I-SceI Endonuclease, a New Tool for Studying DNA Double-Strand Break Repair Mechanisms in Drosophila

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ABSTRACT

As a step toward the development of a homologous recombination system in Drosophila, we have developed a methodology to target double-strand breaks (DSBs) to a specific position in the Drosophila genome. This method uses the mitochondrial endonuclease I-SceI that recognizes and cuts an 18-bp restriction site. We find that >6% of the progeny derived from males that carry a marker gene bordered by two I-SceI sites and that express I-SceI in their germ line lose the marker gene. Southern blot analysis and sequencing of the regions surrounding the I-SceI sites revealed that in the majority of the cases, the introduction of DSBs at the I-SceI sites resulted in the complete deletion of the marker gene; the other events were associated with partial deletion of the marker gene. We discuss a number of applications for this novel technique, in particular its use to study DSB repair mechanisms.

ONIZING radiation and radiomimetic drugs induce DNA double-strand breaks (DSBs) at random positions in the genome. During mating-type switching in yeast, transposition of P elements in Drosophila, or rearrangements of immunoglobulin genes in vertebrates, DSBs are introduced at specific positions within the genome (Klar 1989; Jackson and Jeggo 1995; Weaver 1995). During evolution, to maintain genome integrity, a number of genetic pathways have been deployed to repair DSBs (reviewed in Haber 1995).

The mechanisms underlying DSB repair have been studied in Drosophila using the P-element transposase as a means to generate the chromosomal breaks (Engels et al. 1990; Kaufman and Rio 1992). These studies have revealed that DSBs, induced by excision of P elements, can be repaired by a conservative mechanism during which the genetic information near a DSB site is copied from a homologous region in the genome (Formosa and Alberts 1986; Engels et al. 1990, 1994; Glower et al. 1991; Nassif et al. 1994; Mueller et al. 1996; Keeler and Glower 1997). The synthesis-dependent strand annealing (SDSA) model has been put forward to describe the mechanisms underlying the repair of those conservative events. This model proposes that the broken ends of DNA invade and displace independently a local loop of homologous regions of DNA during the repair process (Formosa and Alberts 1986; Nassif et al. 1994; Mueller et al. 1996).

Although studies of the repair mechanism of DSBs induced by P-element excision have been very successful, the use of the P element to induce DSBs is technically limiting. P elements are used as transformation vectors; therefore, most of the studies can only analyze DSB events introduced at the extremity of a given transgene. Further, it is possible that there is a bias in the repair process of P-element-induced DSBs caused by the inverted repeat binding protein (IRBP), a homologue of the Ku70 protein that plays a central role in the repair process (Rio and Rubin 1988; Beall et al. 1994; Beall and Rio 1996; Dynan and Yoo 1998). P-element termini are bound by IRBP, and the P-element transposase cuts the P-element termini directly adjacent to the IRBP binding site (Beall and Rio 1996; Beall and Rio 1997). Thus, it is possible that the binding of IRBP to the P element prior to the cut might affect the kinetics of the repair process, and in this way affect the outcome of the repair mechanism, such as the ratio between conservative and nonconservative repair (Staveley et al. 1995; Beall and Rio 1996). Further, it has been proposed that the ratio of imprecise vs. precise repair following P-element-induced DSBs is biased toward imprecise repair because of the unusual 17-bp overhang that is left after cleavage by the P-element transposase (O’Brochta et al. 1991; Engels et al. 1994; Beall and Rio 1997).

The availability of a technique that is different from the use of P elements to induce DSBs in Drosophila would allow the analysis of the SDSA model in a more general manner. A number of recent studies have shown that the yeast I-SceI homing endonuclease can introduce DSBs in the genome of mouse cells or Xenopus oocytes (for review see Jasin 1996). Such studies have confirmed some aspects of DSB repair mechanisms previously ana-

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lyzed by transfection or injection of linear DNA molecules. I-SceI is encoded by an intron of the large mitochondrial rRNA (Dujon 1988). Biochemical studies have shown that this restriction enzyme has an 18-bp specificity and leaves a 4-bp 5’ overhang after the cleavage (Colлеaux et al. 1988). In this article, we show that expression of the yeast I-SceI endonuclease in Drosophila can be used as a general method to induce DSBs at I-SceI target sites in the Drosophila genome. We discuss the use of this novel technique to study SDSA and to analyze the functions of mutagen-sensitive (mut) mutations (Dusenbery and Smith 1996) that have been implicated in DNA repair mechanisms.

