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Molecular analysis of a candidate gene for the reproductive isolation between sibling species of *Drosophila*

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Abstract. The X-linked gene *Hmr* in *Drosophila melanogaster*, when mutated, rescues otherwise inviable interspecific hybrids from crosses between *D. melanogaster* and any of its three most closely related species *D. simulans*, *D. mauritiana* and *D. sechellia*. DNA from the site of a breakpoint at the putative locus of the gene has been cloned, and results of transcription and sequence analyses are presented. Three distinct mRNAs are transcribed from this locus, two of which are abundantly expressed throughout life. A third transcript, which is larger but rarer, appears to be disrupted by at least one of the two known mutations of *Hmr*. The gene encodes a mitochondrial ADP/ATP translocator protein, which plays an essential role in maintaining metabolic energy. Analysis of several cDNAs suggested that the rescue of hybrids may be dependent on mutations in the variable 3′ end region of this gene, affecting the level and/or the stability of the largest messenger RNA.
Key words. *Hmr*; reproductive isolation in *Drosophila*; ADP/ATP translocator protein (ANT); speciation.

The biological species concept is defined in terms of the mechanisms operating on reproductive isolation. These are thought to stem from incidental genetic changes which eventually act as barriers, preserving the integrity of novel species-specific genetic systems. The fundamental question of exactly how reproductive isolation arises between two subpopulations of a species, resulting ultimately in what is described as a speciation event, awaits a more satisfactory answer. Although most researchers[1–4] have traditionally emphasized the importance of allopatric genetic divergence in order to explain the establishment of postmating reproductive barriers (i.e. hybrid sterility and hybrid inviability), the 'key' or 'trigger' genetic events underlying these processes at the time of speciation remain difficult to identify. Indeed, neither the exact circumstances under which species formation originates nor the factors instrumental in the process can be deduced from the fixed genetic patterns that recent species possess. The neodarwinian scenario of speciation, based on polygenic changes which are a by-product of differing selective regimes or a result of genetic drift, has probably discouraged workers from searching for major genes responsible for the inviability or sterility of interspecific hybrids. Nevertheless, some evidence has recently come to light, indicating that the genetic factors involved in reproductive isolation may not be scattered across the genome. Indeed a restricted number of genes or gene clusters have been shown to play an important role in hybrid sterility in *Drosophila*[5–9], in mice[10], and in hybrid inviability between *Mimulus* species[11] or between *Xiphophorus* species[12]. Interestingly, there is a possibility that factors affecting male sterility in *Drosophila* are tightly clustered, as shown by introgressions of small pieces of chromosome from a closely related species in an otherwise pure species background[9, 13, 14], and such a pattern may also be relevant to the data presented in this article. Besides, there are now a few established cases, in both plants and insects, where one component of the reproductive isolation between sibling species has been found to be overridden by single gene mutations (see later for references), implying that a single biochemical pathway may underlie these reproductive barriers.

In *Drosophila*, the early stages of speciation appear to start from recessive changes at genes on the X-chromosome, followed by divergence at autosomal loci[15]. It has been known for a long time that the inviability of *Drosophila* hybrids is one important component of reproductive isolation[16–18], and this reproductive barrier appears to occur at an early stage of an incipient process of speciation[15]. Furthermore, within the genus *Drosophila*, the number of genes which cause hybrid inviability between closely related species appears to be less than 1/10 of those for hybrid sterility[14]. Among species of the *Drosophila melanogaster* species subgroup, four mutations affecting hybrid viability have already been discovered either in natural populations or as laboratory mutants; these mutations allow the rescue of the normally inviable interspecific hybrids, which die either as embryos or as third instar larvae.

Early observations[19, 20] and later studies[21] indicated that *D. melanogaster* and *D. simulans*, when intercrossed, essentially produce unisexual progeny of the sex of the *melanogaster* parent. Indeed, progeny of the sex of the *simulans* parent regularly die before metamorphosis. Moreover, as recently reported[22], the heterogametic hybrids from the above crosses have a reduced fitness because they lack an X-chromosome that is "compat-

ible" with the autosomes of one parental species, thus supporting the traditional explanation for Haldane's rule[16] for hybrid inviability. Hybrids between these two species have been found in the wild[23, 24]. The first mutation (*Lhr*) affecting the viability of the hybrids between these species was discovered on a second chromosome of *D. simulans* from a Japanese wild population[25]. This autosomal allele rescues the otherwise lethal hybrid sons from the cross between *D. simulans* fathers and *D. melanogaster* mothers. A second mutation (*mhr*) was recently identified also on the second chromosome of *D. simulans*[26], which, if not an allele of *Lhr*, further points to a probable multigenic basis for the inviability hybrids between species. The *mhr* mutation rescues the usually poorly viable daughters from the reciprocal cross between *D. melanogaster* fathers and *D. simulans* mothers. The third mutation (*hmr*) was discovered on the X chromosome of *D. melanogaster*, also from a wild population[27]. When *D. melanogaster* females carrying this mutation are crossed to males of *D. simulans*, *D. mauritiana* or *D. sechellia*, the otherwise lethal hybrid sons[28, 29] are rescued by *hmr* (hybrid daughters from these crosses survive anyway). Normally lethal hybrid daughters from the cross of compound-X *D. melanogaster* females to males of its sibling species are also rescued by this mutation[30]. Furthermore, normally lethal hybrid females from the reciprocal cross of *D. simulans* females to *D. melanogaster* males, are rescued by a dominant mutation[30], probably allelic to *hmr* (*Hmr*, see below). Hybrid sons from this latter cross normally survive. The fourth mutation (*Zhr*) was recently discovered in the centric heterochromatin of the X chromosome of *D. melanogaster*[31, 32], and rescues the normally embryonic lethal hybrid daughters from the cross between *D. simulans* females and *D. melanogaster* males.

Even though it is formally possible that the five mutations listed above represent major genes determining hybrid inviability, it seems very likely that additional minor genes implied in hybrid inviability will soon be identified, in a way analogous with hybrid sterility genes[33]. This once more points out the complexities of understanding the genetic basis of genes involved in reproductive isolation between species which have not diverged in the very recent past, as genetic analysis probably overestimates the number of changes actually responsible for reproductive isolation. Indeed a multigenic basis of any component of reproductive isolation is likely to be a common feature for species which have diverged a long time ago. Recent studies on sterility of hybrids between members of the *Drosophila* clade[13, 34] have suggested that epistatic interactions between conspecific genes (of minor or no effect individually) in a hybrid background, are probably quite common. Given that an imbalance between genes on the X chromosome and genes on the autosomes is most probably crucial to genes causing hybrid inviability[14, 16], it is not surprising that the prevalent mode of hybrid inviability also involves major as well as several minor genes.

Earlier observations, from an analysis of the viabilities of 'synthetic partial hybrids' between *D. melanogaster* and *D. simulans*[35], had suggested that nine or more genes might be implicated in the inviability of the interspecific hybrids. Besides, recent results[36] have confirmed that the Y chromosome from *D. simulans* plays no role in the above hybrids' deaths. Still, the genetic analysis of the two putative alleles of *Hmr* has led to a model essentially involving a mere two-locus interaction to account for at least the initial genetic basis of hybrid inviability[30]. The model is based on a lethal action of the X-linked gene Hmr^+ in *D. melanogaster*, whose effect is normally suppressed within *D. melanogaster* itself by a suppressor on chromosome 2. This suppressor may be homologous to the *Lhr* gene of *D. simulans*. The model predicts that any interspecific hybrid which is hemizygous (in males) or homozygous (in compound-X females) for Hmr^+, but necessarily heterozygous for its autosomal suppressor, should be lethal. This hypothesis is consistent with all the current genetic data. According to this model, Hmr^+ is expected to encode a deleterious product, normally offset by the product of the *melanogaster Lhr* allele[37], and the work described in this article was aimed at investigating this possibility through a molecular analysis of the *Hmr* locus. Moreover, there is now good evidence for a direct interaction between *Hmr* on the X chromosome of *D. melanogaster* and *Lhr* on the second chromosome of *D. simulans* (M. Ashburner, pers. commun.).

