluorescent *in situ* hybridization: Polytene chromosome *in situ* hybridizations were carried out by standard means. Chromosomes were stained with DAPI and probes were labeled by extension of random hexamer primers with either fluorescein-12-dUTP (*D. affinidisjuncta Adh*) or tetramethylrhodamine-6-dUTP (*D. melanogaster Adh*) supplied by Boehringer Mannheim. Chromosome preparations were



CGTTCGAGGTCGACTCTAGAGGATCT**ATAACTTCGTATAGCATACATTATACGAAGTTAT**AGATCCCCGGGTACCGAGCTCGAATTCTAGTATGTATG

AGATGCAGGTCGACTCTAGAGGATCT**ATAACTTCGTATAGCATACATTATACGAAGTTAT**AGATCCCCGGGTACCGAGCTCGAATTCCTGCATCTCAA

CGTTCGAGGTCGACTCTAGAGGATCT**ATAACTTCGTATAGCATACATTATACGAAGTTAT**AGATCCCCGGGTACCGAGCTCGAATTCCTGCATCTCAA

FIGURE 2.—Excision product of Cre recombination. Excision of mini-*white* from $P[^{\wedge}w^{+}{}^{\wedge}]$ yields the deletion product encircled and shown enlarged at right. Positions of primers 3′*P* and 5′*P*, which amplify a 0.75-kb product on the recombined template, are shown. Nucleotide sequences flanking *loxP* sites in the original and recombined constructs are given below the diagram with individual sites identified by different shading of the arrowhead symbols. Bold text indicates the 34-bp *loxP* site. Underlined text identifies those sequences that comprise (or are destined to comprise) the recombined template. *P*-element sequences are shown shaded.

visualized under a fluorescent microscope (Olympus Corporation, Lake Success, NY) equipped with DAPI, FITC, and TRITC filters.

RESULTS

Cre-induced somatic and germline excision: To assay Cre function in transgenic Drosophila, we performed a series of genetic crosses in which a source of Cre was brought together with the reporter target. The two sources of Cre, $P[y^{+}, cre]$ and $P[w^{+}, cre]$, are shown in Figure 1, along with the *loxP*-flanked mini-*white* target, $P[^{\wedge}w^{+}{}^{\wedge}]$. The caret (^) notation for *loxP* sites in the latter construct is patterned after that of GOLIC and LINDQUIST (1989), who use a ">" to represent the *FRT* target site of FLP recombinase. The notation allows direct *loxP* repeats to be denoted "^ ^" and inverted repeats to be denoted "^ v" For ease of reference, we use the following notation:

- *cre*y1a and *cre*y1b denote two independent insertions of $P[y^{+}, cre]$ into the *X* chromosome
- *cre*w2 denotes an insertion of $P[w^{+}, cre]$ into chromosome *2*
- $^{\wedge}w^{+}{}^{\wedge}1$ denotes an insertion of the $P[^{\wedge}w^{+}{}^{\wedge}]$ reporter into the *X* chromosome
- $^{\wedge}w^{+}{}^{\wedge}2$ denotes an insertion of the $P[^{\wedge}w^{+}{}^{\wedge}]$ reporter into chromosome *2*

Other insertions, into each major chromosome, of the recombinase and its target, were also examined and found to behave in a manner completely consistent with those listed above. In total, 10 insertions of $P[y^{+}, cre]$, six insertions of $P[w^{+}, cre]$, and five insertions of $P[^{\wedge}w^{+}{}^{\wedge}]$ have been tested.

Cre activity was assayed in the soma, by scoring eye color as w^{+} (no apparent excision), w^{-} (complete excision), or mosaic (excision in part of the eye), and in the germline, by scoring progeny eye color as w^{+} (not excised), w^{-} (excised), or $w^{+} \cdot w^{+}$ (apparently duplicated). This last category corresponds to an eye phenotype darker than w^{+} and is reminiscent of the FLP-induced duplications observed by GOLIC and LINDQUIST (1989). As explained by those authors, such duplications are presumably produced either by unequal sister-chromatid exchanges at mispaired recombinase target sites or by integration of a recombinase-excised circular DNA molecule into one of the recombinase target sites of the sister chromatid. In addition to a genotypic effect of *cre*, a maternal effect of *cre* was observed in the cre^{-} progeny of cre^{+}/cre^{-} females. Therefore, scored flies were classified according to which parent was heterozygous for *cre* and to whether *cre* was inherited from this parent. Including the formal possibility of a paternal effect due to Cre activity supplied by a cre^{-} sperm, there are four potential modes of Cre transmission: "genotypic + paternal" (*cre* inherited from male parent), "genotypic + maternal" (*cre* inherited from female parent), "paternal" (*cre* not inherited from male

