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TECHNICAL NOTE

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Reduction of Cre recombinase toxicity in proliferating *Drosophila* cells by estrogen-dependent activity regulation

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Abstract The Cre/*loxP* site-specific recombination system has been used successfully for genome manipulation in a wide range of species. However, in *Drosophila melanogaster*, a major model organism for genetic analyses, the alternative FLP/FRT system, which is less efficient at least in mammalian cells, has been established, primarily for the generation of genetic mosaics for clonal analyses. To extend genetic methodology in *D. melanogaster*, we have created transgenic lines allowing tissue-specific expression of Cre recombinase with the *UAS/GAL4* system. Surprisingly, chronic expression of Cre recombinase from these transgenes (*UAST-cre*) was found to be toxic for proliferating cells. Therefore, we also generated transgenic lines allowing the expression of Cre recombinase fused to the ligand-binding domain of the human estrogen receptor (*UASP-cre-EBD*). We demonstrate that recombination can be efficiently dissociated from toxicity by estrogen-dependent regulation of recombinase activity of the *UASP-cre-EBD* transgene products.

Keywords Cre recombinase · *loxP* · Clones · Chromosomal aberration · Toxicity

Introduction

The Cre recombinase from bacteriophage P1 has proven to be a powerful tool for manipulating pro- and eukaryotic genomes (for a review see Nagy 2000). Cre is a member of the integrase family of site-specific recombinases and catalyzes recombination between *loxP* sites. The *loxP* site is a 34-bp consensus sequence consisting of an 8-bp core spacer sequence flanked by an inverted 13-bp repeat. The demonstration that Cre recombinase is active in eukaryotic cells combined with the fact that a specific 34-bp sequence is not expected to occur by random chance within even large vertebrate genomes has encouraged the development of a large variety of genetic strategies (Nagy 2000). In particular, Cre/*loxP*-mediated conditional excision of defined sequences resulting in elimination, modification or activation of gene function in mice is now very widely used. In addition, defined chromosomal aberrations (deletions, inversions, translocations) have been created successfully, and strategies for efficient site-specific insertion of transgenes have been described.

Siegal and Hartl (1996) have recently demonstrated that the Cre/*loxP* system is applicable in *Drosophila melanogaster* as well. In this organism, however, an alternative site-specific recombination system, FLP/FRT, originally identified in yeast, was established earlier (Golic and Lindquist 1989) and is much more widely used. This FLP/FRT system has been used in *Drosophila* with great success, predominantly for the generation of genetic mosaics for clonal analyses. These experiments depend on a low recombination efficiency, just sufficient to induce recombination in a few isolated cells of a progenitor cell population followed by clonal expansion of these recombined cells. Low efficiency of recombination, however, is a severe limitation for other experimental strategies. Quantitative recombination in all cells is required for the inactivation of an allele in adult post-mitotic tissues like the *Drosophila* brain for example. In mammalian cells, the Cre/*loxP* system has proven to be far more effective than FLP/FRT (Nakano et al. 2001). The Cre/*loxP* system, therefore, might also be the system of choice for experiments requiring maximal recombination efficiency in *Drosophila*.

To expand genetic methodology in *Drosophila*, we have established transgenic lines allowing tissue-specific expression of Cre recombinase with the *UAS/GAL4* system (Brand and Perrimon 1993). Our *UAST-cre* transgene, therefore, can be combined with the existing large collection of *Drosophila* lines expressing the yeast tran-

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scription factor Gal4 in defined tissues and at defined stages. Surprisingly, however, *UAST-cre* expression was found to be highly toxic especially in proliferating cells. To minimize these toxic effects, we have constructed additional transgenes (*UASP-cre-EBD*; EBD = estrogen-binding domain) allowing expression of Cre recombinase fused to the ligand-binding domain of the human estrogen receptor. The activity of similar fusion proteins has previously shown to be dependent on the presence of estrogen in mammalian cells (Metzger et al. 1995). Here we show that the activity of our Cre recombinase-EBD fusion proteins can also be regulated by estrogen in *Drosophila*. Thereby, activity levels can be fine-tuned allowing *loxP* site-dependent recombination without apparent toxicity.

Materials and methods

Fly stocks

The following *GAL4* lines were used: *ey-GAL4* (2/8; kindly provided by B. Dickson), *GMR-GAL4* (12; Freeman 1996), *dpp.blk1-GAL4* (40C.6; Staehling-Hampton et al. 1994), *en-GAL4*, *prd-GAL4* (Brand and Perrimon 1993), *MS1096* (Capdevila and Guerrero 1994), *F4* (Weiss et al. 1998), and *sev-hs-GAL4* (K25; kindly provided by K. Basler). *GAL4* expression in the last line is controlled by a regulatory region containing two copies of a *sevenless* enhancer and a heat-inducible promoter fragment from the *Hsp70* gene.

Transgenic lines allowing *GAL4*-dependent expression of Cre recombinase were obtained by germ line transformation with three different P element constructs. The vector pUAST (Brand and Perrimon 1993) was used for the first construct. A DNA fragment containing the complete coding sequence of Cre recombinase was amplified by polymerase chain reaction (PCR) from the plasmid pRH200 (kindly provided by Mark Siegal and Daniel Hartl, Harvard University; Mack et al. 1992) using primers (5′-GGA AGA TCT GAA TGC AAA ATG TCC AAT TTA CTG ACC-3′ and 5′-GCG GTA CCT ATC AAC TAA TTA TAG CAA TCA TTT AC-3′) introducing *Bgl*II and *Kpn*I sites at the 5′ and 3′ ends, respectively, followed by insertion into the corresponding sites of pUAST. Sequence analysis of the amplified region confirmed the presence of the correct sequence. Several independent *UAST-cre* lines were established and analyzed.

