

A Hybrid Incompatibility Factor in *Tribolium castaneum*

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Hybrid incompatibility and infertility were observed between strains of *Tribolium castaneum* from India (T-1) and Canada (C). The severity of the syndrome depended on cross direction and temperature. When reared at 32°C, adult hybrids with T-1 fathers produced a high frequency of somatic abnormalities, reduced fertility, and shortened life span. Hybrids with T-1 mothers were normal. At 25°C, abnormalities were more severe and occurred in both reciprocal crosses: hybrids with T-1 fathers died as larvae or pupae, whereas hybrids with T-1 mothers showed a syndrome similar to that produced by T-1 fathers at 32°C. Among a set of strains representing diverse, world-wide geographic origins, eight produced inviable hybrids and nine produced normal hybrids when females were crossed to T-1 males. One of the latter also produced normal hybrids when males were crossed to C females. The syndrome was associated with a dominant factor, H (Hybrid incompatibility factor), which mapped to the ninth linkage group.

Studies of hybrid inviability and hybrid sterility have revealed a diversity of phenomena relevant to evolution and population genetics. Examples include the identification of X-linked genes and their suppressors, associated with speciation in *Drosophila* (Coyne and Orr 1989; Hutter et al. 1990); microorganism-mediated cytoplasmic incompatibility (Breeuwer and Werren 1990); selfish genes (Beeman et al. 1992); and transposable element-mediated hybrid dysgenesis (Kidwell et al. 1977). Because each of these examples involves a unique mechanism, investigating new cases of hybrid inviability and sterility should be a productive enterprise.

Studies of hybrid dysgenesis in *Drosophila* have led to the discovery of transposable elements that have been developed into useful tools for molecular genetic manipulations (Bingham et al. 1981; Blackman et al. 1989; Cooley et al. 1988; Gloor et al. 1991; Robertson et al. 1988; Rubin and Spradling 1982; Wilson et al. 1989). With the aim of developing similar tools for use in the flour beetle, *Tribolium castaneum*, we assembled a large collection of wild strains from globally diverse geographic origins and performed intercrosses to search for reduced hybrid fertility. In addition to revealing a maternally acting selfish genetic element (Beeman et al. 1992), this effort led to the discovery of a novel incompatibility

syndrome, which we describe in this report.

Materials and Methods

Unless noted otherwise, all *T. castaneum* strains came from the World Strain Collection maintained by the Agricultural Research Service, U.S. Department of Agriculture, U.S. Grain Marketing Research Laboratory (USGMRL). These strains were provided by individual collectors and scientists in 1989. The T-1 strain originated from a single pair from the T strain collected in Puranpur village, Bareilly District, Uttar Pradesh, India, June 1988 by S. N. Tiwari. The C strain was collected in Argyle, Manitoba, Canada, October 1988 by N. D. G. White. GA-1 and Lab-S are standard laboratory stocks that originated in the United States and have been kept in culture for >10 years. The *T. confusum* strain, b-yugo, was acquired from Michael Wade, University of Chicago. Several *T. castaneum* strains bearing genetic markers were used for recombinational mapping (Table 1).

Mass crosses involving the hybrid incompatibility factor (H) were performed by placing five beetles of each sex on 5 g of medium (19:1 v/v wheat flour/brewer's yeast) in a 118-ml glass bottle. The beetles were allowed to mate and oviposit for 7 days at 30–32°C or 14 days at 25°C. Parents

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Table 1. Strains and their visible markers used for recombinational mapping of the H factor in *T. castaneum*

Strains	Visible markers	Linkage group
mms	<i>missing abdominal sternite</i> (A^{mas})	2
	<i>sooty</i> (<i>s</i>)	4
	<i>ruby</i> (<i>r</i>)	5
	<i>antennapedia</i> (<i>ap</i>)	8
	<i>Reindeer</i> (<i>Rd</i>)	2
Rd mas p	A^{mas}	2
	<i>pearl</i> (<i>p</i>)	9
baptsac	<i>alate prothorax</i> (<i>apt</i>)	2
	<i>black</i> (<i>b</i>)	3
	<i>chestnut</i> (<i>c</i>)	7
	<i>short antennae</i> (<i>sa</i>)	unknown
Se p	<i>Short elytra</i> (<i>Se</i>)	9
	<i>p</i>	9
T(2;9)mxp ^{Dch-1} /A ^{Ea-1}	<i>short legs and antennae</i> (T(2;9)mxp ^{Dch-1})	2,9
	<i>Extra sclerite</i> (A^{Ea-1})	2

