Targeted Chromosomal Cleavage and Mutagenesis in Drosophila Using Zinc-Finger Nucleases

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ABSTRACT

Zinc-finger nucleases (ZFNs) are hybrids between a nonspecific DNA-cleavage domain and a DNA-binding domain composed of Cys_2His_2 zinc fingers. Because zinc fingers can be manipulated to recognize a broad range of sequences, these enzymes have the potential to direct cleavage to arbitrarily chosen targets. We have tested this idea by designing a pair of ZFNs that recognize a unique site in the *yellow* (*y*) gene of Drosophila. When these nucleases were expressed in developing larvae, they led to somatic mutations specifically in the *y* gene. These somatic mosaics were observed in approximately one-half of the males expressing both nucleases. Germline *y* mutations were recovered from 5.7% of males, but from none of the females, tested. DNA sequences were determined and showed that all of the mutations were small deletions and/or insertions located precisely at the designed target. These are exactly the types of alterations expected from nonhomologous end joining (NHEJ) following double-strand cleavage of the target. This approach promises to permit generation of directed mutations in many types of cells and organisms.

COMPLETE genomic sequences have been determined for a number of experimental organisms, and more are in progress. Knowledge of these sequences does not lead directly to understanding of the underlying gene functions, which must come from a combination of genetic, biochemical, cytological, and physiological analyses. Genetic approaches would be greatly facilitated by the ability to direct mutations to chosen genomic targets. A number of procedures have been introduced with this aim in mind (Vasquez *et al.* 2001).

In fungi (Rothstein 1983), in cultured mammalian cells (Capecchi 1989), and recently in Drosophila (Rong and Golic 2000, 2001), gene targeting by homologous recombination has proved effective in replacing resident sequences with experimentally manipulated ones. This approach is not available for many organisms, and it can be quite inefficient even when it works. In murine embryonic stem cells, only ~1 cell among 10^6 treated with donor DNA incorporates it at the target locus (Capecchi 1989). With powerful selection or screening procedures (Mansour *et al.* 1988; Koller and Smithies 1992), these low frequencies can still be very effective, but improved efficiency would be quite useful, as would extension to other organisms.

Alternative approaches have utilized oligonucleotides with the capability of recognizing specific target sequences. These include triplex-forming oligos (TFOs) that can bind *in vivo* to polypurine tracts. When linked to a DNA-damaging agent, such as psoralen (Havre and Glazer 1993; Wang *et al.* 1995), and even when used alone (Wang *et al.* 1996), TFOs can direct mutations to their target sequences. DNA-RNA hybrid oligos, called chimeraplasts, have also been used to introduce specific sequence alterations (Cole-Strauss *et al.* 1996), but with variable outcomes. We have endeavored to develop procedures that are more general, more efficient, and more reproducible than any of these techniques. The basis of our approach is the directed cleavage of specific genomic targets.

Introducing a double-strand break (DSB) in a eukaryotic chromosome stimulates DNA repair by both homology-dependent and nonhomologous mechanisms (Jeggo 1998; van Gent *et al.* 2001). Nonhomologous end joining (NHEJ) in particular can produce localized mutations due to deletion and/or insertion of short sequences at the break (Jeggo 1998). Investigating these processes in detail has required insertion of the recognition site for a meganuclease, such as I-*Sce*I (Jasin 1996), or mapping of an excisable transposon (Gloor *et al.* 1991; Keeler *et al.* 1996) before cleavage could be induced. We intend to bypass this limitation with nucleases based on zinc-finger DNA-recognition domains. Because of their modular design (Pavletich and Pabo 1991), zinc fingers can be manipulated to recognize a wide range of DNA sequences (Isalan *et al.* 1998; Segal *et al.* 1999; Dreier *et al.* 2001; Pabo *et al.* 2001). This should permit cleavage by zinc-finger nucleases (ZFNs) to be directed

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to different genomic sequences without the need to alter those sequences in advance.

