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Interspecific DNA transformation in *Drosophila*

(*Drosophila melanogaster*/*Drosophila simulans*/rosy gene/P element/transposable element)

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ABSTRACT **A DNA fragment that includes the wild-type rosy (*ry*$^+$) gene of *Drosophila melanogaster* has been introduced by microinjection into the germ line of the reproductively isolated species *Drosophila simulans* and incorporated into the *D. simulans* genome. Transformation was mediated by the transposable element P, which occurs in the genome of most natural populations of *D. melanogaster* but not in *D. simulans*. Rubin and Spradling [Rubin, G. M. & Spradling, A. C. (1982) *Science* 218, 348–353] have previously shown that the *ry*$^+$ DNA fragment, which is flanked by recognition sequences of P element, can transform the germ line of *D. melanogaster*. Successful transformation in *D. simulans* indicates that the P element continues to function as a transposable element in the *D. simulans* genome. Moreover, the *ry*$^+$ gene of *D. melanogaster* functions in the genome of *D. simulans* to produce normal eye color, despite the estimated 1 to 5 million yr of reproductive isolation since the evolutionary divergence of these species. Interspecific DNA transformation provides a useful method for the study of genetic differences affecting gene expression among related but reproductively isolated species.**

Drosophila melanogaster Meigen (1830) and *Drosophila simulans* Sturtevant (1929) are sibling species in the subgenus *Sophophora*, which together with six other described species form the *melanogaster* species subgroup. The present biogeographical distribution of the subgroup implies that it almost certainly originated and diversified in Africa (1, 2). *D. melanogaster* and *D. simulans* are morphologically nearly indistinguishable except for the male genitalia, and their only major karyotypic difference is in a large inversion in the right arm of the third chromosome (3). Both species are cosmopolitan in distribution and often exist sympatrically, but they are completely isolated reproductively.

The time elapsed since the evolutionary separation of *D. melanogaster* and *D. simulans* is very uncertain. One estimate (4) is based on a comparison of the genetic distance between the species in terms of electrophoretically detected enzyme differences (5, 6) and the rate at which genetic distance has accumulated in the *planitibia* subgroup of Hawaiian drosophilids, which is known because of the exceptionally well understood geological history of the Hawaiian Islands (7). This estimate is 0.8 ± 0.2 million yr, but the estimate is of uncertain validity because it is based on the rate of genetic divergence in a distantly related subgroup of species, which may have evolved at an unusually rapid rate because of the island habitat; it is also uncertain because substantial genetic divergence occurs among local populations of *D. melanogaster* (8). Much greater times since separation are estimated from comparison of differences in nucleotide sequence between the *D. melanogaster* and *D. simulans* gene coding for alcohol dehydrogenase and the average rate of nucleotide substitution that occurs among genes in other organisms whose evolutionary history is known from the fossil record (4). Use of an average rate of synonymous substitutions in coding regions of $5.1 \pm 0.3 \times 10^{-9}$ per nucleotide site per yr (9, 10) results in an estimated time since separation of 4.1 ± 0.2 million yr, but analogous estimates based on rates of nucleotide substitution in intervening sequences range from 3.8 to 6.8 million yr. These estimates are uncertain because average rates of substitution in other organisms may have little relevance to insects, and because evolutionary change in the gene coding for alcohol dehydrogenase may be atypical.

Although *D. melanogaster* and *D. simulans* are reproductively isolated, the species can be hybridized. Indeed, *D. simulans* was first recognized as a group of strains that produced nearly unisexual progeny in crosses with *D. melanogaster* (11). However, interspecific mating is difficult to achieve even under laboratory conditions, and most of the hybrids that survive are of the same sex as their *D. melanogaster* parent (11, 12). Interspecific hybrids usually have reduced viability, and they are invariably sterile (13, 14). The cross of triploid *D. melanogaster* with heavily irradiated *D. simulans* results in offspring having various combinations of chromosomes from the parental species, but virtually all of the hybrids are sterile (15).

