Neighboring genes for DNA-binding proteins rescue male sterility in *Drosophila* hybrids

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Crosses between closely related animal species often result in male hybrids that are sterile, and the molecular and functional basis of genetic factors for hybrid male sterility is of great interest. Here, we report a molecular and functional analysis of *HMS1*, a region of 9.2 kb in chromosome 3 of *Drosophila mauritiana*, which results in virtually complete hybrid male sterility when homozygous in the genetic background of sibling species *Drosophila simulans*. The *HMS1* region contains two strong candidate genes for the genetic incompatibility, *agt* and *Taf1*. Both encode unrelated DNA-binding proteins, *agt* for an alkyl-cysteine-*S*-alkyltransferase and *Taf1* for a subunit of transcription factor TFIID that serves as a multifunctional transcriptional regulator. The contribution of each gene to hybrid male sterility was assessed by means of germ-line transformation, with constructs containing complete *agt* and *Taf1* genomic sequences as well as various chimeric constructs. Both *agt* and *Taf1* contribute about equally to *HMS1* hybrid male sterility. Transgenes containing either locus rescue sterility in about one-half of the males, and among fertile males the number of offspring is in the normal range. This finding suggests compensatory proliferation of the rescued, non-dysfunctional germ cells. Results with chimeric transgenes imply that the hybrid incompatibilities result from interactions among nucleotide differences residing along both *agt* and *Taf1*. Our results challenge a number of preliminary generalizations about the molecular and functional basis of hybrid male sterility, and strongly reinforce the role of DNA-binding proteins as a class of genes contributing to the maintenance of postzygotic reproductive isolation.

postzygotic reproductive isolation | hybrid male sterility | gene conflict | transcription factor

Sterility, lethality, or other abnormalities observed among the offspring of crosses between species are known as hybrid incompatibilities. Usually ascribed to the dysfunction of parental coadapted gene complexes, hybrid incompatibilities have intrigued geneticists since almost the beginning of modern genetics (1). Hybrid incompatibilities are important evolutionarily because they act as reproductive barriers that can both promote speciation in sympatric populations and maintain the integrity of the species following allopatric divergence. They are also paradoxical because they cannot usually arise as a direct result of natural selection in the diverging parental lineages; rather, they are incidentally acquired in the ordinary course of evolutionary divergence and only manifest in the unique hybrid genomic background (2–4).

In the century since hybrid incompatibilities were first called out as an intriguing and important issue in genetics and evolutionary biology, the identification of the causal genes and their molecular mechanisms has been hampered by hybrid incompatibility itself, because any sterile or lethal hybrid individual constitutes a virtual dead end for genetic analysis. Additionally, even when only one sex is sterile or lethal, the number of genes contributing to hybrid incompatibility is usually large and their interactions complex (5–8). There is also the inherent difficulty of distinguishing when during the course of speciation incompatibilities may have arisen, especially because postspeciation incompatibilities may accumulate exponentially in time (9).

Recent reviews detail virtually all analyzed cases of hybrid incompatibility in a variety of organisms (flowering plants, yeast, copepods, fruit flies, fish, and mouse), which altogether still contribute fewer than two dozen genetic factors (3, 10). Considerable discussion has focused on whether particular classes of genes are overrepresented in this limited number of examples, with a high proportion of genes involved in internal genomic conflicts, especially those involving genes encoding proteins that bind to DNA or chromatin (3, 10). Among the genetic factors identified to date in *Drosophila*, the latter category includes the homeobox-containing gene *OdsH* (11–13), two heterochromatin proteins *Lhr* (14) and *Hmr* (15), and the coding gene *Ovd* (16).

Most empirical evidence about hybrid incompatibility comes from *Drosophila* owing to the special advantages and resources available for genetic analysis in this organism (5, 17). Among the well-studied drosophilid species are *Drosophila simulans* and its island-endemic sibling species *Drosophila mauritiana*, which diverged ~250,000 y ago (18). Among the hybrids, males are sterile but females are fertile, in accordance with Haldane's rule that the heterogametic sex manifests hybrid incompatibilities sooner than the homogametic sex (9, 19, 20), and makes genetic studies by backcrossing feasible. Short genomic regions introgressed from *D. mauritiana* in an otherwise isogenic *D. simulans* genetic background identify numerous regions associated with hybrid incompatibilities (21–24). Many of these

Significance

Hybrid sterility is a frequent outcome of crosses between closely related plant and animal species because of incompatibilities that have evolved in the parental genomes. Here, we show that a small region associated with hybrid male sterility between two closely related species of *Drosophila* contains two genes, both encoding DNA-binding proteins, each of which contributes to the hybrid male sterility. These results emphasize that hybrid incompatibility between well-established species is the result of numerous genetic factors, each contributing quantitatively to the incompatibility. Among these factors, DNA-binding proteins are disproportionately represented. Each incompatibility is complex, resulting from interactions between nucleotide sites in different regions of the gene, and is likely to have evolved long after the initial establishment of reproductive isolation.

regions show complex nonadditive epistatic interactions modulating male fertility (25). Although genomic conflicts over sex chromosome transmission contribute significantly to the evolution of reproductive isolation (23, 24, 26–28), the autosomes alone contain ~40 genetic regions that contribute significantly to hybrid male sterility (29).

