The genetics of *Drosophila* transgenics

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**Summary**

In *Drosophila*, the genetic approach is still the method of choice for answering fundamental questions on cell biology, signal transduction, development, physiology and behavior. In this approach, a gene’s function is ascertained by altering either the amount or quality of the gene product, and then observing the consequences. The genetic approach is itself polymorphous, encompassing new and more complex techniques that typically employ the growing collections of transgenes. The keystone of these modern *Drosophila* transgenic techniques has been the Gal4 binary system. Recently, several new techniques have modified this binary system to offer greater control over the timing, tissue specificity and magnitude of gene expression. Additionally, the advances in post-transcriptional gene silencing, or RNAi, have greatly expanded the ability to knockdown almost any gene’s function. Regardless of the growing experimental intricacy, the application of these advances to modify gene activity still obeys the fundamental principles of genetic analysis. Several of these transgenic techniques, which offer more precise control over a gene’s activity, will be reviewed here with a discussion on how they may be used for determining a gene’s function. *BioEssays* 26:1243–1253, 2004. © 2004 Wiley Periodicals, Inc.

**Introduction**

Genetics is a question that man poses to nature, obliging her to answer. The question is simple: what effects will be brought by altering a gene’s function? Animals harboring a mutation are analyzed for instructive phenotypes (e.g. changes in morphology, behavior, cellular identity, gene expression, etc.). One can begin with a phenotype and search for mutations that have the effect—forward genetics. Alternatively, one can start with a gene of interest, and see what phenotypes are produced from mutations within this gene—reverse genetics. Yet for any phenotype to be instructive, it is necessary to understand what effect the mutation has on the wild-type gene function. A description of these effects was codified by Hermann Muller in an astonishingly insightful treatise, over seven decades ago.(1)

Mutations that are informative will result in either a loss-of-function or a gain of-function effect. The loss-of-function mutations include amorphs, hypomorphs and antimorphs (also known as dominant negatives), which all act to reduce or eliminate the normal gene function.(1) The gain-of-function mutations include the hypermorphs (resulting in increased gene function) and the neomorphs (resulting in a new gene function(1)). A hypermorphic phenotype would occur if the gene was expressed at levels exceeding a tolerable cellular range or if the gene product was constitutively activated, in many cases resulting in a phenotype that is opposed to the amorph phenotype. In contrast, the neomorphic phenotype might result if the gene is ectopically expressed at the wrong time or in a wrong cell type allowing for promiscuous gene activation, aberrant phosphorylation, or other abnormal cellular events. The phenotype of an amorph or null mutation (lacking any gene function) is the easiest to interpret as it directly indicates the requirement for the gene. The logic is that, if the absence of a gene (e.g. the *eyeless* gene of *Drosophila*) results in the absence of a function (e.g. eye development), then the gene is required for that function. Since gain-of-function mutations affect gene activity in different ways, deducing the wild-type gene function from the resulting phenotypes is necessary more complex. This is especially true for neomorphic mutations, which can result in changes that lie outside of the normal gene function; interpreting these phenotypes should be approached with resolute caution. Even so, the characterization of neomorphic mutations can frequently reveal exciting glimpses into the function of regulatory genes.(2–4)

Knowing which of these classes of mutations you have is not always easy. The hypermorph and the antimorph are
typically dominant. The neomorph can also be dominant but is more frequently semi-dominant. Hypomorph and amorph mutations are generally recessive. These latter two classes can be distinguished by the phenotypes when placed over a deficiency for the locus. The phenotype of the hypomorph will become more severe, and there will be no change in the phenotype of an amorph. The nature of a dominant mutation becomes less severe, the hypermorphic phenotype becomes more severe, and there is generally no change in the neomorphic phenotype.

In more recent years, Drosophila geneticists have increasingly turned to transgenes to modify gene activity. In principle, changes in a gene’s activity corresponding to any of Muller’s classes of mutations may be achieved through transgenesis. With P element vectors, it is possible to increase the copy number of either wild-type or mutant genes in almost any genetic background. These vectors are most commonly used to either complement loss-of-function mutations or to generate hypomorphic conditions through the overexpression of a gene of interest. In 1993, the Gal4 binary system was introduced into Drosophila, giving rise to a quantum leap in the experimenter’s control of gene activity. In this system, the yeast Gal4 transcription factor is expressed in specific tissues either through enhancer detector P-elements or through the use of defined promoter or enhancer sequences. A second construct, the UAS responder, is also required; in this vector, a gene of interest is cloned behind a Gal4 responsive promoter. Gal4 can then drive the transcription of this gene in the desired tissue. A modicum of control over the levels of gene expression can be obtained by either altering the copy number of either or both P-elements, or by changing the incubation temperature of the flies; Gal4 is more active at 29°C than at 19°C.

