Genetic Analysis of the *ADGF* Multigene Family by Homologous Recombination and Gene Conversion in Drosophila

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ABSTRACT

Many Drosophila genes exist as members of multigene families and within each family the members can be functionally redundant, making it difficult to identify them by classical mutagenesis techniques based on phenotypic screening. We have addressed this problem in a genetic analysis of a novel family of six adenosine deaminase-related growth factors (*ADGF*s). We used ends-in targeting to introduce mutations into five of the six *ADGF* genes, taking advantage of the fact that five of the family members are encoded by a three-gene cluster and a two-gene cluster. We used two targeting constructs to introduce loss-of-function mutations into all five genes, as well as to isolate different combinations of multiple mutations, independent of phenotypic consequences. The results show that (1) it is possible to use ends-in targeting to disrupt gene clusters; (2) gene conversion, which is usually considered a complication in gene targeting, can be used to help recover different mutant combinations in a single screening procedure; (3) the reduction of duplication to a single copy by induction of a double-strand break is better explained by the single-strand annealing mechanism than by simple crossing over between repeats; and (4) loss of function of the most abundantly expressed family member (*ADGF-A*) leads to disintegration of the fat body and the development of melanotic tumors in mutant larvae.

OVER 5000 genes in the Drosophila genome appear to have arisen by gene duplication and are now members of multigene families (Rubin *et al.* 2000), raising the possibility that members of a family may have overlapping functions. Such functional redundancy could preclude many types of conventional genetic analysis based on screening for phenotypes caused by loss-of-function mutations, since other family members with overlapping functions could potentially rescue the effects of such mutations. These problems of functional redundancy may be a significant problem in the genetic analysis of three recently discovered families of growth-controlling molecules: imaginal disc growth factors (*IDGF*s—six members; Kawamura *et al.* 1999), insulin-like peptides (seven members; Brogiolo *et al.* 2001), and adenosine deaminase-related growth factors (*ADGF*s—six members; Maier *et al.* 2001; Zurovec *et al.* 2001, 2002).

Growth factors are generally considered to function by interacting with specific cell-surface receptors, initiating signal transduction mechanisms that result in changes in gene transcription. However, other mechanisms of growth stimulation are possible. For example, the *ADGF*s appear to stimulate cell growth by an indirect mechanism, depleting the levels of extracellular adenosine that otherwise have a negative effect on growth of several cell types (Zurovec *et al.* 2002). *ADGF*s have close homologs in organisms ranging from the slime mold Dictyostelium to humans, and the human homolog *CECR1* is strongly implicated in cat-eye syndrome (Riazi *et al.* 2000). The identification of the *ADGF*s in Drosophila therefore provides a useful model for studying the effects of adenosine and its role in genetic disease.

From sequence analysis of *ADGF* genes, Maier *et al.* (2001) suggested that individual *ADGF* members might have different subcellular localizations (mitochondrial, membrane, and secreted). Matsushita *et al.* (2000) have shown membrane localization of the *ADGF-A2* protein, and we have shown that *ADGF-A* and *ADGF-D* are secreted while *ADGF-E* is not (Zurovec *et al.* 2002). Northern blot analysis and *in situ* hybridization showed distinctive expression patterns of *ADGF* genes (Zurovec *et al.* 2002). Although all *ADGF* sequences contain a predicted adenosine deaminase domain, some of them have amino acid substitutions at residues critical for ADA activity and are therefore probably not active deaminases. These data suggest that individual *ADGF*s might have different roles in Drosophila. In the cell-culture experiments mentioned above we were not able to distinguish between the roles of individual *ADGF* genes. However, genetic studies of mutant phenotypes have the potential to provide definitive answers.

Because of the possibility of genetic redundancy

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within the *ADGF* gene family, it was necessary to use a mutagenesis system that recovers knockout mutations independent of phenotype. We therefore used the method of homologous recombination (HR) recently developed for Drosophila (Rong and Golic 2000; Rong *et al.* 2002) to obtain mutations in individual members of the *ADGF* family. With the completion of the Drosophila genome project, HR is particularly useful for targeting genes of interest. The originally described "ends-in" targeting method allows the introduction of any specific mutation into the Drosophila genome (Rong and Golic 2000). Indeed, several groups have successfully implemented ends-in targeting for the disruption of important genes (Rong and Golic 2000, 2001; Rong *et al.* 2002; Seum *et al.* 2002; Egli *et al.* 2003). In this work, we used ends-in targeting to introduce mutations into five of the six *ADGF* genes. We took advantage of the fact that five of the family members are encoded by a three-gene cluster and a two-gene cluster (Figure 1). We designed two constructs (Figure 2) for ends-in targeting to introduce loss-of-function (LOF) mutations in all five genes as well as different combinations of multiple mutations in just two independent sets of crosses (Figure 3). Thus, we were able to analyze unique recombination events in detail. We found that LOF mutations in *ADGF-A* cause a larval lethal phenotype, whereas double mutants in *ADGF-C* and *ADGF-D* show semilethality during larval and pupal stages. The effect of both mutations seems to be cumulative since mutants in either the *ADGF-C* or the *ADGF-D* gene have similar phenotypes, but with lower penetrance than that seen in the double mutants.

MATERIALS AND METHODS

Design of homologous recombination constructs: We used the ends-in targeting procedure (Rong and Golic 2000) in which an I-*Sce*I site is engineered into the HR construct. This procedure produces a duplication in the homologous genomic region, with the wild-type copy on one side and the mutated copy on the other, and the marker gene (*miniwhite* in the pTV2 version) in the center. In the second step of the targeting scheme, this duplication is reduced to a single copy carrying only the mutated version. We chose ends-in targeting because it allowed us to produce mutations in all the genes of each cluster in one procedure. We designed two constructs (Figure 2): one for the ADGF-A region (containing *ADGF-A*, *-A2*, and *-B* genes; Figure 1) and one for the ADGF-C+D region (containing *ADGF-C* and *ADGF-D* genes; Figure 1). A +1 frameshift mutation was introduced into the *ADGF-A2* gene, where it also created a new *Sma*I restriction site. In *ADGF-A*, *ADGF-B*, and *ADGF-C* we created stop codons with new *Nhe*I sites. In the *ADGF-D* gene we added a new *Xho*I site associated with a stop codon. Each mutation was placed upstream of the most conserved domain of the gene. Thus we expected that all mutations should cause LOF. For the ADGF-A cluster, we placed an I-*Sce*I site between the *ADGF-A* and *ADGF-A2* genes (see Figure 2). With this combination, we could recover the duplication with mutated copies of *ADGF-A* and *ADGF-B* genes on the left side and the mutated *ADGF-A2* on the right side. The I-*Sce*I site was placed 1 kb downstream from the mutation in the *ADGF-A2* gene and 1.3 kb upstream from the mutation in the *ADGF-A* gene. For the ADGF-C+D region, we inserted the I-*Sce*I site 3.5 kb downstream from the mutation in the *ADGF-D* gene. Exchange near the double-strand break (DSB) can lead to gene conversion between mutant and wild-type copies in either direction during the repair process. Since the average size of gap expansion is ~1.3 kb (Gloor *et al.* 1991), the probability of reversion to the wild-type sequence by gene conversion during recombination is higher for *ADGF-A* and *ADGF-A2* than for *ADGF-C+D*. The inserted *miniwhite* gene serves as a marker for screening, and the I-*Cre*I site allows us to produce a DSB between repeats to reduce the duplication to single copy during the second step of the HR procedure.

Constructs: The *pTV2(ADGF-A2$^-$, -A$^-$, -B$^-$)* plasmid for HR in the ADGF-A region was constructed as follows (Figure 2A): four fragments containing all three genes were PCR amplified from Oregon-R genomic DNA using the following primers: 5′-CTTCGCTCCTGGTGGTGGTC and 5′-CCCGGGCAAATC CAGCAAAGAAATTCG (introducing a +1 frameshift mutation and a new *Sma*I restriction site into the *ADGF-A2* gene, 1299 bp downstream of the start codon); 5′-CACGCTAGCT TAACACCTTTGGGGACGAG and 5′-GGGTTTGGAGCTAG CCAGTTACGC (introducing an in-frame stop codon and a new *Nhe*I restriction site into the *ADGF-A* gene, 1160 bp downstream of the start codon); 5′-GCGTAACTGGCTAGCTCC AACCC and 5′-CTCGCTAGCTCTTGAACGCCGTGGGTGAC (introducing an in-frame stop codon and a new *Nhe*I restriction site into the *ADGF-B* gene, 125 bp downstream of the start codon); and 5′-AGAGCTAGCGACAAGCAATCGCCAAGGTG and 5′-CAGGTACCGGCCAGGCTTTTGAGGAACC (creating a *Kpn*I restriction site at the end of construct for later cloning into pTV2). All four fragments were cloned step by step into the modified pBLUESCRIPT II KS− (the *Xba*I-*Eco*RI fragment was replaced by a *Xba*I-*Eco*RI fragment from a pSLfa1180fa plasmid containing an *Nhe*I site) using restriction sites added to amplified fragments. An I-*Sce*I site was added by the annealing and cloning of the complementary oligonucleotides 5′-TCGAATTACCCTGTTATCCCTA (containing an *Xho*I cohesive end) and 5′-CGCGTAGGGATAACAGGGTAAT (containing a *Mlu*I cohesive end) into *Mlu*I and *Xho*I sites originally present 151 and 167 bp upstream of the *ADGF-A* start codon. The entire fragment was then cut from the modified pBLUESCRIPT by *Not*I and *Kpn*I and cloned into the pTV2 plasmid (provided by Yikang Rong and Kent Golic).