Most of the hybrid incompatibility regions are relatively large and may contain more than one contributing genetic factor. Here, we report the genetic analysis of a 9.2-kb region in chromosome 3 of *D. mauritiana*, denoted hybrid male sterility one (*HMS1*), which is associated with hybrid male sterility when introgressed into the genome of *D. simulans* (30). By means of germ-line transformation with a number of constructs of sequences within or near *HMS1*, we show that *HMS1* contains two neighboring genes for unrelated DNA-binding proteins, each of which contributes quantitatively to hybrid male sterility. One gene, *Taf1*, encodes a component of transcription factor TFIID with a testes-enriched alternatively spliced isoform. The other gene, *agt*, encodes a DNA alkyltransferase. In both cases, the incompatibility is not due to a single-nucleotide replacement in the gene but to interactions among multiple sites along the gene. These results emphasize the quantitative, multifactorial basis of hybrid male sterility and strongly support DNA-binding proteins as a prominent class of genes contributing to genetic conflicts resulting in reproductive isolation.

Results

Identification of *HMS1*. *HMS1* refers to a region in the right arm of chromosome 3 of *Drosophila mauritiana* that is associated with hybrid male sterility between *D. mauritiana* and its sibling species *D. simulans*. When the *HMS1* region from *D. mauritiana* (*HMS1[mau]*) is homozygous in an otherwise *D. simulans* genetic background, the males show a dramatic reduction in fertility. *HMS1* is one of about 20 HMS factors in chromosome 3 originally mapped as quantitative-trait loci affecting male fertility (29). Most HMS factors have relatively small effects so that generally at least two or three different factors are required to produce complete sterility (29). In contrast, males homozygous for *HMS1* alone are usually quasisterile (29), although the effect of *HMS1* is strongly dependent on genetic background (30).

Originally localized to a region of 1.26 Mb between the genes *Regena* (*Rga*) and *Antennapedia* (*Antp*) in chromosome 3R within the overlap between *P*-element introgressions *P32.110* and *P45.6* (Fig. 1*A*) (29), the *HMS1* interval was significantly narrowed by four successive rounds of recombination to a region of 9.2 kb (Fig. 1*B*) corresponding to band 84A1 in the polytene salivary-gland chromosomes (30). Within this region are four genes, two of which exhibit significant coding and noncoding differences between the species, making them candidate genes for hybrid male sterility (30). The candidate genes are *Taf1* (CG17603) and *agt* (CG1303) (Fig. 1*B*), both of which encode DNA-binding proteins The genetic mapping and initial analysis left it unclear, however, which one of these genes, or possibly both, contributed to the male-sterility phenotype.

HMS1 male sterility is manifested in a *D. simulans* genetic background (strain SimB) into which the *HMS1* region of *D. mauritiana* (strain Mau12) has been introgressed. For simplicity, we will refer to the genotype of the SimB introgression genotype as *HMS1[mau]/HMS1[mau]* (see refs. 29 and 30 for details of the SimB and Mau12 strains and the introgression methods). Homozygous *HMS1[mau]/HMS1[mau]* males are almost completely sterile: 97.9% of tested males are sterile ($n = 109$), and among those with progeny the average number is 1.5 ± 0.71 (Fig. 1*C* and Table S1). The sterility is restored in heterozygous *HMS1[mau]/HMS1[sim]* males, among which all are fertile ($n = 183$) with an average offspring number of 82.6 ± 52.1 (Fig. 1*C* and Table S1).

To ascertain the genetic basis of *HMS1* sterility, we carried out germ-line transformation using various constructs inserted into the *piggyBac* vector (31). The strategy was to create *HMS1[mau]/HMS1[mau]* male-sterile genotypes carrying an extra, transgenic, *piggyBac* copy of all or part of the *HMS1* region from SimB (*HMS1[sim]*) to look for evidence of complementation, which would be manifested as partial or complete recovery of male fertility. One problem with this strategy is that *HMS1[mau]/HMS1[mau]* is male sterile and so cannot be transformed directly. Instead, we transformed SimB with *piggyBac* constructs containing all or part of *HMS1[sim]* (symbolized *pB::HMS1[sim]*) (Fig. S1) to generate transgenic *HMS1[sim]/HMS1[sim]; pB::HMS1[sim]* animals. In each case, the location
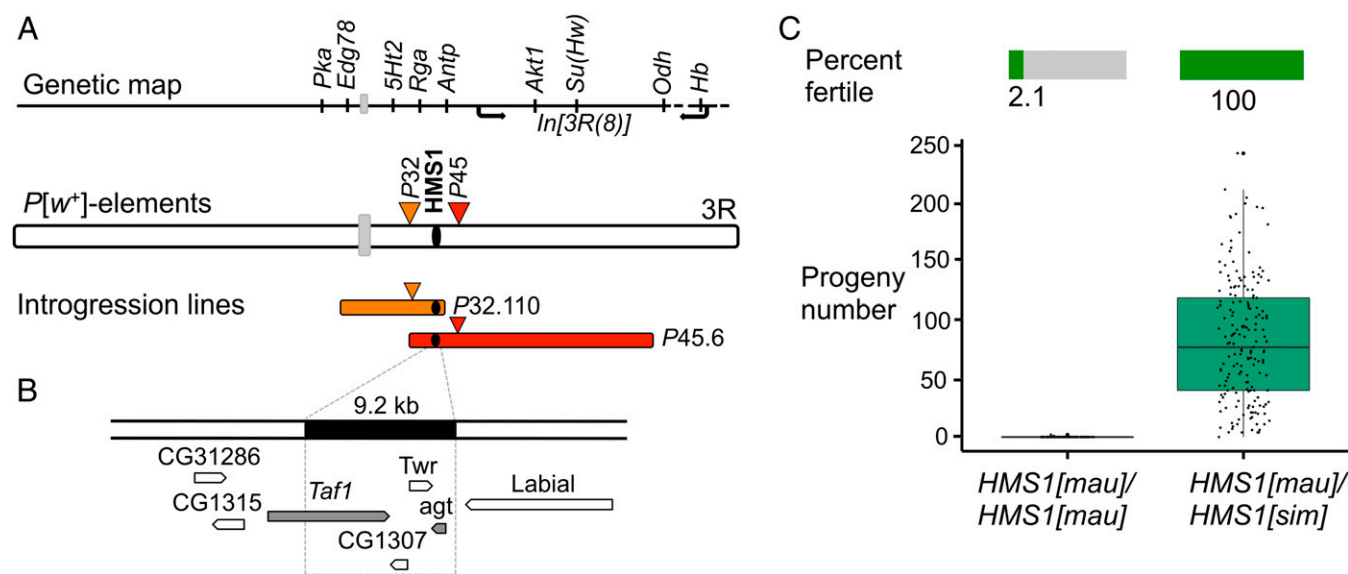