As outlined above, three mutations are known in *D. melanogaster* to rescue the above interspecific hybrids. All three mutations act zygotically, are X-linked and two of them are probably allelic at the *Hmr* locus. The first allele found at this locus acts as a recessive by genetic criteria and maps meiotically to the 9E region[27]. This allele will be referred to as *hmr* throughout this article, whereas the second allele, which will be referred to as *Hmr*, behaves as a dominant mutation and was discovered on an X-chromosome carrying an inversion with one breakpoint first erroneously localized in 9E 1.2 (ref. 30). Results from in situ hybridization to polytene chromosomes of a clone spanning the breakpoint site suggested that the break lies more proximally, most probably in the very thin bands around 9E6, which is more in line with the original localization in 9 E 7.8 (ref. 38). This observation is further confirmed by the fact that the above clone was obtained from a chromosomal walk extending proximally from an entry point at 9E3.4. The chromosome carrying the *hmr* allele was found to harbour a P transposable element inserted at 9E1.2, along with a few other P elements elsewhere in the genome. However, it must be stressed that, if the two mutations are indeed allelic, the 9E1.2 site appears

to be irrelevant to *Hmr*, as this site is known to be separated from 9E6 at least by the *pur1*, *gua1*, *ras* complex unit whose genetic function is related to purine metabolism[38,39]. We have analyzed the genetic activity at both sites and we present data which indicate, by exclusion, that *Hmr* may lie at 9E6, where it would encode an adenine nucleotide translocator (ANT) protein.

Materials and methods

Construction of genomic libraries. Genomic libraries of mutants were constructed by *Sau*3A partial digestion of high molec. wt total genomic DNA. Size-selected DNA (15–30kb) fractionated through sucrose gradients was ligated to the *Bam*HI sites of lambda phage EMBL3[40]. Ligated DNA was packaged using commercially available extracts and phages were propagated in KH802 cells.

Chromosome walks. Chromosome walks were performed according to standard procedures[41,42], with phage lambda genomic libraries from the Canton-S Maniatis standard library in Charon 4 phage, from libraries made in our laboratory in EMBL3 phage and in a Lorist 6 cosmid vector.

Preparation of phage DNA. Phage DNA preparation was as described in ref. 42.

Preparation of *Drosophila* nucleic acids. Total genomic DNA was extracted from adults homogenized in a dounce homogenizer in *Drosophila* grinding buffer and treated as described in ref. 42.

Total RNA was obtained from second, third instar larvae and adults, homogenized in a polytron in the following RNA extraction buffer: 100mM Tris-HCl pH 9, 100mM NaCl, 20mM EDTA, 1% Sarcosyl-30[43]. RNA was extracted in phenol/$CHCl_3$/isoamylalcohol (50:50:1). Polyadenylated RNA was selected by oligo (dT)-cellulose.

Southern transfer and hybridization. Digests of phages were electrophoresed in 0.9% agarose gels and transferred to Nytran membranes in 0.4 M NaOH according to standard procedures[44,45]. Probes were made either from isolation of elecrophoresed digested fragments[46] or from subclones in pEMBL9 vectors. Probes were labelled by random priming with hexanucleotides[47]. Prehybridization and hybridization were performed under appropriate conditions[48]. Filters were washed four times 30 min, firstly twice in 2X SSPE and 0.2 %SDS at 65 °C, then twice in 0.2X SSPE and 0.2% SDS at 65 °C.

Northern transfer and hybridization. Electrophoresis of poly (A^+) RNA and total cellular RNA (1 and 10 microgram respectively) took place in 1% agarose non denaturing gel in Na_2HPO_4/NaH_2PO_4 buffer[48]. Prehybridization and hybridization were carried out at 42 °C in 50% formamide and washing conditions were as for Southern transfer[44]. RNA sizes were estimated by reference to RNA markers run in parallel to poly(A^+) and total RNA.

DNA sequencing and analysis. DNA sequencing was performed utilizing the dideoxy-chain termination method[49] on single-strand DNA preparations of clones in pEMBL or pBluescript II vectors (Stratagene). The GenBank and the EMBL data libraries were searched for nucleotide sequence homology. GenBank searches for homologies were conducted with the 'basic local alignment search tool' programme for nucleotides (BLASTN). The Poisson P-value represents the probability of a high score occurring by chance, given the number of residues in the query sequence of database.

In situ hybridization to polytene chromosomes. In situ hybridizations to polytene chromosomes were done both with ^3H probes[50] and with biotinylated probes detected by the horseradish peroxidase reaction[51] using reagents from ENZO Biochemical.

Interspecific crosses. Interspecific crosses were done in vials with three 1-day-old virgin females and 6 to 8 males aged for 5 days as virgins; crosses were set up at 25 °C (for 2 days) then cultured at 20 °C. All hybrid flies were bred on yeast-glucose medium seeded with live yeast.

Construction of *Df(1) hmr*$^+$ and *Df(1) hmr* chromosomes. Gamma-ray irradiation was carried out at an exposure of 4500 R on 3- to 5-day-old males. A genetic screen for deletions of *hmr*$^+$ was performed as follows: irradiated y^1 *hmr*$^+$ males were crossed to ras^2 females and ras^2 daughters were searched for in the F1 generation. A genetic screen for breakpoints/deletions in *hmr* was carried out as outlined in figure 1.

Stocks. The various mutations as well as the two balancer chromosomes *FM6* and *CyO* referred to in the text are commonly used mutations[52]. These are visible markers easy to score as they affect physical structures such as the colour of the body (*yellow*), the colour of the eyes (*vermilion*), or the shape of the wings (*Curly*). The duplication used to cover the *Hmr* locus was *Dp* $(1;2)v^{+75d}$ which extends from 9A2 to 10C2 cytological bands.

Results

Genetic screens. In order to find new dominant alleles of *hmr*, two mutagenesis experiments were carried out on both *hmr*$^+$ and *hmr* chromosomes (see 'Materials and Methods' and fig. 1). Both screens were aimed at inducing deletions in the 9E region and five putative *hmr* deletions were recovered out of 72,300 chromosomes screened. These were covered by $Dp(1;2)v^{+75d}$ (a duplication for 9A2-10C2) and tested for altered hybrid rescue in *melanogaster/mauritiana* hybrids. No new alleles of *hmr* were obtained. Therefore, in the absence of additional mutants, we decided to attempt to analyze DNA from the possible *hmr* locus by cloning both the P insert region at 9E1.2 and the putative *Hmr* locus of the distal breakpoint of the *In(1)AB*, at 9E6. In

