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Genomic Deletions of the *Drosophila melanogaster Hsp70* Genes

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ABSTRACT

Homologous recombination can produce directed mutations in the genomes of a number of model organisms, including *Drosophila melanogaster*. One of the most useful applications has been to delete target genes to generate null alleles. In Drosophila, specific gene deletions have not yet been produced by this method. To test whether such deletions could be produced by homologous recombination in *D. melanogaster* we set out to delete the *Hsp70* genes. Six nearly identical copies of this gene, encoding the major heat-shock protein in Drosophila, are found at two separate but closely linked loci. This arrangement has thwarted standard genetic approaches to generate an *Hsp70*-null fly, making this an ideal test of gene targeting. In this study, ends-out targeting was used to generate specific deletions of all *Hsp70* genes, including one deletion that spanned ~47 kb. The *Hsp70*-null flies are viable and fertile. The results show that genomic deletions of varied sizes can be readily generated by homologous recombination in Drosophila.

GENE targeting by homologous recombination has only recently been developed as a viable technique for modifying the Drosophila genome. The method depends on the transgenic expression of the FLP recombinase and I-*Sce*I endonuclease in whole flies. These enzymes act on another transgenic element to produce an extrachromosomal donor DNA molecule with a double-strand break (DSB) that stimulates recombination between the donor and the homologous target region. When such recombination occurs in germline cells, the new alleles generated by gene targeting may be recovered by mating. Initially, the feasibility of the method was demonstrated in experiments that converted a mutant allele of the *yellow* gene into *yellow*$^+$ (Rong and Golic 2000). A DSB within the *yellow*$^+$ gene of the donor stimulated recombination that integrated the donor DNA, producing a duplication of the target locus, with one of the copies being wild type. This arrangement of donor is generally referred to as ends-in targeting.

Most applications for gene targeting involve the conversion of a wild-type allele to mutant. Several methods for the production of mutant alleles by gene targeting in Drosophila have been developed and reported (Rong and Golic 2001; Rong *et al.* 2002; Gong and Golic 2003). One shortcoming of these methods has been that part or all of the target locus remained intact, leaving open the possibility that the modified alleles may not be entirely null. In organisms where gene targeting has been in use for many years, homologous recombination is often used to completely delete genes. Typically an ends-out donor arrangement is used, where breaks are provided at the outer ends of a linear DNA molecule. Recombination within flanking homologous regions is used to replace the target gene with a marker gene. The work reported here demonstrates that ends-out gene targeting can be used to generate such target gene deletions in Drosophila.

To test the capability of ends-out targeting (Gong and Golic 2003) to produce gene deletions in Drosophila we chose to delete the *Hsp70* genes. The task of producing classical loss-of-function *Hsp70* mutants has been virtually impossible because of the complex and repetitive organization of the *Hsp70* genes. *Drosophila melanogaster* has six nearly identical genes that encode Hsp70, the major heat-shock protein in Drosophila. They are located at cytological loci 87A and 87C on the right arm of chromosome *3* (Figure 1). The two genes at 87A, *Hsp70Aa* and *Hsp70Ab*, are transcribed in divergent directions and are separated by 1.7 kb, which includes a transposable element, the *S* element. At 87C the proximal *Hsp70Ba* gene copy is transcribed toward the centromere, while the three other genes, *Hsp70Bbb*, *Hsp70Bb*, and *Hsp70Bc*, are transcribed away from the centromere. Making the task more difficult, the three distal genes are separated from *Hsp70Ba* by 38 kb of αβγ-repeats consisting of transposable elements (Figure 1). The αβγ-repeats are transcribed upon heat shock but are not translated, and no function has been associated with them (Leigh-Brown and Ish-Horowicz 1981). These repetitive sequences are also found in centric heterochromatin (Lis *et al.* 1978).

Because of the multiple gene copies and their arrangement in separate but closely linked groups, *Hsp70*-null flies have not been produced. Thus, this seemed like an ideal test of the utility of ends-out targeting to generate mutants that were unattainable by classical

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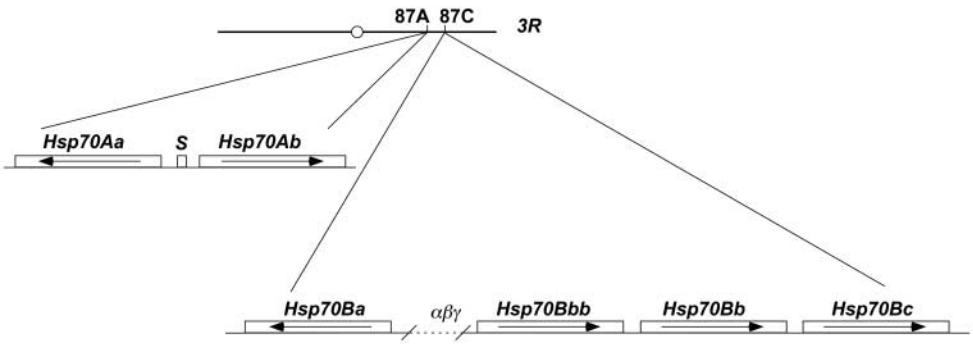

FIGURE 1.—Organization of the *D. melanogaster Hsp70* genes.

means. Prior to the work that we report here, the existing alleles of *Hsp70* were those with genetic modifications affecting only one or two *Hsp70* genes (UDVARDY *et al.* 1982; BETTENCOURT *et al.* 2002; LERMAN *et al.* 2003), large chromosomal deficiencies that deleted many other genes as well (ISH-HOROWICZ *et al.* 1977, 1979; ISH-HOROWICZ and PINCHIN 1980), or transgenic constructs providing copies of the wild-type gene under its own or alternate promoters (SOLOMON *et al.* 1991; FEDER *et al.* 1992; WELTE *et al.* 1993) or expressing an antimorphic form of the protein (SOLOMON *et al.* 1991). Although these alleles have been useful, *Hsp70*-null flies are clearly desirable for a complete genetic analysis. The generation of such flies is a task that seems perfectly suited to the use of homologous recombination for directed modification of the genome.

