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Ectopic mitotic recombination in *Drosophila* probed with bacterial β-galactosidase gene-based reporter transgenes

Stephan Bärtsch, Klaus Dücker[1], Friederich E. Würgler and Christian Sengstag*

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, Schorenstrasse 16, 8603 Schwerzenbach, Switzerland and [1]Institute of Zoology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

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ABSTRACT

Plasmids were constructed to investigate homologous mitotic recombination in *Drosophila* cells. Heteroalleles containing truncated but overlapping segments of the bacterial β-galactosidase gene (*lacZ*) were positioned either on separate plasmids or as direct repeats on the same chromosome. Recombination reconstituted a functional *lacZ* gene leading to expression of LacZ$^+$ activity detectable by histochemical staining. High extrachromosomal recombination (ECR) frequencies between unlinked heteroalleles were observed upon transient co-transfection into *Drosophila melanogaster* Schneider line 2 (S2) cells. Stably transfected cells containing the *lacZ* heteroalleles linked on a chromosome exhibited intrachromosomal recombination (ICR) frequencies two orders of magnitude lower than ECR frequencies. Recombination was inducible by exposing the cells to ethyl methanesulphonate or mitomycin C. Recombination products were characterized by multiplex PCR analysis and unequal sister chromatid recombination was found as the predominant mechanism reconstituting the *lacZ* gene. To investigate recombination *in vivo* imaginal disc cells from transgenic larvae carrying the reporter gene on the X chromosome were isolated and stained for LacZ$^+$ activity. The presence of a few LacZ$^+$ clones indicated that mitotic recombination events occurred at frequencies two orders of magnitude lower than the corresponding event in cultured cells and late during larval development.

INTRODUCTION

During their life most cells undergo a variety of DNA rearrangements which are often attributed to recombinational activities. Homologous recombination between identical or similar sequences occurs both in meiosis and mitosis and mitotic recombination between ectopic repeated sequences may be responsible for genome rearrangements which can either lead to cell death or to the generation of cells with tumourigenic potential (1,2).

Mitotic recombination is the molecular basis of sister chromatid exchange (3), DNA sequence amplification (4), excision of small circular DNAs from chromosomes (5) and generation of chromosomal deletions, such as those observed during rearrangement of immunoglobulin genes (6). Mechanisms of extrachromosomal (ECR) and intrachromosomal (ICR) recombination have been studied in detail in bacteria, yeast, mammals and plants and have been reviewed (7–10). In *Drosophila* recombination between repeated elements such as the *Suppressor of Stellate Su* (*Ste*) locus (11), the *Bar* eye mutation (12), retrotransposons (13) and ribosomal DNA sequences (14) have been studied.

Various approaches to study mitotic recombination are based on artificial recombination substrates, introduced into the cells by transformation/transfection with plasmids bearing different mutant alleles of selective or non-selective marker genes which may recombine to reconstitute a functional gene. The mutations were due to linker insertions, single nucleotide alterations or deletions that render the marker genes non-functional and these experimental systems have proven to be valuable in dissecting mechanisms of recombination.

To our knowledge no *Drosophila in vitro* model for the study of mitotic recombination has been described so far, whereas different *Drosophila in vivo* models have been developed (15). Green and colleagues (16) have described a somatic assay system to investigate repeat recombination in the eye of *Drosophila melanogaster* using a tandem quadruplication of the *white-ivory* (w^i) eye color mutation, which reverts to w^+ by recombination. Also, to permit *in situ* detection of recombination in different tissues of *Arabidopsis thaliana* by histochemistry, Swoboda *et al.* (17) developed a system based on direct repeated heteroalleles of the non-selective β-glucuronidase gene.

We have used a similar strategy based on the *lacZ* gene. Plasmids were developed bearing two defective *lacZ* alleles with overlapping regions of homology as extrachromosomal or genomic recombination substrates. By recombination a functional *lacZ* gene may be restored, giving rise to cells expressing β-galactosidase, which can be detected by histochemical staining. Using this system we found that: (i) mitotic recombination occurs in transiently and stably transfected *Drosophila* S2 cells; (ii) unequal sister chromatid exchange (USCE) is the major cause for the rearrangement; (iii) recombination is inducible by treating the cells with carcinogens. Moreover, when the *lacZ*-based recombination

*To whom correspondence should be addressed. Tel: +41 1 825 7461; Fax: +41 1 825 0476; Email: sengstag@toxi.biol.ethz.ch

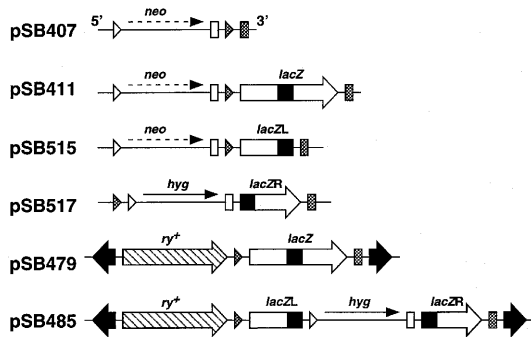

Figure 1. Schematic representation of recombination reporter plasmids (pSB515, pSB517 and pSB485) and negative and positive control vectors (pSB407, pSB411 and pSB479). Plasmids are not drawn to scale. Black boxes indicate regions of 288 bp homology. A dashed arrow represents the neomycin resistance gene (*neo*) and a lined arrow the hygromycin resistance gene (*hyg*), both regulated by the *Drosophila copia* LTR (open triangle). The 3′-ends of the *neo* and *hyg* genes are fused to the simian virus 40 (SV40) polyadenylation signal (open small box). The 3′-deleted *lacZ* genes (open and black box) in pSB515 and pSB485 and the complete *lacZ* in pSB411 and pSB479 are preceded by the *Drosophila actin5C* promoter (shaded triangle). The *actin5C* polyadenylation signal sequence (shaded small box) is positioned downstream of all *lacZ* alleles. The P element plasmids pSB479 and pSB485 contain the *Drosophila* eye colour marker *rosy* (*ry*$^+$), indicated by a striated boxed arrow, and the 31 bp inverted repeats (filled arrowed boxes) from a *Drosophila* P element.

reporter was stably introduced into the genome of *Drosophila* by P element-mediated transformation, LacZ$^+$ activity was detected in a few cells of dissected imaginal discs.