Fig. 1. *HMS1* structure and phenotype. (*A*) Genetic map of *HMS1* region showing location of *P32* and *P45* *P*-element insertions as well as the extent of *D. mauritiana* Mau12 genome present in the *P32.110* and *P45.6* introgressions obtained by repeated backcrossing. *P*-elements *P45* and *P32* localize to polytene bands 83A and 84E8, respectively (76). (*B*) Expanded map of *HMS1* region showing the genes in and near the region. (*C*) The phenotype of the *HMS1[mau]/HMS1[mau]* homozygous males showing that the great majority of males are completely sterile, whereas only a few are quasisterile; the phenotype of the *HMS1[mau]/HMS1[sim]* heterozygous males is complete fertility rescue with high numbers of progeny among most of the fertile males.

of the transgene in the genome was determined by the inverse PCR (32). The *pB-HMS1[sim]* transgene was then transmitted among genotypes by a stepwise crossing design to create the required *HMS1[mau]/HMS1[mau]; pB::HMS1[sim]* genotypes to assay for fertility (Fig. S2). The genetic markers for the *HMS1[mau]* introgression and also the *pB::HMS1[sim]* constructs all affect eye color; however, the copy number effects on phenotype usually allow the genotypes to be distinguished (Fig. S3). All assayed males, both fertile and sterile, were unambiguously genotyped postmating by means of PCR, and in the case of fertile males the reliability of the molecular genotyping was further verified by observing segregation of the eye color phenotypes among the progeny.

Both *agt* and *Taf1* Rescue *HMS1* Sterility, But Neither Wholly. Among the *piggyBac* constructs created to dissect the genetic basis of *HMS1* were *agt[sim]* and *Taf1[sim]* (Fig. S4). The former includes the entirety of the *agt* gene, and the latter, the entirety of *Taf1*, both derived from the SimB strain of *D. simulans*. We tested three independent insertions of *pB::agt[sim]* and two of *pB::Taf1[sim]* in a genetic background of *HMS1[mau]/HMS1[mau]* to detect possible position effects of the insertions. Although introgressed *HMS1[mau]/HMS1[mau]* males are virtually all sterile (Fig. 1C), all three *pB::agt[sim]* and both *pB::Taf1[sim]* transgenes restore fertility to some extent (Fig. 2). Among transgenic males that are fertile, the level of fertility is high and not significantly different overall among all *pB::agt[sim]* and *pB::Taf1[sim]* transformants (t test on progeny number: $t = 0.369$, df = 152, $P = 0.713$). The fertility rescue is only partial, however, as indicated by the relatively high proportions of sterile males among all transgenic lines (Fig. 2). The percentage of sterile males averages $42 \pm 4\%$ among *pB::agt[sim]* transformants and $54 \pm 5\%$ among *pB::Taf1[sim]* transformants (Fig. 2). For *agt[sim]*, the number of progeny does not differ significantly for the three genomic transgene insertions (one-way ANOVA: $F = 2.65$, df = 2, $P = 0.075$) and the fractions fertile are also homogeneous [$P = 0.172$ ($X^2 = 3.523$, df = 2, $n = 185$)]. For *Taf1[sim]*, the number of progeny differs somewhat from one insertion site to the next (t test on progeny number: $t = -3.030$, df = 43, $P = 0.004$) as

indicated by the asterisk in Fig. 2, but importantly the fraction fertile remains homogenous [χ^2 test on number fertile: $P = 0.063$ ($X^2 = 3.453$, df = 1, $n = 94$)], which attests to the limited impact of positional effects at the sites of insertion studied. From the standpoint of rescuing complete sterility, *agt[sim]* is thus not significantly different from *Taf1[sim]* [$P = 0.079$ ($X^2 = 3.076$, df = 1, $n = 279$)]. In both cases, the partial rescue is specific to the *agt[sim]* or *Taf1[sim]* transgene, because control males carrying an empty *piggyBac* vector with no insert fail completely to rescue *HMS1* sterility (Table S2).

To further investigate the mechanism by which fertility is restored in the transgenic lines, we assayed whether decreased expression levels would account for the sterility at either candidate locus. We first quantified mRNA levels in male reproductive tissue of both *agt* and *Taf1* loci in fertile (*HMS1[mau]/HMS1[sim]*) and sterile (*HMS1[mau]/HMS1[mau]*) introgression males, which are isogenic except for a small region including *HMS1* (*SI Materials and Methods* and Fig. S5A). These males yield baseline levels of *agt* and *Taf1* expression for males bearing either one or two *sim* alleles of each gene. In each of two genetic backgrounds, SimB and w501, our quantitative PCR results indicate no significant difference in mRNA level of either *Taf1* or *agt* between fertile (*HMS1[mau]/HMS1[sim]*) and sterile (*HMS1[mau]/HMS1[mau]*) males (Fig. S5B and Table S3). This finding supports the inference that the hybrid male sterility is not mediated by differences in gene expression of either *agt* or *Taf1*.