MATERIALS AND METHODS

Ends-out vector constructs: Two pairs of oligos, 5′-ATAACTTCGTATAGCATACATTATACGAAGTTATCTAGACTAGTCTAGGGTACCGCATG-3′ and 5′-CGGTACCCTAGACTAGTCTAGATAACTTCGTATAATGTATGCTATACGAAGTTATCATG-3′ and 5′-GATCACGTACGGGCGCGCCCTAGACTAGTCTAGATAACTTCGTATAGCATACATTATACGAAGTTAT-3′ and 5′-GATCATAACTTCGTATAATGTATGCTATACGAAGTTATCTAGACTAGTCTAGGGCGCGCCCGTACGT-3′, were annealed and cloned into the *Sph*I and *Bam*HI sites of vector pW35 (GONG and GOLIC 2003), respectively. By DNA sequencing, a plasmid was chosen with the arrangement as *Not*I-*Sph*I-*Acc*65I-Stop-*lox*-w^{hs}-*lox*-Stop-*Asc*I-*Bsi*WI, where Stop represents the six-frame stop codons (New England Biolabs, Beverly, MA; CTAGACTAGTCTAG) and *lox* is the recognition site of Cre recombinase. The two *lox* sites lay in the same direction and generated the vector pW25. The unique feature of pW25 is the *lox* sites, which make it feasible to remove the w^{hs} marker by expression of Cre recombinase (SIEGAL and HARTL 1996).

The *Hsp70A* (*Hsp70Aa* and *Hsp70Ab*) deletion construct: Primers 5′-AGCAGCGGCCGCAACTAGAGCGCCAGCAATCCTCCAT-3′ and 5′-TACCGGTACCATTGGACGAGGCTGACAAAGAACTCC-3′ were used in the PCR with BAC clone BACR48E12 (Berkeley *Drosophila* Genome Project) as template, to add *Not*I and *Acc*65I termini to a 3.7-kb DNA fragment (46647–50367 nucleotides of BACR48E12 sequence), which was then cloned into those sites of pW25. Next, primers 5′-AGCAGGCGCGCCACGAGGCTGACAAGAACTCCGTCTT-3′ and 5′-TACCCGTACGATCAGATATATGCGCACGTCGTCGT-3′ were used to add *Asc*I and *Bsi*WI termini to a 3.6-kb DNA fragment (56217–59857 nucleotides of BACR48E12 sequence) by PCR, and then this fragment was cloned into the corresponding sites downstream of w^{hs}.

The *Hsp70B* deletion construct: Primers 5′- AGCAGCGGCCGCGTGCCACCAAGTTGTCGAGGAAGGA-3′ and 5′- TACCGGTACCTCATGACCAAGATGCATCAGCAGGG-3′ were used to add *Eag*I and *Acc*65I termini to a 5.1-kb DNA fragment (102552–107652 nucleotides of BACR28I14 sequence) by PCR with BACR28I14 (Berkeley *Drosophila* Genome Project) as template, which was then cloned into the *Not*I and *Acc*65I sites of pW25, destroying the *Not*I site. Oligos 5′-CGCGGCGGCCGCGGATCCGG-3′ and 5′-CGCGCCGGATCCGCGGCCGC-3′ were annealed and cloned into the *Asc*I site, and the plasmid with *Asc*I-*Bam*HI-*Not* I-*Bsi*WI was selected. Next, primers 5′-TACCGGCGCGCCGTATAGTTCCGGGGCAGCATTGTCC-3′ and 5′-AGCAGCGGCCGCGCCACTGTTCAAGCGCAAAGATAGC-3′ were used in the PCR to add *Asc*I and *Not*I termini to a 4.25-kb DNA fragment (154363–158608 nucleotides of BACR28I14 sequence) with BACR28I14 as template, and the resulting fragment was cloned into the corresponding sites of the plasmid above.

The *Hsp70Ba* deletion construct: Primers 5′-AGCAGGCGCGCCGCGATAATCTCAACCTTGCCATGCT-3′ and 5′-TACCCGTACGTAGAGTATTCATCTTGCGGCGTGGG-3′ were used to add *Asc*I and *Bsi*WI termini to a 4.3-kb DNA fragment (109380–113666 nucleotides of BACR28I14 sequence) by PCR with BACR28I14 as template, which was then cloned into pW25. Another pair of primers 5′-AGCAGCGGCCGCGCCTTTGCTTGTGTGGAGTGTGTCA-3′ and 5′-TACCGGTACCTCATGACCAAGATGCATCAGCAGGG-3′ were used to add *Not*I and Acc65I termini to a 4.1-kb DNA fragment (103592–107652 nucleotides of BACR28I14 sequence) by PCR and the resulting fragment was cloned into the corresponding sites upstream of w^{hs}.

Crosses and heat shocks: Flies were raised at 25° on standard cornmeal-agar medium. Fly lines bearing the *Cre* transgene were obtained from the Drosophila Stock Center (Bloomington, Indiana). All DNA constructs were transformed into the germline of *D. melanogaster* by standard methods (RUBIN and SPRADLING 1982). Crosses for targeting were carried out using the rapid scheme described by RONG and GOLIC (2001). The w^{hs} marker gene was then mapped to identify cases in which w^{hs} was located on the target chromosome (chromosome *3*). Heat shocks were performed in circulating water baths as described by GOLIC and LINDQUIST (1989).