The sterility of the hybrids between *D. melanogaster* and *D. simulans* and their reduced viability undoubtedly results in part from the fixation of genetic differences between the species that accompanied the process of speciation 1–5 million yr ago and in part from genetic differences that have accumulated since that time. To study genetic differences between related but reproductively isolated species, it would be useful to have a method for transferring individual genes between them. As Muller and Pontecorvo pointed out long ago (15), "the genetic analysis of species differences is of especial interest in precisely those cases where the sterility or inviability of the hybrids between the species stands in the way of such analysis."

A method of DNA-mediated transformation of germ cells of *D. melanogaster* has recently been developed that uses the transposase enzyme of a transposable element (P element) to catalyze the transposition of a genetically engineered element consisting of the DNA sequence of interest flanked by the recognition sequences of P element (16–20). We have investigated whether this system of intraspecific transformation can successfully catalyze interspecific transformation between *D. melanogaster* and *D. simulans*. The result is that interspecific transformation does occur, which implies that the P element also functions as a transposable element when present in the germ cells of *D. simulans*. This finding has implications regarding the potential for transposable elements such as the P element to be disseminated across interspecific reproductive barriers. Moreover, the method of interspecific gene transfer will be of general use in studies of the molecular biology and evolution of gene regulation in related but reproductively isolated species of *Drosophila*.

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Abbreviation: kb, kilobase(s).

MATERIALS AND METHODS

Recovery of a rosy (*ry*) Mutation in *D. simulans*. A strain of *D. simulans* carrying a vermilion (*v*) mutation was provided by Victoria Finnerty. Males of this strain were subjected to mutagenesis with ethyl methanesulfonate (21) prior to mating with *v* females. Approximately 300 F_1 offspring from treated males were mated individually with *v* females, and the F_2 offspring were sib-mated. The resulting F_3 progeny were screened for orange-eyed flies (the *v ry* double mutant has orange eyes), and two putative *ry* mutants were recovered. One of these was identified as a *ry* mutation because it failed to complement a known *ry* mutation in interspecific hybrids with *D. melanogaster*. The mutation was also found to lack xanthine dehydrogenase activity, the gene product of *ry*. The *v ry* strain was freed of the *v* mutation by crossing with wild-type *D. simulans* followed by inbreeding to reextract the *ry* mutation. The resulting strain was designated iso-1, and the *ry* mutation in this strain was designated ry^{i83}.

Protein Electrophoresis. Extracts of individual flies were subjected to electrophoresis in 7% polyacrylamide gels with 40 mM Tris·glycine buffer (pH 8.3) at 150 V for 4.5 hr. Gels were stained for xanthine dehydrogenase in 100 mM Tris·HCl, pH 7.1/0.7 μM phenazine methosulfate/5 μM NAD^+/2 μM nitro blue tetrazolium/7 μM hypoxanthine.

Transformation. Transformation mediated by the P element was carried out as described by Rubin and Spradling (16, 17). The injected DNA solution contained plasmid Carnegie-20 (300 μg/ml), which carries a 7.2-kilobase (kb) fragment including the ry^+ gene from *D. melanogaster* (17, 22), and plasmid pπ25.1 (75 μg/ml), which carries a complete P element (17, 23).

The transformation apparatus consists of a Nikon S-Ke II phase contrast microscope and a Narishige micromanipulator. Injection needles made from triangular glass (Glass Company of America) were partly filled with 2–5 μl of DNA solution and then with light weight oil (Norton Lubricant). The end of the needle was inserted into oil-filled tubing attached to a peristaltic pump, which provided the pressure for injection.

Injections were carried out with embryos from the strain iso-1, which carries ry^{i83}. Embryos harvested from eggs laid within a 30-min period were rinsed, dechorionated, and placed in a row on a microscope slide in a small quantity of dissolved adhesive. After suitable dehydration, embryos were covered with series 700 halocarbon oil (Halocarbon Products, Hackensack, NJ) and viewed with the microscope at ×200 magnification. The injection needle was inserted into the clear cap space at the posterior pole of the embryo, and injection pressure was applied until a sufficient volume of DNA solution was introduced to counteract the volume of fluid lost by dehydration. Injected embryos were covered with a thin layer of oil and placed overnight in a humidified and oxygenated chamber. Larvae obtained in the following 24–36 hr period were placed in instant *Drosophila* medium (Carolina Biological, Burlington, NC) at 25°C until eclosion. Flies that survived injection (the G0 generation) were mated singly with iso-1 flies (ry^{i83}) to produce the G1 generation, and individual strains were derived from phenotypically wild-type G1 offspring.