**MATERIALS AND METHODS**

**Plasmid constructions:** P{FRT-I-SceI-y’-Δ(w)::XEN-I-SceI-FRT}: Two direct flip recombinase target (FRT) repeats of the J32 vector (Struhl and Basler 1993) were PCR amplified using the oligonucleotides 5’-GCCTAACTCGAGGTACC CAGTCTCAAGGCGCTCT-3’ and 5’-AGTGAATTCGAGCTCTGC TGATCCCGGG-3’. They were cloned at the SnaI and PsiI site of a P-CarY vector (Patton et al. 1992) in which the NotI site has been deleted by blunt-end ligation. Two I-SceI sites were then added in direct orientation by subcloning two double-strand oligonucleotides, 5’-CTAGCTAGGATAACAGGTATG/3’-GATCCCTATTGTCCCATTACAGCT and 5’-TGCGGCCGCTAGGGATAACAGGGTAATG/3’-GGGCATGATGATCAATTAATATTCTAGTCTCAGTGTGATC at the NheI and BamHI sites, creating the P{2xFRT-I-SceI} vector. The two double-strand oligonucleotides also contain a SalI site, as well as a NotI site between two I-SceI sites. The 5.2-kb Sall fragment of the yellow gene from the Y.S.C vector (Patton et al. 1992) was then cloned into the Sall site of the P{2xFRT-I-SceI} vector, creating the P{FRT-I-SceI-y’-FRT-I-SceI} vector. A 9-kb HindIII fragment from the white gene containing two-thirds of the first exon and 5 kb of 3’ untranslated region (0’Hare et al. 1984) was modified by insertion of the double-strand oligonucleotide 5’-CCGGTAGTCTGAGGGAAATCGCGATGATCTCGGTAATG/3’-CGGACGATCCCTATCGGCTGATCGTGTGATTCTCCGCTGAC at the BspMI I (11063) site (0’Hare et al. 1984). The insertion introduces a frameshift and three new restriction sites for EcoRI, NruI, and Xho1 in the white sequence. This fragment was then flanked by NotI linkers and cloned at the NotI site of the P{FRT-I-SceI-y’-FRT-I-SceI} vector. The resulting plasmid was named P{FRT-I-SceI-y’-Δ(w)::XEN-I-SceI-FRT}.

P{b2-tubulin-3nls-I-SceI}: The BamHI/HindIII fragment, which contains a DNA sequence from −511 to +156 of the b2-tubulin promoter from the PWMelpvu vector (Michielss et al. 1993), was cloned between the BamHI and EcoRI sites of the pPGK3Xnls-I-SceI vector (Donoho 1996). This vector contains the 3nls-I-SceI sequence cloned between the EcoRI and SalI sites of the pBluescriptKs+ vector (Strategene, LaJolla, CA). The NotI, Sall fragment, which contains the promoter and 3nls-I-SceI, was then cloned with a Sall, NotI fragment from the 3’ sequence of the hsp70 gene from the pCasperHsp70 vector at the NotI site of the pDM30 transformation vector. The pDM30 transformation vector contains the y’ gene.

**Molecular methods:** Genomic DNA was prepared as described in Ashburner (1989), and Southern blot analysis was conducted as described in Sambrook et al. (1989). PCR amplifications of genomic fragments were carried out with the Ready-To-Go kit (Pharmacia, Piscataway, NJ) using the following primers: TTTCACGCCGCGATTATTAGCC or ATATGC GTATTATACGTCG for the 3’ end of P element, and CACG TTTGCTTGTGAGGAG or AAAGCCTGTGCGGCTCAT for the 5’ end of P element. PCR products were subcloned into a Promega pGEM-T vector and sequenced using the Sequenase2 kit (United States Biochemical/Amersham).