Southern blotting: Genomic DNA were prepared and digested and then separated by agarose gel electrophoresis and transferred to nylon membranes. Probes were prepared by PCR. Primers 5′-GTCATCGCACGTTCGCCCTCATACA-3′ and 5′-TCGGGGTGGAGTATAAGGGTGAGTC-3′ were used to prime PCR from the template pDM300 (MCGARRY and LINDQUIST 1986) to produce a 1.04-kb *Hsp70* coding sequence probe. DNA downstream of *Hsp70Aa* was amplified by PCR using primers 5′-GACAGAAAAGTGCCGAAAGCGTAGC-3′ and 5′-

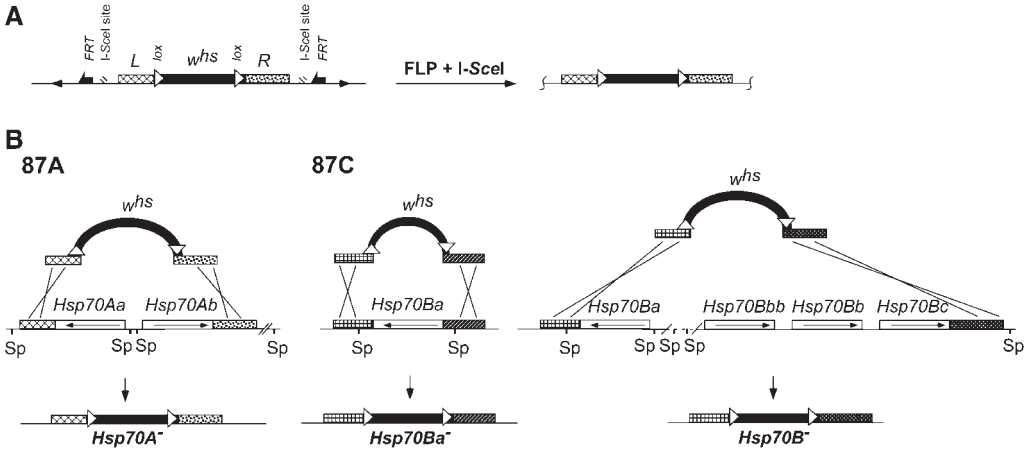

FIGURE 2.—The targeting scheme. (A) Regions flanking the locus to be deleted, indicated as L and R, were cloned into the *P*-element vector, pW25, with the indicated features. FLP and I-*Sce*I generate the extrachromosomal linear donor, as shown. (B) Recombination of the *Hsp70A*, *Hsp70Ba*, and *Hsp 70B* donors should generate the indicated deletions. The approximate locations of *Sph*I (Sp) sites used in subsequent analyses are indicated.

TTCCCGTATTCCCGTATGTCCAACT-3′ with BACR48E12 as template. Primers 5′-GCAAACGAAAAACCCGCCTACAAAT-3′ and 5′-TGTCTCCGTTTTCAGCTCCTTGGTC-3′ were used to amplify DNA downstream of *Hsp70Ba* with BACR28I14 as template. A 1.7-kb w^{hs}-specific band was amplified by PCR using primers 5′-GTCCGCCTTCAGTTGCACTT-3′ and 5′-TCATCGCAGATCAGAAGCGG-3′ with pW25 as template. All the PCR products were labeled with digoxigenin (Dig) using the Genius kit (Roche). Membranes were probed with these Dig-labeled DNA fragments, and hybridization was detected with Dig antibodies and chemiluminescence using the system as described by the manufacturer. In the figures that present the results of Southern blotting, uninformative lanes were deleted, in some cases bringing together lanes that were not immediately adjacent on the gel.

Cytology: Third instar larvae that were homozygous for the unmarked *Hsp70A*$^-$, *Hsp70B*$^-$, or *Hsp70*$^-$ deletion chromosomes or wild type were heat-shocked at 37° for 25 min and allowed to recover for 15 min at room temperature. Salivary gland polytene chromosomes were then prepared as described by LEFEVRE (1976).

RESULTS

Three different donor constructs were built to delete different sets of *Hsp70* genes. In general, a segment of DNA immediately to the left of the target locus and another to the right of the target were cloned into pW25, a modified version of pW35 (GONG and GOLIC 2003) carrying a w^{hs} marker gene flanked by *lox* sites and by stop codons in all six reading frames (Figure 2). After transformation, crosses were used to bring this element together with transgenes that express the FLP site-specific recombinase and I-*Sce*I endonuclease. The action of these enzymes generates a linear extrachromosomal donor molecule that can undergo homologous recombination with the target locus. Targeting events that occur in the germline are recovered by genetic schemes that have been described previously (RONG and GOLIC 2000, 2001; RONG *et al.* 2002). A common method is to use testcrosses that can detect linkage of the w^{hs} marker gene that is part of the donor and to screen for its movement from a chromosome where it was initially inserted by transformation to the target chromosome. In these examples, homologous recombination is expected to replace *Hsp70* genes with a w^{hs} marker gene (Figure 2). In pW25 the w^{hs} marker is also flanked by *lox* sites. These are recognized by the Cre site-specific recombinase, which can be used after targeting to remove the w^{hs} marker, if desired (SIEGAL and HARTL 1996).

***Hsp70* deletions:** The targeting schemes were designed to produce three different deletions, ranging from a 1.7-kb deletion of the *Hsp70Ba* gene to a 46.7-kb deletion of all four *Hsp70B* genes (Figure 2). Putative targeting events were initially recovered by the rapid targeting scheme described previously (RONG and GOLIC 2001) and confirmed by linkage mapping of the w^{hs} marker and by genomic Southern blotting.

Deleting the 87A genes: For the deletion of *Hsp70* genes at 87A, 20 independent targeting events were recovered in 479 vials using four different donors at an average (unweighted) rate of ~1/16 vials (Table 1). In this work we refer to a targeting event as any recombinant between the donor DNA and the target locus, whether or not it was precisely homologous. The molecular analysis shows that the majority of targeted recombinants were not precisely homologous. When digested with *Sph*I, the *Hsp70* coding region produces four *Hsp70*-containing bands in the w^{1118} strain. The 6.3- and 14.7-kb bands are produced by the 87A locus, and the 4.5- and 21.0-kb bands are from the 87C locus. By Southern blotting we found that the *Hsp70Aa* and *Hsp70Ab* genes have been deleted in only 5 of those events (one example is shown in Figure 3A). Twelve recombinants showed only partial loss of the target locus, with nine deletions of *Hsp70Aa* and three deletions of *Hsp70Ab* (Table 2). The Southern blots for these events were missing either the 6.3- or the 14.7-kb band with no other alterations (not shown). Three other recombinants were more complex, showing absence of some bands and new bands of altered size (not shown).