were then removed, and an additional 25 g of medium was added. Five weeks (30–32°C) or 11 weeks (25°C) later, progeny were collected using a U.S. standard sieve no. 25, and all retained insects were scored. By this method, we assessed adults, pupae, and late larval instars, but ignored early instars that passed through the 25-mesh screen. Single pair crosses were performed in the same fashion with smaller amounts of medium in 8-dram shell vials.

We conducted drug treatments to test for a possible role of microbial symbionts. Beetles were reared from egg to adult stage in medium containing 0.125% or 1.25% (w/w) tetracycline-HCl (Sigma), and then aged for an additional 20 days in a fresh preparation of tetracycline-treated medium before testing. Other beetles were treated in a similar way with 0.11% (w/w) of the anti-protozoan, bicyclohexylammonium fumagillin (5% w/w, Mid-Con Fumidil B). However, these insects were aged as adults in drug-free medium.

Results

Previously, we screened >3,000 crosses involving >100 strains from 25 countries for reduced hybrid fertility. These crosses were done at the standard rearing temperature of 32°C. Several cases of reduced fertility were recovered. One of these was a hybrid incompatibility syndrome (HI) involving a cross between males of the T strain of India and females of the C strain of Canada. Among hybrids with T fathers, one or more of the following was observed: deformed elytra (not meeting at mid-line, wings unfolded), tremor and incoordination, infertility, atrophied gonads, and short adult life span (death at eclosion or shortly thereafter). Male and female hybrids were affected equally. Because results from this cross were variable, we subsequently tested single pair lines from the T strain and identified one, T-1, which consistently produced HI in outcrosses to C females. This line was used for further studies.

Table 2 shows the effects of cross direction and rearing temperature on the severity of HI. At 32°C, all inter- and intra-strain crosses produced adult progeny in roughly the same numbers. However, 75% of the progeny produced by the cross T-1 males × C females were abnormal (malformed elytra or tremor or both) and/or short-lived. In contrast, no more than 17% of the progeny of the reciprocal or intra-strain crosses displayed abnormalities. Twelve progeny of each sex without obvious morphological or behavioral abnormalities were selected from each of the two interstrain crosses. Each of these hybrid beetles was mated in single pairs to both the T-1 and C strains. Nine female and nine male hybrids with T-1 fathers were sterile, whereas only one female hybrid with a C father was sterile. At 25°C, the severity of HI increased dramatically and was detected (albeit unequally) in each of the reciprocal crosses. All hybrid progeny with T-1 fathers died before reaching the pupal stage (Table 2). Judging by the number of dead late instar larvae collected, and assuming that this cross had normal fertility, most hybrid progeny must have died as embryos or early instars. At 25°C the reciprocal cross (C male × T-1 female) did express HI, although it was less severe, producing normal numbers of adults, over half of which were abnormal. Experiments involving temperature shifts between 32 and 25°C revealed no discrete temperature sensitive period. Rather, the severity of HI depended on the proportion of development at the lower temperature (data not shown).

Role of Microbial Symbionts

Infections with rickettsiae have been associated with unidirectional hybrid incompatibility in a diversity of insect species (Breeuwer and Werren 1990). These incompatibilities have been effectively removed by treating the insects involved with antibiotics such as tetracycline (Wade and Stevens 1985). To test the role of microorganisms in HI, we treated the T-1 and C strains with 1.25% (w/w) tetracycline-HCl. As a positive control, we also treated a rickettsia-infected *T. confusum* strain, b-yugo, that shows incompatibility when crossed to strains lacking the infection. Beetles from the b-yugo strain, either untreated or treated with 0.125% tetracycline-HCl (10-fold less concentration than *T. castaneum* treatment) were checked for the characteristic effects of tetracycline on cytoplasmic incompatibility (Wade and