The ZFNs consist of a DNA-binding domain composed of three Cys_2His_2 zinc fingers linked to the nonspecific DNA-cleavage domain from *Fok*I (Kim *et al.* 1996; Figure 1). The cleavage domain must dimerize to cut DNA, so efficient cleavage requires two zinc-finger-binding sites in close proximity (Smith *et al.* 2000; Bibikova *et al.* 2001). Each finger contacts primarily 3 bp; the component sites are 9 bp in length; and the optimum arrangement of paired sites is an inverted orientation with a spacer of 6 bp (Bibikova *et al.* 2001). The two sites need not be identical, as long as ZFNs that bind both sites are provided. If all positions in the target sites are contacted specifically, these requirements enforce recognition of an 18-bp sequence, which is long enough to be unique, even in a complex genome (Bibikova *et al.* 2001).

We demonstrated previously that ZFNs could find, cleave, and stimulate recombination of extrachromosomal targets in Xenopus oocyte nuclei (Bibikova *et al.* 2001). In this report we extend our observations to specific cleavage of a chromosomal target in *Drosophila melanogaster*. We demonstrate targeted mutagenesis due to NHEJ as a result of ZFN-induced DSBs. This approach should also stimulate gene targeting by homologous recombination in flies and in many other organisms.

MATERIALS AND METHODS

Redesign of the zinc fingers: Coding sequences for zinc fingers that recognize the DNA sequences 5′-GCGGATGCG-3′ and 5′-GCGGTAGCG-3′ were obtained from Drs. David Segal and Carlos Barbas (Scripps Research Institute, La Jolla, CA; Segal *et al.* 1999). They were modified using mutagenic PCR primers, so they would recognize the component 9-mers of the *y* gene target (Figure 2). The resulting three-finger sets were cloned in frame with the *Fok*I cleavage domain in the pET15b expression plasmid with no linker between the domains (Bibikova *et al.* 2001). Both proteins were expressed, purified by Ni-affinity chromatography, and tested for cleavage activity *in vitro* by methods described previously (Smith *et al.* 2000; Bibikova *et al.* 2001) using the pS/G plasmid (Geyer and Corces 1987), which carries the complete *y* gene.

***P*-element vectors and transformation:** The yA and yB ZFN coding sequences were cloned separately behind the Drosophila Hsp70 heat-shock promoter by insertion between the *Bam*HI and *Sal*I sites of a modified phsp70 plasmid (Petersen and Lindquist 1989). A fragment carrying the promoter and ZFN sequences was excised by partial *Hin*dIII and complete *Apa*I digestion and cloned between these same sites in pBluescript. After verification of the sequence of the insert, it was excised by digestion with *Not*I and inserted into the ry^+ *P*-element vector pDM30 (Mismer and Rubin 1987). The resulting yA and yB plasmids were injected separately into *v ry* embryos, along with the *P*-transposase expression plasmid pπ25.1wc, and eclosing adults were mated to screen for ry^+ germline transformants. The ry^+ insertion was mapped to a specific chromosome for multiple independent transformants with each ZFN. Both balanced and homozygous stocks were created for several lines carrying yA and yB without viability problems in most cases. Genes for the two ZFNs were brought together with appropriate crosses, and the offspring were heat-shocked 4 days after the initiation of mating by immersing the glass vials in a water bath at 35° for 1 hr. As adults eclosed they were screened for evidence of somatic *y* mutations. Control vials from crosses involving each nuclease separately were subjected to the heat shock, and yA + yB flies that had not been heat-shocked were also screened.

Recovery of germline mutants: All flies emerging from the heat-shock protocol and carrying both the yA and yB nucleases were mated to reveal potential germline mutations. Males were crossed with two or three attached-X [*C(1)DX*] females, and male offspring were screened for yellow body color. Females were crossed with two or three *y* (*FM6*) males, and both male and female offspring were screened. The identified mutants (all of which were males from male parents) were crossed again to *C(1)DX* females to produce additional progeny with the same mutation.