The vector pUASP (Rorth 1998) was used for the other constructs which contained fusion genes of Cre recombinase and the EBD of the human estrogen receptor. The primer EBD251 (5′-GAA GTG CGG CCG CTG AAA GGT GGG ATA CGA AAA G3′) in combination with the primer EBD3′ (5′-GTC GAC GGA TCC GAA TTC AGG-3′) was used to amplify the sequences encoding the D linker domain followed by the E ligand-binding domain of the human estrogen receptor from a cDNA plasmid (pG/ERG; kindly provided by D. Picard, University of Geneva). The resulting fragment was digested with *Not*I and *Bam*HI and inserted into the corresponding sites of pUASP. For a second construct, the ligand-binding domain without the D linker domain was amplified with the primer EBD304 (5′-CGC TCG CGG CCG CAC AGC CTG GCC TTG TCC CTG-3′) in combination with EBD3′ and inserted analogously into pUASP. In a second step the sequence encoding Cre recombinase was amplified from pRH200 using primers (5′-TCC GGT ACC CTT TAC TTA AAA CCA TTA TCT G-3′ and 5′-CGT TAG CGG CCG CTC GCC ATC TTC CAG CAG GC-3′) introducing *Kpn*I and *Not*I sites at the 5′ and 3′ end, respectively. Using these sites, the Cre recombinase fragment was introduced upstream of the estrogen receptor fragment and several independent *UASP-cre-EBD*251 and *UASP-cre-EBD*304 lines were established with the resulting constructs.

To detect Cre recombinase activity, we used the transgene ^w^+^2 (Siegal and Hartl 1996). In this transgene insertion on the second chromosome, which we will designate as *lox-w-lox* in the following, a mini-*white*$^+$ gene is flanked by *loxP* sites. Cre recombinase activity therefore results in excision of the mini-w^+ gene from the chromosome.

Estrogen application

Standard fly food containing brewer's yeast, corn meal, soy meal, molasses and agar was melted in a microwave oven and cooled to about 55°C before addition of β-estradiol. Water soluble β-estradiol (E 4389, Sigma) from a 15-mg/ml stock solution was added to obtain the desired final concentration. To evaluate dose dependencies, we used final concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1.0 mg/ml. For brief incubation periods, we used instant *Drosophila* food (Schlüter, Wimenden, Germany) which was dissolved in water containing β-estradiol at the desired concentration. Larvae were added to the resulting food paste on apple agar plates for the desired period. To re-isolate the larvae, food paste was scraped from the apple agar plates and mixed with 10 volumes of glucose solution (30% in H_2O). After sedimentation of food particles, the floating larvae were isolated, washed and transferred to bottles with standard fly food.

PCR assay for Cre recombinase activity

Progeny carrying *lox-w-lox*, *sev-hs-GAL4* and either *UAST-cre*, *UASP-cre-EBD*251, *UASP-cre-EBD*304, or no *UAS* transgene were collected from appropriate crosses during 24 h at 25°C. After aging for 24 h at 25°C, some of the bottles were transferred for 45 min into a 37°C water bath. After a 22-h recovery period at 25°C, larvae were isolated and genomic DNA was prepared essentially as described previously (Pirrotta et al. 1983). Genomic DNA was then used as a template for PCR with three different primers. Primer A (5′-CCT TAG CAT GTC CGT GGG GTT TGA AT-3′) was derived from a sequence present in the *lox-w-lox* transgene downstream of the 3′ *loxP* site. Primer B (5′-ATA TAT CCA TGG CAA CAC TAT TAT GCC CAC CAT TT-3′) was derived from a sequence present in the *lox-w-lox* transgene upstream of the 5′ *loxP* site. Primer C (5′-AAG TTC AAT GAT GTC CAG TGC AG-3′) was derived from a sequence present in the *lox-w-lox* transgene in between the *loxP* sites. With the combination of primers A and C, therefore, a 335-bp fragment is amplified only from the non-recombined *lox-w-lox* gene. The combination of primers A and B, in principle, allows the amplification of fragments from both the non-recombined and the recombined *lox-w-lox* transgene. However, amplification from the non-recombined *lox-w-lox* transgene is much less efficient than from the recombined *lox-w-lox* transgene because of the extensive fragment size difference (4,475 bp vs 283 bp). Amplification of the 4,475-bp fragment, therefore, was not detected and the amplification of the 283-bp fragment reflects the abundance of recombined *lox-w-lox* copies. PCR products were resolved on agarose gels and visualized with ethidium bromide. For a semi-quantitative estimation of the ratio of non-recombined and recombined *lox-w-lox* copies in the genomic DNA isolated from larvae, we performed parallel PCR experiments using standard mixtures with known amounts of the 335-bp and 283-bp products amplified with primers A and B or A and C, respectively, as template for enzymatic amplification. Moreover, in the PCR assays with genomic DNA from larvae we used template amounts resulting in comparable levels of PCR products as observed in the assays with the standard mixtures.

