Genetic Analysis of the *Hybrid male rescue* Locus of Drosophila

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ABSTRACT

Several hybrid rescue mutations—alleles that restore the viability of normally lethal hybrids—have been discovered in *Drosophila melanogaster* and its relatives. Here we analyze one of these genes, *Hybrid male rescue* (*Hmr*), asking two questions about its role in hybrid inviability. (1) Does the wild-type allele from *D. melanogaster* (Hmr^{mel}) cause hybrid embryonic inviability? (2) Does Hmr^{mel} cause hybrid larval inviability? Our results show that the wild-type product of *Hmr* is neither necessary nor sufficient for hybrid embryonic inviability. Hmr^{mel} does, however, appear to lower the viability of hybrid larvae. The data further suggest (though do not prove) that Hmr^{mel} acts as a gain-of-function poison in hybrids. These findings support previous claims that hybrid embryonic and larval lethalities are genetically distinct and suggest that Hmr^{mel} is at least one of the proximate causes of hybrid larval inviability.

ONE of the most important and surprising findings in the genetics of speciation has been the discovery of "hybrid rescue" mutations. At least since Dobzhansky (1937), evolutionary biologists have imagined that speciation involves the gradual accumulation of many slight incompatibilities between populations. Reproductive isolation should therefore be essentially irreversible: because taxa will come to differ at many sets of genes, hybrids will suffer many developmental problems and it is implausible to think that evolution could ever retrace its steps, restoring the fitness of hybrids. In Muller's (1939) words, genetic divergence between populations leads to an "ever more pronounced immiscibility" that cannot be undone.

The discovery by Watanabe and by Ashburner and their colleagues of at least five different hybrid rescue mutations comes as a surprise, then (reviewed in Hutter *et al.* 1990 and Sawamura *et al.* 1993). These mutations, when introduced singly into species hybrids, restore the viability of normally lethal hybrids. Some mutations appear to have a nearly complete effect on hybrid fitness, *i.e.*, a lethal sex is restored to a 50:50 sex ratio. Most of the known rescue mutations occur in the *Drosophila melanogaster* group (Hutter 1997), rescuing hybrids formed when *D. melanogaster* ("*mel*") is crossed to its relatives *D. simulans*, *D. mauritiana*, or *D. sechellia* (these three sibling species are hereafter referred to as "*sib*"). Rescue mutations will presumably turn out to be widespread. The fact that they are best known in Drosophila surely reflects the intense scrutiny to which these hybrids have been subjected.

The discovery of rescue mutations raises several questions. First, how many different genes can rescue hybrids? Although the screens performed so far have not been exhaustive, four (and perhaps five) rescue loci have been found and each new screen seems to uncover new ones, *e.g.*, Sawamura *et al.*'s (1993a,b) discovery of *maternal hybrid rescue* (*mhr*) and *Zygotic hybrid rescue* (*Zhr*). Second, are rescue genes alleles of "speciation genes"? In other words, are rescue genes mutant alleles of the genes that normally kill hybrids? This question is important because, if the answer is yes, the study of rescue genes may provide a shortcut to the molecular characterization of the genes causing speciation. Rescue mutations need not, however, be alleles of speciation genes. They might instead be second-site suppressors—loci that, while not causing hybrid inviability, can, when mutated, override the effects of the genes actually killing hybrids. (Rescue genes might, for instance, allow use of an alternative metabolic pathway that sidesteps the primary problematic pathway.) Under the second-site hypothesis, rescue mutations may even occur at loci that have not diverged molecularly between the relevant species.

Third and most important, does the recovery of rescue genes imply that reproductive isolation has a simple developmental basis? After all, if inviability reflects many independent developmental problems, the chance of recovering single mutations that simultaneously correct all of these problems would seem vanishingly small. A simple developmental basis, in turn, suggests a simple genetic basis. If many genes cause hybrid inviability, why do they all affect the same developmental pathway?

Hutter *et al.* (1990) argued that hybrid inviability in the *mel-sib* species cross is, in fact, simple and proposed an elegant genetic model to explain it. This model had two important features. First, it posited that hybrid inviability is caused by wild-type alleles at known rescue genes

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(*i.e.*, rescue mutations are alleles of speciation genes). Second, it posited that hybrid inviability reflects a single incompatibility between the wild-type alleles of two hybrid rescue genes, *Hybrid male rescue* (*Hmr*) and *Lethal hybrid rescue* (*Lhr*).

