"Parahomologous" Gene Targeting in Drosophila Cells: An Efficient, Homology-Dependent Pathway of Illegitimate Recombination Near a Target Site

Lucy Cherbas and Peter Cherbas

Department of Biology, Indiana University, Jordan Hall, Bloomington, Indiana 47405

Manuscript received July 17, 1996
Accepted for publication October 30, 1996

ABSTRACT

Drosophila cells in culture can be transformed by introducing exogenous DNA carrying a selectable marker. Here we report on the fate of plasmids that contain an extended fragment of Drosophila DNA in addition to the selectable marker. A small minority of the resulting transformants appear to arise from homologous recombination at the chromosomal target. However, the majority of the insertions are the products of illegitimate events in the vicinity of the target DNA, and they often cause mutations in the targeted region. The efficiency of this process, its homology dependence, and the clustering of the products define a novel transformation pathway that we call "parahomologous targeting."

EXOGENOUS, replicatively incompetent DNA transforms cells by integrating into the host genome either homologously or nonhomologously. The first process, which may involve both recombination and gene conversion, leads to precise sequence replacements or the generation of precise direct repeats, and it is an efficient process in some organisms including yeast. The latter process generates illegitimate or nonhomologous insertions at random sites in the host genome, and its efficiency does not depend on homology. This is the predominant pathway in cultured mammalian cells. (for reviews, see ROTH and WILSON 1988; SEDIVY and JOYNER 1992).

When Drosophila cells are transformed with plasmids that contain a selectable marker and at most a few kilobases of Drosophila DNA, the plasmids undergo extensive homologous recombination *inter se,* and the resulting tandem arrays can be detected as chromosomal integrants (BOUROUIS and JARRY 1983; MOSS 1985; CHERBAS *et al.* 1994). In S2 cells, these tandem arrays are extremely long, containing hundreds of unit repeats; in Kc cells, the arrays are much shorter (L. CHERBAS and K. LEE, unpublished data; however, see BOUROUIS and JARRY 1983). In none of these experiments have the sites of integration been characterized. Here we report that when Kc cells are transformed with plasmids containing long targeting fragments of Drosophila DNA, the integration events are efficiently localized to the neighborhood of the target. However, despite the strong homology dependence of this process, the recombination itself is illegitimate.

It should be noted that the literature contains several suggestions of homology-driven associations between exogenous DNA and Drosophila chromosomes which do not lead to homologous recombination. Early work in the FOX laboratory on germ-line transformation demonstrated a stable, noncovalent association between transfected DNA and its chromosomal homologue (FOX *et al.* 1970, 1971; FOX and VALENCIA 1975); these observations remain poorly understood. More recently, germ-line transformation of flies has been carried out extensively by *P*-element transposition; the insertion sites of a P transposon are, in general, scattered throughout the genome. However, in a few cases transposition is clustered in the vicinity of a chromosomal sequence homologous to the sequence contained in the *P* transposon (HAMA *et al.* 1990; KASSIS *et al.* 1992; FAUVARQUE and DURA 1993).

The experiments reported in this paper began as an attempt to mutagenize genes in the near-diploid Drosophila cell line Kc by homologous recombination, using techniques in common use for mammalian cells. The two targeted genes were chosen largely for their differing technical virtues: (1) *Eip71CD* (formerly known as *Eip28/29*), is an ecdysone-inducible gene of unknown function (SAVAKIS *et al.* 1984) contained in a region whose extensive molecular characterization facilitated Southern analysis of the transformants. The 9-kb region we targeted (located in polytene region 71CD) is sequenced, a 25-kb restriction map of the Kc cell alleles is available, as are probes for the surrounding 200 kb (CHERBAS *et al.* 1986; EICKBUSH 1987). (2) *EcR,* an ecdysone receptor gene located at polytene position 42A (KOELLE *et al.* 1991) was chosen because *EcR*-null cells are expected be resistant to ecdysone, a phenotype which permits screening for targeted events in the strongly ecdysone-responsive line Kc. Experiments reported here indicate that Kc cells contain a single functional copy of *EcR,* permitting elimination of function by a single targeted event. Ecdysone-resistant cells arise spontaneously in Kc cell populations at a significant

Corresponding author: Lucy Cherbas, Department of Biology, Jordan Hall, Indiana University, Bloomington, IN 47405.
E-mail: lcherbas@bio.indiana.edu

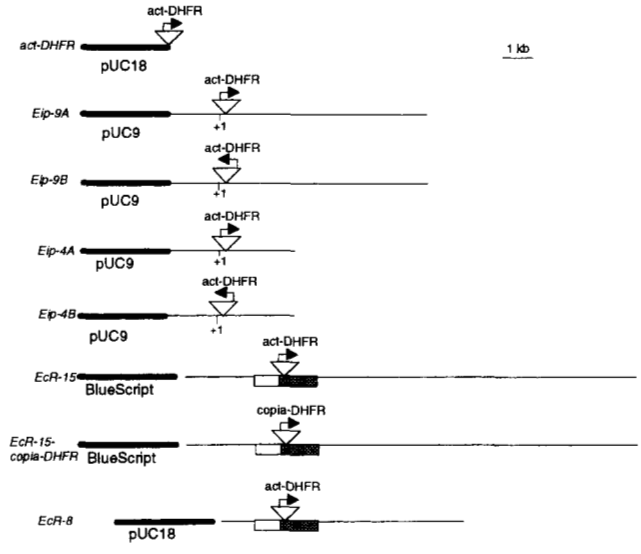

FIGURE 1.—Maps of plasmids used for transformation. Each plasmid name indicates the length of the genomic fragment in kb; "A" and "B" are used to distinguish plasmids differing only in the orientation of the selectable marker. *Eip71CD* transcriptional initiation is indicated as "+1"; exon II of the *EcR*-B1 transcription unit is shown as a box with the coding region hatched. *Eip71CD* and *EcR* transcription is left to right. Arrows indicate the DHFR transcription unit. Unless explicitly stated otherwise in the text, each plasmid was transfected in the linear conformation shown in the drawing. Note that act-DHFR and the Eip plasmids are cut at a single site, yielding a linear molecule that includes both vector and insert. EcR plasmids are cut at both ends of the insert, yielding the vector and insert in separate linear molecules.

frequency (around 2% of cells are resistant in the subline used for these experiments), but this spontaneous resistance is in general derived from mechanisms other than a defect in EcR; thus, the ecdysone response cannot be fully restored by expression of EcR from a plasmid source in most spontaneous ecdysone-deficient lines (CHERBAS and CHERBAS 1996). Ecdysone-resistant lines induced by targeting *EcR* should be readily distinguishable by the ability of an EcR-expressing plasmid to restore full hormone responsiveness.

MATERIALS AND METHODS

Vectors: The transformation vectors we employed are illustrated in Figure 1. All are pUC-derived plasmids carrying both a dihydrofolate reductase (DHFR) selectable marker and, save for the control vector act-DHFR, a "targeting segment" from the Drosophila genome. The DHFR marker is a methotrexate-resistant (MTX^R) DHFR gene taken from the plasmid p8HCO (REBAY *et al.* 1991). In one vector, we retained the *copia* promoter used in p8HCO (REBAY *et al.* 1991), but for most, we substituted a 300-bp *actin 5C* promoter (a gift of M. SCOTT).