Detection of apoptotic cells and scanning electron microscopy

Wing imaginal discs were dissected from wandering stage third instar larvae in Ringer's solution. Vital staining of the dissected discs with 1.6 µM acridine orange was performed (Wolff 2000)

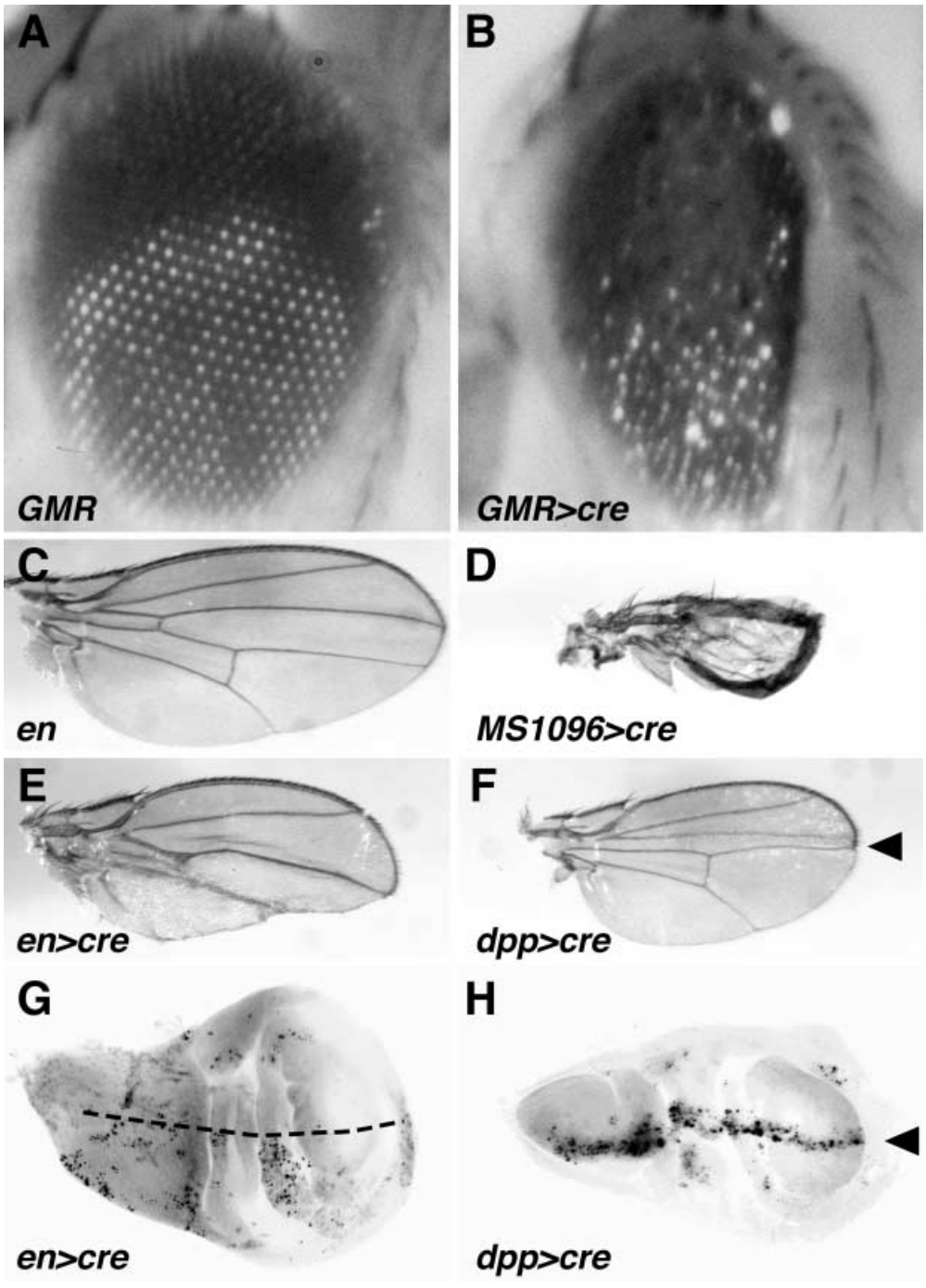

Fig. 1A–H Toxicity of Cre recombinase expression in *Drosophila*. While adult *GMR-GAL4*/+ flies have wild-type eye morphology (**A**), severe abnormalities are present in eyes of *GMR-GAL4*/+; *UAST-cre* III.4/+ flies (**B**). Similarly, while adult *en-GAL4*/+ flies have normal wings (**C**), severe truncations of the posterior wing compartment are present in *en-GAL4*/+; *UAST-cre* III.4/+ flies (**E**). Abnormal wing phenotypes were also observed in *MS1096*/+; *UAST-cre* III.4/+ (**D**) and in *dpp-GAL4*/+; *UAST-cre* III.4 flies (**F**) where they were restricted to the *GAL4*-expressing stripe anterior to the compartment boundary (*arrowhead*). Acridine orange staining (shown in *black*) revealed increased numbers of apoptotic cells at the third instar in imaginal discs of *en-GAL4*/+; *UAST-cre* II.3/+ flies (**G**) posterior to the compartment boundary (*dashed line*) or in imaginal discs of *dpp-GAL4*/+; *UAST-cre* II.3 flies (**H**) in a stripe anterior to the compartment boundary (*arrowhead*)

and analyzed immediately on a Leica TCS SP confocal microscope. For analysis of adult eye morphology by scanning electron microscopy (SEM) we followed standard procedures (Basler et al. 1991).