DNA isolation, amplification, and sequencing: Individual flies were homogenized in 100 μl of a 1:1 mixture of phenol and grind buffer (7 M urea, 2% SDS, 10 mM Tris, pH 8.0, 1 mM EDTA, 0.35 M NaCl) preheated to 60°. Each sample was extracted with 50 μl of chloroform, the organic phase back-extracted with 100 μl of grind buffer, and the combined aqueous phases re-extracted with 50 μl of chloroform. DNA was precipitated with ethanol and redissolved in 20 μl of 10 mM Tris, pH 8.5. A 600-bp fragment was amplified by PCR with primers flanking the yA + yB recognition site: YF2 (5′-ATTCC TTGTGTCCAAAATAATGAC-3′) and YR3 (5′-AAAATAGGCAT ATGCATCATCGC-3′). For the larger deletions, YR3 was used in combination with a more distant sequence, YF1 (5′-ATTTT GTACATATGTTCTTAAGCAG-3′). Amplified fragments were recovered after gel electrophoresis, and DNA sequences were determined at the University of Utah DNA Sequencing Core Facility with an ABI3700 capillary sequencer and the YR3 primer.

RESULTS

Design of the ZFNs: Zinc fingers generally prefer to bind G-rich sequences, and an extensive study has been performed of fingers that bind all 5′-GNN-3′ triplets (Segal *et al.* 1999; Dreier *et al.* 2000). Because the binding sites must be in inverted orientation (Bibikova *et al.* 2001), we searched the *y* gene on the X chromosome of *D. melanogaster* for inverted sequences of the form $(NNC)_3 \ldots (GNN)_3$. As shown in Figure 1, we identified a site in exon 2 with a 6-bp separation between the component 9-mers, which is the optimal spacer for specific recognition and cleavage by ZFNs that have no added linker between the binding and cleavage domains (Bibikova *et al.* 2001). We constructed coding sequences for fingers designed to bind each of the component triplets on the basis of the results of Segal *et al.* (1999), as diagrammed in Figure 2. These were linked to the *Fok*I cleavage domain in ZFN expression constructs, and the yA and yB nucleases were expressed in bacteria and purified. Together they made a single DSB at the expected site in a 10.7-kb plasmid DNA carrying the *y* gene (not shown).

Induction of *y* mutations: The yA and yB ZFNs were cloned separately under the control of a heat-shock promoter and introduced into the Drosophila genome by *P*-element-mediated transformation. We found that

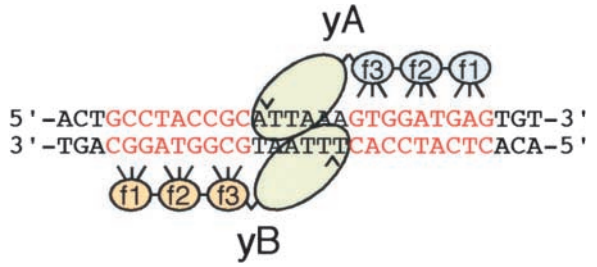

FIGURE 1.—Diagram of the zinc-finger nucleases yA and yB and the target site in exon 2 of the *y* gene. Each nuclease is composed of three zinc fingers (f1–f3) linked to the DNA-cleavage domain of *Fok*I (green). Each finger contacts three consecutive base pairs of DNA; thus, each of the component sites is 9 bp (red). When both sets of fingers are bound, the cleavage domain can dimerize to form an active nuclease and cleave the DNA at the sites indicated by carats. The first base pair shown is 3053 bp from the transcription start and 120 bp from the start of exon 2. It is also the first base pair of codon 120, out of 541 total.

the levels of expression of yA induced at 37°, in several independent transformants, were lethal when applied at larval and embryonic stages. This may be due to excessive cleavage or to simple binding by the yA nuclease (see DISCUSSION). We found that moderating the heat shock to 35° allowed survival of a good proportion of the yA-carrying flies. The yB ZFN did not affect viability at any temperature tested.