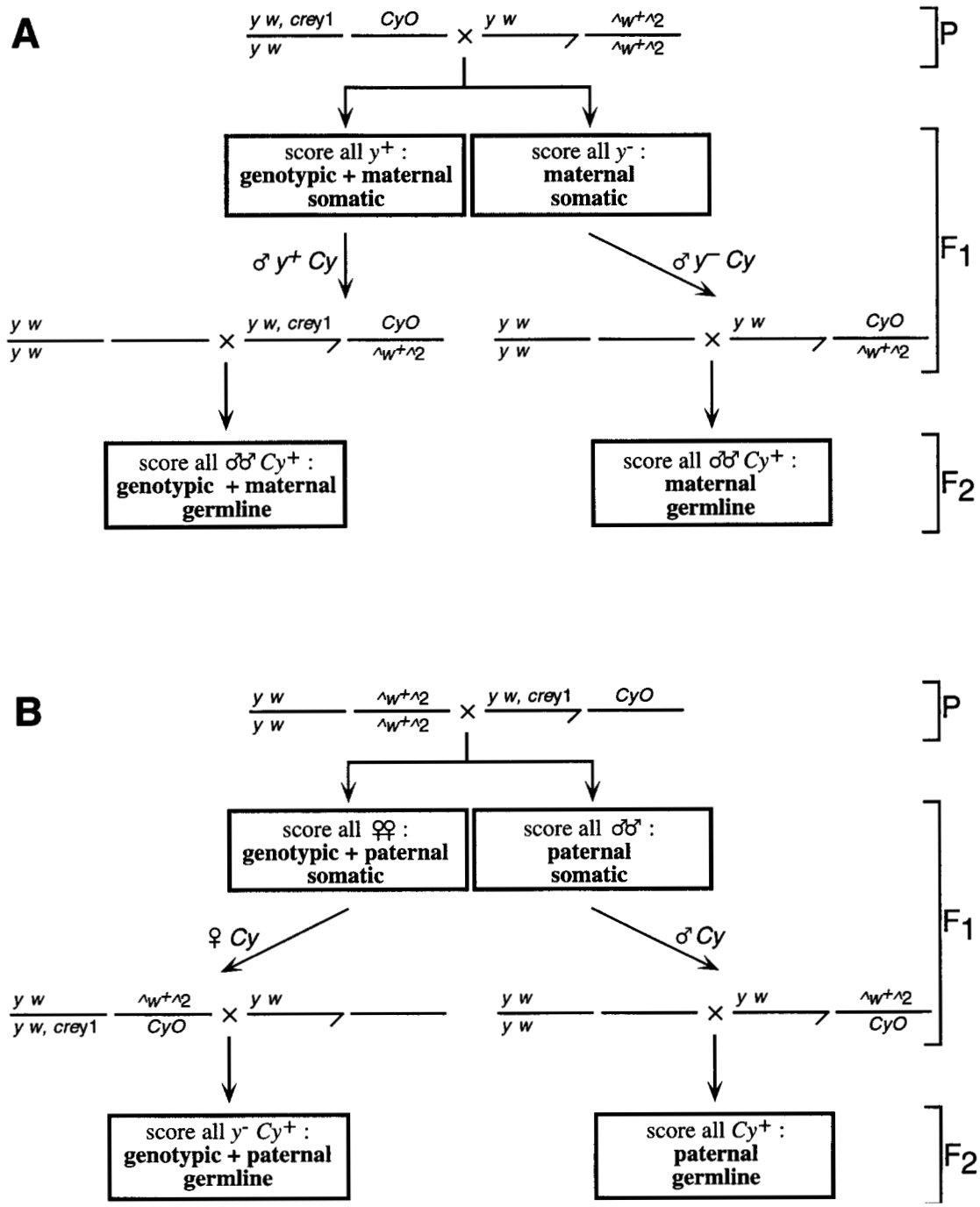


FIGURE 3.—Crosses to assay somatic and germline activity of $P[y^+, cre]$. Crosses described in the text are diagrammed. Flies scored for either somatic or germline activity of Cre are characterized by mode of Cre transmission. As described in the text, A and B depict reciprocal experiments used to assay these various modes of transmission. Data from these crosses are tabulated in Table 1, and representative flies from the somatic assay are shown in Figure 5.

heterozygote), and "maternal" (*cre* not inherited from female heterozygote).

As shown in Figure 2, excision of the $^w+^$ cassette yields a characteristic footprint. PCR amplification of single-fly genomic DNA with primers directed inward across each *loxP* site in the $P[^w+^]$ construct was used to confirm site-specific recombination in flies selected by phenotypic assay (refer to Figure 6 for PCR data from the following crosses). In addition, several independent excision events were characterized by nucleotide sequencing of cloned amplification products. In each case, the sequence flanking and including the single remaining *loxP* site exactly matched that expected upon site-specific Cre-mediated recombination (see Figure 2).

The crosses, diagrammed in Figures 3 and 4, were as follows. The data for these and all subsequent crosses are tabulated in Table 1, and representative flies are shown in Figure 5. Females of genotype *y w, crey1/y w;CyO/+* were crossed with males of genotype *y w/Y;^w+^2/^w+^2* (Figure 3A, P generation). All y^+ F_1