Recently, two new techniques have emerged that advance the Gal4 binary vector method by combining the existing spatial control of gene expression with experimenter control over the timing of gene expression. These techniques, the hormone-inducible Gal4 systems and the temperature-sensitive Gal80 TARGET system, also provide for improved control over the level of transcription from UAS responder transgenes. The ability to further delimit the expression in both space and time with the newly developed technologies will increase the specificity of transgenic approaches to gene activity modification, and provide for within genotype controls. Furthermore, the promising techniques for post-transcriptional gene silencing (PTGS) may vastly expand the ability to reduce gene activity. Our usage of these techniques is firmly based upon the genetic principles learned over the last century. In this review, I seek to illustrate, with relevant examples, these modern transgenic techniques and how they may be used for reduction-of-function and gain-of-function experiments.

**Hormone-inducible Gal4 systems**

The hormone-inducible Gal4 fusion proteins contain a ligand-binding domain of a mammalian nuclear hormone receptor, the Gal4 DNA-binding domain, and a transcriptional activation domain; these hybrids can activate transcription from a Gal4 promoter in a ligand-inducible manner. Two such fusion proteins have been moved into Drosophila for use in binary transgenic systems. The Gal4–estrogen receptor fusion protein (Gal4ER) can activate transcription from UAS responders after flies are fed β-estradiol, while the Gal4–progesterone receptor fusion protein (Gene-Switch) will activate transcription in the presence of the anti-progestin RU486 (Fig. 1). The levels of RU486 used to obtain maximum induction appear, so far, to have no inherent effect on Drosophila development, behavior or aging, thereby averting a possible confound. The critical features of these new hybrid Gal4 drivers to be considered here are the availability of lines with defined spatial expression patterns, and the control over the level of gene activation.

A major advantage to the hormone-inducible Gal4 systems is that they will work with the thousands of extant UAS responder lines, but one drawback is that new driver lines will have to be produced. Enhancer detector P-elements have been generated for both the Gal4ER and Gene-Switch fusion proteins. These P-elements utilize transcriptional enhancers located in the flanking genomic DNA to drive the expression of the hybrid Gal4s. The different enhancer detectors can be further mobilized to generate new lines with distinct insertion sites and expression patterns. Small collections of these lines have already been generated, from which many independent expression patterns have been found. A much larger screen of the P(Switch2) enhancer detector element has recently taken place in the laboratories of Ronald Davis and Haig Keshishian (personal communication; Baylor College of Medicine and Yale University respectively) increasing the number of independent lines. Another successful strategy for producing a desired spatial expression pattern is to drive the hybrid Gal4s with defined promoters or transcriptional enhancers. The torso-like (tsl) regulatory region was used to drive expression of Gal4ER within the border cells and a subset of posterior cells of the follicle. Both pan-neuronal and pan-muscular Gene-Switch lines were generated using the elav and mhc promoters, respectively. Furthermore, a series of ten vectors designed for the rapid cloning of regulatory regions 5’ to Gene-Switch coding sequences have been constructed. These vectors have been used to make eye-specific and mushroom-body-specific Gene-Switch lines.

The level of gene expression in the uninduced state is a crucial consideration for experiments that use the inducible techniques. In both the Gal4ER and Gene-Switch systems, the level of transcriptional activation in the absence of ligand appears in most lines to be generally quite low and frequently
undetectable. In several Gal4ER-expressing lines, both the detection of β-galactosidase reporter activity and the stimulation of cell death through the induction of an UAS-diphtheria toxin A responder were completely dependent on the presence of β-estradiol. Similar experiments have also shown most Gene-Switch lines to be "off" in the uninduced state. Exceptions to this low level of basal activity come from Gene-Switch lines that also have very high levels of induced expression. In one elav-Gene-Switch line, a 51- to 60-fold increase in reporter gene levels was obtained after induction. Thus, it may be that RU486 increases Gene-Switch-dependent transcriptional activation by greater than 50 fold and, in most lines, the basal uninduced level of activation remains either below the detection threshold or low enough to have little impact on cellular functions.

A clear benefit to the hormone-inducible Gal4 systems is the ability to turn "on" a gene of interest. However, one of the beautiful, if not wholly appreciated, aspects of inducible gene expression system, is that the level of the gene of interest can be controlled by manipulating the ligand concentration or the time after induction. The amount of gene product may then accumulate at this rate until equilibrium is reached. It should be possible, therefore, to examine different levels of the gene of interest by sampling different times after induction or by using different levels of the inducer and sampling at the same time. The amount of RU486 required for saturation of Gene-Switch activation may depend on the level of Gene-Switch fusion protein or on the tissue being analyzed. The principles behind Gene-Switch are very similar to those of the Gal4-ER fusion protein, where the inducer is β-estradiol.