To express both ZFNs simultaneously, individuals carrying the yA and yB nucleases on the same chromosome were crossed and their progeny were heat-shocked (Figure 3). Our expectation was that the paired ZFNs would generate a DSB at the target site in the y^+ gene in at

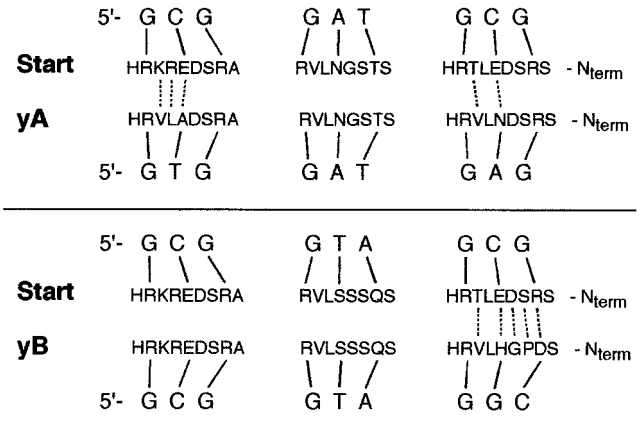

FIGURE 2.—Modification of zinc-finger coding sequences to correspond to sites in the *y* gene. In each set the starting amino acid sequences (Start) are given at the top, along with the DNA triplets they recognize. The sequences shown represent residues −2 to +7 of each zinc finger (PABO *et al.* 2001). Polarities of both protein and DNA are indicated. Contacts between specific amino acid residues and individual base pairs are denoted by solid lines. The changes made in the protein sequences are shown with dotted lines. Two fingers were modified in yA, but only one in yB.

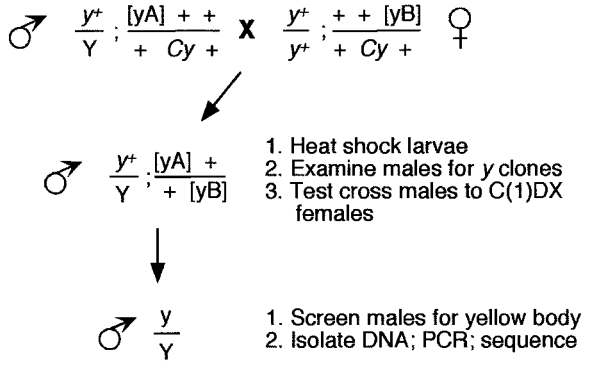

FIGURE 3.—Scheme for expressing the ZFNs, screening for *y* mosaics, and isolating germline mutations. An example using the yA and yB transgenes on chromosome 2 is shown, each balanced by a chromosome bearing a dominant *Cy* mutation.

least some cells. In females the break could be repaired by recombination with the uncut homolog (ENGELS *et al.* 1990); furthermore, the second y^+ gene would obscure any recessive mutation, unless the cleavage efficiency was high enough to cut both loci simultaneously. In males (except following DNA replication), only simple religation or NHEJ would be available to repair the damage. In Drosophila, as in many other eukaryotes, NHEJ frequently produces deletions and/or insertions at the joining site (TAKASU-ISHIKAWA *et al.* 1992; STAVELEY *et al.* 1995; BEALL and RIO 1996; DRAY and GLOOR 1997; GLOOR *et al.* 2000). Since the DSB is targeted to protein coding sequences in y^+, most such alterations would lead to frameshifts or to deletion of essential codons. Therefore, we predicted that some males emerging from heat-shocked larvae would show patches of *y* mutant tissue.

Somatic yellow mosaics were identified in multiple

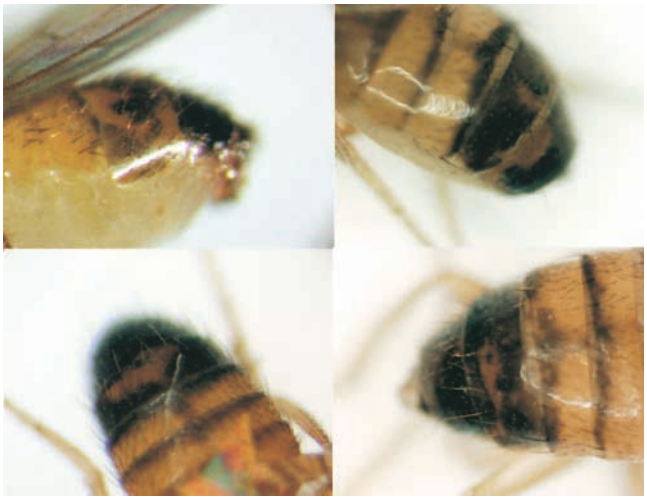

FIGURE 4.—Examples of yellow somatic mosaics in four different male flies. In each case yellow cuticular patches and yellow bristles can be seen in the otherwise dark posterior abdomen.