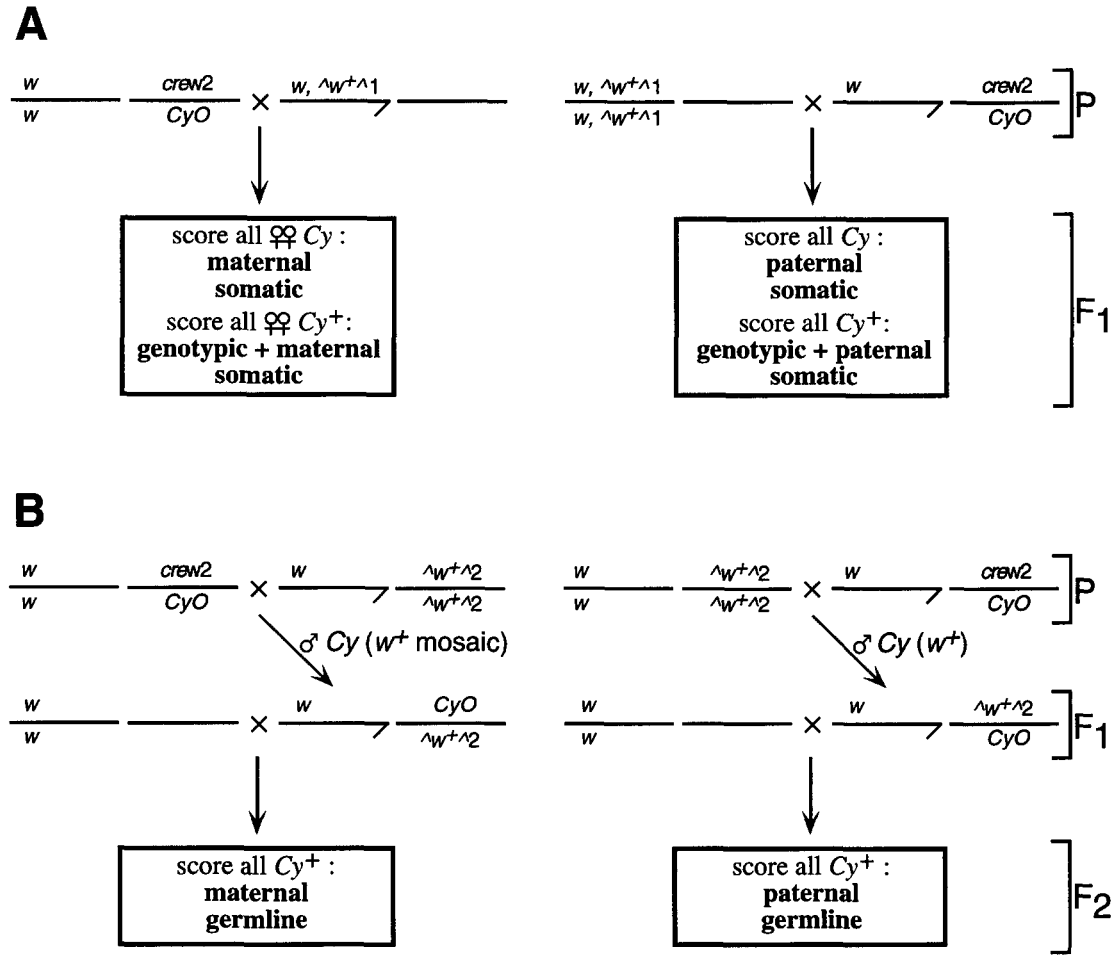


FIGURE 4.—Crosses to assay somatic and germline activity of *P*[w^+, *cre*]. Crosses described in the text are diagrammed. (A) Reciprocal crosses shown that were used to assay the somatic effect of various modes of Cre transmission. Representative flies are shown in Figure 5. (B) Experiment to assay the maternal effect of *P*[w^+, *cre*] in the germline (the grand-maternal effect) shown as well as a reciprocal experiment to assay the grand-paternal effect. Data from the crosses in A and B are tabulated in Table 1.

progeny were scored for genotypic + maternal effect of Cre in the soma; all y^- F_1 progeny were scored for maternal effect of Cre in the soma. Individual curly-winged male F_1 progeny were crossed with *y w* females to assay germline activity of Cre. Genotypic + maternal effect of Cre in the germline was determined from the Cy^+ male F_2 progeny of *y w, cre*y1*/Y;CyO/*^w^+^2 males; maternal effect of Cre in the germline was determined from the Cy^+ male F_2 progeny of *y w/;CyO/*^w^+^2 males (Cy^+ female progeny were not scored in the latter cross because w^+ females could not reliably be distinguished from $w^+ \cdot w^+$ females).

A set of crosses reciprocal to those just described were performed to assay the somatic and germline effects of the other modes of Cre transmission. Females of genotype *y w/y w;*^w^+^2/^w^+^2 were crossed with males of genotype *y w, cre*y1*/Y;CyO/+* (Figure 3B, P generation). Female F_1 progeny were scored for genotypic effect of Cre in the soma; male F_1 progeny were scored for paternal effect of Cre in the soma. Individual curly-winged female F_1 progeny, of genotype *y w/y w, cre*y1*;*^w^+^2*/CyO,* were crossed with *y w* males, and all y^- Cy^+ F_2 progeny were scored for genotypic + paternal Cre effect in the germline. Individual curly-winged F_1 males, of genotype *y w/Y;*^w^+^*/CyO,* were crossed with *y w* females, and all Cy^+ F_2 progeny were scored for paternal Cre effect in the germline. The maternal somatic effect did not interfere with scoring the genotypic + paternal germline effect because the maternal effect of *P*[y^+, *cre*] is weak and has not been observed to abolish reporter expression completely (Table 1).

As can be seen in Table 1, the *P*[y^+, *cre*] source of Cre has a potent genotypic effect in both the soma and germline. Reporter expression is completely lacking in virtually all flies possessing *cre,* with rare mosaic exceptions having small patches of reporter expression on an otherwise completely white eye. The maternal effect is less pronounced, with only a small fraction of flies displaying sectors of white in the eye, and a slightly larger fraction of flies having germline excision events. This is in marked contrast to the strong maternal effect of *P*[w^+, *cre*], crosses for which are described below. Another interesting contrast is that the only observed duplications of the mini-*white* cassette were for the inter-