Results

To achieve tissue-specific expression of Cre recombinase with the *UAS/GAL4* system in *Drosophila*, we generated transgenic lines carrying a *UAST-cre* transgene. Unexpectedly, *UAST-cre* expression with various *GAL4* lines driving distinct temporal and spatial expression programs was found to result in severe phenotypic abnormalities. Figure 1 illustrates the abnormalities resulting from expression in eye imaginal discs (Fig. 1B, *GMR-GAL4*), or in various regions of wing imaginal discs (Fig. 1D–F, *MS1096*, *en-GAL4*, *dpp-GAL4*). Staining for apoptotic cells clearly revealed increased cell death in the *UAST-cre* expressing regions of wing imaginal discs (Fig. 1G, H). The finding that the *sev-hs-GAL4* expression resulting from a single 30 min heat shock during the first larval instar caused complete lethality in *UAST-cre*

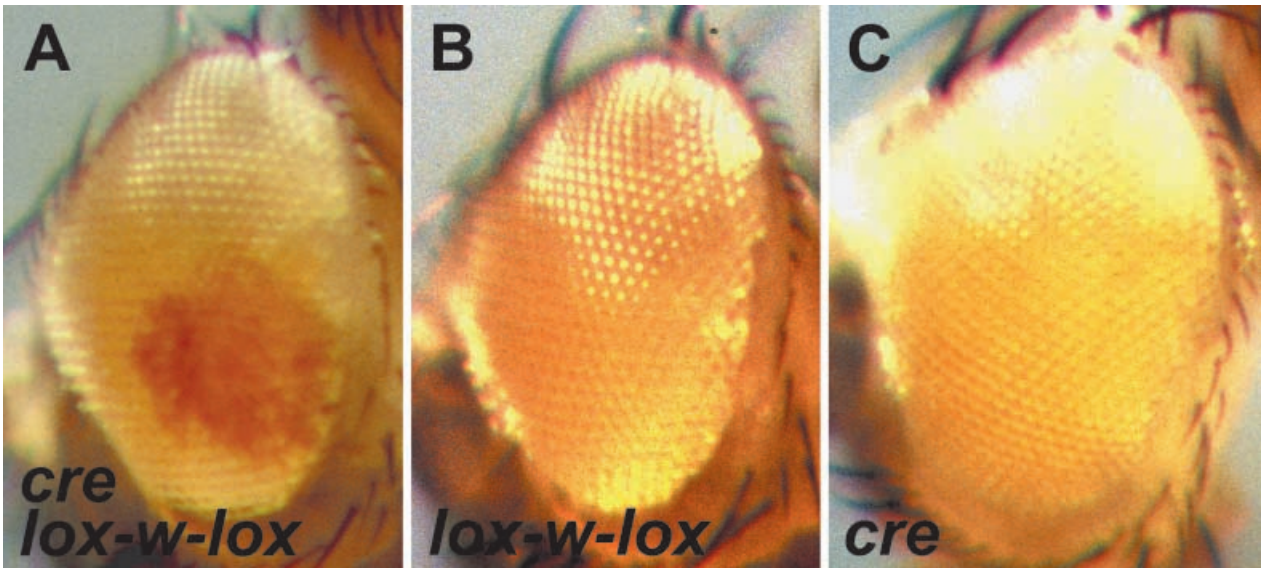

Fig. 2A–C *GAL4*-independent recombinase activity from *UAST-cre*. Eyes of flies carrying both *UAST-cre* III.4 and *lox-w-lox* (**A**) display a patchy distribution of dark and light eye color, while uniform eye color is observed in flies carrying only *lox-w-lox* (**B**) or only *UAST-cre* III.4 (**C**)

progeny further emphasized the toxicity of Cre recombinase.

Interestingly, after *UAST-cre* expression in salivary glands throughout late embryonic and larval development (with the *GAL4* enhancer trap line *F4* which results in expression levels comparable to the other *GAL4* lines used) we were unable to detect morphological abnormalities and increased apoptosis at the third instar stage. Salivary gland cells do not divide during larval development although they progress through up to ten endoreduplication cycles. In contrast, imaginal disc cells proliferate mitotically. It appears therefore that *UAST-cre* expression is especially toxic for dividing cells. Moreover, we also expressed *UAST-cre* during embryogenesis with *prd-GAL4* which directs transient expression in alternating segments during the cell division cycles 14–16 (stage 7–11). DNA labeling of embryos fixed during these and subsequent stages did not reveal differences when the extent of cell death, proliferation and differentiation in *UAST-cre* expressing and non-expressing regions was compared (data not shown). *prd-GAL4* directed *UAST-cre* expression also did not cause lethality. We conclude therefore that Cre recombinase toxicity is primarily apparent after prolonged expression in mitotically proliferating cells.

The severe toxicity resulting from prolonged *UAST-cre* expression was unexpected based on the work of Siegl and Hartl (1996). These authors have successfully established transgenes (*hsmos-cre*) which express Cre recombinase from a hybrid promoter containing elements from *Hsp70* and the mariner transposable element *Mos1*. This hybrid promoter is thought to drive constitutive ubiquitous expression. Since the *hsmos-cre* transgene does not result in phenotypic abnormalities (Siegal and Hartl 1996; and data not shown), we assumed that our *UAST-cre* transgenes result in higher expression levels. This interpretation was supported by the observation (Fig. 2) that *GAL4*-independent, basal *UAST-cre* expression resulted in a mosaic inactivation of a mini-*white*$^{+}$ transgene (*lox-w-lox*), in which essential sequences are flanked by *loxP* sites.

To evaluate whether toxicity results from high levels of Cre recombinase activity we generated transgenes allowing expression of a Cre protein version with inducible recombinase activity. Fusion proteins of Cre recombinase and the ligand-binding domain of the human estrogen receptor are known to display estrogen-inducible activity in mammalian cells (Metzger et al. 1995). Therefore, we generated analogous *UASP* transgenes. Two different transgenes, *UASP-cre-EBD*251 and *UASP-cre-EBD*304, were constructed. The former contains a longer spacer domain between Cre recombinase and the EBD. In analogous fusions of FLP recombinase with *EBD*, the presence of the longer spacer domain has been shown to result in a higher estrogen-induced maximal recombinase activity and a higher estrogen-independent basal activity (Nichols et al. 1997).