yA + yB males. Some examples are shown in Figure 4. Most of the patches were in the distal abdominal cuticle and bristles, but some examples in leg, wing, and scutellar bristles were also observed. No other phenotypic defects have been seen on a regular basis. The frequency of somatic mosaics was quite high. In pooled data from crosses involving a number of independent yA and yB lines, 105 of 228 candidate males (46%) showed obvious *y* patches. For some yA + yB combinations the frequency was >80%. No yellow mosaics were observed in controls with a single nuclease or without heat shock. This indicates that the yA + yB ZFNs are capable of inducing somatic mutations at their designated target.

Characterization of germline *y* mutations: To isolate germline *y* mutations, all yA + yB males from several heat-shock experiments were crossed to females carrying an attached-X chromosome [*C(1)DX/Y*], so sons received their father's X chromosome (Figure 3). In total, 228 males yielded 5870 sons; 26 of these, from 13 different fathers, were clearly *y* throughout their bodies. Thus, 5.7% of the yA + yB males produced at least one germline mutant, and 0.44% of all the candidate progeny were *y* mutants. Of the 13 fathers, 6 had been identified as having *y* somatic patches, while the other 7 appeared to be entirely y^+ in diagnostic features. No *y* flies were isolated among 7050 progeny of 125 heat-shocked yA + yB females crossed to *y* males. Considering the fact that the yA + yB females were typically not virgins, the actual number of candidate progeny was probably closer to 5000. We conclude that the ZFNs appear to be effective in inducing mutations via NHEJ most efficiently in the male germline.

DNA was isolated from 18 *y* germline mutants (those above and 5 isolated subsequently), and a 600-bp fragment including the expected cleavage site was amplified by PCR. In three cases, the binding site for one of the primers had been deleted, and amplification was accomplished with a more distant primer. Sequence analysis of these fragments revealed unique alterations precisely at the target site (Figure 5). Nine of the sequenced mutants had simple deletions; 5 had deletions accompanied by insertions; and 3 were simple, short duplications. Three of the deletions extended for hundreds of base pairs to one side of the target (the two shown in Figure 5 and one that was characterized by PCR but not sequenced). Exactly these types of mutation are expected to result from NHEJ after cleavage by the yA + yB ZFNs, and they are very similar to those produced after *P*-element excision (Takasu-Ishikawa *et al.* 1992; Staveley *et al.* 1995; Beall and Rio 1996; Dray and Gloor 1997; Gloor *et al.* 2000). Some of the frameshift *y* mutations created a stop codon within a short distance of the alteration, while one inserted an asparagine codon into the normal reading frame (Figure 5). We cannot predict how this will affect the yellow protein, since its function is not known. It seems essentially certain, however, that this alteration is responsible for the *y* phenotype, as it is very unlikely that a second, independent mutation occurred elsewhere in the gene.

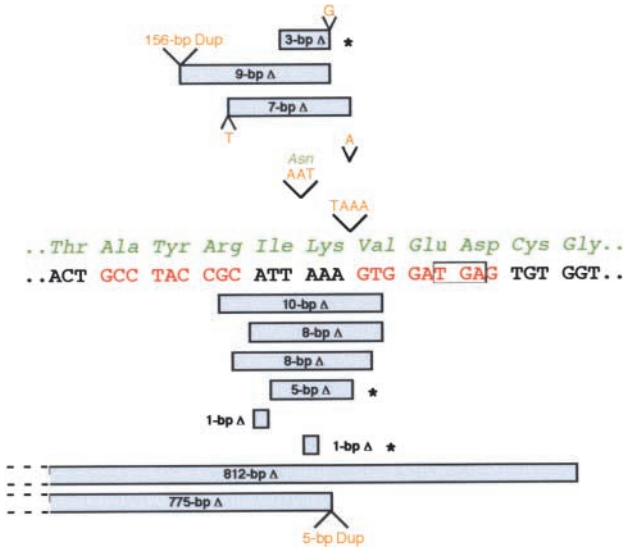

Figure 5.—Sequences of ZFN-induced germline *y* mutations. The wild-type sequence is shown with the normal translation (green); the yA and yB recognition sequences are red. Deletions found in the mutants are indicated with blue boxes, and insertions (some of which are duplications, Dup) are orange. Six of the mutations result in frameshifts that encounter the stop codon that is boxed. The three mutations indicated with asterisks were recovered twice from independent parents. The 156-bp duplication is of sequences just to the left of the cleavage site.