Targeting segments from the *Eip71CD* region were taken from the plasmid pCg101-1, which originated from a Canton S genomic library (CHERBAS *et al.* 1986). The 9-kb fragment (*SalI-Hin*dIII) contains, in addition to *Eip71CD,* the genes *z600, gdl,* and *mex1* (SCHULZ and BUTLER 1989; SCHULZ *et al.* 1991). The smaller 4-kb *SalI-PmlI* fragment contains *z600, gdl,* and the 5′ portion of *Eip71CD.* In each case, the selectable marker, from act-DHFR, was inserted (using *Sac*I linkers) at a *Sac*I site *ca.* 40 bp 3′ of the *Eip71CD* translational start site.

Targeting segments from the *EcR* region were derived from Kc167 cell genomic DNA so that they are isogenic to one chromosomal copy in the host cells. A partial *Bam*HI library of Kc167 DNA was prepared in the vector lambda FIX-II (Stratagene) and *EcR* region clones were identified by hybridization to the *EcR* cDNA clone pMK1 (KOELLE *et al.* 1991), followed by restriction mapping. A single clone served as source for all the constructs used in this paper. Segments included in the transformation vectors contain exon II (isoform B1-specific) and adjacent intronic DNA. Exon II contains the translational start for isoform B1 (KOELLE *et al.* 1991; TALBOT *et al* 1993). Selectable markers derived from either act-DHFR or p8HCO (copia-DHFR) were inserted (using *Nco*I linkers) into a *Nco*I site *ca* 200 Np downstream of the translational start site.

Transformation: Kc167/M3 cells (CHERBAS *et al.* 1988) were maintained in HyQ-CCM3 medium (Hyclone, Inc.) and transfected by electroporation, following precisely the detailed protocol given in CHERBAS *et al.* 1994. Two days after transfection, the cells were transferred to medium containing methotrexate (MTX) at 2×10^{-7} M final concentration; 4 days later, they were cloned in 96-well plates in the presence of MTX (CHERBAS *et al.* 1994). For cloning, transfected cells were plated at concentrations of 10^2, 10^3, and 10^4 cells/well; in most cases plates chosen for scoring MTX^R clones and for picking clones for further analysis contained 1 clone/3–10 wells.

Southern analysis: For most experiments DNA was prepared from batches of 2×10^7 cells using Qiagen tips. Larger-scale preparations (5×10^8 cells) for a few transformed lines followed the procedure of MANIATIS *et al.* (1982). For Southerns, *ca* 10 μg of restriction enzyme-digested was displayed in each lane of a 1% agarose gel. Gels were blotted onto GeneScreen Plus membranes and the blots hybridized with ^{32}P-labeled probes prepared by random-primer labeling (Prime-It II kit, Stratagene, Inc.). Conditions for the hybridizations and washings have been described previously (CHERBAS *et al.* 1986). Size markers in each gel were visualized by ethidium bromide staining and marked by injecting India ink into each band. The ink was transferred to the membrane along with the DNA, and its position traced onto the autoradiogram after development of the X-ray film.

Assays of ecdysone responsiveness: Clones of transformed cells were tested by treating 1-ml cell cultures with 10^{-6} M 20-hydroxyecdysone; after 2 to 3 days, cells were compared visually with control (untreated) cultures. Clones which had neither responded morphologically (CHERBAS *et al.* 1981) nor ceased to proliferate (CHERBAS and CHERBAS 1981) were judged to be ecdysone-resistant. In accord with our previous observations, the two responses were strictly linked: cells either responded both morphologically and proliferatively, or not at all.

A second assay measured the transcriptional response (induction) of a reporter gene linked to an ecdysone response element (EcRE). For these transient assays cells were electroporated (20 μg plasmid/10 ml plate) with H1X-188-cc-cat, a plasmid which contains a single *hsp27* EcRE and an *Eip71CD*-derived promoter linked to the reporter chloramphenicol acetyltransferase (CAT) (CHERBAS *et al.* 1991). Hormone treatments and enzyme assays were performed according to procedures described previously (CHERBAS *et al.* 1991).

A third assay measured the sensitivity of the transcriptional response to increased levels of functional EcR. For those assays, cells were cotransfected with H1X-188-cc-cat and act-EcR (20 μg/plate each). act-EcR expresses *EcR* isoform B1 in Drosophila cells: it was constructed by substituting the *actin5C* promoter for the metallothionein promoter of Mt-EcR (D'AVINO *et al.* 1995). act-EcR/plate (20 μg) is adequate to confer maximal ecdysone responsiveness on Kc cells in which EcR is limiting (SWEVERS *et al.* 1996).

Finally, we assayed functional receptor activity by measuring the ability of a crude cell sonicate to bind the hormone analog ^{125}I-iodoponasterone (CHERBAS *et al.* 1988).

RESULTS

Transformations using *Eip71CD*-targeted vector: We transfected cells with plasmids containing a methotrexate resistance (MTX^R) marker under the control of a 300-bp promoter fragment from Drosophila actin 5C. One plasmid (act-DHFR) contained no additional Drosophila sequences; in the others, the actin-DHFR marker fragment was embedded within a 4- or 9-kb fragment of DNA from the *Eip71CD* region of Drosophila. The transfected cells were then cloned in the presence of MTX. Surprisingly, the frequency of MTX^R transformants was significantly elevated when the transfected plasmid contained a 9-kb fragment of *Eip71CD* DNA (see below). We therefore examined the DNA of the transformed clones, looking for evidence of homologous recombination with the chromosomal copies of *Eip71CD*.

Southern analysis of transformed clones: We began by preparing miniprep DNA from ~200 transformed clones, and screening them with a single restriction digest and a single probe ("3′ probe") taken from the chromosomal region immediately adjacent to the longer (9 kb) *Eip71CD* fragment used for transformation. Thus, the probe does not detect the transforming DNA, but instead reports on the structures of chromosomal regions adjacent to the target. (See Figure 3 for maps of the region and the probe.) Figure 2 (top) shows one of these Southern blots. The probe hybridizes to four bands in *Sal*I-cut DNA from untransformed cells (lane U); in this experiment, a fifth band generated by partial digestion is also present. The two upper bands (labeled 2 and 3, corresponding to fragments Sal 2 and Sal3 in the map of Figure 3) are of particular interest here. These two allelic fragments differ by the presence of a 5-kb insertion near *Eip71CD* in one chromosome (Figure 3; CHERBAS *et al.* 1986). Bands 2 and 3 each contain the 9-kb fragment found in the Eip-9 plasmids and would therefore be altered by homologous recombination with the plasmid. The two heavy bands at the bottom of the gel represent a region further 3′ (see DISCUSSION and map in Figure 3); we believe that they are also allelic variants, but the structures of the two chromosomes in this region have not yet been clearly mapped. The middle band in this lane is absent in other blots from the same DNA; it represents a partial digestion product.