It is clear that these 15 imprecise events are not simply insertions at unrelated sites since the w^{hs} marker had moved from the donor chromosome to the target chromosome and, as judged by Southern blotting, they all

TABLE 1

Recovery of *Hsp70* targeting events

Donor	*N*	Precise	Imprecise	*NT*	Rate
Hsp70A deletion construct (7.36 kb), 5.85-kb deletion					
1A on *2*	273[a]	2	8	1	1/27
3A on *2*	112[a]	3	3	0	1/19
4A on *X*	72[a]	0	1	0	1/72
5A on *2*	22[a]	0	3	0	1/7
Total	479	5	15	1	1/16
Hsp70Ba deletion construct (8.35 kb), 1.73-kb deletion					
3A on *2*	702	7[b]	0	4	1/65[c]
Hsp70B deletion construct (9.35 kb), 46.7-kb deletion					
1A on *2*	2000	7[d]	15	47	1/38[e]

The number of vials with precise, imprecise, and nontargeted events are reported here. Each vial contained four to seven pairs of heat-shocked females and males. The total length donor homology is given in parentheses. *N*, total number of vials scored for targeting; *NT*, number of vials with nontargeted events on chromosome *3*. The approximate targeting rates are shown (per vial).

[a] Only half of the tested females carried the donor, so the number of vials tested has been multiplied by 0.5 for correction.

[b] Six additional events were recovered but were not analyzed by Southern blotting.

[c] The targeting rate was calculated by making the assumption that the rate of targeting events in unexamined flies is the same as in flies examined by Southern blotting. In this case the calculation was $17 \times T/(T + NT)/N$, where T represents the precise plus imprecise targeting events.

[d] Ninety-four more events are yet to be examined.

[e] The targeting rate was calculated as $163 \times T/(T + NT)/N$.

exhibit alterations of the target locus. In most previous targeting experiments imperfect recombination events have been observed (Rong and Golic 2000; Rong *et al.* 2002; Seum *et al.* 2002; Dolezal *et al.* 2003; Egli *et al.* 2003; Lankenau *et al.* 2003; Sears *et al.* 2003). But such events were usually in the minority. In this case the majority of events were imperfect.

A possible explanation for the high rate of imperfect targeting in this case derives from a molecular characterization of the *Hsp70* loci. Genomic DNA from four of the fly strains used in this work was digested with *Sph*I and, after Southern blotting, probed with 1.04 kb of *Hsp70* coding sequence (Figure 4). Each strain shows a different pattern of *Hsp70*-hybridizing bands. In other words, the *Hsp70* region is highly polymorphic. Although the coding sequences of the six *Hsp70* genes are nearly identical, their flanking regions are rich in transposable elements, including the *S* element at 87A and the αβγ-sequences at 87C, and the 5′-and 3′-UTRs of *Hsp70* genes are inclined to diversification (Leigh-Brown and Ish-Horowicz 1981; Bettencourt and Feder 2002). Although isogeny does not appear to strongly influence targeting efficiency in Drosophila (Egli *et al.* 2003), it is possible that imprecise matching between the donor and target tends to produce imprecise recombination events, resembling the parahomologous events in cultured cells described by Cherbas and Cherbas (1997). We cannot exclude the possibility that the 5.85-kb gap, the segment to be deleted, hinders the ability of the two donor DNA segments to simultaneously pair with the matching regions of the target. One end of the donor may recombine by homology with the remaining end undergoing nonhomologous or illegitimate recombination.

Deleting the 87C genes: Two different deletions were generated for the *Hsp70* genes at 87C. In the first case, the *Hsp70Ba* gene was deleted. Seven targeted recombinants were recovered and confirmed to be deletions (one is shown in Figure 3B). For this deletion the homologous segment on the right side contained αβγ-repeats, which are also present in the chromocenter (Lis *et al.* 1978). This result demonstrates the feasibility of targeting genes that are flanked by repetitive DNA.

In the second case, all four *Hsp70* genes at 87C, contained in a 46.7-kb span of genomic DNA, were deleted. To our knowledge, this is the largest deletion produced by gene targeting in Drosophila to date and is comparable to genomic deletions that have been produced in mouse targeting (Mombaerts *et al.* 1991; Zhang *et al.* 1994; Tsuda *et al.* 1997; Muller 1999). As was the case with targeting *Hsp70A*, most events were not precise, with 7 of the 22 target locus recombinants having the desired deletion (one example shown in Figure 3C). The remaining 15 have only partial deletions of the *Hsp70B* locus: 8 were missing the *Hsp70Ba* only, and 7 were missing the *Hsp70Bbb, b, c* gene cluster (not shown).

In addition, unlike the previous two targeting experiments, the majority of putative events were actually not targeted as judged by the lack of alteration in the *SphI* bands from the 87C locus. This may be related to the large size of the chromosomal DNA to be deleted, to polymorphism at this locus, or to specific effects of the donor DNA. Because only 22 of the 69 events that were examined were actually recombinants with the target locus, and only 7 of those were the desired deletions, this result may indicate that it is necessary to collect more than the usual number of targeting events to produce large deletions.