MATERIALS AND METHODS

Plasmid constructions

Plasmids are illustrated in Figure 1. DNA modifying enzymes were obtained from Boehringer (Mannheim, Germany). DNA was prepared and modified using standard molecular methods (18).

Plasmid pSB515. To obtain a 3′-deleted *lacZ* allele (*lac*ZL) flanked by *Not*I sites an *Eco*RV fragment was isolated from pSB341 (19) carrying a *lacZ*-derived *Eco*RI–*Xba*I fragment of pC4AugβGal (20). A *Drosophila* translation initiation site was fused to the 5′-end of *lacZ* in pC4AugβGal. The *Eco*RV fragment was subcloned into the *Eco*RV site of pBLSK-NotI-NotI (21). The re-isolated *Not*I–*Not*I *lacZ* fragment was further subcloned between the *Drosophila actin5C* promoter sequence and *actin5C* polyadenylation site (22) of pSB407 (19), which carries the *Eco*RI–*Bam*HI fragment of the neomycin resistance gene (*neo*) of pcopneo (23) in its *Bgl*II and *Eco*RI sites. In the resulting plasmid pSB515 the 5′-end of the truncated *lacZ* allele is linked to the 3′-end of the *actin5C* promoter. The *neo* gene in pSB515, regulated by the constitutive *Drosophila copia* long terminal repeat (LTR) element (24), allowed selection for the plasmid in *Drosophila* cells.

Plasmid pSB517. To obtain plasmid pSB517, where a 5′-deleted *lacZ* allele (*lac*ZR) is preceded by a *copia* LTR and a hygromycin resistance gene (*hyg*) (23), the *Sal*I–*Xba*I fragment of pSB425 (19) was gel purified and inserted between the *Sal*I and *Xba*I sites of pBLSK-NotI-NotI. The *Not*I–*Not*I fragment was rescued from this plasmid and subcloned into pNpAc (25). The LTR–*hyg*–*lac*ZR fragment was now flanked by the *actin5C* promoter sequence on the 5′-side and by the *actin5C* polyadenylation site upstream of the *lacZ* sequence. The *hyg* gene was used as a selection marker for stable integrants after *Drosophila* cell transfection.

Plasmids pSB407, pSB411, pSB479 and pSB485. Construction of these plasmids has been described elsewhere (19) and a detailed description will be provided upon request. Plasmids pSB411 and pSB479 harbour a *lacZ* gene, whereas the recombination reporter plasmid pSB485 contains physically linked truncated *lacZ* heteroalleles with overlapping homologies. The dominant *Drosophila* eye color marker *rosy*$^+$ (*ry*$^+$) (26) and the 31 bp inverted repeats from a *Drosophila* P element flank the *lacZ* alleles in pSB479 and pSB485.

Cell culture, transfection and cloning

S2 cells (27) were maintained in culture in Schneiders *Drosophila* medium (SDM) supplemented with 10% fetal bovine serum (FBS) and passaged as described (28). Transfection of cells was performed with supercoiled plasmid DNA, purified according to the protocol from Qiagen (Chatsworth, CA) by the calcium phosphate technique (29). Cloning of cell line 485AD1 transgenic for the recombination reporter construct pSB485 was performed as described (19).

Drosophila germline transformation

Plasmid pSB485 or pSB479, purified by caesium chloride/ethidium bromide equilibrium density gradient centrifugation (18), was used to transform *Drosophila* embryos of the ry^{506} (26) genotype by standard procedures of microinjection (30). Inserts in emerging transgenic flies were made homozygous. Matings were done at 25°C. All stocks were maintained on standard cornmeal/molasses/agar medium (28). The following lines were established and used in this study: 485#1, in which the reporter sequence of pSB485 was genetically mapped to the X chromosome; 479#4-C, which contains a functional *actin5C* promoter–*lacZ* gene insertion on the X chromosome.

Southern blots

Isolation of genomic DNA from flies was carried out essentially as described (28), but slightly modified. To extract DNA, 50 flies were homogenized with a Teflon pestle in 750 µl homogenization buffer (0.1 M Tris–HCl, pH 9.0, 1 M EDTA, 1% SDS). Digested genomic DNA was electrophoresed in 0.5% agarose. The DNA was Southern blot hybridized as described (19). The filter was probed with a 288 bp *Eco*RV–*Cla*I *lacZ* fragment complementary to the region of homology in pSB485. The probe was labelled as described (31).

Multiplex PCR-based screening for recombined *lacZ* gene sequences

For PCR analysis recombinant LacZ$^+$ cells were isolated by limited dilution as follows. Exponentially growing 485AD1 cells were seeded into five 96-well microtiter plates containing SDM supplemented with 20% FBS at a density of 200–400 cells/well. After 2 weeks plates were replica plated and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (32). Populations containing LacZ$^+$ cells were re-diluted and expanded. Genomic DNA was isolated from wells containing ~50% LacZ$^+$ cells as described (19) and subjected to PCR analysis.