Most of the transformed clones in the Southern blot of Figure 2 have patterns of hybridization different from that of the untransformed control. To eliminate the possibility that the new bands are merely partial digestion products, the blot was washed and reprobed with a smaller probe containing no homology to the small, heavily hybridizing 3′ fragments (Figure 2, bottom); this probe consists of the 5′ 200 bp of the "3′ probe."

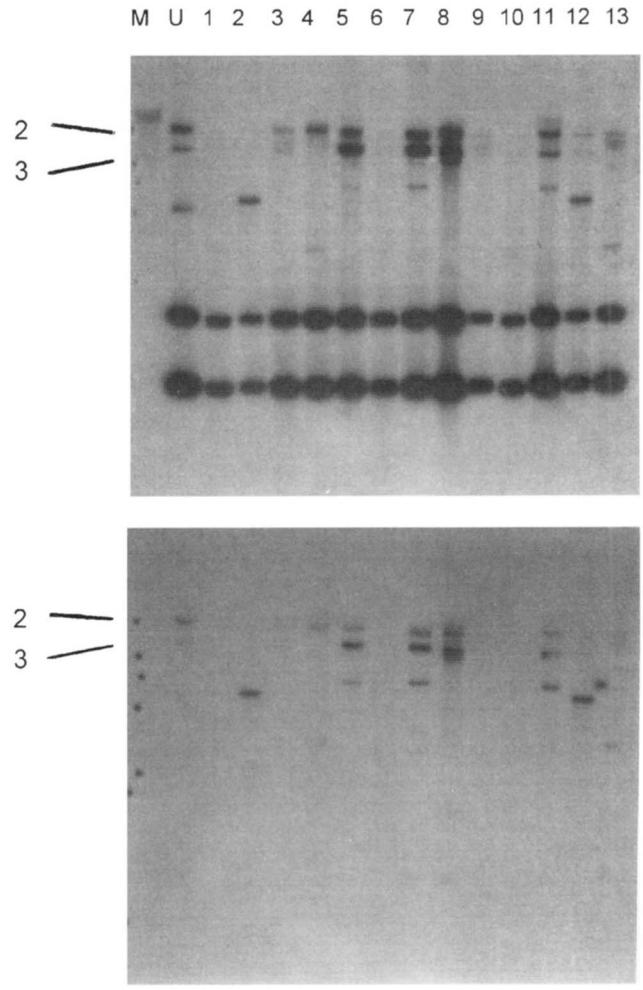

FIGURE 2.—Example of a Southern blot in which miniprep DNAs from transformed clones were screened for rearrangements at *Eip71CD*. DNA was digested with *Sal*I. The blot was probed first with the "3′ probe" of Figure 3 (top), and then with a 200-bp *Hin*dIII/*Sal*I fragment, consisting of the 5′ 200 bp of the 3′ probe (bottom). Lane M: λ/*Hin*dIII size markers; dots represent the positions of India ink marks (see MATERIALS AND METHODS). Lane U: untransfected Kc167 cells. Lane 1–13: clones transformed with 16 μg/plate uncut Eip-9A (1 and 2), uncut Eip-9B (3 and 4), linear Eip-9A (5–12), or linear Eip-9B (13). Bands marked as "2" and "3" are shown in the map of Figure 3 as Sal2 and Sal3. Clones in lanes 2, 3, 4, 5, 7, 8, 9, 11, 12, and 13 were scored as rearranged. Clones in lanes 1, 6, and 10 were not scored.

The use of this probe eliminated the heavy bands at the bottom of the gel and the minor band in the control lane, which we have already attributed to partial digestion, but few if any of the novel bands in the lanes corresponding to transformed clones. Since these novel bands are in most cases smaller than the wild-type bands, we conclude that they are not partial digest products from a wild-type chromosome.

Altogether, 159 clones transformed with *Eip71CD* fragments gave interpretable patterns in this Southern screen. Remarkably, 52% of these showed altered restriction patterns (Table 1). Rearrangements were qualitatively and quantitatively similar whether the transformants were made with Eip-4 or with Eip-9 and

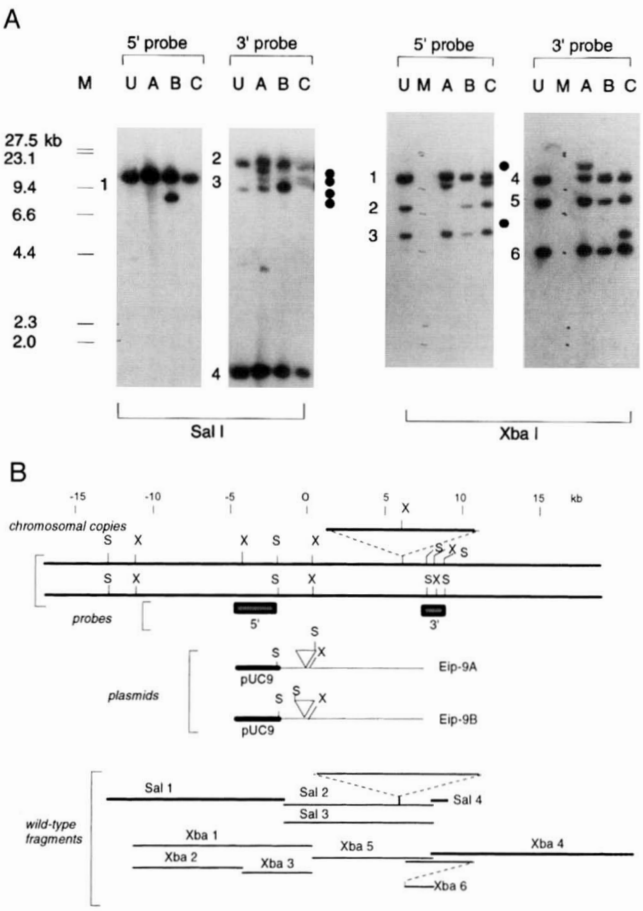

FIGURE 3.—(A) Southern analysis of some *Eip71CD*-targeted transformants. M: size markers (λ/*Hin*dIII). U: untransformed cells. A, B, C: individual transformed clones. Clone A was transformed with Eip-9B (32 μg/plate) and is estimated to contain four copies of DHFR. Clone B, transformed with Eip-9A (16 μg/plate), contains 12 copies of DHFR. Clone C, transformed with Eip-9B (16 μg/plate), contains two copies of DHFR. (B) The diagram below shows: (1) Maps of both chromosomal homologues, as deduced from genomic clones and Southern patterns (CHERBAS *et al.*, 1986). S = *Sal*I; X = *Xba*I. (2) Probe fragments. (3) Maps of the transforming plasmids (see Figure 1). (4) Wild-type *Sal*I and *Xba*I fragments which hybridize to the two probes; thicker lines indicate fragments present in both homologues. All maps are aligned, with the *Eip71CD* transcription unit beginning at 0 and proceeding rightward. Numbers identify bands in the untransformed DNA lanes with the fragments diagrammed below. Filled circles next to the *Sal*I/3′ and *Xba*I/5′ panels show positions for bands predicted to replace fragments Sal2, Sal3, Xba1, or Xba3 as a result of homologous recombination.

whether linear and supercoiled DNA was used. By contrast, the same test detected no sequence alterations in the *Eip71CD* region in any of 32 tested clones transformed with the control vector act-DHFR.

