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Chapter 10

Sex-Specific Selection Using Chimeric Genes,

Applications to Sterile Insect Release

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Application of recombinant DNA technology to isolate, manipulate, and transfer genetic material to a pest insect may offer an efficient and cost effective means of achieving sex-specific selection of males for use in sterile insect release programs. Central to the implementation of genetic modification using recombinant DNA technology is the ability to efficiently transfer and integrate genes into the genome of a pest insect. An embryonic excision assay was developed to assess the potential of utilizing a P-element transposable vector for genetic transfer in insects other than Drosophila melanogaster (Meigen). When tested in various Drosophila species, the excision assay indicated normal P-element function in embryos of D. melanogaster, D. simulans, and D. grimshawi. However, excision events were not observed in embryos of the Caribbean fruit fly, Anastrepha suspensa (Loew), or the Indianmeal moth, Plodia interpunctella (Hübner). Once gene transfer techniques have been established for an insect, conditional sex-specific selection of males can be achieved by transforming the insects with chimeric genes that impart sex-specific sensitivity. The proposed structures of chimeric genes that may be useful in genetic sexing schemes will be presented. The chimeric genes will consist of promoter sequences from sex-specific genes such as yolk protein genes and will either express structural genes that impart chemical sensitivities to the females or produce antisense sequences to sex determining genes to disrupt development of females.

The combination of restrictions on the use of agrochemicals, the costs of developing and registering new chemicals, and the development of chemical resistance by pest insects has placed farmers and agribusiness in a position whereby they must rely to an ever increasing extent on biorational methods as supplements to chemical measures to achieve effective insect pest management. The greatest successes in achieving biological control of pest

insects have appeared in the use of autocidal programs. The sterile insect technique has been an effective means of limiting dipteran populations for nearly 20 years and has been examined as a means of controlling all pest insects (1). Although the sterile insect technique can be used very effectively to control pest insects, as evidenced by the screwworm eradication program (1), sterile insect release (SIR) in its present form is not applicable to all pest insects. Because lepidopterous insects require greater doses of radiation than dipterans to achieve sterility, the use of substerilizing dosages has been considered. Although release of insects receiving substerilizing doses has an impact on a population, larger releases of insects would be necessary to achieve effective control of a pest and would add considerably to the cost of the program (1).

A major limitation to SIR programs is the requirement for the availability of large numbers of reproductively competent sterile males (2). Because there is no efficient mechanism for the specific selection of males, the current SIR programs rear, sterilize, and release both sexes. The presence of the females results in added costs to the programs for rearing and in reduced efficiency of sterile male matings after release. Implementation of improved procedures for the selection of males or removal of females early in development would offer measures necessary to reduce the costs and increase the efficiency of SIR programs.

A number of classical genetic manipulations theoretically applicable to any insect have been developed in the fruit fly Drosophila melanogaster (Meigen) and several other insects that result in breeding populations that produces only one viable sex. Typically, the selection relies on the disruption of normal sex ratios by induced or naturally occurring mutants of the sex determination genes, chromosomal translocations, maternal effect lethals, or aberrant chromosomes (i.e. compound-X). However, these methods have not been generally applicable to agriculturally important insects because there is a paucity of significant genetic information on these insects. Ultimately, it has been the lack of basic genetic information that has precluded the development of genetic selection schemes applicable to SIR programs.

One scheme utilizing conventional genetic techniques, which has been developed for use in the control of the Mediterranean fruit fly, Ceratitis capitata Wiedemann, has not been completely successful (3). A portion of the chromosome carrying the gene for wild type pupal color was attached to the Y chromosome, which is carried only in males. The wild type color-attached Y chromosome was then mated into a white strain of flies. This composite mutant strain is being tested to determine the efficacy of utilizing a color marker for selecting males under large scale rearing conditions. While similar schemes may eventually prove useful, the preliminary experiments with the pigmentation marker revealed a problem with the breakdown of the marker chromosome by recombination (3). In addition to the breakdown of the marker chromosome, similar genetic manipulations in Drosophila routinely result in decreased viability of the mutant strain when compared with wild type strains (4).