Sequences from reporter genes, from recombination products and from *Drosophila* ribosomal protein 49 genes (*rp49*) (33) were simultaneously amplified using the following pairs of oligonucleotide primers, purchased from Microsynth (Balgach, Switzerland) (see legend to Fig 3.): P1, 5′-GTGAAACGCAGGTCGCCAGC-3′; P2, 5′-ATGCCGCCAACAACAGTTTCC-3′; P3, 5′-AAAACCT-CCCACACCTCCCCC-3′; P4, 5′-TGATTCATTCCCCAGCG-ACC-3′; P5, 5′-CCTACCAGCTTCAAGATGACCATC-3′; P6, 5′-CGTAACCGATGTTGGGCATC-3′.

All primers had annealing temperatures between 57 and 61°C applying the Oligo® program (MedProbe, Oslo, Norway). PCR reactions contained 500 ng genomic or 10 ng plasmid DNA, 50 mM Tris, pH 9.0, 50 mM KCl, 0.01% gelatin, 0.2 mM dNTPs, 500 nM each primer, 6 mM magnesium chloride and 2.5 U Taq polymerase. Reactions were performed in a Hybaid thermal cycler (Hybaid, Teddington, UK) in 100 µl after overlaying the reaction mixtures with 100 µl mineral oil (Sigma, St Louis, MO) and started with a denaturation step at 94°C for 5 min. Further denaturation steps (94°C, 2 min), annealing steps (59°C, 1.5 min) and extension steps (72°C, 0.5 min) were repeated for 40 cycles. PCR fragments were resolved on a 2% agarose gel containing 1 µg/ml ethidium bromide.

Exposure of cultured cells to chemical and physical mutagens

One day prior to exposure to chemicals 485AD1 target cells were seeded at a density of $2–3 \times 10^7$ cells/10 ml SDM, 10% FBS in 15 ml falcon tubes. Cells were harvested by centrifugation (900 *g*) and resuspended in SDM lacking FBS. Aqueous solutions of mitomycin C (MMC) (Syntex Pharm, Allschwil, Switzerland) and ethyl methanesulphonate (EMS) (Eastman Kodak, Rochester, NY) were added to the cells for 2 h at 24°C, gently shaking the suspension every 15 min. Reactions were terminated by replacing the medium with SDM, 10% FBS.

UV irradiation of cells was done in 0.1 M sodium phosphate buffer, pH 7.25, using a short wave length (254 nm) UV lamp (Ultra Violet Products, San Gabriel, CA) at a fluence of 1.76 J/m^2/s. Cells were washed twice and transferred to fresh SDM, 10% FBS medium. After an expression phase of 5 days at 24°C cells were stained (see below) and counted under a light microscope. RFs were determined by dividing the number of recombinant (LacZ$^+$) cells by the total number of viable cells. Cytotoxicity data were determined by comparing suspension growth curves of treated and untreated cultures as previously described (34).

X-Gal staining of transgenic S2 cells and larval tissue

Staining of cultured *Drosophila* cells with X-Gal (Stehelin, Basel, Switzerland) dissolved in *N*,*N*′-dimethylformamide (Merck, Darmstadt, Germany) was done according to MacGregor *et al*. (32). Imaginal discs dissected from wandering third instar larvae were fixed, washed and X-Gal stained overnight (35) and were then mounted under coverslips in 80% glycerol, observed and photographed under a light microscope.

RESULTS

Experimental strategy

The rationale behind the *Drosophila* system used in these studies of recombination involved stable transfection of S2 cells and transformation of embryos with recombination reporter plasmids. The constructs contain deleted *lacZ* genes with overlapping regions of homology of 288 bp. Recombination between the regions of homology of the heteroalleles confers a LacZ$^+$ phenotype to recombinants, which can then be identified by histochemical staining for LacZ$^+$ activity. The *lacZ* gene is expressed under control of the *Drosophila actin5C* promoter, which exhibits a wide range of tissue type expression in *Drosophila*. Plasmids pSB515 and pSB517 (Fig. 1) were used to detect ECR and carry a selection marker for hygromycin (pSB517) or G418 resistance (pSB515) respectively, allowing selection for S2 cells harbouring both plasmids simultaneously or recombinant derivatives thereof. In addition, both plasmids contain physically unlinked, but overlapping *lacZ* heteroalleles and intermolecular recombination within the region of homology may reconstitute the wild-type gene. In plasmid pSB485 the same heteroalleles are linked as direct repeats separated by a hygromycin resistance gene, allowing for selection of stable integrants upon transfection of cells. The control vectors pSB479 and pSB411 contain a functional *lacZ* gene, whereas plasmid pSB407 is the parental vector of pSB411 and lacks the *lacZ* gene (Fig. 1). Inverted repeats derived from the *Drosophila* transposable P element encompass, in addition to *ry*$^+$, either the reporter in pSB485 or *lacZ* in pSB479, allowing integration of the *lacZ* and the *ry*$^+$ alleles into the germline of flies by P element-mediated transformation of embryonic germline cells.

As depicted in Figure 2, different recombinational events occurring extrachromosomally between plasmids pSB515 and pSB517 (Fig. 2A) or between homologous sequences within integrated plasmid pSB485 (Fig. 2B–E) respectively may lead to reconstitution of a wild-type *lacZ* gene. Thus expression of a functional *lacZ* may be an indicator of reciprocal and non-reciprocal DNA exchanges, such as simple cross-over (CO), deletion (DEL), unequal sister chromatid exchange (USCE), single strand annealing (SSA) and gene conversion (GC).