Table 2. Effect of rearing temperature on hybrid incompatibility in *T. castaneum*

	T-1 × C	C × T-1	C × C	T-1 × T-1
Rearing temperature = 32°C				
Total adults	186 ^a ± 8	223 ± 40	222 ± 24	251 ± 53
Normal (%)	25 ± 16	83 ± 12	97 ± 1	85 ± 7
Abnormal ^b (%)	50 ± 3	10 ± 5	1 ± 1	6 ± 3
Dead (%)	25 ± 13	7 ± 8	2 ± 2	9 ± 5
Rearing temperature = 25°C				
Total adults	0 ^c	183 ± 56	149 ± 34	186 ± 13
Normal (%)	NA	45 ± 18	96 ± 3	80 ± 6
Abnormal ^b (%)	NA	20 ± 5	1 ± 1	8 ± 2
Dead (%)	NA	35 ± 23	3 ± 3	12 ± 4

^a Data are expressed as mean number of adult progeny produced by three mass crosses of 5 males × 5 females each, followed by standard deviation. The male parent is shown first in the cross, followed by the female parent.

^b Malformed and/or incapable of coordinated walking.

^c Although no progeny survived to adulthood, this cross produced a mean of 78 ± 31 dead, late instar larvae. In contrast, no more than three larvae or pupae were recovered from other crosses.

NA = not applicable.

Table 3. Geographic distribution of *T. castaneum* strains producing hybrid inviability when crossed to T-1 males

Strain	Country of origin	Severity of incompatibility ^a
N	Canada	+
Lab-s	U.S.A.	-
Ga-1	U.S.A.	-
CR-1	Costa Rica	++
ab	Colombia	++
Brz-1	Brazil	++
Kent	England	-
Pruz-1	Poland	-
Ug-3	Uganda	-
Solet	Israel	+
Dwi-3	India	-
Tiw-1	India	-
Ho-tcs	Singapore	++
Rej-1	Philippines	++
CTC-486	Australia	-
<i>T. freemani</i>		- ^b

^a At 25°C: ++ = most hybrids die as embryos or young larvae; + = most hybrids survive to late larval or pupal stage, but die prior to adult eclosion; - = all hybrids are fully viable.

^b Although hybrids were viable, all hybrids tested (nine males, 12 females) were sterile.

Stevens 1985) by intrastain crosses. In the *T. confusum* control, crosses between treated males and treated females were fertile, whereas crosses between untreated males and treated females were infertile. Thus, tetracycline had the expected effect on infected *T. confusum*. However, tetracycline had no effect on HI (data not shown), despite the 10-fold greater dose given to *T. castaneum*. Treatment with the antiprotozoan bicyclohexylammonium fumagillin at a concentration of 0.11% (wt/wt) also failed to affect HI (data not shown).

Strain Variation

A geographically diverse collection of *T. castaneum* strains was surveyed by crossing single T-1 males to three virgin females in duplicate at 25°C. We also tested the closely related species, *T. freemani*, that normally produces sterile but viable hybrids when crossed to *T. castaneum*. Three distinctly different outcomes were observed (Table 3): (1) no HI; (2) moderate HI (similar to T-1 male × C female; i.e., >40 hybrid progeny recovered, these being dead or dying pupae or larvae); and (3) extreme HI (≤13 hybrid progeny recovered, these being dead or dying larvae). All results were highly reproducible. One of the maternal strains, ab, that typified the third and most severe outcome, was mass crossed to T-1 males, and the progeny were reared at 31°C. This cross produced inviability that was equivalent to that produced by the cross T-1 male × C female at 25°C (data not shown). We in-

fer that the crosses resulting in severe HI at 25°C were fertile, but that almost all hybrid progeny died as embryos or young larvae. Thus, strains incompatible with the T-1 strain were found to be distributed worldwide, and varied in the severity of HI they produced. One of the T-1-compatible strains, GA-1, was crossed to the C strain (six single pair crosses of GA-1 male to C female) to test for HI. GA-1 males did not produce HI when crossed to C females (data not shown). Therefore, at least one strain exists that is "neutral" (compatible with both the T-1 and C strains).