Figure 3 illustrates three clones that were selected for more detailed Southern analysis using larger-scale DNA preparations. The *Sal*I digests probed with the 3′ probe correspond to the Southern blot illustrated in Figure 2; the shortest hybridizing band seen in Figure 3 has run off the end of the gel in the blot shown in Figure 3. To aid in the interpretation of the Southern patterns, we have indicated in Figure 3 the predicted positions for bands that would be generated by homologous recombination between the transfected plasmid sequence and a chromosomal sequence. In the case of *Sal*I, we have indicated two possible recombinant positions for each allele, corresponding to the two plasmids Eip-9A and Eip-9B; for *Xba*I, there is a single predicted recombinant band for each allele. Note that homologous recombination would not alter the pattern detected by hybridization of the 5′ probe to a *Sal*I digest or by hybridization of the 3′ probe to an *Xba*I digest.

The characteristics of these rearrangements can be summarized as follows: (1) None is the product of a simple homologous recombination. This is illustrated by reference to Figure 3. *Sal*I digestion results in two allelic wild-type bands that contain the *Eip71CD* fragment used for transformation; these are detected by the 3′ probe as band 2 (14 kb) and band 3 (9 kb) The MTX^R fragment contains an asymmetrically placed *Sal*I site; hence if homologous recombination with Eip-9A occurred downstream of the MTX^R insertion, it would result in a shortening by 2 kb of the band hybridizing to the 3′ probe (12 or 7 kb for the recombinant band), and similarly homologous recombination with Eip-9B would result in a shortening of the recombinant band by ~1 kb (13 or 8 kb). Thus the expected pattern for homologous recombination would consist of the replacement of band 2 with a 12 or 13 kb band, or replacement of band 3 with a 7 or 8 kb band. While some of the new bands in clones A and C are of approximately the predicted size, in no case was a wild-type band replaced by a recombinant band. (2) The rearrangements vary widely among the transformed clones. Aberrant patterns include apparent duplications of wild-type bands (*e.g.*, the increased intensity of band Sal3 in clone B), shifts in position of wild-type bands (*e.g.*, band Xba2 in clone A), and the appearance of unexplained new bands (*e.g.*, clone B, *Sal*I digest, 5′ probe). In some cases, the pattern implies a rearrangement outside the region of plasmid homology. For example, the *Sal*I band detected by the 5′ probe (Sal1) contains no overlap with the region homologous to the transforming DNA. Yet in clone B, the 5′ probe detects a new *Sal*I band. Similarly, a 7-kb *Xba*I band detected by the 5′ probe (Xba2) is separated from the plasmid homology by ~2 kb. Yet in clone A, this band has been shifted to ~10 kb. (3) The rearrangements are restricted to the *Eip71CD* region. Blots were prepared containing *Xba*I-digested DNA from 26 clones transformed with Eip-9A or Eip-9B, each of which displayed rearrangements at the chromosomal *Eip71CD* locus (polytene region 71CD); the blots were probed with a 15-kb fragment from *EcR* (42A), washed, and reprobed with a 15-kb fragment from *Scr* (84B). In no case did the *EcR* or *Scr* pattern deviate from that of untransformed cells (data not shown). (4) In most cases, it is not possible to tell from the Southern data which allele has undergone rearrangements, or whether both alleles are affected.

TABLE 1

Transformation and rearrangement frequencies in *Eip71CD* targeting

Plasmid	Conformation	Units[a]	Rearrangement Frequency[b]	Relative frequency[c] MTX^R clones
act-DHFR	Linear	1	0/3 (0)	0.2 (17)
act-DHFR	Linear	2	0/6 (0)	0.2 (16)
act-DHFR	Linear	5	0/17 (0)	[1.0] (110)
act-DHFR	Linear	10	0/3 (0)	1.8 (16)
act-DHFR	Linear	20	0/3 (0)	—
Eip-9A	Linear	1	0/2 (0)	—
Eip-9A	Linear	2	2/5 (40)	—
Eip-9A	Linear	5	20/41 (49)	2.4 (44)
Eip-9A	Linear	10	6/22 (27)	—
Eip-9A	Linear	20	12/22 (55)	—
Eip-9B	Linear	1	2/3 (67)	—
Eip-9B	Linear	2	7/10 (70)	—
Eip-9B	Linear	5	10/20 (50)	2.3 (101)
Eip-9B	Linear	10	6/7 (86)	—
Eip-4A	Linear	5	6/10 (60)	0.7 (14)
Eip-4B	Linear	5	3/3 (100)	0.6 (11)
act-DHFR	Uncut	5	ND	0.3 (7)
Eip-9A	Uncut	5	4/7 (57)	0.7 (15)
Eip-9B	Uncut	5	ND	1.1 (12)

Cells (2×10^7) were transfected with the indicated amount of plasmid. MTX^R clones were scored 2 wk after plating in 96-well plates. Southern analysis was then performed on a sample of the clones. Many experiments included a transfection with 5 units of act-DHFR, as a standardization control. In those cases where this control transfection was done and enough clones were counted to permit a statistically significant comparison, the relative frequency is reported.

[a] One unit of transfecting plasmid is defined as the molar equivalent of 1 μg of act-DHFR. Thus 1 unit of act-DHFR = 1 μg, 1 unit of Eip-9 = 3.2 μg, and 1 unit of Eip-4 = 2 μg.

[b] MTX^R transformed clones were tested by Southerns and scored as "rearranged" if a new band appeared in a test using *SalI* digestion and the 3′ probe (Figures 2 and 3). Values in parentheses are percentages. ND, not determined.

[c] Relative frequency is the frequency of MTX^R clones yielded by the test vector divided by the frequency yielded by 5 units of act-DHFR in the same experiment. RF for 5 units act-DHFR is 1.0 by definition. In experiments where the act-DHFR control was omitted or was lost to contamination or where an insignificant number of clones was scored, no relative frequency is reported (—). Values in parentheses are the number of MTX^R clones counted. Because we scored different numbers of wells in different experiments, this number does not reflect the frequency of MTX^R clones; it is provided only as an indicator of the statistical significance of the calculated frequency.

The number of integrated copies of the plasmid DNA was estimated for some clones by reprobing the Southern blots with pUC18. In general, clones contained 1–10 copies per cell of pUC sequences; in some cases, the pattern suggested that some of the plasmid sequences were arranged in a head-to-tail array (data not shown).

Transformation frequency: The frequency of clones recovered after MTX selection was significantly increased by the presence of a 9-kb *Eip71CD* fragment. Experiment-to-experiment variations in transformation efficiency make these comparisons difficult; this is a common problem with transformation experiments (see SEDIVY and JOYNER 1992). However, the number of clones recovered from a transformation with Eip-9A was almost invariably higher than the number of clones recovered from a parallel transformation with an equimolar amount of act-DHFR; Table 1 includes frequency data from two such experiments in which we counted enough clones to make the data statistically meaningful. It is likely that these numbers underestimate the effect of the *Eip71CD* fragment on transformation frequency, because it is likely that the transfection efficiencies for Eip-9A and Eip9B are significantly lower than that of the much smaller act-DHFR.