TABLE 1

Efficiency of somatic and germline excision of $P[\hat{}w^{+}\hat{}]$

Mode of Cre transmission	*cre* source	w^{-}	w^{+} mosaic	w^{+}
		Somatic excision		
Genotypic + paternal	*cre*y1a	301 (98.0)	6 (2.0)	0 (0.0)
	*cre*y1b	335 (100.0)	0 (0.0)	0 (0.0)
	*crew*2	321 (99.1)	3 (0.9)	0 (0.0)
Genotypic + maternal	*cre*y1a	176 (100.0)	0 (0.0)	0 (0.0)
	*cre*y1b	198 (100.0)	0 (0.0)	0 (0.0)
	*crew*2	315 (99.4)	2 (0.6)	0 (0.0)
Maternal	*cre*y1a	0 (0.0)	12 (6.3)	177 (93.7)
	*cre*y1b	0 (0.0)	0 (0.0)	192 (100.0)
	*crew*2	1 (0.3)	302 (99.7)	0 (0.0)
Paternal	*cre*y1a	0 (0.0)	0 (0.0)	314 (100.0)
	*cre*y1b	0 (0.0)	0 (0.0)	328 (100.0)
	*crew*2	0 (0.0)	0 (0.0)	306 (100.0)
Mode of Cre transmission	*cre* source	*N*	w^{-}	w^{+}	$w^{+} \cdot w^{+}$
		Germline excision			
Genotypic + paternal	*cre*y1a	5	181 (97.3)	3 (1.6)	2 (1.1)
	*cre*y1b	5	180 (100.0)	0 (0.0)	0 (0.0)
Genotypic + maternal	*cre*y1a	5	175 (100.0)	0 (0.0)	0 (0.0)
	*cre*y1b	5	189 (100.0)	0 (0.0)	0 (0.0)
Maternal	*cre*y1a	5	34 (19.3)	135 (76.7)	7 (4.0)
	*cre*y1b	5	7 (3.1)	220 (96.9)	0 (0.0)
	*crew*2	10	726 (90.4)	77 (9.6)	0 (0.0)
Paternal	*cre*y1a	5	0 (0.0)	306 (100.0)	0 (0.0)
	*cre*y1b	5	0 (0.0)	309 (100.0)	0 (0.0)
	*crew*2	5	0 (0.0)	285 (100.0)	0 (0.0)

Values are absolute numbers with percentages in parentheses for each data class. For germline excision, the number of individual parents tested is given in the column labeled *N*. Mode of Cre transmission and *cre* source are explained in the text.

mediate maternal effect of *cre*y1a, suggesting that a high dose of Cre (provided either genotypically or maternally) favors excision over reintegration.

Context-dependent maternal effect of the *cre* transgene: A second *cre* transgene, using mini-*white* as the selectable marker instead of *yellow*, gives a pronounced maternal effect in addition to a potent genotypic effect. The insertion of $P[w^{+}, cre]$ used, *crew*2, yields a pale-yellow eye color, upon which orange or red $P[\hat{}w^{+}\hat{}]$ expression is superimposed. To assay somatic activity of Cre from *crew*2, reciprocal crosses were performed with genotypes *w; crew*2/*CyO* and *w*, $\hat{}w^{+}\hat{}1;+/+$ (Figure 4A, P generation). Cy^{+} F_1 progeny of *crew*2/*CyO* male parents were scored for genotypic + paternal effect; *Cy* F_1 progeny of *crew*2/*CyO* male parents were scored for paternal effect. Female Cy^{+} F_1 progeny of *crew*2/*CyO* female parents were scored for genotypic + maternal effect; female *Cy* F_1 progeny of *crew*2/*CyO* female parents were scored for maternal effect. The results, shown in Table 1, indicate a strong maternal effect in addition to the potent genotypic effect. Virtually all flies scored for maternal effect had mosaic eye color, examples of which are shown in Figure 5. Note that the existence of white patches in these mosaic eyes confirms that the *cre* transgene is not present, and therefore that the effect is truly maternal. In addition, the absence of an observed paternal effect in the reciprocal cross argues against the presence of a cryptic *cre* transgene segregating in the *crew*2 stock and, for that matter, every other $P[w^{+}, cre]$ stock tested. Consistent with the observation of apparent $\hat{}w^{+}\hat{}$ duplications in the germline and with the observations of GOLIC and LINDQUIST (1989), some mosaic eyes exhibited patches of darker color, indicative of duplication of the mini-*white* cassette in the corresponding cell lineage. Such mosaics account for ~1% of the maternal-effect flies.

To assay the maternal effect of $P[w^{+}, cre]$ in the germline (*i.e.*, a grand-maternal effect), females of genotype *w*/*w; crew*2/*CyO* were crossed with males of genotype $w/Y;\hat{}w^{+}\hat{}2/\hat{}w^{+}\hat{}2$ (P generation) and curly-winged male F_1 progeny were crossed with *w* females (Figure 4B). In the absence of Cre activity, Cy^{+} F_2 progeny are expected to be w^{+} (Figure 6). However, only 9.6% of such progeny retained the mini-*white* reporter gene (Table 1), indicating a strong grand-maternal effect of $P[w^{+}, cre]$. As expected, in the reciprocal assay for a "grand-paternal" effect shown in Figure 4B, 100% of Cy^{+} F_2 progeny were w^{+}.

Transgene coplacement with two site-specific recombinases: To create Drosophila strains in which either