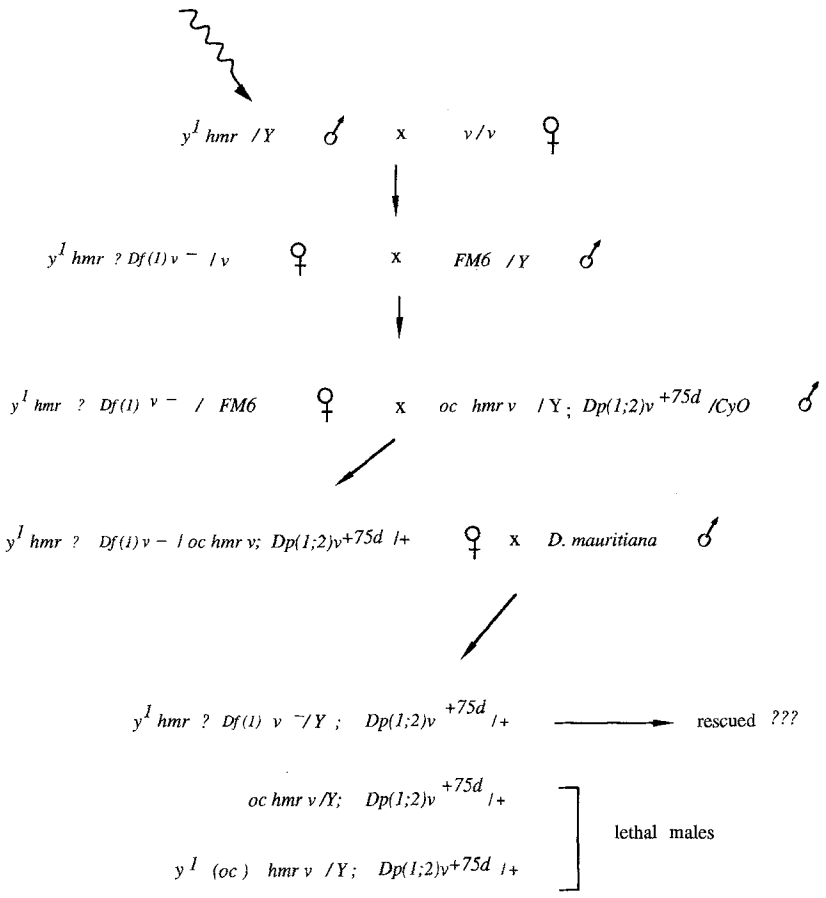


Figure 1. Genetic screen for breakpoints/deletions in *hmr*: irradiated y^1 *hmr* males were crossed to *v* females and the putative deletions were balanced over *FM6*. Balanced females were crossed to *oc hmr v*/*Y*; $Dp(1;2)v^{+75d}$/*CyO* males. From this cross, y^1 (*hmr*?) $Df(1)v^-$/*oc hmr v*; $Dp\ (1;2)^{v+75d}$/+ females were crossed to *D. mauritiana* males. In the next generation, y^1 (with break in *hmr*?) hybrid males were searched for, and both *oc* and y^1 (*oc*) hybrid males (the last two classes in the figure) were used as internal controls for whether or not $Df(1)\ v^-$ included *hmr*.

parallel we attempted to more accurately map *hmr* by further mapping analysis.

Re-mapping *hmr*. Earlier mapping experiments[27] had placed the *hmr* allele between 1–31.58 and 1–32.10. Nevertheless precise meiotic mapping in sometimes poorly viable interspecific hybrids, of a gene whose only visible phenotype is viability versus inviability, is not easy. The main difficulty comes from the observation that the rescue of hybrids by the recessive allele of *Hmr* tends to decline in nearly all stocks carrying the mutation. Therefore, the ambiguous position of this allele, with respect to the closely linked *raspberry* (*ras*) gene at 1-32.35, has led us to an additional mapping experiment. The table shows the results from a recombination experiment using both a y^1 *hmr* and a y^2 *hmr* chromosome against a *ras v* chromosome, which indicate that *hmr* is very close to *ras*, maybe located more proximally towards the centromere than previously thought. Indeed, no *hmr ras v* recombinant male was found among 807 rescued hybrid males. In these experiments less than 10% of the hybrid males carrying *hmr* were rescued.

Table. Mapping of *hmr* from crossing *y hmr*/*ras v D. melanogaster* females to *D. mauritiana* males

y^1 *hmr* females Phenotype of sons	No.	y^2 *hmr* females Phenotype of sons	No.
y	483	*y*	129
+	156	+	32
yv	5	*yv*	2
ras	0	*ras*	0

The numbers of adult males of each phenotype are shown. The standard map positions of the markers are *y* 1-0.0, *ras* 1-32.35 and *v* 1-33.0. y^1 *hmr* mothers produced 14,167 hybrid daughters (i.e. at most 9% of the *hmr* sons were rescued), whereas y^2 *hmr* mothers produced 6,680 hybrid daughters (i.e. at most 5% of the *hmr* sons were rescued).

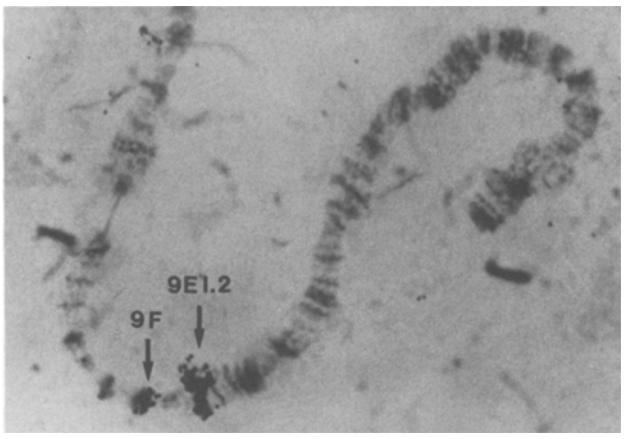
Cloning the *hmr* locus. As a P hybridizing site had consistently been seen at the 9E1.2 doublet of chromosomes carrying *hmr*[30], we first examined the possibility that the mutation results from this insertion. For this purpose we searched for genetic activity around the insert region by several independent criteria.

Firstly, a line of *hmr* flies carrying a P element inserted at 9E1.2 was outcrossed in order to be 'cleaned' of extraneous P elements carried on the autosomes. A genomic lambda phage library was then constructed from this cleaned stock and the 9E1.2 insert region cloned by screening the library with a probe made from the internal *Hin*dIII restriction fragment from the P element. Seven phages were selected and mapped by in situ hybridization to 9E1.2. Homologous wild-type DNA was then cloned from a P-elements-free reference Canton-S library[53], and a heteroduplex EM analysis between mutant and non mutant DNA revealed the presence of a defective 800 bp P insert in the mutant flies. The precise localization of the transposon was confirmed by restriction mapping as 51 kb of genomic DNA surrounding the site of insertion has been cloned in phage and orientated along the physical map of the X-chromosome. Surprisingly, an undetermined sequence contained in phages that hybridized to 9E1.2, when used as probes on the polytene chromosomes, also hybridized more distally to 9C and more proximally to the 9E-9F boundary (fig. 2). Interestingly, the latter site is located just proximal to the break of *In(1)AB* at 9E6, and may be very near the 5′ end of the ADP/ATP translocator gene analyzed in this paper (see below). This sequence showed no additional hybridization site elsewhere in the genome by the criterion of in situ hybridization.

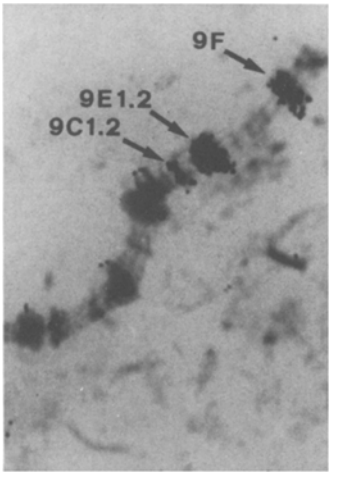
A transcription analysis of the 51 kb genomic region was then carried out with poly $(A)^+$ and total RNAs from second and third instar larvae of both hmr^+ and *hmr melanogaster/mauritiana* hybrid males. These developmental stages were preferred because the *hmr* allele has been found to be temperature-sensitive, with a sensitive period during the second larval instar[27]. Experiments failed to reveal any transcriptional activity throughout the region.

We further examined the possibility that the mutation might result from the insertion of the P transposon at 9E1.2, by mobilizing the transposon in a y^1 *hmr v* strain that had consistently shown a full rescue activity. This was done in sons of a cross of y^1 *hmr v* females to P[$ry^+(\Delta\ 2-3)$](99B)males. 326 X-chromosomes were assayed for partial or total loss of rescuing activity by introducing each chromosome individually into *melanogaster/mauritiana* hybrid males. In one such line the transposon appeared to be excised by genomic Southern criteria, but the X chromosome did not behave as a revertant by genetic criteria.

These results suggested that the P-containing region at 9E1.2 of the *hmr* strain is unlikely to harbour the hybrid rescuing gene. Nevertheless, the possibility that a copy of a sequence repeated in a cluster around the 9E region is present near the 5′ end of the gene described below is intriguing. By and large, these observations led us to focus on the alternative candidate region, that is to say



(*A*)



(*B*)

Figure 2. In situ hybridization to polytene chromosomes of a 13 kb lambda phage from the Canton-S Maniatis library, containing the homologous site of a P element insertion at 9E1.2 in flies mutant for *hmr*. Two additional sites of hybridization can be seen, one at 9F (*A*), and one at 9C (*B*), consistently visible when preparations were exposed for more than three days.

the very thin cytological band at 9E6 where the site of the distal breakpoint of the *In(1)AB* inversion lies.