The smaller Eip-4 plasmids gave slighter fewer transformants than an equimolar concentration of act-DHFR (Table 1). While these data are relatively unreliable because of the small numbers of clones counted and the unknown effect of plasmid size on transfection efficiency, it seems clear at least that the 4-kb *Eip71CD* fragment stimulates transformation less effectively than the 9-kb fragment. Table 1 also shows that linear plasmids are more effective than supercoiled. In contrast to prior observations in yeast (ORR-WEAVER *et al.* 1981), plasmids linearized at a restriction site within the *Eip71CD* sequence did not give rise to significantly more transformants than those linearized at the junction between insert and vector (data not shown).

Transformations using *EcR*-targeted vectors: *Transformation frequency and Southern analysis:* Transforma-

tion with *EcR* fragments gave somewhat different results. The *EcR* transcription unit is ~70 kb in length, encoding at least three protein isoforms (TALBOT *et al.* 1993); we targeted exon II, the first translated exon of isoform B1, which is the major isoform in Drosophila cell lines (TALBOT *et al.* 1993). *EcR*-homologous plasmids yielded approximately the same number of MTX^R clones as an equimolar amount of act-DHFR (data not shown).

Southern analysis revealed that untransformed Kc cells contain four copies of the *EcR* region (Figure 4). Two hybridizing bands are seen in *Bam*HI-digested DNA from untransformed cells. The lower molecular weight band migrates in the same position as the corresponding cloned fragment and is designated "I" (isogenic); the larger fragment differs by the presence of a 5-kb insertion and is designated "N" (nonisogenic). The I band is three times as intense as the N band, and therefore apparently includes three copies of the region, one of which is isogenic to the transforming DNA; since the three copies are indistinguishable in Southern blots, we refer to all of them as I alleles. This interpretation has been confirmed in several other restriction digests (data not shown). The 5-kb insert that defines allele N has not been precisely mapped.

A Southern screen of 40 MTX^R clones transformed with EcR-8 indicated two cases (5%) of homologous recombination, in both cases with an I allele; one of these clones (L78-7-9) is illustrated in Figure 4. Several other restriction digests of DNA from the same clones also gave patterns consistent with homologous recombination, and phosphoimager analysis confirmed the predicted change in the relative intensities of the I and N bands (data not shown). Nonetheless, the conclusion that recombination was homologous must be considered tentative until the rearranged region has been sequenced. No nonhomologous rearrangements in these 40 clones were revealed by Southern analysis using the two *EcR* probes shown in Figure 3. This implies that in most cases the exogenous DNA has inserted outside the ~15-kb region sampled in the Southerns. A third clone, recovered from a different experiment for which no frequency data are available, appears to have undergone homologous recombination in the N allele.

Altogether then, we have recovered three putative homologous recombinants at *EcR*, one in the N allele and two in I alleles; all contain insertions of the transfected DNA, despite the fact that the plasmid was designed to be a "substitution vector" and was linearized at a site outside the Drosophila fragment. We note that insertional integration of substitution vectors is also commonly seen in mouse cells (HASTY *et al.* 1991).

The number of plasmid copies incorporated into the genome was estimated by reprobing Southern blots with the insert of act-DHFR. Since the DNA has been digested with *Bam*HI, which cuts between the Drosophila act5C fragment and the bacterial DHFR coding se-

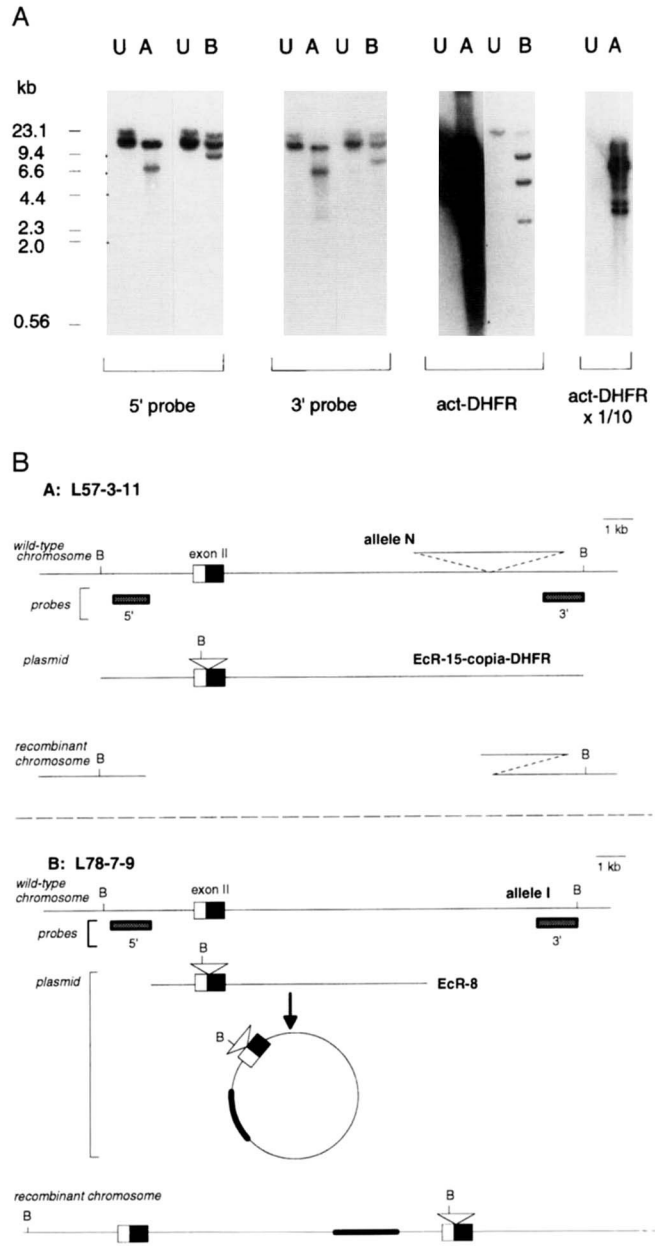

FIGURE 4.—(A) Southern analysis of some *EcR*-targeted transformants. U: untransformed cells. A: clone L57-3-11, transformed with EcR-15-copia-DHFR. B: clone L78-7-9, transformed with EcR-8. The "act-DHFR" probe is the insert of the plasmid act-DHFR; hybridization to the chromosomal copy of *actin5C* is seen in the U lanes. Note that the 5′ and 3′ probe fragments are contained in EcR-15-copia-DHFR, but not in EcR-8. The diagrams below are an interpretation of the Southern patterns. Exon II is represented by a box, with the coding portion shaded. B = *Bam*HI. (B) In L57-3-11, exon II of the N allele has been deleted; ~400 copies of copia-DHFR are inserted at an unknown site(s). The end points of the deletion in L57-3-11 have not been mapped accurately. In L78-7-9, one copy of the transfected plasmid has apparently undergone homologous recombination with a copy of allele I. The inserted DNA includes an unidentified fragment, perhaps pUC18 (indicated by the heavy line). We postulate recircularization of the plasmid to explain the insertional integration. Autoradiograms were exposed for 20 hr with an intensifying screen, except for the panel marked act-DHFR × 1/10, which was exposed for 2 hr. Size markers are λ/*Hin*dIII.