Inheritance

In order to assess if HI has a genetic component, hybrids derived from the viable cross, C males × T-1 females reared at 32°C, were backcrossed to each parent strain for two generations in single pairs. Some of these crosses were repeated using hybrids with a T-1 father and the neutral strain, GA-1, mother. Cross results were scored as either (A) 100% inviable progeny or (B) several dozen viable progeny produced. None of the first generation of backcrosses (F₁ × parental) were 100% inviable (Figure 1). However, three of the six backcross categories in the second generation (F₂ × parental) included some 100% inviable crosses. As discussed below, this pattern can be explained by the segregation of an autosomal hybrid incompatibility factor (H), the effect of which is modified by maternal factors.

Mapping the H Factor

Recombinational mapping of the H factor was performed using genetically marked females from strains incompatible with T-1. Seven of the nine autosomal linkage groups were represented in these crosses (Table 1). Normal hybrids were produced by mating T-1 females with marker strain males and rearing at 30°C. Male hybrids were then backcrossed to females of the marker strain or a wild-type incompatible strain and reared at 25°C, conditions that are expected to produce 100% preadult mortality in those progeny receiving a paternal chromosome bearing the H factor. Linkage to the H factor in trans is expected to result in preferential recovery of the marker. As a control to confirm normal segregation ratios in the absence of HI, female hybrids were backcrossed to males of the marker strain or wild-type incompatible strain, and the progeny were reared at 30°C. Meiotic recombination occurs equally in both sexes in *T. castaneum*. Markers from six of the seven linkage

groups tested showed no evidence of linkage to H. Lack of sex linkage was indicated by absence of sex ratio distortion in the crosses in which the H factor was segregating and expressing. However, the H factor showed linkage to *Se* and *p*, closely linked markers on the ninth autosomal linkage group. Recombination percentages between H and *Se* or *p* were 19 and 21, respectively (Table 4). In addition, the H factor showed no recombination (*N* = 228) with *mxp*^{Dch-1}, a T(2;9) translocation that shows ~20% recombination with *p* (Sokoloff et al. 1983; Beeman RW, et al., unpublished data).

Discussion

We have described a case of interstrain hybrid incompatibility (HI) in *T. castaneum* that is characterized by somatic abnormalities and low fertility. This incompatibility is nonreciprocal and temperature sensitive. We suggest that HI occurs when a dominant autosomal H factor is present, modified by the presence of a particular maternally contributed cytotypic. With respect to the initial observations, HI occurred at 32°C when hybrids received the H factor from a T-1 father (homozygous for H) and cytotypic from a C mother (lacking H), but not in the reciprocal cross, in which progeny of the same genotype received a T-1 cytotypic. HI is more severe in progeny of a T-1 male and a C female raised at 25°C. At this lower temperature, HI can also be detected in the progeny of the reciprocal cross, albeit to a lesser degree. This model is consistent with the observation that intrastain crosses are compatible: in the T-1 strain, the H factor is inactive because of the T cytotypic, whereas no H factors are present in the C strain.

Segregation of the H factor was observed in the progeny of the cross shown in box 1 of Figure 1. Among the progeny of this cross, some males (presumably homozygous for H) test-crossed to +/+ females gave no viable progeny, whereas their siblings (presumably heterozygous) gave some viable progeny. Segregation was also observed in the G2 of a neutral strain mother, GA-1, crossed to a T-1 father (Figure 1A, box 3). The observation of segregation is consistent with our interpretation that H is a chromosomal factor.

Other results, summarized in Table 2, suggested that additional chromosomal factors act zygotically to modify HI. Although hybrids with T-1 mothers were completely protected from HI at 32°C, this