Although most recent proposals for utilizing recombinant DNA technology in insects have focused on measures that would allow for direct genetic control of pest insects (5, 6), the potential for developing effective genetic-sexing procedures for SIR programs through application of recombinant DNA technology is being explored as a more immediate solution to the problem of SIR efficiency. The technology of molecular biology offers the ability to construct chimeric genes that encode for a readily selectable gene product that is expressed in a specific sex, stage, or tissue (7). Once introduced into the host genome, a sex-specifically expressed chimeric gene would offer a means of selecting for a desired sex. For example, combining a gene for a chemical or drug resistance with a male-specific promoter would confer resistance to males and females could be selected against when using a chemical treatment. Alternatively, females could be selected against by utilizing a chimeric gene containing a structural gene that imparts chemical sensitivity with a female-specific promoter that again would make the females lethally sensitive to chemical treatment. Chimeric gene constructs that result in chemical sensitivity in females are being produced and tested in D. melanogaster. However, application of these genetic selection systems to insects other than Drosophila requires the ability to introduce these genes efficiently into the genomes of pest insects, something that has not been achieved.

P-Element Excision Assay in Insect Embryos

The only efficient means of introducing genes stably into the genome of an insect is the modified transposable P-element vector system from D. melanogaster developed by Spradling and Rubin (8,9). The P-elements were found in P strains of D. melanogaster and were lacking in M strains. The presence of the transposable element could be demonstrated by the development of hybrid dysgenesis due to the movement of the P-element when males of a P strain were mated to females of an M strain (10). The DNA sequence of the P-element was cloned and inserted into a bacterial plasmid (8). When the P-element plasmid is injected into embryos of M strain flies, i.e. those flies that do not contain P-elements, the P-element transposes from the plasmid and stably integrates into the genome of the host fly. The ability to identify genetically transformed Drosophila initially depended upon "rescuing" a mutant host fly with a copy of a wild type gene carried by a P-element vector (9). Although genetic rescue can be useful in a well defined genetic organism such as D. melanogaster, the P-element vector required a more generally selectable element to identify transformed flies. The inclusion of the neomycin phosphotransferase gene linked with a heat shock promoter (hspneo) in the P-element vector supplies a dominant marker for selection of germline transformants (11). Other laboratories have utilized the hspneo P-element vector to test the ability of P-elements to effect germline transformation in a variety of insects without success. However, failure to impart neomycin resistance to an

insect does not give an adequate assessment of P-element transposability in an insect or a direct test of the utility of the P-element as a transformation vector in that insect. A simple more direct assessment of P-element functionality in heterologous germline transformation systems therefore is essential.

P-elements have been used as gene vectors in the closely related species D. simulans (12). In addition, the P-elements were found to undergo transposition in the more distantly related species D. hawaiiensis, although the capacity to act as a gene vector could not be demonstrated in this species (13). However, the transposable functionality of P-elements has not been demonstrated for insects outside the genus Drosophila.

Insertion of a P-element into a gene often results in the partial or complete inactivation of the gene and precise or reading-frame-conserved excision of the P-element restores gene function. In D. melanogaster, P-element excision appears to rely on the same enzymatic activity of the P-element transposase as does P-element insertion, and the excision activity is not genomic site dependent but extends as well to P-element sequences in plasmids (14). The excision of a P-element from a gene that results in restoration of gene activity is a means of monitoring the functionality of the P-element vector system, and forms the basis of an assay to test P-element activity in preblastoderm insect embryos.

The P-element excision assay was a modification of the assay used by Rio et al. (14) to assess P-element activity in tissue culture cells. The plasmid pISP (14) (Fig. 1) contains an internally deleted P-element that interrupts the lacZ alpha peptide coding region of the plasmid pUC8 and was used to monitor P-element excision. The pISP plasmid was co-injected into preblastoderm embryos of each species with the plasmid pUChsπΔ2-3, which was used as a helper plasmid to provide a source of P-element transposase by heat shock (15). Precise or reading-frame-conserved excision of the P-element from a pISP plasmid restores lacZ alpha peptide complementing ability to the plasmid, which can be determined easily by transforming the recovered plasmids into a $lacZ^-$ E. coli.

The excision assay plasmid and the helper plasmid were introduced into preblastoderm embryos of the various flies using the well established embryo injection procedures developed for D. melanogaster (8). The fly eggs were dechorionated in dilute bleach, immersed under oil, injected at 22°C, and incubated under O_2 for 15-18 hr. However, the injection procedures were modified for the embryos of the Indianmeal moth because they did not survive dechorionation or immersion in oil. The Indianmeal moth embryos, 1-2 hr old, were injected without dechorionation close to the micropyle and sealed with two layers of Krazy® glue. The embryos were incubated under O_2 for either 20 or 44 hr at 30°C. After incubating all embryos in O_2, both dipteran and lepidopteran embryos were heat shocked at 37°C for 1 hr to induce the P-element transposase activity. The flies were allowed to recover for 1 hr at 22°C and the moths for 2 hr at 30°C, under O_2. The embryos were collected and low molecular weight DNA,