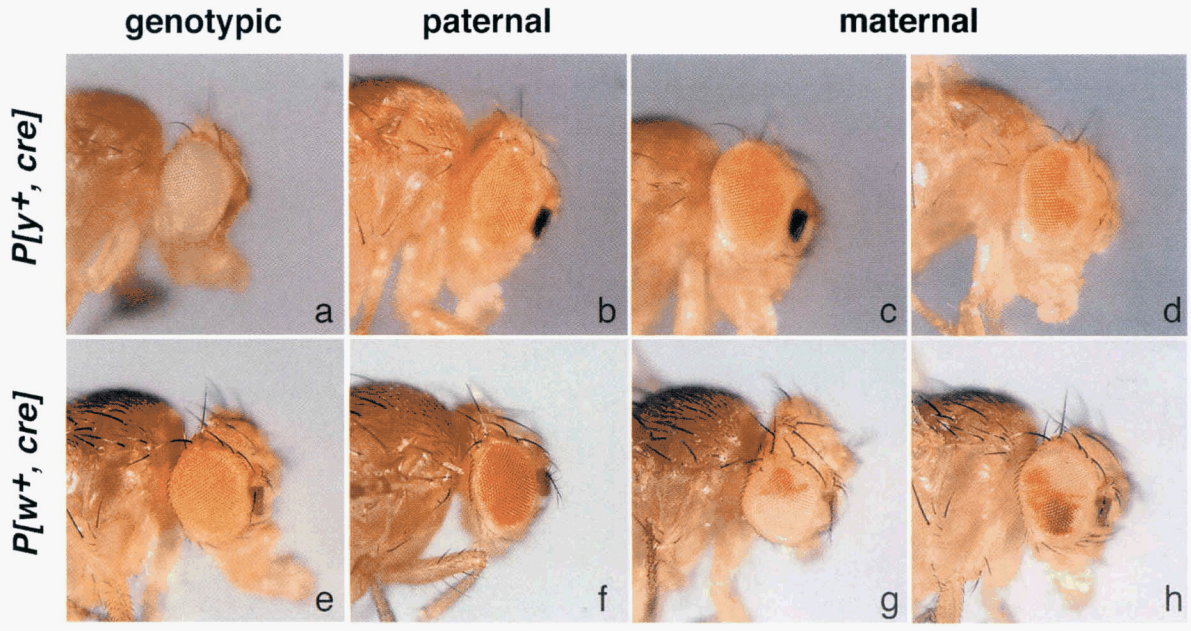


FIGURE 5.—Genotypic and maternal effects of Cre activity in the soma. Shown are representative flies derived from crosses of one parent heterozygous (or hemizygous) for *cre* and the other parent homozygous (or hemizygous) for $P[\hat{}w^{+}\hat{}]$. Mode of Cre transmission and source of Cre (see text) are indicated along the top and left edges of the figure, respectively. "Paternal" *cre* transmission effectively represents absence of Cre activity, so that the flies in the second column demonstrate the eye color associated with $^{\wedge}w^{+}{}^{\wedge}2$ (b) and $^{\wedge}w^{+}{}^{\wedge}1$ (f). The genotypic effect of $P[y^{+}, cre]$ yields an overwhelming majority of white-eyed flies (a), whereas the similarly potent $P[w^{+}, cre]$ produces yellow-eyed progeny (e), which lack the strong mini-*white* expression of $^{\wedge}w^{+}{}^{\wedge}1$ but have weak expression from the mini-*white* marker linked to *cre*. The maternal effect of $P[y^{+}, cre]$ is not strong—most flies show no sign of $^{\wedge}w^{+}{}^{\wedge}$ excision in the eye (c) but some have sectors of white (d). In contrast, the maternal effect of $P[w^{+}, cre]$ is considerable—most flies retain only small sectors of red (g), and some eyes manifest apparent duplications of the mini-*white* target (h).

the *D. melanogaster* or the *D. affinidisjuncta Adh* gene is present at a given locus of *P*-element insertion, we constructed a *P*-element vector into which both *Adh* genes could be cloned, such that Cre-mediated recombination would excise one gene, whereas FLP-mediated recombination would excise the other. In addition, as shown in Figure 7, the vector, p*P*[*wFl*], contains the mini-*white* selectable marker, which is excised upon recombination by either recombinase. Thus, mini-*white* serves as a marker both for transformation and for excision. The *loxP* and *FRT* sites are configured so that the same sequences flank whichever transgene remains after recombinase-mediated excision. The system thus permits the efficient generation of strains carrying one or the other of two transgenes in the same genomic position, flanked by the same sequences. The ability easily to construct such strains will benefit research into such phenomena as position-effect variegation and transvection and will advance our own studies of the evolution of regulatory variation by allowing estimation of the fitness effects associated with replacing a *D. melanogaster* gene with its homologue from another Drosophila species.

Our waffle vector with *Adh* insertions, $pP[^{\wedge}a{>}w^{+}{}^{\wedge}m{>}]$, was introduced into flies by standard *P*-element-mediated germline transformation. The *D. affinidisjuncta* gene is represented by "*a*" and the *D. melanogaster* gene is represented by "*m*." Out of 133 fertile G_0 adults, 17 (12.8%) produced G_1 progeny transformed with the 20-kb construct. Homozygous third-chromosome transformant lines were subjected separately to Cre and to FLP by crossing with males of genotype *w;MKRS, hsFLP/TM6B, cre*w3. Progeny inheriting the *MKRS, hsFLP* chromosome were used to derive excisions of $>w^{+}{}^{\wedge}m>$, leaving $P[\hat{}a>]$; progeny inheriting the *TM6B, cre*w3 chromosome were used to derive excisions of $^{\wedge}a{>}w^{+}{}^{\wedge}$, leaving $P[^{\wedge}m>]$. Both excision events were scored by reversion of the w^{+} phenotype. The $P[^{\wedge}a>]$ and $P[^{\wedge}m>]$ lines were then made homozygous, and heterozygous flies were constructed by crossing the two homozygous lines together. Seven out of seven of the original third-chromosome $P[^{\wedge}a{>}w^{+}{}^{\wedge}m>]$ lines were successfully "waffled" to yield derivative $P[^{\wedge}a>]$ and $P[^{\wedge}m>]$ lines. Hybridization of fluorescent probes derived from the *D. melanogaster* and *D. affinidisjuncta Adh* genes to polytene chromosomes, as shown in Figure 8, demonstrates the successful removal of the desired gene in each homozygote, and the allelism of the remaining genes in the heterozygote.