The Hutter *et al.* model elegantly explains the complex results obtained when *mel* and *sib* are crossed. Postzygotic isolation between these taxa involves both larval and embryonic lethality. When *mel* females are crossed to *sib* males, only hybrid females appear; males die as third-instar larvae. The reciprocal cross produces only hybrid males, with females dying as embryos (Hadorn 1961). Interestingly, the cross of *mel* attached-*X* females to *sib* males produces only males, with females dying as late larvae. [See Hutter *et al.* (1990) for a summary of these and other crossing results.] Because all hybrids, viable and inviable, from these crosses carry a haploid set of autosomes from each species, differences in survival must involve the sex chromosomes and/or cytoplasm. It is now clear that hybrid larval and embryonic lethality involve genes on the *mel X* chromosome (Orr 1991; Yamamoto 1992). Hutter *et al.* suggested that both forms of lethality involve the same *X*-linked locus, the rescue gene *Hybrid male rescue* (*Hmr*). According to their model, "the product of the l^+ [*i.e.*, Hmr^{mel}] allele would lead to death" (Hutter *et al.* 1990, p. 918) when brought together in hybrids with the wild-type allele of another rescue gene, *Lethal hybrid rescue* (*Lhr*), from *sib*. They further suggested that the rescue allele, *Hmr*, is a loss-of-function mutation: removal of the lethal Hmr^{mel} product restores hybrid viability. (Their data suggest that *Hmr* is a hypomorph, not an amorph.)

Later work, however, called this simple model into question. Sawamura *et al.*'s (1993c) extensive analysis of several rescue mutations suggested that embryonic and larval lethalities have different genetic bases, with *Hmr* rescuing larval fitness only. Sawamura and colleagues thus proposed an alternative model of *mel-sib* hybrid inviability. This model also had two salient features. First, like Hutter *et al.*'s model, it posited that postzygotic isolation is caused by wild-type alleles at hybrid rescue genes. But second, it posited that one set of loci (mhr^{sib} and Zhr^{mel}) causes hybrid embryonic lethality, while another set (Hmr^{mel} and Lhr^{sib}) causes larval lethality.

The Sawamura model explains a remarkable range of experimental results and is now widely accepted. Several findings, however, suggest that this "separate compartment" view of hybrid embryonic *vs.* larval lethality might be too simple. First, Hutter *et al.* (1990) presented strong evidence that addition of Hmr^{mel} as a duplication to hybrid males causes embryonic, not larval, lethality. Moreover, several groups have repeatedly found that the mutation *Hmr* appears to rescue hybrid male embryonic lethality, at least at low temperatures. In the cross of *D. simulans* attached-*X* females to *mel* males, X^{mel}-bearing hybrid males die as embryos (Sawamura *et al.* 1993a). But Orr (1991), Hutter *et al.* (1990), and Sawamura *et al.* (1993a) all showed that such males are weakly rescued in crosses to *Hmr*. The latter authors thus concluded that "[a] possibility remains that *Hmr* can sometimes rescue . . . embryonic lethality" (Sawamura *et al.* 1993a, p. 305).

These findings might be legitimately explained away under the Sawamura model. [For example, the *Hmr* stock might by chance carry a *Zhr* mutation. This does not, however, seem likely as several stocks were used; see also Sawamura *et al.* (1993b), whose results suggest that their *Hmr* stock was Zhr^+.] In any case, such special pleading is unnecessary. As we will see, definitive tests of *Hmr*'s role in hybrid embryonic *vs.* larval lethality are possible.

One of the sources of uncertainty about the precise roles of rescue genes in hybrid inviability is clear. Much of the literature involves attempts to infer the action of wild-type alleles from the behavior of a small number (usually one per locus) of poorly characterized mutant alleles. (We sometimes do not know if a mutation is an amorph, hypomorph, hypermorph, or neomorph.) Although this strategy may often succeed, it is of course entirely possible that the wild-type allele at, *e.g.*, *Hmr* may affect both embryos and larvae, while the particular lesion producing a *Hmr* mutation only restores larval viability. In such a case, inferences from the behavior of the mutant allele would mislead.

Here we attempt to obtain clearer evidence about the role of rescue genes in postzygotic isolation by manipulating wild-type alleles at a rescue gene. In particular, we investigate the role of Hmr^{mel} in hybrid inviability by testing whether its removal (by deficiency) from species hybrids rescues embryonic viability and whether its addition (by duplication) to species hybrids causes embryonic or larval lethality. Our results provide strong additional support for the Sawamura model. In particular, they reveal that wild-type product of *Hmr* is neither necessary nor sufficient for hybrid embryonic inviability. Hmr^{mel} does, however, appear to affect hybrid larval viability. The wild-type allele at *Hmr* may therefore be a "speciation gene."