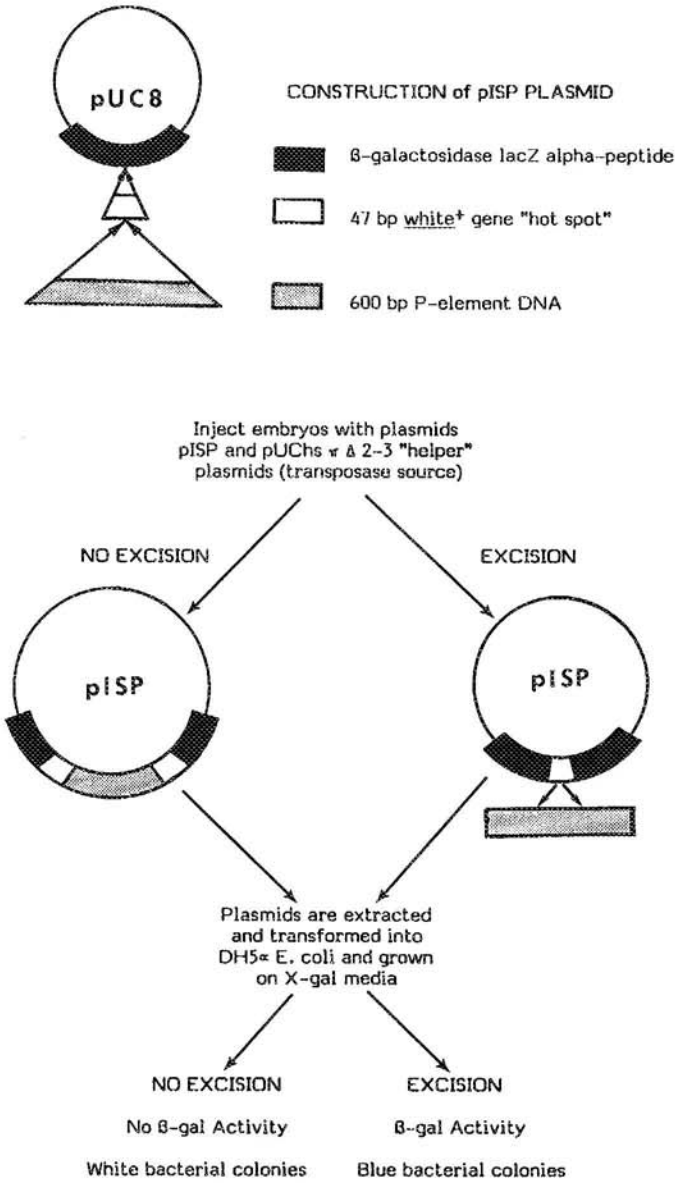

Figure 1. Diagrammatic representation of the P-element excision assay in embryonic soma.

which contained the injected excision assay plasmids, was isolated using the method of Hirt (16). The low molecular weight DNA recovered from injected embryos was used to transform the E. coli strain DH5α made competent according to the methods of Hanahan (17). E. coli transformants were plated on LB plates containing ampicillin (70 μg/ml) and X-gal (50 μg/ml). Transformants containing pISP plasmids that had lost the resident P-element through excision appeared blue on this medium.

Table 1. P-element excision in insect embryos

Insect species	pISP2 Plasmids recovered	$lacZ^+/amp^R$ Colonies	Frequency
D. melanogaster (M strain)	6.0×10^4	109	1.8×10^{-3}
D. melanogaster (P strain)	6.6×10^4	41	0.6×10^{-3}
D. simulans	4.0×10^4	40	1.0×10^{-3}
D. grimshawii	5.0×10^3	6	1.2×10^{-3}
A. suspensa	1.4×10^5	0	0.0
P. interpunctella	3.8×10^5	0	0.0

The functionality of the excision assay was initially tested in preblastoderm embryos of the M and P strains of D. melanogaster. The isolation of $lacZ^+/amp^R$ transformants, as indicated by blue colonies (see Fig. 1), showed that the excision assay was functional in the embryos as it was in cell lines as reported previously (14). The rates of excision for both the M and P strain embryos (Table 1) were equivalent to those observed for genomic P-elements and about ten times higher than the rates observed in cell lines. The latter result is somewhat surprising because P-element transposition is absent or greatly reduced in P strains. Restriction endonuclease mapping of plasmids recovered from these transformants indicated that the plasmids were pISP plasmids that had lost the resident P-element (data not shown). P-element excision in the embryos was observed only when the pISP plasmid was co-injected with the helper plasmid, i.e. when P-element transposase was present at high levels.