TABLE 2

Frequency of ecdysone resistance in transformed clones

Transforming DNA	Frequency of ecdysone-resistant clones (%)	Statistical significance
None	1.8 (63/4000)	—
act-DHFR	5.9 (3/51)	NS[a]
EcR-8	8.8 (11/128)	$P < 0.001$
EcR-15	25.4 (17/67)	$P < 0.001$

The frequency of ecdysone resistance in untreated cells was measured by cloning aliquots of a single cell suspension in the presence and absence of 10^{-6} M 20-hydroxyecdysone; rate is given in parentheses. Frequencies of resistance in transformed cells was measured by assaying the ecdysone response in individual clones. Each datum represents pooled results from two to seven independent transfections. Each frequency was compared with the frequency in untreated cells, and the significance of the difference was measured by a chi-square test with Yates correction. The same test indicates that the frequencies for EcR-8 and EcR-15 differ significantly ($P = 0.013$). NS, not significant.

[a] $P = 0.075$.

quence in act-DHFR, each copy of the transfected plasmid gives rise to two bands in the Southern; an additional band corresponds to the endogenous actin5C gene located on the single *X* chromosome in Kc cells. In most cases, MTX^R clones transformed with *EcR* fragments contained one to five copies of the transfected DNA, with no evidence of array formation (*e.g.*, L78-3-9, Figure 4).

When cells transfected with EcR-15-copia-DHFR were selected for MTX resistance, relatively few transformed cells survived; those few survivors included many copies of the copia-DHFR sequence incorporated either by recombination or replication. This result is consistent with other observations that a single copy of act-DHFR, but not of copia-DHFR, can render Kc cells resistant to MTX (SEGAL *et al.* 1996). L57-3-11 (Figure 4) is an example of an EcR-15-copia-DHFR-transformed clone; in this case, several hundred copies of the DHFR fragment are present. Surprisingly, more distal portions of the transforming DNA fragment, which are homologous to the probes used in this Southern blot, are not overrepresented at all in L57-3-11. As shown in Figure 4, L57-3-11 carries a large deletion in the targeted region of allele N; the large number of copies of the selectable marker appear to be inserted elsewhere in the genome.

Ecdysone resistance: Despite the relatively low frequency of homologous recombination, transformation with *EcR* fragments caused a marked increase in the frequency of ecdysone resistance, defined as the absence of morphological and proliferative responses to the hormone (Table 2). Approximately 2% of untransformed clones are spontaneously ecdysone-resistant. Following transformation with act-DHFR, the frequency of ecdysone-resistant clones was not significantly altered, but the fraction rose in clones transformed with EcR-8 (9%) and EcR-15 (25%).

This induced ecdysone resistance differs qualitatively from the spontaneous resistance of untransformed cells (Table 3). In both spontaneously arising and induced ecdysone-resistant clones, we found reduced but variable levels of induction of an ecdysone response element-linked reporter. Ecdysone responsiveness was increased in some of the spontaneously arising clones by transfection with an EcR expression plasmid, but we never observed a restoration of the wild-type level of response. By contrast, all of the ecdysone-resistant clones arising after transformation with *EcR* fragments could be restored to full ecdysone responsiveness by transfection with the EcR expression plasmid. Thus the lesion in the induced ecdysone-resistant cells is in EcR function, while the lesion in the spontaneously occurring resistant cells is in general in some other component of the hormone response.

The phenotype of one induced ecdysone-resistant clone, L57-3-11, was characterized in more detail. Of all the induced clones that we tested, L57-3-11 displayed the lowest reporter induction (Table 3). In this clone, the N allele is deleted for the isoform B1-specific exon (see Figure 4). The ecdysone-binding capacity of these cells was ~10% that of the parental line (data not shown). Reporter induction in this line can be restored to wild-type levels by co-transfection with plasmids expressing either Drosophila EcR-B1 or a *Bombyx mori* EcR homologue (SWEVERS *et al.* 1996; Table 3).

Unlike L57-3-11, most of the ecdysone-resistant clones derived from transformation with EcR-8 or EcR-15 showed no abnormality in the Southern patterns revealed by the probes shown in Figure 4. This apparent inconsistency between the Southern results and the phenotypic results can be explained by hypothesizing that ecdysone resistance is caused by rearrangements of DNA within the very large *EcR* transcription unit at some distance from the region of plasmid homology. These illegitimate recombinations apparently are less tightly clustered than in the case of *Eip71CD,* since we did not observe them in the Southern screen.

In two of the homologous recombinants (*e.g.*, L78-5-9, Figure 4) the exogenous DNA has recombined with an I allele; both of these clones are ecdysone-sensitive. In the third homologous recombinant (L75-5-28; Table 3), the plasmid sequence is inserted into the N allele; this clone is ecdysone-resistant. L57-3-11, in which exon II has been deleted from the N allele, is strongly ecdysone-resistant (Table 3). Thus, in the four transformed clones where a rearrangement at *EcR* has been detected, there is an absolute correlation between mutation of allele N and ecdysone resistance. Therefore of the four copies of *EcR* that are present in Kc167 cells, probably only one—allele N—is fully functional.

DISCUSSION

Transfection of Kc cells with a long linear fragment of genomic DNA results in a high frequency of illegitimate

TABLE 3

Rescue of ecdysone response by exogenous EcR in induced and spontaneous ecdysone-resistant lines

Line	Source	Induction	
		−exogenous EcR	+exogenous EcR
Kc167	Wild type	50 ± 30 (N = 23)	44 ± 20 (N = 2)
SB1-5-25R	Spontaneous	2.9	10.7
Kcβ2	Spontaneous	14	16
Kcβ3	Spontaneous	2.9	10.5
Kcβ4	Spontaneous	5.4	22
L57-3-11	Transformation with EcR-15-copia-DHFR	2.0 ± 0.4 (N = 7)	32 ± 17 (N = 7)
L69-4-12	Transformation with EcR-15	52	100
L69-4-16	Transformation with EcR-15	20	45
L69-5-3	Transformation with EcR-15	6.6	38
L69-5-29	Transformation with EcR-15	4.5	40
L69-5-29	Transformation with EcR-15	5.0	36
L75-5-28	Transformation with EcR-8	23 ± 2 (N = 2)	56 ± 32 (N = 2)