Figure 1. The Gene-Switch fusion protein offers temporal, spatial and quantitative control of transgene expression. Gene-Switch is a hybrid transcription factor composed of the Gal4 DNA-binding domain, the human progesterone receptor ligand-binding domain and the human p65 transcriptional activation domain. In this system, spatial expression can be achieved by driving transcription of Gene-Switch with a tissue-specific enhancer (TSE) or promoter. In the P(Switch) enhancer detector elements, the TSEs are located in the flanking genomic DNA, while the minimal promoter is from the P-transposase. Since the Gene-Switch activation of a Gal4-responsive promoter (UAS) is strongly dependent of the presence of the inducer RU486, the experimenter can control the onset of gene expression. The expression level of the gene of interest (GOI) can be controlled by sampling at different times after induction or by using different levels of the inducer and sampling at the same time. The amount of RU486 required for saturation of Gene-Switch activation may depend on the level of Gene-Switch fusion protein or on the tissue being analyzed. The principles behind Gene-Switch are very similar to those of the Gal4-ER fusion protein, where the inducer is β-estradiol. 

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Hence, different levels of gene activity are achievable by sampling different times after induction. Interestingly, feeding starved adults a solution of 500 μM RU486 in 2% sucrose for as little as 1 hour provided for maximum levels of induction at 24 hours. Thus, for saturating concentrations of RU486, the rate-limiting step of Gene-Switch transcriptional activation appears not to be the uptake and distribution of this ligand.

**Target**

The TARGET (Temporal And Regional Gene Expression Targeting) system has the potential of working with any existing GAL4 driver and UAS responder (Fig. 2). TARGET utilizes a temperature-sensitive Gal80 molecule to repress Gal4 activity. In yeast, Gal80 will repress Gal4 activity in the absence of galactose by binding and inhibiting its transcriptional activation domain. Gal80 was first introduced into Drosophila to facilitate mosaic analysis; the loss of Gal80 from a daughter cell following mitotic recombination allows Gal4 to specifically mark patches of homozygous mutant cells in an otherwise heterozygous organism. The Gal80<sup>ts</sup> protein was imported into Drosophila and shown to maximally repress Gal4 activity at 19°C, while being permissive for Gal4 activity at 32°C. This temperature preference is the converse of Gal4's own temperature sensitivity. Thus Gal80<sup>ts</sup> and Gal4 act as opposing forces, a sort of yin–yang of transcriptional activation, where lower temperatures inhibit transcription and elevated temperatures push transcription levels of the UAS responder higher. One strong benefit for TARGET is that temperature, as an inducer, is both rapid and easily controlled, but one potential pitfall for TARGET is that elevated temperatures are not always neutral and can have undesired effects on the induced flies. For example, culture temperatures of 30°C result in significantly more axonal branches and varicosities in the larval neuromuscular junction than at 25°C. Furthermore, Drosophila melanogaster show reduced mating, pupariation and oviposition when cultured above 32°C, and males are typically infertile above 30°C.

The utility of the trinary TARGET system comes in part from the ubiquitous, high level of expression of Gal80<sup>ts</sup>, such that Gal4 can be conditionally inhibited globally, and throughout development. In the TARGET system, the Gal80<sup>ts</sup> gene is driven to high levels by the Tubulin 1α-promoter. TARGET offers great flexibility since two or more independent Gal80<sup>ts</sup> transgenes can also be added to the final genotype to boost the expression of Gal80<sup>ts</sup>. Additional Gal80<sup>ts</sup> transgenes may more fully repress transcriptional activation in lines that have particularly high levels of Gal4 or if the levels of the responder need to be extremely low in the uninduced state. Yet, the ability of a single Gal80<sup>ts</sup> to repress Gal4 at low temperatures is quite profound. One Gal80<sup>ts</sup> transgene appears to be able to completely repress a GFP reporter driven by an elev-Gal4 transgene. Additionally, a Gal80<sup>ts</sup> transgene was capable of fully suppressing the lethality produced when the GMRGal4 is combined with the UAS-head involution defect pro-apoptotic responder.

The kinetics of TARGET induction have been determined at the level of GFP mRNA levels, and are therefore not directly comparable to the kinetics of the hormone-inducible Gal4 systems for which protein accumulation or reporter activity has been assayed.