DISCUSSION

Site-specific recombination systems have many applications in genetics (for a review, see KILBY *et al.* 1993). These include selective activation or inactivation of genes (SAUER 1987; SAUER and HENDERSON 1988; GOLIC and LINDQUIST 1989; ODELL *et al.* 1990; LASKO *et al.*

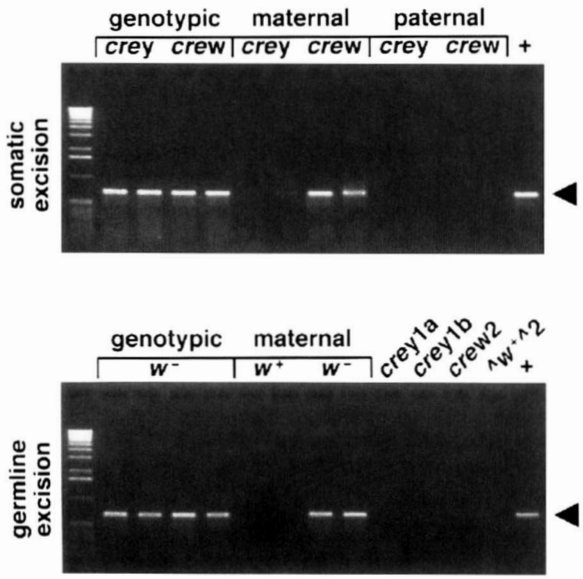


FIGURE 6.—PCR assay for somatic and germline excision. The solid arrowhead points toward the characteristic 0.75-kb amplification product associated with Cre-mediated excision of the mini-*white* from $P[^{\wedge}w^{+}{}^{\varphi}]$. Top: PCR evidence of site-specific somatic excision; Bottom: PCR evidence of site-specific germline excision as well as several negative controls. Identity of single-fly templates are indicated above each lane and are characterized according to mode of Cre transmission and source of Cre (see text). In both panels, the leftmost lane contains 1-kb ladder and the rightmost lane contains positive-control DNA. In the somatic assay, presence of the characteristic amplification product corresponds well with eye-color phenotype. The potent genotypic effect is reflected in the ability of the corresponding flies to support amplification of the 0.75-kb product. The strong maternal effect of $P[w^{+}, cre]$ also yields amplifiable templates. Interestingly, the resolution of the PCR assay matches that of the phenotypic assay in that the weak band in the second maternal *crey* lane derives from a fly with a single sector of white in one eye, whereas the lack of a band in the first maternal *crey* lane derives from a fly with no apparent excision in the eye. Consistent with the phenotypic assay is the absence of an amplification product from flies subject to "paternal" transmission of Cre. As expected of Cre-mediated excision of mini-*white* in the germline, the characteristic amplification product segregates with w^{-} phenotype for both genotypically and maternally induced excisions. Negative controls, labeled diagonally, are PCR reactions performed on flies taken from recombinase or target lines that had never been exposed to the other.

1992; ORBAN *et al.* 1992; KÜHN *et al.* 1995), clonal analysis via inducible mitotic recombination (GOLIC 1991; CHOU and PERRIMON 1992; XU and RUBIN 1993), deletion or inversion of chromosome segments with known endpoints (MATSUZAKI *et al.* 1990; GOLIC 1994; OSBOURNE *et al.* 1995), site-specific translocation between nonhomologous chromosomes (MATSUZAKI *et al.* 1990; VAN DEURSEN *et al.* 1995), and targeted integration of exogenous DNA (SAUER and HENDERSON 1990; FUKUSHIGE and SAUER 1992; BAUBONIS and SAUER 1993; ALBERT *et al.* 1995). Except in the case of gene replacement by *P*-element-induced gap repair (GLOOR *et al.* 1991), which has restricted applicability due to the limited size of conversion tracts and the requirement of a well-placed *P*-element insertion, targeted integration of exogenous DNA into the Drosophila germline has yet to be reported. We have introduced the Cre recombinase of bacteriophage P1 and its *loxP* recombinase target sites into *D. melanogaster* and demonstrate that site-specific recombination takes place at high efficiency. We also demonstrate a novel system of transgene coplacement in which two different systems, namely, Cre/*loxP* and FLP/*FRT*, are used to generate Drosophila lines in which transgenes are inserted at exactly allelic positions in a pair of homologous chromosomes.

Our data show that the chimeric *cre* transgene is a potent source of recombinase that effects intramolecular excision at an exceptionally high frequency in Drosophila. The system is similar in many respects to the FLP/*FRT* system. GOLIC and LINDQUIST (1989) introduced into Drosophila the FLP/*FRT* site-specific recombination system from the 2μ plasmid of yeast. They showed that a heat-shock-inducible source of FLP catalyzes site-specific recombination. GOLIC (1994) used local transposition of *FRT*-carrying *P* elements to induce site-specific deletions and duplications of *FRT*-flanked genomic DNA. Site-specific recombination between distant *FRT* sites was inferred to occur at a very low frequency.

The Cre recombinase system appears to act more efficiently than that with FLP recombinase but, because different regulatory sequences were used with Cre than with FLP, it is difficult to say whether it is regulation or catalytic activity that predominantly determines the level of expression. It is clear, however, that Cre recombinase enters the Drosophila nucleus and catalyzes exchanges between *loxP* sites in genomic DNA packaged into chromatin. Under the control of an inducible, nonleaky promoter and tissue- and developmental-stage-specific enhancers, such as the GAL4 system (FISCHER *et al.* 1988; BRAND and PERRIMON 1993; HALDER *et al.* 1995), a *cre* gene may allow very specific, yet virtually complete, elimination of function of a *loxP*-flanked gene of interest. In addition, the strong maternal effect of the *cre* transgene may be useful in crossing schemes because the recombinase gene need not be present in the *loxP* genotype of interest.

The transgene coplacement system takes novel advantage of the synergistic benefits of two site-specific recombinases used in combination. The "waffle" technique allows comparison of two transgenic alleles while controlling for position effects. Thus, subtle differences in gene expression that would otherwise be overwhelmed by position-effect "noise" may be made manifest. In addition, the study of those very position effects may be made more exact by comparing different genes in an identical genomic context. Other studies that require transgenes to be true alleles, such as those involving transvection or those involving estimation of relative fitness by population-cage competition experiments, will be feasible.