DISCUSSION

Targeted cleavage and mutagenesis: Our results clearly show that designed ZFNs can produce targeted DSBs in the Drosophila genome. Other investigators have produced hybrid transcription regulators by fusing zinc fingers to activation or repression domains (Choo *et al.* 1994; Beerli *et al.* 1998; Zhang *et al.* 2000; Xu *et al.* 2001), but this is the first example of permanent genetic alterations directed by zinc-finger chimeras. The frequency of observed somatic mutation was quite high, and the real number of somatic mosaics may be higher, since *y* mutations have no effect on many visible features. This is corroborated by the recovery of germline mutations from phenotypically y^+ parents.

Germline mutations were recovered only in males, but less frequently than somatic mosaics. The lower frequency in the germline could be due to a lower efficiency of heat-shock induction in these cells, to a higher frequency of cell death after cleavage, or to a greater efficiency of accurate repair of the break. Although there is no homologous meiotic crossing over in Drosophila spermatocytes, homology-dependent repair could still operate in germline cells that are in the G2 phase of their cell cycle. Rong and Golic (2000) observed a higher level of gene targeting in the female

germline than in males, but the events they scored depended on homologous recombination, while the *y* mutations described here depended on nonhomologous repair. We calculate that the frequency of *y* mutations in the male germline induced by ZFN expression is ~1/250 gametes, which is comparable to the targeting frequency of 1/500 observed at *y* in females in the earlier study (Rong and Golic 2000).

In subsequent experiments we have recovered *y* mutations resulting from NHEJ in the female germline, but always at a lower frequency than that in males. In the experiments analyzed in detail here, the difference between the yields of mutations from males and females is quite significant ($P = 0.0053$, by Fisher's exact test). This does not indicate, however, that the frequency is zero in females. The observation of female germline mutations makes possible the recovery of alterations that extend well beyond the *y* gene, including ones that confer recessive lethality or male sterility. Furthermore, this suggests that accurate repair from the homolog is not fully efficient, and the induction of germline mutations in autosomal genes should also be possible by ZFN cleavage.

In connection with the gene targeting experiments of Rong and Golic (2000), a caution was raised that the *y* gene might be exceptional in its ability to undergo homologous recombination due to its proximity to the end of the X chromosome and the possibility of repair by a replicative mechanism (Engels 2000). Demonstration of targeting at other loci has minimized this concern (Rong and Golic 2001). Furthermore, the mutagenesis we observe at *y* depends on ZFN cleavage, but not on homologous recombination by any mechanisms, so this approach should be applicable to many other Drosophila genes, independent of chromosomal location. An issue we have not addressed is the possibility that ZFN cleavage will be limited by chromatin structure at the target (Liu *et al.* 2001). We have no information about nucleosome locations in the vicinity of the yA + yB sequences in either somatic or germline cells. It seems likely that the effects of the heat-shock induction persist for many hours, so it is conceivable that the affected cells have passed through S phase and made the target transiently accessible to the nucleases.