![Figure 2](image-url)
been measured. Nevertheless, GFP reporter mRNA was
detected after three hours at 32°C, and reached maximal
levels after 6 hours at 32°C. This induction resulted in a 42- to
48-fold increase in GFP message levels. These data sug-
gest that the high temperature induction of TARGET may be
slightly faster than the Gene-Switch induction by feeding
RU486. Like Gene-Switch, the TARGET system offers a
rheostat capability. The level of induction can also be
controlled either by the time from the period of induction or by
the temperature used for inactivating the Gal80P suppressor
(Fig. 2).

Reduction-of-function transgenics
In these experiments, a gene’s function is eliminated or
inhibited by dominant transgenes. The need of a gene’s
activity may then be inferred through the resulting phenotypes.
There are several available methods that can disrupt a gene’s
activity including dominant negative approaches, through the
expression of selective peptide toxins, or through the use of
post-transcriptional gene silencing. A gene is often required at
several times and places during development. One challenge
to these techniques is to limit the effect of the reduction-of-
function to a given developmental time point or restricted to a
specific tissue or cell type.

In a classic paper, Ira Herskowitz(22) discussed methods of
achieving the disruption of gene activity through the expres-
sion of dominant negative (antimorphic) mutations. This
approach has been quite productive in Drosophila and is still
in wide use (e.g.23–25). Dominant negative alleles will
typically contain mutations that allow for the assembly of the
altered protein into multimeric complexes, but leave the
protein functionally inactive, thereby poisoning the entire
complex(22). As a consequence, this technique is limited to
those genes whose products form such complexes. One such
gene is shibire (shi), which encodes the GTPase dynamin.(26)

Dynamin selfassembles into large macromolecular spirals
that form collars around membranes pockets and regulates
endocytosis.(27,28) In the shibireP (shiP) mutation, synaptic
vesicle recycling fails at restrictive temperatures, which leads
to a rapid depletion of synaptic vesicles and the inhibition of
synaptic signaling.(29) Toshihiro Kitamoto(30) generated a UAS
responder that contains this temperature-sensitive allele of
shibire. The targeted expression of the UASshiP transgene
has the extremely useful property of dominantly inhibiting
synaptic signaling at temperatures above 29°C, while having
little effect on neuronal physiology at the lower permissive
temperatures. These properties have been exploited by
several groups to dissect the neural and temporal elements of
complex behaviors.(30–33)

The limited expression of bacterial peptide toxins has also
been used to interfere with discrete cellular functions. In most
cases, these toxins will ablate the cells in which they are
expressed; similar results can also be obtained through the
targeted expression of the pro-apoptotic genes reaper, head
involution defect, or grim.(34) Early developmental expression
of these genes frequently leads to the death of the organism,
interfering with the analysis of later stages of development. To
overcome this problem, conditional alleles of the ricin A chain
and the diphtheria A toxin were identified and corresponding
UAS responder lines made.(35,36) Both of these toxins disrupt
cellular functions by interfering with protein translation.
Although the conditional alleles proved useful, cellular ablation
can still occur at permissive temperature when crossed with
most Gal4 lines, presumably due to residual activity at
permissive temperatures combined with high levels of
expression. As stated earlier, when used in combination with
the-inducible Gal4 systems however, the effect of the
uninduced toxin is often minimal to non-existent.(12–14,16,18)

The transgenic expression of the tetanus toxin light chain
(TNT) has been an extremely useful technique for disrupting
neural functions.(37) TNT specifically disrupts neurotransmis-
sion by cleaving synaptobrevin, a protein required for fusion of
synaptic vesicle to the plasma membrane.(38) A novel condi-
tional allele of TNT was recently generated by Keller
et al.(39) to disrupt neural functions in adult flies. The approach
of Keller et al.(39) was to insert a heterologous gene with a stop
codon between the UAS promoter and the TNT coding
sequences. The heterologous gene was also flanked by
FRT-site-specific recombination sites. The transcriptional stop
located between the UAS promoter and the TNT gene was
then removed in vivo by heat-shock-inducible FLP recombi-
nase, resulting in an intact UASTNT transgene.(39) Im-
portantly, the FLP catalyzed activation of TNT could be induced in
some, but not all, postmitotic cells. These conditional TNT
alleles also proved to be somewhat leaky, perhaps limiting the
general usefulness of this technique. It is possible, however,
that using a different inducible system to control the recombi-
nase expression may lower background activity. It may also
be possible that Cre recombinase could be active in cells that
are refractory to FLP recombinase. A possible solution to
both these problems may have come from Heidmann and
Lehner.(40) These authors have developed an inducible Cre
recombinase system by fusing the human estrogen receptor
ligand-binding domain to the Cre recombinase protein. In lines
expressing this hybrid protein, recombination is dependent on
the addition of estrogen.