In all the targeting experiments described above, there were a number of events in which the w^{hs} marker of the donor had not moved to another chromosome, but had lost at least one of its flanking *FRT*s (12 with the *Hsp70A* donors, 33 with the *Hsp70Ba* donor, and 50 with the *Hsp70B* donor). These were identified because the rapid screening method that we employed uses a lack of FLP-induced mosaicism as a first-round screen for targeting. The most likely explanation for these events is that an I-*Sce*I cut was followed by degradation of the broken ends, leading to loss of an *FRT*, and then the broken ends were repaired by nonhomologous end

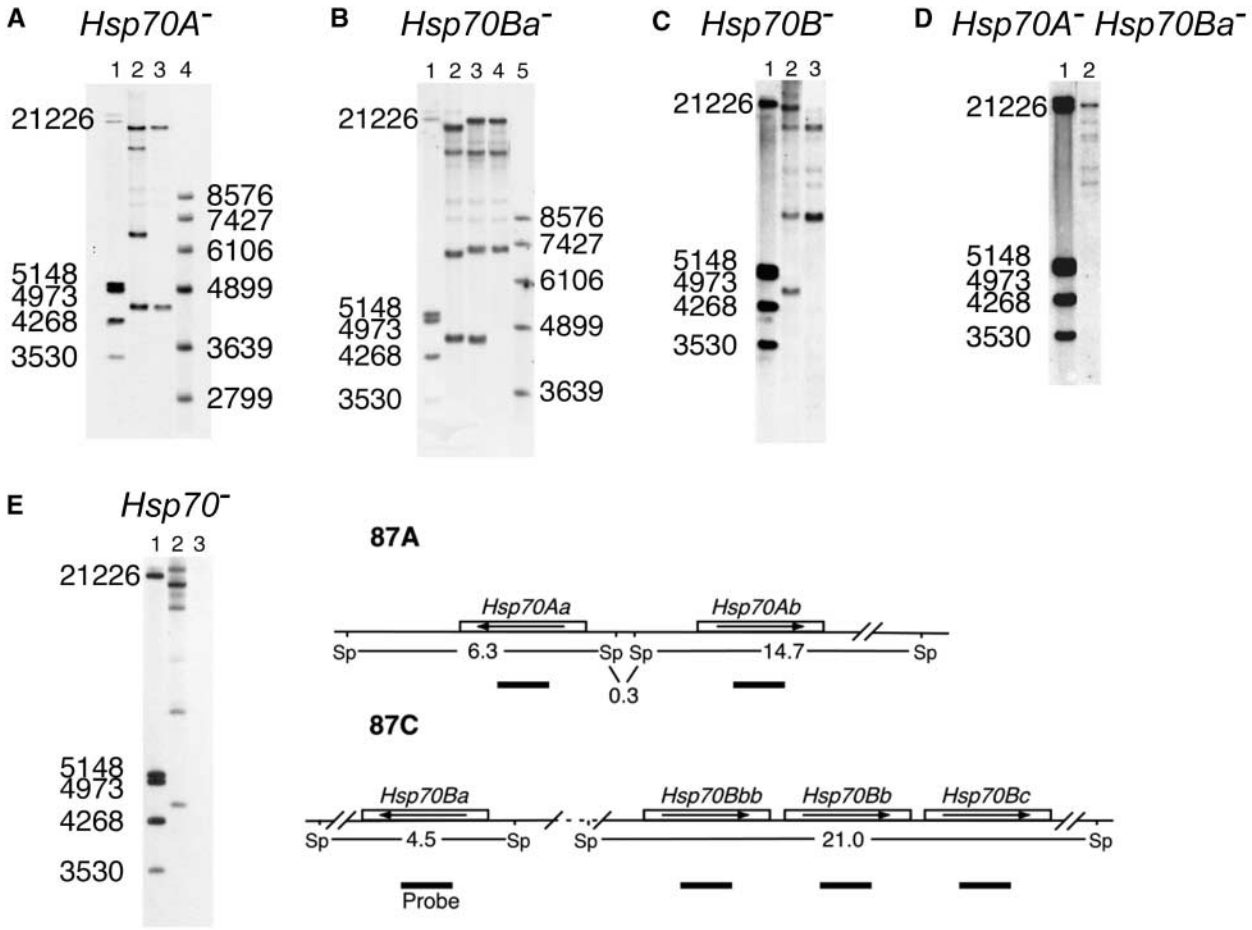

FIGURE 3.—Molecular verification of *Hsp70* mutants. The structures of the *Hsp70* genes are indicated at the bottom right. Genomic DNAs were digested with *Sph*I (Sp). The *Hsp70* coding region that was used as a probe is indicated as the solid line below each diagram. The expected structures of *Hsp70* loci are derived from *D. melanogaster* genome sequence (http://www.fruitfly.org), with the sizes (in kilobases) indicated beneath each fragment. In each case, lane 1 contains molecular weight markers. Additional molecular weight markers are shown in A, lane 4 and in B, lane 5. Sizes are indicated next to the bands. (A) Lane 2, w^{1118}; lane 3, w^{1118}; *Hsp70A*$^{-}$. (B) Lane 2, w^{1118}; lane 3, w^{1118}; *Sco/S*^{2}*CyO*; lane 4, w^{1118}; *Hsp70Ba*$^{-}$. (C) Lane 2, w^{1118}; lane 3, w^{1118}; *Hsp70B*$^{-}$. (D) Lane 2, w^{1118}; *Hsp70A*$^{-}$ *Hsp70Ba*$^{-}$. (E) Lane 2, w^{1118}; lane 3, w^{1118}; *Hsp70*$^{-}$. The *Hsp70* banding patterns from the w^{1118} and w^{1118}; *Sco/S*^{2}*CyO* strains are polymorphic (see Figure 4).

joining. This points out the utility of mapping potential targeting events prior to carrying out more tedious Southern blotting.