The *pTV2(ADGF-C$^-$, -D$^-$)* construct for HR in the ADGF-C+D region was constructed as follows (Figure 2C): the entire region containing both genes and flanking sequences was amplified in four fragments from Oregon-R genomic DNA. Fragment I was amplified using the oligonucleotides 5′-AGGGT ACCATGTCAAGGACGTGGAGGT (introducing a *Kpn*I restriction site) and 5′-CAATGCTAGCTCTGCACTTTTTCAA GGC (containing an *Nhe*I restriction site). Fragment II was amplified using oligonucleotides 5′-GTCCTCGAGTTACAG ATTATTGGTGGTGGTCA (containing an *Xho*I restriction site and introducing a stop codon into the *ADGF-D*, 453 bp downstream of +1 site) and 5′-CATGGCTAGCCCGCATTTTGC TCCGCATTC (containing a *Nhe*I restriction site and introducing a stop codon into the *ADGF-C* gene, 412 bp downstream of +1 site; the original *Not*I restriction site was removed in this step). Fragments I and II were cloned together into the pSLfa1180fa vector. Fragment III was amplified using primers 5′-CGACTAGTGCCATCTGTTCGACTGCTCC (containing a *Spe*I restriction site) and 5′-GCACTCGAGTCGCAATGTGGA TGGACTG (containing an *Xho*I restriction site) and cloned into pBLUESCRIPT II KS−. Fragment IV was amplified using primers 5′-AAGGAAAAGCGGCCGCTTCCCTTTGAACTTAC CTCTGG (containing a *Not*I restriction site) and 5′-CCGCTC GAGGTCCATTCCGAATGGCAAATC (containing an *Xho*I re-

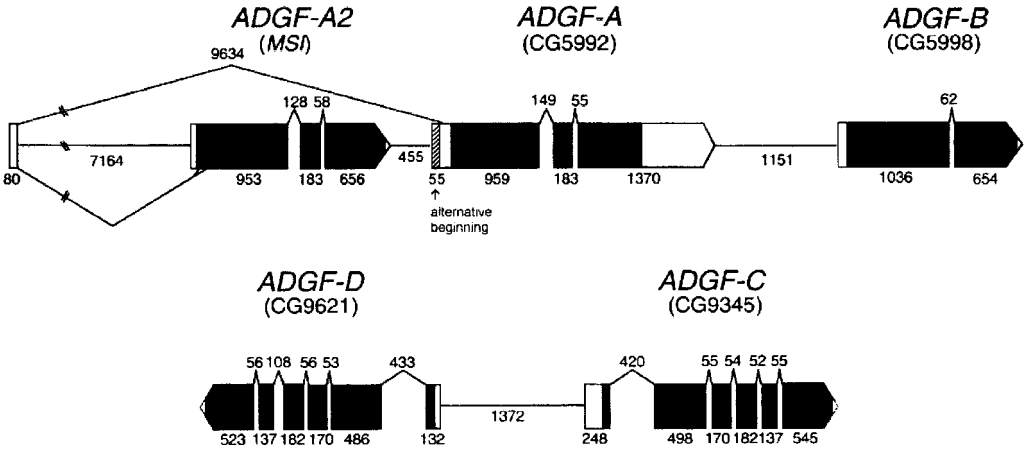


FIGURE 1.—Genomic organization of the *ADGF* genes (modified from MAIER *et al.* 2001). Numbers in the left corners represent chromosome localizations. Solid boxes represent coding exons and open boxes indicate 5′ and 3′ untranslated regions. Introns are represented by chevron-shaped lines, intergenic distances appear as horizontal lines, and exon and intron lengths are indicated below boxes and above chevrons, respectively.

striction site) and added together with an oligonucleotide providing an I-*Sce*I recognition site to pBLUESCRIPT II KS− that already carried fragment III. An I-*Sce*I recognition site was made by the annealing of the complementary oligonucleotides 5′-TCGAATTACCCTGTTATCCCTA (containing *Xho*I cohesive end) and 5′-CGCGTAGGGATAACAGGGTAAT (containing a *Mlu*I cohesive end). Fragments I and II were then recloned (using *Kpn*I and *Xho*I restriction endonucleases) from the pSLfa1180fa vector into pBLUESCRIPT II KS− with fragments III, IV, and I-*Sce*I oligo already present. After assembling the mutated *ADGF-C* and *ADGF-D* region in pBLUESCRIPT II KS−, the construct was recloned (using *Kpn*I and *Not*I restriction endonucleases) into pTV2 plasmid (provided by Yikang Rong and Kent Golic).

Production of transgenic lines and mutagenesis crosses: DNA constructs for HR were injected into *y w* fly embryos using the modified *P*-element-mediated transformation procedure (PARK and LIM 1995). Injected flies were crossed to *y w; Xa/Cyo;MKRS* for mapping and establishing transgenic stocks. Only stocks carrying constructs on the X or the second chromosome (not on the third chromosome where the targeted genes are localized) were used for targeting crosses. Thus, we avoided the complication of a targeting event on the same chromosome as the remaining FRT sequence plus the *P*-element inverted repeats.

In the first step, we used the rapid targeting scheme (RONG and GOLIC 2001; Figure 3) to induce HR. Transgenic lines carrying the constructs (five for the ADGF-A region, two for the ADGF-C+D region, respectively; see Table 1) were crossed to flies carrying *FLP recombinase* and *Sce*I *endonuclease* genes on the second chromosome. The progeny were heat shocked (38°, 1 hr) twice in the first 3 days of development. For further crosses, we selected females, since the recombination frequency is much higher in the female than in the male germline (RONG and GOLIC 2000). Females without the CyO balancer, *i.e.*, carrying *FLP recombinase* and *Sce*I *endonuclease* and the donor construct (usually with mosaic eyes but often with completely white eyes due to the high rate of somatic excision and loss of the donor), were crossed to flies carrying constitutively active *FLP recombinase.* Flies with a nonmosaic eye color were selected from the progeny, the new insertion was mapped to the chromosome by crossing to *y w; Xa/Cyo;MKRS*, and a balanced or homozygous stock was established.

In the second step, selected lines (with targeted events) were crossed with the line carrying the gene encoding I-*Cre*I endonuclease on the X chromosome to induce a DSB in the recognition sequence between the two components of the duplication. This DSB induces reduction of the duplication to a single copy. Flies with such a reduction were scored by loss of the *miniwhite* marker.

Fly stocks: The stocks *y w (v); P[ry$^+$, 70FLP]4 P[v$^+$, 70I-SceI]2B Sco/S^2 CyO* and *w^{1118}; P[ry$^+$, 70FLP]10* (constitutively active FLP recombinase, homozygous on the second chromosome) for the rapid targeting scheme (Figure 3) in the first step and *v P[70I-CreI, v$^+$]2A; ry* for reducing the duplication in the second step were provided by Yikang Rong and Kent Golic. The stocks *y w; Xa/Cyo;MKRS* and *w; TM3 Sb Ser/TM6B* were used for mapping to chromosome and for establishing the recombinant lines.

Molecular characterization of the targeted events: The first step of HR was analyzed using standard methods of DNA isolation, digestion, and Southern blot analysis (SAMBROOK *et al.* 1989), taking advantage of the novel restriction sites associated with the engineered mutations. For the identification of targeted events in the ADGF-A region, genomic DNA from all tested lines was digested with the restriction enzymes *Hpa*I, *Nhe*I, and *Sma*I, and a Southern blot containing this DNA was hybridized with a 1.6-kb *ADGF-A* cDNA probe. For the identification of targeted events in the *ADGF-C+D* region, genomic DNA from all lines was digested with restriction enzymes *Nhe*I, *Nde*I, and *Xho*I, and a Southern blot containing this DNA was hybridized with a 2.4-kb probe from genomic sequence covering part of the *ADGF-D* gene plus adjacent DNA. To verify the results obtained for the *ADGF-C+D* region, genomic DNA from all lines was digested using the restriction enzymes *Nhe*I, *Spe*I, and *Xho*I, and a Southern blot containing this DNA was hybridized with the same 2.4-kb probe (data not shown).