DNA Fractionation. DNA was extracted as described (24) with some modifications. Single adult flies were homogenized in 1.5-ml Eppendorf tubes in 500 μl of extraction buffer [0.1 M Tris·HCl/0.2 M sucrose/50 mM EDTA/0.5% (vol/vol) sodium lauryl sulfate, pH 9.2] and heated at 65°C for 10 min, followed by addition of 75 μl of 8 M potassium acetate, incubation on ice for 15 min, and two centrifugations of 5 min each at room temperature. DNA was precipitated with 0.2 M NaCl and ethanol, and the pellet was dried and resuspended in 20 μl of buffer A (10 mM Tris·HCl/1 mM EDTA, pH 8.0). After extraction with phenol and with chloroform/isoamyl alcohol (25:1), the DNA was precipitated as described above and resuspended in 20 μl of buffer A.

Restriction endonuclease digests were carried out according to supplier's instructions with 20 units of enzyme in a final vol of 50 μl. Samples were subjected to overnight electrophoresis at 40 V in 0.7% agarose gels in 40 mM Tris acetate/2 mM EDTA, pH 8.0, and were stained with ethidium bromide.

DNA Hybridization. Transfer of DNA to nitrocellulose filters (Schleicher & Schuell BA85) and hybridization at 65°C was carried out as described (25). Fragments used as probes were isolated by a modification of the glass powder method (26) and nick-translated with [α-^{32}P]dATP (New England Nuclear) to a specific activity of $>10^8$ dpm/μg (27).

RESULTS

Identification of *ry* Mutation in *D. simulans*. Fig. 1 shows a protein electrophoresis polyacrylamide gel stained for xanthine dehydrogenase, the gene product of the *ry* locus. The indicated lanes contain protein extracts of ry^{506} [a *D. melanogaster* strain carrying a deletion of part of the *ry* locus (17)], wild-type *D. simulans*, the *D. simulans* strain iso-1, and wild-type *D. melanogaster*. Based on the absence of detectable xanthine dehydrogenase activity in the iso-1 strain and the inability of the mutation in this strain to complement a known *ry* mutation in interspecific hybrids with *D. melanogaster*, we conclude that the mutation in iso-1 is in the *ry* locus, and we designate the mutation as ry^{i83}.

Interspecific Transformation. Results of interspecific transformation experiments with iso-1 were as follows. Among 60 iso-1 embryos injected with a mixture of the ry^+-containing plasmid Carnegie 20 and the element P-containing helper plasmid pπ25.1, 27 hatched into larvae, 21 of these survived to produce G0 adults, and 15 of the G0 adults were phenotypically ry^+. All G0 adults were backcrossed with the iso-1 strain; 16 of the backcrosses were fertile, and 4 produced 1 or more ry^+ offspring. From these ry^+ offspring, 17 strains (designated A1–A10, B1–B2, C1, and D1–D4) were established in January, 1984, by backcrossing with iso-1 followed by sib-mating and maintenance at 25°C. In the initial and subsequent crosses, each of the strains produced segregation ratios of ry/ry^+ expected for autosomal inheritance of the ry^+ gene. After ≈14 generations (January, 1984, to July, 1984), all transformed strains remain viable and fertile and apparently stable for ry^+. However, in strain A8, one individual has recently been found to have an X-linked ry^+ gene, apparently the result of transposition within the strain since it was initially established. Such transposition suggests that