Flies were grown on standard cornmeal media. Mutations and chromosome aberrations not described in the text can be found in Lindsley and Zimm (1992). P-element transformation was performed using either the yw; Delta2-3, Sb TM 6 stock, or the p=25.1 helper plasmid (Ashburner 1989).

**RESULTS**

As part of an attempt to develop a general technique to induce gene knockouts in Drosophila, we have developed a new system to induce DSBs. We first describe the gene knockout strategy to provide the background for which the new technique to induce DSBs was developed.

**Gene targeting in Drosophila:** One of the technical limitations of Drosophila as a system to study specific gene functions is the absence of a general gene-targeting system to allow systematic, reverse genetic studies. Such an approach to analyze gene functions is greatly needed, especially in light of the growing amount of information generated by the Drosophila Genome Project. A technique to target specific gene conversion events close to a preexisting P-element insertion site using P-element-induced DSBs has been developed (Gloor et al. 1991). However, this system does not allow a systematic analysis of every gene in the genome since P-element insertions are not evenly distributed throughout the genome. To circumvent this problem, we have attempted to develop a homologous recombination system for Drosophila genes. One of the critical steps of this system involves the linearization of a circular plasmid in the male germ line. To achieve this, we have used the yeast mitochondrial I-SceI enzyme to cut a circular piece of DNA generated by the FLP-out event (Figure 1). The I-SceI endonuclease recognizes a specific 18-bp sequence and leaves a 4-bp 5’ overhang following the cut (Colleaux et al. 1988). We expected that few, if any, I-SceI sites would be present in the Drosophila genome since, theoretically, a single I-SceI site should be found in a genome equivalent to 350 Drosophila genomes (Ashburner 1989). A number of studies have shown that I-SceI efficiently cleaves genomic DNA or extrachromosomal DNA in plants and mammalian cell lines (reviewed in Jasin 1996). However, no studies have assessed the activity of a rare cutting endonuclease in a whole organism. Thus, we decided to conduct a detailed analysis of the activity of I-SceI in Drosophila.

**Expression of I-SceI in Drosophila induces DSBs:** To promote nuclear localization of the I-SceI enzyme, we used a fusion between the SV40 nuclear localization signal (nls) and the I-SceI coding sequence: this construct is referred to as 3nls-I-SceI (Donoho 1996). We generated four independent, P-element-transformed lines that carry the 3nls-I-SceI sequence downstream of
the \( \beta \)-tubulin promoter. This promoter drives expression in postmitotic spermatids (Fuller 1995) such that progeny from a single male should be derived from a number of independent repair events. None of the transgenic insertions affected male fertility since stocks mutant flies in the progeny is strictly dependent on insertions can be maintained. Similar results were obtained using different combinations of the two direct FRT repeats, and linearization of the circular plasmid is catalyzed by I-ScI that recognizes and cuts an 18-bp restriction site. (B) Outline of the homologous recombination screen for the white locus. We introduced an inactive version of the white gene into the fly genome via P-element transformation. The \( \beta \)-2 tubulin promoter drives expression of reporter genes in the male germ line cells during the late stages of spermatogenesis; therefore, the progeny of a single male will derive from a number of independent, I-ScI-induced excision events.

We designed a P-element vector in which a yellow \( ^{+} \) marker and an inactive version of the white gene are flanked by the two FRT and the two I-ScI sites (see also Figure 2 for details). The yellow \( ^{+} \) marker is used as a P-element transformation marker, and it can be used to monitor the excision of the inactive white gene and to analyze the reintegration events. This construct is named P\{FRT-I-ScI-y\(^{+}\)-\( \Delta \)(w)::XEN-I-ScI-FRT\}. We expressed both the FLP and I-ScI enzymes under the control of the \( \beta \)-2 tubulin promoter. The \( \beta \)2 tubulin promoter drives expression of reporter genes in the male germ line cells during the late stages of spermatogenesis; therefore, the progeny of a single male will derive from a number of independent, I-ScI-induced excision events.