In initial experiments, we tested whether estrogen-inducible toxicity resulted from expression of *UASP-cre-EBD*251 and *UASP-cre-EBD*304. Using *ey-GAL4* expression was restricted to eye imaginal discs. Multiple independent insertions of each transgene were analyzed and gave similar results in general, although position effects were observed as well. The majority of *UASP-cre-EBD*251 lines (16 of 26) did not result in abnormal eye morphology when larvae were raised on normal food without estrogen (Fig. 3E). In contrast, when estrogen was added, an aberrant roughened eye appearance was observed with all the tested lines (n=8; Fig. 3H). Penetrance and expressivity of this rough eye phenotype was variable with different lines. Lower concentrations of estrogen resulted in a reduced severity of the phenotypes

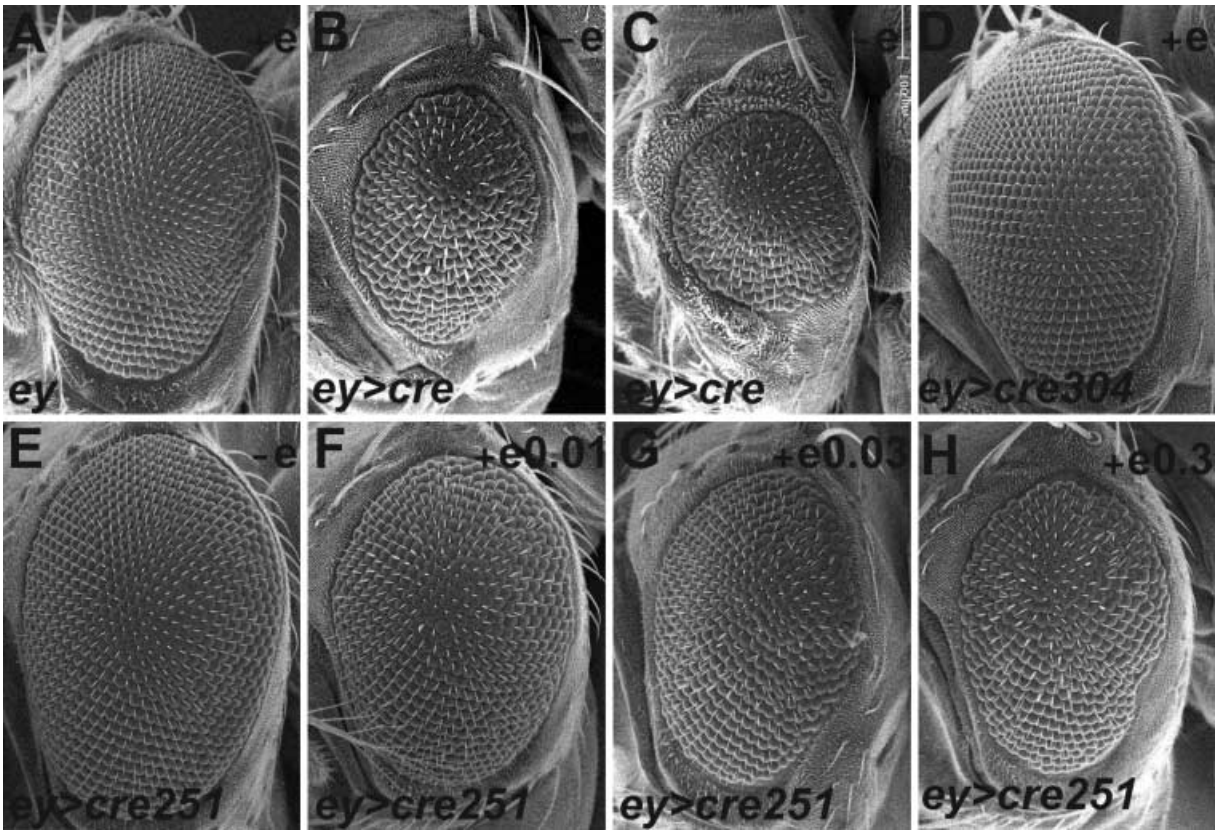

Fig. 3A–H Estrogen-dependent toxicity of *UASP-cre-EBD* expression (*EBD* estrogen-binding domain). Larvae were raised on a diet with (**A, C, D, F–H**) estrogen (+*e*) at a concentration of 0.3 mg/ml (**A**, **C**, **D**, **H),** 0.01 mg/ml (**F**) or 0.03 mg/ml (**G**), or without (**B**, **E**) estrogen (–*e*). All larvae had an *ey-GAL4* transgene and either *UAST-cre* (**B**, **C**), *UASP-cre-EBD*304 III.1 (**D**), or *UASP-cre-EBD*251 III.2 (**E**–**H**), or no *UAS* transgene (**A**). The abnormal eye morphology observed by SEM in the resulting flies reflects toxicity of Cre recombinase