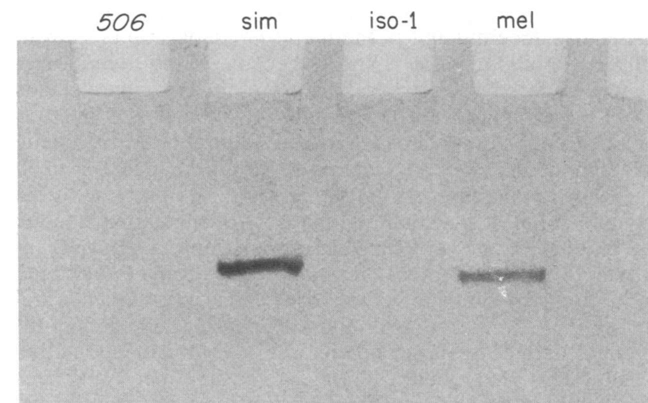

FIG. 1. Polyacrylamide gel stained for xanthine dehydrogenase. Lanes, from left to right, contain extracts of *D. melanogaster* carrying ry^{506} mutation, wild-type *D. simulans*, strain iso-1 of *D. simulans*, and wild-type *D. melanogaster*.

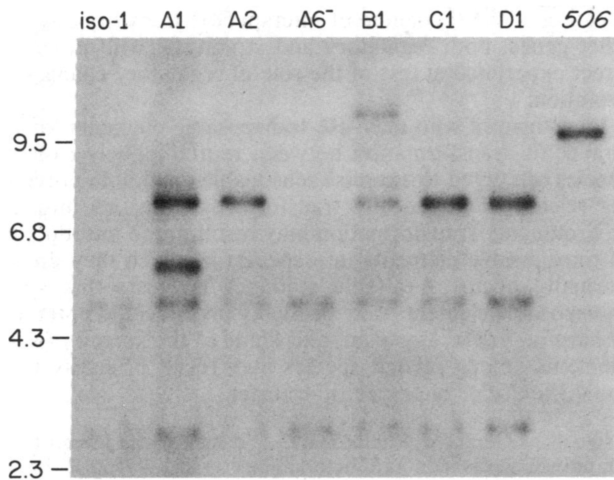

FIG. 2. Hybridization of *Hin*dIII digests of genomic DNA with the 7.4-kb *ry* DNA sequence from Carnegie 20. The fragments of 4.7 and 2.7 kb originate from the *D. simulans ry* gene. Lanes labeled iso-1 and *506* contain DNA from *D. simulans* (ry^{i83}) and *D. melanogaster* (ry^{506}), respectively, and A6⁻ represents a ry^{i83} segregant from transformed line A6. Molecular weight markers ($\times 10^{-3}$) represent positions of λ *Hin*dIII standards. (The 4.7- and 2.7-kb *ry* fragments from *D. simulans* are present in all lanes except *506*, but in some lanes they may not be apparent in the photograph as reproduced.)

the strain carries intact P elements in addition to ry^+, which has been confirmed directly by DNA hybridization with a P-element probe (data shown below).

Fig. 2 shows the pattern of hybridization obtained with *Hin*dIII digests of genomic DNA of single flies after ≈12 generations when probed with a 7.4-kb *ry*-containing *Hin*dIII fragment isolated from Carnegie 20 (22). The lane labeled *506* is a control containing DNA from a *D. melanogaster* strain carrying the ry^{506} mutation. The lane labeled iso-1 is another control containing DNA from the *D. simulans* strain iso-1, and it indicates the occurrence of a *Hin*dIII site within the ry^{i83} mutation in this strain, resulting in two *Hin*dIII *ry* fragments of 4.7 and 2.7 kb being produced. This *Hin*dIII site also occurs in the wild-type *D. simulans* ry^+ gene (data not shown), and it is a useful feature with which to distinguish the *D. melanogaster* and *D. simulans ry* genes. The strain designated A6⁻ is a ry^{i83} segregant from transformed strain A6. Individuals from the transformed lines A1, A2, B1, C1, and D1 have the expected 7.4-kb *ry*-containing DNA sequence from *D. melanogaster*, in addition to the hybridizing sequences present in ry^{i83}. The remaining 12 transformed lines also show both of the ry^{i83} fragments and an additional 7.4-kb fragment that hybridizes with *ry* DNA (data not shown). However, individuals from transformed lines A1 and B1 each have an additional fragment (of ≈5.6 and 11.3 kb, respectively), which represents a rearrangement of the *ry* gene evidently resulting from imprecision in the process of transposition. Such rearrangements have also been observed in *D. melanogaster* (35).