Figure 1.—Homologous recombination strategy. (A) General strategy: the general scheme for gene targeting in various organisms involves the transfection of a linear molecule of DNA containing a modified version of an endogenous gene. Following a homologous recombination event, the modified gene will replace the endogenous sequence. To adapt a similar system to Drosophila, we introduced an inactive version of the white gene into the fly genome via P-element transformation. The mutant version of the white gene is subsequently released and linearized in the nucleus. The excision step is achieved by FLP-mediated recombination between the two direct FRT repeats, and linearization of the circular plasmid is catalyzed by I-ScI that recognizes and cuts an 18-bp restriction site. (B) Outline of the homologous recombination screen for the white locus. We introduced an inactive version of the white gene into the fly genome via P-element transformation. The \( \beta \)-2 tubulin promoter drives expression of reporter genes in the male germ line cells during the late stages of spermatogenesis; therefore, the progeny of a single male will derive from a number of independent, I-ScI-induced excision events.

Anatomy of the repaired DNA: To characterize in more detail the molecular events following DSBs, we randomly selected 25 independent, phenotypically yellow lines generated in the previous experiments and analyzed them by Southern analysis. These lines fall into two major classes on the basis of analysis of the sequences present after induction of DSBs at the I-ScI sites (Figure 3). In class 1 events, which represent 22 cases, a complete deletion of the sequences encoding both the yellow and white sequences was observed. In class 2 events, which represent the other 3 cases, only partial deletion of the yellow gene was detected.

We analyzed class 1 lines in more detail, and found that both the 3’ and 5’ P-element termini were present. These results suggest that the cut was not followed by
Figure 2.—Test of the activity of the I-SceI endonuclease in the male germ line. (A) The P{β2tubulin-3nls-I-SceI} expression construct: the I-SceI sequence was fused with the SV40 nuclear localization signal (3nls-I-SceI) to promote localization of the enzyme into the nucleus. The signal 3nls-I-SceI was cloned downstream of the β2-tubulin promoter in a P-element vector marked with the rosy1 gene. Four independent P{β2tubulin-3nls-I-SceI} insertions were recovered on the third chromosome. Two of these, insertions 1 and 2 are associated with zygotic lethality, while insertions 3 and 4 are homozygous viable insertions. (B) The P{FRT-I-SceI-y1-D(w)::XEN-I-SceI-FRT} reporter construct: this construct contains a yellow1 gene placed between two recognition sites for I-SceI. Three independent P{FRT-I-SceI-y1-D(w)::XEN-I-SceI-FRT} insertions were recovered, one on the first chromosome and two on the second chromosome. (C) To show that the I-SceI can induce DSBs in the male germ line, y, P{FRT-I-SceI-y1-D(w)::XEN-I-SceI-FRT}/Y; 1/1; P{β2tubulin-3nls-I-SceI} males were crossed with FM7a, y homozygous virgin females, and their progeny were scored for yellow females. To demonstrate that the loss of the yellow marker was dependent on the expression of the I-SceI enzyme, we scored the progeny of y, P{FRT-I-SceI-y1-D(w)::XEN-I-SceI-FRT}/Y; +/++; P{β2tubulin-3nls-I-SceI} males crossed with FM7a, y homozygous virgin females, and their progeny were scored for yellow females. We also determined the occurrence of DSB events using a reporter construct located on the second chromosome. In this experiment, progeny of single y, P{FRT-I-SceI-y1-D(w)::XEN-I-SceI-FRT}/CyO; P{β2tubulin-3nls-I-SceI} males crossed to y w; CyO/ Sco virgin females were analyzed and scored for CyO or Sco yellow progeny. (D) Percentage of yellow progeny obtained in various experiments. Numbers on top of the bars indicate the number of flies scored in each cross. The results are given for different combinations of reporter and expression transgenes. A total of 4–10% of yellow progeny (mean value, 6.47%) were recovered. In the absence of the P{β2tubulin-3nls-I-SceI} expression transgene, no phenotypically yellow flies were recovered (1900 progeny scored in six independent experiments). ND, not determined.