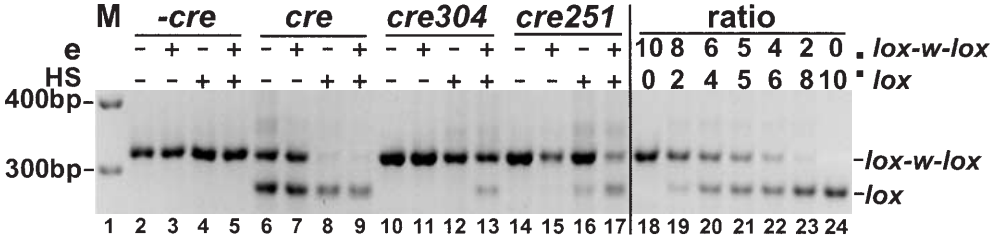

Fig. 4 Estrogen-dependent recombinase activity of *UASP-cre-EBD*. DNA was isolated from *lox-w-lox* larvae carrying only *sev-hs-GAL4* (*lanes 2–5, –cre*), or *sev-hs-GAL4* in combination with either *UAST-cre* III.4 (*lanes 6–9, cre*), or *UASP-cre-EBD*304 II.2 (*lanes 10–13, cre304*), or *UASP-cre-EBD*251 III.2 (*lanes 14–17, cre251*). Before DNA isolation these larvae were raised either in the absence (-) or presence (+) of estrogen (*e*) and either not exposed (-) or exposed (+) to a heat shock (*HS*) to express *sev-hs-GAL4*. The DNA was used for PCR reactions resulting in the amplification of a 335-bp fragment from the non-recombined *lox-w-lox* allele (*lox-w-lox*) and of a 283-bp fragment from the recombined *lox-w-lox* allele (*lox*). For quantitative comparisons of the extent of *lox-w-lox* recombination we started PCR reactions in parallel using purified PCR products from non-recombined and recombined *lox-w-lox* mixed at the indicated ratios (*ratio*) as template DNA (*lanes 18–24*). *M* Molecular weight marker lane

(Fig. 3F, G). Control experiments demonstrated that estrogen has neither an effect on the wild-type eye morphology observed in flies without *cre* transgenes nor on the abnormal morphology observed in flies expressing *UAST-cre* (Fig. 3A–C). Our results demonstrate therefore that the toxicity resulting from expression of *UASP-cre-EBD*251 is estrogen-dependent.

In comparison with the *UASP-cre-EBD*251 lines, the *UASP-cre-EBD*304 lines in general resulted in a somewhat milder phenotype at a given estrogen concentration. Moreover, some *UASP-cre-EBD*304 lines did not, even at the highest estrogen concentrations, result in toxicity (Fig. 3D).

To determine whether *UASP-cre-EBD*251 and *UASP-cre-EBD*304 expressed estrogen-inducible Cre recombinase activity, we developed a semi-quantitative PCR assay

estimating the ratio of non-recombined and recombined *lox-w-lox* alleles in transgenic larvae. These larvae carried a transgene allowing heat-inducible expression of *GAL4* and *UASP-cre-EBD*251 or *UASP-cre-EBD*304 in addition to *lox-w-lox*. Larvae were raised either with or without estrogen in the food. Moreover, while one half of the larvae was kept continuously at 25°C, the other half was briefly exposed to 37°C for 45 min during the first larval instar before analysis by PCR during the second larval instar. Cre recombinase activity was found to be expressed from both *UASP-cre-EBD*251 or *UASP-cre-EBD*304 in a *GAL4*-dependent manner, and Cre recombinase activity was found to be estrogen-inducible. With *UASP-cre-EBD*304 lines (*n*=4) we did not observe recombinase activity in the absence of estrogen (Fig. 4, lane 12, and data not shown). In contrast, with the *UASP-cre-EBD*251 lines (*n*=4) we observed some estrogen-independent Cre recombinase activity (Fig. 4, lane 16, and data not shown). However, estrogen clearly stimulated recombinase activity expressed from both *UASP-cre-EBD*251 and *UASP-cre-EBD*304 (Fig. 4, lanes 13 and 17, and data not shown). This stimulation of Cre recombinase activity by estrogen appears to be dependent on the *EBD* since control experiments with *UAST-cre* did not reveal estrogen inducibility. These control experiments also confirmed that *GAL4*-independent, basal *UAST-cre* expression already results in significant Cre recombinase activity (Fig. 4, lanes 6, 7; also shown in Fig. 2).

To evaluate whether Cre recombinase activity can be adjusted to a level which is not noticeably toxic and yet allows efficient *loxP* site-specific recombination, we analyzed the eye phenotype in flies carrying *lox-w-lox* in combination with *ey-GAL4* and *UASP-cre-EBD*304 II.6. When these flies were raised on food lacking estrogen, all the adult eyes were found to have a normal morphology and a color reflecting the additive function of the three mini-w^+ copies present in the *lox-w-lox*, *ey-GAL4* and *UASP-cre-EBD*304 II.6 insertions (Fig. 5A). In contrast, when these flies were raised on food containing intermediate concentrations of estrogen (0.03 mg/ml), 90% of the adult eyes were predominantly of a lighter color with some darker patches (Fig. 5C), and 10% were exclusively of this lighter color (Fig. 5B) which corresponded to the eye color observed in flies carrying only *ey-GAL4* and *UASP-cre-EBD*304 II.6 but not *lox-w-lox*. Moreover, most of the eyes (76%) also had a completely normal morphology (Fig. 5B). These findings demonstrate therefore that efficient *loxP* site-specific recombination can be obtained without major toxic side effects by estrogen-dependent regulation of Cre recombinase-EBD activity.