Each line was transfected with H1X-188-cc-cat, with or without act-EcR, an expression vector for EcR-B1. Induction is the ratio of reporter activity in aliquots treated with ecdysone to reporter activity in untreated aliquots from the same transfection. Except for Kc167, the parental ecdysone-responsive line, all of the lines listed are ecdysone-resistant as defined by their ability to grow in the presence of the hormone and their inability to undergo the typical morphological response to ecdysone. Lines identified as "spontaneous" were generated by selecting untreated cells for several weeks in 10^{-6} M 20-hydroxyecdysone; Kcβ2, Kcβ3, and Kcβ4 were cloned from a single wild-type population and therefore are not necessarily derived from independent events. SB1-5-25R was selected from an independent ecdysone-responsive clone. In L57-3-11, the isoform B1-specific exon is deleted from allele N (see Figure 3); in L75-5-28, the transforming DNA has inserted by homologous recombination into the N allele. Chromosomal lesions have not been identified in the other ecdysone-resistant clones. In those cases for which multiple independent tests were carried out, variation is indicated as SD ($N > 2$) or range ($N = 2$).

recombinations in the vicinity of the chromosomal homologue. We propose the term *parahomologous targeting* to describe these events, and we offer the following model to explain the occurrence of parahomologous targeting in Drosophila cells: (1) Exogenous DNA associates with its chromosomal homologue with high efficiency. We hypothesize that this association is related to the pairing of somatic chromosomes. (2) Once associated with its chromosomal homologue, the plasmid DNA can have any of three fates: It can undergo homologous recombination with the chromosome, it can be incorporated by illegitimate interactions with chromosomal sites nearby, or it can be lost.

The role of a somatic pairing mechanism is supported by several lines of evidence. First, somatic pairing is prevalent in Drosophila but not in mammals; this is correlated with the fact that parahomologous targeting is not observed in mammalian cells. Chromosome pairing is obvious in polytene cells and has been demonstrated by a variety of techniques in unpolytenized somatic cells of Drosophila (*e.g.*, LEWIS 1954; KOPCZYNSKI and MUSKAVITCH 1992; HIRAOKA *et al.* 1993). Second, a phenomenon similar to parahomologous targeting has been observed in the germ-line transposition of *P* elements harboring either of two independent fragments, one from *engrailed* and one from *polyhomeotic*; both of these fragments are also capable of mediating a pairing-dependent depression of w^+ expression (HAMA *et al.* 1990; KASSIS *et al.* 1991, 1992; FAUVARQUE and DURA 1993). The correlation between targeted *P*-element transposition and a transvection-like effect suggests a role for pairing in this phenomenon. Third, early Drosophila transformation experiments by FOX and his colleagues suggested, on both genetic and morphological grounds, that a noncovalent association may form between transfected DNA and its chromosomal homologue (FOX *et al.* 1970, 1971; FOX and VALENCIA 1975).

The efficiency of parahomologous targeting, presumably a reflection of the efficiency of pairing between a plasmid fragment and its chromosomal homologue, increases with increasing length of the homology. A 15-kb *EcR* fragment causes a higher frequency of ecdysone resistance than an 8-kb fragment. A 9-kb *Eip71CD* fragment causes a larger elevation in transformation frequency than a 4-kb fragment. Preliminary results indicate that the 300-bp act5C fragment in the selectable marker does not cause rearrangements near the chromosomal act5C locus at a detectable rate (data not shown).

Pairing may lead to either homologous or illegitimate recombination. The relative frequencies of these pathways differ in the *EcR* and *Eip71CD* targeting experiments. The reason(s) for the difference (*e.g.*, differences in extent and position of mismatch, global chromosomal/nuclear location) remain to be determined.

Illegitimate recombination is accompanied by rearrangements in the surrounding chromosomal region. The mechanism of these rearrangements is unknown, but similar phenomena have been observed to accompany illegitimate recombination in mouse cells (COVARRUBIAS *et al.* 1986; WILKIE and PALMITER 1987).

The small number of plasmid copies integrated into the chromosome in these experiments contrasts with earlier observations of long arrays of plasmid DNA in transformed Drosophila cells (BOUROUIS and JARRY 1983; MOSS 1985). The failure of plasmid DNA to form long arrays in these experiments may be correlated with the presence of long Drosophila sequences or with the use of electroporation rather than calcium phosphate-DNA coprecipitation for transfection; we have not tested these possibilities. The linearity of the input DNA is probably not the critical variable, since linearized and supercoiled plasmids produce very similar arrays in S2 cells (MOSS 1985).

In the course of these experiments, we found an unexpected degree of variation between the chromosomal homologs in Kc cells. These cells are near-diploid; in particular, the second and third chromosomes, containing *EcR* and *Eip71CD*, respectively, show no obvious karyotypic abnormalities (CHERBAS *et al.* 1980). Yet there are four copies of *EcR*, rather than the expected two, and one of those copies is 5 kb longer than the others. Similarly, one of the two copies of *Eip71CD* contains an extra 5 kb, and the sequence difference between the two alleles is sufficient that hybridization of a 200-bp probe to the Sal2 and Sal3 fragments gives very different intensities of hybridization for the two alleles (see Figures 2 and 3). The source of this large amount of variation is not known; the parental line of flies was lost several decades ago (G. ECHALIER, personal communication). However, it is known that the formation of a permanent line is associated with a high degree of mobilization of middle-repeat elements (POTTER *et al.* 1979), and we suspect that transposition of these elements is responsible for the heterozygous insertions seem at both *Eip71CD* and *EcR*.

In general, the fate of exogenous DNA in Drosophila cells resembles that in mammalian cells, with one critical difference: In mammalian cells, most integrations consist of illegitimate recombinations scattered throughout the genome and accompanied by rearrangements of nearby chromosomal sequences. In Drosophila, most integrations also consist of illegitimate recombination accompanied by rearrangements of nearby chromosomal sequences, but these integrations are clustered near the chromosomal homologue. Parahomologous targeting in Drosophila cells therefore induces apparently random mutations in a narrowly targeted region of the genome. Its potential as a tool for mutagenesis in cell culture and in flies remains to be explored.

We thank R. SADOWSKI for technical assistance, Drs. M. CAPECCHI, W. M. GELBART, J. KASSIS, and T. KORNBERG for useful discussions and an anonymous reviewer for many helpful suggestions. This work was supported by U.S. Public Health Service grant GM-37813.

LITERATURE CITED

BOUROUIS, M., and B. JARRY, 1983 Vectors containing a prokaryotic dihydrofolate reductase gene transform *Drosophila* cells to methotrexate resistance. EMBO J. **2:** 1099–1104.

CHERBAS, L., and P. CHERBAS, 1981 The effects of ecdysteroid hormones on *Drosophila melanogaster* cell lines. Adv. Cell Culture **1:** 91–124.

CHERBAS, L., C. D. YONGE, P. CHERBAS and C. M. WILLIAMS, 1981 The morphological response of Kc-H cells to ecdysteroids: Hormonal specificity. Wilhelm Roux's Arch. **189:** 1–15.

CHERBAS, L., R. A. SCHULZ, M. M. D. KOEHLER, C. SAVAKIS and P. CHERBAS, 1986 Structure of the *Eip28/29* gene, an ecdysone-inducible gene from *Drosophila*. J. Mol. Biol. **189:** 617–631.

CHERBAS, L., K. LEE and P. CHERBAS, 1991 Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. Genes Dev. **5:** 120–131.

CHERBAS, L., R. MOSS and P. CHERBAS, 1994 Transformation techniques for *Drosophila* cell lines. Methods Cell Biol. **44:** 161–179.