Cotransformation with the Intact P Element. Tests for the presence of P element sequences within the transformed lines after ≈12 generations are shown in Fig. 3. In this case, a *Sal* I digest of genomic DNA was probed with a 1.5-kb *Sal* I/*Hin*dIII fragment isolated from the P element in plasmid pπ25.1, which does not overlap the P sequences present in Carnegie 20 (22, 23). Each P element in the genome appears as a distinct fragment. The five lanes at the right contain DNA from controls: ry^{506} (lane 8); iso-1 (lane 9); wild-type *D. simulans* (lane 10); 4th A2, a strain of *D. melanogaster* obtained from William R. Engels, which contains a single complete P element (lane 11); and a wild-type Oregon R strain of *D. melanogaster*, which lacks P elements altogether (lane 12). Transformed strains A1 and A8 obviously contain P sequences in addition to those occurring in Carnegie 20 that were introduced in the process of transformation to ry^+. Indeed, at least six fragments that hybridize with the P element DNA are present in strain A1. In addition, all transformed strains in Fig. 3 contain an anomalous hybridizing fragment of ≈3.8 kb. We conclude from Fig. 3 that cotransformation of the P element and ry^+ occur frequently in *D. simulans* and that the P element is an active transposable element in this species.

DISCUSSION

The attempt at interspecific gene transfer between *D. melanogaster* and *D. simulans* was based on the presumed validity of several hypotheses. First, we assumed that the P element would retain its ability to code for transposase when present in germ cells of *D. simulans*. Second, we assumed that transposition catalyzed by the transposase would require no host functions supplied by the genome of *D. melanogaster* that would not also be supplied by the genome of *D. simulans*. Third, we assumed that the transposase would have the same sequence specificity in *D. simulans* as in *D. melanogaster*. Fourth, we assumed that the transposase would have sufficient activity in *D. simulans* to catalyze incorporation of ry^+ DNA flanked by recognition sequences of the P element into chromosomes in the *D. simulans* germ line. And fifth, we assumed that, once incorporated into the genome, the

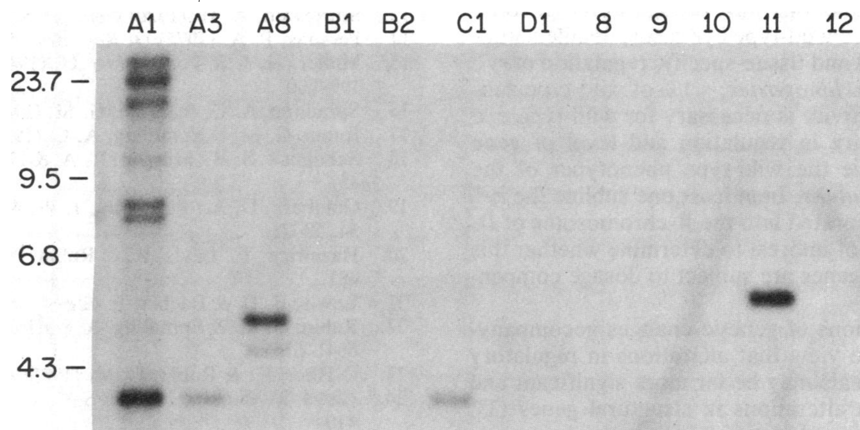

FIG. 3. Hybridization of *Sal* I digests of genomic DNA with the 1.5-kb *Sal* I/*Hin*dIII fragment of the P element from plasmid pπ25.1. Each P element in a strain results in a different size hybridizing fragment. Lanes 8–12 contain DNA from the following control strains (left to right): *D. melanogaster* ry^{506}, *D. simulans* iso-1, wild-type *D. simulans*, *D. melanogaster* carrying a single P element, and wild-type *D. melanogaster* Oregon R. Molecular weight standards ($\times 10^{-3}$) represent positions of λ *Hin*dIII fragments.

ry^+ gene would function well enough in *D. simulans* to compensate for the ry^- genotype and restore eye color to the wild-type phenotype.