ECR occurred at high frequencies in cultured *Drosophila* cells

We asked whether plasmids pSB515 and pSB517 could recombine extrachromosomally to generate an intact *lacZ* gene. Each plasmid (1 µg/ml cell suspension containing 5×10^5 cells/ml) was transfected separately or in combination into S2 cells. Cells were then maintained in non-selective medium for 48 h at 24°C to allow expression of antibiotic resistance followed by growth for two cell generations in selective medium containing G418 (2 mg/ml) and/or hygromycin (0.3 mg/ml). One aliquot of the cells was then X-Gal stained for LacZ$^+$ activity and another for viability with fluorescein diacetate. Neither plasmid alone was able to confer a LacZ$^+$ phenotype on S2 cells, whereas co-transfection of both plasmids resulted in a fraction of LacZ$^+$ cells (data not shown). From counting 500–2500 viable cells per experiment ($n = 4$) and determining the ratio of LacZ$^+$ cells over the total number of viable cells, a RF of 1.8 ± 0.4% was estimated. Although the structure of the recombination products was not analysed at the molecular level, it was concluded from this experiment that a functional *lacZ* gene could be formed from the two truncated *lacZ* alleles. This encouraged us to study recombination between both *lacZ* alleles stably integrated into the genome.

Histochemical monitoring of mitotic recombination events in clonal transgenic cultured *Drosophila* cells

Cell clone 485AD1 containing ~40 copies of plasmid pSB485 integrated as concatemers in its genome as well as positive and negative control strains (polyclonal populations) constructed by introducing either pSB411 or pSB407 have been described (19).

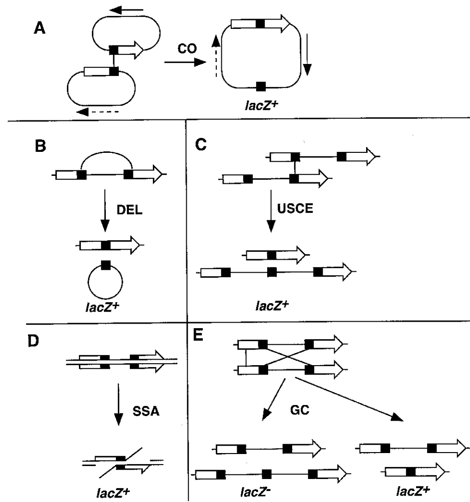

Figure 2. Different recombination events may lead to reconstitution of a functional *lacZ* gene. Black boxes represent regions of homology. A single line represents duplex DNA, except in (D) where each line represents a single DNA strand. (**A**) Extrachromosomal recombination (cross-over, CO) between pSB515 and pSB517 gives rise to a *lacZ* gene and the 288 bp region newly flanked by both selective markers. (B–E) Possible recombination mechanisms for genomic rearrangements. (**B**) The deletion recombination (DEL) event generates a functional *lacZ* gene by deleting the sequence that separates the repeats and producing an unstable non-replicating extrachromosomal element. (**C**) Unequal sister chromatid exchange (USCE) gives rise to a duplication of the *hyg* gene and reconstitution of a *lacZ* gene on the other chromatid. (**D**) According to the single strand annealing model (SSA), DNA ends at a double-strand break between the sequences of homology are degraded by exonucleases such that complementary sequences may be exposed as single strands. By removal of non-homologous ends these sequences may anneal and by DNA synthesis and ligation a functional *lacZ* gene may be reconstituted. (**E**) Possible gene conversion (GC) events.

The three cell lines were expanded and subjected to histochemical X-Gal staining. No blue colour was detectable in pSB407 (*lacZ*$^-$)-transfected cells after overnight incubation in X-Gal solution. In contrast, every pSB411 (*lacZ*$^+$)-transfected cell immediately stained blue (data not shown). Thus, no endogenous LacZ$^+$-like activity was present in S2 cells and the *actin5C* promoter allowed expression of sufficient amounts of LacZ$^+$ from the bacterial *lacZ* gene to be detected by X-Gal staining. In clone 485AD1 rare putative recombination events giving rise to LacZ$^+$ cells were identified by their blue colour against a LacZ$^-$ background. The molecular nature of such *lacZ*$^+$ recombinants was investigated further.

Analysis of spontaneous genome rearrangements at the molecular level

Nine individual, randomly chosen subpopulations that expressed *lacZ* were isolated from 485AD1 cells and genomic DNA was prepared and subjected to PCR analysis. As had been observed before (19), such lacZ$^+$ derivatives give rise to a 599 bp fragment, corresponding to the recombined reporter construct, upon amplification with primers P1 and P4. To further discriminate between the different mechanisms that may give rise to a functional *lacZ* gene (Fig. 2B–E), multiplex PCR was performed using *lacZ*-specific primers P1–P4 (Fig. 3, left) and internal control primers (P5 + P6) (not shown), allowing monitoring of successful PCR conditions by amplifying a 252 bp fragment from the endogenous *rp49* gene.

Figure 3 (right) shows the result of agarose gel electrophoretic analyses (upper two panels) and Southern blot analysis (bottom panel). As is visible in the top panel, 599 bp PCR fragments were detected in PCR reactions using P1–P6 performed with (i) genomic DNA from spontaneous LacZ$^+$ cells (lane 1–9), (ii) genomic DNA from pSB411-transfected cells containing a *lacZ* gene (lane 11) and (iii) with DNA from plasmid pSB411 (lane 13). Additional PCR fragments of 649 and 745 bp, corresponding to amplification products of primer pairs P1 + P2 and P3 + P4, were present in lanes 1–9 (recombinants) and lane 14 (pSB485, positive control) and were diagnostic for the unrearranged recombination reporter. The co-amplified 252 bp PCR fragments, corresponding to DNA amplified from the cellular *rp49* gene (lanes 1–12), reflected successful PCR conditions in reactions containing genomic DNA. The intensity of the fragments diagnostic for deletions (599 bp, P1 + P4) was reduced compared with that of others. This product of recombination within the array of 40 copies of the reporter gene provides a lower amount of DNA target compared with the unrearranged reporter construct amplified by P1 + P2 and P3 + P4. Fragments indicative of duplications of *hyg* (795 bp, P2 + P3) were not clearly detectable.