In its current form, the waffle vector does not insulate

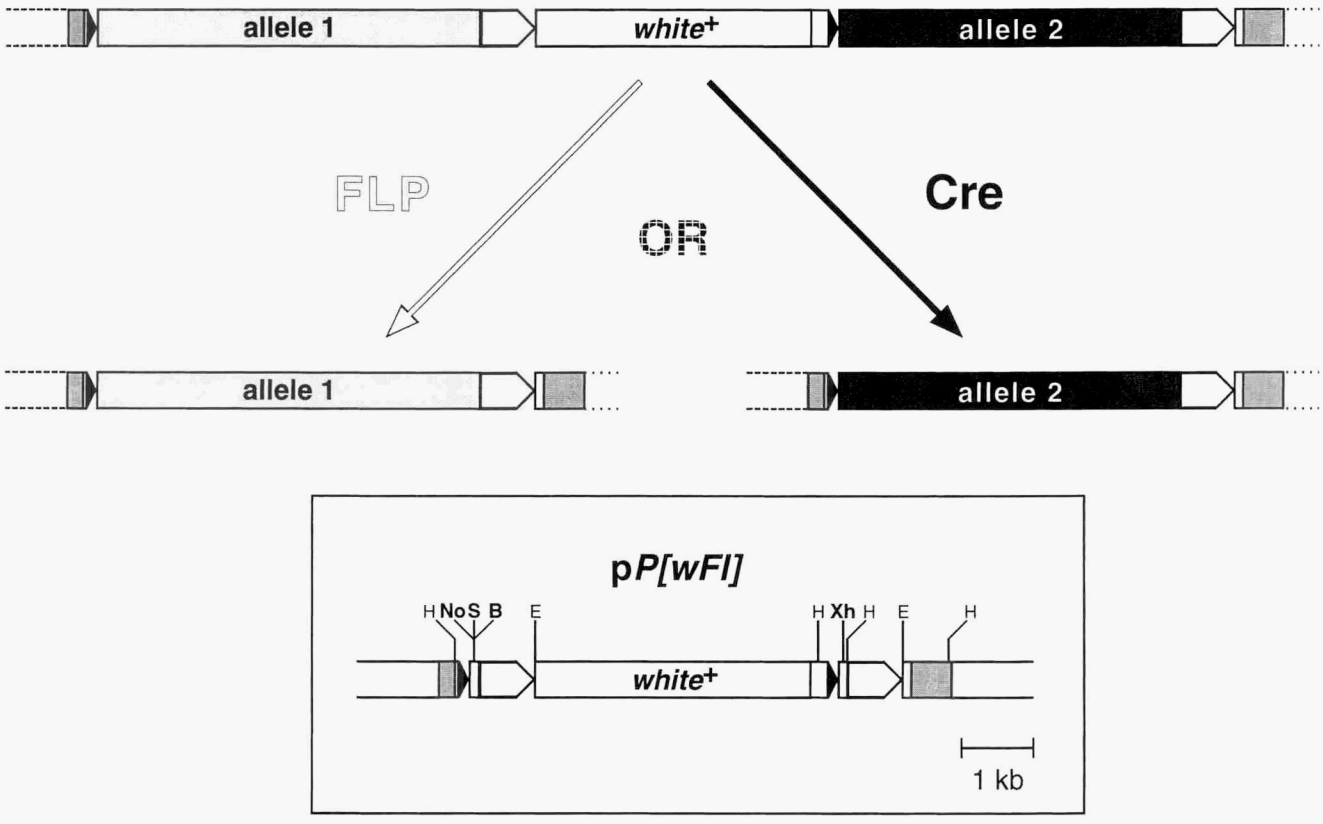


FIGURE 7.—The "waffle" technique for generating allelic transgenes. Using *P*-element-mediated transformation and the excision reactions of two site-specific recombinases, one can generate pairs of Drosophila strains that contain, at a given locus of *P*-element insertion, one or the other of two transgenic alleles. The recombination target sites of each recombinase are configured within *P*-element ends (intermediate shading) such that *loxP* sites (solid arrowheads) flank both allele 1 and a mini-*white* marker, and *FRT* sites (open arrowheads) flank both allele 2 and the same marker. Germline transformants of a w^- strain are recognized by gain of w^+ phenotype. After homozygous single-insertion lines are constructed, the waffle construct is exposed either to Cre or to FLP by crossing with an appropriate recombinase strain. In the presence of FLP, allele 2 and mini-*white* are excised, yielding eye-color revertants and leaving allele 1 flanked on one side by a *loxP* site and on the other side by a *FRT* site. Similarly, in the presence of Cre, allele 1 and mini-*white* are excised, yielding eye-color revertants and leaving allele 2 flanked by the same sequences with which allele 1 is left. The result is two strains of flies with inserts of allele 1 or allele 2 in the same genomic position, flanked by the same sequences. Shown within the box at bottom is the "waffle" vector, p*P*[*wFl*], which has unique cloning sites (boldface) for inserting two alleles. Restriction sites indicated are H, *Hin*dIII; No, *Not*I; S, *Spe*I; B, *Bam*HI; E, *Eco*RI; and Xh, *Xho*I.