MATERIALS AND METHODS

Mutations and stocks: *Hmr*, which was described by Hutter and Ashburner (1987; Hutter *et al.* 1990) and Zhang *et al.* (1999), resides at 32.0 on the *X* chromosome within polytene bands 9D1-9E4.

The experiments below use three *X* chromosome deletions. The first is *Df(1)HC133* (9B9; 9F2-5), which Hutter *et al.* argue includes *Hmr*. The distal breakpoint of this deficiency extends far to the left of the likely position of *Hmr*. The proximal breakpoint is to the right of *fliK* (9F3-5) which, according to the mapping of Hutter *et al.* (1990), is to the right of *Hmr* (see also Zhimulev *et al.* 1987 and Lindsley and Zimm 1992). *Df(1)HC133* includes *raspberry* (*ras*; *1*-32.35), which is very tightly linked to *Hmr* (Zhang *et al.* 1999). Two other deficiencies, *Df(1)ras-v-17* (9D1-2; 10A2-3) and $Df(1)v^{-L15}$ (9B1-2; 10A1-2),

were also used. The breakpoints of these large deficiencies suggest they include *Hmr* (also D. Barbash, personal communication).

Hutter *et al.* also argue that the duplication $Dp(1;2)v^{+75d}$ (9A2; 10C2) includes *Hmr*. This duplication extends far past *Hmr*'s likely position on both the left and right. More important, Hutter *et al.* show that males bearing the rescue allele *Hmr* are not rescued if they also carry $Dp(1;2)v^{+75d}$, a result that we confirm below.

D. mauritiana Synthetic is a mixture of six isofemale lines collected by O. Kitagawa on Mauritius in 1981; the lines were pooled in 1983. This stock, as well as *D. mauritiana* v^C, *D. simulans v m*, and *D. simulans Ro*/+ were kindly provided by J. A. Coyne. All other mutations are described by Lindsley and Zimm (1992).

Crosses: Although *Hmr* rescues the viability of hybrids from all of the *mel-sib* species crosses, the present experiments focus on the *D. melanogaster-D. mauritiana* ("*mel-maur*") and *D. melanogaster-D. simulans* ("*mel-sim*") hybridizations. These species crosses differ in one important respect. While the *maur* female × *mel* male cross gives unambiguous results (females almost never appear), the *sim* female × *mel* male cross is leaky, *i.e.*, hybrid females sometimes appear, depending on temperature and the particular *sim* stock used (Orr 1993a; Sawamura *et al.* 1993).

Some of the species crosses below involved the *CyO* balancer. Because *Cy* sometimes overlaps wild type, we scored the phenotype of 217 *Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO* males at 18° and 22°; all but one were phenotypically Cy. *Cy*'s reliability was further confirmed in several preliminary crosses, *e.g.*, *mel v* females × *Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO* males. All phenotypically Cy males (69) were vermillion-eyed while all phenotypically Cy^+ males (91) were wild-eyed, as expected. This result of course also confirms the presence of the v^+-bearing duplication in the *Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO* stock.

Flies were typically aged for several days before species crosses were set up. All crosses were performed on standard cornmeal medium at 22° or 18°, except as indicated.

RESULTS

Deletion/duplication tests: A direct test of Hmr^{mel}'s role in embryonic lethality requires its deletion from species hybrids. If, as Hutter *et al.* suggested, Hmr^{mel} product causes hybrid inviability—and the rescue allele *Hmr* is a loss-of-function mutation—deletion of Hmr^{mel} must rescue embryonic lethality. Conversely, addition of the putatively poisonous Hmr^{mel} product to hybrids that do not normally carry it must cause lethality.

Figure 1 shows how Hmr^{mel} can be deleted from normally lethal hybrids and added to other, normally viable hybrids. In particular, one can delete Hmr^{mel} by deficiency from hybrid females (which normally die as embryos), giving them an *Hmr* genotype that is identical to that of viable hybrid males. Simultaneously, one can add Hmr^{mel} by duplication to hybrid males (which normally survive), giving them an *Hmr* genotype that is identical to that of lethal hybrid females.