Therefore, to increase the sensitivity for detection of *hyg* duplications, one of the two products of USCE, a new PCR reaction was performed on the same DNA batches with primer pairs P2 and P3 alone and the products analysed by Southern blot hybridization (Fig. 3, middle and bottom panels). Amplicons of 795 bp hybridized to a *lacZ* probe which covers the repetitive *lacZ* region in pSB485 in six of nine analysed recombinants (lanes 1–9). Note that the respective diagnostic bands were not formed in a PCR with linearized pSB485 (lane 14). A band of 0.4 kb (*) was observed the nature of which remained unexplained, however, it exhibited no hybridization with the *lacZ* probe. The *lacZ* gene could be reconstituted by either USCE, intrachromatid recombination or SSA and can segregate from the sister chromatid during subsequent cell division. The former event, USCE, concomitantly produces a duplication of the *hyg* gene on one chromatid and a deletion of the *hyg* gene on the other chromatid, whereas the latter two events delete the *hyg* gene without giving rise to a duplication. Sister chromatid conversion (Fig. 2E) leading to LacZ$^+$ cells would require conversion of large (4.3 kb long) regions of heterology. For this reason conversion was considered a minor mechanism for generation of LacZ$^+$ cells.

Detection of recombined *lacZ*$^+$ sequences concomitantly with duplicated *hyg* sequences in cell extracts of six of the nine recombinants argues for USCEs as the predominant cause of rearrangement. The events that occurred in the remaining three populations containing LacZ$^+$ cells could be due to either intrachromosomal deletion or SSA, which cannot be distinguished in our experimental set-up.

Induction of direct repeat recombination in cultured *Drosophila* cells by model mutagens

UV, the monofunctional alkylating chemical EMS and the bifunctional alkylating clastogen MMC are well-characterized mutagens known to be recombinagenic in yeast, plants and mammalian cells. Since biological effects cannot be compared on the basis of applied dose, these mutagens were compared on the basis of equal cytotoxicity. Survival data were obtained by performing the cytotoxicity experiments as described in Materials and Methods and doses giving 50% survival were chosen.

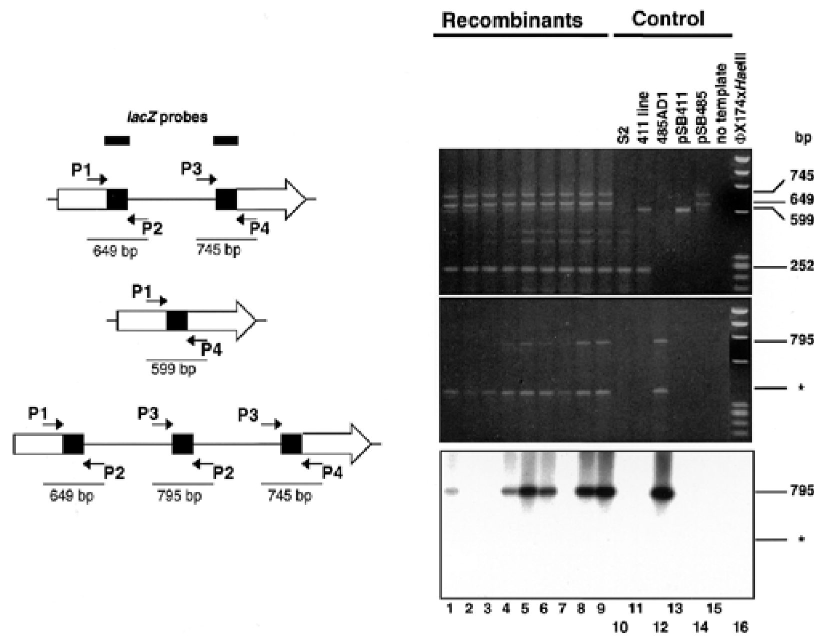

Figure 3. PCR and Southern blot analysis of recombination products in 485AD1 cells. (Left) The diagram depicts the sites of primer hybridization in the recombination reporter construct pSB485 (top). Boxes and arrowed boxes represent the *lacZ* heteroalleles in different recombination products (middle and bottom). Black boxes designate the region of homology. The small arrows correspond to the position of PCR primers P1–P4. Primers P5 and P6 (not shown) were used to amplify a fragment from the endogenous *rp49* gene that served as an internal control template. The sizes of amplified fragments are indicated by the labelled thin bars. The radiolabelled probe (thick bar) used for Southern blot analysis is complementary to the *lacZ* repeat. (Right) Genomic (lanes 1–12) and plasmid (lanes 13–14) DNA from the indicated sources was PCR amplified, using equimolar mixtures of primers, and fragments analysed by agarose gel electrophoresis (top, P1–P6 in the PCR mixture; middle, P2 and P3 alone). DNA was subsequently transferred to a nylon filter and subjected to Southern blot analysis (bottom). A size marker is shown in lane 16.

Table 1. Spontaneous and induced mitotic recombination frequencies (RFs) of *lacZ* alleles in *Drosophila* 485AD1 cells

Mutagen	No. of LacZ$^+$ cells detected[a]	No. of cells screened[a]	RF[b]
None	19	19331	
	29	22059	
	43	21717	$1.44 \pm 0.51 \times 10^{-3}$
UV (1.76 J/m^2/s × 180 s)	16	12249	
	29	16254	
	26	12555	$1.74 \pm 0.39 \times 10^{-3}$
EMS (75 mM × 2 h)	52	12996	
	65	13635	
	56	12879	$4.38 \pm 0.38 \times 10^{-3}$
MMC (0.5 µM × 2 h)	28	11511	
	27	10071	
	31	9537	2.76 ± 0.41 x 10^{-3}

[a]As determined by microscopic examination following X-Gal staining, counting the number of cells using a haemocytometer.
[b]RFs were calculated from the number of LacZ$^+$ cells detected divided by the total number of cells screened.