The second-step recombination events were detected by PCR analysis using primers with mutation-specific 3′ ends: *ADGF-A* region—5′-GAATTTCTTTGCTGGATTTGCC (last CC specific for *ADGF-A2* mutation), 5′-CCTTTATTTGTT TAAGGGTTTGGAGC (last GC specific for *ADGF-A* mutation), and 5′-CCACGGCGTTCAAGAGC (last GC specific for *ADGF-B* mutation); *ADGF-C+D* region—5′-ATGCGGAGCA AAATGCGGGCTA (last G and TA specific for *ADGF-C* mutation) and 5′-CACCAATAATCTGTAACTCGAG (last ACT and GAG triplets specific for *ADGF-D* mutation). The PCR analysis of the reduction events was confirmed in selected samples by Southern blot. In each case the sample DNA was a mixture of genomic DNA from heterozygous and homozygous adult flies. The reduction events in the ADGF-A region were analyzed using *Hpa*I, *Nhe*I, and *Sma*I restriction enzymes, and the membrane was hybridized with two probes covering the whole region prepared by PCR from genomic DNA (Figure 7). The reduction events in the ADGF-C+D region were analyzed us-

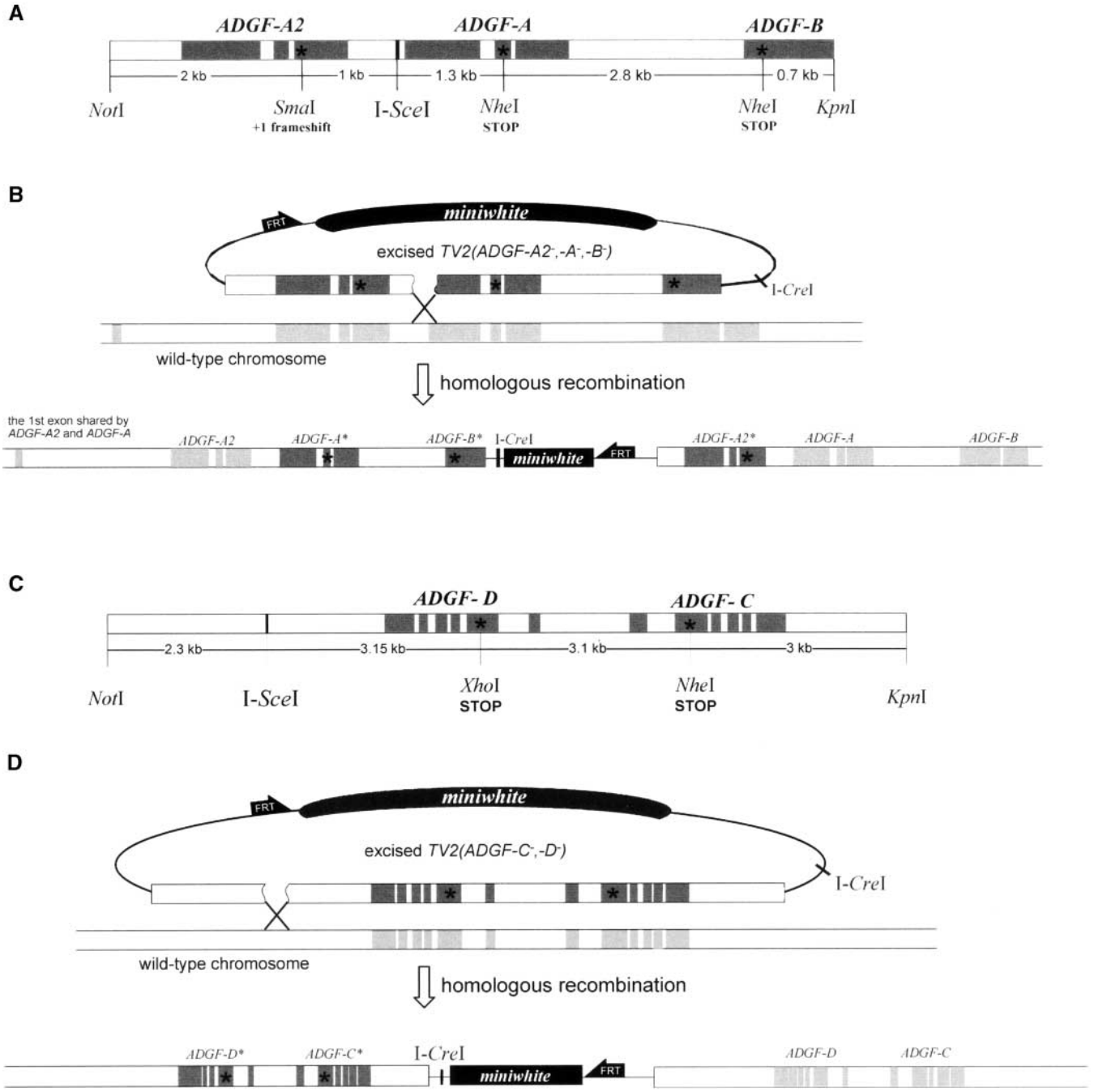


FIGURE 2.—Design of HR constructs and the first recombination step. (A and C) Donor constructs for ADGF-A and ADGF-C+D regions. Genomic sequences were cloned into the pTV2 plasmid using the end restriction sites *Not*I and *Kpn*I. Mutations and additional restriction sites were introduced as 2- to 6-bp changes (asterisked) into all genes (coding sequence is darkly shaded). The new restriction site introduced with the mutation and the type of mutation are indicated below each asterisk. *ADGF-A*, *ADGF-A2*, *ADGF-C*, and *ADGF-D* genes contain all exons except the shared first one of *ADGF-A* and *-A2*; *ADGF-B* contains only the first exon. A recognition site for I-*Sce*I meganuclease was cloned between the *ADGF-A* and *ADGF-A2* genes and 1.7 kb downstream from the *ADGF-D* 3′ end. (B and D) Scheme of ends-in targeting with *TV2(ADGF-A2*$^{-}$, *-A*$^{-}$, *-B*$^{-}$*)* and *TV2(ADGF-C*$^{-}$, *-D*$^{-}$*)* donors, respectively. The circularized construct after excision by FLP recombinase with a DSB in the I-*Sce*I site recombines with homologous sequences on the wild-type chromosome, producing a duplication with the combination of alleles as shown (light shading, originated from wild-type chromosome; dark shading, originated from donor construct) and the *miniwhite* marker in the center.

ing *Xho*I and *Nhe*I restriction enzymes and a PCR-amplified probe for each of the two genes (Figure 7).

Two alternative start sites of the *ADGF-A* gene were analyzed by RT-PCR: total RNA from homozygous lines 1 and 14 and from wild-type third instar larvae was isolated using an RNAeasy kit (QIAGEN, Chatsworth, CA). The first strand was produced by Superscript reverse transcriptase (Invitrogen, San Diego) using the *ADGF-A*-specific primer 5′-AGGTTCTCATC CACAGTGG. Primers 5′-GAAGATCGCGGCGAGGAAGT and 5′-CGAACGCGTGTTAAATCAAAG, specific for the first non-

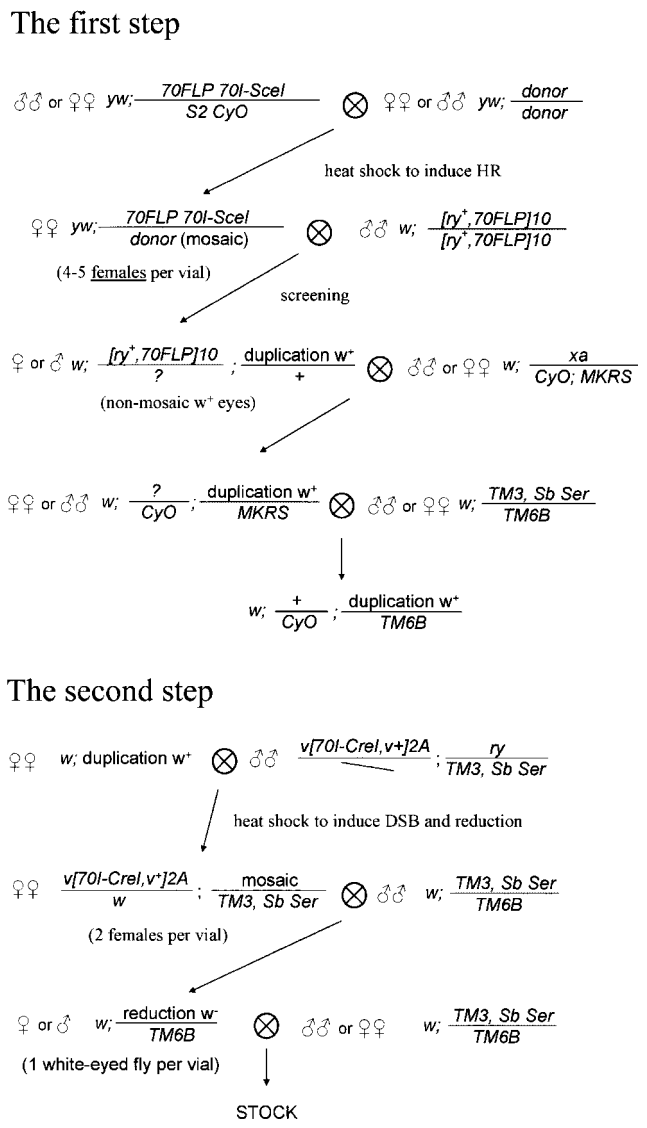


FIGURE 3.—Rapid targeting scheme used for the first step of homologous recombination and for the second step of reduction of the duplication to a single copy (for details see text).

coding exon of *ADGF-A* and an alternative transcriptional start site of *ADGF-A*, respectively, were used for PCR with the first strand as a template.