Impact of a Candidate Amino Acid Replacement in *agt*. A previous analysis had suggested that an amino acid replacement in *agt* might contribute to hybrid male sterility (30). In *D. simulans*, amino acid position 121 is occupied by aspartic acid, whereas in *D. mauritiana* position 121 is an asparagine. This D121N difference is the only fixed amino acid difference then known between the species (30), hence making it a strong candidate for an incompatibility. To test this hypothesis, we created an *agt[simD121N]* sequence (Fig. S4) that is identical to *agt[sim]* except for a G-to-A nucleotide change in codon 121 resulting in D121N. A transgenic *agt[simD121N]* *piggyBac* transformant was obtained, crossed into an *agt[mau]/agt[mau]* genetic background (Fig. S2), and tested for male fertility. The results are shown in Fig. 3. If D121N were solely responsible for the *agt[mau]* incompatibility, then transgenic *pB::agt[simD121N]* males should be sterile. However, 27% of the males are fertile (Fig. 3), and their average number of offspring does not differ significantly from that observed for *pB::agt[sim]* ($t = 0.184$, df =121, $P = 0.89$) (Fig. 2). Nevertheless the fertility rescue of *pB::agt[simD121N]* is significantly less than that of *pB::agt[sim]*, with $73 \pm 7\%$ sterile in the former and $42 \pm 4\%$ in the latter [$P = 0.014$ ($X^2 = 6.09$, df = 1, $n = 225$)]. These results apply to a single genomic insertion; however, the observation that position effects for degree of rescue are not observed for distinct genomic insertion sites for agt[sim] (Fig. 2), together with the absence of transcriptional difference in *agt* between *HMS1[mau]/HMS1[sim]* and *HMS1[mau]/HMS1[mau]* males, suggest that the reduced fraction of fertile males in *pB::agt[simD121N]* may be heavily attributable to the coding mutation.

Follow-Up Analysis of *agt[mau]* Incompatibility. The findings with *agt[simD121N]* suggest that the *agt[mau]* incompatibility is due only in part to D121N and in part to other nucleotide differences in *agt* between the SimB and Mau12 strains. A total of 16 nucleotide differences are positioned across the gene (Table 1), or 132 differences if those in the intergenic regions are also considered; hence there is considerable opportunity for interactions among sites. To test for such interactions, we created an *agt[simP_mauCDS]* construct that includes the promoter region from *D. simulans* and the ORF from *D. mauritiana* (*agt* contains no introns), and the reciprocal *agt[mauP_simCDS]* construct (Fig. S4). Two independent transgenic *piggyBac* lines were obtained and tested from each construct (Table S1), yielding the grouped results shown in Fig. 3.
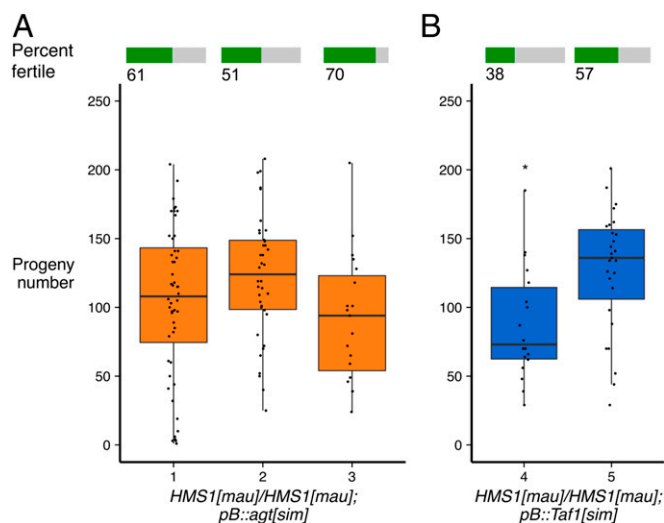

Fig. 2. Phenotypes of *HMS1/HMS1* sterile males with *agt[sim]* or *Taf1[sim]* transgenes. (A) Males carrying a transgene of *piggyBac* containing the complete *agt[sim]* gene show partial rescue of male sterility with nearly normal numbers of progeny compared with males that are fertile in Fig. 1C. (B) Males carrying a transgene of *piggyBac* containing the complete *Taf1[sim]* gene show a similar phenotype, with partial rescue of sterility and nearly normal number of progeny yielded by fertile males except for insertion 4 that has a reduced fecundity (t test on progeny number: $t = -3.030$, df = 43, $P = 0.004$), as indicated by the asterisk (B). All of these *piggyBac* insertions are in introns or intergenic regions.
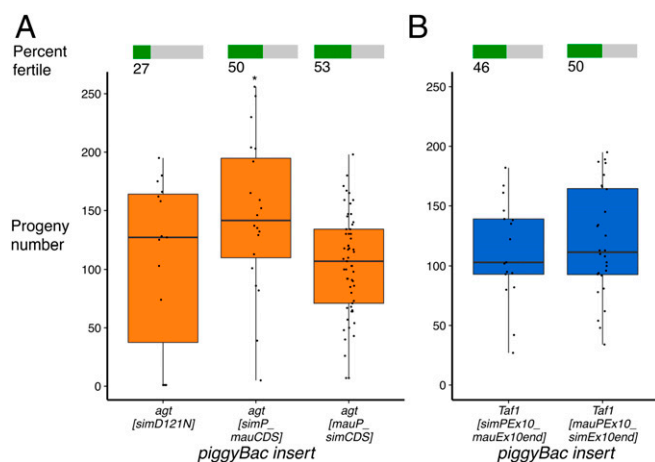
Fig. 3. (*A* and *B*) Phenotypes of *HMS1/HMS1* sterile males with *agt* or *Taf1* chimeric genes. The *agt[simD121N]* transgene contains the *agt[sim]* sequence from SimB with a D121N amino acid replacement, which is a fixed difference between *D. simulans* and *D. mauritiana*; this transgene rescues sterility significantly less than does *agt[sim]*; however, progeny production of the fertile males is in the normal range. The *agt[simP_mauCDS]* and *agt[mauP_simCDS]* are reciprocal chimeras carrying the *agt* promoter from one species and the *agt* coding sequence from the other. Both rescue fertility to the same extent as *agt[sim]*, whereas *agt[simP_mauCDS]* exhibits higher fecundity than *agt[sim]* (one-way ANOVA: $F = 2.998$, df = 5, $P = 0.012$, Tukey post hoc test), as indicated by the asterisk (*A*). The *Taf1[simPEx10_mauEx10end]* and *Taf1[mauPEx10_simEx10end]* are reciprocal chimeric constructs containing *Taf1* of one species from the promoter through an AvrII site in exon 10 fused to *Taf1* of the other species from the AvrII site in exon 10 through to the end of the gene. Both chimeras complement sterility and productivity to the same extent as does *Taf1[sim]*. All of these *piggyBac* insertions are in introns or intergenic regions.