We emphasize, however, that we were unable to obtain full penetrance of complete *lox-w-lox* recombination in the absence of morphological defects. While defects were completely absent at the lowest estrogen concentration analyzed (0.001 and 0.003 mg/ml), some eyes (6%) contained rough regions when 0.01 mg/ml was applied. The percentage of eyes with pattern abnormalities, as

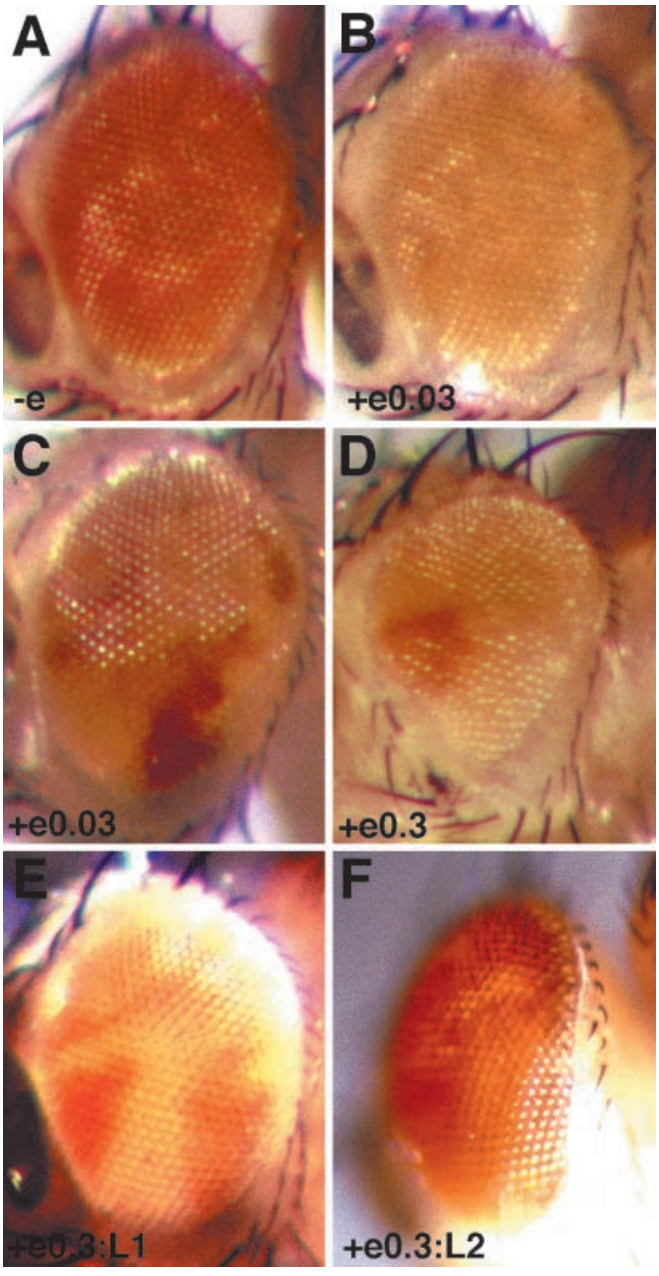

Fig. 5A–F Regulation of Cre recombinase activity and toxicity by estrogen dose and application time. Larvae with *ey-GAL4* and *UASP-cre-EBD*304 II.6 were raised in a diet without estrogen (**A**, *–e*) or with estrogen at 0.03 mg/ml (**B**, **C** *+e0.03*), or at 0.3 mg/ml (**D**, *+e0.3*) or only transiently for 3 h with estrogen during the first larval instar (**E**, *+e0.3: L1*) or during the second larval instar (**F**, *+e0.3: L2*). While *lox-w-lox* recombination does not occur without estrogen application (**A**), it can occur completely in all cells of the eye without accompanying toxicity after application of estrogen at 0.03 mg/ml (**B**). Many eyes however still contain regions with cells that have not recombined *lox-w-lox* at this concentration (**C**). Higher estrogen concentration (0.3 mg/ml) increase the efficiency of *lox-w-lox* recombination minimally but the accompanying toxicity considerably (**D**). Large continuous sectors of dark eye colors can be observed after early pulses of estrogen application during the first larval instar (**E**), while small patches of dark and light regions (see *arrow*) are observed after late pulses of estrogen application during the second larval instar (**F**)

well as the size of the affected regions within these eyes and the severity of the abnormalities, increased with higher estrogen concentrations. Interestingly, while the extent of *lox-w-lox* recombination appeared to plateau at an estrogen concentration of 0.01 mg/ml, the morphological abnormalities increased up to 0.3 mg/ml. Moreover, the morphological abnormalities often affected precisely those regions which were of a dark color (see Fig. 5D).

In an additional experiment we addressed whether Cre recombinase-*EBD* activity can be temporally controlled. In contrast to all of the previous experiments, therefore, larvae were removed from the estrogen-containing diet after a brief incubation period (3 h) and transferred to food lacking estrogen after extensive washing. As expected, small patches of lighter color were observed when the estrogen pulse was applied late during development (Fig. 5F) and very large regions of lighter color were observed after an early estrogen pulse (Fig. 5E). Importantly, the regions of darker color in the latter eyes were mostly continuous with relatively straight borders (Fig. 5E). Thus, these patches had an appearance similar to that observed for the clonal progeny of a single eye imaginal disc cell marked early during larval development. The appearance of the dark patches remaining after transient exposure of estrogen early in development was clearly distinct from the more irregular and less uniform dark patches observed after continuous exposure to estrogen. These findings indicate that Cre recombinase activity declines after removal of the larvae from the estrogen-containing food, since an extended persistence of Cre recombinase activity beyond the time of estrogen withdrawal should effectively prevent the appearance of large uninterrupted dark patches and lead to few irregular dark regions containing many smaller light clones. Moreover, the fact that Cre recombinase activity declines after removal from estrogen-containing food was further indicated by the observation that perturbation of eye morphology was no longer detected after transient feeding of estrogen at concentrations which result in obvious toxicity when present continuously (compare Fig. 5D, E).