Post-transcriptional gene silencing (PTGS) is rapidly
becoming one of the most powerful techniques for reducing
gene activity. PTGS, also known as RNAi, is a conserved
cellular process that exists in plants, nematodes, flies and
mammals in which double-stranded RNA (dsRNA) induces the
degradation, and thus silencing of homologous mRNAs.(41–44)
PTGS is mechanistically linked to the protection against virus
proliferation and to the production of the microRNA transla-
tional regulators.(45,46) In this process, the dsRNA is recog-
nized by the RNase III-like enzyme dicer and cleaved into
~21 nucleotide fragments known as small interfering RNAs (siRNAs; Fig. 3\(^{47}\)). The siRNAs are used as guides by the RNA interference silencing complex (RISC) to recognize and cleave homologous sequences, reducing target transcripts.\(^{48,49}\) The dicer RNase physically associates with the RISC complex.\(^{50}\) Members of the RISC complex, including dicer are cytoplasmically localized in both vertebrates and \textit{Drosophila}.\(^{51,52}\) The separate dicer and RISC enzymatic activities are also localized in the cytoplasm.\(^{46,53}\) Therefore the nuclear export of the dsRNA substrate is an essential feature of PTGS and nuclear RNAs appear immune.

Recently many laboratories have generated dsRNA producing transgenes to reduce or eliminate gene function (e.g.\(^{54–57}\)). In this technique, mRNAs that contain inverted repeats are induced from UAS transgenes (Fig. 3). The expressed inverted repeats (EIR) form dsRNA that becomes a template for siRNA formation and PTGS. There are several features of PTGS in \textit{Drosophila} that make this an incredibly powerful tool for reduction-of-function studies. The sequence targeted can be highly specific, the effect appears to be cell autonomous, and can be easily modulated experimentally. The rules for producing effective expressed inverted repeat constructs are becoming clearer and, in most cases, are quicker and require less work than generating mutations de novo in the gene of interest.

In plants and \textit{C. elegans}, PTGS produces a general systemic effect where the knockdown of targeted messages can occur very far away from the initial site of siRNA induction, although neurons of \textit{C. elegans} and the guard cells of plants appears to escape systemic PTGS response.\(^{41,58,59}\) In \textit{C. elegans}, the transmembrane protein product of \textit{sid-1} is required for the spreading of PTGS,\(^{60}\) a \textit{Drosophila} homolog of this protein has not been found. The systemic PTGS response appears to be absent in both \textit{Drosophila} and mouse. The evidence supporting cell autonomy for PTGS in \textit{Drosophila} came initially from a failure to see spreading of the phenotypic effects outside of regions defined by Gal4 expression (e.g.\(^{56,61–63}\)) found, by examining the PTGS of GFP variants using laser scanning confocal microscopy, that the effect could be strictly limited to engrailed defined embryonic segments. An important point from this study was that GAP junctions present between epidermal and ectodermal cells would not support PTGS spreading.\(^{64}\) Similarly, Roignant et al.\(^{65}\) using immunohistochemistry, failed

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**Figure 3.** Expressed inverted repeats result in post-transcriptional gene silencing through the production of siRNAs. In \textit{Drosophila}, an inverted repeat is cloned behind a Gal4 responsive promoter. These two repeats are separated by an intron. The inverted repeats are transcribed in cells that are also expressing Gal4. The expressed inverted repeats (EIR) are shown in blue and purple, separated by an intron shown in white. The transcribed intron engages the splicesome complex, which increases the export of the expressed inverted repeat from the nucleus. The EIR folds back on itself to form a long stretch of double-stranded RNA. This dsRNA is a substrate for the Dicer RNase, which is a component of the RNA interference silencing complex (RISC), located in the cytoplasm. The Dicer RNase cleaves the exported dsRNA into smaller, ~21 nucleotide small inhibitory RNAs (siRNAs). The siRNAs are used by the RISC complex as a template for destroying homologous messages. Through this mechanism, specific messages can be targeted for knockdown in a cell-autonomous manner.
to find any spreading of EIR-induced PTGS of the *batman, Ecr-B1* and *Pcaf* genes into adjacent cells. In this study, *batman* expressed inverted repeat clones were made within third instar tissues, and again PTGS spreading went undetected in the surrounding cells. Thus it appears that EIR-induced PTGS is generally a cell-autonomous process in *Drosophila*.