The success with interspecific transformation seems to confirm the essential correctness of all five postulates. Although presently available data are inadequate to compare quantitatively the frequency of transposition and the efficiency of transformation in the two species, the qualitative features of our results in *D. simulans* are not markedly different from what we have found (unpublished) and what has been reported for *D. melanogaster* (17, 28). Efficient transposition of the P element is also inferred from the occurrence of cotransformation, in which intact P elements are incorporated into the genome along with ry^+ DNA. It is yet to be determined whether P elements will continue to accumulate in the genome, whether they will eventually induce some or all of the manifold symptoms of hybrid dysgenesis as observed in suitable crosses in *D. melanogaster* (29, 30), and whether they will eventually induce in *D. simulans* a cytoplasmic state analogous to the P cytotype in *D. melanogaster* (30).

The P element is evidently capable of transposition in a variety of drosophilid genomes. The element has been introduced into *Drosophila hawaiiensis* by Brennan *et al.* (36), and we were pleased to learn recently that S. B. Daniels, R. Armstrong, and L. D. Strausbaugh (personal communications) have independently succeeded in introducing P elements into *D. simulans*. Furthermore, the recent discovery of P elements in *Drosophila paulistorum* and related species (31) suggests that P elements can not only catalyze transposition in many species but can also be maintained in the genome in natural populations. It will therefore be of some interest to determine whether P elements are absent from the genome of natural populations of *D. simulans*, because the genome was never infected with the element by means of some retrovirus-like process of horizontal transmission, or because there is some as yet unrecognized feature of transposition in *D. simulans* or some effect of the P element that is highly detrimental to the fitness of the host. The finding that some insertions of ry^+ DNA are accompanied by rearrangement of part of the *ry* gene implies an imprecision in the process of transposition in *D. simulans*, but analogous defects have also been found in *D. melanogaster* by Daniels *et al.* (35), and it is not possible at present to compare the rates at which such rearrangements occur in the two species. However, it is possible that one of the determinants of the ability of the P element to successfully invade a genome is the rate of defective transposition that occurs in the genome, and this may differ even among closely related species.

Although ry^+ DNA functions well enough in the genome of *D. simulans* to produce wild-type eye color, detailed studies of the developmental and tissue-specific regulation of ry^+ are still needed. In *D. melanogaster*, <1% of wild-type xanthine dehydrogenase activity is necessary for wild-type eye color (32), so a diversity in regulation and level of gene expression may underlie the wild-type phenotypes of the transformants in *D. simulans*. In at least one subline the ry^+ gene has become incorporated into the X-chromosome of *D. simulans*, and it will be of interest to determine whether this and other X-linked ry^+ genes are subject to dosage compensation.

Many recent discussions of genetic changes accompanying speciation favor the view that alterations in regulatory genes or regulatory signals may be far more significant and far more frequent than alterations in structural genes (33, 34). A rigorous experimental test of this hypothesis is made possible by the ability to carry out transformation between related but reproductively isolated species. Quite apart from whether the *D. melanogaster* ry^+ gene shows evidence of altered regulation in the genome of *D. simulans*, further application of the technique of interspecific transformation to other genes, both regulatory and structural, will provide a direct experimental test of the role of regulatory changes in evolution.

Investigators who may use transposable elements for interspecific transformation between reproductively isolated species are urged to exercise reasonable caution in preventing release of genetically transformed organisms into the environment. Transformation may result in the introduction of transposable elements into species in which they do not normally occur. Available evidence suggests that some transposable elements may be highly invasive and potentially harmful to the organism, and limits to the spread of such elements among related species may result primarily from the difficulty of interspecific transfer.

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