RESULTS

Recombination—first step: To induce HR, we established 315 vials (containing four to five females each) for the ADGF-A region and 350 vials for the ADGF-C+D region, and we recovered 18 potential recombinant lines for the ADGF-A region and 19 for the ADGF-C+D region. All potential recombinant lines were tested by Southern blot (Figures 4 and 5), and Table 1 summarizes the overall frequencies of targeted and nontargeted events. For the ADGF-A region, the duplication with the expected combination of mutations (Figure 2B) was detected in 5 of 14 targeted events (lines 6, 10, 13, 14, and 16; Table 2). Two triplications (lines 7 and 8), containing an additional insertion of the original donor carrying all three mutations (a class IV event according to RONG and GOLIC 2000), were found in the ADGF-A region. In one of them (line 8), a deletion of ~450 bp was detected in the *ADGF-A2* wild-type copy (a class III event). The remaining seven lines with targeted events carried the expected duplication but with various gene conversions (see Figure 4 and Table 2 for details) in which we assume that the introduced mutation was converted back to wild type using the wild-type chromosome as a template or that the wild-type copy in the duplication was converted to a mutant using the donor DNA as a template. In line 9, the mutation in the *ADGF-A* gene was found on the opposite side of the duplication than expected. Two other lines carried the mutation in both copies of *ADGF-A* in the duplication (lines 1 and 15 are also called *karel* and *gerda*, respectively).

The two lines carrying both mutated copies of *ADGF-A* (*karel* and *gerda*) showed a larval lethal phenotype in the homozygotes (described below). All other lines with targeted events were homozygous viable. However, we expected that a mutation in only one copy of the *ADGF-A* gene (on the left side, Figure 2) would cause the same phenotype as a mutation in both copies of this gene. This was because the construct was designed so that the wild-type copy of the *ADGF-A* gene on the right side of the duplication lacks the first noncoding exon, which in the normal genome is shared by *ADGF-A* and *ADGF-A2* (see Figures 1 and 2). By searching the expressed sequence tag (EST) database, we found one EST clone of *ADGF-A*, which has an alternative transcriptional start site 55 bp prior to the second exon (the first exon is not present in this clone). We confirmed this alternative transcriptional start site by RT-PCR using primers specific to each transcriptional start site (see MATERIALS AND METHODS). Both variants were overexpressed in larvae homozygous for the *ADGF-A* mutation (line Karel; data not shown). This overexpression may be a regulatory response to the lack of functional ADGF-A protein, suggesting that the expression of ADGF-A is tightly controlled. The transcriptional start using the first exon seems to be used more than the alternative transcriptional start site; there are 16 clones with the first-exon transcriptional start in the EST database compared to only 1 clone with the alternative transcriptional start site. Lines with the expected duplication in the *ADGF-A* region showed that the *ADGF-A* mutant phenotype is fully suppressed by the presence of a wild-type *ADGF-A* transcript starting from the alternative start site. Unfortunately, none of the combinations we obtained allowed us to test whether the transcript containing the first exon, when expressed alone, would also be sufficient to suppress the phenotype.

For the ADGF-C+D cluster we obtained 19 fly lines with a nonmosaic eye color, which was the marker for potentially targeted duplications. In genetic crosses we excluded lines with the insertion on X or the second

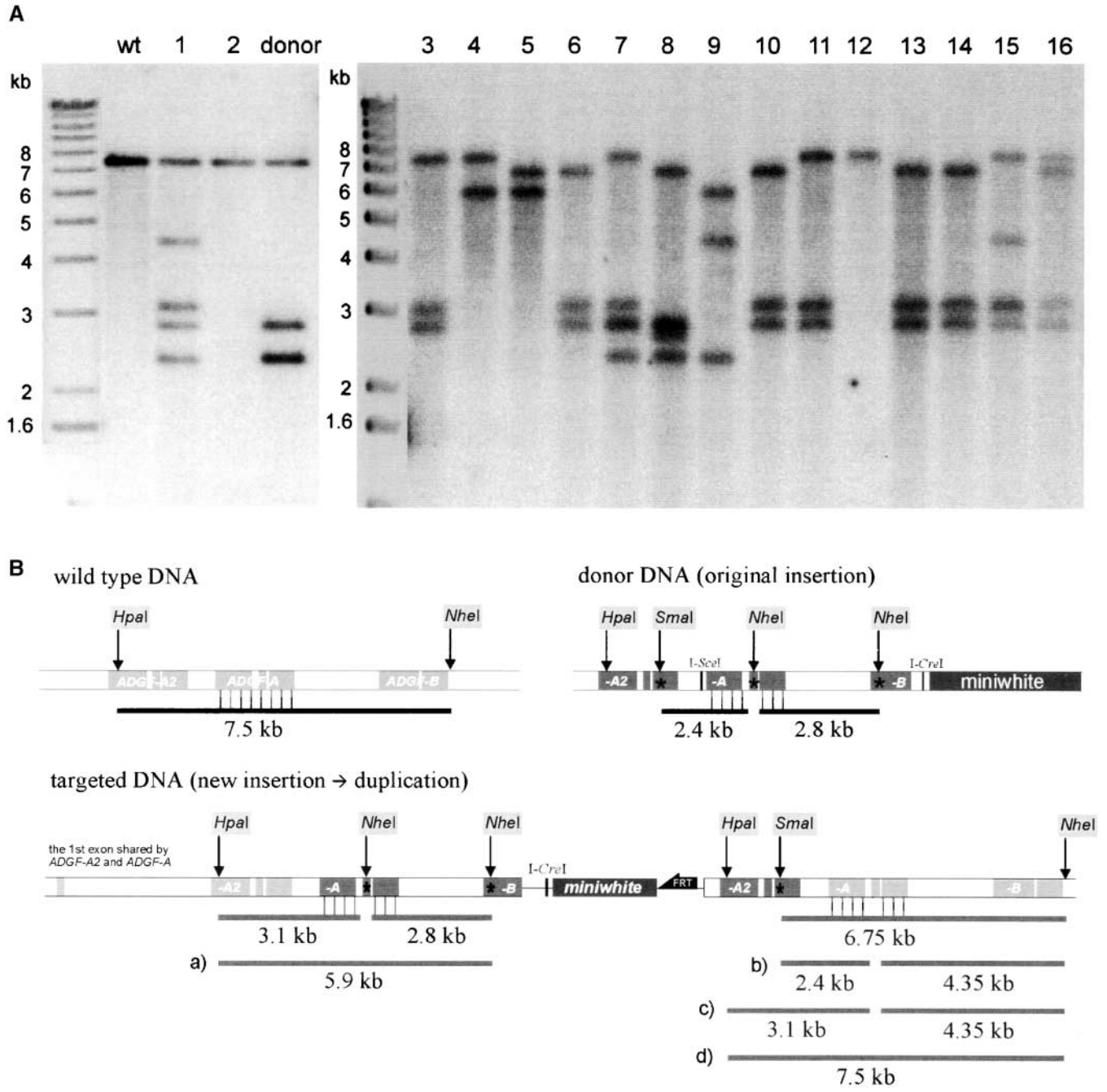


FIGURE 4.—Southern blot analysis of targeting events in the ADGF-A region. All genomic DNA was isolated from adult flies homozygous for the chromosome carrying the targeting event except for lines 1, 2, and 15 in which the targeting event was lethal so heterozygous flies were used, and line 16 in which some heterozygous individuals were present. DNA was digested with *Hpa*I, *Nhe*I, and *Sma*I restriction enzymes and the membrane was hybridized with a 1.6-kb *ADGF-A* cDNA probe. (A) Two membranes showing molecular markers with indicated sizes, wild-type DNA (wt), DNA isolated from donor transgenic line 3A (Table 1), and DNA from 16 analyzed events (Table 2). (B) Schematic diagram showing genomic organization with restriction sites and sizes of fragments hybridized to probe. In wild-type DNA, only one 7.5-kb fragment is detected. In the randomly positioned donor construct carrying three introduced point mutations, two fragments, 2.4 and 2.8 kb in length, are hybridized to an *ADGF-A* probe; a 7.5-kb wild-type fragment is also present in the DNA of the transgenic fly carrying the donor construct. The targeted DNA diagram shows the expected duplication with 2.8-, 3.1-, and 6.75-kb fragments (lines 6, 10, 13, 14, and 16 on the membrane). Other combinations that occurred due to gene conversion are also shown. A mutation in the *ADGF-A* gene converted to wild type in lines 4, 5, and 9, producing the 5.9-kb fragment (a). A wild-type copy of the *ADGF-A* gene converted to a mutated copy in lines 1 and 9, producing the 2.4- and 4.35-kb fragments (b); the case (b) with the simultaneous conversion of the *ADGF-A2* mutation to wild type in line 15 produced 3.1- and 4.35-kb fragments (c). In lines 3 and 11 (homozygous), the *ADGF-A2* mutation converted to wild type, producing a 7.5-kb fragment similar to the wild-type fragment (d). A triplication (class IV event discussed in RONG and GOLIC 2000) occurred with the conversion of the *ADGF-A2* mutation to wild type in line 7 and with an ~450-bp deletion in the *ADGF-A2* wild-type copy in line 8, respectively, producing an additional 2.4- and 2.8-kb fragment originating from the donor construct inserted between the two parts of the duplication.