In plants and *C. elegans*, there is also a transitive process in which secondary siRNAs appear that are homologous to sequences on the mRNAs that are either 5’ or 3’ to the original targeted sequence.\(^{66,67}\) Thus PTGS can “spread” to include targets upstream or downstream of the original siRNA. This can limit the effectiveness of EIR-induced PTGS by eliminating entire classes of mRNAs that share sequence with the target siRNAs. In both plants and *C. elegans*, the transitive attribute of PTGS requires an RNA-dependent RNA polymerase\(^{68–70}\).

*Drosophila*, however does not appear to have any sequences homologous to this RNA-dependent RNA polymerase gene family.\(^{71}\) The question of transitivity in *Drosophila* was addressed experimentally by Roignant et al.\(^{65}\). These authors found that PTGS could effectively work against an individual splice form of the ecdysone receptors by targeting exons unique to a specific isoform. The failure to directly identify siRNAs from common exons and the failure to silence mRNAs unique to a specific isoform indicates that transitive “spreading” did not occur. This point was again demonstrated by looking for transitivity in larvae expressing a *batman–GFP* fusion and an EIR–GFP construct under the *daughterless–Gal4* driver.\(^{65}\) In this experiment, GFP but not *batman* siRNAs were detected by RNase protection. Additionally, there was no suppression of the native *batman* gene in these animals.\(^{65}\)

Taken together, these data suggest that extreme specificity of EIR-induced PTGS can be achieved in *Drosophila* through the appropriate selection of target sequences.

The effectiveness of EIR-induced PTGS as a research tool appears to be increased by the ability to modulate the response experimentally. The severity of the PTGS response is sensitive to the level of EIR.\(^{56,57,72}\) Control of EIR levels can be relatively easily achieved by using independent transgenes with different position effects, or by increasing the copy number of EIR transgenes present in the animal. Furthermore, this dose-dependent response suggests that PTGS should work quite well with inducible expression systems. Lam and Thummel\(^{73}\) found that hsp70Bb promoter provided significant control over the timing of EIR-induced PTGS of the *EcR* and *pFTZ-F1* genes. In another experiment, hsp-Gal4 was used to induce yellow EIR.\(^{72}\) Simply by varying the number heat-shock treatments, the response would range from very weak to very severe PTGS of yellow.\(^{72}\) The Tet-on-inducible system has also been used to knockdown the levels of *phosphoglucomutase*.\(^{74}\) In these experiments, induction of the *pgm* EIR with doxycycline resulted in a 1.3- to 24-fold reduction in mRNA levels, while a significantly more modest reduction in enzymatic levels were found.

A second method to modulate EIR levels is through temperature. Fortier and Belote\(^{56}\) found that the EIR-induced PTGS of *transformer-2* was temperature dependent using three independent Gal4 drivers—more severe at 29°C and practically absent at 22°C. One possibility for this temperature dependence is that Gal4 is more active at 29°C than at lower temperatures,\(^{77}\) so the EIR is expressed at higher levels resulting in greater PTGS. However, results from PTGS in plants suggest that it may be a more general phenomenon. The pathological effects of virus infection can be masked by higher temperature.\(^{72,76}\) The temperature-masking effect can be attributed to the loss of virus-specific siRNA formation at temperatures below 22°C, whereas at 24°C siRNA formation was significantly enhanced.\(^{77}\) Therefore, the temperature-sensitive event occurs at or before the dicer processing step and, in plants, higher temperatures lead to substantially more PTGS than lower temperatures.