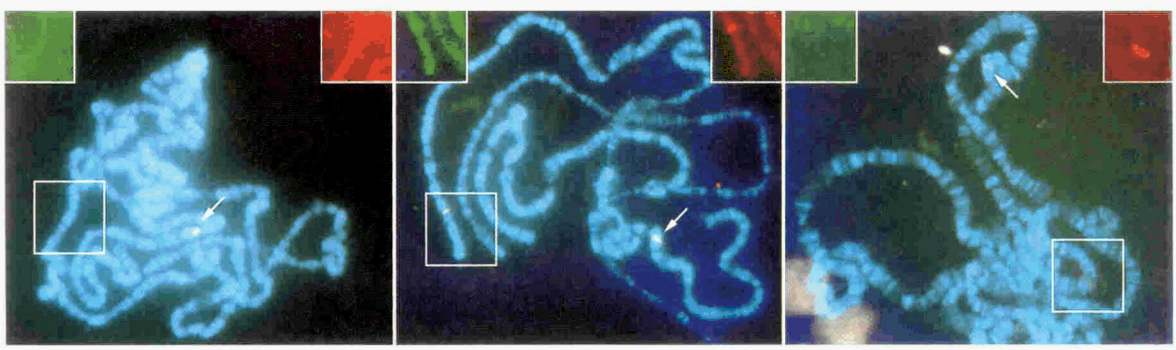


FIGURE 8.—Fluorescent *in situ* hybridization to polytene chromosomes of excision derivatives of a "waffle" insertion. The three panels contain polytene chromosome preparations of larvae derived from flies carrying excision derivatives of an *Adh*-carrying waffle insertion, $P[\hat{}a{>}w^{+}\hat{}m{>}]$, into region 99B on chromosome *3*. The left and right panels show the FLP and Cre derivatives, $P[\hat{}a{>}]$ and $P[\hat{}m{>}]$, respectively, in homozygous condition. The center panel shows $P[\hat{}a{>}]$ and $P[\hat{}m{>}]$ in heterozygous condition. Each preparation was probed with both fluorescein-labeled *D. affinidisjuncta Adh* sequence (green signal) and rhodamine-labeled *D. melanogaster Adh* sequence (red signal). An image taken with a composite (DAPI, FITC, TRITC) filter is shown in each panel. The region boxed in white highlights the location of the waffle insertion derivative. This region is duplicated in the upper left and right corners of the panel, showing only FITC (left) and TRITC (right) filter images. The arrow in each panel points to the red signal from the endogenous *D. melanogaster Adh* gene.

from position effects, but instead controls for such effects by placing genes in the same genomic context. Some applications may benefit both from allelism and from insulation from position effects, as provided by the *suppressor of Hairy-wing* [su(Hw)] protein (PATTON *et al.* 1992). Thus, the potential exists for a hybrid system comprising a waffle within Su(Hw) binding sites. (One might call this a "soufflé" system because the inside is insulated.)

A formally similar system of transgene coplacement might be imagined in which three directly repeated *FRT* or *loxP* sites flank two transgenes introduced together in a *P*-element construct (*e.g.*, ^A^B^). Then, the single corresponding recombinase could be used to excise *A* but not *B* or *vice versa*. Such a system would have several potentially prohibitive drawbacks. Unless *A* and *B* both correspond to easily scored phenotypes, the presence or absence of each would have to be determined by molecular means, such as PCR screening. Because excision to leave a single transgene is rare compared with excision of both or excision of neither (GOLIC and LINDQUIST 1989), extensive screening might be required. The need for such screening could be obviated by including markers in the original construct, but then, in addition to adding much extra DNA to the *P* element, there is a catch-22: using the same marker linked to both *A* and *B* makes the excision of *A* and the excision of *B* indistinguishable, but using different markers would leave each transgene in a different genomic context. The waffle system provides a convenient alternative that reduces construct size by sharing a marker between the two genes, while at the same time providing selective elimination of each gene by using the appropriate recombinase. In addition, the excision reactions approach 100% efficiency for both Cre (Table 1) and FLP (GOLIC and LINDQUIST 1989; GOLIC 1994), making screening for the desired events trivially easy.

In addition to its highly efficient excisive activity, Cre may be useful in generating stably inherited specific chromosome rearrangements in Drosophila, including deletions and inversions with defined endpoints, as well as translocations between *loxP* sites on nonhomologous chromosomes. Preliminary data (not shown) suggest that this is the case.

Site-specific transformation of *loxP*-containing plasmids into chromosomally integrated *loxP* docking sites has been demonstrated in yeast, plant, and mammalian cells (SAUER and HENDERSON 1990; FUKUSHIGE and SAUER 1992; BAUBONIS and SAUER 1993; ALBERT *et al.* 1995). However, integration of an introduced plasmid into a genomic docking site has not been reported for Drosophila. It is not clear whether this failure is because intermolecular recombination *in vivo* is exceedingly rare or because intermolecular recombinations are quickly reversed by intramolecular excision events, preventing the recovery of stable integrants. In either case, data from Cre- and FLP-mediated integration in yeast and mammalian cells suggest that a great advance in the technology of introducing DNA into Drosophila and the subsequent screening methods will be required before direct site-specific germline integration is possible. With Cre, integration efficiency in yeast is on the order of 10^{-6} transformed cells per electroporated cell, and in mammalian cells it is on the order of 10^{-4} to 10^{-5} (SAUER and HENDERSON 1990; FUKUSHIGE and SAUER 1992). With FLP, integration efficiency in mammalian cells is on the order of 10^{-5} to 10^{-6} (O'GORMAN *et al.* 1991). Mutant *loxP* sites favoring integration over excision have been used for targeted integration in tobacco, but site-specific integration generally occurred at a lower frequency than random integration, and the highest reported transformation frequency was $<10^{-3}$ (ALBERT *et al.* 1995).

Cre-mediated docking of introduced DNA would be exceptionally useful in applications of the phage P1 cloning system. Phage P1 is capable of acting as a cloning vector for inserts $\geq$100 kb (STERNBERG 1990), and each P1 clone contains a single *loxP* site. Thus, without any special manipulation of the existing well-characterized P1 genomic libraries (HARTL *et al.* 1994), Cre-mediated transformation of more than double the amount of contiguous DNA than what is currently feasible might be possible. However, due to the limitations in DNA delivery and transformant screening discussed above, and possibly other unanticipated impediments, the practical implementation of Cre or FLP to mediate targeted integration of exogenous DNA seems a rather remote possibility. In the meantime, the combined Cre/FLP "waffle" system of transgene coplacement provides an efficient, reliable method of situating pairs of transgenes in exactly allelic sites in the genome.

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