Both types of construct show levels of complete fertility rescue comparable to those of *agt[sim]* [$P = 0.257$ ($X^2 = 5.309$, df = 4, $n = 335$)] and, among the fertile males, only *agt[simP_mauCDS]* sire more progeny (one-way ANOVA: $F = 2.998$, df = 5, $P = 0.012$, Tukey post hoc test). These results support a model in which the incompatibility in *agt[mau]* results from epistatic interactions of nucleotide sites within or near the *agt* gene, interactions that the *agt[simP_mauCDS]* and *agt[mauP_simCDS]* constructs disrupt.

Complex Nature of *Taf1[mau]* Incompatibility. Differences between SimB and Mau12 in the *Taf1* region give even more scope for complex interactions than in *agt*. *Taf1* is a much larger gene than *agt* (9,412 vs. 758 nt). Across the *Taf1* gene region, there are 127 nucleotide differences between the SimB and Mau12 strains (Table 1), and 164 differences if the 3′-untranslated and intergenic regions are also taken into account. To test for interactions among these sites, we created two chimeric constructs designated *Taf1[simPEx10_mauEx10end]* and *Taf1[mauPEx10_simEx10end]* (Fig. S4). The first construct joins a DNA fragment containing the SimB *Taf1* promoter through an AvrII restriction site in exon 10 with another DNA fragment containing Mau12 *Taf1* coding sequence from the

AvrII restriction site through to the 3′ end of the gene. The AvrII site was chosen as the joining site because it is near where recombination analysis had suggested the approximate 5′ upstream boundary of *HMS1* was located (30). The second construct is the reciprocal, joining the 5′ end of Mau12 *Taf1* with the 3′ end of simB *Taf1*, again with the joining site at the AvrII site in exon 10.

Results with the reciprocal *Taf1* transformants are shown in Fig. 3. The two constructs do not differ significantly in either the proportion of sterile males or the fecundity of fertile males, nor do they differ significantly from the *Taf1[sim]* insertions in Fig. 2 (χ^2 on number fertile: $P = 0.913$, $X^2 = 0.182$, df = 2, $n = 189$) or fecundity from *Taf1[sim]* insertion 5 (one-way ANOVA on fecundity: $F = 0.612$, df = 2, $P = 0.545$). Considering the limited contribution of position effects at the sites of insertion studied in terms of degree of rescue (although not globally for fecundity; i.e., lower progeny numbers in *Taf1[sim]* insertion 4), these results suggest that the *Taf1[mau]* incompatibility is due to interactions between sites in or near the gene. In the case of *Taf1*, between the strains SimB and Mau12, there are 5 nonsynonymous differences and 44 synonymous differences in the coding sequence preceding the AvrII site, and 9 nonsynonymous differences and 23 synonymous differences following the AvrII site; hence, opportunities for interactions among sites are plentiful.

Tests for Positive Selection. To look for signals of positive selection, we sequenced the ORF of 32 alleles of *agt* and exons 10–14 of 15 alleles of *Taf1* from genomic DNA extracted from individual flies from 17 diverse *D. simulans* populations from South, Central, and North Africa, Europe, Japan, Australia, and North America as well as 16 independent acquisitions of *D. mauritiana* (an island endemic), from Mauritius (Table S4). Statistical tests for selection included comparing the mean number of pairwise differences with the number of segregating sites [Tajima's *D* (33)], as well as comparing old and recent mutations according to where they occur in the gene genealogies using mutation rates either estimated from nucleotide diversity [Fu and Li's *D* (34)] or else estimated from nucleotide polymorphism [Fu and Li's *F* (34)]. We also analyzed nonsynonymous versus synonymous polymorphism and divergence [McDonald–Kreitman test (35)], as well as site-specific models of selection using phylogenetic analysis by maximum likelihood (PAML) (36). None of these tests reached statistical significance, even when uncorrected for multiple testing (Table S4).