CHERBAS, P., L. CHERBAS, G. DEMETRI, M. MANTEUFFEL-CYMBOROWSKA, C. SAVAKIS *et al.*, 1980 Ecdysteroid hormone effects on a *Drosophila* cell line, pp. 278–305 in *Gene Regulation by Steroid Hormones*, edited by A. K. ROY and J. H. CLARK. Springer-Verlag, Heidelberg.

CHERBAS, P., D. A. TRAINOR, R. J. STONARD and K. NAKANISHI, 1988 26-[^{125}I]Iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. Proc. Natl. Acad. Sci. USA **85:** 2096–2100.

CHERBAS, P., and L. CHERBAS, 1996 Molecular aspects of ecdysteroid hormone action, pp. 175–221 in *Metamorphosis: Postembryhonic Reprogramming of Gene Expression in Amphibian and Insect Cells*, edited by L. I. GILBERT, J. R. TATA and B. G. ATKINSON. Academic Press, San Diego.

COVARRUBIAS, L., Y. NISHIDA and B. MINTZ, 1986 Early postimplantation embryo lethality due to DNA rearrangements in a transgenic mouse strain. Proc. Natl. Acad. Sci. USA **83:** 6020–6024.

D'AVINO, P. P., S. CRISPI, L. CHERBAS, P. CHERBAS and M. FURIA, 1995 The moulting hormone ecdysone is able to recognize target elements composed of direct repeats. Mol. Cell. Endocrinol. **113:** 1–9.

EICKBUSH, D., 1987 Molecular characterization of the 71CE region of *Drosophila melanogaster*. Ph.D. thesis, Harvard University, Cambridge, MA.

FAUVARQUE, M.-O., and J.-M. DURA, 1993 *Polyhomeotic* regulatory sequences induce developmental regulator-dependent variegation and targeted P-element insertions in *Drosophila*. Genes Dev. **7:** 1508–1520.

FOX, A. S., and J. I. VALENCIA, 1975 Gene transfer in *Drosophila melanogaster*: cytological alterations in the salivary gland chromosome of transformed stock. Chromosoma **51:** 279–290.

FOX, A. S., W. F. DUGGLEBY, W. M. GELBART and S. B. YOON, 1970 DNA-induced transformation in *Drosophila*: evidence for transmission without integration. Proc. Natl. Acad. Sci. USA **67:** 1834–1838.

FOX, A. S., S. B. YOON and W. M. GELBART, 1971 DNA-induced transformation in *Drosophila*: genetic analysis of transformed stocks. Proc. Natl. Acad. Sci. USA **68:** 342–346.

HAMA, C., Z. ALI and T. B. KORNBERG, 1990 Region-specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. Genes Dev. **4:** 1079–1093.

HASTY, P., J. RIVERA PÉREZ, C. CHANG and A. BRADLEY, 1991 Target frequency and integration pattern for insertion and replacement vectors in embryonic stem cells. Mol. Cell. Biol. **11:** 4509–4617.

HIRAOKA, Y., A. F. DERNBURG, S. J. PARMELEE, M. C. RYKOWSKI, D. A. AGARD *et al.*, 1993 The onset of homologous chromosome pairing during *Drosophila melanogaster* embryogenesis. J. Cell Biol. **120:** 591–600.

KASSIS, J. A. , E. P. VANSICKLE and S. M. SENSABAUGH, 1991 A fragment of *engrailed* regulatory DNA can mediate transvection of the *white* gene in Drosophila. Genetics **128:** 751–761.

KASSIS, J. A., E. NOLL, E. P. VANSICKLE, W. F. ODENWALD and N. PERRIMON, 1992 Altering the insertional specificity of a *Drosophila* transposable element. Proc. Natl. Acad. Sci. USA **89:** 1919–1923.

KOELLE, M. R., W. S. TALBOT, W. A. SEGRAVES, M. T. BENDER, P. CHERBAS *et al.*, 1991 The Drosophila *EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell **67:** 59–77.

KOPCZYNSKI, C. C., and M. A. T. MUSKAVITCH, 1992 Introns excised from the Delta primary transcript are localized near sites of Delta transcription. J. Cell Biol. **119:** 503–512.

LEWIS, E. B., 1954 The theory and application of a new method of detecting chromosomal rearrangements in *Drosophila melanogaster*. Am. Nat. **88:** 225–239.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

MOSS, R. E., 1985 Analysis of a transformation system for *Drosophila* tissue culture cells. Ph.D. thesis, Harvard University, Cambridge, MA.

ORR-WEAVER, T. L., J. W. SZOSTAK and R. J. ROTHSTEIN, 1981 Yeast transformation: A model system for the study of recombination. Proc. Natl. Acad. Sci. USA **78:** 6354–6358.

POTTER, S. S., W. J. BROREIN, P. DUNSMUIR and G. M. RUBIN, 1979 Transposition of elements of the *412, copia* and *297* dispersed repeated gene families in *Drosophila.* Cell **17:** 415–427.

REBAY, I., R. J. FLEMING, R. G. FEHON, L. CHERBAS, P. CHERBAS *et al.*, 1991 Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for notch as a multifunctional receptor. Cell **67:** 687–699.

ROTH, D., and J. WILSON, 1988 Illegitimate recombination in mammalian cells, pp. 621–653 in *Genetic Recombination,* edited by R. KUCHERLAPATI and G. R. SMITH. American Soc. Microbiol., Washington, DC.

SAVAKIS, C., M. M. D. KOEHLER and P. CHERBAS, 1984 cDNA clones for the ecdysone-inducible polypeptide (EIP mRNAs of Drosophila Kc cells. EMBO J. **3:** 235–243.

SCHULZ, R. A., and B. A. BUTLER, 1989 Overlapping genes Of Drosophila melanogaster: organization of the *Z600-gonadal-Eip28/29* gene cluster. Genes Dev. **3:** 232–242.

SCHULZ, R. A., X. XIE, A. J. ANDRES and S. GALEWSKY, 1991 Endosperm-specific expression of the *Drosophila mex1* gene. Dev. Biol. **143:** 206–211.

SEDIVY, J. M., and A. L. JOYNER, 1992 *Gene Targeting.* W. H. Freeman and Co., N.Y.

SEGAL, D., L. CHERBAS and P. CHERBAS, 1996 Genetic transformation Of *Drosophila* Cells in culture by P element-mediated transposition. Somatic Cell Mol. Genetics, **22:** 159–165.

SWEVERS, L., L. CHERBAS, P. CHERBAS and K. IATROU, 1996 *Bombyx* EcR (BmEcR) and *Bombyx* USP (BMCF1) combine to form a functional ecdysone receptor. Insect Biochem. Mol. Biol. **26:** 217–221.

TALBOT, W. S., E. A. SWYRYD and D. S. HOGNESS, 1993 Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell **73:** 1323–1337.

WILKIE, T. M., and R. D. PALMITER, 1987 Analysis of the integrant in MyK-103 transgenic mice in which males fail to transmit the integrant. Mol. Cell. Biol. **7:** 1646–1655.

Communicating editor: R. S. HAWLEY