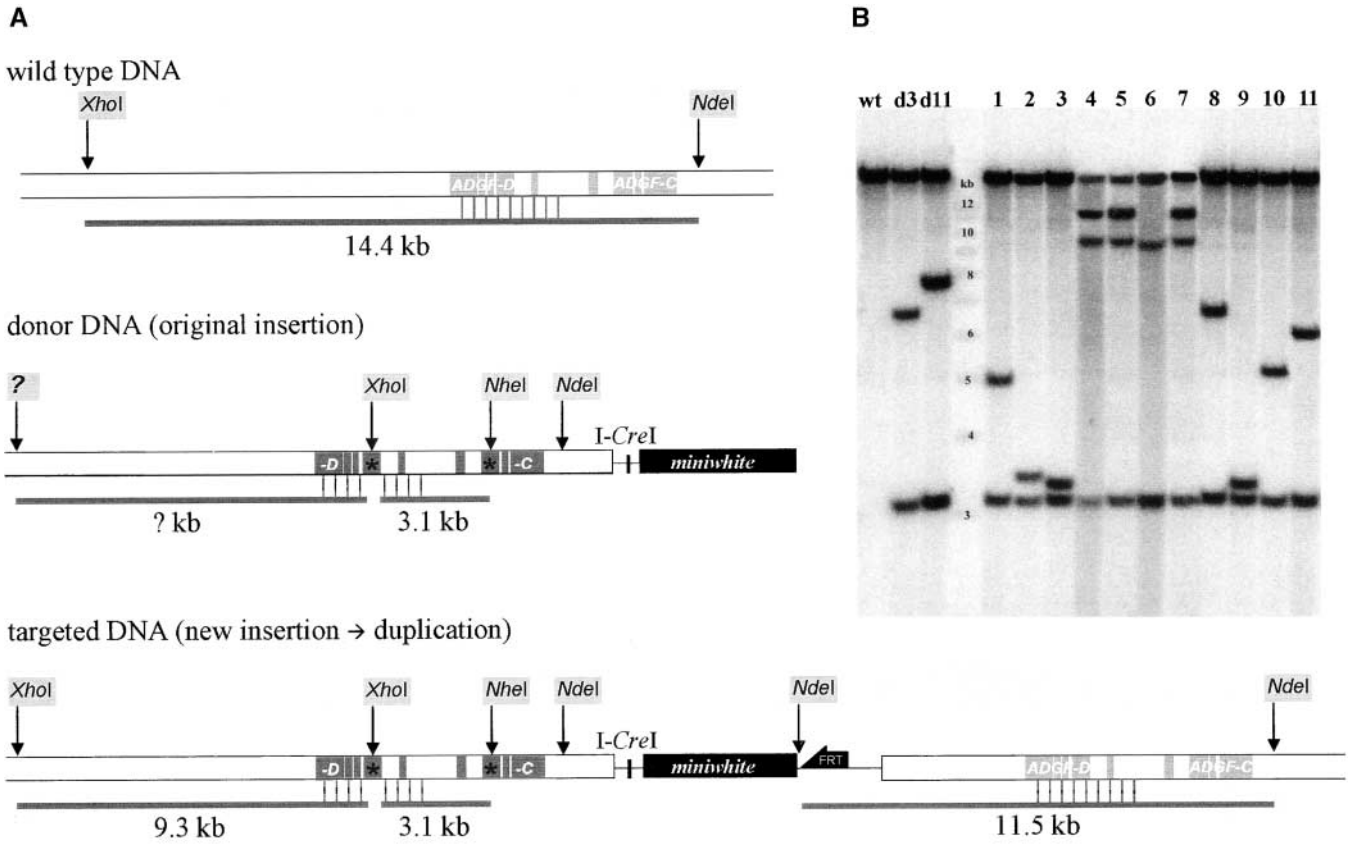


FIGURE 5.—Southern blot analysis of targeting events in the ADGF-C+D region. Genomic DNA was isolated from adult flies heterozygous for the chromosome carrying the potential targeting event. DNA was digested with *Nde*I, *Nhe*I, and *Xho*I restriction enzymes in one reaction mix. The membrane was hybridized with a 2.4-kb probe from genomic sequence covering part of the *ADGF-D* gene plus adjacent DNA. (A) Schemes of genomic organization with marked restriction sites and sizes of fragments recognized by the probe. In wild-type DNA, only one 14.4-kb fragment is detected. In the randomly positioned donor constructs d3 and d11 carrying two introduced mutations, two fragments are hybridized to the probe: a 3.1-kb fragment between introduced mutations and a fragment of unpredicted size depending on the insertion site of the donor construct. In the expected duplication event three fragments should occur: a 3.1-kb fragment between introduced mutations, a 9.3-kb fragment that can occur only if the insertion is targeted as expected, and an 11.5-kb fragment covering the wild-type copy plus part of *TV2* in the duplication. (B) Hybridized membrane with lines: wild-type DNA, DNA isolated from the two donor transgenic lines d3 and d11. Lines labeled 1–11 show analyzed HR events. Lines 4, 5, and 7 represent targeted events.

chromosome (8 lines). The remaining 11 lines, all with the insertion on the third chromosome, were subject to Southern blot analysis. In the first round of Southern blots we used enzymes *Spe*I, *Nhe*I, and *Xho*I and we found three lines with the correct hybridization pattern. Since there was a possibility that in the next 8 lines a deletion had occurred in the DSB area (a class III event), we performed another Southern blot using the *Nde*I restriction enzyme instead of *Spe*I, since this should change the size of DNA fragments in a specific way according to the size of the deletion. Using these two different enzyme sets for Southern blot analysis, we showed that the remaining 8 lines have apparently random insertions into the third chromosome (*i.e.*, they are nontargeted events). The data are summarized in Table 3 and Figure 5, which shows only the Southern blot using *Nde*I, *Nhe*I, and *Xho*I.

Recombination—second step: We crossed lines selected from the previous step with the line carrying the I-*Cre*I endonuclease on the X chromosome to induce a DSB in the recognition sequence between repeats (see Figure 2). The selected lines for the ADGF-A region were line 9, called Amalie, and line 14, called Franta; for the ADGF-C+D region, they were lines 4, 5, and 7. The most frequent repair mechanism of the DSB between direct repeats is reduction to a single copy by either simple crossing over between repeats or single-strand annealing (SSA; PAQUES and HABER 1999). Figure 6 shows the possible combinations of mutations, depending on the site of the recombination event. We chose two different lines (Amalie and Franta) with duplications in the ADGF-A region, having *ADGF-A* mutations in opposite positions so that we could produce more combinations and also a single mutation in either the *ADGF-A2* or the *ADGF-B* genes. Lines established from flies that had lost their miniwhite marker after I-*Cre*I induction by heat shock were tested for the presence of the mutation by PCR using mutation-specific primers

TABLE 1

Summary of targeting events

Donor	No. of screened vials	No. of recombinants (percentage per screened vials) Targeted events	Nontargeted events
ADGF-A (8.5 kb)			
1A (on II)	77	2 (2.6)	2 (2.6)
3A (on II)	84	1 (1.2)	0
4 (on II)	59	3 (5.0)	0
6 (on X)	55	5 (9.0)	2 (3.6)
9 (on II)	40	3 (7.5)	0
Total	315	14 (4.4)	4 (1.3)
ADGF-C+D (11.5 kb)			
d3 (on II)	300	2 (0.67)	15 (5.0)
d11 (on II)	50	1 (2.0)	1 (2.0)
Total	350	3 (0.86)	16 (4.57)

(see MATERIALS AND METHODS; data not shown). A total of 14 lines from Franta and 20 lines from Amalie were established from a single cross of white-eyed progeny in each vial. White-eyed progeny were found in every vial (containing two to three mothers), indicating a high rate of reduction after I-*Cre*I-induced recombination. Figure 6 summarizes simple recombination frequencies in different locations. In six other cases, apparent multiple exchanges produced other mutation combinations (*ADGF-A2** + *ADGF-B** in two cases from the line 14 screen; *ADGF-A2** + *ADGF-A** in two cases, single *ADGF-B** in one case, and *ADGF-A2** + *ADGF-A** + *ADGF-B** in one case from the line 9 screen; an asterisk indicates mutated copy).