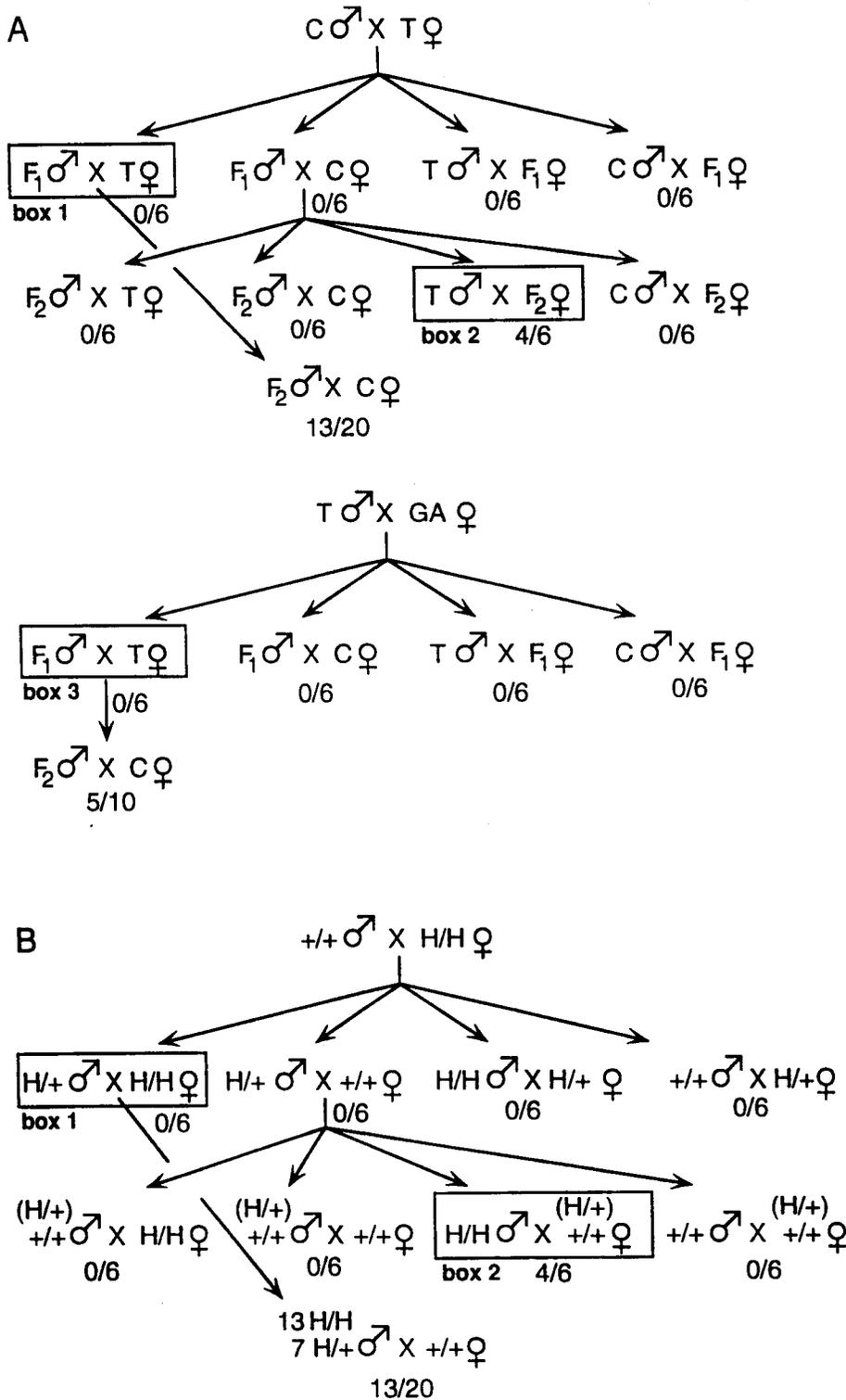


Figure 1. Inheritance of hybrid incompatibility in *Tribolium castaneum*: (A) The C and T-1 strains (the latter abbreviated as T) were mass-crossed under conditions that give viable hybrid progeny, i.e., C males \times T females at 31°C; F₁ progeny were backcrossed to either parental strain for two consecutive generations as shown; similar crosses were done using T males and females of the neutral strain GA-1 (here abbreviated as GA); all backcross generations were reared at 25°C to permit expression of incompatibility; the fraction under each backcross indicates the number of single pair crosses that produced no viable progeny divided by the total number of single pair crosses made: (B) The crosses from A have been redrawn, indicating genotypes that reflect the assumption that the T strain is homozygous for a dominant hybrid incompatibility (H) factor that is usually lethal in the presence of C cytoplasm (note exception in box 2); the H factor is assumed to be absent in the C strain; genotypes in parentheses are assumed to be inviable.