extensive degradation of the broken DNA since the P-element termini are located within 100 bp from the cleavage site. To confirm these results, the genomic DNA located between the two P-element termini was cloned from nine independent, randomly selected lines. Interestingly, sequence analysis of these DNAs revealed that the repair of the DSBs had proceeded in different ways (Figure 4A). In six out of the nine cases, we could identify partial sequences from the FRT and/or I-SceI sites, suggesting that following the cut, the gap was enlarged and repaired by direct end joining. One of the nine lines was found to be associated with a reconstitution of a perfect I-SceI site. This event can be explained either by repair through direct ligation of the two DNA strands following cuts at the two I-SceI sites or by a single cut at one of the I-SceI sites followed by single-strand
annealing (SSA) repair. Finally, sequence analysis of the remaining two DSB events revealed the presence of a unique FRT site. These two events can either be the result of two independent cuts at each I-SceI site or of a single cut at one of the I-SceI sites. In both cases, the repair would have then proceeded via SSA using the direct repeat from the FRT sites. The length of the FRT repeats is compatible with the length necessary for homologous pairing (Haber 1995; Keeler and Gloor 1997).

Southern blot analysis of class 2 events revealed that a partial deletion of the yellow sequence had occurred following the cut by I-SceI. In every case, consistent with the yellow phenotype, the coding sequence of the yellow gene was altered (Figure 4B). Since the white sequence appears intact, we interpret these events as the result of a single I-SceI cut at the I-SceI site located close to the 5′ P-element end. In two cases, the DSB was slightly enlarged and appeared to leave the P-element terminus intact. In a final case, the 5′ P-element end appeared to have been deleted. This suggests that after the cut at one I-SceI site, the cut was enlarged and the yellow sequence was altered. We could not determine whether a part of the yellow sequence was then copied from the other intact chromatid. We suspect that a number of similar events should have taken place at the I-SceI site located close to the 3′ P-element end; however, such events would not have been recovered since the selection was based on the loss of the yellow marker.

**DISCUSSION**

**I-SceI can induce DSBs at a specific position in the Drosophila genome:** We have demonstrated that the I-SceI endonuclease from yeast is able to induce DSBs in the Drosophila genome at positions that contain the 18-bp recognition site for this endonuclease. We found that ~6% of the progeny of males expressing I-SceI in their germ line lose a yellow gene that is flanked by two I-SceI sites. Most of the events resulted in complete deletion of the sequences located between the two I-SceI sites. These events are either the result of two independent cuts occurring at both I-SceI sites or a single cut at one of the I-SceI sites. Following the cut by I-SceI, the DSB was enlarged and then repaired by direct end joining or SSA. An enlargement of the DSB has also been proposed to occur for P-element-induced DSBs (reviewed in Keeler and Gloor 1997).

**Development of a targeting system:** We developed the I-SceI system as a means to linearize a circular piece of DNA in vivo. This method represents one of the steps in a protocol to develop a general gene-targeting system in Drosophila (Figure 1). To test for homologous recombination events, we generated a line containing two P-element constructs expressing FLP-recombinase and I-SceI enzyme under the control of the β2-tubulin promoter, and we conducted the screen described in Figure 1. Although we demonstrate that the 3nls-I-SceI enzyme, expressed under the control of the β2-tubulin promoter, is able to induce DSBs, our attempts to recover homologous recombination events failed; i.e., analysis of >250,000 females derived from Df(1)γ-ac, P[β2-tubulin-FLP]; P[FRT-I-SceI-y-Δ(w)::XEN-I-SceI-FRT]; P[β2-tubulin-3nls-I-SceI] males (see Figure 1B) failed to produce any white mutant females, indicating that no homologous recombination events had occurred.

Two recently published studies may provide an explanation for our failure to induce homologous recombination using a single linear piece of DNA provided by excision. Leung et al. (1997) and Negriotto et al. (1997) have designed a similar system in yeast. This system is
Figure 4.—Characterization of the events. (A) Sequences of the lines that were associated with complete deletions of both the yellow and white reporter sequences. The first sequence represents a perfect restoration of the I-SceI recognition site. The two last cases were recovered twice. (B) Interpretation of the partial deletions based on Figure 3 genomic Southern blots. The lengths of the deletions are approximate and are based on the size of the fragments detected by Southern analysis.