For the ADGF-C+D region, we screened 130 reduction events for the presence of mutations by PCR using mutation-specific primers. We obtained nine lines with mutations in both *ADGF-C* and *ADGF-D*, and seven lines with a mutation in *ADGF-D*. In two cases we detected a single mutation in *ADGF-C*, indicating that multiple exchanges can occur. PCR screening of the reduction events was further confirmed by Southern blot analysis in selected samples (Figure 7).

In summary, all potential combinations of mutations in both ADGF-A and ADGF-C+D regions, *i.e.*, a single mutation in each gene, all double-mutant combinations, and one triple-mutant combination in the ADGF-A region, were obtained from this second step, allowing us to analyze the phenotypes produced by both single mutations and all combinations of multiple mutations in these five members of the ADGF family.

Mutant phenotype: Both *karel* and *gerda* homozygotes (A2 A* B* w A2* A* B and A2 A* B* w A2 A* B genotypes, respectively), as well as heterozygous *gerda/karel* flies, show a clear mutant phenotype, presumably due to LOF for *ADGF-A*. They are mostly lethal in late third instar, and larval development is significantly delayed. They show disintegration of the fat body and most third instar larvae develop melanotic tumors (Figure 8). They rarely pupate, but when they do, they produce abnormal pupae. It is important to note that these two lines are totally independent, since they were produced from different transgenic flies. However, they both carry mutations in both copies of *ADGF-A* (see *Recombination—first step*). The homozygous phenotype is also the same in all lines carrying mutant *ADGF-A* after reduction to single copy. The other confirmation that the phenotype is caused by the mutation in the *ADGF-A* gene is the rescue of the phenotype to wild type by the expression of a transgenic *ADGF-A* gene under either a heat-shock or a UAS promoter (driven by actin-Gal4 driver; data not shown).

Double mutants in *ADGF-C* and *ADGF-D* have prolonged development and show semilethality during larval and pupal stages. They are also lethargic during the first few days after emerging, and their fertility is significantly lower than that of heterozygous siblings. The effect of both mutations seems to be cumulative since mutants in either the *ADGF-C* or the *ADGF-D* gene have similar phenotypes with lower penetrance than that seen in double mutants. A similar phenotype was observed in experiments with RNAi-mediated silencing of *ADGF-C* and *ADGF-D* genes (data not shown).

Mutations in the *ADGF-A2* and *ADGF-B* genes (both single mutants and double mutants) do not express any obvious phenotype, and homozygous adults are fertile. A homozygous triple-mutant combination (A2* A* B*) expresses the same phenotype as does a single homozygous mutation in *ADGF-A* (*karel* and *gerda*).

DISCUSSION

Our work confirms that ends-in targeting is a very powerful tool for introducing specific mutations into the genome, and it adds to our knowledge of the process of HR. Introducing two or three mutations into one construct allowed us to analyze targeting events in detail, especially gene conversions as well as recombination events during reduction of duplication to single copy. We have also shown that it is possible to introduce multiple changes into the genome simultaneously by this method and, in our case, to disrupt a multigene family.

It has already been pointed out that mutations close to DSB can be repaired to wild type by gene conversion using the homologous chromosome as a template. GLOOR *et al.* (1991) reported that the average length of conversion is ~1.3 kb. Our results support this conclusion since conversion was frequent (11 conversions in 14 targeted events) in the case of *ADGF-A2* and *ADGF-A* mutations, which were 1.0 and 1.3 kb, respectively, from

TABLE 2

Targeting events in the ADGF-A region

Line no.	Name of mutant line	Donor line	Donor localization	New position	Targeted/ nontargeted	Molecular characterization	Genotype
1	Karel	3A	II	III	T	Duplication with conversion A → A*	A2 A* B* *w* A2* A* B
2	1A-3	1A	II	II	NT	No mutation present	A2 A B
3	Albert	6	X	III	T	Duplication with conversion A2* → A2	A2 A* B* *w* A2 A B
4	Mojmir	4	II	III	T	Duplication with conversion A2* → A2 and A* → A	A2 A B* *w* A2 A B
5	Drahomira	4	II	III	T	Duplication with conversion A* → A	A2 A B* *w* A2* A B
6	Hermina	9	II	III	T	Duplication as expected	A2 A* B* *w* A2* A B
7	Hubert	1A	II	III	T	Triplication with conversion A2* → A2	A2 A* B* *w* A2* A* B* *w* A2 A B
8	Berta	9	II	III	T	Triplication with 450-bp deletion in A2	ΔA2 A* B* *w* A2* A* B* *w* A2* A B
9	Amalie	6	X	III	T	Duplication with conversion A → A* and A* → A	A2 A B* *w* A2* A* B
10	Viktor	6	X	III	T	Duplication as expected	A2 A* B* *w* A2* A B
11	Rudolf	6	X	III	T	Duplication with conversion A2* → A2	A2 A* B* *w* A2 A B
12	Tonda	6	X	X	NT	No mutation present	A2 A B
13	Teodor	4	II	III	T	Duplication as expected	A2 A* B* *w* A2* A B
14	Franta	1A	II	III	T	Duplication as expected	A2 A* B* *w* A2* A B
15	Gerda	6	X	III	T	Duplication with conversion A → A* and A2* → A2	A2 A* B* *w* A2 A* B
16	Ferda	9	II	III	T	Duplication as expected	A2 A* B* *w* A2* A B
17	Jarmila	6	X	X	NT	Not determined	?
18	Jarda	1A	II	II	NT	Not determined	?

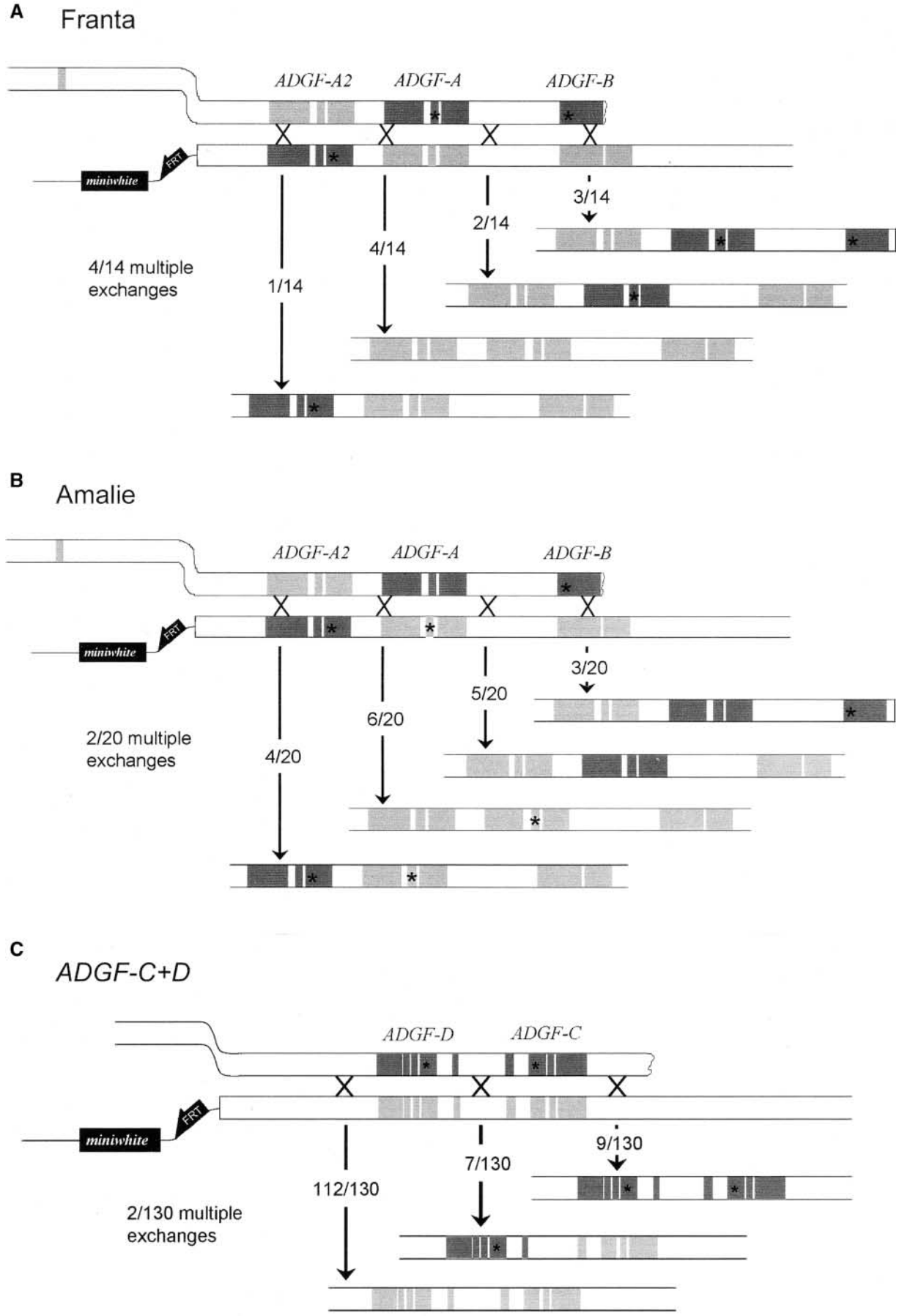
*, mutant; *w*, miniwhite.