We first confirm that the normal species crosses behave as expected. Consider first the results of crosses between *mel* and *maur*. Table 1 shows that all control crosses behave as expected at both 18° and 22°. The cross of *mel* female × *maur* male produces only hybrid daughters, with males dying as late larvae. The reciprocal cross produces only hybrid males, with hybrid females dying as embryos, although a few individuals hatch and die as first-instar larvae (at 18°, one escaper survived to adulthood). Table 1 also shows that the *Hmr* mutation rescues normally inviable hybrid males. Preliminary crosses also confirmed that the *Df(1)HC133* stock carries a deficiency in the expected region (not shown).

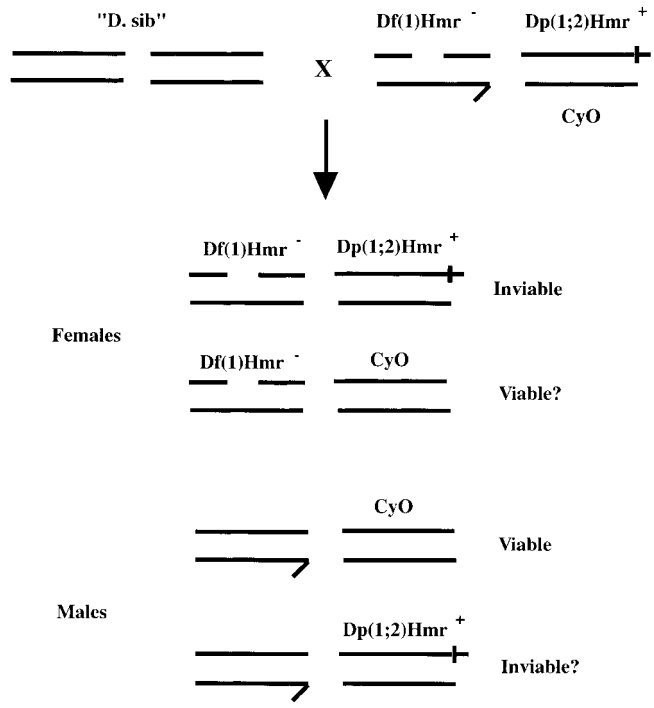

Figure 1.—Deletion/duplication test of *Hmr* in *sib-mel* hybrids. Hutter *et al.*'s (1990) model predicts that hybrid females who have had Hmr^{mel} deleted should be viable, while *Dp*-bearing hybrid males should be embryonic lethal. In most cases, "$Df(1)Hmr^-$" = *Df(1)HC133*, while "$Dp(1;2)Hmr^+$" = $Dp(1;2)v^{+75d}$ (see Table 2).

We now consider the deficiency/duplication tests. The within-species control cross of *mel* females × *mel Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO* males behaves as expected: all genotypes of males and females are recovered in reasonable numbers. The critical cross of *maur* females × *mel Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO* males, however, behaves quite differently. While hybrid females who carry the duplication are lethal as expected, females who carry the balancer chromosome remain completely inviable despite the fact that Hmr^{mel} has been deleted (Table 2). Moreover, these females die as embryos: a large number of brown eggs (and a few dead first-instar larvae) were observed. This result shows that Hmr^{mel} is not necessary for hybrid embryonic lethality. Table 2 further shows that hybrid males who have had Hmr^{mel} added by duplication remain viable. Thus Hmr^{mel} is also not sufficient for hybrid embryonic lethality. Although hybrid females carrying one *maur X* and one *mel X* (in

TABLE 1

Control crosses between *D. melanogaster* and the two *"sib"* species, *D. mauritiana* and *D. simulans*

Cross	Temp.	Females	Males
mel Oregon-R × *maur* Synthetic	22°	704	0
	18°	335	0
maur Synthetic × *mel* Oregon-R	22°	0	410
	18°	1	52
mel y^{1} *Hmr v* × *maur* Synthetic	22°	391	144
	18°	68	11
mel Oregon-R × *sim Ro*/+	22°	409	2[a]
mel Oregon-R × *sim v m*	22°	297	0
sim Ro/+ × *mel* Oregon-R	22°	52	626
sim v m × *mel* Oregon-R	22°	151	388
mel y^{1} *Hmr v* × *sim Ro*/+	22°	253	0[b]
mel y^{1} *Hmr v* × *sim v m*	22°	210	14
mel w$^{-6613.4}$ × *sim Lhr*	22°	227	184

[a] Males presumably resulted from maternal nondisjunction, yielding viable $X^{sim}/0$ males.
[b] *Hmr*'s rescue is much weaker with *sim* than *maur* (Hutter and Ashburner 1987). No males were rescued when using the *sim Ro*/+ stock, although weak rescue occurs with *sim v m*.

a *maur* cytoplasm) are invariably lethal, hybrid males carrying one *maur X* and one piece of the *mel X* including Hmr^{mel} (in a *maur* cytoplasm) are not.