For this purpose a second chromosome walk was undertaken in the Canton-S Maniatis library, starting from a *raspberry* clone (in 9E3.4) as an entry point. A two-way walk across 46kb of genomic DNA was again orientated along the X-chromosome. Unfortunately, the progression towards the site of the distal inversion break at 9E6 was hindered around 9E5 by a repetitive sequence almost entirely contained in a 2.1 kb *Hin*dIII fragment and reiterated across a stretch of at least 11 kb. Two attempts to further advance by switching to either a *D. simulans* or a *D. teissieri* library did not succeed. This obstacle was eventually overcome when DNA from the other side of the repeats was obtained from a cosmid clone (152C3) isolated from a clone originally called 152F6[54]. Both in situ hybridization to polytene chromosomes and Southern analysis (fig. 3) showed that a 4.4 kb *Eco*RI fragment from the 152C3 cosmid contains the site of the distal breakpoint of the

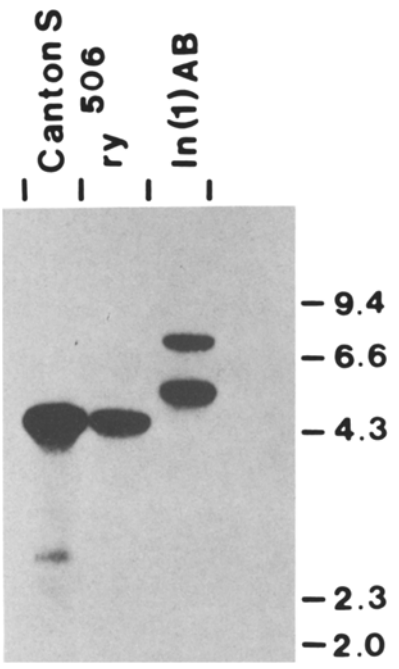


Figure 3. Southern blot of *Eco*RI digests of genomic DNA hybridized with a 4.4 kb *Eco*RI fragment from cosmid 152C3. The faint band of 2.5 kb was always seen on overexposed films and corresponds to the repeat D in figure 4.

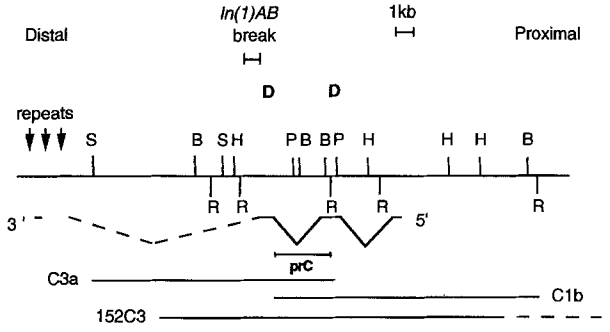


Figure 4. Restriction map of genomic DNA from the 9E5-6 region surrounding the site of the distal breakpoint of *In(1)AB*, and approximate positions of the minimum number of exons. One or more exon(s) is postulated to belong to DNA situated outside the map (see text). The extent of DNA cloned either in lambda phage (C3a, C1b) or in cosmid (152C3) is shown. 45 kb have been cloned distally in phage, including the gene *ras* and 23kb have been cloned proximally in cosmid (see text).
B: *Bgl* II; H: *Hin*d III; P: *Pst* I; R: *Eco*RI; S: *Sal* I. D: sequence duplicated distally. prC: sequence used as probe to select cDNAs.

In(1)AB inversion, and the surrounding region (50 kb in total) was then recloned from the Canton-S Maniatis library. A restriction map of the vicinity of the breakpoint region is given in figure 4, where the extent of the DNA cloned either in cosmid (152C3) or in lambda phage (C1b and C3a) is shown. The 2.1 kb *Hin*dIII repetitive sequence mentioned above was found again about 7 kb distal to the inversion break, which has left uncloned a small gap of DNA between both ends of the repeats.

Transcription analysis. In order to perform a transcription analysis of the region encompassing the distal breakpoint of the *In(1)AB* inversion, a series of contiguous genomic restriction fragments were used as probes on Northern blots. Total cellular RNA and poly (A)$^+$ RNA were prepared from second instar larvae of *Hmr* and *Hmr*$^+$ hybrid (*melanogaster/mauritiana*) larvae as well as from *Hmr* and *Hmr*$^+$ *D. melanogaster* larvae of the same age. These experiments showed that across the whole 50 kb region examined, only three contiguous *Eco*RI restriction fragments, close to the breakpoint site (fig. 4), appear to encode transcripts. Northern blot analysis (fig. 5) shows that the central 4.4 kb *Eco*RI genomic fragment shown in figure 4 detects two quite abundant transcripts of about 1.5 kb and 1.65 kb which are present in embryos, larvae, pupae and adults (data not shown), and one much less abundant (or more tissue specific) transcript of about 2.8 kb, not seen in embryos. Only the latter appears to be altered in mutant larvae, where it was either not detected (fig. 5) or sometimes seen as a very faint and perhaps slightly larger band in mutant larvae, when the cDNA clones described below were used as probes (data not shown). This 2.8 kb transcript was also found to be consistently more abundant in *Hmr*$^+$ second instar hybrid larvae than in larvae carrying the recessive allele of *Hmr*.
A search for cDNAs coding for these RNAs was performed as follows. A 2.5 kb fragment, labelled prC in figure 4, was chosen as probe to screen 4×10^5 phage from a cDNA library made from second instar larvae of

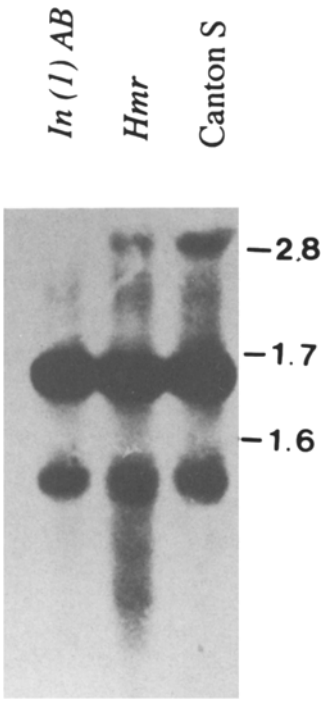
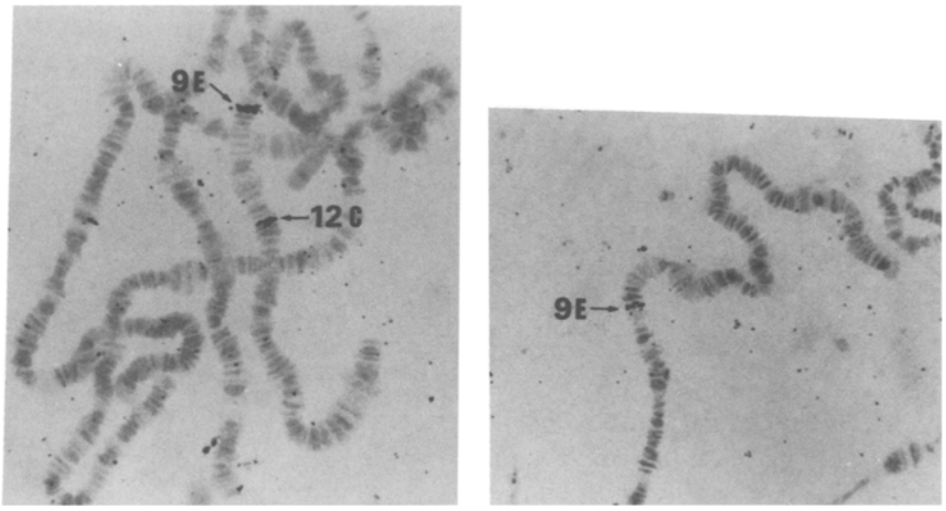


Figure 5. Identification of the mRNA encoded by the candidate *Hmr* gene at cytological band 9E6. Poly(A)$^+$ RNA from second instar larvae was probed with the central 4.4 kb *Eco*RI fragment shown in figure 4. Poly(A)$^+$ RNA is from: *In(1) AB*; mutant for *Hmr*, carrying the P insert at 9E1.2; wild-type Canton-S.