Discussion

The Cre/*loxP* site-directed recombination system has been used for genetic manipulation in a variety of organisms. Many lines of transgenic mice expressing Cre recombinase have been described and *Drosophila* lines with a transgene thought to drive constitutive and ubiquitous expression were successfully established as well (Siegal and Hartl 1996). We were surprised, therefore, by our observation that prolonged expression of Cre recombinase during *Drosophila* development kills proliferating cells effectively. This toxicity, which appears to be dependent on high expression levels, might not be restricted to *Drosophila*. A very recent description of the consequences caused by a particular Cre transgene in mice raises the possibility that in this species Cre recombinase might also be more toxic than previously assumed (Schmidt et al. 2000). In mice, a transgene driving Cre recombinase expression in postmeiotic spermatids was found to cause abortive pregnancies with complete penetrance. A similar but reduced toxicity resulting from Cre expression in somatic cells during mouse development or in adults might perhaps have escaped detection previously because of the extensive regulative capacity of mammalian organisms and the substantial background of programmed cell death already occurring during normal development. The potential toxicity of Cre recombinase which might complicate the interpretation of some phenotypes should certainly be kept in mind.

The toxicity appears to be dependent on Cre recombinase activity. In mice, expression of an inactive Cre recombinase no longer caused male sterility (Schmidt et al. 2000). In our experiments in *Drosophila*, the expression of Cre recombinase fused to the ligand-binding domain of the human estrogen receptor was toxic only in the presence of estrogen. High levels of Cre recombinase activity therefore presumably catalyze recombination between cryptic pseudo-*loxP* sites in both the mouse and the *Drosophila* genome which result in chromosomal aberrations. This suggestion is supported by our observation that mitotically proliferating imaginal disc cells are much more sensitive than endoreduplicating salivary gland cells. Many chromosomal aberrations lead to chromosome loss during mitosis, while they have no consequences during interphase. In vitro studies have revealed a number of pseudo-*loxP* sites in yeast and mammalian genomes (Sauer 1992, 1996; Thyagarajan et al. 2000). These sites often include a number of deviations from the *loxP* consensus and yet they are capable of supporting efficient Cre-mediated recombination in vitro. Searches of the *Drosophila* genome sequence also reveal some regions that might qualify as pseudo-*loxP* sites. However, we have not analyzed the *Drosophila* genome sequence in detail because the best matches to the *loxP* consensus sequence might have been discarded as potential BAC vector sequence contamination during genome sequence assembly. Nevertheless, we emphasize that temporally limited expression of reduced Cre recombinase activity results in efficient *lox-w-lox* recombination which is not accompanied by toxicity. It is very unlikely, therefore, that the *Drosophila* genome contains pseudo-*loxP* sites that promote Cre-mediated recombination in critical regions with comparable efficiency to wild-type *loxP* sites.

As the observed toxicity is dependent on chronic expression of high levels of Cre recombinase activity it can readily be avoided. By controlling the timing and level of activity we were able to dissociate toxicity completely from *lox-w-lox* recombination. We show that inducibility of Cre recombinase activity can be achieved in *Drosophila* by expression of a fusion with an estrogen-binding domain, essentially as previously described in mammalian cells (Metzger et al. 1995).

While the activity of our Cre recombinase-*EBD* fusions could be regulated by estrogen, we were unable to

find conditions resulting in a quantitative recombination of the *lox-w-lox* allele in all of the eye imaginal disc cells in all animals. As expected, increases in estrogen concentration were found to be paralleled by a stimulation of the extent of *lox-w-low* recombination and only in the range of higher estrogen concentrations by enhanced toxicity. Concentrations resulting in a considerable fraction of progeny flies in a complete *lox-w-lox* recombination in all cells of normally patterned eyes could readily be found. Surprisingly, however, the extent of *lox-w-lox* recombination appeared to be saturated before complete penetrance and expressivity was reached. While higher estrogen concentrations further enhanced toxicity, they no longer increased penetrance and expressivity of *lox-w-lox* recombination. These results observed with our Cre recombinase-*EBD* fusions contrast with the findings of Siegal and Hartl (1996) who obtained complete penetrance and expressivity of *lox-w-lox* recombination in the absence of detectable toxicity by expressing wild-type Cre recombinase from a hybrid promoter composed of *hsp70* and *Mos1* sequences. We were readily able to confirm their results. The differences in efficiency and toxicity could reflect variations in the timing of expression. Cre recombinase is present from the onset of development when the *hsp70-Mos1* promoter is used, while we have activated the Cre-EBD fusions beginning in the first larval instar. In addition, it is conceivable that the Cre-EBD fusions have an altered, perhaps less processive, recombinase activity resulting in a higher frequency of illegitimate recombination and chromosomal aberrations. Moreover, mutations resulting within *loxP* sites during recombination attempts might also explain the difficulties in obtaining 100% of *lox-w-lox* recombination.

While the present *UASP-Cre-EBD* lines are unlikely to allow complete recombination of lox target genes, they will nevertheless be excellent tools for instance in clonal analyses. A control of Cre recombinase activity to a level appropriate for clonal analyses might also be achieved simply by using a heat-inducible promoter to regulate the level of Cre expression. However, the *UASP-Cre-EBD* lines offer the possibility to combine temporal control of recombinase activity levels and tissue-specific expression. The Cre/*loxP* system can be readily combined in *Drosophila* with the widely used FLP/FRT system, for example to generate clones within clones. This latter level of sophistication has been reached before and has revealed the highly interesting and still mysterious phenomenon of cell competition (Simpson and Morata 1981). For similar future studies, the *UAS-Cre-EBD* lines will allow for a higher efficiency and flexibility.

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