It is, however, worth noting a point that will become increasingly important. Although males carrying a duplication of Hmr^{mel} are not invariably lethal, they do appear to suffer some inviability: *Dp*-bearing males are typically rarer than non-*Dp*-bearing males. Although the effect is frustratingly variable, recovery ratios as extreme as 210:34 are not uncommon. This recovery bias does not reflect a trivial property of the duplication because no such shortage of *Dp*-bearing flies occurs in within-species controls. Indeed *Dp vs.* balancer males appear in almost perfect Mendelian ratios within *mel* (Table 2, lines 1 and 2). Duplications of Hmr^{mel} may therefore cause some hybrid larval lethality. We will return to this point below.

The deficiency/duplication test was extended in one trivial way: it was repeated using another deficiency, *Df(1)ras-v-17* (9D1-2; 10A2-3), that extends farther proximally. Although the required cross (*maur* Synthetic females × *mel Df(1)ras-v-17* ; $Dp(1;2)v^{+75d}$/+ males) proved extremely difficult and only 10 hybrids were recovered, all were, once again, males.

Table 2 also reveals that all of the above results (both intra- and interspecific) remain essentially unchanged at the two temperatures studied (18° and 22°).

The *mel-sim* hybridization: Because species crosses between *mel* and *sim* proved far more difficult (especially in the critical *sim* female × *mel* male direction), some hybridizations were performed at only one temperature, 22°. Table 1 again shows that control crosses behave as expected. Note that the *sim* female × *mel* male cross is leaky. Although female hybrids are rarer than males, they occur at appreciable frequencies.

Does deletion of Hmr^{mel} boost the frequency of these weakly viable females to much higher "male-like" values? The answer is no. Table 2 shows that females who carry no Hmr^{mel} appear (at most) only one-third as often as males. Once again, hybrid males who have had Hmr^{mel} added to their genome are recovered fairly readily. Also once again, however, these males appear to suffer some decreased fitness as *Dp*-bearing males are consistently rarer than their balancer brothers, although the effect is small and variable.

Table 2 also reports the results of a deletion/duplication test in which a much larger *X* chromosome deletion, $Df(1)v^{-L15}$ (9B1-2; 10A1-2), was employed. The results are nearly identical to those obtained with *Df(1)HC133*.

Effect of Hmr^{mel} on hybrid larval viability: The possi-

TABLE 2

Results of the deletion/duplication test shown in Figure 1

		Females		Males		
Cross	Temp.	*Df*	*Df*; *Dp*	+	*Dp*	% *Dp* males
mel Or-R × *mel Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO*						
	22°	694	951	639	687	51.8
	18°	445	605	400	410	50.6
maur Synth. × *mel Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO*						
	22°	0	0	210	34	13.9
		0	0	155	68	30.5
	18°	1	0	246	39	13.7
		0	1	25	26	51.0
sim Ro/+ × *mel Df(1)HC133*; $Dp(1;2)v^{+75d}$/*CyO*						
	22°	7	4	295	188	38.9
		33	21	101	95	48.5
	18°	9	10	35	23	39.6
sim v m × *mel* $Df(1)v^{-L15}$; $Dp(1;2)v^{+75d}$/*CyO*						
	22°	8	14	109	96	46.8

In some cases, the same cross was repeated at different times. When the results of such crosses differed, they are presented as separate rows of data.

TABLE 3

Fitness effects of duplications of *Hmr*^{mel} in *D. melanogaster*-derived cytoplasm

Cross	Temp.	Females +	Females *Dp*	Males +	Males *Dp*	% *Dp* males
mel C(1)RM, y w f; *Dp(1;2)v*$^{+75d}$/+ × *maur v*^{C}						
	22°	0	0	168	120	41.7
	18°	0	0	132	74	35.9
mel C(1)RM, y w f; *Dp(1;2)v*$^{+75d}$/+ × *sim v m*						
	22°	0	0	37	9	19.6
mel C(1)DX, y f; *Dp(1;2)v*$^{+75d}$/+ × *sim v m*						
	22°	0	0	457	59	11.4