Discussion

Genetic Basis of *HMS1*. Our findings demonstrate the genetic complexity of a small region of 9.2 kb designated *HMS1* that is associated with hybrid male sterility when introgressed from *D. mauritiana* strain Mau12 into *D. simulans* strain SimB. The region contains two likely candidate genes for hybrid male sterility, *agt* and *Taf1*, and we find that both genes contribute to the incompatibility. In both cases, transgenes carrying a compatible allele rescue the fertility of otherwise sterile *HMS1* males. The rescue phenotype has the unusual property that, although only about one-half of the transgenic males regain fertility, those that are fertile produce on average as many progeny as *HMS1[mau]/HMS1[sim]*

Table 1. Nucleotide differences across *agt* and *Taf1* between SimB and Mau12

Gene	Promoter region		Coding sequence		Other	
	Intergenic	5′-UTR	Nonsynonymous	Synonymous	Intron	3′-UTR/intergenic
agt, length (bp)	598*	84	579		N/A	95
agt, no. differences	116	4	6	6	N/A	0
Taf1, length (bp)	1,174	100	6,393		2,440	427
Taf1, no. differences	26	1	14	67	45	11

*Portion of the intergenic region included in the *piggyBac* construct; the total intergenic region between *agt* and *Lab* is 1,421 bp and includes 147 differences. N/A, not applicable.

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heterozygous males (Figs. 1 and 2). We note that position effects cannot be fully excluded from having contributed to some of the results, even if the few replicates noted do not show statistically significant differences after multiple comparisons. In *agt*, a transgene carrying an amino acid replacement that had previously been identified as a candidate incompatibility factor (30) rescued *HMS1* sterility less than controls; however, the full effect of *agt* appears to involve an interaction among two or more nucleotide sites along the gene (Fig. 3). Likewise, the *Taf1* incompatibility seems to result from interactions among nucleotide sites, as chimeric constructs do ameliorate the *HMS1* sterile phenotype (Fig. 3). Sequence analysis of allelic diversity among naturally occurring *agt* and *Taf1* alleles gives no evidence of positive selection.

Reproductive Isolation as a Polygenic Quantitative Trait. Our findings with *HMS1* challenge a number of hypotheses regarding hybrid incompatibilities while they support others. The first is that hybrid incompatibilities are due to single genes of large effect. Although a number of large-effect genes are known that result in partial rescue of hybrid sterility or lethality (13, 16, 37, 38), *HMS1* does not represent a single incompatibility gene but a nearby pair of incomplete incompatibilities that each afford only partial rescue. The partial incompatibility due to each gene is itself attributable to epistatic interactions among sites in or near the gene, as expected of ongoing evolution through slightly deleterious mutations compensated by other mutations elsewhere in the gene (39). The number of interacting sites could be as few as two, but experimental evidence implies that higher-order interactions are more likely (40, 41). Considering the within-gene interactions and the effects of environment and genetic background (30), hybrid male sterility in this case behaves like a classical quantitative trait affected by multiple genes with relatively small effects (42) as well as environmental factors. Although a number of genes associated with hybrid incompatibility show evidence of positive selection (3, 10, 43, 44), this is not the case for either *agt* or *Taf1*; however, our findings do not rule out strong selection in the ancient past or ongoing weak selection because these processes would not necessarily leave statistically detectable signals of selection on contemporary polymorphisms.

Fig. 4 illustrates a threshold model of *HMS1* sterility treated as a quantitative trait subject to environmental effects. The horizontal axis represents a hypothetical fertility potential, and the vertical axis is proportional to the fraction of males in a genetically homogeneous population. Any male with a fertility potential less than some threshold (dashed vertical line) is completely sterile. The *mau/mau* curve on the *Left* corresponds to the recessive hybrid male-sterile *HMS1[mau]/HMS1[mau]* carrying two copies of the *HMS1* introgression, whereas the *sim/mau* curve depicts the fertility potential of SimB male individuals carrying one copy of the *HMS1* introgression (*HMS1[sim]/HMS1[mau]*). Because the incompatibility is recessive, *sim* represents the dominant allele conferring male fertility. Note that a small proportion of *mau/mau* males are fertile, and likewise a small proportion of *sim/mau* males are sterile. The *sim + mau/mau* curve in the *Center* represents the effects of the rescue transgenes *pB::agt[sim]* and *pB::Taf1[sim]* in Fig. 2 and the chimeric genes in Fig. 3. One copy of the dominant fertility allele is sufficient to restore fecundity in the normal range, on average, in 45% of the transgenic males. This kind of fecundity rescue would be expected if, in the transgenic males, any germ cells that survive the incompatibility proliferate more than they otherwise would, thereby compensating for germ cells that succumb to the incompatibility and restoring male fecundity to high levels. It is worth noting that a substantial proportion of males who fail to rescue fertility produce some motile but dysfunctional sperm (Table S1). The proportion of sterile individuals with motile sperm is relatively constant (50%) regardless of their transgenic genotype.

Support for DNA-Binding Proteins. One hypothesis that the genetic analysis of *HMS1* strongly supports is the prominence of internal
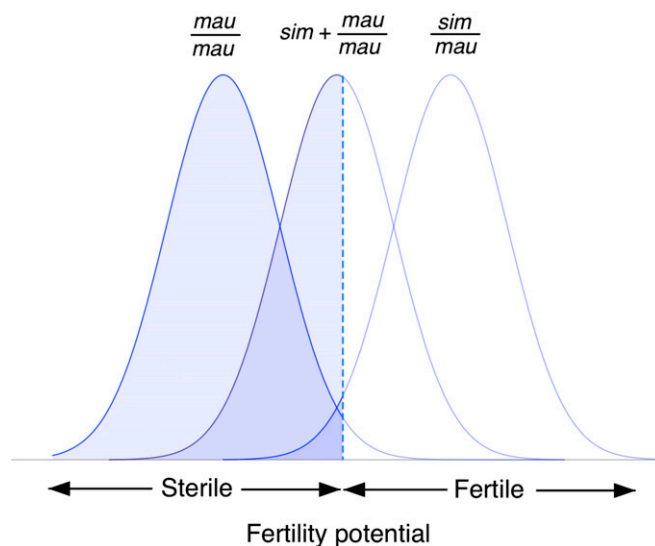