The excision assay was tested also in two other Drosophila species to determine the functionality of the assay in other insects. Excision occurred in both D. simulans and D. grimshawii at nearly the same rate as observed in D. melanogaster (Table 1). The transposition activity of the P-element in the embryos of these species demonstrates that the excision assay can be employed as a reliable indicator of P-element behavior in other insects.

When the excision plasmid was injected into the embryos of A. suspensa and P. interpunctella, no excision events were observed (Table 1). We conclude that these two species lack the cellular

machinery necessary for normal P-element transposition that is present in D. melanogaster and the other Drosophila species tested.

Testing P-element functionality using an excision assay as described here has a number of advantages over testing for germline transformation directly using dominant selectable markers or measuring P-element activity by conducting the excision assay in cell lines. First, because the assay is conducted only in embryos and without having to achieve correct insertion into the host genome, the procedure does not require extensive rearing and selection of injected animals and their progeny, nor does it depend upon achieving genetic transformation. Second, the plasmid excision assay is extremely sensitive to P-element activity. As the assay is currently conducted, we can recover approximately 10^3 pISP plasmids per injected embryo allowing as many as 10^6 plasmids to be screened easily from any species. Finally, since the assay is conducted in insect embryos, the problem of differences in transposon activity observed between cell lines and embryos is obviated. When transformed into cell lines, the Drosophila transposon, copia, undergoes extensive transposition whereas it rarely undergoes transposition in the whole animal (18). When all of these factors are considered, the P-element plasmid excision assay will be useful for determining P-element functionality in heterologous systems, and in addition will permit extensive analysis of the mechanics of P-element excision in D. melanogaster.

The failure to observe P-element excision in A. suspensa and P. interpunctella embryos suggests that P-elements may not be functional in all insects. Although the reason for the lack of functionality has not been identified, the excision assay is designed with adequate latitude for modification so that it is amenable to experimental analysis. With sufficient effort, the biochemical basis for the lack of P-element activity can be identified and potentially be corrected. Preliminary results indicate that the transposase gene carried by the helper plasmid is being transcribed in A. suspensa, and efforts are now underway to determine if all of the posttranscriptional modifications necessary to generate a functional transposase mRNA occur (15).

The rapid and simple assay of P-element functionality described here can be used readily to assess P-element function in a wide variety of economically important insects. The assay also will be used in the development of transformation vectors that are phylogenetically unrestricted, which in the end is the absolute prerequisite for the application of recombinant DNA technology to the genetic modification of pest insects and their integration into insect pest management programs.

Chimeric Gene Constructs for Genetic-Sexing Schemes

Once reliable methods for genetic transformation of pest insects have been developed, several chimeric gene constructs may be useful in genetic-sexing schemes. The schemes presented here employ existing molecular genetic technologies and use promoters

and structural genes that already have been isolated and characterized. The constructs and rationales can be tested in Drosophila species while methods for transformation of other insects are being developed. Thus, when gene transfer is more universally available, the constructs for genetic-sexing can be implemented with greater efficiency.

One general approach that can be considered for genetic-sexing is to impart a chemical sensitivity to the females and then select for the males by chemical treatment. The use of male-limited alcohol dehydrogenase (Adh) activity has been presented as one possible scheme (19). Development of this technique could be accomplished in insects that have a functional alcohol dehydrogenase even using classical genetic techniques but would require genetic transformation in those that lack Adh activity. The rationale is to develop a strain that has a functional Adh gene attached to a Y-chromosome, and then introduce the Adh-bearing Y chromosome into a strain that lacks Adh activity. This would result in a breeding population in which the males would have Adh activity and the females would not. Thus, the females would be lethally sensitive to low ethanol concentrations and could be selected out of the cultures. The male selection scheme described here is of course applicable to those species where the males are heterogametic. For insects such as lepidopterans, where the female is heterogametic, the genetic-sexing scheme would have to be modified to reverse the selection (see below). As pointed out above, the attached Y chromosomes are of limited use because they are subject to breakdown by recombination. In addition, the translocation of autosomal genes onto the Y chromosome can cause decreased viability of the strain. Therefore a more efficient and stable means of establishing the mechanics for genetic selection is required.