Lethality of the yA nuclease: The yA ZFN was lethal when induced with a 37° heat shock, but the yB ZFN was not. If the yA zinc fingers lack complete specificity, they may induce cleavage at multiple genomic sites where related 9-mers are found in close proximity. Alternatively, binding of the yA protein to one or more individual copies of the target 9-mer that lie in a region critical for expression of an essential gene could interfere with binding of important transcription factors. In a search of the Drosophila genome, the only site that matched the target sequence, GCC TAC CGC $(N)_6$ GTG GAT GAG, or its complement was the one at *y*. At no sites in the genome was the yA or yB 9-mer present as an inverted pair with a 6-bp spacer. There were, however, many single occurrences of the half sites: 262 for the yB 9-mer and 774 for the yA 9-mer. These numbers are not far from expectation, given the size (120 Mb) and G + C content (40%) of the Drosophila euchromatic genome (Adams *et al.* 2000). The calculated number for yB (7/9 G + C) is 276, while that for yA (5/9 G + C) is 622. The upshot is that there are more binding sites for the yA monomer where it may interfere with cellular processes or be in sufficient proximity to a closely related site to permit dimerization and cleavage.

These calculations refer to the situation in which the yA zinc fingers bind only the specified 9-mer. It is certainly possible that the fingers do not discriminate completely against closely related sequences. Derivation of new fingers targeted to the yA 9-mer using phage display might improve specificity. In addition, context effects in zinc-finger binding (Isalen *et al.* 1997; Pabo *et al.* 2001), which we have not addressed, can be optimized experimentally.

Future applications of ZFNs: One may reasonably ask how generally applicable specific genomic cleavage by ZFNs will be. Zinc-finger combinations that recognize many different DNA sequences have been identified, but the range of effective targets may still be somewhat limited. In initiating our search for plausible targets in the *y* gene, we restricted ourselves to component sequences of the form $(GNN)_3$. Paired inverted sequences are required, and we prefer separations of precisely 6 bp between component 9-mers. This is not overly restrictive, since a sequence of the form $(NNC)_3N_6(GNN)_3$ should appear at random approximately once every 4 kb (in DNA of 50% G + C), and there is some flexibility in the spacer length (Bibikova *et al.* 2001). Furthermore, fingers showing reasonable selectivity for other triplets, including the ANN series (Dreier *et al.* 2001), have been described. Additional fingers can be added to achieve greater specificity (Liu *et al.* 1997; Beerli *et al.* 1998; Kim and Pabo 1998), and powerful methods of design and selection (Greisman and Pabo 1997; Isalen *et al.* 2001; Moore *et al.* 2001a,b) can be applied to isolate combinations that show high affinity and good discrimination among related DNA sequences. The full range of targetable sequences will be determined only by continuing experimentation.

The mutations induced by the ZFNs are the result of NHEJ after targeted cleavage. Most are small alterations—insertions and/or deletions of small numbers of base pairs—but some are larger deletions. This is reminiscent of the consequences of *P*-element excision, where both small changes and large deletions that remove parts of neighboring loci have been described (Preston *et al.* 1996). The ZFNs have the advantage that a *P*-element insertion near the desired target need not preexist. The larger deletions may be of particular

utility when zinc-finger-binding sites can be identified only some distance from the desired target.

As we have employed them, there are two slow steps in applying the ZFNs to new targets. One step is the derivation of new zinc-finger combinations directed to the chosen DNA sequence; this can be accomplished by design, as we have done, or by selection via phage display. This situation will improve as more finger-target combinations are described and more libraries of candidate fingers are produced. Nonetheless, the labor of generating and testing new ZFNs will be best justified when the envisioned use entails repeated attacks on the same target sequence. The second slow step is composed of the genetic manipulations required to introduce the ZFN coding sequences into the genome and combine them in individual flies. In most cases we presume it will be possible to place both ZFN genes in a single *P* element to abbreviate the process. We found that the yA and yB nucleases could not be cloned on the same high-copy vector in bacteria, which necessitated introducing them individually into Drosophila. Further simplification is possible if the ZFNs could be productively introduced by direct embryo injection, and this is currently being tested.

Since DSBs stimulate mutagenic repair in essentially all organisms, cleavage by ZFNs promises to have utility well beyond Drosophila. In addition to being mutagenic in itself, achieving targeted cleavage of chromosomal DNA is a step on the route to improving the efficiency of targeted gene replacement. Experiments are in progress with a marked donor DNA for the *y* gene to see if it can be used effectively as a partner in homologous repair. Enhancing the efficiency of gene targeting could substitute for powerful selection procedures (Mansour *et al.* 1988) in the isolation of desired genomic alterations.

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