Based on the release of a linear piece of DNA for the \( \text{Leu}2 \) or \( \text{SAM}2 \) genes using the \( \text{HO} \) endonuclease. The two studies show that the efficiency of homologous recombination is low in these systems and, at most, occurs once for every 20,000 excision events. \( \text{Leung et al.} \) (1997) proposed that this effect is due to the preferential correction of a DNA nick with the intact chromosomal DNA. This explanation has been suggested in a number of other assays as well (reviewed in \( \text{Leung et al.} \) 1997). Interestingly, mutations in the mismatch repair genes \( \text{PMS1} \) or \( \text{Msh2} \) have been found to improve by 20- to 40-fold, respectively, the efficiency of homologous recombination. It will be interesting to repeat our homologous recombination screen in a \( \text{PSM1} \) or \( \text{Mhs2} \) mutant background when mutations in these genes become available.

**Generalization of the SDSA model using the I-SceI enzyme:** Studies of DSBs in \( Drosophila \) have been carried out using \( P \) elements as a means to introduce DSBs at specific locations in the genome (\( \text{Kaufman and Rio} \) 1992). These studies have led to the SDSA model to explain the repair mechanism following \( P \)-element excisions (\( \text{Formosa and Alberts} \) 1986; \( \text{Nassif et al.} \) 1994; \( \text{Mueller et al.} \) 1996; \( \text{Keeler and Gloor} \) 1997). The most comprehensive study of DSB repair has been conducted at the white locus. In addition, a number of studies have been conducted at both the vestigial and the Broad Complex loci (reviewed in \( \text{Keeler and Gloor} \) 1997). The generalization of the SDSA model to other induced DSB events represents an important step toward the understanding of DSB repair mechanisms. We believe that I-SceI can be used as a tool to further analyze the parameters of the SDSA model. For example, it will allow the determination of whether the binding of the IRBP protein on \( P \)-element termini influences the repair mechanism (see Introduction), as well as the testing of whether the high rate of imprecise \( P \)-element excisions is caused by the unusual 17-bp overhang left by the \( P \)-element transposase (\( \text{Engel et al.} \) 1990; \( \text{O’Brien et al.} \) 1991, see Introduction; \( \text{Beall and Rio} \) 1997).

**Further applications of I-SceI to dissect genetic path-**
ways involved in the DSB repair: The development of the I-SceI system will allow the characterization of molecules involved in DSB repair. In Drosophila, 15 loci associated with mus or ma repair defects have been isolated on the basis of their sensitivity to specific mutagenic agents (Dusenbery and Smith 1996). Importantly, different studies have shown that this group of genes is involved in the DSB repair process (Sekelsky et al. 1995; Araj and Smith 1996; Beall and Rio 1996). To date, however, no methodology is available to classify these genes in epistatic or genetic groups. Studies in yeast have characterized in more details the function of radiation-sensitive genes (RAD) by comparing their effects on the repair of a DSB induced within a direct DNA repeat to a DSB induced outside of a direct repeat. For example, several studies have shown that RAD51, RAD54, RAD55, and RAD57 are required for mating-type switching, but not for the SSA repair of a DSB introduced between two direct repeats. These results have led to the proposal that these genes are required for gaining access to an intact, constrained region of the chromatid to facilitate the copying of information (Sugawara et al. 1995). In addition, mutations in RAD1 and RAD10 have a slight effect on DSB gap repair at the mating-type locus (MAT). However, in RAD1 and RAD10 mutants, SSA between two direct repeats is completely blocked (Fishman-Lobell and Haber 1992). These results suggest that Rad1 is part of a complex that is necessary for removing nonhomologous sequences before SSA. It has been subsequently demonstrated that Rad1 and Rad10 form a complex that has a single-strand endonuclease activity (Bardwell et al. 1994). Our study shows that the I-SceI system allows the analysis of DSB repair between two direct repeats, an event that cannot be generated using P elements. Because these events occur at relatively high frequency, it should allow experiments similar to those conducted in yeast and permit the assignment of different mus Drosophila genes into epistatic groups.

Finally, we envisage that the I-SceI system could be used to engineer specific changes in the fly genome. For example, one can envisage using this system as a general means to induce a series of deficiencies. Following local duplication of a P element containing a single I-SceI site, the expression of the I-SceI enzyme should generate deletions between the two P elements. In contrast to other systems, such as the FRT/FLP system (Golic 1994; Golic and Golic 1996), the recovery of deletions between the two distant P elements should not be influenced by the distance between the insertion sites.

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