There are now several “rules” or suggestions that may lead to an efficient design of EIR transgenes. The actual orientation of the inverted repeats does not seem to matter since efficient PTGS has been achieved with either sense–antisense or antisense–sense constructs.\(^{56,72}\) The length of the repeat region may be a critical factor, since Riechhart et al.\(^{62}\) demonstrated that EIRs containing 200 nucleotide repeats provided poor PTGS as compared to repeats of greater than 500 nucleotides. The ability to clone the inverted repeat in *E. coli* is markedly increased by the addition of a spacer of at least 20–50 base pairs between the repeats.\(^{72,78}\) Several studies have successfully used intronic spacers to separate the inverted repeats.\(^{61,62,79}\) Kalidas and Smith\(^{61}\) generated *lush, white* and *Gxq* EIRs by cloning into UAS vectors, genomic DNAs containing introns followed by an inverted cDNA sequence immediately after an intron. All three constructs demonstrated significantly stronger and more uniform PTGS than previous reports.\(^{56,57,72}\) Reichhart et al.\(^{62}\) examined the PTGS strength of *necrotic* with EIR constructs containing either a stem loop intervening spacer, or with an intronic spacer between the inverted repeats.\(^{61,62,79}\) Kalidas and Smith\(^{61}\) generated *lush, white* and *Gxq* EIRs by cloning into UAS vectors, genomic DNAs containing introns followed by an inverted cDNA sequence immediately after an intron. All three constructs demonstrated significantly stronger and more uniform PTGS than previous reports.\(^{56,57,72}\) Reichhart et al.\(^{62}\) examined the PTGS strength of *necrotic* with EIR constructs containing either a stem loop intervening spacer, or with an intronic spacer between the inverted repeats. In the resulting transgenic flies, significantly stronger PTGS was seen in the lines containing the intronic spacer.\(^{62}\) What is not clear from these studies is whether the intron needs to be the spacer, or whether the increase potency of these constructs is simply because of the presence of an intron within the transcribed region, regardless of its position. In mammalian systems, the expression of recombinant proteins frequently requires the presence of an intron.\(^{80–82}\) In *Xenopus* oocytes, spliced mRNAs are exported from the nucleus more rapidly and to much higher levels than are the identical mRNAs that did not contain the intron.\(^{83}\) In the C6/36 *Aedes* cultured cells, adding an intron to luciferase increased the levels of expression from 12- to 60-fold, depending on the promoter.\(^{84}\) Thus, it is possible the intron simply promotes the nuclear export of the EIR for more efficient processing by the dicer RNase and
greater PTGS. Interestingly, when Svoboda et al.\(^{(78)}\) varied the position of the SV40 intron within their mos EIR construct, they found that the highest PTGS in mouse oocytes occurred when the intron was 5’ of the inverted repeat; placement of the intron between the inverted repeats actually inhibited mos PTGS relative to the absence of an intron. These data suggest that the position of an intron within the EIR may be very important, and should be further analyzed in Drosophila. In summary, when building an EIR construct for PTGS, the length of the repeat should be greater than 200 nucleotides, the repeats may be separated by a spacer for more efficient cloning, and the construct should contain an intron. However, care should be taken in choosing which intron to add to your EIR construct. Several Drosophila introns have been described that contain complex transcriptional regulatory sequences.\(^{85–88}\) The placement of such an intron within the EIR construct could drive expression of the EIR in an unintended tissue or cell type.

There are a few general provisos to keep in mind since EIR expression is still a young technique in Drosophila. It remains possible that some constructs may suppress translation of more than one gene. Whenever possible, phenotypes induced by EIR transgenes should be compared with those of the loss-of-function mutations. It is also possible that some tissues may be refractory to this technique. In C. elegans, for example, RNAi works very poorly in gonads and in the nervous system.\(^{(89)}\) Lastly, it may even be possible that some postdevelopmental cells may have suppressors of PTGS, or may lack the machinery necessary for PTGS.

**Gain-of-function transgenics**

The use of UAS responders in gain-of-function studies frequently involves the expression of a wild-type cDNA in an otherwise mutant background. These experiments can determine the cells or tissues responsible for a given loss-of-function phenotype. For example, Zars et al.\(^{(90)}\) found that the expression of a wild-type rutabaga (rut) cDNA in the mushroom bodies of a rut mutant was sufficient to rescue the olfactory learning phenotype of this rut mutation, thereby suggesting rut function within this brain region is important for learning and memory. Two important experimental points on gain-of-function experiments were made in this study.\(^{(90)}\) First, the authors drew the comparison of the rut rescue with Gal4 lines to mosaic analysis. All of the Gal4 lines used in the Zars et al. study\(^{(90)}\) also express in cells outside of the mushroom bodies. The positioning of the rut function within mushroom bodies, therefore, was only possible because mushroom body neurons were the only cells in common among all the tested Gal4 lines capable of rescuing the phenotype. The second point is that, in these lines, Gal4 is expressed throughout development. Thus, one could not determine when rut was required for olfactory learning and memory.

The inducible Gal4 systems have been utilized recently to address this specific question of a developmental versus an acute physiological role for rutabaga in olfactory learning and memory. McGuire et al.\(^{(18)}\) using the TARGET system found that rut expression induced within the mushroom bodies posteclosion for 6 days rescued the olfactory learning and memory phenotype of a severe rut mutation. These authors also found that expression of rut during development, but not during adulthood was insufficient for rescuing the rut phenotype. In a similar series of experiments, Mao et al.\(^{(17)}\) used a mushroom-body-specific Gene-Switch line to control the timing of rut expression within mushroom bodies. These authors found that induction of rut expression for as little as 12 hours before training was enough to rescue the olfactory learning and memory phenotype of a severe rut allele, while expression during development, but not during adulthood, failed to improve the learning scores of the rut mutant. These results indicate that the time constraint for rut expression is very close to the time of training and, therefore, rut is most likely to be involved in the physiology of learning.\(^{(17,18,90)}\)