the DSB, whereas no conversion was detected in *ADGF-B*, *ADGF-C*, and *ADGF-D* mutations, which were from 3 to 6 kb from the DSB. Our results also show that the wild-type copy on a chromosome can be converted to a mutant copy, presumably using the donor construct as a template. This was observed in lines 1 and 15 where the wild-type copy of the *ADGF-A* gene was converted to a mutated copy, resulting in a duplication with mutated copies on both sides. It is more difficult to explain the reciprocal exchange of the *ADGF-A* mutation in the line 9 (Amalie) duplication. If the duplication arises by insertion of the donor construct into the I-*Sce*I site, then reciprocal conversion (conversion in the chromosome using the donor as a template and conversion in the donor using the chromosome as a template, respectively) would occur. This would be possible only in the G_2 phase of the cell cycle, when more than one template is available. It could also include a two-copy donor intermediate, perhaps followed by I-*Sce*I cutting and repair after a class IV event is generated by the initial targeting.

TABLE 3

Targeting events in the ADGF-C+D region

Line no.	Donor line	Donor localization	Molecular characterization
1–3	d3	II	Nontargeted events on third chromosome
4	d3	II	Targeted duplication as expected
5	d3	II	Targeted duplication as expected
6	d11	II	Nontargeted event on third chromosome
7	d11	II	Targeted duplication as expected
8–11	d3	II	Nontargeted event on third chromosome
12–19	d3	II	Nontargeted events on X (2) and second (6) chromosome; not shown on Southern blot


A
Franta
ADGF-A2
ADGF-A
ADGF-B
miniwhite
FRT
3/14
2/14
4/14
4/14 multiple
exchanges
1/14
B
Amalie
ADGF-A2
ADGF-A
ADGF-B
miniwhite
FRT
3/20
5/20
6/20
2/20 multiple
exchanges
4/20
C
ADGF-C+D
ADGF-D
ADGF-C
miniwhite
FRT
9/130
7/130
112/130
2/130 multiple
exchanges

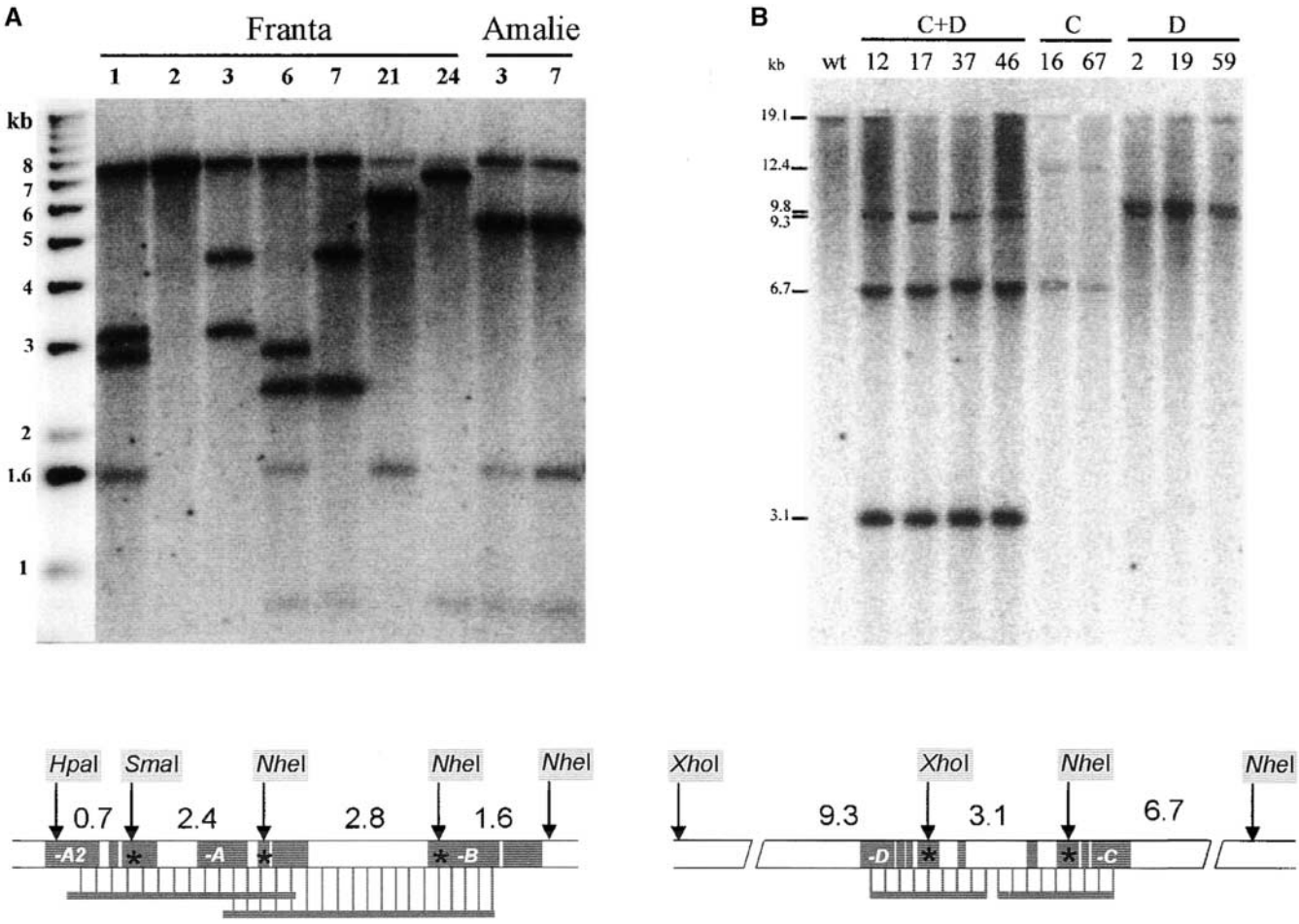


FIGURE 7.—Southern blot analysis of reduction events. In each case, the sample DNA is a mixture of genomic DNA from heterozygous and homozygous adult flies. Numbers above the schematic DNA map show the distances between restriction sites in kilobases, and bars below the map show the extent of probes. (A) Reduction events in the ADGF-A region were analyzed using *Hpa*I, *Nhe*I, and *Sma*I restriction enzymes and the membrane was hybridized with two probes detecting all of the relevant restriction fragments. No mutation was preserved in Franta 2, but the other samples show the following genotypes: *a2*a b* in Fr24, *a2*a*b* in Fr7, *a2*a*b** in Fr6, *a2 a*b** in Fr1, *a2 a b** in Fr21, *a2 a*b* in Fr3, and *a2*a b** in Am3 and Am7. (B) Reduction events in the ADGF-C+D region were analyzed using *Xho*I and *Nhe*I restriction enzymes and a probe for each of the two genes. Lines 12, 17, 37, and 46 contain mutations in both ADGF-C and -D genes, lines 16 and 67 contain mutations in the ADGF-C gene, and lines 2, 19, and 59 contain mutations in ADGF-D gene.

In any case, the data show that gene conversion must be anticipated when designing a donor construct and that this phenomenon can also be used for specific purposes. In our case, we used gene conversion to increase the number of mutation combinations in the following step of reduction to a single copy.