Further examination of the deletions: Because many of the targeting events did not produce the deletions they were designed to generate, we undertook a further examination of the deletion alleles by Southern blotting of *Sph*I-digested genomic DNA (partial results shown in Figure 5; the approximate locations of the *Sph*I sites relative to donor DNA segements are also indicated in Figure 2). This was to ensure that future experiments used only alleles that removed the *Hsp70* genes and no others. Five *Hsp70A* deletions were examined by probing with a segment of DNA adjacent to the genes. All five showed the 20-kb *Sph*I band that is expected to result from replacing those *Hsp70* genes and the central two *Sph*I sites with the w^{hs} marker. For the *Hsp70Ba* deletion, two deletion alleles were examined by probing with adjacent sequence and both showed the 7.5-kb *Sph*I band that is expected to result from the replacement of *Hsp70Ba* with w^{hs}. These most likely resulted from precise recombination. Two other *Sph*I sites are within the αβγ-repeats, so it is possible that some imprecise events could have generated an *Sph*I band of this size, but the likelihood is small. For the *Hsp70B* deletion one allele was examined by probing with w^{hs} sequence, and it showed the 11-kb band that should be produced by replacing all four *Hsp70B* genes with w^{hs}, bringing the flanking *Sph*I sites closer. In sum, eight alleles that had deleted the expected number of *Hsp70* genes were examined, and in all cases the recombination events appeared to be precise.

Heat-shock puffs: Because the polymorphic nature of the *Hsp70* region may slightly complicate the interpretation of the Southern blots, we examined the pattern of polytene chromosome puffing after heat shock to

TABLE 2

Summary of *Hsp70A* and *Hsp70B* targeting

	Partial deletions		
Hsp70A targeting donors	*Hsp70Aa* deletion	*Hsp70Ab* deletion	Complete deletion
1A[a]	3	3	2
3A	3	0	3
4A	1	0	0
5A[b]	2	0	0
Hsp70B targeting donor	*Hsp70Ba* deletion	*Hsp70Bbb, Hsp70Bb, Hsp70Bc* deletion	Complete deletion
1A	8	7	7

[a] Two additional events were more complex.
[b] One additional event was more complex.

further confirm that *Hsp70* deletions had been produced. Two heat-shock puffs are formed by the *Hsp70* loci, representing the transcriptional activation of the genes at 87A and 87C. It was first necessary to remove the w^{hs} marker because its expression is driven by the *Hsp70* promoter, and it can also produce a puff. The Cre recombinase was used to remove w^{hs} by causing recombination between the flanking *lox* sites (Siegal and Hartl 1996). Three or four Cre-mediated excision lines were produced for each selected *Hsp70* deletion line and were examined cytologically. After heat-shock treatment at 37° for 25 min followed by a 15-min recovery, two heat-shock puffs were observed at 87A and 87C in the $Hsp70^{+}$ line. In contrast, the heat-shock puff at 87A was absent in $Hsp70A^{-}$ homozygotes, and the puff at 87C was absent in $Hsp70B^{-}$ homozygotes (Figure 6), confirming the Southern blotting results.

Strategy to generate multiple deletions on the same chromosome: Flies that carry any one of the targeted deletions have light orange eyes, a common phenotype resulting from a lower than wild-type expression of the hypomorphic w^{hs} marker gene. This made it feasible to generate chromosomes that contain deletions at both 87A and 87C by meiotic recombination. When both deletions and their associated marker genes are present on the same chromosome the eye color is expected to be darker than that of either deletion alone. The procedure was performed in females also carrying a heterozygous chromosome *2* balancer, to increase the rate of recombination via the interchromosomal effect (Ashburner 1989). Multiple isolates for both recombinants, $Hsp70A^{-}$ $Hsp70Ba^{-}$ and $Hsp70A^{-}$ $Hsp70B^{-}$ ($Hsp70^{-}$), were recovered. Southern blotting of a number of selected recombinants (20 for $Hsp70A^{-}$ $Hsp70Ba^{-}$ and 10 for $Hsp70A^{-}$ $Hsp70B^{-}$) showed that all had the expected deletions (examples are given in Figure 3, D and E). The *Hsp70*-null genotype was further confirmed by examining heat-shocked larvae for the HS puffs at 87A and 87C after removal of the w^{hs} markers. The *Hsp70*-null flies lacked puffs at both sites (Figure 6).

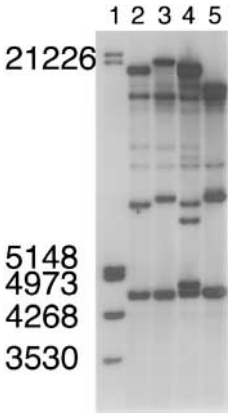

Figure 4.—Polymorphism of the *Hsp70* region in different fly strains. Genomic DNAs were digested with *Sph*I and probed with 1.04 kb of *Hsp70* coding sequence. Lane 1, molecular weight marker; lane 2, w^{1118}; lane 3, w^{1118}; Sco/S^{2}*CyO*; lane 4, w^{1118}; *70FLP10*; *Sb/TM6*; lane 5, w^{1118}; *70FLP10*.

DISCUSSION

The successful deletion of all six *Hsp70* genes in this study demonstrates that ends-out gene targeting is useful for producing specific gene deletions in Drosophila. This was probably not the easiest example that could be chosen for a simple demonstration of ends-out gene deletions. The occurrence of repeated sequences within and about these gene clusters may contribute to a reduced efficiency or fidelity of targeting. In addition, one of the deletions we desired was in excess of 46 kb. Most single-gene deletions are likely to be much smaller than this. The successful production of each of the desired deletions is a strong indicator that this method will be widely applicable.