The ability of the mutagens to induce recombination in 485AD1 cells was assayed in three experiments using an EMS concentration of 75 mM and a MMC concentration of 0.5 µM for 2 h in growth medium lacking serum. In the UV irradiation experiment cells were exposed in sodium phosphate buffer to 370 J/m^2. Untreated controls accompanied each experiment. Prior to staining with X-Gal, the cells were allowed to recover for 5 days (two to three generations) in growth medium supplemented with serum. The data are shown in Table 1. EMS and MMC increased the frequency of LacZ$^+$ cells above the background frequency, which was determined to be $1.44 \pm 0.51 \times 10^{-3}$ from three experiments ($n = 3$). The applied dose of EMS elevated the frequency to $4.38 \pm 0.38 \times 10^{-3}$ ($n = 3$), thus producing a 3-fold increase. The cross-linking agent MMC induced a 2-fold increase. UV irradiation was only able to marginally increase the frequency of LacZ$^+$ cells and the variation in viability of the cells was considerable from experiment to experiment. These results argue that at least two of the used agents, MMC and EMS, were able to increase the RF in the 485AD1 cell line.

Spontaneous chromosomal recombination rarely occurred *in vivo*

To investigate direct repeat recombination *in vivo*, strain 485#1 was established in which the pSB485-derived recombination reporter transgene was genetically mapped to the X chromosome. Since the presence of a functional reporter in the transformants is a prerequisite for its usefulness to study recombination, integrity of the reporter was assessed with Southern blot hybridization (Fig. 4.). Genomic DNA was isolated from 50 adult 485#1 flies which had been cultured for a year after transformation. Restriction enzyme analysis revealed the presence of *Eco*RV fragments of 4.3 and 4.9 kb and a *Not*I fragment of 7.9 kb that hybridized to a probe corresponding to the 288 bp direct repeated region of the *lacZ* alleles *lacZ*L and *lacZ*R respectively. Double digests liberated

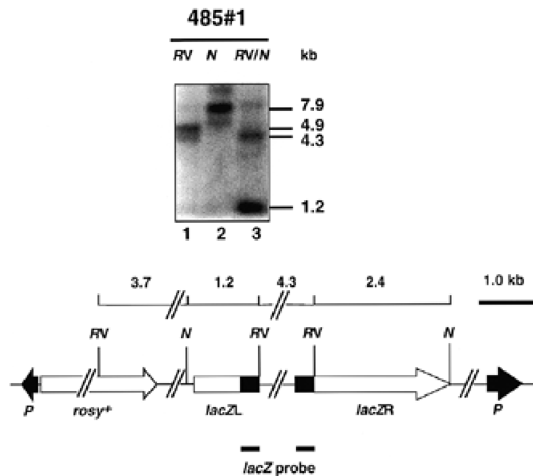

Figure 4. Genomic blot of fly DNA isolated from 485#1 transformants. Genomic DNA samples were separately digested with *Eco*RV (*R*V), *Not*I (*N*) or double digested with the same enzymes (*R*V/*N*), electrophoresed and blot hybridized. Filters were probed with a 288 bp *lacZ* sequence (black bars) corresponding to the direct repeated sequence (black boxes).

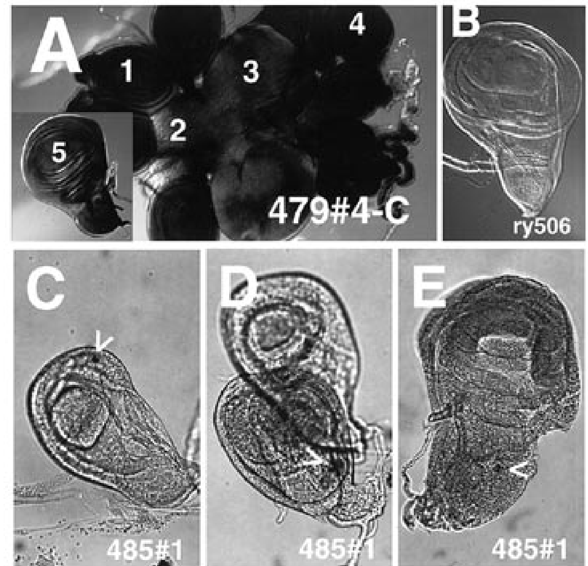

Figure 5. Visualization of recombination events in imaginal discs of transformed *Drosophila* third instar larvae by histochemical X-Gal staining. Imaginal discs prepared from 479#4-C larvae, containing a functional *lacZ* gene, were used as a positive control for tissue type expression: (**A**) (1) leg disc, (2) ventral ganglion, (3) brain hemisphere, (4) eye disc and (5) wing disc. (**B**) A wing disc isolated from ry^{506} mutant larvae. (**C–E**) Imaginal disc preparation of strain 485#1 revealing independent late recombination events resulting in small LacZ$^+$ spots in leg discs (C and D) and in a wing disc (E).

two fragments of 1.2 and 4.3 kb which hybridized to the probe. These results confirmed the correct arrangement of the repeat units within the genome and in addition showed that the repeat on the X chromosome in the 485#1 line was genetically stable over many generations. Some weak additional bands were observed in lane 2 corresponding to the *Not*I digest. Although the nature of these bands remained elusive, we regard them as artifacts related either to suboptimal digestion or to unrelated sequences in the *Drosophila* genome exhibiting weak homology to the probe used. Nevertheless, the absence of a signal corresponding to a 3.6 kb band in the *Not*I digest argues for the absence of a previously recombined reporter cassette in 485#1 flies.