(*a*) (*b*)

Figure 6. Location of the candidate gene *Hmr* by in situ hybridization to polytene chromosomes of *In(1)AB* larvae using ^{3}H cDNA probes. Probes were from HL3 cDNA clone (*a*), showing signals at 9E and 12C (ectopic sequence); and from HE50 cDNA clone (*b*), showing a single signal at 9E.

D. melanogaster. Over 100 positive clones were selected, and the two longest cDNAs (HL37 and HL3), of 1909 and 2245 nucleotides respectively, overlapping for 981 nucleotides, were further studied. Unfortunately, both clones turned out to be chimaeric, containing ectopic DNA at two sites situated 80 nucleotides apart along the 3′ end non coding region of the gene (see below). Both in situ hybridization to polytene chromosomes and sequencing experiments indicated that, in addition to identical sequences hybridizing to 9E, clone HL37 contained an ectopic sequence which hybridized to chromosome arm 2R, and clone HL3 contained an ectopic sequence which hybridized to 12C (fig. 6a). HL3 clone was used as probe to screen a cDNA library made from embryos (4–8 h old) and again the two longest selected clones, HE47 and HE50 of 1625 and 1744 nucleotides respectively, were further studied. The two clones were found to be identical in their overlapping parts (see below). Sequence analysis as well as in situ hybridization to polytene chromosomes of clone HE50 (fig. 6b) indicated that both these clones hybridized only at 9E.

In order to determine the number of different sequences coding for the gene, total genomic DNA was isolated from adult flies, digested by *Eco*R1 and transferred in parallel with *Eco*R1 digests from genomic DNA from the 50 kb chromosomal walk. Several such blots were then hybridized respectively with clones HL37 and HL3 as probes (fig. 7). The results indicated that both the 10 kb and 5.2 kb *Eco*R1 fragments detected by HL3 and HL37 respectively, correspond to the above ectopic sequences included in the cDNAs. Indeed figure 8 shows that when HE50 cDNA is used as probe on wildtype genomic DNA digested by *Eco*R1, this probe detects only three fragments, as expected from the restriction map shown in figure 4 (the largest fragment of about 8 kb was weakly, but consistently detected). Remarkably, at least one additional fragment (one strong of 2.1 kb and perhaps a one weak of 2.7 kb) is detected by both HL3 and HE50 clones as probes on genomic DNA extracted from flies carrying *In(1)AB* (fig. 8), suggesting that a rearrangement affecting the gene may be associated with this inversion, and that flies carrying the inversion appear to have an additonal rearranged copy of the gene. The blot in figure 8 shows that the central 4.4 kb *Eco*R1 genomic fragment of figure 4 becomes a new 5 kb fragment that results from the fusion of the proximal part of the 4.4 kb fragment to DNA on the other side of the inversion at 13E (as seen in figure 3). Nevertheless, the above cDNA probes do not hybridize to the other *Eco*R1 fusion fragment from the distal part of the 4.4 kb fragment (which should be of about 7 kb as seen in figure 3), which suggests that HL3 and HE50 cDNAs do not span the distal breakpoint of the inversion.

Sequence analysis of the cDNAs and exon mapping. The above four cDNA clones were sequenced and all four clones were found to be identical in their overlapping parts. HL37, HE47 and HE50 clones begin at the same nucleotide in their non-coding 5′ end, and extend increasingly further towards their 3′ end. Only HL3 clone, which is truncated at the 5′ end, begins at the *Eco*R1 site situated at the proximal end of the central 4.4 kb *Eco*R1 genomic fragment in figure 4, which lies 564 nucleotides downstream from the start of the composite cDNA sequence. The structure of the composite nucleotide sequence obtained from the four overlapping cDNAs is shown in figure 9. The sequence consists of

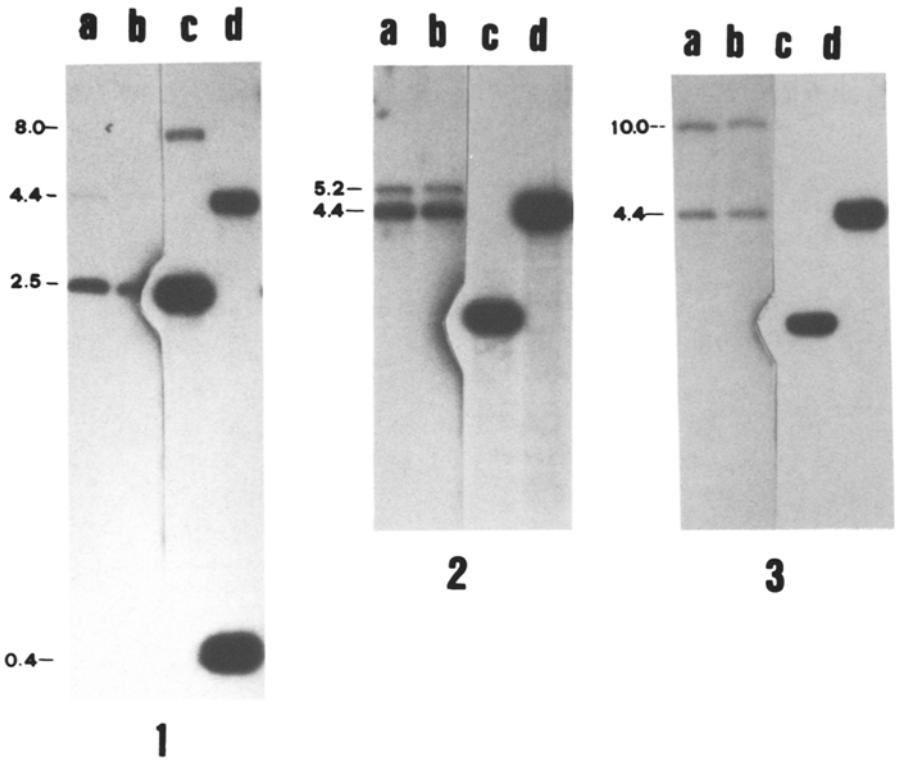
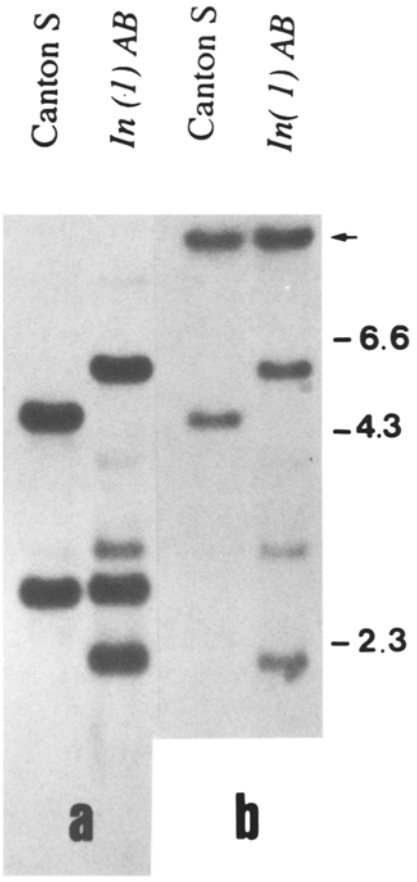


Figure 7. Hybridization of Southern blots of *Eco*RI digests of genomic DNA from both wild-type Canton-S (*a*) and Oregon-R (*b*) flies, in parallel with *Eco*RI digests of lambda phage DNA from C1b (*c*) and C3a (*d*), hybridized with the following cDNA probes: 1 = HL37 (bp:1-565). 2 = HL37 (bp:566-1547) and ectopic cDNA (bp:1548-1909). 3 = complete HL3 with ectopic DNA from bp 1627 to bp 2811.