The efficiency of recombination between the donor and the target in this work was approximately similar to that for ends-in targeting (Rong *et al.* 2002). The

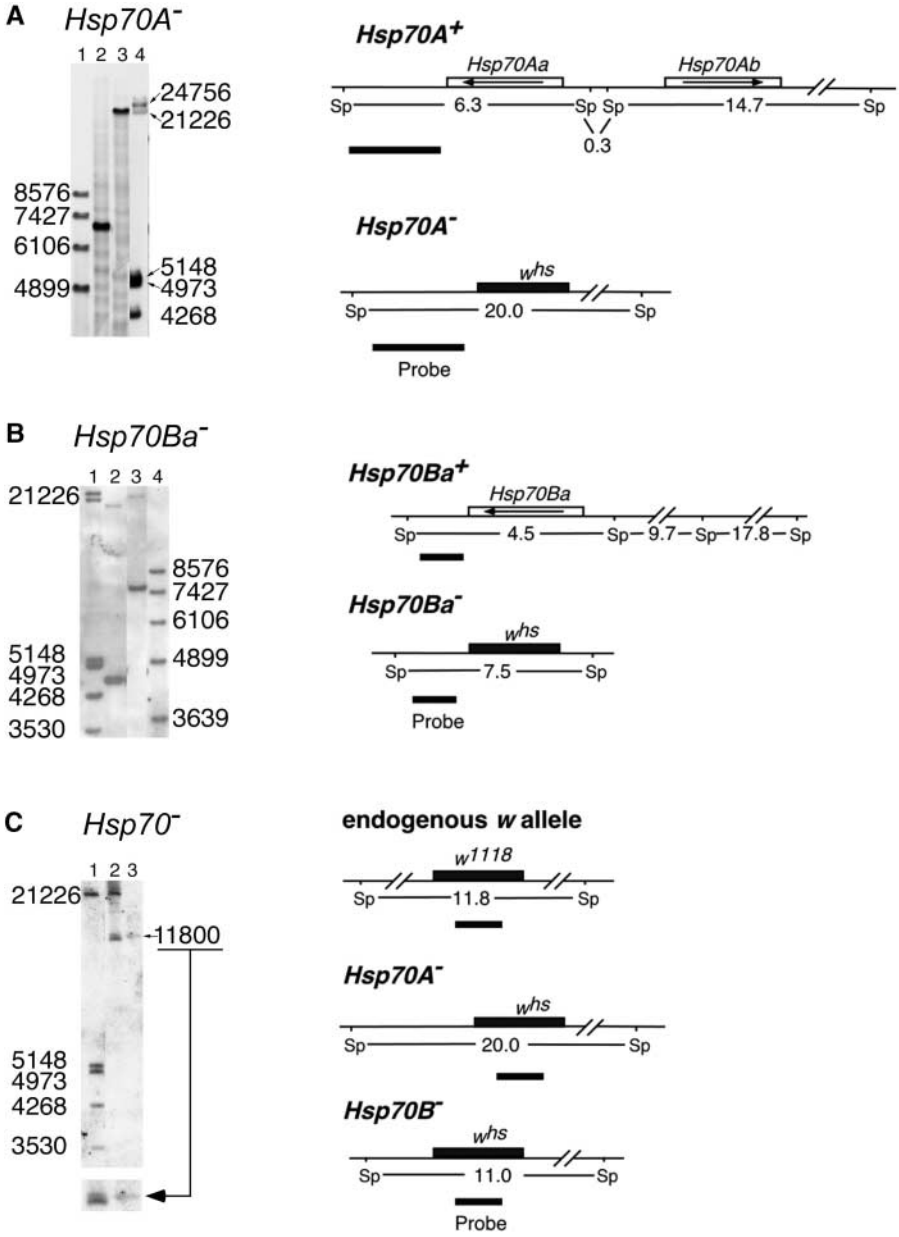

FIGURE 5.—Molecular verification of the structure of *Hsp70* deletions. All genomic DNA was digested by *Sph*I (Sp). The region used as a probe is indicated as the solid line below each diagram. The expected structures of *Hsp70* loci and deletions are derived from the *D. melanogaster* genome sequence and the targeting design, with the sizes (in kilobases) of *Sph*I fragments indicated below. Lanes 1 and 4 represent molecular weight markers. (A) Lane 2, w^{1118}; lane 3, w^{1118}; *Hsp70A*$^{-}$. (B) Lane 2, w^{1118}; lane 3, w^{1118}; *Hsp70Ba*$^{-}$. (C) Lane 2, w^{1118}; *Hsp70*$^{-}$; lane 3, w^{1118}. The bands at ~11 kb from lanes 2 and 3 are enlarged beneath the gel for clarity.

targeting was most efficient at *Hsp70A*, but varied considerably between different insertions of the donor, a common observation for targeting in Drosophila. The much larger deletion of the *Hsp70B* cluster was produced at a frequency that was within the range of frequencies found with the different *Hsp70A* donors, and the difference in these experiments was not significant ($P = 0.09$; 2×2 contingency test of summed data for *Hsp70A vs. Hsp70B*). This is an encouraging finding for investigators desiring to produce large deletions. Similar results have been reported in mouse gene targeting: when different lengths of deletions, ranging from 1.7 to 19.2 kb, were generated at the mouse hypoxanthine phosphoribosyltransferase (*hprt*) gene, the targeting events were obtained at similar rates (ZHANG *et al.* 1994).

It is worth considering whether the targeting efficiency achieved in this work owes anything to the nature of the target locus. The targeting protocol uses a heat shock to induce the expression of FLP and I-*Sce*I. This heat shock also strongly induces the expression of the *Hsp70* genes that are to be deleted. Many studies have made a connection between transcription in a region and the frequency of recombination observed in that region. For instance, KIRKPATRICK *et al.* (1999) found that the meiotic recombination hotspot upstream of the yeast *HIS4* gene depends on the binding of a functional transcriptional activator to that site. Additionally, among the meiotic hotspots that require transcription factor binding, a majority lie between two genes that are transcribed divergently (GERTON *et al.* 2000), similar to the arrangement of the *Hsp70* genes at 87A. Early results with mouse gene targeting suggested that genes that are not transcribed (in ES cells) are targeted with reduced efficiency (MANSOUR *et al.* 1988) and that transcriptional