A selection scheme for males can be developed without classical genetics by employing recombinant DNA technology to construct a chimeric gene between the structural gene for Adh and a female-specific promoter for introduction into an Adh^- insect. This scheme would permit a stronger selection program to be used against the females. Since Adh would be expressed only in females, chemical treatment with 1-pentyn-3-ol or related compounds which are metabolized by Adh to lethal products (20) would result in the elimination of all females in the culture and survival of all males which would lack Adh activity. The yolk protein genes have been cloned from several insect species including D. melanogaster (21), Locusta migratoria (22, 23), Aedes aegypti (24), A. suspensa (A. Handler, unpublished), and P. interpunctella (P. Shirk, unpublished), and offer an easily obtainable female-specific promoter that could be used in the chimeric gene constructs. Unfortunately, it may be necessary to use a homologous cloned promoter for each insect as not all female-specific promoters are regulated correctly in heterologous insects (25).

A second general scheme for developing genetic-sexing, which perhaps has more potential, is the use of antisense RNA to disrupt

normal gene activity. Antisense RNA is a transcript generated from the complementary DNA strand of a gene and therefore codes for an RNA that is complementary to the normal RNA transcript produced during transcription of a gene (26). A chimeric gene that produces antisense RNA to a gene can be made by cutting the original gene from the promoter sequences and resplicing the gene with the promoter at the opposite end thus reversing the polarity of the structural gene relative to the transcriptional signals. By reversing the polarity of the DNA strands, the opposite strand will be transcribed and a complementary or antisense RNA will be produced. When antisense RNA was introduced either in vitro (27) or in vivo (28), the presence of the antisense RNA inhibited the expression of the normal RNA transcript produced by the resident gene. Suppression of RNA translation by antisense RNA may be due to the formation of an RNA heteroduplex between the two complementary RNA strands thus blocking the attachment of ribosomes. An example of this application in the context of the Adh example would be the construction of a chimeric gene containing the antisense sequence for Adh linked with a female-specific promoter. Thus, Adh activity would be eliminated from females and make them lethally sensitive to treatment with ethanol.

Another prospective application of antisense RNA to genetic-sexing being tested in our laboratories involves the use of antisense RNA to control sexual differentiation. In Drosophila, sex determination is controlled by the interaction of several autosomal genes (29). The activity of two of sex determination genes, transformer (tra) and transformer-2 (tra-2), is required to maintain a female state of differentiation. If either of the two genes is mutant or nonfunctional in a chromosomal female (XX vs. XY in males), the individual will develop phenotypically as a sterile male. Because of the required activity of the transformer genes, the phenotype of the insect can be changed from female to male during development if a transformer gene is switched off (30); temperature sensitive tra-2 females are phenotypically female when reared at the permissive temperature but become males biochemically when switched to the restrictive temperature as adults. Chromosomal XY males mutant for tra or tra-2 are not affected phenotypically except that tra-2 males are germline sterile; tra males are fertile. The rationale for utilizing the sex determining genes is to construct a chimeric gene containing an antisense sequence for a transformer gene linked with a promoter that can be controlled conditionally. This would allow XX females to develop as phenotypic females under the nonrestrictive conditions. However, when reared under restrictive conditions, the XX females would develop as phenotypic sterile males as would the XY males. This technique in effect would obviate genetic-sexing by creating a population of sterile males only. For this scheme to be useful, transformed genetic females as well as mutant carrying males would have to mate successfully with wild type females and be competitive in the field. Laboratory testing of Drosophila sex-determination mutants indicate that mutant phenotypic males do court and mate (31).

At present, the tra gene has been isolated from Drosophila (32, 33) and our laboratory is in the process of constructing a chimeric gene with antisense tra linked with a heat shock promoter. This construct will be introduced into D. melanogaster and its ability to inhibit normal tra activity in females will be tested morphologically and biochemically. Since a tra mutation does not cause male sterility, the complete scheme cannot be tested and awaits the isolation of the tra-2 gene. While this scheme may prove functional in Drosophila, the genetics of sex-determination has not been examined extensively in other insects. Possibly, the sex-determination genes in other insects can be identified by hybridization with a tra or tra-2 probe. Although nothing is known about the similarity of structure and function of the sex-determining genes in other species, analogous if not homologous genes are expected to be functioning in other insects. Should experimental disruption of sex-determination prove successful in Drosophila, the high efficiency of this scheme would justify the research necessary to identify the genes and implement similar programs in agriculturally important pest insects.

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