Table 4. Recombinational mapping of the H-factor in *T. castaneum*

Visible marker	Type of backcross	Total number of progeny ^a	Percentage	
			Number with marker phenotype	with marker phenotype
pearl	Incompatible	433	344	79
	Compatible	205	114	56
Short elytra	Incompatible	442	359	81
	Compatible	92	37	40
T(2;9)mxp ^{bch-1}	Incompatible	228	228	100
	Compatible	114	67	59

^a Progeny produced by backcrossing hybrids of T-1 and marker strains to the marker strain (marker is recessive) or wild-type permissive strain (marker is dominant). Compatible backcrosses (female hybrid, reared at 30°C) favored survival of progeny inheriting an H factor. Incompatible backcrosses (male hybrid, reared at 25°C) favored larval death of progeny inheriting the H factor. Data are presented for six independent crosses.

protection was only partial at 25°C, as indicated by a higher incidence of somatic abnormality and shortened adult life span at the lower temperature. Progeny of the T-1 intrastain cross were unaffected by low temperature. Therefore, the T-1 strain must have factors that convey insensitivity to the intensifying effects of low temperature. These factors would be diluted or could exist as unexpressed recessives in heterozygous progeny of the interstrain crosses.

Cytype is apparently modified by factors other than H. Firstly, both T-1 (H present) and GA-1 (H absent) exhibited compatible cytypes in crosses with T-1 males. Secondly, if cytype depends only on the maternal presence of an H factor, we would predict that none of the surviving +/+ female progeny (Figure 1, box 2) should yield viable offspring when backcrossed to T-1 (H/H) males. Although four progeny followed this prediction, two did produce viable progeny. This observation suggests that cytype does not depend exclusively on H. An alternative explanation is that some of the F₂ H/+ hybrids escaped the lethal effect; i.e., H was incompletely penetrant.

One possible mechanism for the model we propose is that the H factor is an autonomous transposable element and that HI is hybrid dysgenesis. Hybrid dysgenesis is a syndrome of genetic instability and reduced fertility seen in the hybrids of some conspecific strains. The syndrome is generally not seen within the pure strains, but only in the hybrids of one cross direction. Most cases are temperature sensitive. Described cases of hybrid dysgenesis

include the P-M (Engels 1989), I-R (Finnegan 1989), and hobo (Blackman and Gelbart 1989; Blackman et al. 1989) systems of *Drosophila melanogaster*, and a system in *D. virilis* (Lozovskaya et al. 1990; Scheinker et al. 1990). In all of these, dysgenesis has been associated with high-frequency mobilization of transposable elements in the germline tissues of hybrids. Infertility has been linked to cell death, presumably caused by lethal rearrangements in dividing cells (Engels 1989; Finnegan 1989). Control of hybrid dysgenesis depends on the presence of autonomous elements and a permissive cytotype associated with the absence of autonomous and nonautonomous elements maternally. Autonomous elements are fully functional, i.e., capable of mediating their own transposition as well as that of nonautonomous elements. Nonautonomous elements lack sequences required to catalyze transposition, but can contribute factors to the maternal cytotype that restrict transposition (Rio 1990). Strains vary in potential for causing hybrid dysgenesis, and some appear to be neutral. Hybrid dysgenesis is often temperature sensitive, although the relationship varies between cases: dysgenesis is more severe at high temperature in the P-M (Engels 1989) and *D. virilis* (Lozovskaya et al. 1990; Scheinker et al. 1990) systems; it is more severe at low temperatures in the I-R system (Finnegan 1989).

HI may be similar to cases in which a single autonomous element (the H factor) mobilizes many nonautonomous elements (factors that modify HI maternally and zygotically). For example, Chomet et al. (1991) described a strain of maize carrying one regulatory locus that controls the excision of the *Mu* family of transposons. The regulatory locus is itself a *Mu* transposon whose presence is required for the activity of other *Mu* transposons in the genome. The *Mos* factor of *Drosophila* was first discovered as a dominant autosomal factor whose presence stimulated excision of *mariner* elements (Bryan et al. 1987). *Mos* was identified subsequently as an autonomous *mariner* element (Medhora et al. 1988).