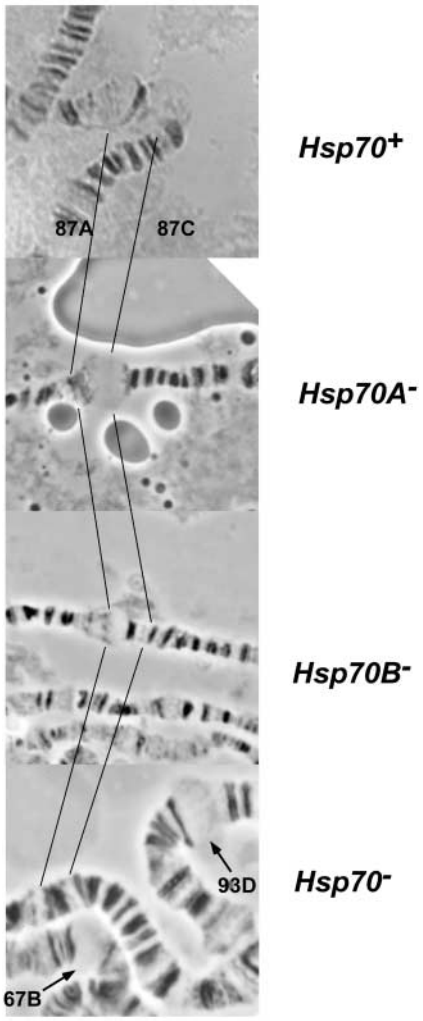

Figure 6.—Cytological verification of *Hsp70* deletions. Salivary gland polytene chromosomes were prepared after heat shock from the homozygous strains indicated to the right. The hairlines indicate the 87A and 87C loci. In the *Hsp70*-null strain (bottom), note that other heat-shock puffs are visible, even though the *Hsp70* puffs are absent.

induction of a target gene can increase the frequency at which it undergoes homologous recombination (Nickoloff and Reynolds 1990; Nickoloff 1992). This increased rate of recombination also applies specifically in the context of gene targeting (Thyagarajan *et al.* 1995). However, the correlation of transcription and high recombination is not universal. Some meiotic hotspots in yeast are not associated with a transcription factor requirement (Kirkpatrick *et al.* 1999; Gerton *et al.* 2000) and transcription does not always lead to higher rates of recombination (Taghian and Nickoloff 1997; Yanez and Porter 2002).

The nature of the transcriptional effect is not well understood. Transcription through a target sequence stimulates homologous recombination, but transcription that begins near the target locus and proceeds away from it does not (Thyagarajan *et al.* 1995). However, as mentioned, transcription that proceeds bidirectionally away from a site is a characteristic of many meiotic hotspots in yeast. It is not possible to know, at this time, whether *Hsp70* targeting was influenced by their high rates of transcription, because the only constructs available for FLP and I-*Sce*I expression use the *Hsp70* promoter. However, we note that we have also targeted the *yellow* gene with an ends-out design (Gong and Golic 2003), and targeting was approximately as efficient as ends-in targeting of the same gene (Rong and Golic 2000). Ends-in targeting has been used for a number of other genes, none of which are induced by heat shock (Rong and Golic 2001; Rong *et al.* 2002; Seum *et al.* 2002; Dolezal *et al.* 2003; Egli *et al.* 2003; Elmore *et al.* 2003; Gao *et al.* 2003; Greenberg *et al.* 2003; Lankenau *et al.* 2003; Liu and Kubli 2003; Sears *et al.* 2003; Watnick *et al.* 2003; Hirosawa-Takamori *et al.* 2004). We have also used ends-out targeting to delete other genes that are not induced by heat shock (H. Xie, R. S. Hawley and K. G. Golic, unpublished results). Although the induced transcription of the target regions might have some influence on recombination, neither ends-out nor ends-in targeting is limited to such highly transcribed genes.

One notable difference between the work reported here and previous work is that most recombinants between the donor and target did not have the expected structure. Instead, they often appeared to have arisen by homologous recombination at one end of the donor combined with nonhomologous or illegitimate recombination at the other end. Such events often generated only partial deletions of the target locus and could provide additional variants for mutational studies. The results obtained for the *Hsp70A* are especially interesting: 12 of the 15 imprecise events appeared to delete one or the other *Hsp70* gene copy in its entirety, leaving the other copy at this site intact. To obtain this result, the illegitimate exchanges that integrated the donor must have occurred in the short region between the *Hsp70* genes, either between the two *Sph*I sites or possibly next to them. Perhaps, as in yeast, the region between two divergently transcribed genes can be a recombination hotspot, although in this case it would be a hotspot for nonhomologous recombination.

Targeting events with one homologous and one nonhomologous exchange are not unique to Drosophila. For instance, homologous/nonhomologous targeting events were recovered between two plasmids in mammalian cells (Sakagami *et al.* 1994). Berinstein *et al.* (1992) also succeeded in gene replacement with a donor vector containing one-sided homology. And, in work to generate a 15-kb deletion at the T-cell antigen receptor β-subunit, some targeted cells contained the expected structure in the 3′ flanking region but not at the 5′ end (Mombaerts *et al.* 1991).

We previously showed that ends-out targeting could generate insertional disruptions (Gong and Golic

2003). In this work we show that deletions can be similarly generated. In either case, the w^{hs} marker gene is placed at the target locus. However, the pW25 vector we used in this work has *lox* sites that flank the marker gene. This allows the gene to be removed in cases where it might interfere with subsequent analysis, as in the analysis of heat-shock puffs reported here. It might also allow for multiple rounds of targeting to the same chromosome using the same marker. This feature could be useful to make several modifications to sites that are too close to be easily recombined onto the same chromosome. In the pW25 vector we also added stop codons in all reading frames outside the *lox* sites, to help ensure that insertional disruption alleles generated with this vector are still mutant after marker removal. Such insertions would, of course, need to be within the gene's coding sequence. It should also be noted that some added sequence does remain at the target site, even after marker gene removal. If a completely clean deletion is desired, with no added sequence remaining at the target site, the ends-in deletion method reported in the accompanying article (Xie and Golic 2004, this issue) should be chosen.

In the course of generating and maintaining the *Hsp70* deletion strains we found that flies with no copies of the *Hsp70* genes are viable and fertile. A detailed characterization of their other phenotypes will be presented elsewhere.

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