TABLE 4

***Lhr* reverses the deleterious effects of duplications of *Hmr*^{mel}**

Cross	Temp.	Females +	Females *Dp*	Males +	Males *Dp*	% *Dp* males
mel C(1)DX, y f; *Dp(1;2)v*$^{+75d}$/*CyO* × *sim v m*						
	24°	0	0	87	68	43.8
mel C(1)DX, y f; *Dp(1;2)v*$^{+75d}$/*CyO* × *sim Lhr*						
	24°	1	21	85	104	55.0

Crosses proved extremely difficult, but proceeded somewhat better at 24°. The rescue of *Dp*-bearing females by *Lhr* is unexpected [particularly as such hybrids should lack most rRNA genes (Takamura and Watanabe 1980)].

bility that *Hmr*^{mel} affects larval viability was tested further by crossing *mel* attached-*X*; *Dp* females to *sib* males and scoring the recovery of *Dp*- and non-*Dp*-bearing hybrid sons. These crosses also allow us to test whether *Hmr*^{mel}'s apparent fitness effect depends on the species origin of the cytoplasm. This is important because, although all of the above crosses produce hybrids carrying *sib* cytoplasm, normally lethal F_1 males carry *mel* cytoplasm.

Table 3 shows that *X*^{sib} hybrid males who carry a duplication including *Hmr*^{mel} are again rarer than their balancer brothers, with dead late larvae and pupae common. Once again, though, the results are variable. In crosses to *maur*, for instance, *Dp*-bearing adults make up ~35–40% of males. In crosses to *sim*, they make up ~10–20% of males.

We can draw two conclusions. First, *Hmr*^{mel} appears to cause some hybrid larval inviability. Indeed, summing across the hybridizations shown in Tables 2 and 3, *Dp*-bearing males are rarer than their balancer brothers in 11 of 12 species crosses, a pattern that is highly significant (Wilcoxon's signed rank test: $z = 2.845$, $P < 0.005$). Second, this fitness effect does not depend on the species identity of the cytoplasm.

These results are consistent with Hutter *et al.*'s (1990) finding that addition of *Hmr*^{mel} as a duplication to otherwise rescued hybrid males carrying the *Hmr* mutation reverses rescue, a result that suggests (but does not prove) that *Hmr*^{mel} produces a product that kills hybrid males. We verified this finding by crossing *mel y Hmr v*/*Df(1)HC133*; *Dp(1;2)v*$^{+75d}$/+ females to *maur* Synthetic males. We recovered 901 hybrid females and 78 non-*Dp*-bearing *Hmr* males (phenotypically v). No *Dp*-bearing males (phenotypically v$^+$) were recovered. As expected, many dead third-instar larvae/pseudopupae were seen. This inviability is not an artifact of an unusual interaction between the *Hmr* mutation and *Dp(1;2)v*$^{+75d}$ because no such lethality occurs within species: the cross of *mel y Hmr v* females to *mel Df(1)HC133*; *Dp(1;2)v*$^{+75d}$/*CyO* males produced 73 non-*Dp*-bearing *Hmr* males (phenotypically Cy and v) and 101 *Dp*-bearing males (phenotypically Cy$^+$ and v$^+$), as well as many females. Together with the above, these results suggest that *Hmr*^{mel} causes (or at least contributes) to hybrid larval lethality between *D. melanogaster* and species belonging to the *D. simulans* clade.

These data are also consistent with Hutter *et al.*'s suggestion that *Hmr* is a loss-of-function mutation. Put conversely, *Hmr*^{mel} appears to act in a gain-of-function manner.

Interaction with *Lhr*: The hypothesis that *Dp(1;2)v*$^{+75d}$'s deleterious effect reflects the action of *Hmr*^{mel} leads to a simple prediction. This effect should be reversible by the hybrid larval rescue mutation, *Lhr*. If, on the other hand, the observed deleterious effect is due to some other factor(s) carried on the duplication chromosome (which acts only in hybrids), the presence *vs.* absence of *Lhr* should be irrelevant. We tested this prediction by crossing *mel C(1)DX, y f*; *Dp(1;2)v*$^{+75d}$/*CyO* females to control sim *v m* and to experimental *Lhr* males. The crosses proved extremely difficult, but 155 hybrids were obtained in the former cross and 209 in the latter (after months of crossing). As Table 4 reveals, the results were consistent with our prediction: *Lhr* allows recovery of significantly more *Dp*-bearing males than in controls ($\chi^2 = 4.24$, $P < 0.05$), although the effect is small.