The efficiency of targeting in these experiments differs between the ADGF-A and ADGF-C+D regions. Although the numbers of recovered events are similar (approximately one event per 18 vials), the frequencies of nontargeted insertions are significantly different. In the ADGF-A region we obtained 14 targeted events out of 18 examined lines, and all nontargeted events were mapped to the same chromosome as the original (donor) insertion. This suggests that the donor construct was not excised by the FLP recombinase but that DSB

FIGURE 6.—Reduction of a duplication to a single copy by induction of I-*Cre*I breakage. The diagrams show the chromosome after a break in the I-*Cre*I site and different possible combinations of genes after recombination. Crosses indicate points where recombination occurred. Numbers on the arrows show the overall frequencies of recombination at the marked points. Asterisks represent introduced mutations. Dark shading represents genes from the donor construct, whereas light shading represents wild-type copies. Loss of the *miniwhite* gene served as a marker for screening. (A) Line 14 screen (duplication in the ADGF-A region as expected). (B) Line 9 screen (duplication in the ADGF-A region with mutation of *ADGF-A* on the other side). (C) Lines 4, 5, and 7 screen (duplication in the ADGF-C+D region as expected).

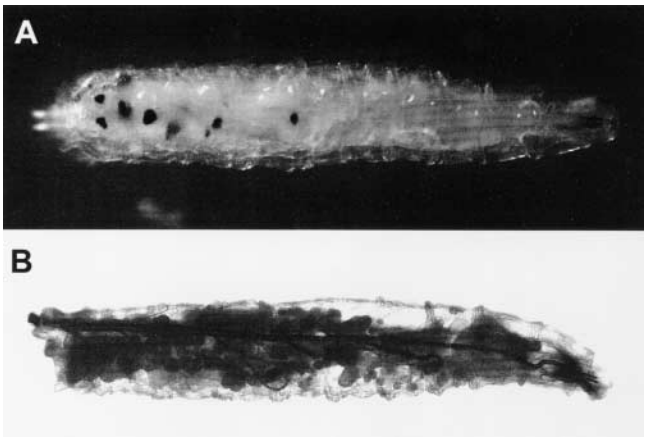


FIGURE 8.—Mutant phenotypes. Homozygous *karel* late third instar larvae develop melanotic tumors (A) and show disintegration of the fat body, in which the tissue is in dispersed fragments rather than in an intact flat layer (B).

occurred at the I-*Sce*I site. This would have been partially repaired using the wild-type template, removing one FRT sequence and leaving the *miniwhite* marker, which is then detected in the rapid targeting screen (only one FRT sequence is present). However, the results for the ADGF-C+D region are different. We found only three targeted events out of 19 examined lines with nonmosaic eyes, and most of the nontargeted events were localized to a different chromosome than was the original insertion. The explanation for such a low efficiency may be that we started the first step of HR with only two different donor lines (Table 1) and that the targeting efficiency for one of them was <12% (2/15). An alternative explanation may be that the ADGF-C+D chromosomal region is somehow protected from any structural changes. This idea is supported by our previous unsuccessful mutagenesis of the ADGF-C+D region using both male recombination and mobilization of a 7-kb-distant *P* element. In conclusion, our results confirm that targeting efficiency depends on the position of the original donor insertion and on the chromosomal structure of the targeted region.

Because in most cases the duplication after the first step contained wild-type copies of the genes, we induced reduction to single copy by I-*Cre*I endonuclease (RONG *et al.* 2002). The reduction can be explained by a simple crossover event between repeats as shown in Figure 6. This explanation is supported by the fact that the expected combinations from simple crossover events predominated in each screen. However, there is another possible mechanism, SSA, that can also account for the repair of DSB between repeats. SSA depends on the resection of the ends of the DSB by an exonuclease to produce long single-stranded tails in which complementary strands of the duplicated sequence are exposed and can reanneal (PAQUES and HABER 1999; Figure 9). PRESTON *et al.* (2002) reported that SSA is the preferred repair pathway in Drosophila for DNA breaks between sequence repeats and RONG *et al.* (2002) suggested that SSA is responsible for the reduction to single copy during the second step of ends-in targeting. There are several reasons to favor SSA as the mechanism for reduction: First, the expected combinations mentioned above can be explained by SSA mechanism as easily as by simple crossing over. Second, since the *ADGF-A* mutation was placed almost exactly in the middle of the homologous sequence, this region would always be single stranded and could make the duplicated sequence available for annealing (see Figure 9). Once the DNA heteroduplex is produced by annealing, random repair of the mismatch would determine whether the mutation or the wild-type sequence would be preserved. If all reductions were produced by SSA, we would expect the *ADGF-A* mutation and the wild-type *ADGF-A* sequence to be recovered equally frequently, and we recovered 18 *vs.* 16 cases, respectively. Third, the simple crossing-over explanation would require triple crossing over to have occurred in 6 of 34 cases in the ADGF-A region, which seems very unlikely. If the area containing mutations is single stranded, then any combination is possible because of the random repair of the mismatch. Fourth, recombination frequency is linearly related to distance between markers. However, we found that the mutation in the *ADGF-B* gene, which would have required recom-

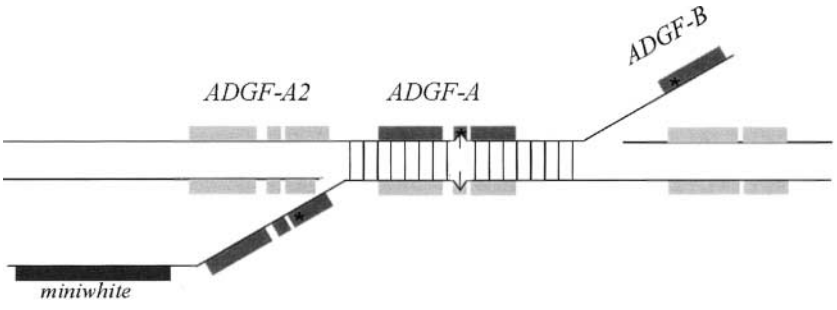


FIGURE 9.—Single-strand annealing in the ADGF-A region. This shows the situation when the exonuclease produces single strands in the area of *ADGF-A* but does not reach the wild-type copy of *ADGF-A2* on one side or the wild-type copy of *ADGF-B* on the other side. Annealing then occurs only in the *ADGF-A* gene, producing heteroduplex DNA. The wild-type *ADGF-A2* and *ADGF-B* are single stranded less often because they are farther from the DSB and thus mutations in these genes are retained with lower frequency than the mutation in *ADGF-A* (in the middle of the figure), which is always single stranded.

bination to take place in the 0.7-kb region (see Figure 6), was detected with a frequency comparable to that of the *ADGF-A2* mutation, which would have required recombination to occur in a 2-kb region. Both mutations were placed at approximately equal distances and closer to the DSB than the *ADGF-A* mutation was placed. Thus the SSA mechanism best explains the detected frequencies (see Figure 9) since the farther *ADGF-A* mutation was retained with higher frequency (18 of 34) but the *ADGF-A2* and *ADGF-B* mutations were retained with similar frequencies yet lower than that of the *ADGF-A* mutation (11 of 34 and 10 of 34).

The ADGF-C+D region behaved significantly differently from the ADGF-A region. Mutations in either one or both *ADGF-C* and *ADGF-D* genes were preserved in only 13% of reduction events. The recovery of such a small number of mutant alleles may indicate that the heterozygous combination can influence the length of development or the viability of mutation carriers. During the screening for mutants after the second step of HR, we took only one fly from each vial to get the highest possible variability. We usually selected the first emerged fly with the loss of red eye color. Thus we could have accidentally favored wild-type alleles. This could also explain why we did not succeed with *P*-element mutagenesis of the ADGF-C+D region.

The results of the reduction screen are consistent with our molecular analysis of the duplications since we recovered the expected combinations (Figure 6). In addition, the reduction of *karel* with both copies of *ADGF-A* mutated produced only flies with the *karel* phenotype (data not shown).

The mutant phenotypes that we discovered correspond to the expression patterns of individual genes. Since the *ADGF-A* gene is expressed at all developmental stages (Zurovec *et al.* 2002), it is not surprising that mutations in this gene cause the strongest phenotype and the earliest lethality among the mutated members. Both *ADGF-C* and *ADGF-D* are the closest homologs in the family and they also show very similar expression patterns starting in the third instar larvae, and they are expressed in both adult males and females. The mutant phenotype, which becomes visible at later stages compared to mutant *ADGF-A* and seems to be cumulative in the double mutant, is consistent with the characteristics mentioned above. *ADGF-A2* (previously described as male-specific insect-derived growth factor, or *MSI*) is expressed exclusively in testes (Matsushita *et al.* 2000) and the expression of *ADGF-B* is also restricted to males. This expression pattern strongly suggests a role for these genes in spermatogenesis. Homozygous mutants in either of these genes as well as in the double mutant are fertile. However, the last member of the family, *ADGF-E*, which was not mutated in our screen and for which no mutation is known, is also expressed exclusively in adult males, leaving the possibility of redundancy of these three genes.

Detailed analysis of phenotypes and of the ADGF-A and ADGF-C+D region mutant combinations are under further investigation. Our results show that by classical mutagenesis methods based on phenotype screening only the *ADGF-A* gene among six members of the ADGF family would have been identified. However, using the HR method for gene targeting we were able to recover five mutants out of six members of the ADGF family, which will allow us to perform further investigations into the roles of individual family members.

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