Other gain-of-function studies utilize either ectopic or overexpression of non-mutant genes in a wild-type fly to examine that gene’s function. While these studies mostly give rise to neomorphic phenotypes, they also are frequently quite informative: an accurate and developed interpretation of these experiments will require information on the wild-type gene function. This information may come from: (1) a phenotype from an amorphic mutation or (2) biochemical information on gene function, which could possibly be presumed through sequence similarity to a known gene product. The phenotypes arising from ectopic expression of the eyeless gene is an excellent example of the former. Loss-of-function mutations in eyeless lead to a deletion of the eyes.\(^{(91)}\) Yet when ectopically expressed in imaginal discs, eyeless can drive eye formation, indicating that the expression of this gene is sufficient for eye formation in some contexts.\(^{(92,93)}\)

In another classic example of this gain-of-function experimental approach, Störtzhl and Kettler\(^{(84)}\) utilized the Gal4 system to increase the number of olfactory receptor neurons that expressed the or43a gene. Since this gene encoded a putative G protein-coupled receptor and was expressed in a small subset of olfactory receptor neurons, it was assumed to be an odorant receptor.\(^{(95,96)}\) The flies in which or43a expression was increased gained an increased olfactory sensitivity to the odorant benzaldehyde as measured by electroantennagrams, suggesting that OR43a is activated by this odorant.\(^{(84)}\) A companion paper confirmed that, when expressed in Xenopus oocytes, OR43a can activate G proteins in response to benzaldehyde.\(^{(97)}\) There are significant advantages to these gain-of-function experiments. Information can be obtained about the function of a gene in the absence of a mutation, such as with or43a.\(^{(94)}\) Gain-of-functions studies of this kind may also provide a phenotype when, due to functional
redundancy, the loss-of-function will not produce one. For instance, if Drosophila perceives benzaldehyde through multiple receptors, which is almost certainly the case, then an or43a loss-of-function mutant would have a very subtle to imperceptible behavioral phenotype.

The ability to perform gain-of-function studies on the genomic scale was made possible by the development of the specialized EP transposable element.(98) This P-element construct contains a Gal4 UAS promoter at the terminus of the P-element-facing toward the 3' flanking sequence; when inserted within a proximal promoter region, it brings the flanking gene under Gal4 control. A large collection of EP lines has spawned a cottage industry of gain-of-function genetic screens, by allowing the overexpression or misexpression of hundreds of genes in tissues defined by the extant Gal4 driver lines.(88) A phenotype arising from these screens would most likely be neomorphic. Still, the responsible gene would be identified by the site of the EP-element insertion, and the sequence of this gene may provide insights into its cellular function. Additionally, the EP element will generally be a hypomorphic mutation, allowing for the characterization of a loss-of-function phenotype if one was not already available.

The use of an inducible Gal4 system in these gain-of-function screens would offer a few theoretical advantages over the usual Gal4 system. First, a critical time for the emergence of the phenotype could be directly determined by modifying the time of induction. By inducing gene expression from the EP element at defined times, the resulting phenotype would be less likely to be caused by earlier, undesired and potentially unknown sites of expression. The induction may also suggest whether the phenotype arises as the end point of a cascade of gene-activation events, which may take some time to develop, or whether the phenotype is the direct result of the ectopically produced gene. A second benefit is that the level of ectopic activation may be modulated. The use of the inducible system’s rheostat function allows for an allelic series within a single genotype. From this type of experiment, one may discern whether there are phenotypic thresholds and whether the induced phenotype is a linear response to levels of ectopic expression. The single genotype allelic series would be particularly instructive for genes that function through concentration gradients (e.g. the gap genes(90)), or for genes that operate as part of a counting mechanism (e.g. runt, vap-33a(100,101)).

Conclusions
It has now passed 103 years since William Castle, at Harvard University, first used Drosophila melanogaster in biological experimentation.(102) During this past century, a fairly detailed understanding of this organism’s biology has developed. As a consequence of this understanding, the questions asked are also becoming progressively more refined, and more highly developed tools are required to answer these new questions. Yet, there is also a universal benefit to controlling as many variables as possible in experimentation. When it comes to altering gene activity, the primary variables that should be controlled are how, when, where and to what degree. The recent technical breakthroughs of the inducible Gal4 systems and the PTGS techniques hold the promise of allowing us to control these variables to a much greater extent than was previously possible. As a result, a gene’s function may be determined faster, more precisely, and with significantly less effort than ever before.

References