Fig. 4. Threshold model of *HMS1* sterility treated as a quantitative trait subject to environmental effects. The horizontal axis represents the magnitude of a hypothetical fertility potential, and the vertical axis is proportional to the fraction of males in a genetically homogeneous population. Males homozygous for a Mau12 introgression containing *HMS1* (*HMS1[mau]/HMS1[mau]*) are virtually sterile, whereas the heterozygous SimB lines *HMS1[sim]/HMS1[mau]* are fertile.

genomic conflicts in hybrid incompatibilities, especially those conflicts involving genes for DNA- or chromatin-binding proteins (3, 10). In *Drosophila*, spermatogenesis proceeds through a mitotic phase of stem cell renewal and differentiation followed by meiosis and posttranscriptional spermatid maturation into fully motile, individualized sperm (45). Germ cell development is influenced by genotypic variability as well as microenvironmental perturbations that are ultimately reflected in quantitative differences in the function of mature spermatocytes and spermatozoa within and among individual males.

Several lines of evidence suggest that alternatively spliced isoforms of *Taf1* transcripts regulate spermatogenesis in a tissue-specific manner. *Taf1* encodes a TAF (TATA-box–binding associated factor) constituting one subunit of transcription factor TFIID broadly required for transcription by RNA polymerase II (46, 47). The product of *Taf1* serves as a multifunctional transcriptional regulator operating in the normal cell cycle, the assembly of other TAFs and TBPs (TATA-binding proteins), promoter activities, histone acetylation, and other chromatin modifications that can modulate chromatin structure enabling it to access transcriptionally repressed chromatin (48). In mice, the product of the *Taf1* homolog *Bdrt* associates with hyperacetylated histone H4 and functions in chromatin remodeling following histone hyperacetylation as postmeiotic germ cells mature into fully differentiated sperm (49). In *Drosophila*, the primary transcript of *Taf1* is alternatively spliced into at least four isoforms whose protein products contain AT hooks that directly bind with DNA (50). One of the isoforms (Taf1-2) is enriched in *Drosophila melanogaster* testes where it accounts for 45% of *Taf1* mRNA versus 10% of *Taf1* mRNA in adult male whole flies (50). Among other possible functions, the Taf1-2 product can bind promoters of testes-specific genes including sperm-specific dynein intermediate chain (*Sdic*), heat shock protein 70bc (*hsp70*), β2-tubulin (*β2t*), and don juan (*dj*) (51), and the role of Taf1-2 as transcriptional activator in the testes is likely conserved across *Drosophila*. At 10 kb, *Taf1* is a relatively large gene for *Drosophila*, and there are about 1.5% nucleotide sequence differences in *Taf1* between SimB and Mau12 (Table 1). Several fixed nucleotide differences are located in the gene region around to exon 12a, which is a spliced exon

required for isoform Taf1-2. The sequence divergence might therefore affect alternative splicing efficiency (52), promoter-binding affinity, chromatin modification, or other processes that could contribute to hybrid male sterility as a quantitative trait (53, 54). The role of *Taf1* in hybrid male sterility is perhaps not surprising in light of the rapid evolution and functional diversification of testes-specific TAFs in *Drosophila* (55).

The mechanism by which *agt* contributes to *HMS1* sterility is more obscure. The gene is induced by genotoxic stress, and the protein is an alkyl-cysteine *S*-alkyltransferase that removes alkyl groups from DNA, notably from O^6-methylguanine (56), a repair process observed in eukaryotes, notably in postmeiotic male germ cells in *Drosophila* (57). A much smaller gene than *Taf1*, with an ORF of only 192 codons and no introns, *agt* is 2.4% different between SimB and Mau12 (Table 1). It is also widely divergent in the species subgroup, with nearly every species having a different amino acid at position 121 (30). The D121N difference between SimB and Mau12 clearly contributes to the hybrid male sterility (Figs. 2 and 3); however, interactions among sites in *agt* are also implicated (Fig. 3). Although the role of *agt* in hybrid male sterility in *Drosophila* is uncertain, in the mouse the DNA repair gene *Ercc1* is essential for functional integrity of germ cell DNA and normal spermatogenesis (58), and a histone methyltransferase encoded by *Prdm9* is necessary for meiosis (59). This emphasizes the intriguing relationships between gametogenesis and the variety of DNA repair enzymes functioning in parallel pathways in spermatogenesis. Also, it suggests that in eukaryotes dysfunction of genes involved in any DNA repair mechanism in male germ cell stages may influence the degree of fertility (60, 61).

Temporal Origins of Reproductive Incompatibility. It is unlikely that either *Taf1* or *agt* was involved in the origin of reproductive isolation between *D. simulans* and *D. mauritiana*. Recent experimental evidence (62) and theory (63–67) imply that certain allele combinations causing partial reproductive incompatibility can be found segregating in natural populations, which suggests that, on an evolutionary timescale, reproductive isolation can evolve very rapidly from standing genetic variation for deleterious allelic mutations causing partial reproductive isolation, even in sympatric populations (68, 69).

A quick, heuristic calculation also suggests that the incompatibilities of *Taf1* and *agt* are both likely to have evolved more recently than the species divergence time of 250,000 y. Between *D. simulans* and *D. mauritiana*, there are an estimated 40 autosomal genes that contribute to hybrid male sterility (29); however, the X chromosome, which evolves incompatibilities faster than the autosomes, has about 100 such factors (24). Each factor reduces male fertility by about 10%, and, taken together, they constitute 15 "HMS equivalents," where each HMS equivalent includes a sufficient number of incompatibilities to result in hybrid male sterility (24). In effect, hybrid males of *D. simulans* and *D. mauritiana* are sterile 15 times over. The factors do not act independently, however: epistasis among incompatibility genes is pervasive (7, 29, 70).