1744 residues which contains one 891 nucleotide long open reading frame, thus encoding a polypeptide 297 amino acid long. The putative amino acid sequence is also shown in figure 9, with the AUG initiation codon at nucleotide 105 and a TAA termination codon at nucleotide 996. HE47 and HE50 clones were polyadenylated at nucleotide 1626 and 1744 respectively, 23 and 24 nucleotides downstream from an AATATA sequence in both cases. Besides, three potential polyadenylation signals (two with sequence ATTAAA and one with sequence AATACA) are present at 1, 9 and 32 nucleotides respectively, downstream from the termination codon. Several A rich regions extend from the termination codon to about nucleotide 1160 but no typical polyadenylation signal AATAAA[55] was found in the determined sequence. It is possible that HE47 and HE50 cDNAs correspond respectively to the 1.5 kb and 1.65 kb messenger RNAs seen on our Northern blots (see fig. 5), implying that the size of these transcripts has been slightly underestimated. In order to confirm

Figure 8. Hybridization of Southern blots of *Eco*RI digests of genomic DNA from both *In(1)AB* and wild-type Canton-S, with HE50 cDNA clone (*a*) and HL3 cDNA clone (*b*). Two additional bands of 2.1 kb and 2.7 kb can be seen with DNA carrying the inversion. The 10 kb fragment indicated by an arrow corresponds to the ectopic DNA contained in HL3 clone (see text).

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                                                                                                                      116
GACTTTACGA ATTTCATTCG CCAAGTGAGC ACGCGGCTTC GTCTGATTCT GCAAAAGAAG TTTTTATTCT GTGACTTTTG TGAGAAGTTA TCAACTGAAG CAAA ATG GGC AAG GAT
                                                                                                                     M   G   K   D
                                                                                                                      212
TTC GAT GCT GTT GGA TTC GTC AAG GAT TTC GCT GCC GGA CAG GTC TCC GCA GCT GTC TCC AAG ACT GCC GTC GCC CCC ATT GAG CGT GTG AAA CTG
 F   D   A   V   G   F   V   K   D   F   A   A   G   Q   V   S   A   A   V   S   K   T   A   V   A   P   I   E   R   V   K   L
                                                                                                                      308
CTC CTG CAG GTT CAG CAC ATC TCG AAA CAA ATC AGC CCC GAC AAG CAG TAC AAG GGC ATG GTT GAT TGC TTT ATC CGC ATT CCA AAG GAG CAG GGA
 L   L   Q   V   Q   H   I   S   K   Q   I   S   P   D   K   Q   Y   K   G   M   V   D   C   F   I   R   I   P   K   E   Q   G
                                                                                                                      404
TTC TCG TCC TTC TGG CGC GGC AAC TTG GCC AAC GTC ATC AGA TAC TTC CCA ACC CAG GCT CTG AAC TTT GCC TTC AAG GAC AAG TAC AAG CAG GTC
 F   S   S   F   W   R   G   N   L   A   N   V   I   R   Y   F   P   T   Q   A   L   N   F   A   F   K   D   K   Y   K   Q   V
                                                                                                                      500
TTC CTG GGT GGC GTG GAC AAG AAC ACC CAG TTC TGG CGC TAC TTC GCC GGC AAC TTG GCC TCC GGT GGT GCC GCT GGT GCT ACC TCT CTG TGC TTT
 F   L   G   G   V   D   K   N   T   Q   F   W   R   Y   F   A   G   N   L   A   S   G   G   A   A   G   A   T   S   L   C   F
                                                                                                                      596
GTC TAC CCC TTG GAC TTT GCC CGT ACT CGC TTG GCT GCT GAT ACT GGC AAG GGT GGT CAG CGT GAA TTC ACC GGT CTG GGC AAC TGC TTG ACC AAG
 V   Y   P   L   D   F   A   R   T   R   L   A   A   D   T   G   K   G   G   Q   R   E   F   T   G   L   G   N   C   L   T   K
                                                                                                                      692
ATC TTC AAG AGC GAC GGC ATC GTT GGA TTG TAC CGT GGT TTC GGA GTG TCC GTG CAG GGC ATC ATC ATC TAC CGT GCC GCC TAC TTC GGC TTC TAC
 I   F   K   S   D   G   I   V   G   L   Y   R   G   F   G   V   S   V   Q   G   I   I   I   Y   R   A   A   Y   F   G   F   Y
                                                                                                                      788
GAT ACC GCT CGC ATG CTG CCC GAC CCC AAG AAC ACA CCC ATC TAC ATC AGC TGG GCC ATC GCC CAG GTT GTG ACA ACC GTC GCT GGC ATC GTG TCC
 D   T   A   R   M   L   P   D   P   K   N   T   P   I   Y   I   S   W   A   I   A   Q   V   V   T   T   V   A   G   I   V   S
                                                                                                                      884
TAT CCC TTC GAT ACC GTG CGT CGT CGC ATG ATG ATG CAG TCT GGT CGC AAG GCC ACC GAG GTC ATC TAC AAG AAC ACA CTG CAC TGC TGG GCC ACC
 Y   P   F   D   T   V   R   R   R   M   M   M   Q   S   G   R   K   A   T   E   V   I   Y   K   N   T   L   H   C   W   A   T
                                                                                                                      980
ATC GCC AAG CAG GAG GCA CCG TGC TTC TTC AAG GGC GCC TTC TCC AAC ATT CTC AGA GGA ACT GGT GGC GCT TTC GTG CTT GTG TTG TAC GAT GAG
 I   A   K   Q   E   A   P   C   F   F   K   G   A   F   S   N   I   L   R   G   T   G   G   A   F   V   L   V   L   Y   D   E
                                                                                                                 1090
ATC AAG AAG GTC TTG TAAAT TAAATTATTA AATTATTCAA AACAAGCAAA ATACAACAAC ATACAACAAC AAAACAAAAC AAAAGAAGTA AAACTAAACA CTACCAGCTA
 I   K   K   V   L   -
                                                                                                                         1210
ATTATGTTAG ATAACTGAAT GAGAAAAGCT AAAACAACAG CAATAGCAAC AGCAAAGGCA TAAGCTAAGT TTGATCTGCA ACCAACACCT CTTACCACGA ACAACGGCAA GCAATAACAG
                                                                                                                         1330
CAAGAACCCT TCCTTCCTCA CCCCGATATA ATTATGAAAT TATATTATTT ATAAAAACTG GGAACTAATT TACGGAACGA TGGCCAGCGT CAATAGAACA ACAATACTAC AGCAAACAGC
                                                                                                                         1450
AATGCTCGGG GCAGAGAGAG GATGCGACTC AGCACATGAG ACTAGACCCA CTACACTGAA AGACGAACGA ACATAGCGAC GATATCGATT AGTATTGTTA AGTTTCGGTT AGGCTATATA
                                                                                                                         1570
CTATAGATCC AGTCATGTTG CTGGGTCGTC CCACATCTCC CCAGCCAAAT CAGTGTTATC GATCGGATGC AAAGAATCGA ACCGAAGACC TGTAGATTCT TTCGCCTCCG AAACGACAAA
                                                                                                                         1690
CCAACAAACA AACTCAACTT CGGTTGGTTT AAAAATATAT AAAAATTGAT AAAAATAGCA CAATGTTGTG TTTTTCTTTC TGCGCACGTA CATATATATG TATGTATATC GATCTCACAT

ATTGCGACTT GAACCCAATT ATTGCAATGA ATATACATAT GTGTGTGTCA TTCA
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Figure 9. Nucleotide and predicted amino acid sequences of the *Drosophila melanogaster* ADP/ATP translocase. The first 565 bp were sequenced from HL37, HE47 and HE50 cDNA clones, and the next 1179 bp were sequenced from parts of the same clones as well as from HL3 truncated cDNA clone (see text). The three potential polyadenylation signals near the termination codon are underlined.

the orientation of the cDNA on the genomic map, both ends of the central 4.4 kb *Eco*RI genomic fragment were also sequenced.