We aimed to quantitate RFs in larval tissue. As a prerequisite for a crude quantitation of recombination events in cells of imaginal discs, it was necessary to show that a recombined *lacZ* gene was expressed in sufficient amounts to be detected by X-Gal staining. For that purpose strain 479#4-C was constructed, which harbours an X chromosomally linked *lacZ* gene originating from plasmid pSB479. Females and males of the transformed strain were crossed and imaginal disc cells isolated from the larval third instar progeny were analysed for LacZ$^+$ activity. As shown in Figure 5A, the *lacZ* gene was strongly and abundantly expressed in leg discs (1), in ventral ganglion cells (2), in cells of the brain hemispheres (3), in eye discs (4), in wing discs (5) and in halteres (not shown).

A few brain and ventral ganglion cells as well as some epithelial cells and cells of the fat body isolated from 10 $lacZ^- ry^{506}$ mutant larvae stained non-specifically for LacZ$^+$ activity (results not shown). This suggested that not all organs of *Drosophila* are suitable for detection of mitotic recombination by the histochemical staining method applied due to the presence of endogenous β-galactosidase-like activities. Non-specific staining was not observed in the cells of 17 wing discs and 10 eye discs isolated from ry^{506} larvae. A wing disc incubated overnight in the X-Gal solution is shown in Figure 5B. Thus a quantitative analysis of recombination seemed restricted to wing, leg and eye imaginal discs.

For analysis of recombination in tissues of 485#1 larvae five adult males were crossed to five females in each experiment. Imaginal discs were dissected from third larval instar progeny. As summarized in Table 2, a total of 36 wing discs were X-Gal stained and analysed for blue spots. Three small LacZ$^+$ spots (arrow) were each found on separate wing or leg discs dissected from different larvae (examples are shown in Fig. 5C–E). Due to the small clusters and/or individual blue cells the recombination events presumably occurred late in development of the particular larvae.

Table 2. Recombination events detected in imaginal discs of 485#1 larvae

Imaginal disc[a]	No. of disks analyzed		No. of LacZ$^+$ spots detected	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Wing	19	17	2	1
Leg	4	2	2	0
Eye	6	2	0	0
Haltere	4	3	0	0

[a]One wing imaginal disc (larval third instar) consists of ~30 000 cells, a leg disc of 10 000, an eye disc of 20 000 and a haltere of 5000.

Assuming that a wing disc contains ~30 000 cells (15), an approximate RF (number of LacZ$^+$ spots/number of imaginal discs analysed/30 000 cells) of 2.8×10^{-6} was calculated, a frequency two to three orders of magnitude lower than determined in the *in vitro* system with cell line 485AD1.

DISCUSSION

Much of our current understanding of mitotic recombination in eukaryotes has been obtained using recombination reporters containing two different mutant alleles of a selective marker gene. However, an evident limitation using selective marker genes is based on the fact that only cells containing the recombined marker gene are selected and other products of recombination are lost.

To overcome these limitations we have constructed vectors based on the non-selective bacterial *lacZ* gene, allowing us to monitor recombination *in vivo* and *in vitro* in *Drosophila*. Truncated *lacZ* heteroalleles retaining overlapping regions of homology were placed either on different plasmids (pSB515, pSB517), to monitor interplasmid ECR *in vitro*, or positioned in tandem on plasmid pSB485, which was stably integrated into the genome of *Drosophila* cells to study ICR. Rearrangements that created a functional *lacZ* gene were identified by histochemical staining for $LacZ^+$.

Most mammalian ECR assay systems employ selection for the recombination product and high RFs of 1–20% have been found (36–38). ECR in plant cells has also been analysed and frequencies of 0.1–10% have been reported (39–41). We found that ECR between physically unlinked *lacZ* heteroalleles occurred at similarly high frequencies (2%) in *Drosophila* cells when the respective plasmids were transiently co-transfected. Since selection was applied a few days after transfection, intermolecular recombination presumably occurred extrachromosomally. The high frequency of $LacZ^+$ cells detected directly after transfection might reflect facilitated recombination of extrachromosomal DNA free of adherent proteins.

Ectopic homologous recombination was monitored between the same heteroalleles integrated in the chromosome of cell line 485AD1, which contained ~40 arrayed copies of the reporter sequence. At early passages this line gave rise to $LacZ^+$ subclones at a frequency of 2.85×10^{-4} (19), which is comparable with values described by Hellgren *et al.* (42), who determined a frequency of 5.8×10^{-4} for Chinese hamster ovary (CHO) cells bearing 34 truncated *neo* alleles with a common homology of 400 bp at one integration site. A frequency of 2.8×10^{-4} was reported for another clone carrying 26 copies of the recombination reporter integrated at an independent locus. An increased ability of CHO cells to take up and integrate DNA in the genome has been described, which may be related to an elevated recombination activity detectable in protein extracts (43). This might also hold true for S2 cells.

Much lower RFs were observed by Lin and Sternberg (44), who used a substrate consisting of two partially defective herpes simplex virus thymidine kinase (*tk*) genes cloned into bacteriophage λ DNA, which was transferred into mouse $LMtk^-$ cells. The two alleles, sharing homologous 984 bp sequences, are separated by 3 kb largely vector DNA and are present in the transformants at ~40–100 copies. From two cell lines they obtained spontaneously occurring tk^+ recombinants at frequencies of $2–2.5 \times 10^{-6}$. Using a closely related assay involving ICR in mouse L cells, Wang *et al.* (45) observed a background frequency of tk^+ recombinants of 18×10^{-6}. These cells contained a single integrated copy of the recombination reporter. Recombination frequencies might not only be influenced by differences in copy numbers of the reporter gene but also by differences in DNA methylation. Such DNA modification can indeed lead to inactivation of recombined intact genes in mammalian cells (46), whereas in *Drosophila* cells DNA is not methylated (47).