If hybrid incompatibility factors evolve at a rate that is linear in time, and those in the X chromosome evolve at a rate 2.5 times that of those in the autosomes (24), then each HMS equivalent would entail, on average, 9.3 total incompatibility factors of which 2.7 would be autosomal. Because 40 autosomal factors have evolved in 250,000 y, then with a linear increase in number through time, 2.7 autosomal factors would be expected to have evolved in 17,000 y. This number is less than 7% of the total time since species divergence, so any incompatibility factor between *D. simulans* and *D. mauritiana* is very likely to have evolved since their divergence. On the other hand, population genetics theory suggests that the accumulation of incompatibility factors might be exponential rather than linear (9). In the exponential case, the situation for early evolution is a bit more optimistic, but not by much. With exponential increase from 0 to 40 autosomal factors in 250,000 y, 2.7 factors would be expected to accumulate in 27,000 y, or about

10% of the total time since divergence. Both estimates imply that most hybrid incompatibility factors identified in well-established, reproductively isolated species are likely to have arisen since speciation. Unveiling the genetic origins of species, "that mystery of mysteries" (71), requires an untangling of complex combinations of the evolutionary forces that create incompatibilities and the reproductive isolating mechanisms that result.

Materials and Methods

Fly Lines. All flies were reared on cornmeal–molasses–agar medium sprinkled with yeast grains (*SI Materials and Methods*). The *D. simulans* stocks used in this study are *w501* (University of California, San Diego, line 14021-0251.011), *w;e* (*w;II:e*), and simB (*w;nt;III*), where *II* and *III* represents isogenic second and third chromosomes, respectively, from *D. simulans* line (13*w* × JJ) (24). The *D. mauritiana* × *D. simulans* heterozygous introgression lines used in this study are designated *P45.6* and *P32.110* (72); the creation of these lines has been described in detail earlier (72, 73). *P* represents the immobile *P*-transposon element *P[w⁺]* marking the portion of *D. mauritiana* [Mau12, a white-eyed inbred laboratory stock (14021 0241.60)] on the right arm of the third chromosome. *P[w⁺]*-elements are semidominant markers sensitive to the location and copy number of the miniwhite *w⁺* gene (Fig. S3 and *SI Materials and Methods*). All crosses were performed at room temperature (20–22 °C), except the cross that generated *HMS1[mau]/HMS1[mau]; pB::HMS1[sim]* ("3P") progeny, which was performed at 18 °C. Absence of recombination in *Drosophila* males allows maintaining the original introgression lines by backcrossing each generation of *P[w⁺]*-males with virgin SimB females.

Cloning and Germ-Line Transformation. Germ-line injections were carried out by BestGene. *D. simulans w⁻* embryos were injected with the purified MW-FPNS piggyBac (*pB*) plasmid carrying either agt[sim], Taf1[sim], agt[simD121N], agt[simP_mauCDS], agt[mauP_simCDS], Taf1[simPEx10_mauEx10end], Taf1 [mauPEx10_ simEx10end], or a control MW-FPNS plasmid with no insert. Full details of cloning procedures are provided in *SI Materials and Methods*. Injected embryos were subsequently raised in the laboratory, and emerging individual virgin adult *w⁻* flies were backcrossed to SimB. Progeny were screened via presence or absence of the *w⁺* eye marker (74), and those containing the *pB[w⁺]* transposed DNA insert were selected to establish stable male transgenic lines. Altogether, the progeny of 429 (agt) and 160 (Taf1) injected adult flies was scored, all constructs considered, and yielded similar transformation efficiencies of 2.79% and 3.1%. Flanking genomic insertions were determined via inverse PCR (Table S1 and *SI Materials and Methods*). Stable male transgenic lines representing eight unique agt insertions, four unique Taf1 insertions, and two control lines were used to assess the role of each candidate gene in fertility rescue crosses (Fig. S1).

Phenotyping of Rescue Hybrid Males. Rescue hybrid males bearing a copy of the SimB allele for either agt or Taf1 (*HMS1[mau]/HMS1[mau]; pB::HMS1[sim]*), or a chimeric agt or Taf1 construct, were assayed for their ability to sire progeny in single-mating fertility assays with three virgin *w;e* females before scoring of sperm motility and genotyping of all *P[w⁺]*-elements. Full details of these procedures are provided in *SI Materials and Methods*.

Molecular Analysis. The genomic region comprising agt or Taf1 and their respective regulatory regions were amplified for *D. mauritiana* Mau12 and *D. simulans* SimB using overlapping oligonucleotide primers, sequenced, and analyzed in Geneious, version 9.0.5 (75). The sequences have been deposited in the GenBank database under accession nos. KX225407–KX225410.

Tests of Positive Selection. Patterns of DNA divergence were calculated to detect departure from neutral models of molecular evolution among sequenced agt and Taf1 alleles from 17 *D. simulans* populations spanning all continents, as well as 16 independent isogenic lines of *D. mauritiana* collected on Mauritius island (see *Results, Tests for Positive Selection*). Various specific site models from the PAML, v4.8, package (36) were used to test for interspecific site-specific positive selection. Likelihood ratio tests were used to evaluate whether the model pairs allowing positive selection provided a significantly better fit to the data. Results from theses comparisons were consistent, all supporting neutral evolution in and around the *HMS1* region.

Models for the Accumulation of Hybrid Male-Sterility Factors. The linear model used for the accumulation of 40 hybrid male-sterility factors in 250,000 y was $f(t) = 40 \times (t/250,000)$; the exponential model used was $f(t) = 40 \times [\text{Exp}(t/250,000) - 1]/[\text{Exp}(1) - 1]$.

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