The effect of extra doses of *Hmr*^{mel}: Although the above results suggest that *Hmr*^{mel} plays a role in larval but not embryonic hybrid inviability, at least one loose end remains. Hutter *et al.* presented suggestive evidence that *Hmr*^{mel} affects embryonic viability. They argued that hybrid males that are normally destined to die as larvae will instead die as embryos if forced to carry an extra copy of *Hmr*^{mel}. In particular, they showed that the cross of *mel Dp(1;2)v*$^{+75d}$/*CyO* females × *maur* males—which is expected to produce males that die as late larvae—instead produces many lethal embryos (Hutter *et al.* 1990, their Table 7). They guessed that these dead embryos were *Dp*-bearing hybrid males; lethal embryos were not seen when using other duplications that do not include *Hmr*.

We performed a similar experiment. Through a series of crosses, we produced *mel* females of the genotype *y*^{2} *cv v f*/*y*^{2} *cv v f*; *Dp(1;2)v*$^{+75d}$/+. In the experimental

TABLE 5

Effects of duplications of *Hmr*^{mel} on embryonic viability

Cross	Fraction	No. dead eggs
mel y^2 *cv v f*; *Dp(1;2)*v^{+75d}/+ × *maur* Synth.	10/10	21.9 ± 9.5
mel y^2 *cv v f*; *Dp(1;2)*v^{+75d}/+ × *mel* Or-R	10/10	20.3 ± 13.5
mel y^2 *cv v f* × *maur* Synth.	6/15	0.53 ± 0.74

"Fraction" is the proportion of crosses that produced any lethal embryos. Last column gives the mean ± standard deviation number of lethal embryos seen in each cross. Data were taken only from those crosses that were successful (*i.e.*, that later produced larvae, showing that females had been inseminated). Lethal embryos were counted on days 5–7.

cross, these *Dp*-bearing *mel* females were crossed to *maur* males (Table 5, line 1). This cross, like Hutter *et al.*'s, should produce dead embryos. In an intraspecific control, *Dp*-bearing *mel* females were crossed to *mel* males (Table 5, line 2). This cross controls for any deleterious effect the duplication (or any other factor in the duplication stock) may have within species. In a second control, non-*Dp*-bearing *mel* females were crossed to *maur* males (Table 5, line 3). This cross controls for the effect of the species cross *per se* on embryonic viability (*i.e.*, the traditional description of this hybridization could be wrong, with lethal embryos routinely appearing).

Our results confirm that lethal embryos appear when *Dp*-bearing females are crossed to *maur* males (Table 5). However, dead embryos also appear when *Dp*-bearing females are crossed within species (Mann-Whitney *U*-test against the intraspecific control: $z = -0.76$, $P > 0.40$). As expected, lethal embryos are very rare in the "normal" species cross in which no *Dp* is used (Mann-Whitney *U*-test comparing lines 1 and 3 in Table 5: $z = -4.27$, $P < 0.0001$). Although these results must be viewed as rough (as we have no guarantee of equal fertilization rates), the embryonic lethality seen in Hutter *et al.* and in the present Table 5 (line 1) may have little to do with the genetics of hybrid inviability. Instead, some factor in the *Dp(1;2)*v^{+75d} stock clearly causes embryonic lethality, whether in hybrids or within species (although we cannot rule out the possibility that different levels of lethality occur within *vs.* between species). This embryonic lethality, however, does not appear to involve the duplication *per se* as intraspecific controls show that duplication and balancer flies appear in Mendelian ratios, at least among males (Table 2, lines 1 and 2).

DISCUSSION

Two conclusions follow from these experiments. First, the wild-type allele *Hmr*^{mel} does not cause hybrid embryonic lethality. This result is essentially proved by the finding that hybrid females who have had the putatively poisonous *Hmr*^{mel} allele deleted remain embryonic lethal. [It is worth noting that Hutter *et al.* also introduced deletions of *Hmr*^{mel} into hybrids. Unfortunately, they only considered the *mel* female × *sib* male direction of the cross (their Table 8). Because hybrid females are already viable in this direction of the cross, deletion of *Hmr*^{mel}, for our purposes, is uninformative.] The present finding thus militates against the original Hutter *et al.* model of hybrid inviability, providing strong support for the later Sawamura *et al.* (1993c) one. Although a good body of data already suggested that *Hmr* (as well as *Lhr*) play no role in embryonic lethality, this inference was based on the behavior of a small set of mutant alleles. The present results, on the other hand, derive solely from manipulations of the wild-type allele at *Hmr*. Fortunately, the mutant and deficiency/duplication approaches reach identical conclusions: hybrid embryonic lethality has some cause (or causes) other than *Hmr*^{mel}.