Several physical and chemical agents are known to induce recombination. The induction experiments with UV, MMC and EMS were done with 485AD1 cells that had proceeded through ~30 generations. This led to accumulation of spontaneous $LacZ^+$ subclones in the culture, for which reason the frequency of $LacZ^+$ cells in the controls was 5-fold higher (1.44×10^{-3}) compared with that determined at early passages (2.85×10^{-4}). Nevertheless, recombination between the truncated *lacZ* genes was inducible by EMS and MMC 2- to 3-fold. In our experiments 0.5 µM MMC applied for 2 h killed 50% of the cells. Wang *et al.* (45) reported a MMC-induced 2.8-fold increase in tk^+ recombinants after 1 h exposure to 6 µM, with 59% survival. Lin and Sternberg (44) observed a 3- to 4-fold increase in tk^+ colonies at 0.3 µM MMC for 48 h with the transgenic mouse cell line $LMtk^-aprt^-$. However, the authors do not report percentage survival at the appropriate concentration. Hellgren *et al.* (48) showed that MMC induced a 2-fold increase in the frequency of G418-resistant recombinants upon treating the CHO:5 cell line for 24 h with 0.15 µM MMC (40% survival).

EMS is known to predominantly induce nucleotide transitions (49), but it also induces direct repeat recombination in yeast (50). The exposure of yeast cells for 17 h to 8 mM EMS (relative survival of 57%) induced a 7-fold increase in RF (51). Preliminary results reported by Murti *et al.* (52) indicate that it induced a marked response in gene conversion observed in germ cells of mice. In our experiments a 3-fold increase in RF was observed in *Drosophila* cells exposed for 2 h to 75 mM EMS, further evidence for the recombinagenicity of EMS.

In contrast, UV irradiation barely induced homologous recombination in 485AD1 cells at a dose resulting in 50% cell killing and transgenic S2 cells were relatively insensitive to UV radiation. Yet UV efficiently induces mitotic recombination in yeast (50) and in mammalian cells (53). Thus our results might be related to photorepair activation of damaged DNA and the efficient repair of UV-induced lesions found in *Drosophila* cells (54) argues for this assumption. In addition, UV-irradiated cells might die, show delayed cell cycling or be prevented from entering S phase, allowing photorepair and/or nucleotide excision repair to occur prior to replication. This might reduce recombinagenicity and, to a lesser extent, also cytotoxicity to background levels in cultured cells. Such a phenomenon has been observed for mutagenicity/cytotoxicity in cultured human fibroblasts (55).

To gain information on the recombination mechanism preferentially used by 485AD1 cells, a PCR-based analysis of genomic DNA was performed. Using different primer pairs evidence was obtained that in six out of nine cases a functional *lacZ* gene was reconstituted by USCE. The remaining three events were either due to deletion or SSA. The observation that *hyg* duplication products were frequent (six of nine) is consistent with models for gene amplification based on USCE (56). Using similar DNA substrates, Bollag and Liskay (57) also observed a predominance of USCE over other recombination mechanisms.

The preponderance of USCE was not observed in yeast. Deletion and duplication products of direct repeat recombination could also be explained by a sister chromatid conversion (SCC) mechanism (58). In contrast, SCC could not readily explain the recovery of deletion products in mouse L cells (57). In this particular set-up SCC would have required conversion of large regions of heterology of 4.4 kb and previous studies done by the same group (59) indicated inefficient conversion even at 1.5 kb long regions of heterology. Since the regions of heterology in our

substrate are 4.3 kb in size, we do not favour gene conversion as a significant mechanism leading to duplications and deletions.

In addition to the *in vitro* system we present, to our knowledge, the first *in vivo* system for the study of direct repeat recombination in *Drosophila*. P Element-transformed 485#1 larvae, carrying the recombination reporter integrated in the X chromosome, exhibited a frequency of 2.8×10^{-6} for direct repeat recombination. Similarly low frequencies have been reported for the *Drosophila* system developed by Green *et al*. (16), where somatic reversion of w^{i} (26) within the w^{i} quadruplication occurred at a frequency of 1.14×10^{-5} and a single copy w^{i} allele reverted at a frequency of 1.07×10^{-6}. Furthermore, >1 000 000 mice have been screened for phenotypic reversion at the *dilute* (*d*) coat colour locus (60) and one displayed the expected phenotype, reflecting a frequency of 0.9×10^{-6} for recombination between repeated 520 bp LTR elements separated by ~7.0 kb. To keep the direct repeat RF low by suppression of recombination between reiterated sequences makes sense with regard to the presence of large amounts of repetitive DNA in the eukaryotic genome and to the deleterious effects which recombination events may have (loss or amplification of information).

In conclusion, the presented *lacZ*-based systems provide advantages over selective systems since (i) no selection phase is required to identify recombination events and (ii) cells can be stained for enzymatic activities indicative of recombination at any time of interest. It will be interesting to see whether feeding carcinogens to DNA repair-proficient 485#1 larvae will result in an increase in recombination events in imaginal disc cells and, for comparison, to repeat the experiments with otherwise isogenic but recombinational repair-deficient fly strains, once such strains are available. So far a *RAD1* homologue has been identified in *Drosophila* as the *Mei-9* gene (61). Since yeast *rad1* mutants are also defective in direct repeat recombination (62), it would be interesting to also test our substrates in this *mei* genetic background.

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