The localization of the minimum number of exons on the genomic map is shown in figure 4. In situ hybridization to polytene chromosomes of *In(1)AB* flies with either HL37, HL3 or HE50 cDNAs as probes (fig. 6), as well as Southern analysis (figs. 7 and 8), indicated that these cDNAs do not span the distal breakpoint site of the inversion, unless they do so for a very short sequence. Besides, a radioactively labelled synthetic oligonucleotide extending from nucleotide 1501 to nucleotide 1519 of the cDNA sequence reported in figure 9 hybridized to the central 4.4 kb *Eco*R1 fragment, probably just proximal to the distal breakpoint of the inversion. Moreover, a set of PCR experiments were aimed at amplifying, under standard conditions, the genomic DNA (from both a Canton S and an Oregon R strain) which extends from the above 19 bp proximal sequence to the distal *Eco*R1 site of the 4.4 kb fragment. The results indicated that the two sites are about 1 kb apart, therefore the poly(A) site of HE50 must be very close to the distal breakpoint of the *In(1)AB* inversion. As these PCR experiments consistently amplified an additional weak product of about 1.3 kb, and as we found a repeated sequence in the same region by Southern blot experiments (see below), the existence of either a nearby pseudogene or even a tandem (transcriptionally active) copy of the gene cannot be formally excluded, a situation already observed for yeast ADP/ATP translocases[56]. Indeed it is worth noting that an undetermined coding sequence D, comprised in a 350 bp *EcoRI-PstI* fragment (fig. 4), was found to be weakly homologous to a sequence near the site of the breakpoint (contained in the 2.9 kb *Eco*RI-*Bgl* II fragment shown in figure 4), as revealed by Southern blot analysis (fig. 10) – though present nowhere else in the genome by the same criterion.

The probable localization of at least three exons is shown in figure 4, although this does not account for the consistently observed 2.8 kb messenger RNA transcribed from the same genomic region. Therefore one (or more) exon is postulated to belong to a genomic sequence located more distally, outside the central 4.4 kb *Eco*R1 fragment, probably near the uncloned 9E5 region, where a DNA stretch of repeated sequences was found (see 'Discussion').

A GenBank search for nucleotide sequence homology with BLASTN indicated strong sequence similarity with several mitochondrial ADP/ATP translocator proteins. The sequence is over 99% identical to a cDNA clone from *D. melanogaster*, encoding the above protein[57], which was isolated from an adult head cDNA library with a probe from a region of the gene highly conserved from yeast to humans. The authors also failed to find a typical poly (A) signal, as their sequence did not extend

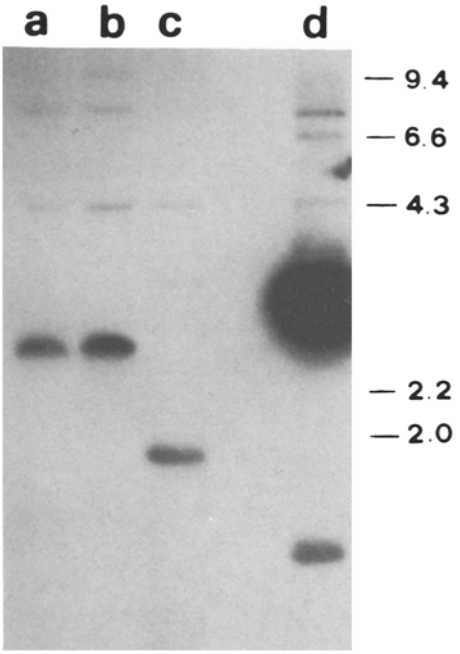

Figure 10. Hybridization of genomic DNA, and DNA subcloned from cosmid 152C3 to a probe containing the first 5′ end 565 bp of HL37 cDNA clone.
a, b DNA from Canton-S and Oregon-R wild-type flies respectively, digested by *Eco*RI.
c DNA from Oregon-R flies digested by both *Eco*RI and *Hin*d III.
d Sub-clone of the central 4.4 kb *Eco*RI fragment from cosmid 152C3 digested by *Eco*RI and *Bgl* II. The strong signal in this lane is due to the repeat D shown in figure 4.

beyond nucleotide 1591 of our sequence[57]. We have been able to sequence an additional 153 bp of the 3′ noncoding region from both HE47 and HE50 cDNA clones. The coding sequence which we determined differs by three amino acids (Ile to Tyr, Ala to Gly, and Cys to Ser, at codons 81, 266 and 268 respectively).

Homologies at both nucleotide and amino acid levels are close to 80% and are highly significant with a Poisson P-value <0.0001 for organisms as distantly related to *Drosophila* as *Saccharomyces cerevisiae*, *Neurospora crassa*, *Caenorhabditis elegans* and *Homo sapiens*[58–61]. In all species studied the mitochondrial ADP/ATP carrier-protein (also named adenine nucleotide translocator, ANT) is the most abundant mitochondrial protein[62] and it plays an essential role in maintaining metabolic energy (see 'Discussion'). ANT proteins appear to be highly regulated proteins, and most are known to be members of a small gene family, whose members are all approximately 300 amino acids long[61]. As shown in figure 9 the deduced amino acid sequence from the coding sequence reveals a 297 amino acid protein.

Discussion

The original meiotic mapping experiment of *hmr*[27], based on a total score of 5365 rescued *melanogaster*/

mauritiana hybrid males had placed the gene just distal to *ras*. Our additional mapping experiment suggested that the gene may be located slightly more proximally, at about 1–32.5, which would be around the 9E6 band, as inferred from the relationship between genetic and cytological map positions.

The chromosome bands at the junction between the cytological divisions 9 and 10 on the X-chromosome have been the subject of detailed cytogenetical analysis, where 32 loci were found in three map units[38]. Just proximally to the *pur 1*, *gua 1*, *ras* complex unit around 1–32.35 (see introduction), one essential locus is known very near the site of the distal breakpoint of *In(1)AB* at 9E6. This is the *stress-sensitive-B* (*sesB*) gene at 1–32.53[38, 52, 63], some alleles of which are lethal, others being very sensitive to mechanical shock[64]. The possibility of allelism between *Hmr* and *sesB* deserves further examination as the *sesB* phenotype appears to be compatible with a mitochondrial disorder.

Our results suggest that the *Hmr* gene is an ADP/ATP translocator gene encoding an ANT protein but conclusive proof is still lacking. Indeed, the demonstration that the right gene has been identified is made particularly difficult by the fact that *Hmr* has no phenotype apart from the rescue of interspecific hybrids. In the absence of new mutants, one critical experiment to settle this issue would be to sequence the homologous cDNA made from larvae carrying the recessive allele of *Hmr*, in order to detect a functionally relevant sequence difference. An alternative experiment would be to carry out more functional analysis by P-transforming *Hmr* mutant flies with a copy of the wildtype ANT coding gene(s), in order to see whether the rescue is suppressed in hybrid sons obtained from such female transformants, when crossed to *D. mauritiana* males. With respect to the possibility of allelism between *Hmr* and the *sesB* gene just mentioned, it may also be worth trying to rescue some lethal *sesB* alleles by transformation with the ADP/ATP translocator gene(s). As already mentioned, several observations suggest that the lethality of the hybrids investigated in this study cannot have a simple monogenic basis. Firstly, at least two other loci have been identified as containing genes interacting with *Hmr* (see introduction). Secondly, most of the laboratory stocks of *D. melanogaster* carrying the recessive allele *hmr* have been found to progressively loose their rescue activity through time, which clearly suggests the existence of modifier genes. This observation is unlikely to result simply from an incomplete penetrance of the mutation since this phenomenon was also observed in the original stock of *hmr*, which at first showed complete penetrance. The data in the table, for instance, indicate that the rescue activity had dropped to about 5% of its original value, after several years. A third indication of a probably multigenic basis of the rescue activity comes from the difficulties we encountered in obtaining new alleles of *hmr* by inducing deletions of the 9E region. Indeed, none of three recovered deletions which were interpreted cytologically as spanning the 9E region exhibited the rescue. Similarly, when *melanogaster/mauritiana* hybrid females heterozygous for $Df(1)v^{L15}$ (=Df(1)9B1-10A1) were constructed, the rescue by the deleted chromosome was only partial (unpubl. data). Lastly, if the ADP/ATP translocator gene is involved in the rescue activity, it must be emphasized that because there may be more than one copy of the gene in the genome (see below), this may also contribute to complex genetic interactions.

With regard to the identification in general of genes possibly involved in reproductive isolation, the following point needs to be made here. Discovering a locus where mutant alleles show some rescue activity of interspecific hybrids does not necessarily mean that the wildtype gene is directly responsible for hybrid inviability. Such genes may sometimes be for instance 'second-site suppressors', for which mutant alleles may be able to override the effects of other genes which normally cause hybrid lethality. Thus rescue mutations may not always represent genes directly relevant to reproductive isolation.