The hybrid dysgenesis model of HI presumes that observed somatic abnormalities and low fertility of inviable beetles are due to high frequency transposition of a mobile element. Similar abnormalities in dysgenic *D. melanogaster* have been ascribed to such a mechanism. For example, it has been suggested that P element mobilization causes dominant lethal chromosome breakage in germline cells, re-

sulting in atrophied gonads (Engels 1989). Similarly, chromosome breaks left by incomplete I element transpositions have been invoked to explain the infertility of females from I-R dysgenic crosses (Finnegan 1989). The somatically active $\Delta 2-3$ P element causes pupal lethality (Engels et al. 1987) that is possibly analogous to the late larval and pupal lethality seen in some T-1 crosses. The poor vigor and variety of abnormalities of HI adults suggest a generalized somatic cell death syndrome reminiscent of X-ray-induced chromosome damage in *Tribolium* larvae (Sokoloff 1977).

In the P-M, I-R, and *D. virilis* systems, hybrid dysgenesis is restricted to the germline. However, restriction of transposition activity to the germline is not a rule for transposable elements in general. For example, the *mariner* element of *Drosophila* is quite active in somatic tissues, producing hypermutability, but without the cell death associated with P-M hybrid dysgenesis (Hartl 1989). As noted above, the $\Delta 2-3$ P element is somatically active (Engels et al. 1987).

Two alternatives to the hybrid dysgenesis mechanism of HI, cytoplasmic incompatibility and interspecific hybrid inviability, are less robust explanations of the phenomenon. Many cases of cytoplasmic incompatibility have been described in insects (Breeuwer and Werren 1990). In these, hybrid death occurs very early and is due to developmental defects in zygote formation (Breeuwer and Werren 1990; O'Neil and Karr 1990). Symbiotic microorganisms are associated with cytoplasmic incompatibility, and the phenomenon can be eliminated by treatment with an antibiotic. Inheritance is entirely cytoplasmic. Although there is a cytoplasmic determinant involved in HI, chromosomal factors clearly are also involved. The insensitivity of HI to treatments with an antibiotic or antiprotozoan agent does not support a role for microorganisms. The observation of HI in both reciprocal crosses at 25°C (albeit unequally) is inconsistent with the strictly unidirectional incompatibility usually associated with rickettsiae.

Inviability associated with interspecific hybrids is similar to HI, but typically shows a different pattern of inheritance. For example, a variety of hybrid defects are known to occur in interspecific crosses between *Drosophila melanogaster* and its sibling species, *D. simulans*, *D. mauritiana*, and *D. sechellia*. These defects may include embryonic death, larval-pupal

death, or adult sterility. Also, these effects have definite zygotic and maternal determinants (Hutter et al. 1990). However, for the *Drosophila* system and for the majority of interspecific hybrids from other taxa, inviability and sterility are associated with factors on the X chromosome (Coyne and Orr 1989). No obvious sex-linked effects have been observed for HI. To our knowledge, there is no previously recognized subdivision of *T. castaneum* into subspecies or races.

The possibility that the H factor may be an autonomous transposable element that mobilizes nonautonomous elements to produce HI suggests several testable predictions. For example, the mapped position of an autonomous transposable element may not be stable. Additional mapping experiments using other isolates from the original T strain may reveal new loci with H factor-like activity. An autonomous element without nonautonomous elements to serve as "ammunition" for chromosome damage is expected to produce a milder hybrid dysgenesis syndrome. Using the tightly linked, homozygous lethal marker *mwp^{Dch-1}*, the H factor can be extracted into an incompatible genetic background (presumably lacking nonautonomous elements) and then tested for ability to produce HI. Finally, an autonomous element should occasionally mobilize to new genomic locations. Currently available balancer chromosomes (unpublished data) could be passed through an HI individual, segregated away from the H factor, and then tested for H factor-like activity that would indicate mobilization of the H factor independent of recombination. These possibilities are now being examined.

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