Second, *Hmr*^{mel} *does* appear to cause hybrid larval lethality. This conclusion, unlike the first, must remain tentative. Our results show that addition of *Hmr*^{mel} to hybrid males who do not ordinarily carry it significantly lowers their viability. Although the magnitude of this effect is frustratingly variable, its direction is consistent. Moreover, the effect is independent of the species origin of the cytoplasm (*mel vs. sib*). D. Barbash, J. Roote and M. Ashburner (personal communication) have recently documented a similar effect: addition of an extra dose of *Hmr*^{mel} to hybrid females lowers their viability relative to balancer controls. Although it is difficult to prove that these duplication viability effects are due to the *Hmr* locus *per se*, this seems likely for two reasons. First, duplications harboring *Hmr*^{mel} have no such viability effect within species (Table 2), suggesting that the effect is tied to the genetics of hybrid inviability, not to some trivial effect of the duplication itself. Second, the deleterious effects of *Hmr*^{mel}-bearing duplications can be reversed by the rescue mutation *Lhr* (Table 4), although the effect is small.

This work supports two key features of the Sawamura model. First, the two forms of hybrid inviability characterizing the *mel-sib* species crosses appear genetically distinct. *Hmr* (and *Lhr*) apparently affect only hybrid larval viability, while *mhr* and *Zhr* apparently affect only hybrid embryonic viability. Second, hybrid lethality appears to result from the action of the wild-type allele at

the *Hmr* locus. Our results thus support the "speciation gene" hypothesis (rescue mutations are alleles at the genes whose wild-type product causes hybrid inviability) and militate against the second-site suppressor hypothesis (rescue mutations are second-site suppressors at genes whose wild-type product does not cause hybrid inviability).

These results also touch on several other properties of *Hmr* that merit discussion. First, the fact that duplications carrying Hmr^{mel} have variable (but deleterious) effects on hybrid viability suggests that hybrid lethality may be a threshold character that is very sensitive to subtle environmental change (see also Orr 1996). This interpretation is strengthened by the fact that duplication viability effects vary considerably even when performing the same cross with the same stocks at different times (see the repeated crosses in Table 2).

Second, Hutter *et al.* argued that the rescue allele *Hmr* is a partial loss-of-function mutation. Conversely, the wild-type allele Hmr^{mel} acts in a gain-of-function manner. The present findings are consistent with this suggestion. Similar results appear to hold at the *Zhr* gene (Sawamura *et al.* 1993b) as well as at the *Tumor* (*Tu*) locus, which causes inviability among certain swordtail-platyfish backcross hybrids (Wittbrodt *et al.* 1989).

It is important to understand what such gain-of-function behavior does and does not imply. Muller (1942) suggested that the genes causing postzygotic isolation often behave as recessives in hybrids. He further claimed that this behavior may explain Haldane's rule [the preferential sterility/inviability of the hemizygous (XY) sex hybrids]. This idea eventually led to the modern dominance theory of Haldane's rule (Turelli and Orr 1995). But as Orr and Turelli (1996) emphasized, "[t]he dominance theory says nothing about *why* the alleles causing hybrid problems act as partial recessives. It merely says that *if* they are recessive, Haldane's rule will follow" (p. 613). Early on, however, Orr (1993b) speculated that the recessivity of speciation genes might imply that they act in a loss-of-function manner when placed on a hybrid background. The above findings suggest that this speculation is wrong. But this conclusion does not affect the dominance theory *per se*. The dominance theory holds only that the fitness of $l/+$ hybrids (where l represents a hybrid lethal/sterile) is closer to that of $+/+$ than l/l hybrids, regardless of the biochemical basis of this relationship. In short, "speculation about mechanism can be distinguished from the dominance theory *per se*" (Orr and Turelli 1996).

In summary, the deletion/duplication tests reported here allow us to rule out the simplest models of how Hmr^{mel} may cause postzygotic isolation. They instead provide strong support for the alternative, and reigning, Sawamura *et al.* (1993c) model. Given that Hmr^{mel} may be a direct cause of hybrid larval inviability, its genetic and molecular characterization may well provide a valuable window on the genetic causes of speciation.

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