Still, one most intriguing feature of the two mutations of *Hmr* dealt with in this study, is the fact that otherwise lethal hybrids can be fully viable when homozygous for either mutation, without any visible effect in *D. melanogaster*. When this is coupled with the lack of alteration of the size of either the 1.5 kb or the 1.65 kb transcripts as detected on Northern blots, it suggests that the DNA lesion is located near or in a sequence encoding a transcript larger than the observed 1.65 kb transcript. Results from hybridization of cDNA clones to both polytene chromosomes and genomic DNA from *In(1)AB* flies indicated that none of the cDNA clones which were isolated substantially spans the distal breakpoint of the inversion at 9E6, unless the inversion is associated with a small deletion of a sequence adjacent (distally) to the above break. Nevertheless, results from PCR experiments suggested that the poly(A) site of HE50 cDNA must be very close to the site of the distal breakpoint of the inversion. From our data one would predict that only a cDNA encoding a mRNA longer than 2 kb can be interrupted by the inversion breakpoint, therefore the rarer 2.8 kb transcript which was observed is likely to be affected by the break, as suggested from Northern blot analysis. Still, although in mutant larvae this transcript consistently appeared to be either absent or less abundant and slightly larger on our Northern blots, than the same transcript from wildtype larvae, it is difficult to characterize precisely the nature of the alteration. This may partly be due to the presence of more than one copy of the ANT encoding gene (see below).

As no typical polyadenylation signal was found in the determined sequence, the presence of three transcripts

on Northern blots can be interpretated in at least two ways. Firstly, as already mentioned, these transcripts may have originated from two or more homologous genes clustered in the 9E region, and these genes may differ in the length of their 5′ and/or 3′ untranslated regions. The observation that an undetermined sequence D (figure 4) is duplicated in the 2.9 kb *Eco*RI-*Bgl* II fragment which comprises the site of the *In(1)AB* distal breakpoint (figure 10), but nowhere else in the genome, is also compatible with the presence of two copies of the gene in this region. The human genome contains at least 10 sequences related to probes from the 5′ and 3′ ends of coding regions of translocases 1 and 2 (ref. 65). The possibility of a repetition of a sequence of the ADP/ATP translocator gene may also be due to the presence of internal repeats in the coding sequence of this gene. Indeed, it has been shown that in ANT 1 six hydrophobic segments of 26 amino acids each, which are thought to form structures folded into a-helices that traverse the mitochondrial membrane, are part of three repeated domains of 100 amino acids each[66]. This suggests that the gene evolved from an ancestral gene by two duplication events.

Secondly, the observation of more than one transcript may be due to a differential polyadenylation/cleavage of the same transcript using different potential signals, perhaps in a tissue-specific regulation. Although we have found two cDNAs polyadenylated at nucleotides 1626 and 1744 respectively, no typical polyadenylation signal was found in the determined sequence, but three potential signals were identified further upstream. The ANT gene is known to have a long 3′ untranslated region in various species and at least in two bovine translocases these regions extensively diverge and are variously expressed in different tissues. In humans, three distinct RNA species, of 1200, 1450 and 1600 nucleotides have been found in HeLa cells when using a bovine ADP/ATP translocase cDNA as a probe under low stringency conditions[59]. Taken together, these results point to a high complexity of organization and expression of the gene(s) in humans, and since the ANT protein appears to be remarkably conserved between *Drosophila* and *Homo*, the *Drosophila* gene is likely to be complex as well.

Although it is still not clear at this stage whether the *Hmr* gene is functionally related to the ANT encoding gene, the data are tantalizing enough to briefly speculate on the possible implication of mitochondria in hybrid inviability. Indeed a relationship between the 2.8 kb transcript and the species-specific origin or composition of the mitochondrion may be critical to a proper oxydative phosphorylation of cells. As inferred from our results, the simplest explanation to account for an effect on rescue of hybrids resulting from mutations in the candidate gene analyzed in this paper would be as follows. The 2.8 kb messenger RNA encoded by an ADP/ATP translocase gene may become deleterious in hybrids as a result of impaired epistatic interactions in a hybrid genotype. This working hypothesis thus postulates that the transcript's organization and/or expression should differ between sibling species. The above interactions presumably include the other genes already mentioned as being involved in hybrid inviability (see introduction). It is worth emphasizing here that the *mhr* gene in *D. simulans* acts maternally[26], and that the postulated autosomal suppressor of *Hmr* in *D. melanogaster* is also expected to have a strong maternal effect[30]. Mutations in the 3′ end region of the cDNA encoding the 2.8 kb transcript in *D. melanogaster* might then lessen the supposedly adverse nucleus-cytoplasm interaction by reducing the level and/or the stability of the transcript.

Although this scenario is entirely based on a pre-translational explanation for the effect of mutations of *Hmr*, the possibility of a post-translational explanation must not be excluded. Indeed, if *Hmr* is a gene implied in mitochondrial ADP/ATP metabolism, this is of special interest with respect to the pattern of hybrid inviability observed in *Drosophila*, which, as already mentioned, often includes strong interactions between the zygotic genotype and maternal factors[15,67–69]. In the *melanogaster* species subgroup the best example of this is provided by the fact that $X_{melanogaster}/X_{sibling}$ hybrid females are viable if the mother is *melanogaster* but are lethal if the mother is a sibling species. A model based on the maternal effect of a dominant lethal gene on the X chromosome of *D. simulans*, normally offset by a recessive suppressor on the second chromosome, has recently been proposed[31]. With respect to this, it may even be worth considering that the observation of exceptional hybrid survivors might not be unrelated to mitochondrial heteroplasmy or rare paternal transmission of mitochondria[70].

With regard to the oxidative phosphorylation process, it must be emphasized that in mammals, although the ANT proteins are encoded in the nucleus, 13 other polypeptides essential to the above process are known to be encoded in the maternally inherited mitochondrial DNA[71]. Therefore, a substantial coevolution of these proteins is bound to take place, as they are highly interdependent. Similarly, it can be argued that if the number of copies of the ANT gene varies between the sibling species studied, this may readily impinge on the general fitness of hybrids between these species. It must also be borne in mind that mitochondrial genes tend to mutate up to 10 times more frequently than nuclear genes[60]. Hence, since nuclear genes and mitochondrial genes must cooperate to ensure a proper oxidative phosphorylation process, the high mitochondrial DNA mutational rate might occasionally affect hybrid survival. This could result, for instance, from a difficulty for the ANT protein to tail into the inner mitochondrial mem-

brane. Considering that about half of the DNA from unfertilized eggs of *Drosophila* is mitochondrial[72,73], it seems reasonable to envisage that an altered nucleus-mitochondrion-interaction in hybrids may readily lead to severe physiological defects.

In humans at least, ANT is known to function as a dimer embedded in the inner mitochondrial membrane[74]. The two subunits form a gated pore through which ADP is moved across the inner membrane into the mitochondrial matrix, and ATP is moved from the matrix into the cytoplasm, in 1:1 stoichiometry. The ADP/ATP carrier RNA levels undoubtedly respond with great sensitivity to a wide range of conditions that affect cell growth[58], and these must include nucleus-cytoplasm-interactions. Thus these interactions can impinge on the rates of both oxidative phosphorylation and energy-consuming processes controlled by the ANT protein.

If the *Hmr* gene is related to the ANT encoding gene, this study will have theoretical implications for evolutionary biologists interested in the study of 'isolating mechanisms', although this work does by no means make the claim that such a result might solve the still fully mysterious 'problem of speciation'. Nonetheless, our data would support the view that a single gene may play an important role in reproductive isolation, as originally postulated when a search for the mutation was undertaken[75]. So far only one gene, possibly involved in animal reproductive isolation (by virtue of its negative effect on hybrid fitness), has been analyzed at the molecular level[12]. This is also an X-linked gene, which codes for a receptor of tyrosine kinase and causes malignant melanoma in hybrids between the platyfish and the swordtail. However, as recently pointed out by Orr[8], this gene may not be representative of the genes that cause hybrid inviability, as this trait is generally unlikely to result from malignancies.

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